TESE DE DOUTORAMENTO

JASIONE L. (CAMPANULACEAE). BIOGEOGRAPHIC HISTORY, EVOLUTIONARY RELATIONSHIPS, SPECIES DELIMITATION, AND NOMENCLATURAL REVISION

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Resumo

Esta tese doutoral ten como obxectivo revelar diferentes aspectos da realidade evolutiva do xénero *Jasione* L. (Campanulaceae), unha liñaxe vexetal distribuída pola conca mediterránea. Leváronse a cabo análises moleculares, citolóxicos, morfolóxicos e ambientais para abordar o seguinte: (i) as relacións filoxenéticas internas en *Jasione*, así como a súa posición entre outros xéneros de Campanulaceae, (ii) a historia bioxeográfica dun xénero cun notable patrón bioxeográfico mediterráneo este-oeste, (iii) os procesos que conducen á especiación e adaptación a novas contornas, (iv) o estudo dos cambios de nicho ambiental asociados con cambios no nivel de ploidía, (v) os métodos utilizados para delimitar entidades crípticas evolutivamente significativas utilizando diferentes liñas de evidencia, e (vi) tipificación de grupos complexos de *Jasione* como un paso no establecemento dunha nova organización taxonómica do xénero baseada en relacións evolutivas, entre outros aspectos.

A familia Campanulaceae é un grupo evolutivo complexo caracterizado polo conflito entre as clasificacións tradicionais e as filoxenias moleculares xeradas nas últimas décadas, conflitos que se agudizan na subfamilia Campanuloideae. Unha das principais razóns das diferenzas entre as clasificacións premoleculares e os resultados filoxenéticos modernos é a escaseza de caracteres morfolóxicos discriminatorios, o que levou a unha énfase excesiva nos tratamentos taxonómicos tradicionais a algúns trazos reprodutivos, a saber, as morfoloxías de flores e froitos. A evolución paralela dos trazos morfolóxicos aparentemente embazou a sistemática do grupo, dificultando a proposta de delimitacións evolutivas significativas entre e dentro dos xéneros.

Neste contexto xeral, *Jasione* é un dos xéneros de Campanuloideae máis homoxéneos desde o punto de vista morfolóxico. En

consecuencia, foi desconcertante con respecto á súa sistemática evolutiva. Existe unha controversia xeneralizada entre os autores en canto ao número de taxones que deben recoñecerse, con ao redor dun centenar de diferentes nomes propostos. Con todo, os tratamentos xerais actuais só recoñecen entre 12 e 16 especies, aínda que este enfoque sintético considerouse demasiado simplista e resulta da dificultade do xénero. En consecuencia, reivindicouse a necesidade dun tratamento moderno con perspectiva evolutiva. O obxectivo desta tese doutoral é lanzar luz sobre a realidade evolutiva e a historia bioxeográfica dun xénero distribuído en Europa e na conca mediterránea, utilizando ferramentas moleculares, citolóxicas e ambientais para lograr este obxectivo.

O xénero *Jasione* L. (Campanulaceae) distribúese pola conca mediterránea e por toda Europa, con dous centros de diversidade nos lados opostos do mar Mediterráneo, un na Península Ibérica e Marrocos e outro nas penínsulas dos Balcáns e Anatolia, en consonancia co patrón bioxeográfico de disxunción do Mediterráneo Occidental-Oriental. O xénero atópase nunha serie de condicións ecolóxicas e substratos xeolóxicos nun amplo rango de altitude, incluídos os hábitats costeiros e alpinos. No **Capítulo 1** revísanse todos os nomes publicados de *Jasione* nas especies, subespecies e rangos de variedades para as poboacións das penínsulas dos Balcáns e Anatolia, incluídos os nomes dos taxones afíns a estes grupos nas áreas circundantes. Abórdanse o estado taxonómico e a tipificación dos nomes. En total, desígnanse once lectotipos, un lectoneotipo e un epítipo. Incluíronse os membros do complexo *Jasione orbiculata* e, por tanto, tamén se trata ao representante do complexo no sur de Italia. A revisión do capítulo presenta os basionimos en orde alfabética, estruturados como unha discusión de cada nome seguido dos seus sinónimos e tipificación, designando tipos cando é necesario. Proporciónase unha lista de nomes actualmente aceptados en checklists recentes e indícanse os sinónimos heterotípicos.

Os grupos con estasis morfolóxica son un marco interesante para abordar o posible recoñecemento de especies crípticas escondidas detrás dos tratamentos taxonómicos tradicionais, particularmente cando os rangos de distribución suxiren patróns bioxeográficos disxuntos e

ambientalmente heteroxéneos. Novas hipóteses de delimitación de unidades evolutivas independentes poden conducir á identificación de diferentes patróns bioxeográficos, sentando as bases para investigar o seu significado histórico e ecolóxico. *Jasione* (Campanulaceae) é un xénero de plantas cunha distribución centrada na conca mediterránea caracterizada por unha estasis morfolóxica significativa. *Jasione sessiliflora* s.l. e taxóns afíns teñen rangos de distribución disxuntos dentro da Península Ibérica, ocupando rexións ambientalmente diversas. Sábese que no grupo ocorren polo menos dous niveis de ploidía, diploide e tetraploide. Estes aspectos motivaron a avaliación da variabilidade interna no grupo con ferramentas filoxenéticas para a delimitación de especies. Os resultados das análises GMYC e ASAP compáranse no **Capítulo 2** con outras liñas de evidencia, incluíndo morfoloxía, citoloxía e fenoloxía. O axuste dos patróns de distribución das entidades inferidas ás subprovincias corolóxicas utilízase como marco bioxeográfico e ambiental para probar a hipótese das especies. A pesar da escaseza de caracteres morfolóxicos diagnósticos no grupo, a delimitación filoxenética apoia a descrición de polo menos unha especie críptica, unha endémica estreita no NE da Península Ibérica, e a separación de *J. sessiliflora* dun grupo de poboacións do ambiente termófilo no leste da Península. Aínda que os rangos disxuntos suxeriron a avaliación de grupos de poboación, non todas as poboacións disxuntas apuntaron a historias evolutivas independentes. As diferenzas de ploidía apoian a reordenación sistemática suxerida pola delimitación de especies. A reorganización taxonómica en *J. sessiliflora* s.l. permitiu interpretacións bioxeográficas dos patróns de distribución que están de acordo coa rexionalización bioxeográfica. Estes resultados suxiren que a diferenciación de especies, xunto co illamento xeográfico e a poliploidización asociouse á adaptación a diferentes ambientes, pasando de condicións máis a menos termofílicas durante a historia evolutiva do grupo *J. sessiliflora*. Por tanto, o recoñecemento de entidades evolutivas ocultas é fundamental para interpretar correctamente os patróns bioxeográficos en rexións cunha historia xeolóxica e evolutiva complexa como a conca mediterránea.

Jasione é un xénero taxonómicamente complexo cun patrón de distribución mediterráneo disxunto este-oeste. Actualmente

recoñécense ao redor de 16 especies, aínda que se propuxeron máis de cen nomes taxonómicos. Debe probarse a hipótese de que a intensa estasis morfolóxica oculta a variabilidade evolutiva relevante e dificulta a comprensión correcta das relacións evolutivas no xénero. Aínda que o xénero distribúese en Europa e ao redor da conca mediterránea, ata o de agora non se realizou unha análise filoxenético completo. Os obxectivos do **Capítulo 3** consisten en probar a monofilia do xénero e determinar a súa posición entre outras liñaxes basales á tribo Campanuleae, un tema conflitivo non resolvido en diferentes estudos. A investigación tamén tivo como obxectivo establecer as relacións filoxenéticas internas mediante unha mostraxe exhaustiva que inclúe diferentes poboacións e a maioría dos nomes propostos, para evitar a exclusión *a priori* de liñaxes evolutivas significativas. Tamén se investigaron os roles do contacto secundario e a variación nos niveis de ploidía na configuración da historia evolutiva do xénero. Investigáronse os procesos subxacentes que conducen ao patrón de distribución disxunto do Mediterráneo este-oeste. Para abordar estas preguntas, construíuse unha filoxenia calibrada no tempo de cinco loci con 111 terminais, 93 dos cales correspondían a *Jasione*. O xénero resolveuse como monofilético, dentro dun clado compartido con outras dúas liñaxes illadas, *Hesperocodon* e *Feeria*. Este clado é irmán da tribo Campanuleae. A liñaxe que leva a *Jasione* orixinouse a finais do Oligoceno (c. 25 Mya) e as liñaxes existentes de *Jasione* derivan dun evento de diversificación moito máis tardío, que ocorreu en c. 9 Mya no Mioceno, dun antepasado orixinado no sur da Península Ibérica ou xa presente nas rexións do Mediterráneo occidental e oriental. Pódense identificar dous clados principais, un só presente no Mediterráneo occidental e que engloba especies con morfoloxías moi diferentes e outro co patrón de distribución disxunto existente. Un episodio de vicarianza é compatible coa apertura mesiniense tardía do estreito de Xibraltar, aínda que en ambos os clados inferíronse varios eventos posteriores de dispersión ultramarina desde a Península Ibérica ao norte de África. O segundo clado comezou a diversificarse a principios do Pleistoceno, probablemente na Península Balcánica, e polo menos dous eventos de dispersión de leste a oeste identifícanse neste clado, con modelos de nichos que identifican o conservadurismo de nicho no

grupo oriental e a expansión de nichos no grupo occidental. A idoneidade do hábitat non se identificou na maioría das rexións europeas intermedias. O patrón de distribución disyunto este-oeste en *Jasione* é un proceso bioxeográfico dinámico no que as dúas áreas albergan liñaxes que brindan a oportunidade tanto de permanecer como de dispersarse ás outras áreas, o que aumenta as posibilidades de que a liñaxe perdure. Finalmente, a filoxenia resultante non é consistente coa taxonomía actual que mostra que a estasis morfolóxica en *Jasione* obstaculizou a comprensión sistemática e suxire a necesidade de máis estudos taxonómicos integradores.

Existe unha tendencia xeral ao conservadurismo de nicho entre taxones estreitamente relacionados, aínda que nos complexos poliploides, a diverxencia de nichos pode permitir que os poliploides se establézan fora do rango de distribución dos seus parentes diploides. A poliploidización conduce á aparición inmediata de barreiras evolutivas e especiación. Con todo, os poliploide aparecen dentro das poboacións diploides e, por tanto, están exposto para os efectos da competencia e os procesos dependentes da frecuencia que, en última instancia, conducen á exclusión reprodutiva do citotipo minoritario. A diverxencia ecolóxica permitiría aos poliploides evitar estes procesos, converténdose así nun mecanismo crave na configuración da distribución xeográfica dos citotipos nos complexos poliploides. Con todo, aínda se necesitan probas que apoien esta hipótese, xa que se informaron moitos achados contraditorios. Ademais, queda por determinar se os poliploides diverxen ecoloxicamente en maior grao que os diploides. O estudo de complexos poliploides que abarcan tanto poliploides como taxones diploides estreitamente relacionados proporciona unha oportunidade para comparar o cambio de nicho poliploide ou o conservadurismo en relación co cambio de nicho dentro dun marco evolutivo apropiado. A hipótese do cambio de nicho avalíase no **Capítulo 4** nun sistema diploide-tetraploide de taxones estreitamente relacionados do xénero *Jasione* L. (Campanulaceae) pertencentes ao grupo *Jasione crispa* (Pourr.) Samp. En dous estudos anteriores, o cambio de nicho hipotético noutros grupos poliploides modelo do xénero *Jasione* foi rexeitado ou non foi totalmente apoiado polos resultados, e os rangos de distribución actuais deses complexos

poliploides deben explicarse por outros factores. Aquí, a distribución a escala fina dos citotipos e os grupos taxonómicos significativos evolutivos determinouse mediante reconto de cromosomas, citometría de fluxo en material secado en xel de sílice e marcadores moleculares do xenoma do plastidio. Aplicáronse técnicas de modelado ambiental e desenvolvéronse modelos de distribución de especies para examinar se se produce un cambio de nicho ambiental ou conservadurismo entre tres taxóns diploides e entre estes e un taxón tetraploide presumiblemente descendente. O proceso de modelado utilizou variables climáticas e edáficas, comparando modelos de distribución de especies con e sen variables edáficas. A inclusión de variables edáficas no modelado deste complexo poliploide proporcionou unha descrición máis precisa da realidade ecoxeográfica dos taxones en estudo. Ademais, os modelos demostraron que o grupo tetraploide experimentou un cambio significativo e expansión de nicho en relación cos parentes próximos diploides, nos que se detecta conservadurismo de nicho. A expansión do nicho cara á tolerancia de ambientes máis fríos deu forma á distribución actual de *J. sessiliflora* tetraploide, mentres que o conservadurismo do nicho restrinxe os diploides a áreas comparativamente térmicas e subhúmedas no centro suroeste e mediterráneo da Península Ibérica. O conservadurismo de nichos diploides parece revelar certa inercia residual nos nichos ancestrais da liñaxe. En resumo, e á inversa doutros sistemas modelo de *Jasione*, a poliploidización probablemente causou unha innovación ambiental exitosa en *Jasione sessiliflora*.

A variabilidade edáfica é un factor importante que promove a microevolución nas plantas, o que pode conducir á formación de novas especies a longo prazo. *Jasione crispa* (Campanulaceae) é un grupo composto maioritariamente por orófitos restrinxidos a solos ácidos. No **Capítulo 5**, a identificación dunha poboación illada e en perigo de extinción de *Jasione* no macizo calcáreo do Mont Caro (Tarragona, España) permite o estudo de novas presións ambientais nunha liñaxe acidófilo e os pasos iniciais do camiño de especiación. Esta poboación incluíuse na variabilidade de *J. sessiliflora*, unha especie tetraploide. O nivel de ploidía da poboación determínase como hexaploide, evidenciando relacións máis estreitas con *Jasione crispa* subsp *crispa*.

O fluxo de xenes reducido por illamento xeográfico identifícase mediante procedementos de clonación do marcador ITS do nrDNA, nun marco filoxenético e filoxeográfico que inclúe poboacións de todas as cadeas montañosas ocupadas polo taxón. A interrupción do fluxo de xenes e a exposición a novas limitacións ambientais son os principais promotores da especiación adaptativa. Para avaliar se a poboación de Mont Caro está a experimentar un proceso de especiación ecolóxica, cultiváronse plantas de Mont Caro e de dúas poboacións de zonas montañosas silíceas (Sistema Central e Pireneos) e diferentes proximidades filogenéticas a Mont Caro en tres tipos de solo. As medicións non destrutivas de cor por reflectancia representadas no sistema de cor CIELAB, utilizando os parámetros cromáticos: a* (asociado con cambios no vermello-verde) e b* (asociado con cambios no amarelo-azul), demostraron ser relevantes para avaliar estes diferentes comportamentos, e por tanto, son unha boa variable proxy para os estudos de especiación de plantas neste grupo de modelos. As concentracións de biomasa e Al, Ca, Fe e Mg estimáronse no momento de finalización do experimento. A poboación de Mont Caro mostrou mellores respostas aos solos básicos (é dicir, maior produción de biomasa, baixa concentración de Mg, equilibrio nas concentracións de Ca nas raíces e follas) que as outras dúas poboacións, aínda que peor desempeño en solos ácidos e mal manexo do aluminio a nivel das raíces, o que parece ser unha compensación evolutiva negativa. Estes resultados suxiren a ocorrencia dun proceso de especiación ecolóxica en curso en *J. crispa* de Mont Caro, o que podería contribuír á descrición desta poboación como un novo taxón.

O **Capítulo 6** baséase parcialmente nunha breve publicación froito da colaboración coa administración ambiental de Andalucía. *Jasione corymbosa* Poir. ex Schult., nun sentido amplo, é un polimórfico anual endémico da zona bético- mauritana, cuxo hábitat preferido son as dunas e areas costeiras do sur da Península Ibérica e o Magreb, desde o oeste de Alxeria ata a costa atlántica de Marrocos. Considerouse extinto na Península Ibérica debido á alteración xeneralizada do hábitat nas costas mediterráneas, aínda que aínda se atopaban poboacións existentes nas costas atlántica e mediterránea do noroeste de África. O coñecemento do hábitat das poboacións africanas guiou unha intensa

mostraxe no Mediterráneo andaluz. Finalmente detectouse unha poboación putativa en hábitats de dunas de area en Manilva, Málaga. A súa identificación como *Jasione corymbosa* foi controvertida entre os botánicos andaluces, e resolveuse definitivamente co uso de ferramentas moleculares, que recuperaron as plantas de Manilva aniñadas filogenéticamente entre as poboacións norteafricanas de *Jasione corymbosa*. Por tanto, *Jasione corymbosa* é unha especie da flora da Península Ibérica en grave perigo de extinción. Unha clasificación seguindo a categorización da UICN levou a consideralo baixo a categoría CR (En Perigo Crítico). Finalmente suxírense diferentes medidas de conservación.

Finalmente, o **Capítulo 7** está dedicado á medición da cor do xénero *Jasione*. A cor dos órganos das plantas, en particular das follas e as flores, é un trazo fenotípico utilizado tradicionalmente como indicador visual do estado fisiolóxico da planta. De feito, a tensión ambiental relacionada coa cantidade e calidade da luz, os nutrientes, a temperatura e a seca provocan un cambio de cor da planta. Estas correlacións son tan informativas que a cor é un dos trazos fenotípicos para monitorar o crecemento das plantas en enfoques de clasificación de alto rendemento. Desafortunadamente, a detección e clasificación de cores polo ollo humano son extremadamente pouco confiables debido á súa dependencia da experiencia e a capacidade do observador. A elección dunha cor por parte dun organismo vivo baséase en criterios psicolóxicos e evolutivos ou de supervivencia. Por exemplo, entre as cores que distingue o ollo humano, o verde percíbese con maior facilidade e co maior cambio de matiz que calquera outro cor, debido á percepción combinada de bastóns e conos. Exponse a hipótese de que isto podería representar unha adaptación á contorna dos primates en busca de alimento. A cor pódese describir de forma obxectiva e precisa mediante o uso de espectrofotómetros, colorímetros ou cromómetros que describen a cor reflectida no sistema de cor estandarizada CIELAB. Funcionan por contacto nunha superficie, como outros dispositivos espectrómetros de contacto, e promedian a luz reflectida desde unha abertura na cabeza do dispositivo. A apertura é circular e ten un diámetro de entre 3 mm e 60 mm. As superficies cunha área cunha forma diferente ou máis pequena que a apertura do dispositivo non

reflicten por completo a luz emitida, o que conduce a unha perda constante de luz e a resultados pouco fiables. Para realizar a medición da cor correctamente, a superficie da mostra debe cubrir por completo a apertura do dispositivo para evitar fugas de luz ou interferencias de luz externa. Por esta razón, a cor non se pode medir cando a área obxectiva é menor de 3 mm e / ou non é circular. Para abordar este tema, no Capítulo 7 proponse reducir nun 50% e 70% a área de apertura dun espectrofotómetro portátil con adaptadores de cartón en cores branco e negro, co fin de describir a cor no espazo CIELAB de pequenas mostras biolóxicas, como follas e pétalos. Por tanto, mellórase aínda máis a usabilidade do dispositivo sensor ao estender a súa aplicación a mostras máis pequenas que aquelas para as que foi deseñado. Isto desbloquea formas previamente inexploradas de abordar problemas actuais no campo da vixilancia ambiental, como a resposta da biodiversidade ao cambio climático. Neste marco mídese a cor de follas de especies do xénero *Jasione* e outras da familia Campanulaceae, validando así esta metodoloxía dentro dun amplo grupo filogenético con especies de diferente ecoloxía e frecuentemente con follas de pequeno tamaño. Este grupo engloba unha serie de especies alpinas, con hábito de densas rosetas con follas pequenas, sendo esta unha característica de adaptación vexetal aos ambientes alpinos. Neste capítulo, o uso de follas o suficientemente grandes para ser medidas coa gran apertura do colorímetro garantiu a confiabilidad desta metodoloxía tamén para follas onde só pódese usar a apertura máis pequena da apertura do colorímetro, como é o caso de moitas especies alpinas. Ademais, inclúense mostras de herbario, onde se conservan tecidos vexetais secos como valiosas evidencias ecolóxicas, sistemáticas e históricas da biodiversidade vexetal. Ao estender a presente metodoloxía a mostras secas conservadas, proponse o uso da colorimetría como ferramenta para describir a cor de follas e pétalos despois dun tempo e monitorear as condicións de conservación destes especímenes. Os resultados mostran que, seguindo criterios colorimétricos, a única configuración que proporciona cores indistinguibles segundo a percepción do ollo humano é o uso dun adaptador de redución do 50% na apertura de 3 mm. Ademais, a análise estatística multivariante, desenvolto polo autor da tese, suxire o uso do adaptador branco. O estudo ofrece unha técnica

de medición de son para recompilar información ecolóxica da cor de follas, pétalos e outras pequenas mostras.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Resumen

Esta tesis doctoral tiene como objetivo revelar diferentes aspectos de la realidad evolutiva del género *Jasione* L. (Campanulaceae), un linaje vegetal distribuido por la cuenca mediterránea. Se llevaron a cabo análisis moleculares, citológicos, morfológicos y ambientales para abordar lo siguiente: (i) las relaciones filogenéticas internas en *Jasione*, así como su posición entre otros géneros de Campanulaceae, (ii) la historia biogeográfica de un género con un notable patrón biogeográfico mediterráneo este-oeste, (iii) los procesos que conducen a la especiación y adaptación a nuevos entornos, (iv) el estudio de los cambios de nicho ambiental asociados con cambios en el nivel de ploidía, (v) los métodos utilizados para delimitar entidades crípticas evolutivamente significativas utilizando diferentes líneas de evidencia, y (vi) tipificación de grupos complejos de *Jasione* como un paso en el establecimiento de una nueva organización taxonómica del género basada en relaciones evolutivas, entre otros aspectos.

La familia Campanulaceae es un grupo evolutivo complejo caracterizado por el conflicto entre las clasificaciones tradicionales y las filogenias moleculares generadas en las últimas décadas, conflictos que se agudizan en la subfamilia Campanuloideae. Una de las principales razones de las diferencias entre las clasificaciones premoleculares y los resultados filogenéticos modernos es la escasez de caracteres morfológicos discriminatorios, lo que llevó a un énfasis excesivo en los tratamientos taxonómicos tradicionales a algunos rasgos reproductivos, a saber, las morfologías de flores y frutos. La evolución paralela de los rasgos morfológicos aparentemente ha empañado la sistemática del grupo, dificultando la propuesta de delimitaciones evolutivas significativas entre y dentro de los géneros.

En este contexto general, *Jasione* es uno de los géneros de Campanuloideae más homogéneos desde el punto de vista morfológico.

En consecuencia, ha sido desconcertante con respecto a su sistemática evolutiva. Existe una controversia generalizada entre los autores en cuanto al número de taxones que deben reconocerse, con alrededor de un centenar de diferentes nombres propuestos. Sin embargo, los tratamientos generales actuales solo reconocen entre 12 y 16 especies, aunque este enfoque sintético se ha considerado demasiado simplista y resulta de la dificultad del género. En consecuencia, se ha reivindicado la necesidad de un tratamiento moderno con perspectiva evolutiva. El objetivo de esta tesis doctoral es arrojar luz sobre la realidad evolutiva y la historia biogeográfica de un género distribuido en Europa y en la cuenca mediterránea, utilizando herramientas moleculares, citológicas y ambientales para lograr este objetivo.

El género *Jasione* L. (Campanulaceae) se distribuye por la cuenca mediterránea y por toda Europa, con dos centros de diversidad en los lados opuestos del mar Mediterráneo, uno en la Península Ibérica y Marruecos y otro en las penínsulas de los Balcanes y Anatolia, en consonancia con el patrón biogeográfico de disyunción del Mediterráneo Occidental-Oriental. El género se encuentra en una serie de condiciones ecológicas y sustratos geológicos en un amplio rango de altitud, incluidos los hábitats costeros y alpinos. En el **Capítulo 1** se revisan todos los nombres publicados de *Jasione* en las especies, subespecies y rangos de variedades para las poblaciones de las penínsulas de los Balcanes y Anatolia, incluidos los nombres de los taxones afines a estos grupos en las áreas circundantes. Se abordan el estado taxonómico y la tipificación de los nombres. En total, se designan once lectotipos, un lectoneotipo y un epítipo. Se incluyeron los miembros del complejo *Jasione orbiculata* y, por lo tanto, también se trata al representante del complejo en el sur de Italia. La revisión del capítulo presenta los basionimos en orden alfabético, estructurados como una discusión de cada nombre seguido de sus sinónimos y tipificación, designando tipos cuando es necesario. Se proporciona una lista de nombres actualmente aceptados en checklists recientes y se indican los sinónimos heterotípicos.

Los grupos con estasis morfológica son un marco interesante para abordar el posible reconocimiento de especies crípticas escondidas detrás de los tratamientos taxonómicos tradicionales, particularmente

cuando los rangos de distribución sugieren patrones biogeográficos disyuntos y ambientalmente heterogéneos. Nuevas hipótesis de delimitación de unidades evolutivas independientes pueden conducir a la identificación de diferentes patrones biogeográficos, sentando las bases para investigar su significado histórico y ecológico. *Jasione* (Campanulaceae) es un género de plantas con una distribución centrada en la cuenca mediterránea caracterizada por una estasis morfológica significativa. *Jasione sessiliflora* s.l. y taxones afines tienen rangos de distribución disjuntos dentro de la Península Ibérica, ocupando regiones ambientalmente diversas. Se sabe que en el grupo ocurren al menos dos niveles de ploidía, diploide y tetraploide. Estos aspectos motivaron la evaluación de la variabilidad interna en el grupo con herramientas filogenéticas para la delimitación de especies. Los resultados de los análisis GMYC y ASAP se comparan en el **Capítulo 2** con otras líneas de evidencia, incluyendo morfología, citología y fenología. El ajuste de los patrones de distribución de las entidades inferidas a las subprovincias corológicas se utiliza como marco biogeográfico y ambiental para probar la hipótesis de las especies. A pesar de la escasez de caracteres morfológicos diagnósticos en el grupo, la delimitación filogenética apoya la descripción de al menos una especie críptica, una endémica estrecha en el NE de la Península Ibérica, y la separación de *J. sessiliflora* de un grupo de poblaciones del ambiente termófilo en el este de la Península. Aunque los rangos disyuntos sugirieron la evaluación de grupos de población, no todas las poblaciones disyuntas apuntaron a historias evolutivas independientes. Las diferencias de ploidía apoyan la reordenación sistemática sugerida por la delimitación de especies. La reorganización taxonómica en *J. sessiliflora* s.l. permitió interpretaciones biogeográficas de los patrones de distribución que están de acuerdo con la regionalización biogeográfica. Estos resultados sugieren que la diferenciación de especies, junto con el aislamiento geográfico y la poliploidización se asoció a la adaptación a diferentes ambientes, pasando de condiciones más a menos termofílicas durante la historia evolutiva del grupo *J. sessiliflora*. Por tanto, el reconocimiento de entidades evolutivas ocultas es fundamental para interpretar correctamente los patrones biogeográficos en regiones con

una historia geológica y evolutiva compleja como la cuenca mediterránea.

Jasione es un género taxonómicamente complejo con un patrón de distribución mediterráneo disyunto este-oeste. Actualmente se reconocen alrededor de 16 especies, aunque se han propuesto más de cien nombres taxonómicos. Debe probarse la hipótesis de que la intensa estasis morfológica oculta la variabilidad evolutiva relevante y dificulta la comprensión correcta de las relaciones evolutivas en el género. Aunque el género se distribuye en Europa y alrededor de la cuenca mediterránea, hasta ahora no se ha realizado un análisis filogenético completo. Los objetivos del **Capítulo 3** consisten en probar la monofilia del género y determinar su posición entre otros linajes basales a la tribu *Campanuleae*, un tema conflictivo no resuelto en diferentes estudios. La investigación también tuvo como objetivo establecer las relaciones filogenéticas internas mediante un muestreo exhaustivo que incluye diferentes poblaciones y la mayoría de los nombres propuestos, para evitar la exclusión *a priori* de linajes evolutivos significativos. También se investigaron los roles del contacto secundario y la variación en los niveles de ploidía en la configuración de la historia evolutiva del género. Se investigaron los procesos subyacentes que conducen al patrón de distribución disyunto del Mediterráneo este-oeste. Para abordar estas preguntas, se construyó una filogenia calibrada en el tiempo de cinco loci con 111 terminales, 93 de los cuales correspondían a *Jasione*. El género se resolvió como monofilético, dentro de un clado compartido con otros dos linajes aislados, *Hesperocodon* y *Feeria*. Este clado es hermano de la tribu *Campanuleae*. El linaje que lleva a *Jasione* se originó a finales del Oligoceno (c. 25 Mya) y los linajes existentes de *Jasione* derivan de un evento de diversificación mucho más tardío, que ocurrió en c. 9 Mya en el Mioceno, de un antepasado originado en el sur de la Península Ibérica o ya presente en las regiones del Mediterráneo occidental y oriental. Se pueden identificar dos clados principales, uno solo presente en el Mediterráneo occidental y que engloba especies con morfologías muy diferentes y otro con el patrón de distribución disyunto existente. Un episodio de vicarianza es compatible con la apertura mesiniense tardía del estrecho de Gibraltar, aunque en ambos clados se infirieron varios eventos posteriores de

dispersión ultramarina desde la Península Ibérica al norte de África. El segundo clado comenzó a diversificarse a principios del Pleistoceno, probablemente en la Península Balcánica, y al menos dos eventos de dispersión de este a oeste se identifican en este clado, con modelos de nichos que identifican el conservadurismo de nicho en el grupo oriental y la expansión de nichos en el grupo occidental. La idoneidad del hábitat no se identificó en la mayoría de las regiones europeas intermedias. El patrón de distribución disyunto este-oeste en *Jasione* es un proceso biogeográfico dinámico en el que las dos áreas albergan linajes que brindan la oportunidad tanto de permanecer como de dispersarse a las otras áreas, lo que aumenta las posibilidades de que el linaje perdure. Finalmente, la filogenia resultante no es consistente con la taxonomía actual que muestra que la estasis morfológica en *Jasione* ha obstaculizado la comprensión sistemática y sugiere la necesidad de más estudios taxonómicos integradores.

Existe una tendencia general al conservadurismo de nicho entre taxones estrechamente relacionados, aunque en los complejos poliploides, la divergencia de nichos puede permitir que los poliploides se establezcan fuera del rango de distribución de sus parientes diploides. La poliploidización conduce a la aparición inmediata de barreras evolutivas y especiación. Sin embargo, los poliploide aparecen dentro de las poblaciones diploides y, por lo tanto, están expuesto a los efectos de la competencia y los procesos dependientes de la frecuencia que, en última instancia, conducen a la exclusión reproductiva del citotipo minoritario. La divergencia ecológica permitiría a los poliploides evitar estos procesos, convirtiéndose así en un mecanismo clave en la configuración de la distribución geográfica de los citotipos en los complejos poliploides. Sin embargo, aún se necesitan pruebas que respalden esta hipótesis, ya que se han informado muchos hallazgos contradictorios. Además, queda por determinar si los poliploides divergen ecológicamente en mayor grado que los diploides. El estudio de complejos poliploides que abarcan tanto poliploides como taxones diploides estrechamente relacionados proporciona una oportunidad para comparar el cambio de nicho poliploide o el conservadurismo en relación con el cambio de nicho dentro de un marco evolutivo apropiado. La hipótesis del cambio de nicho se evalúa en el **Capítulo 4**

en un sistema diploide-tetraploide de taxones estrechamente relacionados del género *Jasione* L. (Campanulaceae) pertenecientes al grupo *Jasione crispa* (Pourr.) Samp. En dos estudios anteriores, el cambio de nicho hipotético en otros grupos poliploides modelo del género *Jasione* fue rechazado o no fue totalmente respaldado por los resultados, y los rangos de distribución actuales de esos complejos poliploides deben explicarse por otros factores. Aquí, la distribución a escala fina de los citotipos y los grupos taxonómicos significativos evolutivos se determinó mediante recuento de cromosomas, citometría de flujo en material secado en gel de sílice y marcadores moleculares del genoma del plastidio. Se aplicaron técnicas de modelado ambiental y se desarrollaron modelos de distribución de especies para examinar si se produce un cambio de nicho ambiental o conservadurismo entre tres taxones diploides y entre estos y un taxón tetraploide presumiblemente descendiente. El proceso de modelado utilizó variables climáticas y edáficas, comparando modelos de distribución de especies con y sin variables edáficas. La inclusión de variables edáficas en el modelado de este complejo poliploide proporcionó una descripción más precisa de la realidad ecogeográfica de los taxones en estudio. Además, los modelos demostraron que el grupo tetraploide experimentó un cambio significativo y expansión de nicho en relación con los parientes cercanos diploides, en los que se detecta conservadurismo de nicho. La expansión del nicho hacia la tolerancia de ambientes más fríos ha dado forma a la distribución actual de *J. sessiliflora* tetraploide, mientras que el conservadurismo del nicho restringe los diploides a áreas comparativamente térmicas y subhúmedas en el centro suroeste y mediterráneo de la Península Ibérica. El conservadurismo de nichos diploides parece revelar cierta inercia residual en los nichos ancestrales del linaje. En resumen, y a la inversa de otros sistemas modelo de *Jasione*, la poliploidización probablemente causó una innovación ambiental exitosa en *Jasione sessiliflora*.

La variabilidad edáfica es un factor importante que promueve la microevolución en las plantas, lo que puede conducir a la formación de nuevas especies a largo plazo. *Jasione crispa* (Campanulaceae) es un grupo compuesto mayoritariamente por orófitos restringidos a suelos ácidos. En el **Capítulo 5**, la identificación de una población aislada y

en peligro de extinción de *Jasione* en el macizo calcáreo del Mont Caro (Tarragona, España) permite el estudio de nuevas presiones ambientales en un linaje acidófilo y los pasos iniciales del camino de especiación. Esta población se ha incluido en la variabilidad de *J. sessiliflora*, una especie tetraploide. El nivel de ploidía de la población se determina como hexaploide, evidenciando relaciones más estrechas con *Jasione crispa* subsp *crispa*. El flujo de genes reducido por aislamiento geográfico se identifica mediante procedimientos de clonación del marcador ITS del nrDNA, en un marco filogenético y filogeográfico que incluye poblaciones de todas las cadenas montañosas ocupadas por el taxón. La interrupción del flujo de genes y la exposición a nuevas limitaciones ambientales son los principales promotores de la especiación adaptativa. Para evaluar si la población de Mont Caro está experimentando un proceso de especiación ecológica, se cultivaron plantas de Mont Caro y de dos poblaciones de zonas montañosas silíceas (Sistema Central y Pirineos) y diferentes proximidades filogenéticas a Mont Caro en tres tipos de suelo. Las mediciones no destructivas de color por reflectancia representadas en el sistema de color CIELAB, utilizando los parámetros cromáticos: a* (asociado con cambios en el rojo-verde) y b* (asociado con cambios en el amarillo-azul), demostraron ser relevantes para evaluar estos diferentes comportamientos, y por lo tanto, son una buena variable proxy para los estudios de especiación de plantas en este grupo de modelos. Las concentraciones de biomasa y Al, Ca, Fe y Mg se estimaron en el momento de finalización del experimento. La población de Mont Caro mostró mejores respuestas a los suelos básicos (es decir, mayor producción de biomasa, baja concentración de Mg, equilibrio en las concentraciones de Ca en las raíces y hojas) que las otras dos poblaciones, aunque peor desempeño en suelos ácidos y mal manejo del aluminio a nivel de las raíces, lo que parece ser una compensación evolutiva negativa. Estos resultados sugieren la ocurrencia de un proceso de especiación ecológica en curso en *J. crispa* de Mont Caro, lo que podría contribuir a la descripción de esta población como un nuevo taxón.

El **Capítulo 6** se basa parcialmente en una breve publicación fruto de la colaboración con la administración medioambiental de Andalucía.

Jasione corymbosa Poir. ex Schult., en un sentido amplio, es un polimórfico anual endémico de la zona bético-mauritana, cuyo hábitat preferido son las dunas y arenas costeras del sur de la Península Ibérica y el Magreb, desde el oeste de Argelia hasta la costa atlántica de Marruecos. Se consideró extinto en la Península Ibérica debido a la alteración generalizada del hábitat en las costas mediterráneas, aunque aún se encontraban poblaciones existentes en las costas atlántica y mediterránea del noroeste de África. El conocimiento del hábitat de las poblaciones africanas guió un intenso muestreo en el Mediterráneo andaluz. Finalmente se detectó una población putativa en hábitats de dunas de arena en Manilva, Málaga. Su identificación como *Jasione corymbosa* fue controvertida entre los botánicos andaluces, y se resolvió definitivamente con el uso de herramientas moleculares, que recuperaron las plantas de Manilva anidadas filogenéticamente entre las poblaciones norteafricanas de *Jasione corymbosa*. Por tanto, *Jasione corymbosa* es una especie de la flora de la Península Ibérica en grave peligro de extinción. Una clasificación siguiendo la categorización de la UICN llevó a considerarlo bajo la categoría CR (En Peligro Crítico). Finalmente se sugieren diferentes medidas de conservación.

Finalmente, el **Capítulo 7** está dedicado a la medición del color del género *Jasione*. El color de los órganos de las plantas, en particular de las hojas y las flores, es un rasgo fenotípico utilizado tradicionalmente como indicador visual del estado fisiológico de la planta. De hecho, el estrés ambiental relacionado con la cantidad y calidad de la luz, los nutrientes, la temperatura y la sequía provocan un cambio de color de la planta. Estas correlaciones son tan informativas que el color es uno de los rasgos fenotípicos para monitorizar el crecimiento de las plantas en enfoques de clasificación de alto rendimiento. Desafortunadamente, la detección y clasificación de colores por el ojo humano son extremadamente poco confiables debido a su dependencia de la experiencia y la capacidad del observador. La elección de un color por parte de un organismo vivo se basa en criterios psicológicos y evolutivos o de supervivencia. Por ejemplo, entre los colores que distingue el ojo humano, el verde se percibe con mayor facilidad y con el mayor cambio de matiz que cualquier otro color, debido a la percepción combinada de bastones y conos. Se plantea la hipótesis de

que esto podría representar una adaptación al entorno de los primates en busca de alimento. El color se puede describir de forma objetiva y precisa mediante el uso de espectrofotómetros, colorímetros o cromómetros que describen el color reflejado en el sistema de color estandarizado CIELAB. Funcionan por contacto en una superficie, como otros dispositivos espectrómetros de contacto, y promedian la luz reflejada desde una abertura en la cabeza del dispositivo. La apertura es circular y tiene un diámetro de entre 3 mm y 60 mm. Las superficies con un área con una forma diferente o más pequeña que la apertura del dispositivo no reflejan por completo la luz emitida, lo que conduce a una pérdida constante de luz y a resultados poco fiables. Para realizar la medición del color correctamente, la superficie de la muestra debe cubrir por completo la apertura del dispositivo para evitar fugas de luz o interferencias de luz externa. Por esta razón, el color no se puede medir cuando el área objetivo es menor de 3 mm y / o no es circular. Para abordar este tema, en el Capítulo 7 se propone reducir en un 50% y 70% el área de apertura de un espectrofotómetro portátil con adaptadores de cartón en colores blanco y negro, con el fin de describir el color en el espacio CIELAB de pequeñas muestras biológicas, como hojas y pétalos. Por lo tanto, se mejora aún más la usabilidad del dispositivo sensor al extender su aplicación a muestras más pequeñas que aquellas para las que fue diseñado. Esto desbloquea formas previamente inexploradas de abordar problemas actuales en el campo del monitoreo ambiental, como la respuesta de la biodiversidad al cambio climático. En este marco se mide el color de hojas de especies del género *Jasione* y otras de la familia Campanulaceae, validando así esta metodología dentro de un amplio grupo filogenético con especies de diferente ecología y frecuentemente con hojas de pequeño tamaño. Este grupo engloba una serie de especies alpinas, con hábito de densas rosetas con hojas pequeñas, siendo esta una característica de adaptación vegetal a los ambientes alpinos. En este capítulo, el uso de hojas lo suficientemente grandes para ser medidas con la gran apertura del colorímetro garantizó la confiabilidad de esta metodología también para hojas donde solo se puede usar la apertura más pequeña de la apertura del colorímetro, como es el caso de muchas especies alpinas. Además, se incluyen muestras de herbario, donde se conservan tejidos vegetales

secos como valiosas evidencias ecológicas, sistemáticas e históricas de la biodiversidad vegetal. Al extender la presente metodología a muestras secas conservadas, se propone el uso de la colorimetría como herramienta para describir el color de hojas y pétalos después de un tiempo y monitorear las condiciones de conservación de estos especímenes. Los resultados muestran que, siguiendo criterios colorimétricos, la única configuración que proporciona colores indistinguibles según la percepción del ojo humano es el uso de un adaptador de reducción del 50% en la apertura de 3 mm. Además, el análisis estadístico multivariante, desarrollado por el autor de la tesis, sugiere el uso del adaptador blanco. El estudio ofrece una técnica de medición de sonido para recopilar información ecológica del color de hojas, pétalos y otras pequeñas muestras.

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Summary

This PhD thesis dissertation aims to reveal different aspects of the evolutionary reality of the genus *Jasione* L. (Campanulaceae), a plant lineage distributed around the Mediterranean basin. Molecular, cytological, morphological and environmental analyses were carried out to address the following: (i) the internal phylogenetic relationships in *Jasione*, as well as its position among other genera of the Campanulaceae, (ii) the biogeographical history of a genus with a remarkable east-west Mediterranean biogeographical pattern, (iii) the processes leading to speciation and adaptation to new environments, (iv) the study of environmental niche shifts associated with changes in ploidy level, (v) the methods used to delimit evolutionarily significant cryptic entities by using different lines of evidence, and (vi) typification of complex groups of *Jasione* as a step in establishing a new taxonomic organization of the genus based on evolutionary relationships, among other aspects.

The family Campanulaceae is a complex evolutionary group characterized by conflict between traditional classifications and the molecular phylogenies generated in the last decades, conflicts that exacerbate in the subfamily Campanuloideae. One of the main reasons of the differences between pre-molecular classifications and modern phylogenetic results is the scarcity of discriminatory morphological characters, that led to overly emphasis given in the traditional taxonomic treatments to some reproductive traits, namely the flower and fruit morphologies. Parallel evolution of morphological traits has apparently clouded the systematics of the group, hampering the proposal of evolutionary significant delimitations between and within the genera.

In this general context, *Jasione* is one the genera of the Campanuloideae more homogeneous from the morphological point of view. Consequently, it has been puzzling regarding its evolutionary systematics. There is a general controversy among authors in the number of taxa that must be recognized, with about one hundred of different proposed names. However, current general treatments only recognize between 12 and 16 species, although this synthetic approach has been considered overly simplistic and resulting from the difficulty of the genus. Consequently, the need of a modern treatment with evolutionary perspective has been claimed. It is the aim of this PhD thesis dissertation to cast light on the evolutionary reality and biogeographic history of a genus distributed in Europe and around the Mediterranean basin, using molecular, cytological, and environmental tools to achieve this objective.

The genus *Jasione* L (Campanulaceae) is distributed around the Mediterranean basin and throughout Europe, with two centres of diversity at the opposite sides of the Mediterranean Sea, one in the Iberian Peninsula and Morocco and another in the Balkan and Anatolian peninsulas, consistent with the West-East Mediterranean disjunction biogeographical pattern. The genus occurs in a number of ecological conditions and geological substrates across a wide elevational range, including coastal and alpine habitats. In the **Chapter 1** all the published names of *Jasione* in the species, subspecies and variety ranks for populations from the Balkan and Anatolian peninsulas are reviewed, including names of taxa allied to these groups in surrounding areas. The taxonomic status and typification of the names are addressed. In total, eleven lectotypes, one lectoneotype and one epitype are suggested. The members of the *Jasione orbiculata* complex were included, and the representative of the complex in southern Italy is therefore also treated. The review presents basionyms in alphabetical order, structured as a discussion of each name followed by its synonyms and typification, designating types when required. A list of currently accepted names from recent checklists is provided, and the heterotypic synonyms are indicated.

Groups with morphological stasis are an interesting framework to address the possible recognition of cryptic species hidden behind

traditional taxonomic treatments, particularly when distribution ranges suggest disjunct and environmentally heterogeneous biogeographic patterns. New hypotheses of delimitation of evolutionary independent units can lead to the identification of different biogeographic patterns, laying the foundation to investigate their historical and ecological significance. *Jasione* (Campanulaceae) is a plant genus with a distribution centered in the Mediterranean basin characterized by significant morphological stasis. *Jasione sessiliflora* s.l., and allied taxa have disjunct distribution ranges within the Iberian Peninsula, occupying environmentally diverse regions. At least two ploidy level, diploid and tetraploid, are known to occur in the group. These aspects motivated the assessment of the internal variability in the group with phylogenetic tools for species delimitation. The results from GMYC and ASAP analyses are compared in the **Chapter 2** with other lines of evidence, including morphology, cytology and phenology. The fitting of distribution patterns of the inferred entities to chorological subprovinces is used as a biogeographical and environmental framework to test species hypothesis. Despite the scarcity of diagnostic morphological characters in the group, phylogenetic delimitation supports the description of at least one cryptic species, a narrow endemic in NE Iberian Peninsula, and the separation from *J. sessiliflora* of a group of populations from thermophile environment in eastern Iberia. Although disjunct ranges suggested the assessment of population groups, not all disjunct populations pointed to independent evolutionary histories. Ploidy differences support the systematic rearrangement suggested by species delimitation. Taxonomic reorganization in *J. sessiliflora* s.l. allowed biogeographic interpretations of distribution patterns that are in accordance with biogeographical regionalization. These results suggest that species differentiation, together with geographic isolation and polyploidization was associated to adaptation to different environments, shifting from more to less thermophilic conditions during the evolutionary history of the *J. sessiliflora* group. Thus, the recognition of concealed evolutionary entities is essential to correctly interpret biogeographical patterns in regions with a complex geologic and evolutionary history such as the Mediterranean basin.

Jasione is a taxonomically complex genus with a disjunct east-west Mediterranean distribution pattern. Around 16 species are currently recognized, although more than one hundred taxonomic names have been proposed. The hypothesis that intense morphological stasis is concealing evolutionary relevant variability and hampering correct understanding of the evolutionary relationships in the genus needs to be tested. Although the genus is distributed in Europe and around the Mediterranean basin, no comprehensive phylogenetic analysis has been carried out until now. The aims of the **Chapter 3** were to test the monophyly of the genus and determine its position among other lineages basal to the tribe Campanuleae, an unresolved conflicting topic in different studies. The research also aimed to establish the internal phylogenetic relationships by comprehensive sampling including different populations and most of the proposed names, to prevent a priori exclusion of evolutionary significant lineages. The roles of secondary contact and variation in ploidy levels in shaping the evolutionary history of the genus were also investigated. The underlying processes leading to the disjunct east-west Mediterranean distribution pattern were investigated. To address these questions, a five loci time-calibrated phylogeny was constructed with 111 terminals, 93 of which corresponded to *Jasione*. The genus was resolved as monophyletic, within a clade shared with two other isolated lineages, *Hesperocodon* and *Feeria*. This clade is sister to the *Campanuleae* tribe. The lineage leading to *Jasione* originated in the late Oligocene (c. 25 Mya) and the extant lineages of *Jasione* derive from a much later diversification event, occurring in c. 9 Mya in the Miocene, from an ancestor either originated in the southern Iberian Peninsula or already present in the western and eastern Mediterranean regions. Two main clades can be identified, one only present in the western Mediterranean and encompassing species with very different morphologies and another with the extant disjunct distribution pattern. One episode of vicariance is compatible with the late Messinian aperture of the Gibraltar strait, although several posterior overseas dispersal events from the Iberian Peninsula to North Africa were inferred in both clades. The second clade began to diversify at the beginning of the Pleistocene, probably in the Balkan Peninsula, and at least two east to west dispersal

events are identified in this clade, with niche modelling identifying niche conservatism in the eastern group and niche expansion in the western group. Habitat suitability was not identified in most of the intermediate European regions. The disjunct east-west distribution pattern in *Jasione* is a dynamic biogeographic process in which the two areas harbour lineages providing the opportunity both to remain and to disperse to the other areas, increasing the chances of the lineage to endure. Finally, the resulting phylogeny is not consistent with current taxonomy showing that morphological stasis in *Jasione* has hampered the systematic understanding and suggesting the need for further integrative taxonomic studies.

There is a general tendency for niche conservatism among closely related taxa, although in polyploid complexes niche divergence may enable polyploids to become established outside the range of distribution of their diploid relatives. Polyploidization leads to the immediate emergence of evolutionary barriers and speciation. However, the new polyploid appears within diploid populations and is thus exposed to the effects of competition and frequency-dependent processes that ultimately lead to reproductive exclusion of the minority cytotype. Ecological divergence would enable polyploids to avoid these processes, thus becoming a key mechanism in shaping geographical distribution of cytotypes in polyploid complexes. However, evidence supporting this hypothesis is still needed, as many contradictory findings have been reported. Moreover, it remains to be determined whether polyploids diverge ecologically to a greater degree than diploids. Study of polyploid complexes encompassing both polyploids and closely related diploid taxa provides an opportunity to compare polyploid niche shift or conservatism in relation to niche shift within an appropriate evolutionary framework. The niche shift hypothesis is evaluated in the **Chapter 4** in a diploid-tetraploid system of closely related taxa of the genus *Jasione* L. (Campanulaceae) belonging to the *Jasione crispa* (Pourr.) Samp group. In two previous studies, the hypothesized niche shift in other model polyploid groups of the genus *Jasione* was either rejected or not fully supported by the results, and current distribution ranges of those polyploid complexes must be explained by other factors. Here, the fine scale distribution of cytotypes

and evolutionary significant taxonomic groups was determined by chromosome counting, flow-cytometry on silica-gel dried material and molecular markers of the plastid genome. Environmental modelling techniques were applied and species distribution models were developed to examine whether environmental niche shift or conservatism occurs between three diploid taxa and between these and a presumably descendent tetraploid taxon. The modelling process used climatic and edaphic variables, comparing species distribution models with and without edaphic variables. The inclusion of edaphic variables in modelling this polyploid complex provided a more accurate description of the ecogeographic reality of the taxa under study. In addition, the models demonstrated that the tetraploid group underwent a significant change and niche expansion relative to the diploid close relatives, in which niche conservatism is detected. Niche expansion towards tolerance of colder environments has shaped the current distribution of tetraploid *J. sessiliflora* while niche conservatism restricts the diploids to comparatively thermic and sub-humid areas in the central south-western and Mediterranean Iberian Peninsula. Diploid niche conservatism seems to reveal some residual inertia in the ancestral niches of the lineage. In summary, and conversely to other *Jasione* model systems, polyploidization probably caused successful environmental innovation in *Jasione sessiliflora*.

Soil variability is an important factor promoting microevolution in plants that can lead to the formation of new species in the long term. *Jasione crispa* (Campanulaceae) is a group of cespitose, mostly orophytes, practically restricted to acid soils. In the **Chapter 5**, the identification of an endangered and isolated population of *Jasione* in the calcareous massif of Mont Caro (Tarragona, Spain) allows the study of new environmental pressures in an acidophile lineage and the initial steps of the speciation path. This population has been included in the variability *J. sessiliflora*, a tetraploid species. The ploidy level of the population is determined as hexaploid, evincing closer relationships with *Jasione crispa* subsp *crispa*. Reduced gene flow by geographic isolation is identified by cloning procedures of the ITS marker from the nrDNA, in a phylogenetic and phylogeographic framework including populations from all mountain ranges occupied by the taxon. Gene flow

interruption and exposure to new environmental constraints are main promoters of adaptive speciation. To assess whether Mont Caro population is undergoing a process of ecological speciation, plants from Mont Caro and from two populations from siliceous mountain areas (Central System and Pyrenees) and different phylogenetic proximity to Mont Caro were cultivated on three soil types. The non-destructive reflectance colour measurements, using the chromatic parameters: a* (associated with changes in redness-greenness) and b* (associated with changes in yellowness-blueness) from the CIELAB colour system, proved relevant for evaluating these different behaviours and, thereby are a good proxy variable for plant speciation studies in this model group. Biomass and Al, Ca, Fe and Mg concentrations were estimated at harvest. Mont Caro population showed better responses to basic soils (i.e., higher biomass production, low Mg concentration, equilibrium in root and leaf Ca concentrations) than the other two populations although worse performance on acidic soils and poor aluminium management at root level, what seems to be a negative evolutionary trade-off. These results suggest the occurrence of an on-ongoing ecological speciation process in *J. crispa* from Mont Caro, which could contribute to the description of this population as a new taxon.

Chapter 6 is partially based on a short publication that resulted from the collaboration with the environmental administration of Andalucia. *Jasione corymbosa* Poir. ex Schult., interpreted in a broad sense, is an annual polymorphic endemic to the Betic-Mauritanian area, whose preferred habitat are dunes and coastal sands of southern the Iberian Peninsula and the Maghreb, from the western Algeria up to the Atlantic coast of Morocco. It was considered extinct in the Iberian Peninsula due to generalized habitat alteration in the Mediterranean coasts, although extant populations were still found in the Atlantic and Mediterranean coasts of north-western Africa. Habitat knowledge of the African populations guided an intense sampling in the Andalusian Mediterranean. A putative population was finally detected in sand dune habitats in Manilva, Malaga. Its identification as *Jasione corymbosa* was controversial among the Andalusian botanist, and issue definitely solved with the use of molecular tools, that recovered the plants from Manilva phylogenetically nested among North-African populations of

Jasione corymbosa. Therefore, *Jasione corymbosa* is one severely endangered species of the flora of the Iberian Peninsula. A classification following the IUCN categorization led to consider it under the CR (Critically Endangered) category. Different measures of conservation are finally suggested.

Finally, the **Chapter 7** is dedicated to the colour measurement using the genus *Jasione* as model group. The colour of plant organs, particularly of leaves and flowers, is a phenotypic trait traditionally used as a visual indicator of the plant physiological status. Indeed, environmental stress regarding light quantity and quality, nutrients, temperature and drought result in a change of plant colour. These correlations are so informative that colour is one of the phenotypic traits for monitoring plant growth in high-throughput classification approaches. Unfortunately, colour detection and classification by human eye are extremely unreliable, because of their dependency from the experience and the capacity of the observer. The choice of a colour by a living organism is based on psychological and evolutionary or survival criteria. For example, among the colours distinguished by the human eye, green is perceived more readily and with the largest shift in their hue than any other colour, because of the combined perception of rods and cones. It is hypothesized that this could represent an adaptation to the environment of primates in search for food. The colour can be described in an objective and precise way through the use of spectrophotometers, colorimeters or chroma-meters that describe the reflected colour in the standardized CIELAB colour system. They work by contact on a surface, like other contact-type spectrometer devices, and they average the light reflected from an aperture on the head of the device. The aperture is circular and has a diameter between 3 mm and 60 mm. Surfaces with an area in a different shape or smaller than the aperture of the device do not reflect entirely the emitted light, thus leading to a consistent loss of light and to unreliable results. To perform the colour measurement correctly, the sample surface must cover entirely the aperture of the device to prevent leakage of light or interference from external light. For this reason, the colour cannot be measured when the target area is smaller than 3 mm and/or not circular. To address this issue, in the **Chapter 7**

it is proposed to reduce by 50% and 70% the aperture area of a portable spectrophotometer with cardboard adaptors in white and black colours, in order to describe the colour in the CIELAB space of small biological samples, such as leaves and petals. Thus, the current author further enhances the usability of the sensor device by extending its application to smaller samples than those for which it was designed. This unlocks previously unexplored ways to tackle current issues in the environmental monitoring field, such as the biodiversity response to climate change. In this frame, the colour of leaves from species of the genus of *Jasione* and other of the Campanulaceae family is measured, thus validating this methodology within a broad phylogenetic group with species of different ecology and frequently with small-sized leaves. This group encompasses a number of alpine species, with a habit of dense rosettes with small leaves, being this a characteristic plant adaptation to alpine environments. In this chapter, the use of leaves big enough to be measured with the large colorimeter aperture guaranteed the reliability of this methodology also for leaves where only the smallest aperture of the colorimeter aperture can be used, as is the case of many the alpine species. In addition, herbarium samples, where dry plant tissues are conserved as valuable ecological, systematic and historical evidences of plant biodiversity, are included. By extending the present methodology to conserved dry samples, it is proposed the use of colorimetry as a tool to describe the colour of leaves and petals after some time and monitoring the conservation conditions of these specimens. The results show that, following colorimetric criteria, the only setting providing indistinguishable colours according to the perception of the human eye is the use of a 50%-reducing adaptor on the 3-mm aperture. In addition, statistical analysis suggests the use of the white adaptor. The study offers a sound measurement technique to gather ecological information from the colour of leaves, petals and other small samples.

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General Introduction: General description of the genus *Jasione*

The family Campanulaceae is a complex evolutionary group characterized by conflict between traditional classifications and the molecular phylogenies generated in the last few decades. This conflict is exacerbated in the subfamily Campanuloideae, and e.g. various studies have consistently recovered a polyphyletic genus, *Campanula* L.(Eddie *et al.*, 2003; Lammers, 2007a; Roquet *et al.*, 2008; Crowl *et al.*, 2016; Xu & Hong, 2021). One of the main reasons for the differences between pre-molecular classifications and modern phylogenetic results is the scarcity of discriminatory morphological characters, which has led to overemphasis being given in traditional taxonomic treatments to some reproductive traits, namely the flower and fruit morphologies (Roquet *et al.*, 2008; Haberle *et al.*, 2009). Parallel evolution of morphological traits has apparently clouded the systematics of the group, hampering the proposal of evolutionary significant delimitations between and within the genera.

In this general context, *Jasione* is one the most homogeneous genera of the Campanuloideae from the morphological point of view. The difficulties regarding the evolutionary systematics of this genus are therefore puzzling (Kovanda, 1968; Sales & Hedge, 2001a; Pérez-Espona *et al.*, 2005). There is some general controversy among authors regarding the number of taxa that must be recognized, and about a hundred different names have been proposed (Schmeja, 1931; Kovanda, 1968). However, current general treatments only recognize between 12 and 16 species (Sales & Hedge, 2001a, Greuter *et al*., 1984; Lammers, 2007b; Castroviejo *et al.*, 2010), although this approach has been considered overly simplistic and resulting from the difficulty in identifying the various taxa (Tutin, 1973; Sales & Hedge, 2001a).

Consequently, the need for a modern approach with an evolutionary perspective has been pointed out (Kovanda, 1968, Sales & Hedge, 2001b; Pérez-Espona *et al.*, 2005). It is the aim of this work to cast some light on the evolutionary reality and biogeographical history of a genus distributed in Europe and around the Mediterranean basin, using molecular, cytological and environmental tools to achieve this objective. The following brief synopsis of the genus incorporates information from the studies detailed in the chapters of the Results section together with other information gathered during the research reported in this thesis.

1. THE GENUS *JASIONE* L.

1.1 Systematic position

Jasione montana L. was originally described by Linnaeus (1753) to classify a single species and is therefore the type species of the genus. The first attempt to classify the genus *Jasione* within the subdivisions of the Campanulaceae was carried out by de Candolle (1830), who included *Jasione* in an unnamed subtribe, later identified with the name Wahlenbergieae Endl. (Endlicher, 1838). The other genera grouped in the Wahlenbergeae mostly had Asian distribution ranges (e.g. *Codonopsis* Wall., *Platycodon* A. DC.) or southern hemisphere distributions ranges (e.g. *Wahlenbergia* W. Roth., *Canarina* L., *Prismatocarpus* L'Her.), which made *Jasione* quite unusual in the group, given its European and circum-Mediterranean distribution. De Candolle (1930) also created the other subtribe, identified as Campanuleae Dumort, which mainly encompasses genera with northern hemisphere or Mediterranean distribution ranges and is therefore, similar to *Jasione* in this respect (e.g. *Trachelium* L. *Phyteuma* L, *Campanula* L.). However, de Candolle's subtribal division was based on the mode of capsule dehiscence, which in *Jasione* and the members of the Wahlenbergieae is loculicidal, by apical valves, and in the Campanuleae is poricidal, by lateral pores. Schönland (1889-1894) addressed the internal classification of the family by creating three subtribes and relocating some of the genera of de Candolle's

Wahlenbergeae in the subtribe now called Campanulinae and a new subtribe Platycodinae. Nevertheless, *Jasione* was left in the Wahlenberginae. Schöndland's division was based on aspects like calyx morphology and, again, capsule dehiscence. Fedorov (1957) considered that *Jasione* was distinct enough to be separated in its own tribe, creating the tribe *Jasione*ae Fed. The characters that justified this new classification included the flowers reunited in dense globose heads, the corolla dissected nearly to the base, the anthers connate at base, and once again, the capsule dehiscence by two apical valves (Fedorov, 1957). The first molecular phylogenetic approaches published in the new millennium would later dramatically alter the systematic view of the family, with many genera being recovered as polyphyletic or paraphyletic (Eddie et al., 2003; Roquet et al., 2008). Regarding *Jasione*, the traditional classification within the Wahlenbergieae lost support, although its position remained unresolved, and it was suggested that *Jasione* may form part of a so-called "transitional" group between the Wahlenbergioid and the Campanuloid taxa (Eddie et al., 2003). Cosner *et al.* (2004) studied the chloroplast DNA rearrangements in the family and identified a greater number of affinities between *Jasione* and the Campanuloids than with other groups. These authors also found that the chloroplast genome of *Jasione* was the most heavily rearranged among all the genera studied. The topological position of *Jasione* and the different phylogenetic hypotheses regarding its most closely related lineages have been controversial topics in the different phylogenetic works on the Campanulaceae published in the last decades (Haberle et al., 2009; Roquet et al. 2009; Mansion et al., 2012, Olesen et al., 2012; Crowl et al., 2016; Xu & Hong, 2021). The main conflicting points in these studies are reviewed in detail in the Results section (Chapter 3). The phylogenetic analysis conducted in the research reported in this thesis contributes to resolving the different issues that have affected the topological position of *Jasione* and its closest lineages. In summary, the phylogenetic location of *Jasione* is consistently established in a clade with two monospecific lineages, *Hesperocodon* Eddie & Cupido and *Feeria* Buser. *Hesperocodon* has traditionally been considered a member of the Wahlenbergioids, under the name *Wahlenbergia*

hederacea (L.) Rchb, while Feeria has been considered a member of the Campanuleae, close to *Trachelium* (Eddie & Cupido, 2014). This clade would be sister to a clade grouping the remaining Campanuleae, which would also include some of the previously called "transitional" groups. The *Jasione* and allied genera clade, and the Campanuleae clade would, in turn, form a sister clade of the Wahlenbergioids. Morphological traits with long lasting systematic importance in the Campanulaceae conserve some evolutionary significance in the light of the present results, as the type of capsule dehiscence in *Jasione*, *Feeria* and *Hesperocodon* is apical valvate, which is quite similar to that in the Wahlebergioids (Eddie & Cupido, 2014), although this cannot be used to support a natural group. All of these results emphasize the singularity of *Jasione* and its allied genera *Feeria* and *Hesperocodon*. Among these genera, *Jasione* was the only one that was able to diversify and produce a number of extant lineages. Nevertheless, the internal relationships and taxonomical organization of these lineages is full of complexities and far from being understood, until now.

1.2. General morphology of *Jasione*

Members of the genus *Jasione* are annual, biannual or perennial plants with leafy stems and undivided leaves, ranging from glabrous to densely hairy. The stems are decumbent or erect, branched or not. Some species can develop thin, whitish, subterranean stolons (e.g. *J. laevis* lam.). The leaves are frequently spathulate or lanceolate, sometimes ovate or obovate, normally narrow, sessile or with a petiole-like narrowing at the base, alternate in the stem, in some cases forming sterile rosettes. The leaf margin ranges from entire to crenate, sometimes toothed. Leaf margins have hydathodes, which in one of the two main clades of the genus are modified to become remarkable tubular structures with subapical and submarginal locations (see below). The genus is well characterized by its inflorescence, a capitulum-like globose glomerule of small flowers subtended by a crown of involucral bracts arranged in one to four series. The bract shape can be lanceolate, ovate, obovate, rhomboidal or orbiculate, with entire, crenate or toothed serrate margins. Bract teeth can be aristate

(e.g. *Jasione laevis* Lam.), and in some cases the teeth and the bract apex end in a hardened awn (e.g. *J. heldreichii* Boiss & Orph.). The glomerule is normally pedunculate, although in individuals of some species the leaves can reach the inflorescence (e.g. *Jasione crispa* (Pourr.) Samp.). The flowers can be sessile (as in *J. sessiliflora* Boiss. & Reut.) or more frequently pedicellate. The calyx has a turbinate-ovoid calyx tube and five triangular, linear or subulate calyx teeth. The calyx teeth can be glabrous, ciliate or woolly-hairy. The corolla is divided into five linear linear-lanceolate lobes nearly to the base and therefore does not form a tube. The corollas are blue or lilac, or more rarely white, with whitish colours more frequently found in taxa from high mountain or coastal dune habitats, although an important variation in flower colour is always observed within the populations. Nevertheless, *Jasione mansanetiana* Roselló & Peris consistently has white corollas, sometimes with creamy or lilac shades. Flowering is centripetal, as in the Asteraceae family. Thus, the peripheral flowers mature first with the opening sequence proceeding towards the centre. The stamens have free filaments but a connate anther at base, forming a narrow ring, with one species in the genus having free anthers, i.e. *J. bulgarica* Stoj. & Stef. The ovary is turbinate-ovoid, with one style longer than the lobed corolla and two short stigmas. The upper half of the style is hairy, with hairs in ten longitudinal rows, to facilitate secondary pollen presentation, once the style has passed through the ring formed by the anthers. (Erbar & Leins, 1989). Pollen bluish, pink or purple, very homogeneous in shape among all the species, 3 (4) porate, ranging from prolate-spheroidal to oblate-spheroidal, with microechinate ornamentation (Figure I.1), varying in size between c. 20 and 25 μm (Çelemli, 2020; M. Serrano, unpublished data). The capsule is ovoid, flattened above, bilocular, with a loculicidal mechanism of dehiscence occurring via two small apical valves, located above the insertion of calyx lobes. Seeds are variable in number, small, shiny, brown, with striate coat ornamentation, oblong-elongate, sometimes sub-orbicular, as in some individuals of *Jasione corymbosa* Poir. A caruncle is present in seeds of most populations of *Jasione crispa* subs. *mariana* (Willk.) Rivas Mart. and more rarely in some populations of *Jasione sessiliflora*.

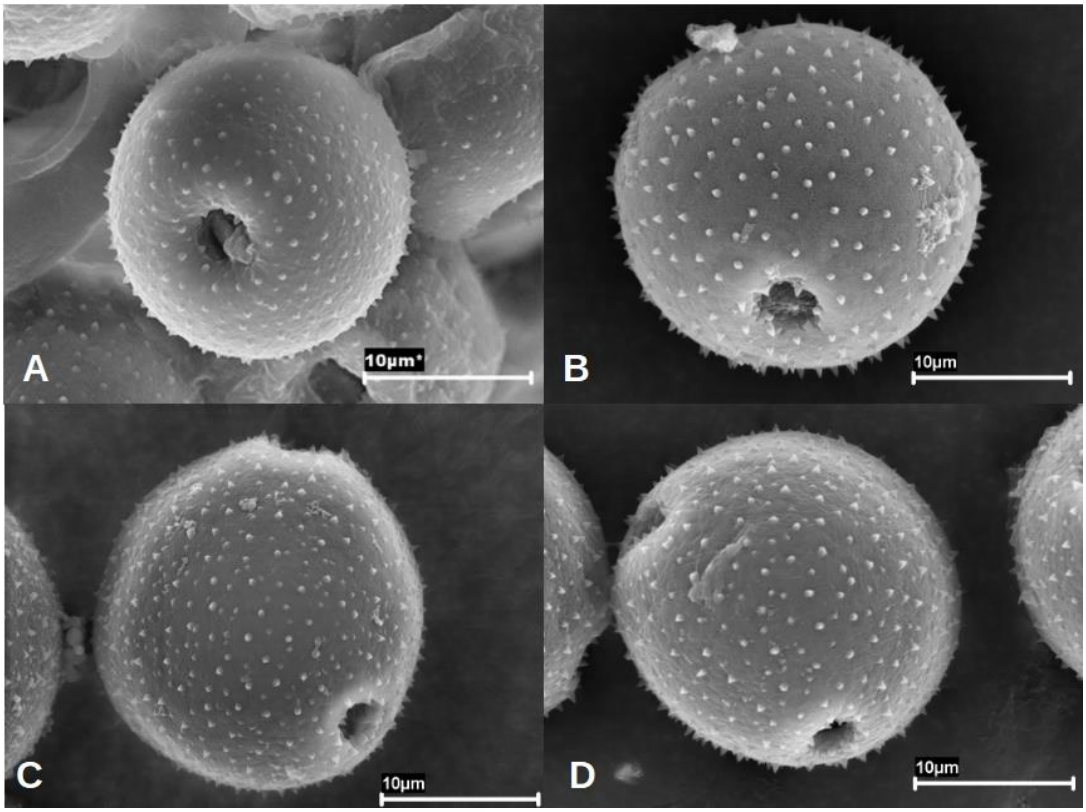

Figure I.1. SEM photomicrographs of pollen grains of *Jasione* species in the four main clades of the genus, showing morphological homogeneity: (A) *Jasione tmolea* Stoj. from Bozdağ, Turkey, (B) *Jasione foliosa* Cav. subsp. *xauenensis* Dobignard from Jbel Tissouka, Morocco, (C) *Jasione crispa* subsp. *mesatlantica* (Emb. & Maire) Dobignard from Jbel Bou Naceur, Morocco, (D) *Jasione blepharodon* Boiss & Reut. from Grazalema, Spain.

1.3. Anatomy

Bokhari & Sales (2001) were the first authors to conduct a relatively extensive anatomical study of the genus, although they focused on the Iberian taxa. Their findings were consistent with the taxonomic treatment reported by Sales & Hedge (2001a) in Flora

Iberica, supporting some disagreements with Tutin (1976), such as the recognition at the species level of *Jasione cavanillesii* C. Vicioso or *Jasione sessiliflora*. (Bokhari & Sales, 2001). These authors identified some anatomical traits that characterized some of the taxa reported by Sales & Hedge (2001a). The most important were epidermal cell shape, differences in adaxial and abaxial epidermal cell sizes, venation of the proximal part of the leaf, presence of stem sclereids in the pith, presence of brachysclereids in the cork of old wood, differentiated or undifferentiated mesophyll and relative distribution of the stomata between abaxial and adaxial surfaces. However, many traits showed variations within the same taxon (e.g. epidermal cell shape or stem sclerification) related to the age of the individual or environmental conditions. Other characters that the authors considered stable and enabled differentiation of species, such as the absence of adaxial stomata in *Jasione laevis*, are not supported by extensive research by the current author in *J. laevis* stomata across all ploidy levels (M. Serrano, unpublished data). On completing their study, Bokhari & Sales (2001) conceded the limited usefulness of the anatomical characters for establishing relationships among taxa, or even distinguishing taxa within species. Some of their conclusions are not supported by the phylogenetic results of this thesis. No anatomical differences were found among varieties of *Jasione montana*, although the varieties encompassed species of distant lineages, such as *Jasione montana* s.str. and *Jasione blepharodon* (sub *Jasione montana* var. *bracteosa* Willk.). Similarly, Bokhari & Sales (2001) found no differences between what they considered populations of *Jasione maritima* (Duby) Merino from France and the Iberian Peninsula, a species distinguished by having brachysclereids in the cork of old wood and two layers of palisade tissue on abaxial and adaxial leaf sides. However, the research findings reported in the present thesis show that the aforementioned authors dealt with at three differentiated evolutionary entities, with three ploidy levels (diploid, tetraploid and hexaploidy) and at least partly different origins, suggesting that anatomical similarities were probably related to the environmental constraints of the maritime dune habitat of these populations. There is, however, one anatomical character whose distribution among the species of the genus is correlated with the main

phylogenetic divisions of *Jasione*, i.e. the modified hydathodes. The adaxial side of the leaves of some species of *Jasione* bears remarkable tubular structures (Figure 2), which have been called "trichoids" (Bokhari & Sales, 2001; Sales & Hedge, 2001a; Pérez-Espona *et al.*, 2005).

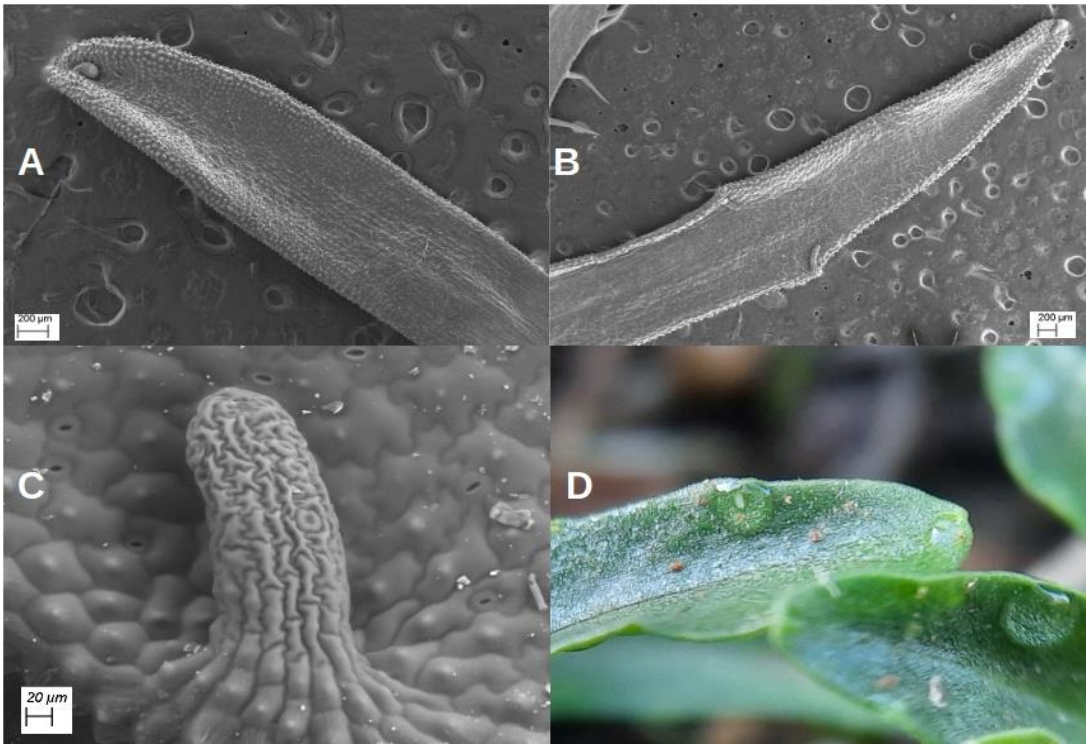

Figure I.2. Modified hydathodes of *Jasione* sect. *Jasione*. SEM photomicrographs of the adaxial leaf surface of (A) *Jasione crispa* subs. tomentosa (A.DC) Rivas Mart. from Mora, Spain and (B) *Jasione* sp. nova from Maçanet de Cabrenys, Spain, showing apical and apical and marginal modified hydathodes, respectively. Scale bars = 10 µm. (C) ESEM photomicrograph of a modified hydathode of *Jasione* carpetana Boiss & Reut. from Miraflores de la Sierra, Spain showing stomata-like pores on the hydathode surface. Scale bar = 20 µm. (D) Fully functional apical and marginal modified hydathodes secreting water by guttation in cultivated *Jasione* carpetana in early morning exterior conditions.

Bokhari & Sales (2001) considered that the function of the "trichoids" was not properly understood, and detailed studies were needed. These authors ruled out the possibility that the "trichoids" could

be hydathodes as they did not find any structural resemblance. Nevertheless, the study of cultivated individuals of different species during the research reported in this thesis revealed that the structures are fully functional hydathodes, allowing guttation, i.e. the release of water in conditions of soil saturation of water and low transpiration conditions (Grundwald *et al*., 2003) such as under high atmospheric humidity or darkness (Figure 2, D). Anatomical analysis showed that the modified hydathodes are unpigmented multicellular structures of length 100-200 μm, vascularized and connected by tracheids to the xylematic system and with several with stomata-like pores on the surface (Figures 2 and 3). Examination of semi-thin sections revealed that the internal structure is similar to the epithem, i.e. thin-walled cells with abundant intercellular spaces in ordinary hydathodes (Figure 3). Transmission electron microscopy images (Figure 4) show that the epithem has vacuolated parenchyma cells with plastids joined to the outer membrane, large intercellular spaces, xylem vessels with tracheids and granules probably containing reserve material, therefore being internally similar to the hydathodes of other plant families (Cerutti *et al*., 2017). In *Jasione* and the Campanulaceae, the reserve material is the polysacharid inulin and not starch as found in most plants (Schmeja, 1931). The epithem is connected to the xylem vascularization of the plant, as vessels and tracheids can be observed (Figure 3). The Campanulaceae is distinguished by presence of laticifers (i.e. latex-containing vessels) in their tissues (Kovanda, 1978). In *Jasione*, the stem and leaves have anastomosed laticifers (M. Serrano, unpublished data), and leaf latex vessels seem to penetrate the modified hydathodes (Figure 4, C and D). The current author agrees with Bokhari & Sales (2001) that the purpose of this specialized structure is far from being understood, and studies addressed to reveal its functionality and evolutionary role in the genus are of major interest. Nevertheless, the name "trichoid" does not seem appropriate, as structurally and functionally the structures are homologous to the ordinary and relatively inconspicuous hydathodes of some species of *Jasione* and all remaining members of the Campanulaceae. Thus, until the function of the structure is fully understood, the name "hydathoids" is suggested for these modified hydathodes.

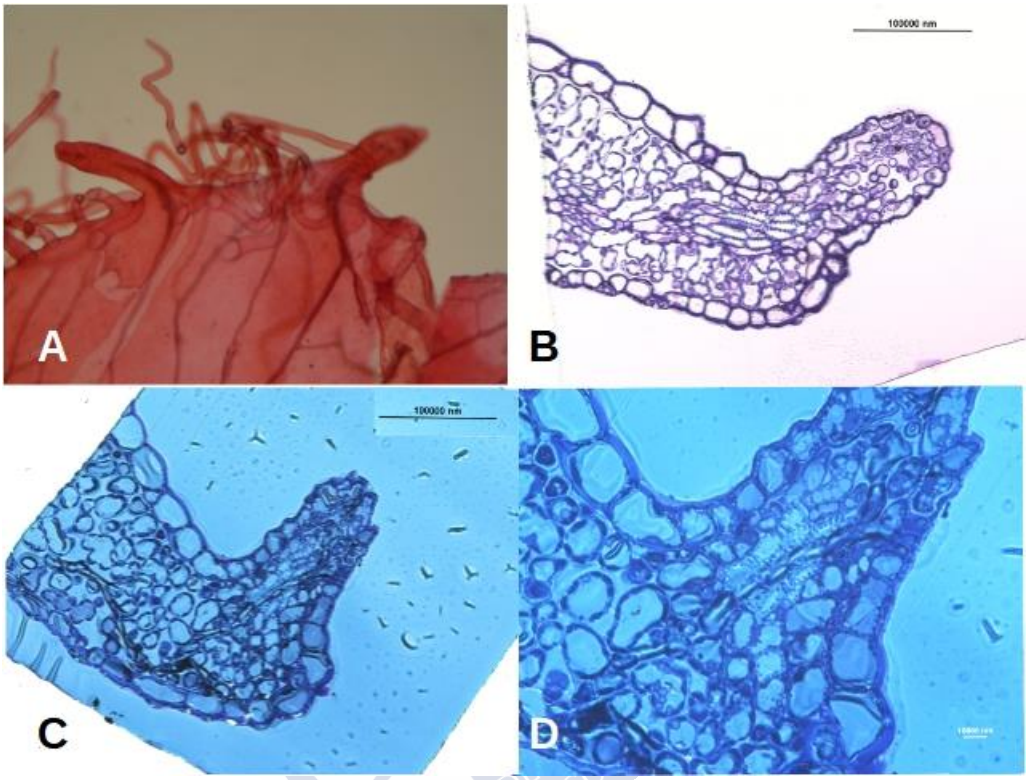

Figure I.3. "Hydathoids" of *Jasione* showing vascularization and epithem organization: (A) Light microscope (x40) photograph of a safranin-stained involucral bract of *Jasione* gr. cripa from Cardaño de Arriba, Spain. (B,C and D) Toluidine blue stained semi-thin sections of hydathoids and the adjacent leaf area of *Jasione* carpetana from Miraflores de la Sierra, Spain. Epithem and tracheid vascularisation can be seen in C, D and D, and apparent laticifers in C, with more detail in D. Scale bars = 100,000 nm in B and C, 10,000 nm in D.

Interestingly, the hydathoids are tubular structures with subapical and submarginal locations, always oriented inwards to the centre of the leaf. Ordinary hydathodes are oriented outwards to facilitate elimination of water from the plant by guttation. The guttation mechanism may only be one of the functions of hydathoids, and several complementary hypotheses should be tested. Preliminary staining experiments showed that fluids can penetrate the leaf through the hydathoids, with internal redistribution being tracked to nearby leaves (M. Serrano, unpublished data). Hydathoids may be associated with mechanisms of leaf water

uptake from dew or fog precipitation. However, observation of water uptake through hydathoids does not necessarily imply that it the uptake is important enough to affect the internal hydric balance of the plants. Robust experimental tests must be conducted to ascertain the physiological importance of foliar water uptake through hydathoids and whether these structures confer any adaptive evolutionary advantage, by comparing the performance of *Jasione* lineages with and without hydathoids. Other possible functions include scent emission by release of volatile secondary compounds or formation of part of the different immune layers of the plant by release of protective compounds by guttation, as identified in hydathodes of other plant families (Cerutti *et al.*, 2017). Hydathoids are only found in one of the two main clades of the genus (sect. *Jasione*), and they are completely absent in the other clade (sect. *Phyteumopsis*). They are present in leaves and involucral bracts and the number may vary. In most taxa of sect. *Jasione* there is one apical and between three and four marginal pairs of hydathoids. However, in some Iberian and North African groups, the hydathoid number is reduced, and only the apical hydathoid is found in some taxa. Species from more mesic environments tend to have more hydathoids on average than taxa from drier or chasmophytic habitats, and the reduction in number may therefore be related to control of water loss.

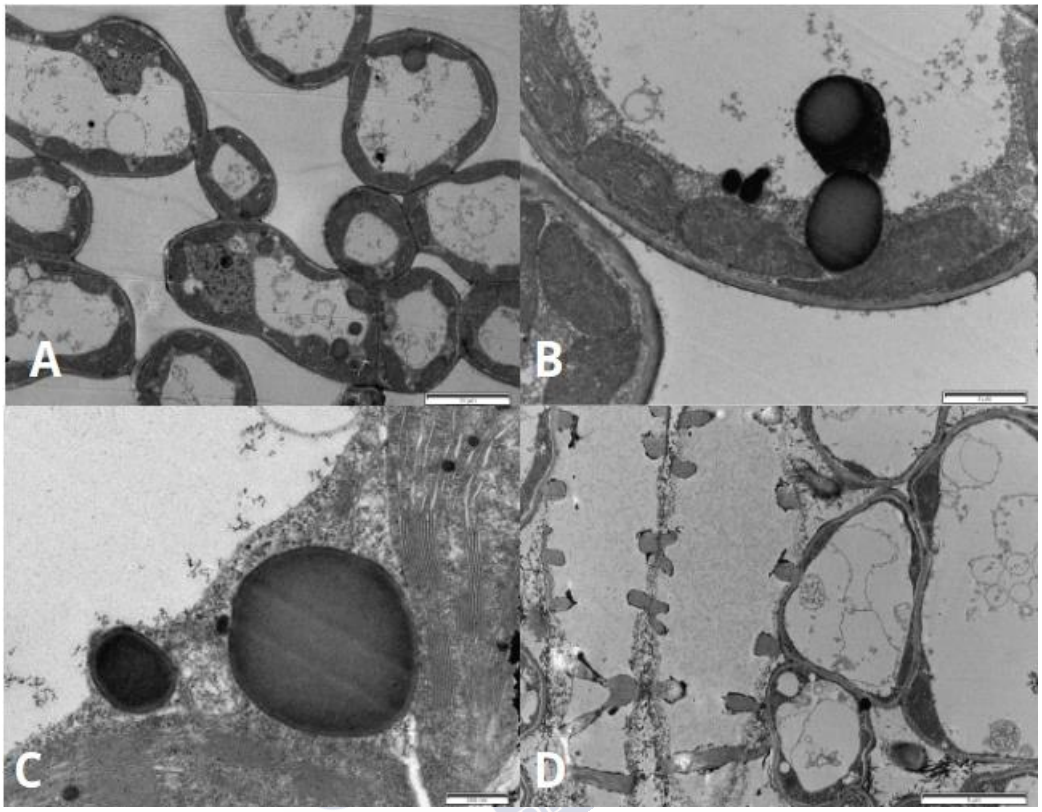

Figure I.4. TEM photomicrograph of an ultrathin section of the epithem below the epidermal layer of *Jasione* carpetana from Miraflores de la Sierra, Madrid. (A, B and C) Large intercellular spaces and vacuolated cells with plastids joined to the outer membrane and reserve (probably inuline) granules. (D) Xylem vessels tracheids showing annular thickenings. Scale bars = 10 µm in A, 2 µm in B, 500 nm in C and 5 µm in D.

1.4. Chromosome number and ploidy level

Changes in chromosome number and ploidy have shaped the evolutionary history of the Campanulaceae, a family with a large variation in chromosome number (Crowl *et al.*, 2016). *Jasione* is the genus with the lowest number of chromosomes in the family, in which base numbers range from 6 (in *Jasione*) to 40 (in *Campanula*) (Lammers, 2007, Hong, 2015). There has been a long-standing debate about the evolution of polyploidy in the family, with two opposing

hypotheses. Lammers (1993) proposed an original low diploid base number and posterior events of whole genome duplication. Conversely, Stace & James (1996) postulated that poplyploidy was ancestral and that variation in the family was explained by events of descending diploidy. The findings of chromosome evolution modelling in a phylogenetic framework are consistent with Lammers' hypothesis, indicating an ancestral chromosome number of nine for the family (Crowl *et al.,* 2016). These results indicate a general tendency in the family for an increase in chromosome number, but that the base number of *Jasione* can only be the result of descending diploidy. Base numbers of eight and nine are found in the closely related clades of the Wahlerbergeae and the Campanuleae s.str. tribes, albeit *Feeria* and *Hesperocodon*, the allied lineages of *Jasione*, are polyploid, with 2n=34 and 2n= 36, respectively (Hong, 2015; Eddie & Cupido, 2014). In the Campanulaceae, polyploidy has played a role in the evolution of montane species, insular endemics and soil specialists (Crowl *et al*., 2016, Antonelli, 2009). In *Jasione*, polyploidy led to the identification of five ploidy levels in different lineages, with 2n = 12 (14), 24, 36, 48 and 60. This process basically occurred in an Iberian Pleistocene scenery. Autopolyploidy and allopolyploidy have probably both played a role in the evolution of the genus (Castro *et al.,* 2019, Castro *et al*., 2020). Polyploidy is restricted to two important lineages, the *Jasione crispa* clade, in which diploid, tetraploid, hexaploid and octoploid populations have been identified (Küpfer, 1969; Favarger *et al*., 1980, Küpfer, 1981), and the *Jasione montana-Jasione laevis* clade, in which diploid, tetraploid and decaploid populations have been identified (Küpfer, 1969; Küpfer, 1971; Küpfer, 1974; Leitão & Paiva, 1988; Rubido-Bará *et al*., 2010). In this thesis, the decaploid and hexaploid levels were identified for the first time in populations of *Jasione* gr. *crispa* and *Jasione* gr. *laevis*, respectively.

1.5. Internal systematics of *Jasione*

As already pointed out, Jasione has traditionally been considered a taxonomically difficult genus (Kovanda et al., 1968, Sales & Hedge, 2001). The main reasons for this difficulty are the

homogeneity of features among the species, due to evolutionary morphological stasis and the high degree of polymorphism within species, making it difficult to separate environmental plasticity from evolutionary differences. After the description of *Jasione montana* by Linnaeus (1753), the first treatment of the genus was carried out by De Candolle (1830), who recognized eight species. Since then, more than one hundred names have been proposed, although the validity of many of these is rather questionable. For example, Sennen (1926) described a number of names as species and "races" from individuals of a population of declaploid *Jasione laevis* from the Pyrenees. Other contributions have been more valuable, such as those of Boissier, who addressed the description of plants from the eastern and western Mediterranean areas (Boissier, 1839-1845; Boissier, 1875; Boissier & Reuter, 1852). Willkomm (1968) created the section *Phyteumopsis* to accommodate the species *Jasione foliosa*, on the basis of the long pedicels and involucral bracts resembling leaves, while in the other species of *Jasione* the bracts are very different from normal leaves. This author considered the species distinct enough to doubt whether it deserved a segregated genus (Willkomm & Lange, 1968). Stojanov (1926) made an important taxonomical contribution by working on the montane populations of what he considered the "*Jasione supina* Sieb. group" from Anatolia, the Balkan Peninsula and southern Italy, describing new taxa and summarizing relevant and previously scattered information. Since De Candolle's work (1830), there has only been one other attempt to deal with the genus as a whole (Schmeja, 1931). This was an excessively summarised approach for species, although not for other taxonomic ranks. Schmeja (1931) recognized only 13 species, but described two subgenera, two sections and five subsections and also suggested the first phylogenetic tree for the genus, although its organization is not supported by the phylogenetic results of this thesis. The subgenera recognised by Schmeja (1931) were based on Willkomm's sections, and therefore *Jasione foliosa* was included in the subgenus *Phyteumopsis*, while the subgenus *EuJasione* encompassed the remaining species. Schmeja's monograph recovered the approach of Stojanov (1926) regarding the taxa of the *Jasione supina* group from the Easter Mediterranean. However, when dealing with other groups,

Schmeja (1931) reduced most of the names to synonyms of *Jasione montana* L., *Jasione crispa* (sub *Jasione humilis* Loisel.) and *Jasione. laevis* (sub *Jasione perennis* Lam.). This approach diminishes the utility of the Schemja's treatment. Since Schmeja's work, there have been no other monographs on *Jasione* until now. Two species have been segregated as independent genera. Stojanov & Stefanov (1933) segregated the species *Jasione bulgarica* as a new genus *Jasionella* Stoj. & Stef. *Jasione bulgarica* is the only species in the genus with free not connate, anthers. Three years later, Stefanov (1936) segregated *Jasione foliosa* as the new genus *Urumovia* Stef., based on supposed differences in the anther shape and the number of ovules (2-3 versus about 20 in other species) and the lanceolate leaf-like bracts of the inflorescence. It is possible that Stefanov had access to scarce, unrepresentative material of *Jasione foliosa* regarding the anther and ovule traits.

This introduction tried to include a new synopsis of the genus based on the results of the different chapters of the thesis. Unluckily, this part was finally excluded of the document in its present form for lack of time to include a complete synopsis. Nevertheless, this work is currently under preparation, including a complete taxonomic revision that will largely transform the current systematics of the genus.

Figure I.5. *Jasione penicillata*. Benalmádena-Torremolinos, Spain. Picture: Quini Escalona, Reproduced with author's permission.

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General Objectives

This thesis aims to reveal different aspects of the evolutionary reality of the genus *Jasione* L. (Campanulaceae), a plant lineage distributed around the Mediterranean basin. Molecular, cytological, morphological and environmental analyses were carried out to address the following: (i) the internal phylogenetic relationships in *Jasione*, as well as its position among other genera of the Campanulaceae, (ii) the biogeographical history of a genus with a remarkable east-west Mediterranean biogeographical pattern, (iii) the processes leading to speciation and adaptation to new environments, (iv) the study of environmental niche shifts associated with changes in ploidy level, (v) the methods used to delimit evolutionarily significant cryptic entities by using different lines of evidence, and (vi) typification of complex groups of *Jasione* as a step in establishing a new taxonomic organization of the genus based on evolutionary relationships, among other aspects.

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General Methods

In this thesis numerous samples of *Jasione* and other representatives of the Campanulaceae family were used for molecular or cytological analysis, among others. Occurrence data obtained in the field by the author or extracted from herbaria or bibliographic sources have been used to inform statistical and modelling analyses. To prevent an inflation of database tables in this document, all this information, including vouchers and geographic information can be found in a online shared spreadsheet. GenBank accession numbers of the DNA sequences used in this thesis will be included in the shared link.

Link with database tables:

https://docs.google.com/spreadsheets/d/1LVcv9jSMR89TX8hh98_-_ZRFJ-WolXXvLdWQ8FemsT0/edit?usp=sharing

The methods used are structured as the different chapters of the thesis, although similar methods have been used in more than one chapter. The cited bibliography can be referred in the indicated chapters.

M.1. METHODS FOR TYPIFICATION AND TAXONOMIC REVISION (CHAPTER 1)

Currently accepted names of the *Jasione*, including homotypic and heterotypic synonyms, those listed in the Med-Checklist (Greuter *et al.*, 1984), the World Checklist and bibliography of the Campanulaceae (Lammers, 2007) and the Euro+Med Plantbase (Castroviejo *et al.*,

2010) were considered. Other names were obtained from diverse sources (see below), mainly Stojanov (1926) and Damboldt (1978). The protologues and original material of names were analysed in detail and those names lacking a nomenclatural type were typified in accordance with the International Code of Nomenclature for algae, fungi, and plants (Turland *et al.*, 2018). The following herbaria were consulted: B, BR, E, G, FI, K, MPU, P, SO, SOM, GOET and W.

M.2. METHODS FOR DNA EXTRACTION AND AMPLIFICATION (CHAPTERS 2, 3, 4 AND 5)

Total genomic DNA was extracted using either a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990) or a commercial kit Nucleo Spin Plant II (Macherey-Nagel). In both cases 0.6 ul of 2-Mercaptoethanol was added during the first liquid step. Foliar tissue was homogenised either with manual grinder or using Mikro – dismembrator (Sartorius). DNA concentration and quality was assessed with Nanodrop 2000C (Thermo Scientific). In Chapter 2 and 4, four plastid DNA regions were amplified, the psbA-trnH intergenic espacer, the trnL-trnF intergenic spacer, the 3' end of the intron of the ndhF gene and the rps16-trnQ intergenic spacer. After alignment and comparison with plastome sequences of members of the Campanulaceae in the GenBank it turned out that the conserved regions where the primers are anchored belong at the end of the trnL(UUA-trnT(UGU) spacer and the trnT(UGU) gene and the trnQ gene region. This can be related to the massive rearrangements underwent by plastid genomes in *Jasione* and in general the Campanulaceae family (Cosner *et al.*, 2004). Therefore, the amplified marker will be denominated trnT(UGU)-trnQ. In Chapter 3 five regions of the chloroplast genome were amplified: the petD region, including the petB-petD intergenic spacer, the petD 5' exon and the petD intron; the rpl32-trnL(UAG) intergenic spacer; the psbA-trnH intergenic spacer; the 3'extreme of ndhF gene intron; and the trnL-trnF intergenic spacer. The primers used for each marker are listed in Table 3.1, including the relevant

references. One internal primer, called "trnL(UAG)-Jasi" was designed in this study for the rpl32-trnL (UAG) intergenic spacer to enable amplification of the region in some *Jasione* accessions with a huge insertion in the 3' extreme of the region. In chapter 5 the complete ITS region (ITS-1, 5.8S gene, and ITS2) was amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). PCR reactions were carried out in 25 ul with PuReTaq Ready-To-Go PCR BEADS (GE Healthcare) and 80-150 ng of genomic DNA. Thermocycler was programmed for amplification of the ITS region with an initial denaturation step of 95ºC for 2 min, then followed by 31 cycles of 1 min at 94ºC, 1 min at 46-48ºC and 2 min 10 s at 72ºC, with a final extension step of 8 min at 72ºC.

PCR products were firstly assessed on 1.5 % agarose electrophoresis gels stained with the nucleic acid staining solution RedSafe (iNtRON biotechnology) and purified with E.S.N.A. Cycle Pure Kit. (Omega). DNA Sanger sequencing was carried out in an 8-capillary 3500 analyzer (Applied Biosystems) by the DNA sequencing service of the University of Santiago de Compostela, Spain, or Software Sequencing Analysis 5.2 (Applied Biosystems) by STAB VIDA, Portugal.

M.3. METHODS FOR PLOIDY ASSESSMENT BY CHROMOSOME COUNTING (CHAPTERS 2, 3, 4 AND 5)

Direct chromosome counting was carried out in metaphases from root apices. Root tips measuring c. 5 mm were excised from cultivated plants grown from field collected individuals or from germinated seeds. All plants and seedlings were acclimated to outdoor conditions and were directly moved from temperatures around 15ºC or lower to the laboratory to conduct immediate root excision, always at first hours in the morning (before 9:00 a.m.) to avoid high laboratory room temperatures. Root tips were washed in distillate water a pre-treated in 2 MM 8-hydroxyquinoleine for 3 hours, followed by a rinsing step in distillate water and then a fixation step in freshly prepared 3:1 96% ethanol - 1 glacial acetic acid solution for 24 hours. Samples were

thoroughly rinsed in 70% ethanol to remove glacial acetic acid, since it has been described to interfere with later chromosome staining (Norman *et al.*, 2012). Samples were either stored in 70% ethanol at 4ºC for further use or directly used in the following steps. Root tips were rinsed in distilled water for 5 minutes, followed by an additional 5 minutes rinsing step in fresh distilled water. Samples were hydrolised by plunging the root tips in and Eppendorf tube with HCL 1 N for 6-9 minutes in a water bath at 60ºC. The tubes were pre-heated to bath temperature before root tip plunging. Two rinsing steps 5 minutes each in distillate water followed to eliminate all possible HCL remains. For mitotic analysis, root tips were stained in Schiff's reagent for 12 hours in dark conditions. Stained root apices were excised under binocular magnifying glass, and immediately squashed on a slide. Cells were observed in a Leica TCS-SP2 confocal laser microscope (LEICA Microsystems Heidelberg GmbH, Mannheim, Germany) under a HCX PL APO CS 63.0x1.40 OIL objective, 580nm-725nm emission band and excitation wavelength of 561 nm with DPS (561 nm) laser diode.

M.4. METHODS FOR PLOIDY ASSESMENT BY FLOW CYTOMETRY IN SILICA GEL DRIED MATERIAL (CHAPTER 2)

Flow cytometry on silica-gel dried plants was carried in some populations, or in other individuals from populations in which chromosome number had been counted previously in at least one individual. Measurements were conducted on a Cell Lab Quanta Beckman Coulter flow cytometer (Beckman Coulter, USA) with a 100-W Mercury arc lamp and excitation line optimized at 366 nm for UV estimations of DNA content with DAPI fluorochrome. Dried samples conserved in silica gel were prepared by chopping in 600 µL of Galbraith's buffer with 100 µg ml^{-1} RNase A. *Pisum sativum* 'Express Long' (2C = 8·37 pg) was initially used as internal reference, although samples from known diploid level (2n=12) of *Jasione montana* were used for rapid assessment of polyploid populations in other cases. Filtering was conducted in a 33-µm nylon filter and o 2 µg mL^{-1} of DAPI fluorochrome was added to the mixture. 10,000 particles were measured in each sample, and the 2C- values were calculated. As

dehydrated instead of fresh samples were used, precise genome size estimation was ruled out, being the objective to grossly estimate the ploidy level of the sample by comparison of the position of plotted peaks.

M.5. METHODS FOR SPECIES DELIMITATION WITH ASAP AND GMYC (CHAPTER 2)

Assemble Species by Automatic Partitioning (ASAP) is a molecular method for species delimitation that uses group-specific ad hoc thresholds without any a priori species hypothesis, that are estimated looking for the barcoding gap in the frequency distribution of nucleotide distances. It represents a transition between intraspecific and interspecific categories. (Puillandre *et al.*, 2021). ASAP is based on a hierarchical clustering algorithm using only pairwise genetic distances from single locus sequence alignments to produce a species partition scheme ranked by a scored system (Puillandre *et al.*, 2021). The current author used a set of plastid regions that are inherited together and therefore can be considered a single locus for Species Delimitation methods (Fujisawa & Barraclough, 2013). ASAP creates successive partitions from all terminals split to all lumped into a single group and assigns them a probability that quantifies the partition chances of being a single species. Them it computes the width of the barcode gap between the previous and the new partition. Probability and gap width are combined into a single asap-score that is used to rank the groupings, the lower the better (Puillandre *et al.*, 2021). ASAP analyses were conducted in the program web-interface (https://bioin fo.mnhn.fr/abi/publi c/asap) with the three available substitution models, Jukes-Cantor (JC69) (Jukes & Cantor, 1969), Kimura 2P (Kimura, 1980), and simple p-distances. The analyses were run from a dataset with 14 sequences, including all detected haplotypes in the populations of study group plus a sequence from a population of the hexaploidy *Jasione crispa* subsp. *crispa* from the Pyrenees.

Generalized Mixed Yule Coalescent (GMYC) model (Pons *et al.*, 2006) was used to suggest hypothetical species as independent

evolutionary units. GNMYC is an exploratory method using single-locus sequence datasets and a ultrametric phylogenetic tree as input to estimate rates of branching to ascertain which parts of the phylogeny behave following population (coalescent) model, and which parts follow a speciation (Yule) model. The favoured species partition maximizes the likelihood of the transition between branching coalescent rates and branching speciation rates in absolute time, using an ultrametric tree, identifying a threshold between speciation and coalescent rates. This single threshold approach is based on the assumption that species are monophyletic, and therefore clades defined by a Most Recent Commons Ancestor (MRCA) reflect diversification events and can be considered species while branches descending from each of the MRCA nodes reflect coalescent events (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013). The set of nodes with highest Maximum Likelihood is chosen as the best species partition model. There is a version of the methods that allows to infer multiple thresholds, relaxing the assumption that all diversification events must be previous in time than all coalescent events (Monaghan *et al.*, 2009). However, it has been demonstrated that this variant of the method is prone to over-splitting (Fujisawa & Barraclough, 2013; Kekkonen & Hebert, 2014), therefore its use was discarded. From each putative species sequences of at least six individuals have been included in the GMYC analysis. When more than one population was included in the sample, at least two individuals per population has been sequenced (mean 4.1 indiv./population). Fujisawa & Barraclough (2013) observed that increases in the number of samples individuals per species led to better performances of the method. The reason is that greater sampling might increase the detected branching rate and therefore the detection of a threshold in branching. However, greater species sampling, particularly without increasing within species sampling of individuals might reduce the ability of GMYC to detect species. The reason is that species monophyly is more easily inferred if random but incomplete sampling provokes that some close species are not included in the analysis (Bergsten *et al.*, 2012). Hence, with the aim of an earnest procedure and to avoid unrealistic over-split results, it has been sampled the complete geographic range of the taxa investigated, even if it could

hamper the detection of non-monophyletic species. As within species geographic structure could affect the accuracy of the method, causing that some relatively isolated populations could be delimited as separate species (Papadopoulo *et al.*, 2008; Lohse, 2009), the sampling design aimed to include not only the main geographic groups by all linking populations. Nevertheless, simulations of the HMYC method showed that if the effective population size remains low relative to species divergence (that is, relatively low within species variation) the threshold is optimized at the correct point of the tree and the effect of geographic structuring is minimal (Fujisawa & Barraclough, 2013). Moreover, Talavera *et al.* (2013) observed that the remotion of intermediate haplotypes had little effect in the results and did not delimit as different species the most extreme haplotypes. The analyses have been conducted by both including repeated sequences and pruning repeated sequences. GMYC performance is affected by identical sequences because terminal zero-length branches affect the likelihood estimation (Monaghan *et al.*, 2009; Fujisawa & Barraclough, 2013). However, when the tree is produced by a genealogy-based inference, as is the case of BEAST, the program does not assign null lengths to identical terminals, since they are treated as different haplotypes coalescing to a MRCA node, what allows GMYC to handle them. Talavera *et al.* (2013) did not observe differences in GMYC results when using repeated sequences from a tree inferred with BEAST relative to when they were removed, although they recommend collapsing all repeated sequences to only keep different haplotypes to reduce computational times. Conversely, Michanneau (2015) advocated the use of repeated sequences in GMYC analysis using a tree inferred by BEAST. BEAST could overestimate effective population sizes if identical sequences are removed by inferring higher unrealistic levels of genetic diversity (Michanneau, 2015). Since population sizes affect lineage coalescence, the remotion of identical sequences would increase average branch length, hampering the distinction between divergent and coalescent events. Michanneau (2015) considered that collapsing sequences to haplotypes would cause species over-splitting and higher uncertainty in the analysis. While the latter is a probably undesirable outcome, it could also be that keeping identical sequences

from BEAST, with minimal (although not zero) lengths could push to the external nodes the species delimitation threshold. Two datasets have been included, one with all identical sequences collapsed to distinct haplotypes (dataset 1, the same as in ASAP) and other in which were retained different sequences in a proportion similar at how they were found in natural populations across the geographic range of the taxa. When one haplotype was found in only one population, it was represented only once in the dataset, either if it was the only haplotype in the populations or not. The possibility that one species could be represented by only one sequence in the dataset (i.e., a singleton) has no demonstrated negative effects in GMYC analysis (Talavera *et al.*, 2013).

GMYC analyses were carried out from trees estimated with BEAST2. The substitution model was HKY + G, with the Gamma count categories prior set four categories, as small number of categories of gamma distributed rates of evolution is enough to capture rate variation in small dataset (Jia *et al.*, 2014). In the first dataset (collapsed to haplotypes) two BEAST priors were modified to test possible effects on the GMYC results. It is expected that variation in priors as branching pattern and rate of molecular evolution would not affect the inferred topologies, but the branch lengths will vary. Two different priors were used for the expected branching, a Yule model, also called pure birth model (Yule, 1925), and a Coalescent model with constant population size (Griffiths & Tavaré, 1994). The former is expected when the tree has a constant speciation rate while the latter describes branching patterns within the same species. For the Yule model analysis, the rate of molecular evolution was set to "constant clock", meaning that the amount of nucleotidic mutations increases at a constant rate with time, and assumption suitable for small datasets of very related taxa. It was also tested a "relaxed clock" allowing that mutation rates could vary throughout the tree following a log-normal distribution. For the coalescent model with constant population size, it was only used a constant clock prior, as it is expected a within-species behaviour. The impact of prior variation has been studied in several works (Monaghan *et al.*, 2009; Talavera *et al.*, 2013; Michanneau, 2015). It has been indicated that the use of a Coalescent constant population model would

be more conservative than the Yule model because GMYC also uses a coalescent model as null model of expected branching (Monaghan *et al.*, 2009). In fact, these authors estimated higher species number with Yule model than with constant coalescent model. However, since this parameter affects branch lengths the prediction of how it would affect GMYC results is not straightforward and could be influenced by the amount of variation in the data (Michanneau, 2015).

It was tested the effect of prior variation over GMYC results using the LRT test significance. After that, for the GMYC analysis with the second dataset (with repeated sequences) it was inferred a Bayesian phylogeny with the coalescent constant population branching and constant clock molecular rate of molecular evolution priors. Bayesian phylogenies were inferred in BEAST2, with 10 million generation MCMC lengths and two independent runs. Convergence was checked with Tracer v1.6 and after inspection of ESS values in each run a 10% burn-in and posterior sample combination was carried out in LogCombiner. Finally, a maximum credibility clade tree was summarized with TreeAnnotator for each analysis. In the analysis with relaxed clock model prior parameters were left as default in BEAUti2 and clock rate set to 1.0.

BEAST ultrametric topologies and posterior probabilities were inspected in FigTree and exported in newick format, since it is required by the R package splits (Ezard *et al.*, 2009) which was used to apply the GMYC method with single-threshold. Each GMYC analysis was repeated in the GMYC server (https://species.h-its.org/gmyc). Species Delimitations, LRT significances and GMYC (AIC) supports values were compared among the analyses.

M.5. METHODS FOR MORPHOLOGICAL AND PHENOLOGICAL ANALYSES TO SUPPORT SPECIES DELIMITATION (CHAPTER 2)

The putative species delineated by the GMYC and ASAP methods can be interpreted as hypotheses and to determine whether they are biologically sound other lines of evidence need to be investigated.

Looking for delimitating morphological traits can be difficult if not misleading in a genus of which has been said that "there is not any clear morphological discontinuity between two species of *Jasione*" (Tison & de Foucault, 2014). Nevertheless, and with the guidance of GMYC and ASAP results, some subtle morphological characters are investigated here. Phylogenetic analyses in the genus (see Chapter 3) showed that hydathoids (see General Introduction for description of the trait) have a distribution among the species of *Jasione* that correlates with the evolutionary history of the genus. Here, the average number of hydathoids per leaf was compared in the model group with the width of leaf hairs, a measure relative to the size and stiffness of trichomes.
The relative size of the epidermal cells of the adaxial and abaxial sides of the leaf has been pointed out by Bohkari & Sales (2001) as one of the few anatomical characters providing taxonomical information in *Jasione*. In most species studied by these authors adaxial and abaxial epidermal cells were subequal in size. However, in *J. laevis*, *J. sessiliflora*, *J. cavanillesii* and *J. crispa* subsp. *tomentosa*, the adaxial epidermal cells were 1.5–2 times larger than the abaxial epidermal cells. This trait was investigated here within the more homogeneous taxa of the model group, *Jasione sessiliflora* subsp. *appressifolia*, *Jasione crispa* subsp. *tomentosa*, *Jasione crispa* var. *praelittoralis*, and the less similar *Jasione crispa* subsp. *mariana*. Epidermal peelings of leaves from herbarium specimens were stained with blue toluidine and photographed at 20x magnification in a light microscope. Length of cell larger dimension was measured in 25 adaxial and 25 abaxial epidermal cells in each leaf, five leaves per individual.

Phenology data was obtained from two sources, collection date information of herbarium specimens with flowers and the phenological information provided by Pérez Chiscano (1987).

M.5. METHODS TO SUPPORT SPECIES DELIMITATION FROM BIOGEOGRAPHICAL REGIONALISATION (CHAPTER 2)

To test for the adequacy of the delimited entities to a pre-established biogeographical framework, the studied populations were

superimposed to a georeferenced map of the Biogeographic subprovinces of the Iberian Peninsula (M. Serrano, own digitization), based on Rivas-Martínez *et al.* (2017). The haplotypes were organized in six haplogroups, those with high posterior probability in the Bayesian genealogy. The frequency of the chloroplast haplogroups in the Iberian biogeographic subprovinces was surveyed and visualized using heatmaps. The heatmaps were generated in R v.4 with the ComplexHeatmaps package (Gu *et al.*, 2016). Two heatmap analysis were generated. First, a heatmap without statistical internal clustering with subprovinces was ordered according to a thermic gradient. Second, a heatmap with split simultaneously performed by rows and columns, applying k-means partitioning analysis to detect underlying patterns. The k parameter is set to three for rows to test whether the partitioning recovers the most probable delimited species number suggested by GMYC and ASAP methods. The k parameter is set to four for columns as it is the number of major inferred dendrogram clusters of subprovinces obtained by hierarchical clustering. As the k-means analysis uses random start points, which could generate different clusters from different runs, the analysis was repeated 25 times to check for consistency of the results, observing no variation in the results.

M.6. METHODS FOR IDENTIFICATION OF PATTERNS OF SPECIES RICHNESS (CHAPTER 3)

A taxonomic richness map was generated to depict the fit of the genus to the east-west Mediterranean disjunct pattern. The map was not built from those species recognized in general treatments (e.g. Lammers, 2007; Castroviejo *et al.*, 2010), but rather with all evolutionary significant units (Ryder, 1986) of the genus, following the taxonomic criteria used by the current author. Therefore, it includes the species and subspecies recognized in general treatments, but also some varieties only recognized in regional floras, such as those of the *J. orbiculata* group (e.g. Ančev, 2012). Some conflicting taxa or populations were considered sufficiently differentiated and are therefore included in the map, but only when researchers suggested their possible relevance using information of evolutionary significance

(e.g. *Jasione jankae*, sensu Hartvig, 1991). In total, 43 taxonomic entities have been included in the map, most of them narrow endemics. However, more than 100 taxonomic names have been proposed in the genus (Kovanda, 1968), most of which were not considered worthy of recognition in this study, and were therefore not used to construct the taxonomic richness map. The map was generated from reviewed herbarium sheets, the author's own field collections or field collections sent to the current author by collaborators from abroad. General databases such as "www.anthos.es." and "www.gbif.org" were not used due to the large number of misidentifications (e.g. over 65% of misidentifications in *J. sessiliflora* in gbif.org, M. Serrano, own data). The INaturalist database was used in some cases when a given observation could be confidently identified by photograph. The richness map was generated in R v4.1 (R Core Team, 2021) using the packages letsR, rgdal, and sp.

M.7. METHODS FOR PHYLOGENETIC ANALYSES (CHAPTER 3)

Several phylogenetic approaches were applied to the concatenated cpDNA data set to generate a phylogeny. These included two Maximum-Likelihod methods, applied using the software IQ-TREE (Nguyen *et al.*, 2015) and RAxML (Stamatakis, 2014), and two Bayesian approaches, applied using the software MrBayes (Ronquist *et al.*, 2012) and BEAST2 (Bouckaert *et al.*, 2019), the latter of which was used for lineage dating and time divergence analyses.

Maximum-likelihood analyses were first performed with IQ-TREE version 1.6.12 in W-IQ-TREE (Trifinopoulos, *et al.*, 2016). Best-fit substitution models and partition schemes for the IQ-TREE analysis were inferred with ModelFinder (Kalyaanamoorthy *et al.*, 2017) in W-IQ-TREE, with three information criteria: the BIC or Bayesian Information Criterion (Schwarz, 1978), the AIC or Akaike information criterion (AIC) (Akaike, 1974) and the AICc or corrected Akaike information criterion (Hurvich & Tsai, 1989). BIC and AIC criteria selected the TVM+F+G4 model (Transversion model with AG=CT unequal base frequencies, F as empirical base frequencies and G as

discrete Gamma model with four rate categories). AIC selected the same model for the petD and psbA-trnH regions, but selected the model GTR+F+G4 (General time reversible model with unequal rates and unequal base frequencies, empirical base frequencies, and G as a discrete Gamma model with four categories) for the rpl32-trnL (UAG), ndhF and trnL-trnF regions. ModelFinder uses BIC by default as the optimality criterion as it has been observed that AIC and AICc in this software are slightly biased toward more parameter-rich models (Kalyaanamoorthy *et al.*, 2017). Consequently, all loci were treated as unique partitions and a partitioned scheme with TVM+F+G4 for all regions was chosen for the analysis. ModelFinder also indicated that the partitioned model yielded a poorer fit than a single model. Therefore, another maximum-likelihood analysis was performed without data set partition and allowing IQ-TREE to auto-select the substitution model. In this case, AIC, AICc and BIC consistently selected TVM+F+G4 as the best-fit model. Branch support was assessed using the ultrafast bootstrap (BS) approximation (Hoang *et al.*, 2018). Log-likelihood of the tree was better and with fewer parameters in the unpartitioned analysis than in the partitioned analysis, although the tree from the latter analysis yielded better (lower) BIC values, but not better AIC and AICc values (Table 3.3).

RAxMl analyses were performed in the CIPRES Science Gateway portal v3.3 (Miller *et al.* 2010) with RAxML-HPC2 on XSEDE v. 8.212. The best-fit partitioning scheme for the RAxML analysis was selected with PartitionFinder2 v2.1.1 (Lanfear *et al.*, 2016) with the greedy algorithm (Lanfear *et al.*, 2012) and PhyML (Guindon *et al.*, 2010). One subset was defined for each gene region by the BIC, AiC and AICc criteria. PartitionFinder2, considering only substitutions models allowed by RAxML, determined GTR+G as the best-fit model of nucleotide evolution for all subsets. Therefore, RAxML analyses applied the GTRGAMMA model to each subset, and nodes support was quantified by RAxML rapid bootstrap with 1,000 replicates (Stamatakis, 2006).

Bayesian analyses were carried out with MrBayes version 3.2 (Ronquist *et al.*, 2012). MrBayes only allows a relatively a small

collection of models, so PartitionFinder2 v2.1.1 was used to test only these models. Each gene region was treated as unique and unalike partitions with all allowed to evolve at different rates. The model selected with the greedy algorithm and BIC criterion was GTR with gamma-distributed rate variation across sites and a proportion of invariable sites. Four runs with 10 million generations and four Monte Carlo Markov chains (MCMC) were carried out, sampling every 1,000 generations. Convergence in all parameters was evaluated with Tracer v1.7 (Rambaut *et al.*, 2018) and burn-in was set to discard the first 10% trees. Support for internal nodes was quantified by Bayesian posterior probabilities.

Five different aspects of evolutionary significance were mapped onto the phylogeny, including the average number of hydathodes, life history habit, mountain or lowland habitat, ploidy level and signs of introgression. The number of hydathodes was based on average measurements of the current author in rosette and stem leaves of the taxa or putative Evolutionary Significant Units represented by the terminals. Within clade variation was represented by a range. Life history habit was represented by annual or perennial habit. Habitat in relation to elevation was represented by lowland habitats (0-500 m a.s.l.), mid-elevation mountain habitats (500-1,600 m a.s.l) and high elevation alpine or oro-Mediterranean habitat (1,600-3,500 m a.s.l.). Ploidy levels were mainly obtained by the current author following the procedures detailed in the General Methods section. Bibliographic information was used in some cases (e.g.,Lammers, 2007a). Signatures of introgression were identified when a clear reticulate pattern was suggested by the phylogenetic position of a terminal and by discordance between morphology and plastid nucleotide sequences.

M.8. METHODS FOR ANALYSIS OF TIME DIVERGENCE AND TREE CALIBRATION IN THE CAMPANULACEAE (CHAPTER 3)

Bayesian analysis was carried out in BEAST2 (Bouckaert *et al.*, 2019) to simultaneously infer the phylogeny and divergence times. A relaxed lognormal clock model was assigned to each partition of the

concatenated plastid data set. The prior rate of the branches was drawn from a parametric distribution that has as mean a function of the rate of the parent branch (Drummond *et al.*, 2006). Prior distributions of ages are set as calibration points of certain nodes to estimate the time of divergence of the lineages. Different fossil information was used to specify priors and calibrate the phylogeny. However, dating the tree of the Campanulaceae is controversial. The fossil record in the Campanulaceae is particularly poor (Lammers, 2007b), although some reliable fossils are available. Two seed types assigned to *Campanula* from Poland (Nowy Sącz basin) have been dated from the Middle Miocene (Upper Carpathian, 16.0-17.5 Mya) (Lancucka-Srodoniowa, 1977; Lancucka-Srodoniowa, 1979). Pollen grains of the Campanulaceae have been dated to the Miocene epoch (c. 23 Mya) (Mildenhall, 1980; Benton, 1993). More strikingly, pollen grains have been attributed to the Campanulaceae in a Late Cretaceous (Maastrichtian, 72.1-66 Mya) fluvio-lacustrine formation from Inner Mongolia (Van Itterbeeck *et al.*, 2007). These authors offer no indication about the morphological traits of the grains or whether they would belong to the Campanuloideae subfamily or the whole Campanulaceae s.l., i.e. including the Lobelioideae subfamily. In any case, the fossil remains include eight Campanulaceae pollen grains, all from a plant community dominated by gymnosperms and ferns as is typical in plant formations prior to the Cretaceous-Paleogene extinction event, which adds reliability to this early dating (Van Itterbeeck *et al.*, 2007). The Maastrichtian lies temporally far before the inferred split between the Campanuloideae and the Lobelioideae, which has been dated at around 45.6 Mya by Magallón *et al.* (2015) and at around 52 Mya by Wikström *et al.* (2003). Nevertheless, it is posterior to the inferred age of the most recent common ancestor between the Roussaceae family and the long lineage leading to the Campanulaceae s.l., a split inferred between 76.1 Mya (Magallón *et al.* 2015) and 80 Mya (Wikström *et al.*, 2003. Bell *et al.*, 2010). All of these fossils, except the pollen grains from the late Cretaceous, have been used for node calibration in lineage dated phylogenies. Controversially, they have not been used to date the same nodes in the Campanulaceae phylogeny, leading to conflicting results (see Cano-Maqueda *et al.*,

2008; Frajman & Schneeweiss, 2009; Roquet *et al.*, 2009; Cellinese *et al.*, 2009; Mansion *et al.*, 2012; Crowl *et al.*, 2016). The two types of seeds assigned to the genus *Campanula* from the Upper Carpathian are notably different. In one case, they are regular Campanuloid seeds with a smooth seed coat (Lancucka-Srodoniowa, 1977). The other type shows a reticulate coat formed by cells divided by thin walls. The latter resembles the seeds of the extant *Campanula pyramidalis* and is in fact the type specimen of the extinct species *Campanula paleopyramidalis*, interpreted as an immediate ancestor of *C. pyramidalis* (Lancucka-Srodoniowa, 1979). The fossil seeds occurred in a warm and wet environment dominated by peat bogs and swampy forests of tropical-subtropical vegetal communities linked to extant south-eastern Asian flora (Lancucka-Srodoniowa, 1979). However, *C. pyramidalis* and its more closely related species are Italian-Balkan plants occurring in limestone crevices or rocky-garrigue Mediterranean dry habitats (Lakušić *et al.*, 2013). *C. carpatica* is in the same clade as the *C. pyramidalis* complex (Mansion *et al.*, 2012), from the Polish Carpathians, it has reticulate seeds (Olesen *et al.*, 2012), although its habitat is upper mountain limestone cliffs. Moreover, reticulate seed ornamentation is not restricted to the *C. pyramidalis* group in the Campanulaceae. For example, it is not rare within the tribe Wahlenbergeae in the genus *Wahlenbergia* (Plunkett *et al.*, 2009; Cupido *et al.*, 2011). The tribe Wahlenbergeae is mostly distributed in the southern hemisphere and is almost absent in Europe, although the situation may have been different in the paratropical conditions of the European Miocene. However, the absence of past or current evidence of the Wahlenbergeae in Central Europe makes assignment of the fossil seeds to the genus *Campanula* more likely. Several works have used the Nowy Sącz fossil seeds to calibrate phylogenies, although with different perspectives. Roquet *et al.* (2009) and Olesen *et al.* (2012) set a calibration point of 16 Mya at a node ancestral to the tribes Campanuleae, Wahlebergeae, plus *Jasione* and its allied lineages. Cellinese *et al.* (2009) used the same age, but for an much more derived node, the stem node between *C. pyramidalis* and *C. carpatica*, arguing that these are the only two species with a seed similar to *C. paleopyramidalis*. This point was contested by Olesen *et al.*, (2012),

who stated that a reticulate coat is found in other Campanula species, such as the American *C. scouleri*. Mansion *et al.* (2012) followed the approach of Cellinese *et al.* (2009). Although in all of these cases, other calibration points were used (e.g. indirect dating of the root or other basal nodes from published angiosperm phylogenies), which nuanced the effects of the fossil seed calibration, the results from the two approaches are very different. For example, with the former approach, the divergence of two core *Campanul*a clades (*Campanula* s. str. and "Rapunculus") was estimated between 20 Mya (Roquet *et al.*, 2009) and 13.5 Mya (Olesen *et al.*, 2012), while with the latter approach it was estimated between 60 Mya (Cellinese *et al.*, 2009) and 39 Mya (Mansion *et al.*, 2012). Later, in a work focused on a group of eastern Mediterranean *Campanula* species, Crowl *et al.* (2015) still used the Nowy Sącz seeds to calibrate the stem node between *C. pyramidalis* and *C. carpatica*. These researchers used the 16 Mya calibration point as an older offset for a lognormal distribution of time with mean around 5 Mya. The approach used in the present work is different from those used in the previously cited studies. Although the literature dealing with the seed morphology of the Campanulaceae is not very extensive, it is known that seeds with reticulate coats appear in more groups than the *C. pyramidalis* clade (Belyaev, 1984; Shetler & Morin, 1986; Cupido *et al.*, 2011). Within the genus Campanula, reticulate seed coat morphology can be found in either Eurasian species, such as the Anatolian *C. olympica* (Akcin, 2009), or American species, such as *C. divaricata* or *C. prenanthoides*, among other species (Shetler & Morin, 1986), and none of these belong to the *C. pyramidalis*-*C. carpatica* clade. Nevertheless, the position of all these species is nested in all published phylogenies within the "Rapunculus" clade of *Campanula* (Wendling *et al.*, 2011; Mansion *et al.*, 2012).

The approach applied in the present work uses all this evidence and the fossilized seeds of *Campanula* sp. and *Campanula paleopyramidalis* from the Nowy Sącz Miocene to establish the calibration point within the Rapunculus clade, between the early divergence (in the current author's data set) that led to the genera *Hanabusaya* and *Adenophora* and the lineage that led to other Rapunculus groups (represented in the current author's data set) by *C.*

pyramidalis and *Campanula* gr. *rotundifolia*. A late Burdigalian (around 16 Mya) Miocene scenario with the Rapunculus clade already diversified is plausible and supported by *C. paleopyramidalis* seeds. However, the scattered occurrence of reticulate seeds in the Rapunculus clade suggests that using this trait to calibrate the derived clade of *C. pyramidalis*-*C. carpatica* may be somewhat adventurous. Conversely, placing this calibration point at the base of the Campanuleae as in Roquet *et al.* (2009) seems too conservative. Other works, apparently unaware of the existence of the Nowy Sącz fossil seeds, and using other lines of evidence, have dated the early divergence of the Rapunculus clade in the late Burdigalian (16.5 Mya). Park *et al.* (2006) used divergence times inferred in the angiosperm phylogeny of Wikström *et al.* (2003). This approach was also used by Cano-Maqueda *et al*. (2008), who established a maximum age of 23 Mya to the Rapunculus clade based on the occurrence of Miocene Campanulaceae pollen records (Benton, 1993). In the current study, a lognormal prior distribution with mean 16 Mya and median 15.3 Mya and a standard deviation of 0.3 to the stem node was established for the node subtending *C. rotundifolia* and *C. pyramidalis* lineages. The root was also calibrated, as deep calibration points are needed to capture a larger proportion of the overall genetic variation (Duchêne *et al.*, 2014). The root node represents the divergence between the subfamily Lobelioideae and the lineage leading to the Campanuloideae. Wikström *et al.* (2003) and Magallón *et al.* (2015) calculated mean ages for this node of 52 Mya and 46.5 Mya, respectively. Mansion et al. (2015) inferred a mean age of 76.5 Mya, although this inference is strongly influenced by the use of the *C. paleopyramidalis* seeds to date the split between *C. pyramidalis* and *C. carpatica*. Nevertheless, the existence of unassigned Campanulaceae pollen in the Late Cretaceous (72.1-66 Mya, Van Itterbeeck *et al.*, 2007) is not very consistent with the inferred divergence between the Campanulacee and the Rousseaceae calculated to be about the same age by Magallón *et al.* (2015) and slightly earlier, about 80 Mya, by Wikström *et al.* (2003). Consequently, the inferred ages for the split between the Lobeliodieae and Campanuloideae calculated by these authors seem excessively recent. Therefore, in this study an age older than the mean age provided by these studies was used

to calibrate this node and using instead their older boundaries. A normal prior distribution was established for the root, with mean in 56 Mya, standard deviation of 1 and 95% of the distribution of ages between 54 Mya and 58 Mya. The prior substitution model was established as TVM+G4, with a concatenated five partition scheme as the best-fit model selected by ModelFinder. Although BEAST2 does not include TVM as default model, the Site Model tab of BEAUTI2 can be established by selecting the GTR model and setting the AG rate parameter at 1.0. The analysis was preliminarily run with two Tree Models, a Yule model and Birth-Death model. The second would be more realistic when many terminals are absent, as in a data set with only a few representatives of each Campanulaceae group included. On the other hand, the Yule model demands fewer parameters. Nevertheless, no significant differences between the results of both models were observed. Final analyses were carried out with a birth-death tree prior. Five independent runs, each with four Monte Carlo Markov chains (MCMC) and 10 million generations, were carried out, sampling every 1,000 generations. Convergence as effective sample sizes (ESS) values in all parameters was evaluated with Tracer v1.7 (Rambaut *et al.*, 2018) and burn-in was set to discard the first 25% trees, with the conserved trees combined in LogCombiner and summarized in TreeAnnotator (Drummond & Rambaut, 2007). Support for internal nodes was quantified by Bayesian posterior probabilities and node ages were set to median ages.

M.9. METHODS FOR DIVERSIFICATION ANALYSIS (CHAPTER 3)

The maximum-credibility clade tree from the BEAST2 time calibrated Bayesian analysis and a random subsample of 750 trees from the posterior distribution of the Bayesian analysis were used to create lineage-through time (LTT) plots to study diversification in *Jasione*. After the set of randomly sampled trees from the posterior distribution was plotted, a Confidence Interval (CI) was calculated for each tree (CI = (1- α)-percent; α = 0.05) on the number of lineages given times, and plotted on the median LTT (Revell, 2012). The trees were cropped to only include *Jasione* terminals and pruned to conserve differentiated

lineages. The LTT representations of the diversification and statistical tests were generated and performed in R v.4.1 (R Core Team, 2021) with the packages ape (Paradis *et al.*, 2004) and phytools (Revell, 2012). The net rate of diversification under a birth-death model ($r = \lambda - \mu$) was calculated on the LTT from the maximum-clade credibility tree, where λ is the rate at which new species are added through speciation and μ is the rate at which species are lost by extinction. The net rate of diversification and extinction fraction ($\varepsilon = \mu/\lambda$) are key parameters to determine the mean clade diversification over time (Rabosky & Benson, 2021). Pybus and Harvey γ constant-rates tests were performed on the LTT from the maximum-clade credibility tree to detect deviations from a pure birth Yule model, with two-tailed p-value for the γ-test (Pybus & Harvey, 2000). Accounting for unsampled lineages is considered essential, as missing terminals can cause the spurious detection of declines in diversification rates (Pybus & Harvey 2000; Morlon *et al.*, 2012). Incomplete lineage sampling could favour the hypothesis of deceleration of diversification rates, leading to early burst interpretations (Fordyce, 2010). To avoid the effects of incomplete lineage sampling, a Monte Carlo constant rate test was performed, where the 0.05 p-value used to reject constant rate was calculated from a distribution of γ for simulated trees including incomplete lineage sampling comparable to the studied tree (Pybus & Harvey, 2000). Complementarily, any important departures in the obtained significance of the γ statistics between the standard Pybus and Harvey γ-test and the Monte Carlo constant rate γ-test will provide information about the completeness (or otherwise) of sampled lineages in the data set. A plot representing the computed Pybus and Harvey γ statistic through time is generated by slicing the tree in 100 points, with the function gtt in phytools, to facilitate the identification of the most influential evolutionary periods in the measured value of γ.

M.10. METHODS FOR ANCESTRAL AREA RECONSTRUCTION AND BIOGEOGRAPHICAL ANALYSIS (CHAPTER 3)

The ancestral area reconstruction and biogeographical analyses were performed on the cropped and pruned Maximum-Credibility

Clade tree from the Bayesian posterior distribution. Two analyses were conducted, one retaining *Hesperocodon* as an outgroup and other "only *Jasione*". As no major difference on the estimation of the ancestral area was detected, only the results of the latter analysis are shown. Six areas were considered: (A) north-western Africa; (B) the southern Iberian Peninsula; (C) northern and Central Iberian Peninsula, including the Pyrenees; (D) intermediate Europe including Italy and all of central and northern Europe; (E) the Balkan Peninsula; and (F) the Anatolian Peninsula (Figure 3.2). The biogeographical analysis was performed in R v.4 (R Core Team, 2021) with the package BioGeoBEARS (Matzke, 2013).

This R-package implements six biogeographic models in a common likelihood framework: a likelihood version of Dispersal-Vicariance analysis (DIVALIKE; Ronquist, 1997), LAGRANGE Dispersal and Extinction Cladogensis (DEC model, Ree *et al.*, 2005; Ree & Smith, 2008), a likelihood version of BayArea (Landis *et al.*, 2013), and an alternative version for each of the models that includes founder-event speciation (+J). BioGeoBEARS has two primary advantages over other biogeographical programs: (1) the best model is selected with likelihood ratio tests, and (2) founder-event speciation is included, a process ignored by most other methods.

M.11. METHODS FOR ENVIRONMENTAL NICHE MODELLING (CHAPTERS 3 AND 4)

In chapter 3, Species distribution modelling (SDM) was performed to compare niche suitability inferred from the potential distribution of the western and eastern groups under current climate conditions. Only taxa diverged from the common ancestor immediately older than the initial divergence leading to the E-W disjunction were included in the analysis. The approach used here pooled the occurrence data into two sets, the eastern and western groups. These groups are phylogenetically and geographically defined. Pooling of closely related species is one of the options to consider when most of the taxa are narrow endemics with small sample sizes (Ahmadi *et al.*, 2021). A number of taxa of the

eastern and western groups have < 5 occurrences, once the data are thinned to avoid spatial autocorrelation. To diminish excessive contribution of those taxa with more occurrences (e.g. *Jasione sessiliflora* in the west or *Jasione orbiculata* var. *bosniaca* in the east) no taxon had more than 6 occurrences in the pooled data. Nevertheless, the results should be interpreted with caution as pooling can lead to inflation of the predicted niches in each group (Hernández *et al*., 2006). The "eastern group" included the Balkan-southern Italian *Jasione orbiculata* complex, the Anatolian *Jasione supina* complex and those populations of the Balkan-Anatolian *Jasione heldreichii* complex, including populations assigned to *Jasione jankae* that were recovered in the previous clade (see Results). The purely annual populations of *J. heldreichii* recovered in a different clade are not included in any of the groups. The "western group" included the northern African *Jasione cedretorum* and the populations assigned to *Jasione crispa* subsp. *lanuginella* and *Jasione crispa* subsp. *mesatlantica* and all the south-western European groups of *Jasione* sect. *Jasione*, and it excluded the annual-biennial *Jasione montana* and *Jasione maritima*, diploid and tetraploid lineages as forms related to the recently evolved *J. montana* lineage. The entropy algorithm implemented in MAXENT v3.3 (Phillips *et al.*, 2006) was used for suitability modelling. The area of study includes Europe and the Mediterranean region. The occurrence was thinned to 5 km to prevent spatial autocorrelation. Nineteen bioclimatic variables for the area of study with current climatic conditions were downloaded from the WorldClim repository (Hijmans *et al.*, 2005). Seven uncorrelated variables were selected: Bio1 (annual mean temperature), Bio3 (isothermality), Bio4 (temperature seasonality), Bio7 (temperature annual range), Bio9 (mean temperature of the driest quarter), Bio12 (annual precipitation) and Bio 19 (precipitation of the coldest quarter). These variables were used as model calibration predictors in MAXEN. Randomly selected occurrences (75%) from each geographic group were used as training data and the remaining occurrences (25%) were used as test data.

In chapter 4, environmental modelling used nineteen layers of WorldClim bioclimatic variables (WorldClim website), in addition to

five edaphic layers (Table 4.1) reported in the public database ISRIC world soil information (Hengl *et al.*, 2017), because of the importance of the soil factor in the distribution of plant species belonging to acidophilic lineages. For example, soil pH is used as a proxy for the presence of cations in the region, although the spatial pattern of this variable in the environmental layer used is greatly influenced by precipitation (Hengl *et al.*, 2017). In theory, this does not detract from the ecological significance of the variable, as precipitation tends to facilitate the creation of organic soils and leaching of basic cations. However, the soil pH in water is measured at 100-200 cm depth, relatively close to the bedrock, and is indicative of the nature of the substrate. The apparent density provides information about the water storage capacity of a soil, as well as about the structural conditions and degree of compaction (Mukhopadhyay *et al.*, 2019).

The resolution of the climatic and edaphic environmental layers is different (i.e. the pixel size of the data is different), being around 1 km (30" of arc) in the first case and 250 m^2 in the second. In order to combine environmental raster layers in ecological modelling processes, they must have the same resolution, as well as the same geographic extension and cartographic projection. The edaphic layers were adjusted to the resolution of the environmental layer with the R package "raster". The variables finally chosen were selected for their ecological significance in relation to the taxa and because of the low collinearity between them, as indicated by Pearson's correlation coefficients < 0.8. For this purpose, a correlation matrix was generated in R (R Core Team, 2020) using the "gridExtra", "rasterVis" and "reshape2" packages. Two sets of variables, i.e. climatic only and climatic plus edaphic variables, were used

The "spThin" package (Aiello-Lammens *et al.*, 2015) reduces the number of presences until there is only one registered point per information pixel, thus eliminating those points for which the distance was less than 5 km in *J. crispa* subsp. *mariana*, *J. sessiliflora* subsp. *sessiliflora*, and *J. crispa* subsp. *tomentosa*. By contrast, in *J. sessiliflora* subsp. *appressifolia*, the minimum distance between

presences was established at 1 km due to the low number of initial data points. One hundred repetitions were generated for each species, to check whether the number of records remained stable.

The models were constructed with the R Biomod2 package and using Generalized Linear Model (GLM), Generalized Additive Models (GAM), MAXENT and Random Forest (RF), thus enabling the species records to be related to climatic variables. For this purpose, 4 validations and 3 replications of 5000 pseudo-absences per algorithm were run, creating a total of 48 models for each species. GLM and GAM are both regression methods that use the presence and absence of data. However, GAM, unlike GLM, can generate a non-linear model. MAXENT and RF are machine learning methods based on the presence of data on a background. MAXENT is based on the principle of maximum entropy for those models with a more uniform distribution (Philips & Dudik, 2008). The models created with the different algorithms for the taxa under study were evaluated with 20% of the data, and the remaining 80% of the data was used for calibration (Guisan & Zimmerman, 2000). The models were evaluated by threshold-independent statistical analysis: true skill statistic (TSS) and receiver operating characteristic (ROC). Models with TSS and ROC values > 0.7 were accepted, as this indicates that the predictions are more realistic than would result by chance. The different individual models were coupled creating consensus models. This assembly technique uses the weighted average and average statistical values to select the prediction that is closest to the actual observed data (Araújo & New, 2007). The coefficient of variation of the ensemble models was also calculated.

For the model projections, the program QGIS 3.14 (QGIS Development Team, 2020) was used by loading the raster environmental maps, the study environment and the author's own data. SDM maps with inferred grading of presence-absence optics were thus created.

Niches of the four taxa were visualized in the environmental space using environmental PCAs (PCAenv), an ordination approach

calibrated with environmental values of the area of study (Di Cola *et al.*, 2017) using the R package "ecospat" (Broennimann *et al.*, 2012). The PCA computes the occurrence density and environmental factor density for each pixel along environmental axes, maximizing the environmental variance of the distribution ranges of each taxon. PCA scores of the occurrences were projected onto a grid of cells bounded by the maximum and minimum PCA scores, allowing the comparison of niches in terms of breadth and location within the environmental space of the area of study (Di Cola *et al.*, 2017).

As a measure of niche overlap, the D value of Schoener (1970) was selected as a metric that varies between 0 (indicating no overlap between niches) and 1 (indicating complete overlap). The D values observed between pairs of species were used to test niche similarity (Warren *et al.*, 2008; Broennimann *et al.*, 2012). The niche similarity test determines whether the observed overlap value (D) differs from the overlap between the observed niche in the distribution of one species and randomly selected niches in the distribution of the other species. In this case, the centre of the mesh density is randomly selected from the available environmental space in the opposite distribution (Broennimann *et al.*, 2012). The significance value was evaluated using 100 randomizations ($\alpha = 0.05$). Niche similarity was tested for all pairs of taxa with the ecospat package. PCAenv and similarity tests were performed only with climatic variables to avoid enlarging the niche breadth visualization with factors such as pH with low variation across recent temporal scales.

M.12. METHODS FOR CLONING THE ITS REGION OF THE NRDNA (CHAPTERS 5)

PCR products from between two and four individuals from each population were used for cloning procedures, except in some cases when the source material was an herbarium specimen and only one individual was used. PCR amplification products were ligated into the pCR2.1-TOPO vector and cloned according to instructions of the TOPO-TA cloning kit (Invitrogen, Carlsbad, Germany). Plasmid DNA

from 10 separated competent cells colonies from each original PCR product was isolated using the S.N.A.P MiniPrep kit (Invitrogen, Carlsbad, Germany). DNA Sanger sequencing was carried out in at least one direct PCR product from almost all populations and in the then isolated DNAs from each population, with an 8-capillary 3500 analyzer (Applied Biosystems) by the DNA sequencing service of the University of Santiago de Compostela.

Sequences were initially aligned in BioEdit using the MUSCLE algorithm (Edgar, 2004), although manual editing performed equally well. Pseudogenes chimeras and recombinant sequences were exclude from the analysis. They were easily identified by comparison with those sequences obtained by direct PCR sequencing. GC content, extreme divergence, within single sequence mix of ribotypes and rarity where used criteria. Only those combinations of polymorphism occurring in at least three cloned sequences, or two cloned sequences plus one direct PCR product, even in the form of double peak, were considered true ribotypes and included in the analyses.

M.13. METHODS FOR POPULATION GENETICS AND PHYLOGEOGRAPHY (CHAPTERS 5)

Phylogenetic reconstruction was conducted by a median-jonining network in PopART v.1.7 (Population Analysis with Reticulate Trees) considering the six main Iberian Mountain ranges occupied by *Jasione crispa* subsp. *crispa*, to simultaneously represent phylogenetic relationships, ribotypic frequencies and geographic distribution. The considered six main mountain ranges are the Cantabrian-Leonese Mountain range, the Central System, the Moncayo in the Iberian System, the Pyrennes, the Montseny Mountain Range and Mont Caro.

DnaSP v6.1 (Rozas *et al.*, 2017) was used to calculate Haplotypic diveristies (Hd), interpreted as ribotypic diversity, and Pi, nucleotide diversity. An Analisis of Molecular Variance (AMOVA) was performed to estimate the relative contribution to genetic variances of different sets of samples (Excoffier *et al.*, 1992). The AMOVA was

performed in R version 4.1.0 (R Core Team, 2021) with the packages apex, adegenet, pegas, mmod, poppr, and ade4. The analysis was set to consider differences between regions, that is, the six main mountain ranges, between populations within mountain ranges and within populations, following a stratified approach. Randomization Monte-Carlo tests with nreps = 1000 were performed in the R package ade4 to assess the significance of the AMOVA results revealed for the different levels of strata (Grünwald & Hoheseil, 2006).

Pairwise genetic differentiation coefficients (GammaSt) were calculated in DnaSP v6.1 to estimate the relative importance of gene flow among the six Mountain ranges. Gene flow was calculated as Nm by the equation Nm = (1/GammaSt−1)/2 (Zhu *et al.*, 2019).

The Nst nucleotide-based statistics (Lynch &Crease, 1990) and the net number of nucleotide substitution per site (Da) were calculated in DnaSP v6.1 between every population and the remaining set of populations, to estimate the frequency of gene flow affecting each population.

M.14. METHODS FOR PLANT AND SOIL EXPERIMENTS AND ELEMENT MEASUREMENTS (CHAPTER 5)

Jasione crispa subsp. *crispa* plants from two Mountain ranges, Pyrenees and Central System, were micropropagated *in vitro* and outdoor acclimated simultaneously with the Mont Caro plants (M. Serrano, own data), in order to have completely comparable individuals.

Pyrenees and Central System are representative of siliceous populations and Mont Caro of the new calcicole population. A total of 126 plants were used, 42 individuals representing each Mountain Range, were cultivated for five months on three substrates with different chemical characteristics. The three substrates used were, (1) a soil of basic nature from the limestone outcrops of the Serra do Courel (Lugo), here called “Courel”, (2) a soil formed on a granite substrate

from a natural environment in Santiago de Compostela, here called "Pedroso", and (3) a commercial COMPO SANA® substrate, composed of peat, perlite, an amorphous silicate fertilizer enriched with phosphorus, lime and some nutrient elements, to which an extra calcium and magnesium amount was added in the experiment, here called "Composana".

Natural soils from the original population to avoid adaptation and performances related to factors other than soil acid/basic chemical nature, like soil biota or compounds of organic origin from the surrounding plant communities.

Plant phenotypical performance was measured as segmented (root, stem and leaves) final biomass and colour changes measured on leaves of living plants with a colorimetric technique adapted for plants with small leaves developed using *Jasione* as model group (Sanmartín *et al.*, 2020). Soil analyses consisted of measuring pH in water, organic matter, total elements, exchangeable cations and effective CEC. In plants, the total elements in root, stem and leaves were measured, fractions that were separated after determining the total biomass per surviving individual. All measurements were performed in triplicate.

Soils were air dried and passed through a 2-mm sieve before analysis. Soil pH was determined in water and 1M KCl suspensions with 1:2.5 sample: solution ratios. Soil total organic matter was determined by weight loss on ignition of dried ground soil at 450 °C. Total elements from soils and plants were extracted after digestion of dried ground sample with aqua regia (HCl and HNO3 in 3:1 ratio) and the elements were analyzed in the extracts by Inductively Coupled Plasma Spectrometry, ICP-MS (Varian 820-MS), equipped with collision reaction interface (CRI) technology to reduce polyatomic interferences. In soils, exchangeable Al was determined after extraction with 2 N KCl (Bertsch & Bloom, 1996). Cation exchange capacity (CEC) and exchangeable bases were determined after saturation of the soil with 1 N ammonium acetate and washing with 1 N KCl (Pansu & Gautheyrou, 2006). Exchangeable Ca, Mg, Na and K were measured in

the ammonium acetate extracts by flame atomic absorption/emission spectrophotometry (Varian FS220).

M.14. METHODS FOR COLOUR MEASUREMENT (CHAPTERS 5 AND 7)

In chapter 7, the colour was measured with a portable spectrophotometer Konica Minolta CM-700d, a contact-type colour measuring device, commonly used in scientific works. A spectrophotometer is a specific type of spectrometer, designed to measure light over the visible and near-visible portion of the electromagnetic spectrum, i.e. from 360 nm to 740 nm. It uses an optic set up of diffuse illumination geometry (recommended by the CIE, "International Commission on Illumination") accomplished by an integrating sphere covered with a white reflective coating and with 8°/diffuse illumination/viewing geometry (Figure 7.3). The sphere acts both as a means of producing a diffuse light source through a series of reflections off the white sphere wall, and for collection of light from the sample surface, placed in the entrance or sample port. The spectrophotometric method utilizes multiple sensors to measure the spectral reflectance of the sample. The sensor's electronics then calculate the tristimulus values from the spectral reflectance data using integration. The device is equipped with the CM- S100w (SpectraMagicTM NX) software.

The measurements were made in the specular component included (SCI) mode, in which the gloss trap of the spectrophotometer is closed, includes the total reflectance (considering both specular and diffuse reflections), with illuminant D65, observer 2°, and both circular aperture masks: an aperture for medium area view (MAV) with a diameter of 11 mm, measuring a circular area of 8 mm of diameter, and an aperture for small area view (SAV) with a diameter of 6 mm, measuring a circular area of 3 mm of diameter. In addition, eight types of adaptors, four for each aperture mask, have been designed and realized in cardboard: in black or white colour, and decreasing the tested surface by 50% (hereinafter called "black 50%" and "white 50%", respectively) or 70% (hereinafter called "black 70%" and "white

70%", respectively) (Figure 7.4). By employing the adaptors, the sample port opening (hole) is reduced to 50% or to 70% by the template, which expand the sphere wall (Figures 7.3 and 7.4).

Colour measurements were analyzed by considering the CIELAB colour system (CIE 1986), the most widely accepted by both industry and scientific community. It represents each colour by means of three scalar parameters or the Cartesian coordinates: L*, lightness or luminosity of colour, which varies from 0 (absolute black) to 100 (absolute white); a*, associated with changes in redness- greenness (positive a* is red and negative a* is green); and b*, associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue).

Five healthy leaves from each species of Campanulaceae have been randomly picked and, on the surface of each of them, five points have been randomly selected to measure the colour, following previous works (Sanmartín *et al*., 2011a, 2011b). Although the aim of the work is not to define the species colour variability, the number of small leaves and of measurements in each leaf was high enough to average the colour variation in small leaves of the Campanulaceae species, in agreement with previous works that analyzed cumulative averages for parameters L*, a* and b* on heterogeneous surfaces (Prieto *et al.*, 2010; Sanmartín *et al*., 2011a, 2011b). Five different set-up for each of the two aperture masks, MAV and SAV, were used to perform the colour record: no adaptor, black 50%, white 50%, black 70% and white 70%. The colour values obtained from the reduced area have been compared with the recorded colour without adaptor, using colorimetric criteria and statistical tools.

Because the dishomogeneus pattern of the vein network, the convexity of the leaf surface, and the intrinsic features of each leaf in each plant may affect the determination of colour, it should be established if the new method developed and here proposed can be extended to other plant samples. In order to address this concern, four patches homogenously coloured in green (green, foliage, yellow green and bluish green) from the color-checker colour rendition chart with 24

colour patches by Gretag Macbeth (McCamy *et al.*, 1976; Sanmartín *et al.*, 2014) were measured with the same protocol used for the leaves, as described in the previous paragraph.

Partial (ΔL^*, Δa^*, Δb^*) and total (ΔE^*_{ab}) colour differences have been calculated in absolute values with the following equations (1-4):

$$\Delta L^* = L^*_i - L^*_x \quad (1)$$

$$\Delta a^* = a^*_i - a^*_x \quad (2)$$

$$\Delta b^* = b^*_i - b^*_x \quad (3)$$

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (4)$$

where L^*_i, a^*_i, and b^*_i are the colour parameters by using the adaptor and L^*_x, a^*_x, and b^*_x are the same parameters by using no adaptor. Here, the threshold of 3 CIELAB units has been adopted as limit of rigorous colour tolerance or noticeable by an observer with normal colour vision, as in several other studies (Catrysse *et al.*, 1999; Martínez *et al.*, 2001; Molada Tebar *et al.*, 2018; Sanmartín *et al.*, 2012, 2014; Vrhel *et al.*, 1994).

M.15. MULTIVARIATE STATISTIC METHODS IN COLORIMTRIC EXPERIMENTS (CHAPTER 7)

In chapter 7, for a robust evaluation of the effect of the adaptors on the colour measurements, the collected data have been analyzed with three different multivariate approaches: Principal Coordinates Analysis (PCoA), Quadratic Discriminant Analysis of multiple groups (QDA) and a combined approach based on k-means and agglomerative clustering analyses. PCoA was also applied to the measurements obtained from the four patches (green, foliage, yellow green and bluish green) of the Gretag Macbeth color-checker colour rendition chart.

With the PCoA analysis the contribution from each adaptor type (black or white, 70% or 50% reduction, or no adaptor) on both MAV

and SAV to the colorimetric information (L*, a* and b*) was assessed. For each species, data were standardized by subtracting the mean of measurements with no adaptor to preserve the units of deviation from the actual colour and lay out all measurements in the same multivariate space. The measurements were distributed in a 2-dimensional Euclidean ordination space using ggplot2 and Stat R packages (Wickham, 2009).

To assess the similarity of the measurements obtained with and without the different tested adaptors, a QDA was performed. The individual measurements were assigned to predefined or actual groups (SAV and MAV with different adaptors or without adaptors), and the adaptor type most alike to SAV and MAV (i.e., those whose observations are significantly misclassified as observations obtained from SAV and MAV with no adaptors) were quantitatively identified from the results of the QDA confusion matrix. The analyses were run separately for SAV and MAV measurements and, in order to compare all species together, data were standardized by subtracting the mean of measurements, respectively from SAV or MAV with no adaptor. A Leave-Out-Out cross-validation was performed after the analysis to validate the QDA. Multivariate normality was assessed with the MVN R package (Korkmaz *et al.*, 2014) and the MASS R package was used for the remaining analysis (Venables & Ripley, 2002).

To verify the results pointed out by the QDA, an approach combining k-means and agglomerative clustering was performed on the same data from SAV, treated as for QDA. By k-means cluster analysis, similar colour measurements were categorized by defining clusters to minimize the total intra-cluster variation. The optimal number of k-clusters in each species was determined using 30 indices provided by the R package NbClust (Charrad *et al.*, 2014). The agglomerative clustering was conducted by computing a dissimilarity matrix based on Euclidean distances and the Ward.D2 agglomeration method in the Stats R package to produce a hierarchical tree for each species (R Core Team, 2020). The identified k-means groups were then superimposed on the dendrogram and, by means of the pvclust R package (Suzuki *et al.*, 2006), the statistical support of all clusters was calculated with

approximately unbiased (AU) *p*-values by multiscaling bootstrap resampling. Clusters with AU *p*-value equal to or greater than 95% were considered strongly supported by data.

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Results

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CHAPTER 1.
Nomenclature and typification of the *Jasione* L. (Campanulaceae) groups from the eastern Mediterranean basin

ABSTRACT

In this research all the published names of *Jasione* in the species, subspecies and variety ranks for populations from the Balkan and Anatolian peninsulas are reviewed, including names of taxa allied to these groups in surrounding areas. The taxonomic status and typification of the names are addressed. In total, eleven lectotypes, one lectoneotype and one epitype are suggested.

Important note: This chapter is not an effective work of typification, according to the article 29 of the International Code of Nomenclature for algae, fungi, and plants this PhD dissertation this work is not an effective publication since it will not meet the required criteria. Thus, this work will not be published or printed in paper and distributed (through sale, exchange, or gift) to the general public or to scientific institutions with generally accessible libraries. Similarly, this work will not be distributed as a Portable Document Formant (PDF) in an online publication with an International Standard Serial Number (ISSN) or an International Standard Book Number (ISBN). Finally, and advice is included after all suggested types that the actual designation of types will be published elsewhere in a future effective publication.

1. INTRODUCTION

The genus *Jasione* L (Campanulaceae) is distributed around the Mediterranean basin and throughout Europe, with two centres of diversity at the opposite sides of the Mediterranean Sea, one in the Iberian Peninsula and Morocco and another in the Balkan and Anatolian peninsulas, consistent with the West-East Mediterranean disjunction biogeographical pattern (Thompson, 2005; Tarkhnishvili, 2014). The genus occurs in a number of ecological conditions and geological substrates across a wide elevational range, including coastal and alpine habitats. The combination of environmental niche diversity, complex geological history of the Mediterranean basin and ploidy variation, which includes five ploidy levels (Küpfer, 1981; Lammers, 2007; M. Serrano, own data), coupled with relative stasis around a general morphological pattern (i.e. medium-sized or small plants with condensed inflorescences in flower heads surrounded by involucral bracts and small flowers with connate anthers), has produced a large number of polymorphic populations frequently lacking clear morphological distinctiveness. In consequence, many names and different taxonomic ranks have been used for members of the genus (e.g. Sales & Hedge, 2001a; Lammers, 2007; Dobignard & Chatelain, 2011). However, most of these names are of unknown evolutionary significance (Pérez-Espona *et al.*, 2005), leading to difficulties in species delimitation by morphological approaches (Kovanda, 1968). General treatment of the genus thus tends to be overly summarized, with the number of species recognized varying between 12 (Sales & Hedge, 2001b) and 17 (Castroviejo *et al.*, 2010). The evolutionary lineage of *Jasione* seems to be prone to micro-endemism and cryptospeciation, and many of the historically described forms, despite being disregarded by current taxonomic treatments, may therefore deserve further attention. Thus, a correct nomenclatural understanding of the clutter of names described so far is particularly important in studies dealing with evolutionary relationships, species delimitation and biodiversity conservation in such complex groups.

Two important nomenclatural summaries have been provided for the centre of diversity of *Jasione* in areas of the western Mediterranean region. Dobignard & Chatelain (2011) reviewed the synonym names

published for North African populations, while Sales & Hedge (2001a) tackled the revision of the published names and nomenclatural types of the taxa occurring in the Iberian Peninsula. Parnell (1987) had previously addressed the nomenclature of *Jasione montana* L. and other taxa that he considered related, dealing with a huge number of published names associated with the wide range and considerable polymorphism of *J. montana*, which occurs in north-western Africa and most of Europe (although it is almost absent in the Balkan area). These works were later complemented by nomenclatural precisions in the Iberian-North African *Jasione corymbosa* poir. ex Schult complex (Ferrer-Gallego *et al.*, 2015).

By contrast, there are no recent published revisions for the eastern centre of diversity of the genus. The most recent comprehensive work dealing with *Jasione* in the Balkan and Anatolian peninsulas is that by Stojanov (1926), with a taxonomic treatment recognizing 13 species and varieties, one from the Italian peninsula. This author addressed the alpine taxa of what he called the *Jasione supina* Sieb. ex Spreng. group, a number of mostly narrow endemic orophytes occurring across the different mountain ranges in both peninsulas. This group should include the *Jasione supina* complex, the *Jasione orbiculata* Griseb. ex Velen. complex and *Jasione bulgarica* Stoj. & Stef. Nevertheless, the evolutionary coherence of this putative group and its internal relationships remain unknown. Stojanov (1926) did not consider the *Jasione heldreichii* Boiss. & Orph. complex, the other remaining group occurring in the Balkan and Anatolian peninsulas. Parnell (1987) only partly addressed the nomenclature of this complex, describing one variety occurring on the Turkish coasts. A wider study is required that would encompass the nomenclature of the group, within which diverse names with difficult taxonomic interpretation have been described (Hartvig, 1991).

Tutin (1973) subordinated *Jasione orbiculata* as a subspecies of *Jasione laevis* Lam. in his treatment of Flora Europaea (Tutin, 1976). This rather questionable view overshadowed all taxa described or recognized by Stojanov (1926) within this species, probably influencing later general treatments that only listed *Jasione orbiculata* (Greuter *et al.*, 1984; Lammers, 2007; Castroviejo *et al.*, 2010). The fact that these taxa

have the rank of variety has also hampered their modern recognition, although some of them have been included in regional floras (e.g. *J. orbiculata* subsp. *balcanica* Urum. in Ančev, 2012).

Damboldt (1976, 1978) tackled the taxonomic diversity of the Anatolian *Jasione* orophytes, recognizing two species in this group, i.e. *Jasione idaea* Stoj. and *Jasione supina*, the latter of which has four subspecies. He designated nomenclatural types to several names in the Flora of Turkey and the East Aegean Islands (Damboldt, 1978), although in the opinion of the current author a formal typification of some of them is still lacking.

The research presented here aims to present a nomenclatural revision of the genus *Jasione* in the Balkan and Anatolian peninsulas, the eastern centre of diversity of the genus, and complements the current author's studies on taxonomy and evolutionary relationships in the genus *Jasione.* The nomenclatural situation of the Balkan and Anatolian taxa of *Jasione*, including the *Jasione heldreichii*, *Jasione supina* and *Jasione orbiculata* taxonomic complexes, are addressed by summarizing synonyms and suggesting types that will be effectively designated in a future valid publication. The application of the many names described from the aforementioned regions plus one from southern Italy is discussed.

2. MATERIAL AND METHODS

Currently accepted names, including homotypic and heterotypic synonyms, those listed in the Med-Checklist (Greuter *et al.*, 1984), the World Checklist and bibliography of the Campanulaceae (Lammers, 2007) and the Euro+Med Plantbase (Castroviejo *et al.*, 2010) were considered. Other names were obtained from diverse sources (see below), mainly Stojanov (1926) and Damboldt (1978). The protologues and original material of names were analysed in detail and those names lacking a nomenclatural type were typified in accordance with the International Code of Nomenclature for algae, fungi, and plants (Turland *et al.*, 2018). The following herbaria were consulted: B, BR, E, G, FI, K, MPU, P, SO, SOM, GOET and W.

3. NOMENCLATURE AND TYPIFICATION OF THE *JASIONE* COMPLEXES FROM THE EASTERN MEDITERANEAN BASIN

All names described for *Jasione* in the Balkan and Anatolian peninsulas at the species, subspecies and variety ranks were analysed. Thus, the members of the *Jasione orbiculata* complex were included, and the representative of the complex in southern Italy is therefore also treated here. The review presents basionyms in alphabetical order, structured as a discussion of each name followed by its synonyms and typification, suggesting types when required, that will be designated as types in a future effective and valid publication. A list of currently accepted names from recent checklists (e.g. Lammers, 2007) is provided, and the heterotypic synonyms are indicated (Table 1.1). Inclusion of a name in the first column of Table 1.1 only reflects its situation in current general lists, as recognized names may be organized differently after ongoing evolutionary studies on *Jasione*. Those names that would deserve recognition in light of such studies are highlighted in colour in the second column.

Table 1.1. Names currently accepted in general treatments are ordered alphabetically in the first column and marked in bold, with different basionyms indicated. The second column lists the heterotypic synonyms. The names of evolutionary entities deserving recognition are highlighted in blue. Those names that may represent significant evolutionary entities, but for which more detailed studies are required, are highlighted in green.

Accepted name	Heterotypic synonym(s)
***J. bulgarica* Stoj & Stef.** ≡ *Jasionella bulgarica* (Stoj. & Stef.).	= *Jasione orbiculata* var. *orbelica* Vel.
***J. heldreichii* Boiss. & Orph.)** ≡ *Jasione montana* L. f. *heldreichi*i	= *J. glabra* Vel. = *J. heldreichii* var. *microcephala* Vel. = *Jasione heldreichii* var. *papillosa* J.Parn. = *J. montana* var. *dentata* f. *microcephala* (Vel.) Stoj & Stef. = *Jasione jankae* Neilr. = *Jasione montana* var. *dentata* DC
***J. idaea* Stoj.**	

***Jasione orbiculata* Griseb. ex Vel**	**= *Jasione orbiculata* var. *balcanica* Urum.** **= *Jasione orbiculata var. bosniaca Stoj.*** **= *Jasione orbiculata var. italica Stoj.*** **= *Jasione orbiculata* var. *supinoides***
***Jasione supina* Sieber ex Spreng**	= *Jasione supina* var. *hirtula* Stoj.
***Jasione supina* subsp. *akmanii* Damboldt**	
≡ ***Jasione supina* subsp. *pontica* (Boiss.) Damboldt** *Jasione supina* var. *pontica* Boiss (basionym)	= *Jasione pontica* var. *microcephala* Freyn in Bornmüller
≡ ***Jasione supina* subsp. *tmolea* (Stoj.) Damboldt** *Jasione* tmolea Stoj. (basionym)	

3.1. *Jasione bulgarica*

Stojanov & Stefanov (1921) used material from the Rila mountain range to describe this name, indicating in the protologue a flowering period of July to September in 1919 in Rila, but without citing any particular specimen in relation to the name. The protologue includes two illustrations, a photograph with six separated plants (protologue, Image 2 on page 106) that matches several aspects of the description, i.e. glabrous plants with upright stems and creeping roots, and a drawing (protologue, Image 3 on page 107) of a flower showing the character considered most remarkable by the authors, i.e. the free anthers (contrasting with the connate anthers in the remaining species of the genus). The current author has no evidence of a formal description of the type and has therefore selected one of the illustrations to designate a lectotype (to be effectively designated in a future valid publication), according to articles 8.1 and 9.12 of the International Code of Nomenclature. Given the importance the authors attributed to the character of the free anthers, the current author has chosen the second illustration (protologue, Image 3 on page 107) for this purpose. Several examples of the material collected by Stojanov & Stefanov from the

Rila mountains in July and September 1919 are conserved in SOM (e.g. SOM 75792, SOM 75780, SOM 75789, among others). It therefore seems reasonable to assume that the authors used at least some of this material to prepare the description, although they did not cite any of these gatherings in the publication. Given that the authors refer to both July and September 1919 in the protologue, only as an indication of the flowering phenology in Rila and not as specimen collections, no particular collection can be unambiguously associated with the illustrations. After careful analysis of the sheets in SOM, the current author could not match any of these specimens to the stems photographed in the first illustration of the protologue. Stojanov & Stefanov (1921) stated that they had seen sheets of the new species in the herbaria of V. Stříbrný & I. Urumov, labelled as, and sometimes mixed with, *Jasione orbiculata*. However, they did not cite any particular collection that could be chosen as the lectotype. Although in opinion of the current author, none of the specimens conserved in SOM can be designated as lectotypes, they make good candidates for designation as epitypes, as they complement the image in the protologue designated as the lectotype, according to article 9.9 of the International Code of Nomenclature. Therefore, the current author has selected as an epitype specimen SOM75792!, collected by Stojanov on July 25th 1919 in Malyovitsa (Мальовица) in the Rila mountain range (Figure 1.1).

The material suggested as lectotype would, logically, also be the type species of *Jasionella bulgarica* (Stoj. & Stef.) Stoj & Stef., and it is consequently the type species of *Jasionella* Stoj. & Stef (1933), a monotypic genus described on the basis of the free anthers in the flower as the main trait differentiating the genus from *Jasione* L.

***Jasione bulgarica* Stoj. & Stef.**, in Österr. Bot. Z. 70: 105 (1921) ≡ *Jasionella bulgarica* (Stoj. & Stef.), Фл. Бълг. изд. 2: 986 (1933) - Lectotype (will be designated in a future effective publication): [illustration] "*Jasione bulgarica* sp. n. Ganze Pflanzen, etwas verkleinert" in Stoj. & Stef., Österr. Bot. Z. 70: 106, Image 2 (1921).

Figure 1.1. Selected epitype of *Jasione bulgarica* (SOM75792).

3.2. *Jasione glabra*

Velenovský (1884) described this species from material collected in the vicinity of Varna, on the Black Sea coast in Bulgaria, sent to him by A. Javasof. The lectotype is held in PRC (PRC 451934), and it matches the protologue description of a glabrous plant without basal leaves, somewhat undulate stem leaves and bract teeth and apex apparently not ending in hard awns. This plant can be included in the variability of *Jasione heldreichii* Boiss & Orph., as considered by Velenovský (1891), who later lowered its rank by describing it as *Jasione heldreichii* var. *microcephala*. This author (*op. cit.*) also wrote that this name alluded to the same plant that he had described under the name *Jasione glabra*, adding a shorter morphological description, but similar to that included in the protologue of *J. glabra*, giving Varna, Bulgaria as the type locality. According to Art. 6.12 of the International Code, *J. heldreichii* var. *microcephala* Vel. is validated as a replacement name for *J. glabra* Vel., as it is only validated by reference to the earlier name. Thus, it is automatically typified by the type of *Jasione glabra*. The lectotype was designated by Ančev (2012) and is shown here in Figure 1.2.

***Jasione glabra* Vel.** In Oesterr. Bot. Z. 34: 424 (1884) ≡ *Jasione heldreichii* var. *Microcephala Vel.*, Fl. Bulg.: 374 (1891) ≡ *Jasione montana* var. *Dentata f. microcephala* (Vel.) Stoj & Stef., Фл. Бълг. Изд, 1,2: 1094 (1925). – Lectotype (designated by Ančev in Fl. Rep. Bulgaria 11: 158. 2012): Bulgaria, Varna, VIII, A. Javasof s.n. (PRC barcode PRC 451934 [photo!]).

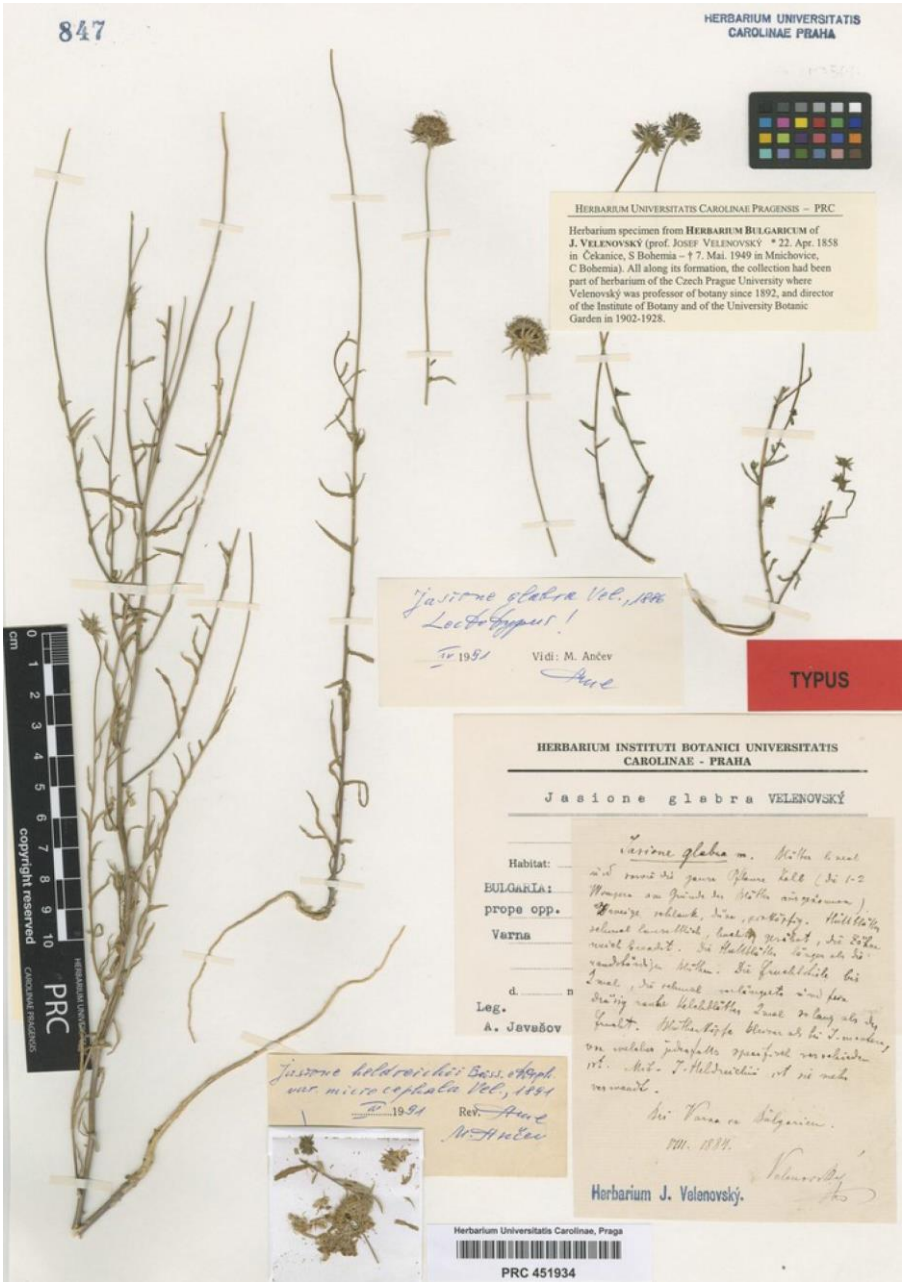

Figure 1.2. Lectotype of *Jasione glabra* (PRC451934).

3.3.*Jasione heldreichii*

The lectotype shows an annual-biennial habit and bracts with aristate teeth, matching the description in the protologue (Boissier, 1859) and the narrower concept used by diverse authors whereby *Jasione heldreichii* is only considered to be annual-biennial. Two main morpho-ecological patterns can be found within the *Jasione heldreichii* complex populations (without taking into account some deviant maritime forms): one of annual-biennial hairy plants without sterile rosettes, mostly occurring at mid-low elevations, and another of perennial glabescent plants with sterile rosettes and occurring at higher elevations or northern latitudes (Hartvig, 1991). Some authors avoid using the name *Jasione heldreichii* for the latter forms, preferring either *Jasione jankae* Neilr. (e.g., Hayek, 1931; Hartvig, 1991; Ciocârlan, 2006) or *Jasione dentata* (DC) Halácsy sensu Josifović (1974) non sensu orig. for these populations. Nevertheless, current treatments synonymize *J. jankae* under *J. heldreichii*, encompassing both annual-biennial and perennial plants (Tutin, 1976; Damboldt, 1978; Greuter *et al.*, 1984; Lammers, 2007; Ančev, 2012). Parnell (1987) designated the lectotype of this name by indicating "Macedonia, 1857, Orph. Lectotype: G!" Unaware of this, Hartvig (1991) apparently used material from the same collection to designate a lectotype, indicating "Macedonia, Mt. Korthiathi prope Thessalonicam, Orphanides 663 (G-Boiss!)". Examination of the type material in G clearly shows that the lectotype designated by Parnell (1987) belongs to a different collection (Orphanides nº 3130) than that used by Hartvig (1991). The former has the G barcode G60078161 and is shown here in Figure 1.3. Regarding the variability in *J. heldreichii*, the current author was able to observe in the type locality that some rare individuals can develop a few sterile rosettes. Indeed, there are some intermediate populations in regard to this trait (personal observations of the current author in Greece and Bulgaria), and populations with some individuals developing sterile rosettes and others without them have been recorded after cultivation of the plants and monitoring of their development (Turrill, 1926).

***Jasione heldreichii* Boiss. & Orph.**, Diagn. Pl. Orient. Ser. 2, 6: 120 (1859) ≡ *Jasione montana* L. f. *heldreichii* (Boiss. & Orph.)

Schmeja in Beih. Bot. Centralbl. 48(2):33 (1931) – Lectotype (designated by Parnell 1987: 265): Macedonia, 1857, Orph. nº 3130 (G barcode G60078161 [photo!]).

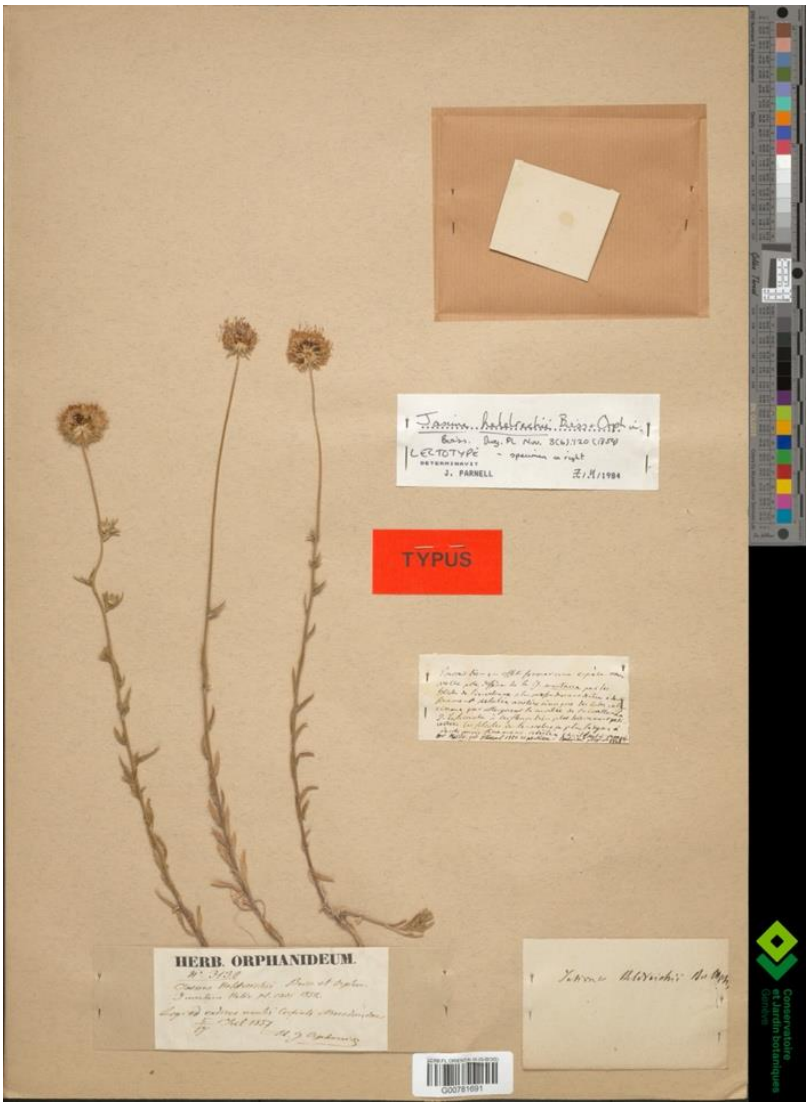

Figure 1.3. Lectotype of *Jasione heldreichii* (G60078161). © Conservatoire et Jardin botaniques de la Ville de Genève

3.4. *Jasione heldreichii* var. *papillosa*

Parnell (1987) used this name to encompass some morphologically deviant populations of the group *J. heldreichii* occurring on the Turkish European coast of the Black Sea. These plants are characterized by densely papillose bracts and leaves with thickened margins, and short-triangular calyx teeth. The Holotype, held in herbarium E, originates from the T. Uslu nº 2070 collection from Istanbul, Terkos Gölü. The specimen has the E barcode E00913661 and is shown here in Figure 1.4. Although these populations may simply be coastal ecotypes of *J. heldreichii*, the evolutionary significance must be studied in greater detail as they display some different traits from other dune populations of *Jasione heldreichii* from the Aegean and Black seas. *J. heldreichii* var. *papillosa* is remarkably distinct from the typical *J. heldreichii*, showing morphological convergence with populations of *Jasione montana* from sand dune habitats in the western Mediterranean region under similar climatic conditions (i.e. subhumid Mediterranean climate). Indeed, this is the reason for the inclusion of *Jasione montana* in the Flora of Turkey and the East Aegean Islands (Damboldt, 1978), an enduring incorrect interpretation of these populations (e.g. Çelemli, 2020).

***Jasione heldreichii* var. *papillosa* J.Parn.,** Watsonia 16 (3): 266 (1987) - Holotype: (Designated by Parnell 1987: 265): Turkey, Istambul, Terkos Gölü, Uslu nº 2070 (E barcode E00913661 [photo!]).

Figure 1.4. Holotype of *Jasione heldreichii* var. *papillosa* (E00913661).

3.5. *Jasione idaea*

This name was published by Stojanov (1926) to describe a narrow endemic species in the Kazdağı, the ancient Mount Ida, in northwestern Anatolia, using one collection of Sintenis (Iter trojanum, 1 Aug. 1993, nº 532). Damboldt (1978) addressed the typification of this name from this collection. However, he designated a holotype by indicating "holo. W! iso. B, M, E!, G! GOET, K! LD". Many specimens exist from the original collection, and the lectotype should be designated in accordance with article X.X of the International Code. Therefore, the current author suggested one of the many specimens from the original collection as the lectotype, the sheet in herbarium E with barcode E00913653 is shown in the Figure 1.5.

***Jasione idaea* Stoj.** in Notizbl. Bot. gart. Berlin 9: 556 (1926) - Lectotype (will be designated in a future effective publication): Turkey, Kazdağı (ancient Mount Ida) 1 August 1883, Sintenis nº532, iter Trojanum (E barcode E00913653 [photo!]).

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Figure 1.5. Lectorype of *Jasione idaea* (E00913653).

3.6. *Jasione jankae*

This name is listed as a synonym of *Jasione heldreichii* Orph. ex Boiss. in Flora Europaea (Tutin, 1976), the Med-Checklist (Greuter *et al.*, 1984) and in the World Checklist of the Campanulaceae (Lammers, 2007), although it is recognised in the Euro+Med PlantBase (Castroviejo *et al.*, 2010), as well as in different regional floras from the Balkan Peninsula (e.g. Velenovský, 1891; Hayek, 1931; Hartvig, 1991), while other floras consider it a synonym of *J. heldreichii* (e.g., Stojanov & Stefanov, 1948; Ghișa 1964; Ciocârlan,1990; Ančev, 2012). Neilreich (1870) described this species from material collected in the rocky areas of Treskovac, NW of Svinica, in Romania (current names in Romanian language, Trescovăţ and Sviniţa). In herbarium C (C barcode C10009017) there is a gathering collected by Viktor Janka in "rupestribus m. Treszkovácz inter Drenkova et Svinieza ad Danubium inferioren Banatus', on 4 July 1870 during his Iter Banaticum. The specimen is identified in the sheet as *Jasione jankae* Neilr. and two manuscript labels dated September 2004 are glued on it (Figure 1.6). One label, by I. C. Hedge, indicates that "the formal lectotypification of *J. jankae* will be published in due course", and the other, by S. Prince, indicates "Lectotype of *Jasione jankae* Neil. = *J. heldreichii* Boiss & Orph." The current author has no evidence of any subsequent formal publication and therefore has suggested this specimen as the lectotype. There is no doubt that this material was used by Neilreich to describe the species, as correctly pointed out by I.C. Hedge and S. Prince, as the label clearly conveys the location cited in the protologue and the specimen matches all points in Neilreich's description.

The status of *Jasione jankae* in relation to *Jasione heldreichii* has been controversial, even in some of the treatments that recognize it as different from *J. heldreichii* (Hartvig, 1991). Ciocârlan (2006) addressed the question by adopting the position of differentiated species, but provided little more evidence than the differing morphological features already described in the protologue. Thus, *J. jankae* may refer to plants similar to *J. heldreichii* but differing in the perennial habit with sterile rosettes, rather than to annual-biennial habit without rosettes in *J. heldreichii*, dentate bracts but with teeth half the

width of the bract teeth rather than pinnate-pectinate bracts with aristate apex. However, as intermediate individuals are found, a wider concept of *Jasione heldreichii*, including plants from mountain or northern areas where sterile rosettes are more frequent, may appear to be more consistent with the current state of knowledge. Nevertheless, more detailed studies are needed to ascertain whether the observed variation can be linked to independent evolutionary lines. Different degrees of introgression in *J. heldreichii* populations from current or past sympatric populations of representatives of the *J. orbiculata* and *J. supina* perennial groups should not be ruled out as a source of morphological and ecological differentiation within the *Jasione heldreichii* complex.

***Jasione jankae* Neilr.** Diagn. Aufz. Ungarn. Slavon, Gefässpfl., Nachtr.: 43 (1870) ≡ *Jasione heldreichii* Janka ex Nyman , Consp. Fl. Eur. 2: 486 (1879) ≡ *Jasione montana* var. *jankae* (Neilr.) Stoj. & Stef., Фл. Бълг. изд, 1,2: 1094 (1925) ≡ *Jasione montana* f. *jankae* (Neilr.) Schmeja in Beih. Bot. Centralbl. 48: 33 (1931) - Lectotype (will be designated in a future effective publication): Romania, Sviniţa, Trescovat peak. 4th July 1870, V. Janka s.n. (C barcode C10009017 [photo!]).

Figure 1.6. Lectotype of *Jasione jankae* (C10009017).

3.7. *Jasione montana* var. *dentata* DC.

De Candolle (1838) described this name from plants collected by P.M.R. Aucher-Eloy in the "Olympo Bithino", i.e. in the UluDag, Turkey. The G-De Candolle herbarium (GDC) holds a gathering collected by Aucher-Eloy from the type location in 1837, number 1880 (G barcode G00329536), matching the morphology described in the protologue (i.e. spiny-dentate acute bracts) shown here in Figure 1.7. The current author is not aware of any formal description of the type species of this name and has therefore suggested this specimen as the lectotype. Another sheet from the Aucher-Eloy nº 1880 collection in K (K barcode K000781177) would be an isolectotype. This name, and the more frequently recognized Halacsy's combination (1902) *Jasione dentata* (DC.) Halacsy has been used for plants from Anatolia and from the Balkan Peninsula (e.g. Halacsy, 1902; Josifović, 1974). Although the most commonly followed current treatments consider this name a synonym of *Jasione heldreichii* (Tutin, 1976; Ančev, 2012), more detailed evolutionary studies are required to clarify this point, given the complexity of the group.

***Jasione montana* var. *dentata* DC**. in Prodr. VII.:415 (1839) ≡ *Jasione dentata* (DC) Halacsy, Consp. Fl. Gre. 2: 280 (1902) - Lectotype (will be designated in a future effective publication): Turkey, Bursa province, Uludağ (Olympo Bithino), 1837, P.M.R. Aucher-Eloy 1880 (G barcode G00329536 [photo!]).

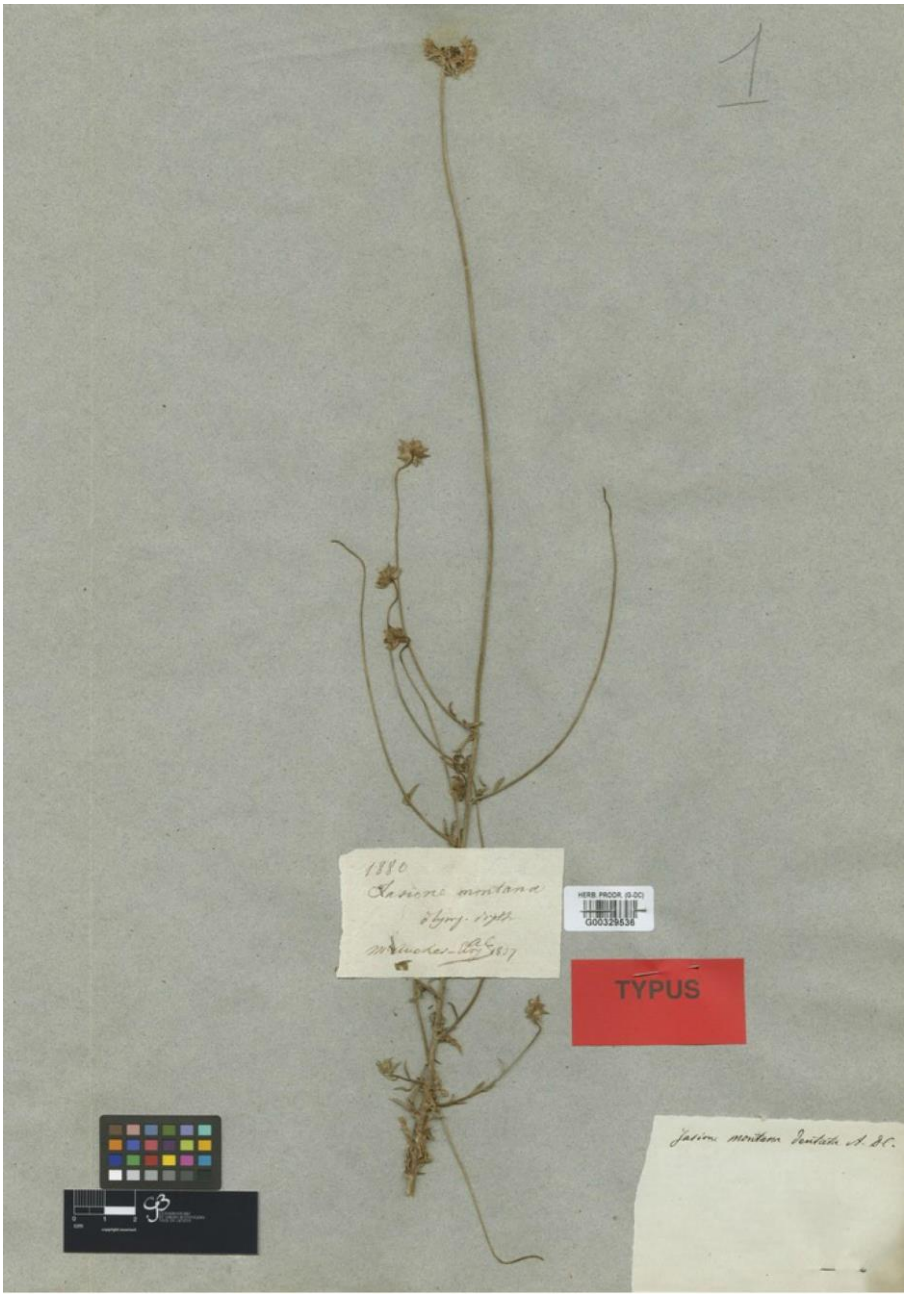

Figure 1.7. Lectotype of *Jasione montana* var. *dentata* (G0032953).

3.8. *Jasione orbiculata*

This name has been generally recognized at the species rank (Greuter *et al.*, 1984; Lammers, 2007; Ančev, 2012), despite its treatment in Flora Europaea subordinated as subspecies of *Jasione laevis* Lam. (Tutin, 1976), which has influenced some subsequent works (Hartvig, 1991; Andreev *et al.*, 1992). Grisebach (1844) created this name in the entry for *Jasione supina* Sieb. in his Spicilegium florae Rumelicae et Bithynicae, although without indicating the morphological features that characterize it and differentiate it from *J. supina*, and without associating this name with any particular location. In the entry he cited several Balkan and Anatolian regions and localities (Macedoniae, Bithyniae, Kobelitza, Peristeri, Olympo in Turkey) but without connecting the new name to any of them. Velenovský (1891) validly described the species, separating the Balkan plant from *J. supina*, recognizing the authorship by Grisebach and explicitly referring to the name proposed in the Spicilegium. Despite having consulted diverse herbaria, including GOET -the herbarium of reference of Grisebach -Velenovský himself (1891) did not see the original Balkan material from Peristeri and Kobelitza cited by Grisebach. Stojanov (1926) also did not cite this material, despite carrying out a huge revision of specimens of the group in different European herbaria. It may be necessary to designate a neotype for this name. However, the current author prefers a conservative approach, while continuing the search for the Grisebach material, leaving the name not typified for now.

***Jasione orbiculata* Griseb. ex Vel.**, in Fl. Bulg.: 375 (1891) ≡ *Jasione laevis* subs. *orbiculata* (Griseb. ex Vel) Tutin, in Bot. J. Linn. Soc. 67: 277 (1973).

3.9. *Jasione orbiculata* var. *balcanica*

This name has been considered by Lammers (2007) as a synonym of *J. orbiculata* s. str. and is not listed in general treatments (e.g. Greuter *et al.*, 1984; Castroviejo *et al.*, 2010), although it has been recognized by Ančev (2012). Urumov (1899) described this name, indicating two

localities and a year "In graminosis alpinis m. Jumruk-Cal, Mara-Gidik legit a. 1897", with the former location being in the Rila mountain range and the latter in the Central Balkan mountain range. The current author has found a gathering collected by Urumov in 1897 in Jumruk-Cal in the SOM herbarium (SOM75664) and that matches the description in the protologue, i.e. leafed ascendent stems, undulate-denticulate obtuse leaves, wide obovate bracts overpassing the flowers and subsessile flowers (Figure 1.8). The current author has not found any evidence of formal typification of this name and has therefore suggested this specimen as the lectotype.

***Jasione orbiculata* var. *balcanica* Urum.** Österr. Bot. Z. 49: 56 (1899) - Lectotype (will be designated in a future effective publication): Bulgaria, Rila mountain range, Jumruk-Cal, 1897, I.K. Urumov s.n. (SOM75664!).

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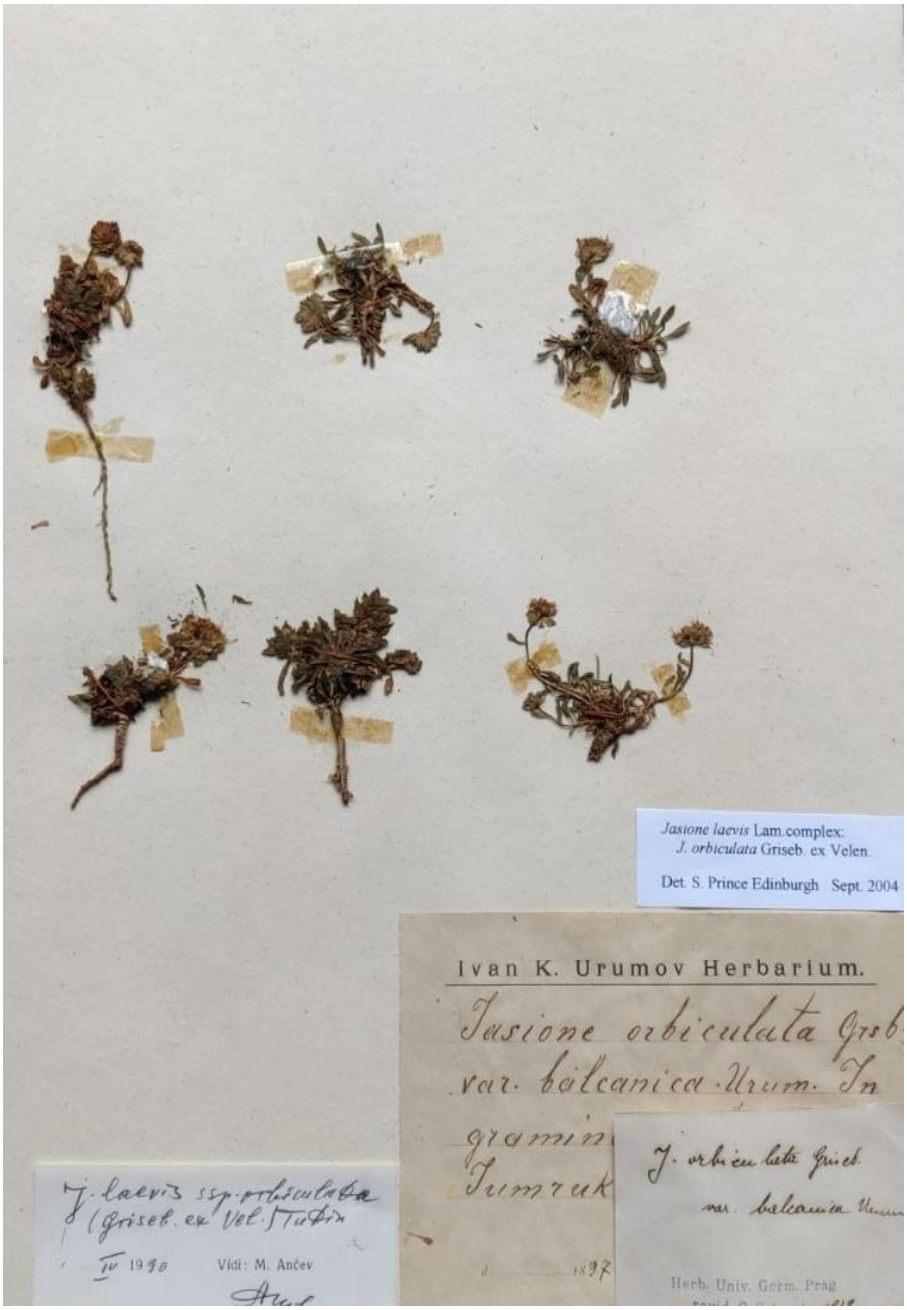

Figure 1.8. Lectotype of *Jasione orbiculata* var. *balcanica* (SOM75664).

3.10. *Jasione orbiculata* var. *bosniaca*

Like all of the varieties of *Jasione orbiculata* described by Stojanov (1926), this is listed as a synonym of *Jasione orbiculata* s. str. by Lammers (2007). Although this plant exhibits a remarkably different morphology than the typical *J. orbiculata*, use of this name is practically absent in the literature (Beck von Mannagetta, 1983; Nikolić, 2000; Barudanović *et al.*, 2007). Stojanov (1926) used diverse collections from a wide range to describe this name, including gatherings from NW Macedonia, Albania, Montenegro and Bosnia-Hercegovina. Among the diverse materials he cited in the protologue, there is one gathering collected by E. Brandis in Bosnia, from Vranika Sekira peak, 15 July 1892. There is one specimen (MPU barcode MPU016152) from this gathering in MPU. The sheet has a glued label, dated 2010 by P. R. Fabre, indicating "isosyntype of *Jasione orbiculata* var. *bosniaca*" (Figure 1.9). The current author has no evidence of a formal typification of this name and has therefore suggested this specimen as the lectotype. This gathering was used by Freyn to designate the name "*Jasione propullulans*" (Freyn & Brandis, 1888), which was invalidly published (see below, in 3.14).

Jasione orbiculata var. bosniaca Stoj. Notizbl. Bot. Gart. Berlin-Dahlem 9; 553 (1926) - Lectotype (will be designated in a future effective publication): Bosnia, Vranika Sekira peak, 1892, E. Brandis 2866. (MPU barcode MPU016152 [photo!]).

Figure 1.9. Lectotype of *Jasione orbiculata* var. *bosniaca* (MPU016152).

3.11. *Jasione orbiculata* var. *italica*

This name is considered a synonym of *Jasione orbiculata* s. str. by Lammers (2007) and is not included in the Checklist of the Italian vascular Flora (Conti *et al.*, 2005) or used in recent works addressing the conservation status of its populations (Fenu *et al.*, 2020), although it is recognized in Pignattti's Italian floras (Pignatti, 1982; Pignatti, 2018). This name refers to a narrow endemic restricted to the Sirino Massif, Basilicata, southern Italy (Conti & Di Pietro, 2004). Stojanov (1926) cited it in three gatherings included in the protologue, one from "Montis Serino del Papa", 14 July 1877 collected by Huter, Porta & Rigo, another from "Monte Serino, pr. Lago Negro", July 1877 collected by Huter, and another from "M. Serino" July 1898 collected by G. Rigo nº493 during his 4th Iter italicum. The current author has found sheets of the first gathering in the FI (FI barcodes FI064103, FI064104, FI064105 and FI064106) and P (P00270615, P00270751, P00270616) herbaria and from the third gathering in the E (E barcode 00913671) and P (P00270620, P00270621, P00279679) herbaria; all are identified as *Jasione supina* by the collectors. The current author has not found any evidence of formal typification of this name and has therefore suggested sheet FI064103 as the lectotype (Figure 1.10). The several individuals on this sheet match the protologue description of prostrate stems and large, wide bracts, a trait that led Stojanov to consider this variety akin to *J. orbiculata* var. *bosniaca*, a taxon with erect stems. Therefore, the sheet captures the variability of the taxon, including the abundance of rosettes on long creeping stems and the absence or scarcity of true stolons.

***Jasione orbiculata* var. *italica* Stoj.** Notizbl. Bot. Gart. Berlin-Dahlem 9; 554 (1926) - Lectotype (will be designated in a future effective publication): Italy, Basilicata, Massicio del Sirino, Monte Papa (in pascuis montis Serino, del Papa 2.000 m) 14 July 1977, Huter, Porta & Rigo, nº 473 (FI barcode FI064103 [photo!]).

Figure 1.10. Lectotype of *Jasione orbiculata* var. *italica* (FI064103). © Museo di Storia Naturale, FI Herbarium, Università degli Studi di Firenze

3.12. *Jasione orbiculata* var. *supinoides*

This name is considered a synonym of *Jasione orbiculata* s. str. by Lammers (2007) and is not recognized in regional floras or taxonomic lists (Hartwig, 1991; Dimopoulos *et al.*, 2013). Stojanov (1926) described the name from material collected in the Kaimaktsalan massif, on the border between North Macedonia and Greece, and from Smolikas peak in the Pindos mountain range in Epirus, Greece. However, as noted by Hartvig (1991), there is significant variation among the Greek populations, with mat-forming plants with creeping, rooting stems and ovate-rhomboidal bracts in the SW populations and more cespitose plants with narrower lanceolate-linear bracts in the northernmost Greek populations. While the epithet "supinoides" (i.e. resembling *J. supina* Sieb., a cespitose plant) would be suitable for populations from the mountains on the border between North Macedonia and Greece, the description in the protologue could encompass all of these Greek forms. However, the mention of oblongo-rhomboidea bracts is more consistent with the plants from the Pindus mountain range. The current author has located two sheets in herbarium P (P barcodes P00270608 and P00270609) of a collection of Antonio Baldacci from Smolikas, one of the three gatherings cited by Stojanov in the protologue. The current author has not found any evidence of previous typification of this name and has therefore suggested one of these sheets as the lectotype (Figure 1.11).

***Jasione orbiculata* var. *supinoides* Stoj.** Notizbl. Bot. Gart. Berlin-Dahlem 9; 554 (1926) - Lectotype (will be designated in a future effective publication): Greece, Epirus, mount Smolika, supra Kerasovo. 18 July 1986, A. Baldacci nº 247 (P barcode P00270608 [photo!]).

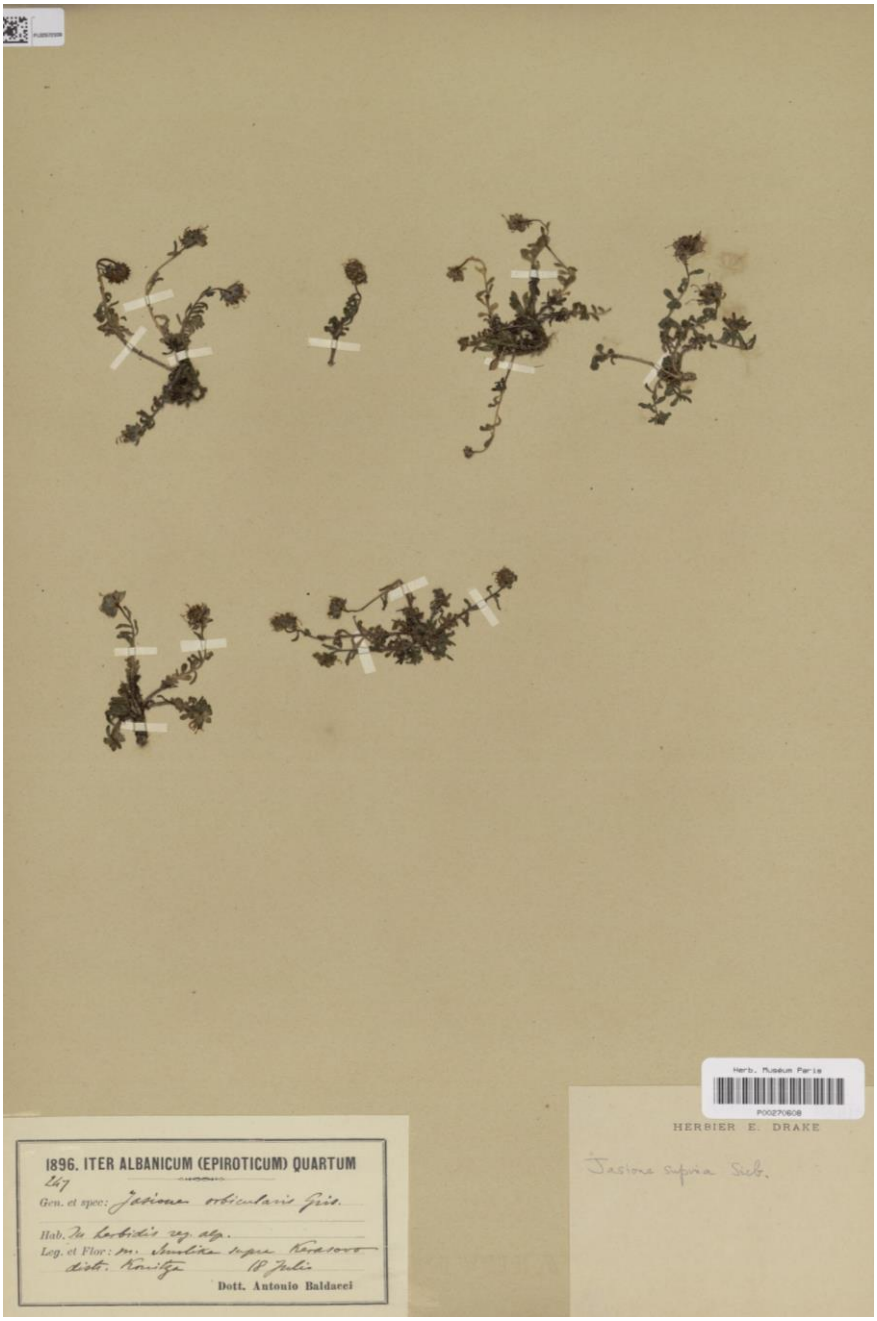

Figure 1.11. Lectotype of *Jasione orbiculata* var. *supinoides* (P00270608).

3.13. *Jasione orbiculata* var. *orbelica*

Velenovský (1898) described this name in supplementum I of Flora Bulgarica. In this work he tackled the morphological description of two varieties of *Jasione orbiculata* Griseb., conveying doubts about which was Grisebach's plant. He left the first variety unnamed, supposedly because he found it was more similar to the typical *J. orbiculata*, although this is not certain. Secondly, the var. *orbelica* was described as a plant with longer, thicker and straight stems, larger denticulate leaves and glabrous acutely denticulate bracts. Stojanov & Stefanov (1921) and Stojanov (1926) noted that the morphological description of this name matched *Jasione bulgarica*. However, as Velenovský (1898) said nothing about one of the main features of *J. bulgarica*, i.e. the free anthers, the description could also match some forms of *J. orbiculata*. Stojanov (1926) thought that this was the reason for Velenovský's doubts about which of the two varieties that he morphologically described would correspond to Grisebach's *Jasione orbiculata*. Nevertheless, the forms of *Jasione orbiculata* that could match Velenovský's description do not occur in Bulgaria, where populations that Stojanov (1926) called *Jasione orbiculata* var. *bosniaca* are found. Velenovský (1898) cited material collected by Stribrný in Gjumrukcal and Musala peaks, by Reiser in the Demir Kapija-Balkan peak, and by Urumov in the Trojan-Balkan mountain range, without linking these localities to any of the varieties. The current author has located two gatherings from Velenovský's collection in the PRC, with manuscript labels by this author indicating "*J. orbiculata* var. *orbelica*": one collected by Stribrný in 1897 in Musala (PRC code PRC451250) and the other collected in 1893 by Reiser in Demir Kapija (PRC code PRC451251). The latter only includes plants matching the description in the protologue, while the former includes two different types of plants, with one at the bottom that does not match the description, but corresponds to *Jasione orbiculata* var. *balcanica* Urum., and five stems in the upper part of the sheet that match the description of var. *orbelica*. To avoid any ambiguity, and in the absence of any evidence of previous formal typification of the variety, the current author has suggested Reiser's sheet annotated by Velenovský

as the lectotype (Figure 1.12). In any case, this name is a synonym of *Jasione bulgarica*.

***Jasione orbiculata* var. *orbelica* Vel**., in Fl. Bulg. Suppl. 1: 188 (1898) - Lectotype (will be designated in a future effective publication): Bulgaria, Demir Kapija peak (Pirin mountain range), 1893, Reiser s.n. (PRC barcode PRC451251 [photo!]).

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Figure 1.12. Lectotype of *Jasione orbiculata* var. *orbelica* (PRC451251).

3.14. *Jasione propullulans*

Freyn & Brandis (1888) equated this name to *Jasione orbicularis* (sic) Griseb., evidently meaning *Jasione orbiculata* Griseb. The species is not recognized in Flora Europaea (Tutin, 1976), the Med-Checklist (Greuter *et al.*, 1984) or the World Checklist of the Campanulaceae (Lammers, 2007) and is not even listed as a synonym. However, it is considered a validly published synonym of *Jasione supina* Sieb. subsp. *supina* in Plants of the World Online (POWO, 2021). The description indicates that the plant is from Vranica Planina, in Bosnia. As *Jasione supina* subsp. *supina* is a plant from eastern Anatolia, the synonym status in POWO (2021) can be ruled out. The origin of this misinterpretation seems to be based on a comment in the protologue stating that this plant is the same as that collected under the name *Jasione supina* by Velenovský in 1887 in western Bulgaria. This name could have been given greater consideration if it had been validly published, in which case the populations of the taxon known as *Jasione orbiculata* var. *bosniaca* Stoj. would have been given a higher taxonomic rank. However, the indication by Freyn & Brandis (1888) does not include any morphological description, and therefore it was not correctly published according to the International Code of Nomenclature.

nom. nud. ***Jasione propullulans*** in Freyn Verh. K. K. Zool.-Bot. Ges. Wien 38: 618 (1888).

3.15. *Jasione supina*

This name was published by Sprengel (1825), who recognized Sieber as the author's name and vaguely indicated “Asia minor?” as the type locality. Damboldt (1976) revised the names of the *Jasione supina* group, adopting De Candolle’s (1838) concept of the species for use in the Flora of Turkey and the East Aegean Islands (Damboldt, 1978). This interpretation of *Jasione supina* is based on the population collected by Aucher (nº 1881) in 1837 from the “Bithynian Olympus”, i.e. the Uludağ mountain in Bursa province, Turkey. Neither Damboldt nor the current author have located any original material collected by Sieber,

although it may exist somewhere. Damboldt (1978) addressed the typification of this name with Aucher's nº 1881 gathering, indicating "Neotype: Turkey, Bursa, Olympus Bithynus (Ulu Da.), 1837, Aucher 1881 G! W!)". Given that two sheets in different herbaria were designated as neotypes there is some ambiguity regarding the actual type. Therefore, according to the International Code, the current author has suggested the sheet in G (G barcode G00329587) as a lectoneotype (Figure 1.13).

***Jasione supina* Sieber ex Spreng.**, Syst. Veg. I :1810 (1825) emend. DC., Prodr. 7:416 (1839). Lectoneotype (will be designated in a future effective publication): Turkey, Bithynian Olympus (Uludağ mountains), 1837 Aucher (nº 1881) (G barcode G00329587 [photo!]).

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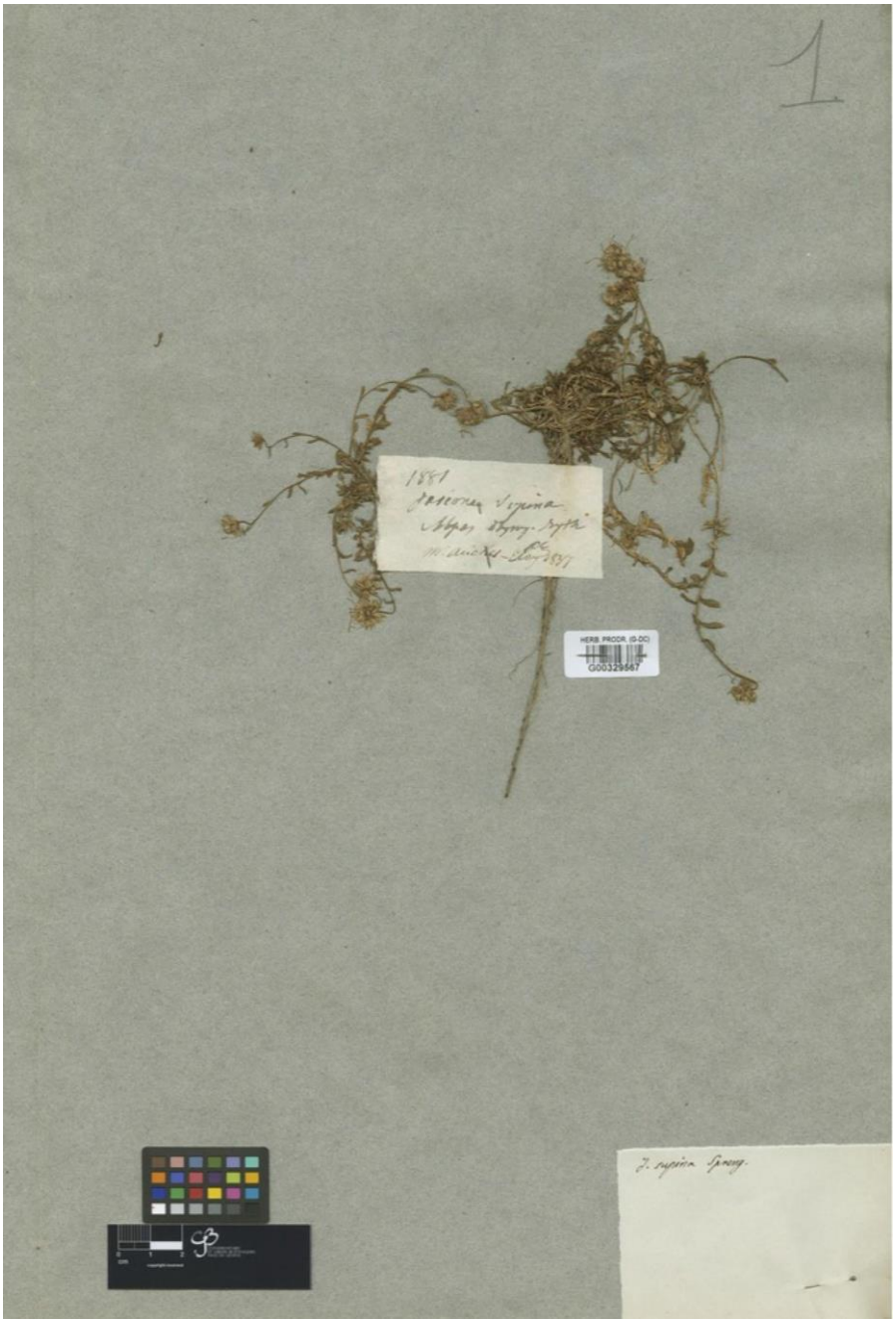

Figure 1.13. Lectotype of *Jasione supina* (G00329587).

3.16. *Jasione supina* subsp. *akmanii*

This name and rank are recognized by Greuter *et al.* (1984) and Lammers (2007), although the close affinity to *Jasione supina* subsp. *pontica* (Boiss.) Damboldt has been noted since inception of this name (Damboldt, 1976). This plant was described as a narrow endemic from the volcanic mountain range of Köroglu, in Bolu province (Damboldt, 1976; 1978), differing from subsp. *pontica* only by the entire bracts instead of dentate bracts. Damboldt only had access to one collection (Akman nº 3051, 14 VII 1975) and in the protologue indicated (holo. ANK), while in Flora of Turkey (Damboldt, 1978) wrote "Type (holo E! iso. ANK). The current author has found one specimen in E (E barcode E00913665) identified as type by a manuscript label by Damboldt. This may imply that the only indication of ANK for the type in the protologue is a typographical error that deleted the reference to "E!". If this were the case, the fact that at least two specimens from the original material collected by Akman exist (in E and ANK) and the unfortunate putative misprint designating as holotype some material explicitly not seen by Dambold, and therefore, not used by him to prepare the protologue, would leave this name without proper typification. Nevertheless, the absence of the "!" after ANK (Damboldt, 1976; 1978), indicating that this material was unseen by this author, could also be attributed to a typographical error. For the sake of precaution, the current author will be conservative and consider that Damboldt properly designated in the protologue a holotype existing in the herbarium ANK. Nevertheless, if after revision of the material conserved in ANK, no indication by Damboldt in the material from the Akman nº 3051 collection is found, the current author considers that a lectotype should be suggested, preferably the specimen in herbarium E (Figure 1.14).

***Jasione supina* subsp. *akmanii* Damboldt** in Notes R. B. G. Edinb. 35: 51 (1976) - Holotype (designated by Damboldt in Notes R. B. G. Edinb. 35: 51. 1976) Turkey, Bolu: summit of the Köroglu Da., volcanic rock, 2000-2400 m, 24 VIII 1975, Akman 3051 (ANK).

Figure 1.14. *Jasione supina* subsp. *akmanii* (E00913665).

3.17. *Jasione supina* var. *pontica*

Boissier (1875) described this name using two collections from the mountains of northern Anatolia, one from "Ponti Armeni ad Aliskeri Khan, Kotschy nº 208" and another from "Bousdouandagh Ponti Lazici supra Khabackar Balansa s.n." This taxon has been treated as a species (Handel-Mazzetti, 1907; Stojanov, 1926) and a subspecies (Damboldt, 1976; 1978), with the latter treatment being followed by Greuter *et al.* (1984) and Lammers (2007).
Damboldt (1978) addressed the typification of the name, indicating "Type: Trabzon, in pascuis alpinis inter Tschayrlar (Çayirlar) et Alischeri Chan, 28 VII 1859, Kotschy 208 (holo, G! iso. W!). Given that Boissier included different collections and specimens in the protologue, the formal typification of the name cannot be achieved until a lectotype is designated. The current author has found specimens from the original Kotschy's collection in herbarium G (G barcode G00781703) and from Balansa's collection (G barcode G00781702). Both match the protologue description of a glabrous plant with smaller but wider calyx lobes than *J. supina* s. str. The current author has therefore suggested one of these as the lectotype (Figure 1.15).

***Jasione supina* var. *pontica* Boiss.** Fl. Or. 3:886 (1875) ≡ *Jasione pontica* (Boiss.) Hand-Mazz. in Ann. Nat. Hofmus. Wien 23:192 (1909). ≡ *Jasione supina* subsp. *pontica* (Boiss.) Damboldt in Notes R. B. G. Edinb. 35: 51 (1976) - Lectotype (will be designated in a future effective publication): Turkey, Trabzon, inter Tschayrlar (Çayirlar) et Alischeri Chan 28 VII 1859, Kotschy 208, (G barcode G00781703 [photo!]).

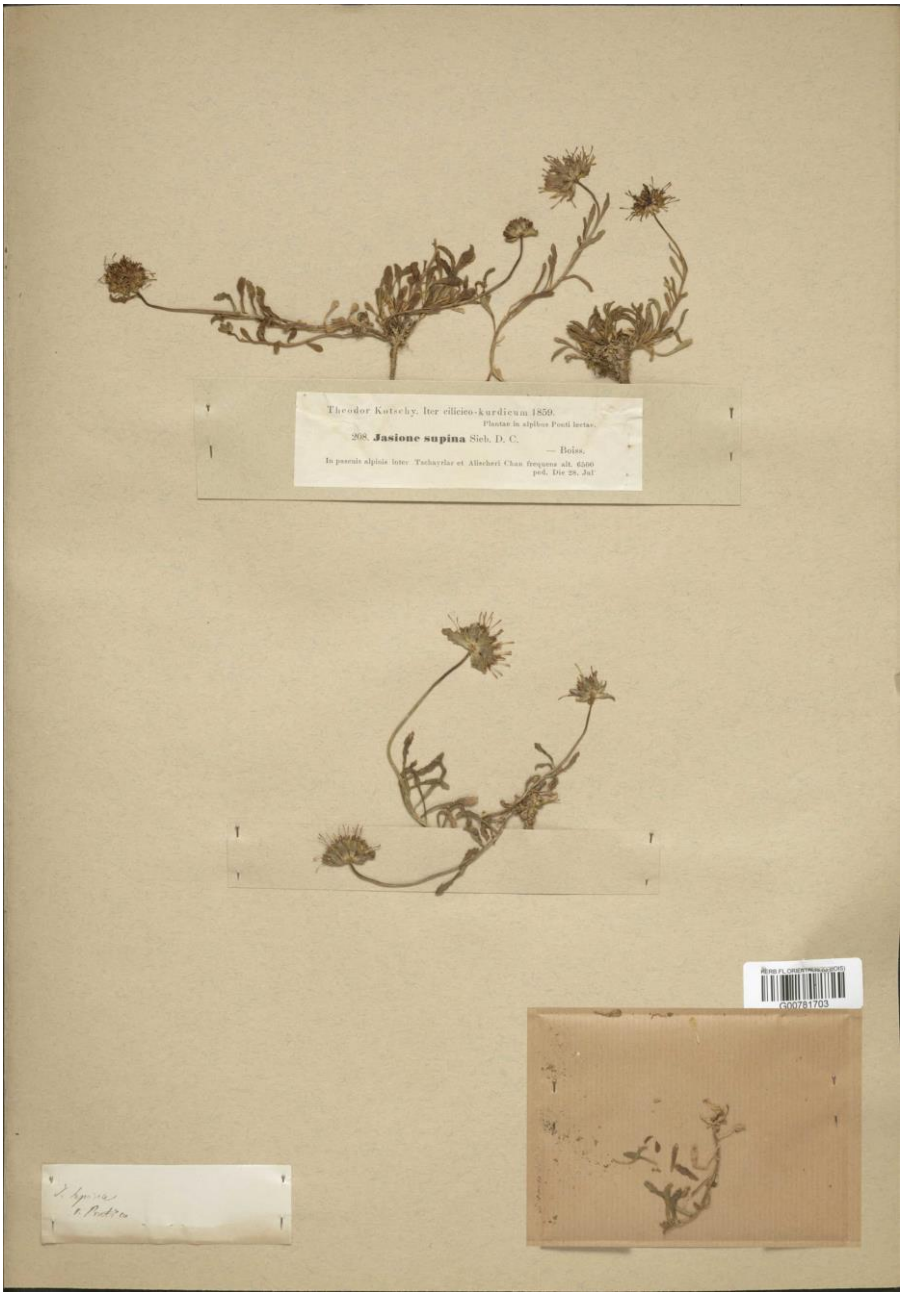

Figure 1.15. Lectotype of *Jasione supina* subsp. *pontica* (G00781703).

3.18. *Jasione tmolea*

This taxon is recognized at the subspecies rank subordinated to *Jasione supina* by Damboldt (1978), Greuter *et al.* (1984) and Lammers (2007), referring to a narrow endemic from mount Bozdagh, the ancient mount Tmolus, in Izmir province, Turkey. Stojanov (1926) described this name citing three different collections, one by Balansa (nº 333 July 1854, Sommet du Tmolus Occidentalis, au dessus de Y'aila de Bozdagh), one by Boissier (s.n., 1866 Smyra: Tmolus) and one by Frivaldsky (s.n. 1845, Smyrna, sub *Phyteuma*). Damboldt (1978) in the Flora of Turkey and the East Aegean Islands listed these three collections and indicated them as "syntypes" in the G and W herbaria. The current author has found several specimens of the Balansa 333 collection in P (P barcodes P00270579, P00270580 and P00270578) and GOET (GOET barcode GOET005895). These specimens match the description in the protologue, and in the absence of any evidence of a previous formal typification of this name, the current author has suggested one of these specimens as the lectotype (Figure 1.16).

***Jasione tmolea* Stoj.** in Notizbl. Bot. Gart. Berlin-Dahlem 9; 550 (1926) ≡ *Jasione supina* subsp. *tmolea* (Stoj.) Damboldt in Notes R. B. G. Edinb. 35: 51 (1976) - Lectotype (will be designated in a future effective publication): Turkey, Izmir, Bozdağ (Tmolus) Balansa nº 333, July 1854 (P barcode P00270580 [photo!]).

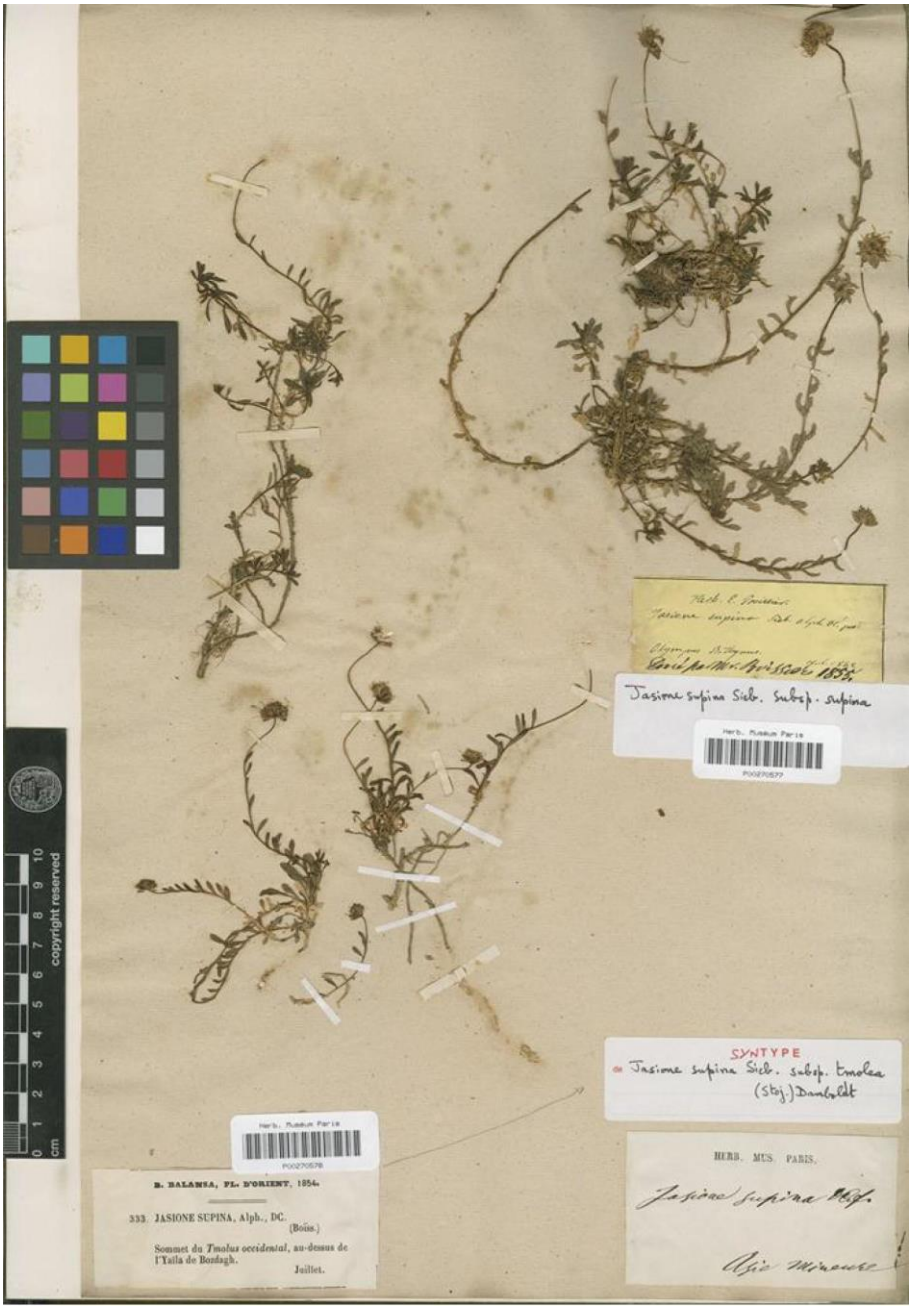

Figure 1.16. Lectotype of *Jasione supina* subsp. *tmolea* (P00270580).

Finally, there remain two names for which the current author was not able to locate any specimens from the original collections, and which therefore remain without any formal typification for the moment. Both names are included in the varietal rank and are synonyms of higher rank names. In both cases, they refer to rare deviant morphs co-existing in populations among the typical forms:

***Jasione supina* var. *hirtula* Stoj**. in Notizbl. Bot. gart. Berlin 9: 550 (1926), Type collection: Olympus Bythinicus (Boissier 1842, in herbario Musei Vindobonensis). A synonym of *J. supina* subsp. *supina* defined by its hairier habit.

Jasione pontica* var. *microcephala Freyn in Bornmüller, Plantae Anatoliae orientalis 1910, 2195 pro forma. Type collection: Paphlagonia, in monte Ilghaz-Dagh alt. 2000 m. 12-VIII-1890 Leg. J. Bornmüller. A synonym of *Jasione supina* subsp. *pontica* defined by its smaller flower heads.

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The images used in this chapter were either taken specifically for this work by the current author's collaborator Lucy Dimitrova (figures 1.1 and 1.8), downloaded from JSTOR database for reproduction in this dissertation, a permitted use for authorized users as the University of Santiago de Compostela (figures 1.2, 1.4,1.6,1.7,1.9,1.11,1.12, 1.13,1. 14, 1.15 and 1.16), or taken under request by the staffs of the Conservatoire et Jardin botaniques de la Ville de Genève (figure 1.3) and Museo di Storia Naturale, Università degli Studi di Firenze (figure 1.10), and reproduced with permission.

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CHAPTER 2.
Species delimitation in the genus *Jasione* L. (Campanulaceae)

ABSTRACT

Groups with morphological stasis are an interesting framework to address the possible recognition of cryptic species hidden behind traditional taxonomic treatments, particularly when distribution ranges suggest disjunct and environmentally heterogeneous biogeographic patterns. New hypotheses of delimitation of evolutionary independent units can lead to the identification of different biogeographic patterns, laying the foundation to investigate their historical and ecological significance. *Jasione* (Campanulaceae) is a plant genus with a distribution centered in the Mediterranean basin characterized by significant morphological stasis. *Jasione sessiliflora* s.l., and allied taxa have disjunct distribution ranges within the Iberian Peninsula, occupying environmentally diverse regions. At least two ploidy level, diploid and tetraploid, are known to occur in the group. These aspects motivated the assessment of the internal variability in the group with phylogenetic tools for species delimitation. The results from GMYC and ASAP analyses are compared with other lines of evidence, including morphology, cytology and phenology. The fitting of distribution patterns of the inferred entities to chorological subprovinces is used as a biogeographical and environmental framework to test species hypothesis. Despite the scarcity of diagnostic morphological characters in the group, phylogenetic delimitation supports the description of at least one cryptic species, a narrow endemic in NE Iberian Peninsula, and the separation from *J. sessiliflora* of a group of populations from thermophile environment in eastern

Iberia. Although disjunct ranges suggested the assessment of population groups, not all disjunct populations pointed to independent evolutionary histories. Ploidy differences support the systematic rearrangement suggested by species delimitation. Taxonomic reorganization in *J. sessiliflora* s.l. allowed biogeographic interpretations of distribution patterns that are in accordance with biogeographical regionalization. These results suggest that species differentiation, together with geographic isolation and polyploidization was associated to adaptation to different environments, shifting from more to less thermophilic conditions during the evolutionary history of the *J. sessiliflora* group. Thus, the recognition of concealed evolutionary entities is essential to correctly interpret biogeographical patterns in regions with a complex geologic and evolutionary history such as the Mediterranean basin.

1. INTRODUCTION

The accelerated global biodiversity decay (Hallmann *et al.*, 2017) urges the need to identify units of conservation representing evolutionary significant entities, being the species the most important recognized one (Richardson & Whittaker, 2010). Species has been defined as independent evolving groups of organisms that are genetically and phenotypically distinct from other such groups, a definition that encapsulates both the pattern of species and the causes of the pattern (Barraclough, 2019). Independent evolution leads to divergence, due to the interruption of cohesive processes related to gene flow. Species would be the most resolved unit of diversity with independent evolution. Although populations could develop in isolation they still are not identified as independently evolving entities on account of insufficient time to accumulate diagnosable signatures (Barraclough, 2019). Accumulation of genetic differences and actual independent evolutionary paths are not always correlated with diagnosable differences in morphological traits, as happens in taxonomic groups with high degree of morphological stasis and, therefore, prone to cryptic speciation. Cryptic species would remain undetected, what hampers ecological approaches studying these

complexes. The reason is that cryptic entities would have evolved without clear morphological differences. However, accumulation of differences in environmental preferences is probable, always within the general evolutionary framework of niche conservatism between sister species (Peterson *et al.*, 1999, Wiens & Graham, 2005). Researchers studying groups with hidden entities inadvertently could mix information that should be treated separately, what could lead to propose meaningless biological hypotheses. Finally, and more importantly, these species are excluded from legal lists and therefore conservation policies will be blind to them. Evolutionary lineages with morphological stasis can be composed by species with few, if any, clearly discriminating morphological characters, although genetic differentiation and interruption of gene flow between the different lineages could have a relatively long evolutionary history. However, other phenotypic traits can be investigated to detect significant differences, among them those related with ecological preferences or habitat (Prada & Helberg, 2013; Michonneau. 2015). The use of molecular information can uncover hidden significant specific divergence supported by Species Delimitation methods. It allows researchers to focus on the detection of other corroborating signatures of speciation, on the knowledge of the expected distribution of discontinuities between populations. The Evolutionary Significant Unit (ESU) concept (Moritz, 1994) defines species as units with reciprocal monophyly in at least one molecular marker plus at least other defining trait, including morphology, distribution or in general reciprocal monophyly in another character (Michonneau, 2015). Although the original ESU concept includes differences in distribution among the defining traits, a direct application of reciprocal monophyly and separated ranges would lead to an inflation of species recognition in many taxa. Nevertheless, it could be advisable to include information from species distribution if it indirectly offers relevant information about the environmental envelope of the putative evolutionary entity, The environmental niche in which a species thrives is ultimately conditioned by intrinsic physiological constraints. Evolved ecological tolerances make up the fundamental niche, that is, the potential environmental space that a species could occupy (Hutchinson, 1957).

However, the realized niche is the one that can be inferred from distribution data, that is also conditioned by biotic interactions and the historical ability of the species to overcome barriers to dispersal (Hutchinson, 1957). This is illustrated by cases of invasive species with clear niche shifts between the native and recently invaded range (Tingley *et al.*, 2014). Thus, distribution ranges are a translation to the geographical space of the evolved optimal environmental preferences of a species that allow it to thrive and successfully interact with other species, conditioned by random opportunities to dispersal. Biogeographical regionalization makes a good hypothetical framework to test hypotheses of evolutionary independent lines (i.e., ESUs or species) as it integrates not only information from the environmental preferences of the geographically encompassed organisms in each sector, but about the occurring communities, and therefore their internal between-species interactions. In this aspect, the adequacy of a species or a lineage to certain biogeographical sectors, but not to others, could provide more information about its singularity than the mere environmental information obtained from geographical occurrences of the species. Nevertheless, there are different approaches to biogeographic regionalization, as those aimed to establish homologous biotic units based of sympatric distributions of endemic species (i.e., Areas of Endemism) together with complementary criteria, although the existence of different concretizations remains a problem (Ebach & Parenti, 2015; Ferrari, 2018; Morrone, 2018). The geobotanic school have proposed a hierarchised organization of biogeographic units widely used in phytosociological research, based on the distribution of species and vegetal communities, in addition to information from other fields, mainly bioclimatology, geology, soil science and geography, establishing typological units to classify the different territories of the Earth (Rivas-Martínez *et al.*, 2017). The hierarchical typologies of organization are normally shared among the different schools in their higher categories, as kingdom, region, province and district, including intermediate categories like subregions or subprovinces (Morrone, 2009). Lower levels of organization are more controversial, and inferior categories of the geobotanic school (e.g., sector, tesela, etc) have not gained support for biogeographical regionalization (Morrone, 2018).

Therefore, the subprovince level of regionalization can be both a consensual typology and at the same time divide the territory in a sufficient number of units to identify patterns in closely related species, in case of absence of strict niche conservatism.

The task of assigning populations to cryptic species must be based on observed patterns. One species' property is that species constitute ecological units (Barraclough, 2019), what makes the species the main unit for ecological and biogeographical models of diversity. Albeit there exist ecotype variation and adaptive polymorphism within species (Hufford & Mazer, 2003; Skúlason & Smith, 1995) the criterion of ecological coherence has especial importance in species definitions, particularly in organisms with scarce morphological distinctiveness (Barraclough, 2019). The present work is based on the conceptual framework that ecological coherence within species and ecological differentiation between species could produce diagnosable biogeographic patterns, and if this is the case, the identified patterns should support cryptic entities inferred from other lines of evidence, namely from the DNA sequence methods. Nevertheless, the accumulation of lines of evidence is needed, as the blind use of molecular methods can led to wrong inferences. The results of empirical methods for species delimitation based on nucleotidic information, regardless of the use of either single-locus sequences or genome-scale data, can be misled by some evolutionary processes specific of the group under study, like hybridization events and introgression, and recent radiations and incomplete lineage sorting (Harrison & Larson, 2014; Jorna *et al.*, 2021). Thus, complex phylogeographic histories and semipermeable species boundaries (Jorna *et al.*, 2021) make important the accumulations of diverse lines of evidence, particularly in close evolutionary entities within the same ploidy level. It is precisely at the beginning of the speciation process when different independent lines of evidence offer frequent conflicting signals, being fundamental to identify those evolutionary independent entities in the group under study not only to unravel the evolutionary events behind the process but to apply conservation polices when necessary due to the accelerated process of global biodiversity loss. The use of sequence-based SD methods could be considered the first exploratory step to set species

hypothesis in an integrative taxonomy process that should be coupled with expert knowledge in the group of interest. Thus, the empirically suggested species are ready to be supported or discarded when faced other lines of evidence, testing if the proposed species fit or behave in a differentiated manner.

In this work it was used as model group a complex of taxa and diverged populations of the genus *Jasione, the Jasione sessiliflora* and some morphologically close taxa, all belonging to the wider *J. crispa* polyploid group. The genus *Jasione* is characterised by a marked morphological stasis that led to conflicting taxonomic treatments (Stojanov, 1926; Kovanda, 1968). The group of study is formed by Iberian endemic taxa, with at least two ploidy levels within, diploid and tetraploid, and whose populations have been assigned to different taxonomic names. The more firmly stablished taxa of the group in current taxonomic treatments are *Jasione sessiliflora*, considered a tetraploid distributed in the Central and northern half of the Iberian Peninsula, and *Jasione crispa* subsp. *tomentosa*, with its allied taxon *J. crispa* subsp. *mariana* (Sales & Hedge, 2001a). These latter taxa have distribution ranges in Central Iberian Peninsula, although in more southernly areas than *J. sessiliflora.* Both are considered diploids, although Sales & Hedge (2001b) included in the variability of the former tetraploid populations that have been described under the name *J. crispa* subsp. *segurensis*. Finally, two Mediterranean groups of populations have been assigned to *J. sessiflora*, *J. sessiliflora* subsp. *appressifolia* in Castellón and Valencia mountains and a population in the Prades mountains, in Tarragona, which was originally described as *J. crispa* var. *praelittoralis*, a treatment never followed since the description (Sales & Hedge, 2001a, 2001b). Although different groups of populations are assigned to different names, the taxonomic resolution has been considered complicated and needed of detailed studies as the diagnostic characters are frequently absent (Sales & Hedge, 2001a, 2001b). Therefore, it makes an excellent model group to use Species Delimitation tools and use the delimited entities as specific hypothesis to test by identifying discontinuous patterns.

In summary, the aim of this study is to detect species in the cryptic diploid/tetraploid group of *J. sessiliflora* and allied taxa by using two complementary methods of molecular species delineation, ASAP and GMYC, whose results are considered hypotheses of species identification. To validate the species hypotheses, different complementary lines of evidence are studied, including ploidy levels, morphological characters and phenology. Finally, a main objective of this work is to appraise whether the suggested species represented by molecular lineages fits into biogeographical subprovinces in a way that clear biogeographical patterns can be identified as an expression of differently evolved realized niches.

2. MATERIAL AND METHODS

2.1. Plant Material

We explored all localities from which type specimens have been described for the treated names. Then, populations covering the whole distribution ranges of the memebres of the group were sampled. (See link in General Methods). Most of the material was sampled in the field, ranging from three to five individuals from each population, although some populations are represented by single individuals from herbarium specimens. Populations from all the biogeographical subprovinces (Rivas-Martínez *et al.*, 2017) with representatives of the group have been included in the study, with a special effort to locate to avoid sampling bias. In addition to representatives of the group of interest, it was included in the Species Delimitation analyses individuals from populations of the hexaploid *Jasione crispa* subsp. *crispa*, one of them from the type locality at Eina mountain (Sales & Hedge, 2001a).

2.2 DNA sequences and phylogenetic analysis

Total genomic DNA was extracted using either a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990) or a commercial kit Nucleo Spin Plant II (Macherey-Nagel). In both cases 0.6 ul of 2-Mercaptoethanol was added during the first liquid

step. Foliar tissue was homogenised either with manual grinder or using Mikro – dismembrator (Sartorius). DNA concentration and quality was assessed with Nanodrop 2000C (Thermo Scientific). Four plastid DNA regions were amplified, the psbA-trnH intergenic espacer, the trnL-trnF intergenic spacer, the 3' end of the intron of the ndhF gene and the rps16-trnQ intergenic spacer. After alignment and comparison with plastome sequences of members of the Campanulaceae in the GenBank it turned out that the conserved regions where the primers are anchored belong at the end of the trnL(UUA-trnT(UGU) spacer and the trnT(UGU) gene and the trnQ gene region. This can be related to the massive rearrangements underwent by plastid genomes in *Jasione* and in general the Campanulaceae family (Cosner *et al.*, 2004). Therefore, the amplified marker will be denominated trnT(UGU)-trnQ. See the General Methods section for primer sequences, primer bibliographic references and PCR conditions. PCR products were firstly assessed on 1.5 % agarose electrophoresis gels stained with the nucleic acid staining solution RedSafe (iNtRON biotechnology) and purified with E.S.N.A. Cycle Pure Kit. (Omega). DNA Sanger sequencing was carried out in an 8-capillary 3500 analyzer (Applied Biosystems) by the DNA sequencing service of the University of Santiago de Compostela, Spain, or Software Sequencing Analysis 5.2 (Applied Biosystems) by STAB VIDA, Portugal.

2.3. Ploidy level assessment

Direct chromosome counting was carried out in metaphases from root apices. Root tips measuring c. 5 mm were excised from cultivated plants grown from field collected individuals or from germinated seeds. All plants and seedlings were acclimated to outdoor conditions and were directly moved from temperatures around 15ºC or lower to the laboratory to conduct immediate root excision, always at first hours in the morning (before 9:00 a.m.) to avoid high laboratory room temperatures. Root tips were washed in distillate water a pre-treated in 2 MM 8-hydroxyquinoleine for 3 hours, followed by a rinsing step in distillate water and then a fixation step in freshly prepared 3:1 96% ethanol - 1 glacial acetic acid solution for 24 hours. Samples were

thoroughly rinsed in 70% ethanol to remove glacial acetic acid, since it has been described to interfere with later chromosome staining (Norman *et al.*, 2012). Samples were either stored in 70% ethanol at 4ºC for further use or directly used in the following steps. Root tips were rinsed in distilled water for 5 minutes, followed by an additional 5 minutes rinsing step in fresh distilled water. Samples were hydrolised by plunging the root tips in and Eppendorf tube with HCL 1 N for 6-9 minutes in a water bath at 60ºC. The tubes were pre-heated to bath temperature before root tip plunging. Two rinsing steps 5 minutes each in distillate water followed to eliminate all possible HCL remains. For mitotic analysis, root tips were stained in Schiff's reagent for 12 hours in dark conditions. Stained root apices were excised under binocular magnifying glass, and immediately squashed on a slide. Cells were observed in a Leica TCS-SP2 confocal laser microscope (LEICA Microsystems Heidelberg GmbH, Mannheim, Germany) under a HCX PL APO CS 63.0x1.40 OIL objective, 580nm-725nm emission band and excitation wavelength of 561 nm with DPS (561 nm) laser diode.

Flow cytometry on silica-gel dried plants was carried in some populations, or in other individuals from populations in which one individual had been counted as previously. See the General Methods section for the flow cytometry methods.

2.4. Species delimitation with ASAP

Assemble Species by Automatic Partitioning (ASAP) is a molecular method for species delimitation that uses group-specific ad hoc thresholds without any a priori species hypothesis, that are estimated looking for the barcoding gap in the frequency distribution of nucleotide distances. It represents a transition between intraspecific and interspecific categories. (Puillandre *et al.*, 2021). ASAP is based on a hierarchical clustering algorithm using only pairwise genetic distances from single locus sequence alignments to produce a species partition scheme ranked by a scored system (Puillandre *et al.*, 2021). The current author used a set of plastid regions that are inherited together and therefore can be considered a single locus for Species Delimitation

methods (Fujisawa & Barraclough, 2013). ASAP creates successive partitions from all terminals split to all lumped into a single group and assigns them a probability that quantifies the partition chances of being a single species. Them it computes the width of the barcode gap between the previous and the new partition. Probability and gap width are combined into a single asap-score that is used to rank the groupings, the lower the better (Puillandre *et al.*, 2021). ASAP analyses were conducted in the program web-interface (https://bioin fo.mnhn.fr/abi/publi c/asap) with the three available substitution models, Jukes-Cantor (JC69) (Jukes & Cantor, 1969), Kimura 2P (Kimura, 1980), and simple p-distances. The analyses were run from a dataset with 14 sequences, including all detected haplotypes in the populations of study group plus a sequence from a population of the hexaploidy *Jasione crispa* subsp. *crispa* from the Pyrenees.

2.5. Species delimitation with GMYC

Generalized Mixed Yule Coalescent (GMYC) model (Pons *et al.*, 2006) was used to suggest hypothetical species as independent evolutionary units. GNMYC is an exploratory method using single-locus sequence datasets and a ultrametric phylogenetic tree as input to estimate rates of branching to ascertain which parts of the phylogeny behave following population (coalescent) model, and which parts follow a speciation (Yule) model. The favoured species partition maximizes the likelihood of the transition between branching coalescent rates and branching speciation rates in absolute time, using an ultrametric tree, identifying a threshold between speciation and coalescent rates. This single threshold approach is based on the assumption that species are monophyletic, and therefore clades defined by a Most Recent Commons Ancestor (MRCA) reflect diversification events and can be considered species while branches descending from each of the MRCA nodes reflect coalescent events (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013). The set of nodes with highest Maximum Likelihood is chosen as the best species partition model. There is a version of the methods that allows to infer multiple thresholds, relaxing the assumption that all diversification events must

be previous in time than all coalescent events (Monaghan *et al.*, 2009). However, it has been demonstrated that this variant of the method is prone to over-splitting (Fujisawa & Barraclough, 2013; Kekkonen & Hebert, 2014), therefore its use was discarded. From each putative species sequences of at least six individuals have been included in the GMYC analysis. When more than one population was included in the sample, at least two individuals per population has been sequenced (mean 4.1 indiv./ population). Fujisawa & Barraclough (2013) observed that increases in the number of samples individuals per species led to better performances of the method. The reason is that greater sampling might increase the detected branching rate and therefore the detection of a threshold in branching. However, greater species sampling, particularly without increasing within species sampling of individuals might reduce the ability of GMYC to detect species. The reason is that species monophyly is more easily inferred if random but incomplete sampling provokes that some close species are not included in the analysis (Bergsten *et al.*, 2012). Hence, with the aim of an earnest procedure and to avoid unrealistic over-split results, it has been sampled the complete geographic range of the taxa investigated, even if it could hamper the detection of non-monophyletic species. As within species geographic structure could affect the accuracy of the method, causing that some relatively isolated populations could be delimited as separate species (Papadopoulo *et al.*, 2008; Lohse, 2009), the sampling design aimed to include not only the main geographic groups by all linking populations. Nevertheless, simulations of the HMYC method showed that if the effective population size remains low relative to species divergence (that is, relatively low within species variation) the threshold is optimized at the correct point of the tree and the effect of geographic structuring is minimal (Fujisawa & Barraclough, 2013). Moreover, Talavera *et al.* (2013) observed that the remotion of intermediate haplotypes had little effect in the results and did not delimit as different species the most extreme haplotypes. The analyses have been conducted by both including repeated sequences and pruning repeated sequences. GMYC performance is affected by identical sequences because terminal zero-length branches affect the likelihood estimation (Monaghan *et al.*, 2009; Fujisawa & Barraclough, 2013).

However, when the tree is produced by a genealogy-based inference, as is the case of BEAST, the program does not assign null lengths to identical terminals, since they are treated as different haplotypes coalescing to a MRCA node, what allows GMYC to handle them. Talavera *et al.* (2013) did not observe differences in GMYC results when using repeated sequences from a tree inferred with BEAST relative to when they were removed, although they recommend collapsing all repeated sequences to only keep different haplotypes to reduce computational times. Conversely, Michanneau (2015) advocated the use of repeated sequences in GMYC analysis using a tree inferred by BEAST. BEAST could overestimate effective population sizes if identical sequences are removed by inferring higher unrealistic levels of genetic diversity (Michanneau, 2015). Since population sizes affect lineage coalescence, the remotion of identical sequences would increase average branch length, hampering the distinction between divergent and coalescent events. Michanneau (2015) considered that collapsing sequences to haplotypes would cause species over-splitting and higher uncertainty in the analysis. While the latter is a probably undesirable outcome, it could also be that keeping identical sequences from BEAST, with minimal (although not zero) lengths could push to the external nodes the species delimitation threshold. Two datasets have been included, one with all identical sequences collapsed to distinct haplotypes (dataset 1, the same as in ASAP) and other in which were retained different sequences in a proportion similar at how they were found in natural populations across the geographic range of the taxa. When one haplotype was found in only one population, it was represented only once in the dataset, either if it was the only haplotype in the populations or not. The possibility that one species could be represented by only one sequence in the dataset (i.e., a singleton) has no demonstrated negative effects in GMYC analysis (Talavera *et al.*, 2013).

GMYC analyses were carried out from trees estimated with BEAST2. The substitution model was HKY + G, with the Gamma count categories prior set four categories, as small number of categories of gamma distributed rates of evolution is enough to capture rate variation in small dataset (Jia *et al.*, 2014). In the first dataset (collapsed to

haplotypes) two BEAST priors were modified to test possible effects on the GMYC results. It is expected that variation in priors as branching pattern and rate of molecular evolution would not affect the inferred topologies, but the branch lengths will vary. Two different priors were used for the expected branching, a Yule model, also called pure birth model (Yule, 1925), and a Coalescent model with constant population size (Griffiths & Tavaré, 1994). The former is expected when the tree has a constant speciation rate while the latter describes branching patterns within the same species. For the Yule model analysis, the rate of molecular evolution was set to "constant clock", meaning that the amount of nucleotidic mutations increases at a constant rate with time, and assumption suitable for small datasets of very related taxa. It was also tested a "relaxed clock" allowing that mutation rates could vary throughout the tree following a log-normal distribution. For the coalescent model with constant population size, it was only used a constant clock prior, as it is expected a within-species behaviour. The impact of prior variation has been studied in several works (Monaghan *et al.*, 2009; Talavera *et al.*, 2013; Michanneau, 2015). It has been indicated that the use of a Coalescent constant population model would be more conservative than the Yule model because GMYC also uses a coalescent model as null model of expected branching (Monaghan *et al.*, 2009). In fact, these authors estimated higher species number with Yule model than with constant coalescent model. However, since this parameter affects branch lengths the prediction of how it would affect GMYC results is not straightforward and could be influenced by the amount of variation in the data (Michanneau, 2015).

It was tested the effect of prior variation over GMYC results using the LRT test significance. After that, for the GMYC analysis with the second dataset (with repeated sequences) it was inferred a Bayesian phylogeny with the coalescent constant population branching and constant clock molecular rate of molecular evolution priors. Bayesian phylogenies were inferred in BEAST2, with 10 million generation MCMC lengths and two independent runs. Convergence was checked with Tracer v1.6 and after inspection of ESS values in each run a 10% burn-in and posterior sample combination was carried out in LogCombiner. Finally, a maximum credibility clade tree was

summarized with TreeAnnotator for each analysis. In the analysis with relaxed clock model prior parameters were left as default in BEAUti2 and clock rate set to 1.0.

BEAST ultrametric topologies and posterior probabilities were inspected in FigTree and exported in newick format, since it is required by the R package splits (Ezard *et al.*, 2009) which was used to apply the GMYC method with single-threshold. Each GMYC analysis was repeated in the GMYC server (https://species.h-its.org/gmyc). Species Delimitations, LRT significances and GMYC (AIC) supports values were compared among the analyses.

2.6. Other lines of evidence: Morphology and phenology

The putative species delineated by the GMYC and ASAP methods can be interpreted as hypotheses and to determine whether they are biologically sound other lines of evidence need to be investigated. Looking for delimitating morphological traits can be difficult if not misleading in a genus of which has been said that "there is not any clear morphological discontinuity between two species of *Jasione*" (Tison & de Foucault, 2014). Nevertheless, and with the guidance of GMYC and ASAP results, some subtle morphological characters are investigated here. Phylogenetic analyses in the genus (see Chapter 3) showed that hydathoids (see General Introduction for description of the trait) have a distribution among the species of *Jasione* that correlates with the evolutionary history of the genus. Here, the average number of hydathoids per leaf was compared in the model group with the width of leaf hairs, a measure relative to the size and stiffness of trichomes.
The relative size of the epidermal cells of the adaxial and abaxial sides of the leaf has been pointed out by Bohkari & Sales (2001) as one of the few anatomical characters providing taxonomical information in *Jasione*. In most species studied by these authors adaxial and abaxial epidermal cells were subequal in size. However, in *J. laevis, J. sessiliflora, J. cavanillesii* and *J. crispa* subsp. *tomentosa*, the adaxial epidermal cells were 1.5–2 times larger than the abaxial epidermal cells. This trait was investigated here within the more homogeneous taxa of

the model group, *Jasione sessiliflora* subsp. *appressifolia*, *Jasione crispa* subsp. *tomentosa*, *Jasione crispa* var. *praelittoralis*, and the less similar *Jasione crispa* subsp. *mariana*. Epidermal peelings of leaves from herbarium specimens were stained with blue toluidine and photographed at 20x magnification in a light microscope. Length of cell larger dimension was measured in 25 adaxial and 25 abaxial epidermal cells in each leaf, five leaves per individual.

Phenology data was obtained from two sources, collection date information of herbarium specimens with flowers and the phenological information provided by Pérez Chiscano (1987).

2.7. Biogeography

To test for the adequacy of the delimited entities to a pre-established biogeographical framework, the studied populations were superimposed to a georeferenced map of the Biogeographic subprovinces of the Iberian Peninsula (M. Serrano, own digitization), based on Rivas-Martínez *et al.* (2017). The haplotypes were organized in six haplogroups, those with high posterior probability in the Bayesian genealogy. The frequency of the chloroplast haplogroups in the Iberian biogeographic subprovinces was surveyed and visualized using heatmaps. The heatmaps were generated in R v.4 with the ComplexHeatmaps package (Gu *et al.*, 2016). Two heatmap analysis were generated. First, a heatmap without statistical internal clustering with subprovinces was ordered according to a thermic gradient. Second, a heatmap with split simultaneously performed by rows and columns, applying k-means partitioning analysis to detect underlying patterns. The k parameter is set to three for rows to test whether the partitioning recovers the most probable delimited species number suggested by GMYC and ASAP methods. The k parameter is set to four for columns as it is the number of major inferred dendrogram clusters of subprovinces obtained by hierarchical clustering. As the k-means analysis uses random start points, which could generate different

clusters from different runs, the analysis was repeated 25 times to check for consistency of the results, observing no variation in the results.

3. RESULTS

3.1. Species delimitation with ASAP

ASAP generated five possible partitions, but only the two best asap-scores according to Puillandre *et al.* (2021), were considered for discussion, as in Magoga *et al.* (2021). The first and second best scored partition schemes divided the dataset in six and four species, respectively. Both partitions had similar values, with identical asap-scores (Table 2.1) and slightly better probability for the four species partition (0.71 vs. 0.73, averaged probability values of the three substitution models in each partition). ASAP ranked first the four species partition scheme because its better probability. Moreover, it overpasses the reference threshold distance (0.0005). The distance inferred by ASAP between panmictic populations and diverged species was 0.00061 for the four species partition and 0.000454 for the six species partition (Table 2.2). The four groups identified as distinct species in the best-ranked partition were, 1- *Jasione crispa* var. *praelittoralis*, 2- *Jasione crispa* subsp. *crispa*, 3- all tetraploid representatives (*Jasione sessiliflora*, *Jasione crispa* subsp. *segurensis* and tetraploid populations of *Jasione crispa* from Serra da Estrela *and Jasione "tomentosa*" 4x), 4- all remaining diploid taxa (*Jasione crispa* subsp. *tomentosa*, *Jasione crispa* subsp. *mariana*, *Jasione sessiliflora* subsp. *appresifolia* and other unassigned diploid populations). The six-partition scheme was similar but separated two diploid populations of *Jasione tomentosa* with somewhat divergent sequences. (Figure 2.1). Other biologically meaningful schemes, like a partition with three species also identified as independent species *J. crispa* subsp. *crispa* and *J. crispa* subsp. *praelittoralis*, lumping the remaining in a group although with a considerably worse ASAP-score of 5. Given the comparable results between both partitions it is favoured the four species partition, since it has better probability values and higher threshold distances, considering the six species partitions an oversplit

resulting for a phenetic, not phylogenetic method (Puillarde *et al.*, 2021).

Table 2.1. First two best-raked ASAP partitions, indicating ASAP-scores for each substitution model, probability for panmixia (p-val), relative gap width metric (W) and inferred threshold distance among species * indicates partitions above the reference range of distances.

Species number	Substitution model	Asap-score	p-val	W Rank	Threshold dist.
4*	JC69	2	7.23e-01	4.69e-05	0.000681
	K80	2	6.89e-01		
	p-distances	2.5	6.07e-01		
6	JC69	2	7.37e-01	6.48e-05	0.000454
	K80	2	7.09e-01		
	p-distances	2	7.47e-01		

The results favour a 4 species partition, followed by a 6 species partition with similar asap-scores.

Table 2.2. First two best-raked ASAP partitions, indicating ASAP-scores for each substitution model, probability of panmixia (p-val), relative gap width metric (W) and inferred threshold distance among species * indicates partitions above the reference range of distances.

Species number	Substitution model	Asap-score	p-val	W Rank	Threshold dist.
4*	JC69	2.00	5.45e-01	6.21e-06	0.000681
	K80	2.00	5.31e-01		
	p-distances	2.00	5.37e-01		
6	JC69	2.00	6.37e-01	1.02e-05	0.000454
	K80	2.00	6.87e-01		
	p-distances	2.00	6.51e-01		

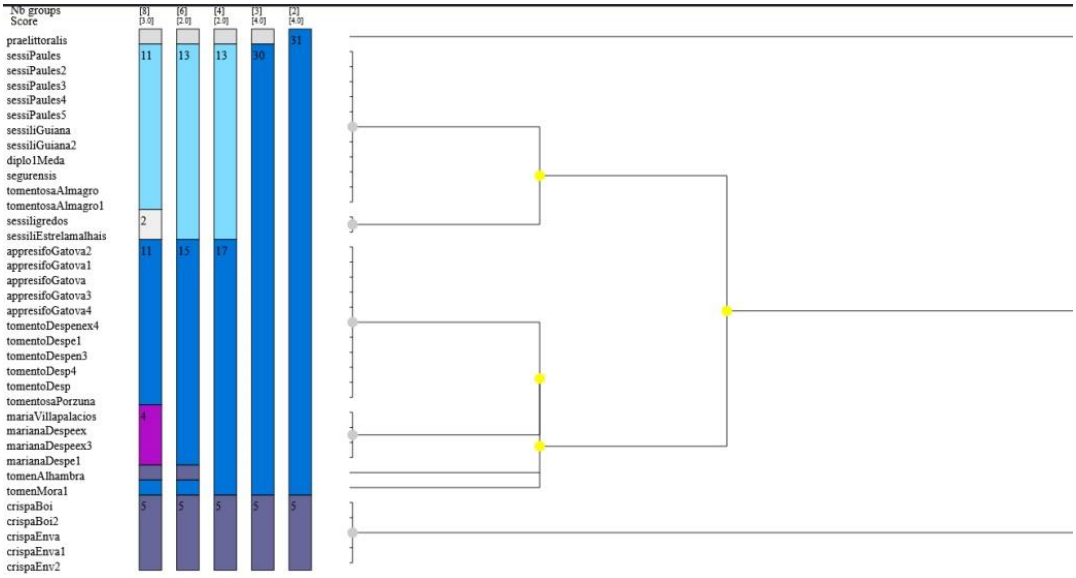

Figure 2.1 ASAP species delimitation tree calculated with the Kimura-2-parameter model. The third column is recovers the most likely species delimitation hypothesis: four species.

stances calculated with the Kimura-2-parameter model (K2P). Results of species delimitation analyses are reported in I (ASAP)

ASAP delimitation was defined evaluating both the partitions with first and the second best asap-score according to Puillandre *et al.* (2021). ASAP analyses species groupings differed whether run from the matrix without repeated sequences or from the matrix with repeated sequences. Besides, the results in the latter case where inconsistent among the different substitution models, with Jukes-Cantor model yielding the most diverging results, and conflicting with species partitions suggested by other lines of evidence. However, the groupings from the matrix without repeated sequences were similar between the three substitution models and congruent with species partition offered by GMYC and other lines of evidence.

3.2. Species delimitation with GMYC

The Species Delimitation scheme estimated by the GMYC single-threshold method in the dataset with sequences collapsed to unique haplotypes varied from two to four species (Table 2.3). The topologies inferred from constant clock rate prior led to four maximum-likelihood entities (*praelittoralis*, *crispa* Pyrenean, tetraploid haplotypes, remaining diploid haplotypes), while in the genealogy inferred from a relaxed-clock rate the GMYC method estimated two maximum-likelihood entities (*crispa* Pyrenean, all other haplotypes). Maximum-likelihood confidence intervals were narrower when the expected branching pattern was based on a constant coalescent model than on a Yule model. The LRT results were no significant in the three cases, although were closer to significant values for the topology inferred with coalescent prior. Conversely, the, GMYC (AIC support) for the basal diverging node was highest in the Yule model.

Table 2.3. Summary of the GMYC maximum-likelihood estimates of Species Delimitation. (1) GMYC support (AIC values) averaged from all nodes of the estimated putative species.

Dataset type	**Haplotypes**			**Populations**
Priors	Yule, constant clock	Yule, relaxed clock	Coalescent constant clock	Coalescent constant clock
Species number	4	2	4	4
LRT result	0.8815751n.s.	0.9209454n.s.	0.1572022n.s.	0.0003451632***
ML entities, Confidence interval	1-13	1-13	1-7	4-9
GMYC support (1)	0.44	0.24	0.60	0.86

Nevertheless, given the narrower confidence intervals and better likelihood ratios of the analyses on the topology from the coalescent branching and constant clock rate priors, the current author used for the populations dataset (with repeated sequences) a topology inferred with

these prior parameters. The resulting maximum-likelihood estimates recovered four species (*praelittoralis*, *crispa*, tetraploid haplotypes, remaining diploid haplotypes), with the narrowest confidence intervals (4-9 species), comparatively good GMYC support and highly significant LRT results. (Table 2.3). Therefore, the species scheme is congruent with two of the three analyses carried out with the "haplotypes" dataset, but well supported by Likelihood Ratios Test. The nodes leading to the four lineages estimated as putative species had high posterior probability support in the Bayesian inference, but not all clades with high posterior probability support are recovered by GMYC as putative species (Fig. 3.2). The profile of the likelihood and the number of lineages through time, expressed as accumulated mutation since the genealogy is not time calibrated, with the inferred threshold between divergence (speciation) and coalescent (populational) events, are showed in Figure 2.3.

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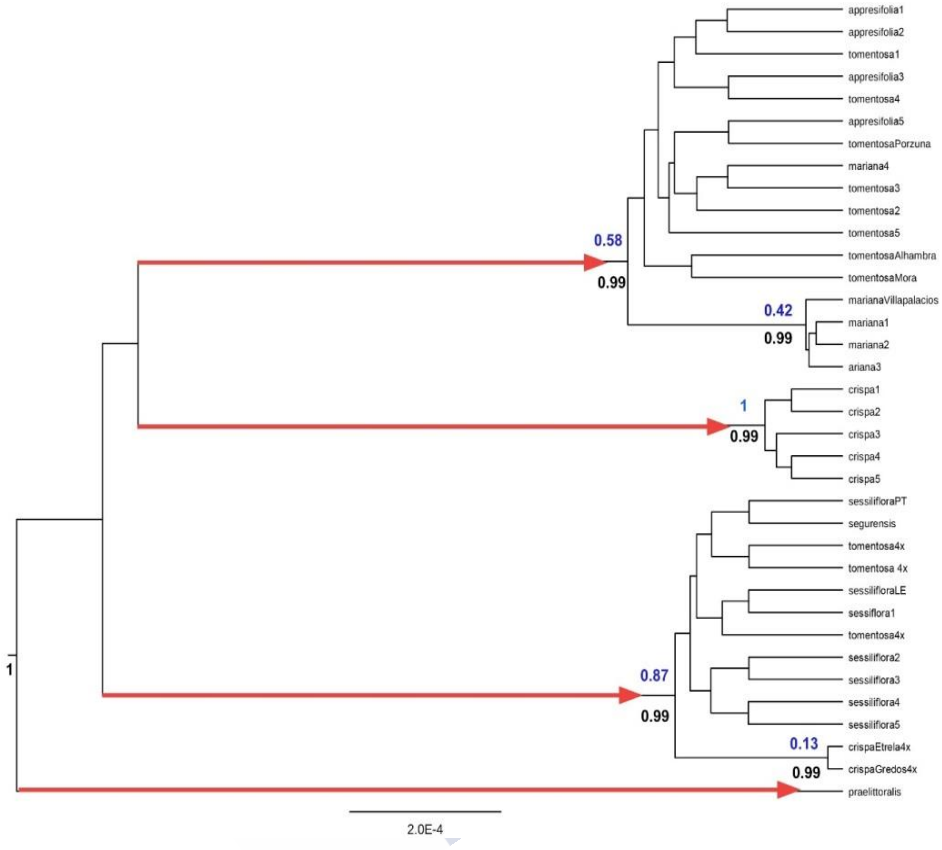

Figure 2.2. GMYC Species Delimitation in a Bayesian genealogy inferred from coalescent and constant clock rate priors, and "populations" dataset (repeated sequences in a proportion similar to its occurrence in natural populations). The four lineages suggested as putative species are indicated by red arrows. Posterior probabilities of well-supported nodes in the Bayesian phylogeny are indicated in black under branches. GMYC support (AIC values) of the same nodes are indicated in blue above branches.

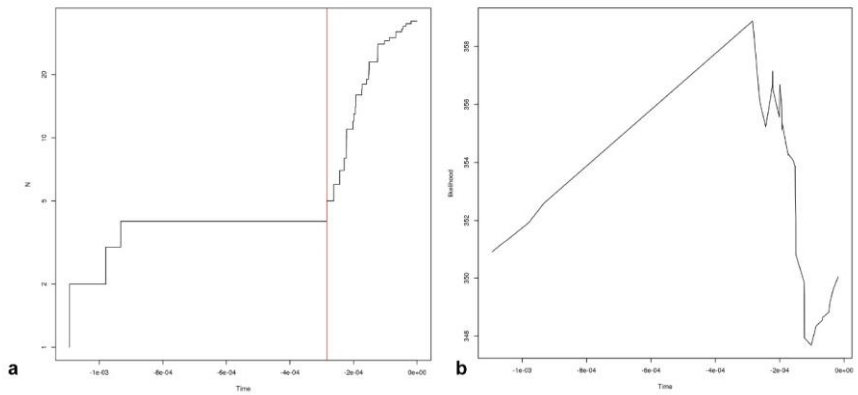

Figure 2.3. GMYC Species Delimitation from the "populations" dataset analysis. a. Number of lineages through time (as accumulated mutations) and inferred threshold for Species Delimitation. b. Profile of the estimated likelihood through evolutionary time.

3.3. Other lines of evidence: Cytology, morphology, and phenology.

The cytological analyses revealed that *Jasione crispa* var. *praelittoralis* is a diploid taxon with 2n=12 chromosomes (Figure 2.4). *Jasione sessiliflora* var. *appressifolia* is also diploid, with 2n=12 chromomes in the studied populations. *Jasione crispa* subsp. *tomentos*a showed variation in ploidy level among populations, with most its populations being diploid (2n=12) and four tetraploid populations. (2n=24). *Jasione sessiliflora* was tetraploid (2n=24). Six populations from the Central System with conflicting taxonomic assignation, either considered *Jasione sessiliflora* or *Jasione crispa* subsp. *tomentosa*, were identified as diploids (2n=12). These populations show morphological differences with typical *Jasione* sessiliflora and are considered "unassigned diploids" in this work hereinafter. Nevertheless, according to the knowledge of the current author they are close or deviant forms of *Jasione crispa* subsp. *tomentos*a. The two investigated populations of *Jasione crispa* subsp. *segurensis* resulted tetraploids (2n=24), as expected.

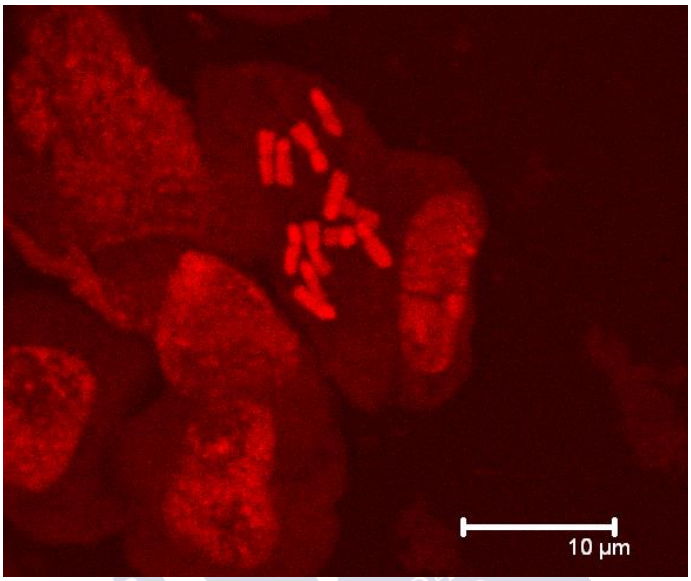

Figure 2.4 Diploid metaphase (2n=12) of *Jasione crispa* var. *praelittoralis* from Prades, Tarragona.

Measurements of morphological traits allowed the identification of informative variation between the taxa. Although the distribution of variability within each taxon is not completely unimodal in both hydathoid average number and hair width, the dispersion of values is enough illustrative to reveal interesting discontinuities (Figure 2.6). Some correlation seems to exist between ploidy level and hairy width, with higher values occurring in tetraploid individuals. *Jasione crispa* subsp. *praelittoralis* tends to have larger hair widths despite being diploid, but still shows some overlap in traits with some individuals with one hydathoids/leaf of "*appressifolia"*. Identification of *Jasione sessiliflora* subsp. *appressifolia* as diploid and molecular identity between "*tomentosa*" and "*appressifolia*" chloroplast markers, suggest thar they form slightly divergent form of the same species. As "*appressifolia*" is a preferent name over "*tomentosa*" individuals of both groups are treated under the label "gr. *appressifolia*" in Figure 2.6

to reduce figure complexity and after checking that no differences occurred between them in the investigated traits. The name "*tomentosa* 4x" is reserved to those populations with intermediate characters between *J. sessiliflora* and *J. crispa* subsp. *tomentosa* identified as tetraploids.

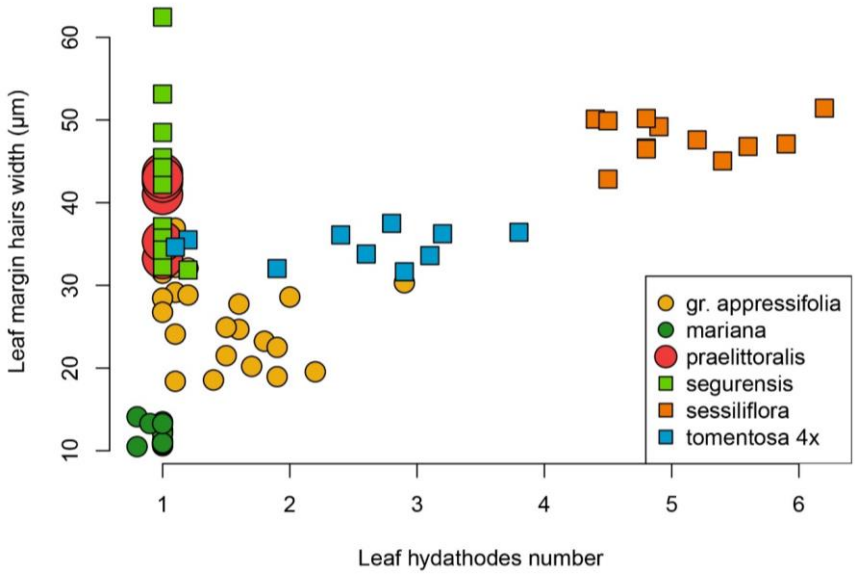

Figure 2.6. Comparison in the model group of leaf marging withd with average numer of leaf hydathoids."gr. *appressifolia* includes *J. sessiliflora* subsp. *appressifolia* and typical diploid *J. crispa* subsp. *tomentosa*.

The analysis of adaxial and abaxial epidermal cell sizes by the measurement of cell larger dimension allows the identification of morphological differences between the populations of the cryptic taxon *Jasione crispa* var. *praelittoralis* and the diploid populations of *Jasione crispa* subsp. *tomentosa* and *Jasione sessiliflora* subs. *appressifolia* (Figure 2.5). These results contributes to support the proximity of the two latter taxa, that would be treated as the "*appressifolia-tomentosa*" group hereinafter.

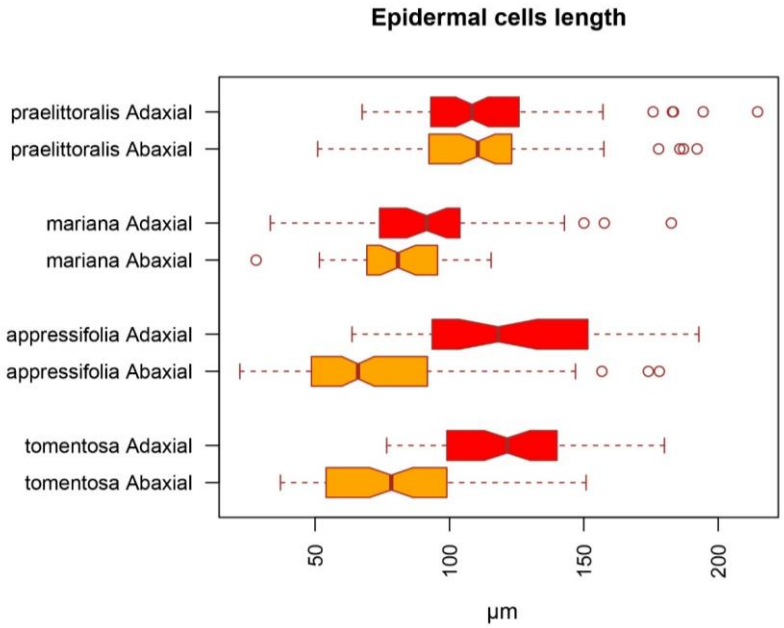

Figure 2.5 Length of the maximum dimension of adaxial and abaxial epidermal cells.

The annual distribution of the flowering period shows clear differences between the taxa of the model group (Figure 2.7). *Jasione crispa* subsp.*mariana* populations are the earlier in flowering. The overlap with plants of the "*appresifolia-tomentosa*" group is not negligible however. The group "*appresifolia-tomentosa*" shows and a bimodal pattern with two flowering peakes, the second one completely overlaped by the flowering distribution of *Jasione sessiliflora*. This striking pattern does not seem explained by ploidy variation with *Jasione* crispa subsp. tomentosa, as many diploids populations are included in the second peak.

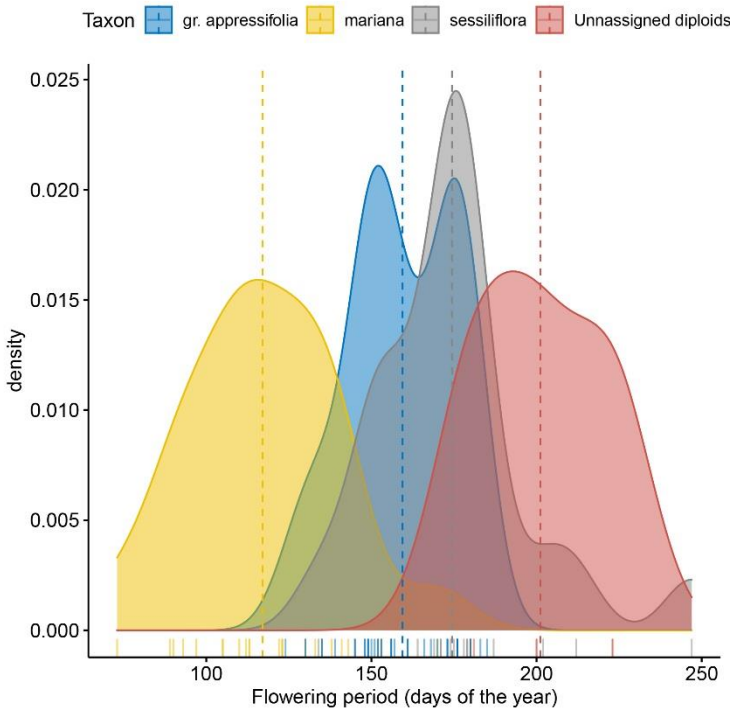

Figure 2.7. Phenology

3.4. Biogeography

The results show a high degree of concordance between the used biogeographical regionalisation, the geobotanic subprovinces of the Iberian Peninsula (Rivas-martínez et al., 2017) and the geographic distributions of the significant evolutionary entities identified in this work. In Figure 2.8 geographic occurrences of the different taxonomic and ploidy groups are shown. The map maximizes differentiation, showing all previously recognized groups even if not delineated as species by ASAP or GMYC. It also shows within taxa ploidy differentiations.

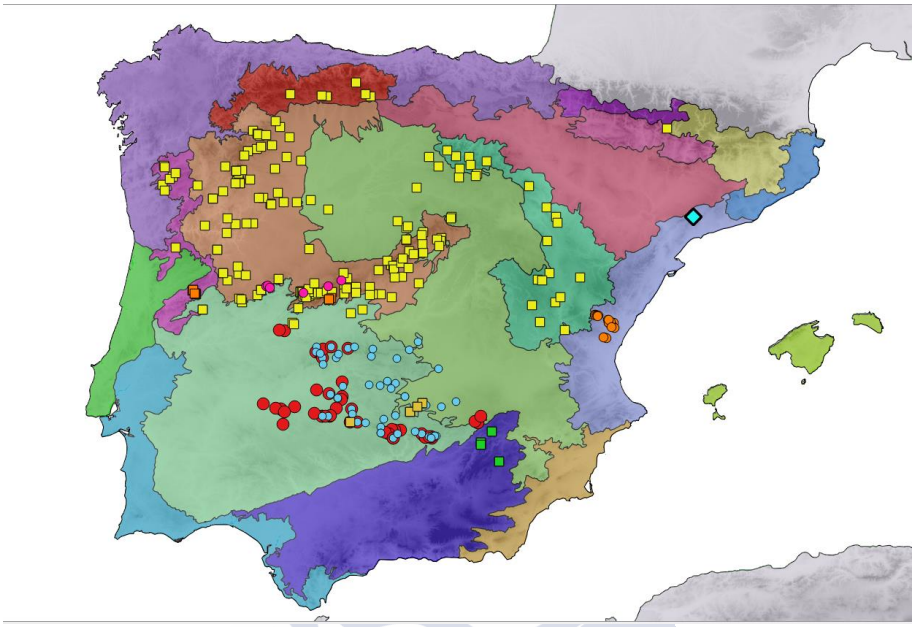

Figure 2.8. Biogeographical subprovinces of the Iberian Peninsula (Rivas-Martínez et al., 2017) and populations of the different groups of *Jasione*. Diploids: J. *praelittoralis* (blue diamond), *J.appressifolia* (orange circles), *J. tomentosa* (blue circles), unassigned diploids of the Central System (pink circles). Tetraploids: *J. sessiliflora* (light yellow squares), *J. tomentosa* 4x (dark yellow squares), *J. crispa* 4x from the Central system (orange squares)

The heatmap analyses made on the haplogroups with GMYC support show a clear biogeographic structure (Figures 2.9 and 2.10). Diploid groups, represented by "*praelittoralis*", "*mariana*" and "*appresifolia*" haplogroups are concentrated in the Luso-Extremadurense and Valencian subprovinces, and in all cases within the Mediterrnean region. These are the most thermic of all subprovinces with occurrences of represeantives of the model group (Figure 2.9). The tetraploids represented by "*sessiliflora*" and "*crispa* 4x" haplogroups show a preference for colder subprovinces, penetrating in the Eurosiberian region.

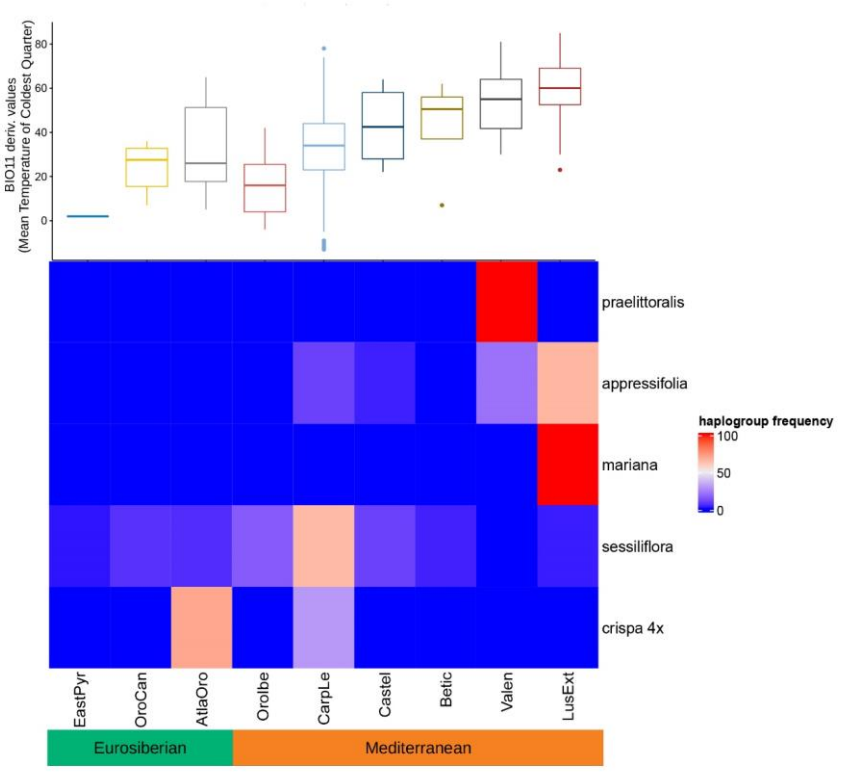

Figure 2.9. Heatmap of haplotype frequencies of the four haplogroups with posterior probability support and GMYC support in the biogeographic subprovinces of the Iberian Peninsula (Rivas-Martinez *et al.*, 2017). The scatter plot of the mean temperature values of the coldest quarter of the year represents the values obtained from populations of the model group in each subprovince.

The k-means clustering analysis based of haplotype frequencies stablished a clear hierachy strikingly coincident with the GMYC and ASAP methods (Figure 2.10). Thus, haplogroups are clustered in three main groups based on their biofgeographic similitudes supporting the Species Delimitation suggested by the SD tools. The three groups are "*praelittoralis*", "*appressifolia*" and "*sessiliflora*". The analysis also split and clustered the biogeographic subprovinces based on the haplotype frequencies indentifiying fouir main geographical groups in the Iberian Peninsula (North-West, Central, South-West and East).

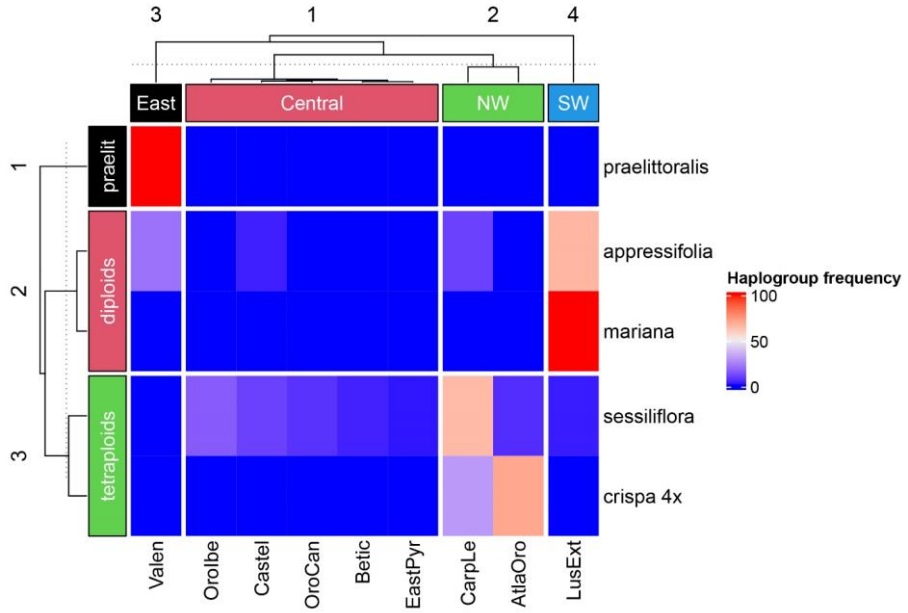

Figure 2.10 Double K-means split and clustering heatmap analysis based on frequencies of each haplogroup with Bayesian posterior probability and GMYC supports (indicated on the left) in the biogeographic subprovinces of the Iberian Peninsula (indicated at botton). Geographical groups are represented on the top. Species Delimitation suggested by biogeographic clustering is based on the occurrence of the haplogroups in the different subprovinces (represented on the right).

4. DISCUSSION

Species delimitation is a complex task in recently diverged groups characterized by morphological stasis, and the results could vary in relation to the lines of evidence used (Michonneau, 2015). The greater the number of concordant lines of evidence the more reliable the results. In this work two Species Delimitation methods were used (ASAP and GMYC), that agreed suggesting the recognition of at least three species withing the diploid-tetraploid complex of *J. sessilifilora*. These results were complemented with multiple concordant lines of evidence form cytology, morphology, and phenology, so assigning populations to

species could be confidently based on observable patterns of relative discontinuity, although not immediately evident.

The ploidy level of diverse taxa is published for the first time in this work, with the ploidy distribution also investigated at the population level. Thus, *Jasione sessiliflora* subsp. *appressifolia* and *Jasione crispa* var. *praelittoralis* are identified as diploids, what supports ASAP and GMYC species delimitation hypotheses. In summary, the three main identified specific groups are: One first group including the tetraploid populations of typical *J. sessiliflora*, some tetraploid populations of the western Central System previously assigned to *J. crispa* subsp. *crispa* (Sales and Hedge, 2001b), tetraploid populations with intermediate characters between *J. sessiliflora* and *J. crispa* subsp. *tomentosa* and *J. crispa* subsp. *segurensis*. One second group included most diploid populations, with *J. crispa* subsp. *tomentosa*, *J. crispa* subsp. *mariana* and *J. sessiliflora* subsp. *appressifolia*. One third group exclusively included the narrow endemic *J. crispa* var. *praelittoralis*, unveiling as a cryptic diploid species.

The multi-evidence approach supported the inferred three groups, buts also suggest that internal variability within the first and second groups requires taxonomic reorganization. As an example, the results do not support the inclusion proposed by Sales & Hedge (2001a) of *J. crispa* subsp. *segurensis* within the variability of *J. crispa* subsp. *tomentosa*. Similarly, the proposal of Mateo Sanz & Crespo (2008) of including the populations of coastal mountains of Valencia and Castellón in *J. sessiliflora* (as *J. sessiliflora* subsp. *appressifolia*) was not supported either.

Reciprocal monophyly is a property of those groups of populations that become different evolutionary units, albeit the differentiation degree can be not important enough to designate species, particularly when secondary contact allows generalized introgression. This pattern was identified in *J. crispa* subsp. *mariana* within the diploid group, and also in "*J. crispa* 4x populations", within the tetraploid, as both groups formed statistically well supported subclades in the Bayesian phylogenetic analysis used to generate a tree for GMYC (Figure 2.2).

Albeit none of them was recognized as different species by ASAP or GMYC, their evolutionary significance probably makes these lineages worthy of more detailed studies. In particular, the maintenance of important phenological and morphological specificities, probably due to phenological assortative mating, in *J. crispa* subsp. *mariana* despite pervasive sympatry with *J. crispa* subsp. *tomentosa*, could justify a higher taxonomic rank.

Ecological unity is one of the properties that characterize species (Barraclough, 2019). Differentiated suitability to contiguous or proximate biogeographic units of the delimited species is considered here be considered a complementary line of evidence to support evolutionary differentiation in neighboring lineages. This work aimed to test whether the delimited species showed internal coherence in realized niches using as proxy its adequacy to biogeographical regionalization. The results consistently supported this theoretical conceptualization. The distribution of frequencies in the subprovinces of five haplogroups, labelled with names from traditional taxonomy (Figure 2.10), was assessed by non-aprioristic K-means split analysis. The results fulfilled the best expectations of species unity in realized niche identified as proxy from biogeographical coherence. Haplogroup were biogeographically organized in complete accordance with the delimited species proposed by ASAP and GMYC methods. Biogeographical coherence would probably result of adaptive processes to different niches to reduce the cohesive effects of gene flow. As in this case a polyploid system is involved, complementary explanations of niche shift to scape minority-cytotype exclusion should be considered (Levin, 1975 and other references in Chapter 4). Finally, the results could be considered a bi-directional validation, as the matching of reciprocally monophyletic lineages identified as species modestly contributes to support the biological significance of a biogeographical regionalization not universally followed (Morrone, 2018).

5. CONCLUSIONS

Species Delineation tools based on co-inherited multilocus information from the chloroplast genome were useful to delineate undetected evolutionary significant entities (i.e., cryptic species) in a plant group with high levels of morphological stasis. Different complementary lines of evidence supported the main conclusion of the GMYC and ASAP species delimitation analyses. Analysis based on biogeographic regionalization, as synthesis of environmental and historical information, also supported the suggested species delineation, contributing to make the species hypothesis biologically sound.

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CHAPTER 3.
Biogeographical history of *Jasione* L. (Campanulaceae), a genus with a disjunct east-west Mediterranean distribution

ABSTRACT

Jasione is a taxonomically complex genus with a disjunct east-west Mediterranean distribution pattern. Around 16 species are currently recognized, although more than one hundred taxonomic names have been proposed. The hypothesis that intense morphological stasis is concealing evolutionary relevant variability and hampering correct understanding of the evolutionary relationships in the genus needs to be tested. Although the genus is distributed in Europe and around the Mediterranean basin, no comprehensive phylogenetic analysis has been carried out until now. The aims of this work were to test the monophyly of the genus and determine its position among other lineages basal to the tribe Campanuleae, an unresolved conflicting topic in different studies. The research also aimed to establish the internal phylogenetic relationships by comprehensive sampling including different populations and most of the proposed names, to prevent a priori exclusion of evolutionary significant lineages. The roles of secondary contact and variation in ploidy levels in shaping the evolutionary history of the genus were also investigated. The underlying processes leading to the disjunct east-west Mediterranean distribution pattern were investigated. To address these questions, a five loci time-calibrated phylogeny was constructed with 111 terminals, 93 of which corresponded to *Jasione*. The genus was resolved as monophyletic, within a clade shared with two other isolated lineages, *Hesperocodon* and *Feeria*. This clade is sister to the *Campanuleae* tribe. The lineage leading to *Jasione* originated in the late Oligocene (c. 25 Mya) and the

extant lineages of *Jasione* derive from a much later diversification event, occurring in c. 9 Mya in the Miocene, from an ancestor either originated in the southern Iberian Peninsula or already present in the western and eastern Mediterranean regions. Two main clades can be identified, one only present in the western Mediterranean and encompassing species with very different morphologies and another with the extant disjunct distribution pattern. One episode of vicariance is compatible with the late Messinian aperture of the Gibraltar strait, although several posterior overseas dispersal events from the Iberian Peninsula to North Africa were inferred in both clades. The second clade began to diversify at the beginning of the Pleistocene, probably in the Balkan Peninsula, and at least two east to west dispersal events are identified in this clade, with niche modelling identifying niche conservatism in the eastern group and niche expansion in the western group. Habitat suitability was not identified in most of the intermediate European regions. The disjunct east-west distribution pattern in *Jasione* is a dynamic biogeographic process in which the two areas harbour lineages providing the opportunity both to remain and to disperse to the other areas, increasing the chances of the lineage to endure. Finally, the resulting phylogeny is not consistent with current taxonomy showing that morphological stasis in *Jasione* has hampered the systematic understanding and suggesting the need for further integrative taxonomic studies.

1. INTRODUCTION

The Mediterranean basin offers one of the most challenging global scenarios for interpreting the evolutionary patterns of the lineages occurring in the region. It is considered one of the 25 global hotspots of biodiversity (Myers *et al.*, 2000), encompassing more than 24,000 plant species, 10% of all described species and 13,000 endemic species (Suc, 1984; Quézel, 1985). The region underwent intense geological and climatic instability during the second half of the Cenozoic. These

changes included the diverse Alpine orogenies (Mather, 2009), the movement of several African microplates, first the Iberian – which collided with Eurasia in the Late Eocene (35 Mya) – and then others such as Betic-Riffian, Balearic-Kabylies and Apulian microplates, which were initially adjoined, then separated and moved westwards. The horizontal movement caused either the isolation or the transfer of terranes, and therefore of biotas, between the Eurasian and African plates (Blondel, 2009; Meulenkamp & Sissingh, 2003; Rosenbaum *et al.*, 2002). Among the most dramatic effects of the westward movement of the Betic-Riffian microplate to its current position between Iberia and Africa was the almost complete desiccation of the Mediterranean Sea at the end of the Messinian age (5.9-5.3 Ma) (Hsü, 1972; Tzedakis, 2009). At the opposite side of the sea, the Eastern Mediterranean basin is a relict of the Mesozoic Neo-Thethys Ocean (Garfunkel, 2004), and the Balkan/Antolian region roughly corresponds to the land mass that separated the Tethys Ocean from the Paratethys during the Middle Miocene (14-13 Ma) (Rögl & Steininger, 1983), although the territories in the eastern Mediterranean region began to form earlier, during the Oligocene (34-30 Mya) (Krijgsman, 2002). The influence of marked climatic changes, characterized since the last paratropical climatic optimum in the Miocene (17-15 Mya) by a general trend of cooling and culminatig in the Pleistocene glaciations, was also dramatic for the diverse biotas (Tzedakis, 2009). The cooling process was coupled with increasing aridification, a factor as important as temperature in determining the fate of many regional lineages. Grassland expansion during the late Oligocene is the first sign of the aridification process (Miller *et al.*, 2008), a trend definitively established around 9-8 Mya (van Dam, 2006). The above referred period of desiccation of the Mediterranean Sea, between 5.9-5.3 Mya, known as the Messinian Salinity Crisis (MSC), caused by the closure of the straits connecting the Mediterranean Sea and the Atlantic Ocean, probably led to a decrease in the regional humidity over time (Hsü, 1972; Krijgsman, 2002; García-Castellanos *et al.*, 2009). Undoubtedly, the onset of the Mediterranean climate in the Pliocene, developed between 3.2-2.8 Mya, characterized by cool wet winters and a period of drought coincident with the hot season, was not the most minor of these changes (Suc,

1984). Again, the Pleistocene glaciations not only dramatically affected the temperature regimes, but also the global circulation of humidity. Reduced evaporation and retention of continental water in the form or glaciers led to lower precipitation in general, making the Pleistocene glaciations cold and dry periods (Rull, 2020).

This eventful history has configured a complex evolutionary and biogeographic framework with a mixture of biotas. In some cases, the endurance of ancient Tertiary lineages was possible in certain areas (Quézel, 1985; Oosterbroek & Arntzen, 1992) while in others complete extinction and substitution of entire biotas prevailed, particularly during the quaternary glaciations (Quézel, 1985; Médail & Diadema, 2009; Hewitt, 2011). The role played by glacial refuge areas has been revealed by their coincidence with the current regional hotspots of plant and animal diversity (Médail & Diadema, 2009; Habel *et al.*, 2010), identified by high rates of narrow endemisms. In summary, the complex geological and climatic history of the Mediterranean area has repeatedly separated and joined diverse land masses, causing intermittence in the efficiency of barriers to dispersal, and recurrently allowing or interrupting the exchange of biotas between areas. This changing process led to reticulate biogeographical patterns of difficult interpretation (Sanmartín, 2003; Nieto-Feliner, 2014).

1.1. The east-west Mediterranean disjunction

This biogeographical pattern is one of the most striking in the Mediterranean basin. Sometimes named the Kiermack disjunction, the prevailing idea is that it has a pre-Pleistocene origin, having arisen during in the Tertiary (García-Vázquez *et al.*, 2016; Fačkovcová *et al.*, 2020). It is well known in many groups of organisms, including animals (Oosterbroek & Arntzen, 1992; Lumaret & Lobo, 1996; Sanmartín, 2003; García-Vázquez *et al.*, 2016), plants (Willkomm, 1896; Braun-Blanquet & de Bolòs, 1957; Kadereit & Yaprak, 2008; Nieto-Feliner, 2014) and lichen-forming fungi (Barreno, 1991; Fačkovcová *et al.*, 2020). The pattern is characterized by disjunct distributions of current

ranges at both extremes of the Mediterranean longitudinal axis, and a gap of occurrences in-between. As pointed out above, the pattern has frequently been explained as result of the fragmentation of wider pre-Pleistocene continuous distributions, with lineages surviving in refugia with suitable climatic conditions, in some cases undergoing new Holocene range expansion (Taberlet *et al.*, 1998), but also as a result of long distant dispersal from one of the sides of the Mediterranean. In the latter case, the newly colonized area would be characterized by being smaller and less variable (Thompson, 1999). The pattern is found across different taxonomic levels, between sister genera, sister clades within a genus or within one species (Calleja *et al.*, 2009; Rosselló *et al.*, 2007, Casimiro-Soriguer *et al.*, 2010). However, the occurrence of the pattern at different taxonomic levels would indicate recurrent biogeographic processes within different temporal frameworks. Pleistocene fragmentation of the lauroid circum-Mediterranean Tertiary vegetation has been hypothesized as the main origin of the pattern in plants (Mai, 1989). The pattern would thus apply to relict elements with thermic and mesic environmental preferences (Kalkman, 1965). In these cases, the western part of the disjunction can include different parts of an area encompassing the Iberian Peninsula, north-western Africa, and/or the islands of Macaronesia (Mai, 1989). The eastern side of the disjunction may include the Balkan Peninsula, Anatolia and/or the Caucasus, with occasional outposts in Lebanon (Milne, 2004). These relict elements are usually more widely distributed in the wetter eastern part of the disjunction, where they have encountered large areas with suitable environmental conditions around the Black Sea (Denk *et al.*, 2001). Among the plants, typical elements of this disjunct relict biota include some woody taxa such as *Rhodondendrom ponticum* (Mejías *et al.*, 2002) or the vicariant pair of species *Prunus lusitanicus* and *Prunus laurocerasus* (Calleja *et al*, 2009). Within the Campanulaceae, the *Campanula peregrina/Musschia* clade fits in this pattern, as their members are mostly linked to relict lauroid habitats (Trias-Blasi *et al.*, 2011; Mansion *et al.*, 2012). Nevertheless, this disjunction appears in other groups that do not correspond to mesic relict environmental preferences. This can be observed in plants of Mediterranean sclerophyllous forests, such as *Erophaca baetica*, occurring on dry

slopes of the Iberian-North African area and in the Peloponese (Casimiro-Soriguer *et al.*, 2010), or *Buxus balearica*, with scattered populations in the western Mediterranean region and in southern Anatolia (Rosselló *et al.*, 2007). Nevertheless, both taxa have been interpreted as Tertiary relicts with disjunct distributions associated with eastern and western glacial refugia (Rosselló *et al.*, 2007; Casimiro-Soriguer *et al.*, 2010). Therefore, in these groups thermic factors would have played a more important role than humidity. Other east-west disjunct groups do not fit well in the Tertiary relict pattern, as is the case of the relatively cold-tolerant *Microcnemum coralloides*, a plant from saline habitats in inner Iberian Peninsula, Anatolia and Iran. In fact, its disjunct range has been suggested to be a result of an expansion from the east in cold Pleistocene periods and later fragmentation in a warmer period (Kadereit & Yaprak, 2008). This species may be representative of a group of organisms that arrived the Iberian Peninsula and North African from the Middle East and the Iranian plateau, most probably during the cold and dry periods of the Pleistocene before undergoing range fragmentation. The most likely migration route for these drought-adapted or steppe groups would have been across North-Africa (Quézel, 1985, Pérez-Collazos *et al.*, 2009; Manafzadeh *et al.*, 2013). Nevertheless, the timing of dispersal varies among the groups, ranging between the Pleistocene and the Late Miocene, implying different pulses of biogeographic expansion. In the case of the Late Miocene, the closure of the straits connecting Africa and the Iberian Peninsula would have enabled dispersal into south-western Europe of plant and animal lineages (Davis & Hedge, 1971; Sanmartín, 2003; Barres *et al.*, 2013). Finally, a disjunct east-west pattern associated with the European Mountain System taxa has been described in the butterfly *Erebias ottomana*, with oro-Mediterrameam environmental affinities and different subspecies in the Balkans and Anatolia and in the French Massif Central. This pattern has been interpreted as the result of pre-glacial or inter-glacial connections with each mountain range being a differentiation centre (Varga & Schmitt, 2008).

A correct approach to the east-west Mediterranean disjunction should be able to discriminate different possible causes underlying a common pattern that probably represents different biographic histories.

Otherwise, the interpretation may lead to biogeographic pseudo-congruence, as defined by Cunningham & Collins (1994). Time calibrated phylogenies and knowledge of the environmental determinants of the occupancy of the territory would allow testing of the biogeographic hypothesis. Accumulation of information from different lineages that match the pattern could lead to the eventual unveiling of the different types of biogeographic causes of what seems to be a recurrent disjunction process. Unfortunately, despite its biogeographic interest and importance in shaping the distribution of biodiversity around the Mediterranean hotspot, studies addressing this topic in plants or animals are scarce, and most research has focused on the specific level (reviewed in Nieto-Feliner, 2014). In most cases, and regardless of the colonizing route followed (either a European or an African path), the prevailing view among researchers analysing the pattern has been that the Eastern Mediterranean area acted as a cradle for and main source of lineages and a reservoir of biodiversity and genetic variation during the harsh periods of the Quaternary. This has been related to the hinge geographical position of the region between the Mediterranean basin and the main portion of the Eurasian landmass, and to the milder environmental conditions during the Pleistocene (wetter, warmer) than in the western area (Fady, 2005; Schmitt, 2017). Thus, successive colonization waves would have originated in the eastern areas throughout the Neogene, with lineages preferentially enduring in the eastern Mediterranean basin during the Pleistocene. This paradigm has mainly been inferred at the generic level or among closely related genera, based on specific richness and genetic variability in the eastern zone, and has been identified in plants either in trees of the gymnosperm families Pinaceae and Cupressaceae (Fady, 2005) or in different herbaceous groups, monocots and dicots (Mansion *et al.*, 2008; Mansion *et al.*, 2009, respectively), with the latter group including the genus *Campanula* s.l. (Roquet *et al.*, 2009). However, as pointed out by Nieto-Feliner (2014), further evidence is required to clarify the causes of the prevalence of the eastern area as a cradle for and reservoir of lineages and also the extent to which this idea represents a general pattern. In fact, the opposite model has been proposed for at least groups of ancient origin, during the early-mid

Tertiary, in which the most basal lineages are located in the western Mediterranean area (Oosterbroek & Arntzen, 1992). The east-west disjunct pattern has a variant in which the centres of diversity of a genus concentrated at both extremes of the Mediterranean, but with one or few species with intermediate ranges, including the Italian Peninsula and/or the great Central Mediterranean islands. In these cases, it has been demonstrated that the intermediate areas have been colonized recently. This type of scenario (i.e. ancient lineages in the Mediterranean extremes and young Pleistocene lineages in the Italian Peninsula), as in the case of the *Deronectes* diving beetles (García-Vázquez *et al.*, 2016) has been explained by the geological history of the Italian Peninsula, which remained submerged until the Pliocene (Meulenkamp & Sissingh, 2003), making it irrelevant during most of the evolutionary history of the group. Nevertheless, this interpretation contradicts the occurrence of many relict and endemic taxa in the Apennine Peninsula (Habel *et al*., 2010; Hewitt, 2011). The biogeographic explanation must be inferred in each lineage without *a priori* assumptions, and only later analysis of the inferred history contributes to supporting any of the different patterns within the general east-west disjunct pattern. The case under study here, i.e. the genus *Jasione* in the family Campanulaceae, corresponds to the variant pattern of diversification at both extremes of the Mediterranean basin with few taxa having intermediate ranges. However, which of the sub-lineages are ancient or young, which area was important as a cradle for biodiversity of the genus and the sequence of colonization and divergence have not yet been studied.

1.2. The genus *Jasione* and allied lineages and their phylogenetic position within the Campanulaceae

The topological position of *Jasione* in relation to the main groups of the subfamily Campanuloideae, one of the five main clades of the Campanulaceae, has been intensely disputed (Eddie *et al.*, 2003; Mansion *et al.*, 2012; Crowl *et al.*, 2016; Yoo *et al.*, 2018; Xu & Hong, 2021). The most speciose group (c. 1,050 spp.) in the subfamily Campanuloieae is the tribe Campanuleae, which is mainly distributed

in extra-tropical zones across north Africa, Eurasia and North America (Lammers, 2007a; Roquet *et al.* 2009; Crowl *et al.*, 2016). The geographic origin of this group is not clear and has been suggested to be either in the western Mediterranean region or in this area plus Anatolia (Roquet *et al.*, 2009). The basal position regarding the core Campanuleae of four isolate and pauci-specific lineages with Mediterranean distribution support this dual hypothesis. These isolated lineages have been referred to as "transitional" (Eddie *et al.*, 2003) because they are located between the Campanuleae and the tribe Wahlenbergeae, a clade with a mainly southern-hemisphere distribution and mostly diversified in tropical and southern Africa. *Jasione* is the most diverse among the isolated "transitional" groups, the other two being monospecific generic lineages, the genus *Feeria* (with *Feeria angustifolia* as the only species) and the genus *Hesperocodon* (with *H. hederaceous/Wahlenbergia hederacea* as the only species). The remaining "transitional" group is a lineage leading to a clade that groups five *Campanula* species (*C. peregrina, C. lactiflora, C. axilaris* in the eastern Mediterranean basin, and *C. primulifolia* and *C. alata* in the western Mediterranean basin) plus the Madeiran endemic genus *Musschia* (Mansion *et al.*, 2012). This group is another example of east-west Mediterranean disjunction, in this case encompassing one Macaronesian island. Nevertheless, the topological recovery of these lineages has been conflicting among different studies, being an open question and recently considered a "basal conundrum" to be resolved (Xu & Hong, 2021). The main conflicts lie in the relationships between *Jasione, Feeria* and *Hesperocodon,* and also in their relationships with the three main clades of the genus *Campanula*. These three major clades of *Campanula* are, in the first place, the above-mentioned clade that groups five Campanula species plus the genus *Musschia,* called the "Cam 01 clade" by Mansion *et al*. (2012). The other two clades are tremendously speciose and share a common ancestor and therefore form a sister group to the "Cam 01 clade". They are the *Campanula* s.str. clade, which includes many species of *Campanula* together with *Trachelium, Edraianthus, Symphyandra, Azorina* and other nested genera, and the "Rapunculus" clade, which groups many species of *Campanula* together with *Legousia, Triodanis, Phyteuma, Asyneuma,*

Adenophora and *Hanabusaya* among other nested genera. *Jasione, Hesperocodon* plus the *Campanula-Musschia* group (but not *Feeria*) have been considered relict lineages and possible components of a paleoflora that would have occupied the North-Atlantic area in the early Tertiary, that later evolved and spread throughout North America and Eurasia (Eddie & Cupido, 2014). This statement seems plausible to some point, although these authors also considered these isolated lineages to be ancestral to the Rapunculus clade (Eddie & Cupido, 2014). However, neither the ancestral position to the Rapunculus clade nor the early Tertiary origin of the lineages are supported by other studies (Roquet *et al.*, 2009; Mansion *et al.*, 2012; Crowl *et al.*, 2016). In fact, the topological position of *Jasione* has been recovered in different parts of the Campanuloideae tree, generally with high uncertainty and low statistical support. Thus, it has been recovered between the clade Cam01, sensu *Mansion et al.* (2012) and the other clades of *Campanula* s.l. (Olesen *et al.*, 2012; Mansion *et al.*, 2012; Yoo *et al.*, 2018), in a sister position to all *Campanula* s.l. clades (Crawl *et al.*, 2016) or in an indeterminate position either with the Wahlenbergeae or the Campanuleae (Xu & Hong, 2021). Therefore, a supported phylogenetic hypothesis of the evolutionary location of *Jasione* is needed, as well as its relative position between or within one of the Wahlenbergeae and the Campanuleae lineages. The relationships between *Jasione* and *Feeria*, *Hesperocodon/W. hederaceae* and Cam 01 must also be resolved. Until now, this clarification has been hampered by one of major points of conflict among different studies, related to the position of the lineages of *Feeria* and *Hesperocodon/W. hederacea*.

1.3. The *Wahlenbergia hederacea*/*Hesperodocon hederaceous* and *Feeria* issues

Wahlenbergia hederacea has traditionally been considered a member of the tribe Wahlenbergeae, although several molecular phylogenies placed it in a sister position to *Jasione* (Cellinese *et al.*, 2009; Haberle *et al.*, 2009; Prebble *et al.*, 2011; Olesen *et al.*, 2012). Based on this evidence, a new monotypic genus *Hesperocodon* was

proposed to accommodate this species (Eddie & Cupido, 2014). The lineage of *Hesperocodum* had been topologically located between the core Campanuleae and the Wahlenbergeae tribes in some early molecular phylogenetic approaches regarding the family (Eddie *et al.*, 2003). Roquet *et al.* (2009) recovered this lineage in an unresolved polytomy with *Jasione* and *Feeria* plus some typical representatives of the tribe Whalenbergeae from the southern hemisphere. Relatively close phylogenetic relationships between *W. hederacea* and *Jasione* were again suggested by Olensen *et al.* (2012), who used a combined data set of the atpB, matK, rbcL and trnL-F plastid regions. However, the species-extensive petD phylogeny of Mansion *et al.* (2012), and a more recent work using one nuclear locus and 15 plastid loci (Crowl *et al.*, 2016) recovered this species within the Wahlenbergeae clade. After obtaining these results, Crowl *et al.* (2016) suggested caution and the need for more in-depth research. The need for clarification of the systematic position of this lineage has been claimed by Kadereit *et al.* (2016).

Feeria has been recovered in different positions in the published Campanulaceae topologies. Three works, the ITS phylogeny of Eddie *et al.* (2003), the three plastid regions phylogenetic study of Haberle *et al.* (2009), and the six plastid loci plus ITS of Xu & Hong (2021) recovered *Feeria angustifolia* within the core Campanuleae tribe, nested in a clade with the Macaronesian *Azorina vidalii* and North-African and Southern-Iberian *Campanula* species. Nevertheless, other works dealing with the phylogenetics of Campanulaceae placed *Feeria* close to *Jasione* or at least not within the core Campanuleae (Roquet *et al.*, 2009; Mansion *et al.*, 2012, Crowl *et al.*, 2016). Therefore, the position of *Feeria* must be clarified.

1.4. The genus *Jasione*

The genus *Jasione* includes perennial, biennial and annual plants with characteristic condensed inflorescences in flower-heads and connate anthers (only one species, *J. bulgarica*, has free anthers), with a distribution around the Mediterranean basin. Only one species, the

biennal/annual *Jasione montana*, is widely distributed throughout Europe and north-western Africa. The genus fits in the east-west disjunct pattern, with two centres of diversification at the extremes of the Mediterranean basin. One in the west, including the Iberian Peninsula and the Maghreb, and other in the east, including Anatolia and the Balkan Peninsula (Figure 1).

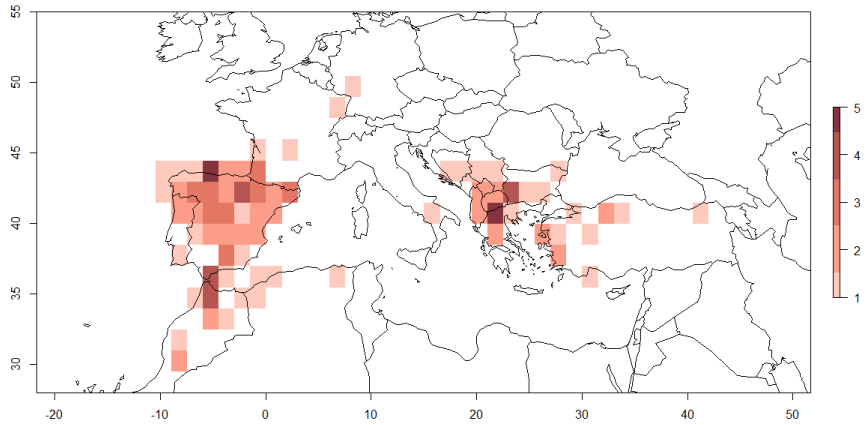


Figure 3.1. Taxonomic richness map for the genus *Jasione*, excluding the widespread species *Jasione montana*. The map depicts the accumulation of putative evolutionary significant entities.

The number of species recognized is relatively low, varying among the different treatments and ranging between 12 and (Sales & Hedge, 2001) and 17 (Castroviejo *et al.*, 2010). However, the number of taxa deserving taxonomic recognition may be much greater, as the genus is characterized by remarkable morphological stasis. Therefore, the scarcity of morphologically discriminating characters, but the relative polymorphism of some species seems to have obscured the recognition of diverse evolutionary significant entities. This is the reason why most treatments tend to be overly synthetic in *Jasione*, although the number of taxonomic names proposed in the genus is close to one hundred (Schmeja, 1931; Kovanda,1968). The genus occupies a number of

ecological niches, ranging from coastal habitats to mountain summits, occurring in mesic meadows in relatively arid sandy or rocky environments. *Jasione* appears in a broad variety of soils, developed from either limestones or siliceous bedrocks. Nevertheless, the edaphic preferences do not seem randomly distributed throughout the genus and may be clade-dependent. Some narrow endemic soil-specialists have adapted to magnesium-rich dolomitic soils in the Iberian Peninsula (e.g. *Jasione penicillata* or *Jasione crispa* subsp. *segurensis*) while some lineages have adapted to ultramafic serpentine soils in the Balkan peninsula (e.g. *Jasione jankae*, sensu Hartvig, 1991). Many taxa are chasmophytes or prefer stony or sandy environments. Divergence seems to have been promoted by polyploidy, with at least five different ploidy levels occurring in the genus (Küpfer, 1981; M. Serrano, own data). Nevertheless, polyploidy is restricted to Western Europe and can be considered a mainly Iberian process (M. Serrano, own data). Narrow endemism seems to shape the biodiversity of the genus, with many morphologically differentiated populations associated with mountain ranges both in eastern and western areas. However, general taxonomic treatments have lumped many forms together (Tutin 1976; Castroviejo *et al.*, 2010), as e.g. most of those described by Stojanov (1926) in the Balkan Mountains and close areas. The *Jasione* genus seems to be prone to cryto-speciation (Chapter 2), and therefore the validity of many unrecognized narrow endemic taxa is worthy of investigation in a molecular phylogenetic framework. The western area accumulates more groups than the eastern area, indicating the western area as the source of lineages of the genus. However, the location of the most basal lineages remains an open question, as *Jasione* species have been segregated in different genera twice, in one case with one western endemic lineage and in other case with an eastern endemic lineage (Lammers, 2007a). The genus *Jasionella* was proposed to accommodate the eastern *J. bulgarica* based on being the only *Jasione* species with free anthers (Stojanov & Stefanov, 1933), while the genus *Urumovia* was created for the western species *J. foliosa* based on it lousy inflorescence and peculiar bract and leaf morphology (Stefanov, 1936). Regardless of the validity of these names as different generic entities, the proposals establish a hypothetical framework regarding the

basal lineages in *Jasione*, which in this case is closely linked to the inference of the ancestral area of the genus. Biogeographic relationships have also been suggested in diverse groups of *Jasione* with supposed disjunct patterns between the two main areas of each Mediterranean side, i.e. between Anatolia and the Balkan peninsula, and between North-Africa and the Iberian Peninsula. On the eastern side, the Balkan and Anatolian mountains would have been the scenario of radiation of ten closely related taxa belonging to a single lineage, the so-called *Jasione supina* group (Stojanov, 1926; Damboldt, 1978), with the main disjunction identified by Stojanov (1926) between both peninsulas, with *Jasione orbiculata* var. *italica*, as an outpost of the Balkan group occurring the southern Italian Peninsula. The Balkan-Anatolian disjunct distribution pattern, sometimes including the Caucasus region, is well known in different plant groups (Caković *et al.*, 2015; Crowl *et al.*, 2015; Eker *et al.*, 2016; Özüdoğru & Mummenhoff, 2020). The biogeographical history of the Balkan-Anatolian distributions has been studied in animal groups (Çıplak *et al.*, 2015; Chobanov *et al.*, 2016; Kaya & Çıplak, 2017) and in some plant groups (Bittkau & Comes, 2005; Crowl *et al.*, 2015; Özüdoğru & Mummenhoff, 2020). Vicariance has suggested to be the result of two main geological events, the break-up of a land mass in the Miocene that united Anatolia and the southern Balkan peninsula until 12 Mya (Dermitzakis, 1990), and the Messinian Salinity crisis (MSC), which reconnected both areas in the period between 5.96 Mya to 5.33 Mya (Hsü, 1972). Both events have been determinant in different groups, promoting two pulses of vicariance, including one representative of the Campanulaceae, the Roucela complex of the genus *Campanula* (Crowl *et al.*, 2015, and reviewed references therein). Posterior land reconnections between the Balkan and Anatolian peninsulas have been suggested, with some land bridges probably lasting until the late Pliocene (3.5-2.6 Mya), a connection that came to an end with the opening of the Sakarya basin strait in the transition between the Pliocene and the Pleistocene (Elmas, 2003; Hill *et al.*, 2017). Özüdoğru & Mummenhoff (2020) suggested a pattern already identified in animals (Kaya & Çıplak, 2017), with Anatolia as the source of Balkan lineages. Older lineages would have remained in Anatolia, while younger lineages would have colonized the Balkans

through two main dispersal corridors: the northern Anatolian Mountains and the Taurus mountains. *Jasione* is indeed present in the northern mountains of Anatolia represented by different taxa, but whether their relationships within Anatolia and with the Balkan Peninsula correspond to this pattern has not yet been investigated.

The biogeographical relationships between the Iberian Peninsula and North Africa are marked by a prolonged common geological history of the Betic and Riffain microplates during long periods in the Tertiary, as well as by their mutual separation in the Miocene and posterior accretion to the Iberian Peninsula, in the former case, and to Africa in the latter, the event that provoked the closure of the Mediterranean straits and subsequent MSC (Rosenbaum *et al.*, 2002). The connecting episode of the MSC between Africana and Europe was followed by the definite opening Gibraltar strait c. 5.3 Mya, creating a barrier that has been considered more important for the exchange of biotas than the Alps or the Pyrenees (Hewitt, 2011). However, its efficacy as a barrier varies greatly among groups, and plant lineages dispersed over the barrier, although no particular correlation with adaptations to dispersal has been found (Lavergne *et al.*, 2013, Martín-Rodríguez *et al.*, 2020). In this respect, different groups of plants were able to cross the strait after the MSC (Fernández-Mazuecos & Vargas, 2011; Gil-López *et al.*, 2017). Several *Jasione* species can be found on both sides of the Gibraltar strait, sometimes considered distinct subspecies. However, no sister relationship between Iberian and African population has been established, and the taxonomical situation may differ among the cases. For example, although the subespecies of *Jasione foliosa* of both continents are morphologically and ecologically similar (Dobignard, 1997), the African populations considered subspecies of *Jasione crispa* (subspecies *lanuginella* and *mesatlantica*) and *Jasione sessiliflora* (subsp. *cedretorum*) more probably represent distinct lineages, despite their relative morphological similarity, on account of their differences in ecology and ploidy with the Iberian populations. Close relationships between the Iberian *Jasione blepharodon* and the Moroccan endemic *Jasione cornuta* have been suggested (Parnell, 1987). Finally, two other species have populations with low differentiation across the strait, the coastal *J. corymbosa* and

the widespread *J. montana*. Quaternary glaciations probably enhanced the permeability to dispersal of the strait of Gibraltar, as the decrease in sea level exposed the continental shelves and created a string of small sandy islands (Gràcia *et al.*, 2008). Under these conditions, plant species from coastal dune habitats may have encountered greater opportunities to thrive and disperse through the strait area. In fact, this is the primary habitat of *Jasione corymbosa* and an ecological niche that *Jasione montana* occupies under certain environmental conditions (Castro *et al.*, 2019; M. Serrano, own data). Nevertheless, the traditional systematics of the taxa of *Jasione* occurring on both sides of the strait should be considered a hypothetical context to be tested. Moreover, the relative importance of each of the areas for the ancestral origin of the genus, the direction of the lineage exchange across the strait, as well as the possibility of separated east-west migratory routes on each shore, must be investigated in a time-calibrated molecular framework.

Mountains seem to have played a major role in the diversification of *Jasione*, with many taxa occupying mountain environments, with several narrow endemics restricted to one or few mountain ranges. Mountains provide altitudinal gradients, microclimatic diversity and habitat heterogeneity. They were major Quaternary refugia during the glaciations, but also in the interglacial periods for cold-adapted species, promoting a dynamic process of vicariance and secondary contact associated with glacial cycles (Kropf *et al.*, 2008; Médail & Diadema, 2009). Mountains also provide shelter from the rigors of the Mediterranean climate, due to higher precipitation and evapotranspiration rates. Many *Jasione* species shows a tendency to occupy sub-Mediterranean environments, a boundary belt between the Euro-Siberian and Mediterranean biomes, better developed in the Mountain ranges of both extremes of the Mediterranean basin. These environmental preferences have also been identified in the north-western Iberian polyploid populations of *Jasione montana* (Castro *et al.*, 2019). Mountains under direct maritime influence (< 50 km far from the Mediterranean coasts) are occupied by different species of *Jasione* (e.g. *J. foliosa, J. mansanetiana* and *J. penicillate*). Frequent formation of orographic fog during the arid-summer period may have played an important role shaping the distribution of some species. This

phenomenon would allow a subhumid and relatively warm environment with an important contribution made by horizontal precipitation. Thus, on the summits of the mid-altitude Mediterranean mountains of the Valencian area, capture of water from orographic fogs provides similar values to those recorded in the relict montane cloud forest of the Canary Islands (Estrela et al., 2008).

Few species occur in the intermediate areas of Central Europe or in the Italian Peninsula (Figure 3.1). *Jasione montana* is widespread throughout Europe, except in the Balkan peninsula, reaching Norway in the north and Kazan and the Urals in the east (Tutin, 1976; Fedorov, 1957). *Jasione maritima* occupies the coasts of western France from the Basque country up to the Charente-Maritime at least in the north. Possibly confounded with coastal forms of *J. montana* in the northernmost recorded localities, it is not clear whether the northern boundary of the species reaches Brittany (Tison & de Foucault, 2014; Dupont, 2015; Castro *et al.*, 2020). Most populations of *Jasione laevis* occur in the northern half of the Iberian Peninsula and the Pyrenees, but some also occur in the Cevennes, the Massif Central and surrounding areas in Southern France, and in the southern Vosges and the upper Rhine valley region, around the Black Forest. Finally, two endemic entities have been described in the southern Italian Peninsula: *Jasione orbiculata* var. *italica*, endemic to the Sirino massif and related to the Balkan orophylous taxa (Stojanov, 1926), and *Jasione sphaerocephala*, with a single locality in Calabria (Brullo *et al*., 1981) and perhaps a form of *J. montana*. Analysis of the phylogenetic framework of these endemic lineages could confirm the apparent irrelevance of the Apennine Peninsula in the evolutionary make-up of *Jasione*. It could also help to reveal the environmental and biogeographical processes underlying the scarcity of *Jasione* in an area that is otherwise a well-known refugium and hotspot of Mediterranean diversity (Cozzolino *et al.*, 2003; Heuertz *et al.*, 2006; Ansell *et al.*, 2008; Canestrelli & Nascetti, 2008; Hewitt, 2011; Španiel *et al.*, 2011).

There has not been any comprehensive phylogenetic work in *Jasione*. Pérez-Espona *et al.* (2005) attempted a phylogenetic approach with a nrDNA ITS phylogeny including a few representatives of the

genus, mostly populations of *Jasione montana* recovered in a polytomy. Although these authors regarded their work as inconclusive, some aspects can be highlighted. Firstly, two clades were inferred, one with few internal resolution and different accessions from the western Europe, including several populations of *Jasione montana* (the type species of the genus), some representatives of the *J. crispa* group, one *J. laevis* and two terminals from the Eastern Mediterranean area, *J. heldreichii* and *J. supina*, both from Turkey. The other clade grouped *Jasione foliosa* with two annual taxa, *J montana* var. *bracteosa* and *J. corymbosa*, which different authors have related to the *Jasione montana* group (Parnell, 1987; Sales & Hedge, 2001, but see Serrano *et al.*, 2009). *J. foliosa* is a perennial chasmophyte from north-faced limestone slopes at the top of low altitude mountains with intense maritime influence, and it is one of the most morphologically divergent species. It occurs in the southern and south-eastern Iberian Peninsula, and a subspecies has been described in northern Morocco (Dobignard, 1997). The grouping of *J. foliosa* with plants that have been considered part of *Jasione montana* highlights how morphology can be deceptive in *Jasione*. Nevertheless, at least one morphological trait is seemingly distributed in accordance with the inferred clades. The leaves of some groups of *Jasione* have remarkable tubular structures at the upper side of the leaf margin. They have been considered noteworthy by other authors (Bokhari & Sales, 2001), who called them "trichoids" and to whom the function remained mysterious. However, these structures are actually modified but fully functional hydathodes, and there is no doubt that they are homologous with the regular hydathodes occurring across the whole Campanulaceae. These structures are named by the current author "hydathoids" (see Introduction). The other main clade recovered by Pérez-Espona *et al.* (2005) show a general lack of resolution, grouping on the one hand the Iberian *Jasione sessiliflora*, with one representative of the group *J. crispa* plus the Anatolian *J. supina*, and on the other, recoversed in polytomy, due to very low nucleotide variation, diverse taxa, mostly from Iberian populations (representatives of *J. crispa, J. montana, J maritima* and *J. laevis*), and one Anatolian species, *J. heldreichii.* These results surprised the authors, who tried to explain them by lack of sufficient time for divergence to accumulate nucleotide mutations (Pérez-Espona *et al.*,

2005). However, other explanations are possible, such as introgression and secondary contact effects, with prevalence of some ITS ribotypes after a process of concerted evolution. Prevailing ribotypes can be extensively distributed across geography and among homoploid taxa, and the ITS homogenization actually underlying the results of Pérez-Espona *et al*. (2005) is a reticulate pattern caused by ribotypic introgression across many diploid taxa from one abundant and widespread species, *Jasione montana* (M. Serrano, own data). Therefore, although the ITS region is an informative marker for studying the phylogenetic ad phylogeographic relationships in some groups of *Jasione*, it may be of no use in other groups if the evolutionary hypothesis to be inferred requires dichotomic ITS topologies for estimating evolutionary species (M. Serrano, own data). The study by Pérez-Espona *et al.* (2005) led to more questions than it solved by drawing evolutionary patterns of difficult interpretation and also because of the scarce sampling of taxa and important biogeographical areas were unrepresented.

This work deals with different questions about the evolution of Jasione and related lineages, using five chloroplast markers. The following main topics are addressed:

Clarification of the conflicting position and time of origin of *Jasione* and its allied lineages, isolated within the Campanulaceae, and how it supports the different biogeographical hypotheses regarding the ancestral area of the core Campanuloideae clades.

The biogeographical and evolutionary history that shaped the east-west disjunct distribution of *Jasione*, unveiling the events that determined the fate of the extant lineages. The underlying processes that allowed the endurance of the genus in some areas and related hypotheses regarding how these processes favoured exchange of lineages between the east and west areas and also within these area and whether distribution ranges can be explained by either vicariance or dispersal opportunities and whether they were conditioned by environmental constraints.

Analysis of the distribution throughout the phylogeny of interesting morphological traits, such as hydathodes, or environments, such as mountain habitats, as well as polyploidization events and signs of possible secondary contact between the species producing reticulate patterns, to explain how diverse processes have affected the evolution of the genus.

2. MATERIAL AND METHODS

2.1. Identification of the biogeographical pattern

A taxonomic richness map was generated to depict the fit of the genus to the east-west Mediterranean disjunct pattern. The map was not built from those species recognized in general treatments (e.g. Lammers, 2007; Castroviejo *et al.*, 2010), but rather with all evolutionary significant units (Ryder, 1986) of the genus, following the taxonomic criteria used by the current author. Therefore, it includes the species and subspecies recognized in general treatments, but also some varieties only recognized in regional floras, such as those of the *J. orbiculata* group (e.g. Ančev, 2012). Some conflicting taxa or populations were considered sufficiently differentiated and are therefore included in the map, but only when researchers suggested their possible relevance using information of evolutionary significance (e.g. *Jasione jankae*, sensu Hartvig, 1991). In total, 43 taxonomic entities have been included in the map, most of them narrow endemics. However, more than 100 taxonomic names have been proposed in the genus (Kovanda, 1968), most of which were not considered worthy of recognition in this study, and were therefore not used to construct the taxonomic richness map. The map was generated from reviewed herbarium sheets, the author's own field collections or field collections sent to the current author by collaborators from abroad. General databases such as "www.anthos.es." and "www.gbif.org" were not used due to the large number of misidentifications (e.g. over 65% of misidentifications in *J. sessiliflora* in gbif.org, M. Serrano, own data). The INaturalist database was used in some cases when a given observation could be confidently identified by photograph. The

richness map was generated in R v4.1 (R Core Team, 2021) using the packages letsR, rgdal, and sp.

2.2. Plant material

Silica-gel field collected dried samples or material from herbarium sheets of 93 individuals of *Jasione* plus 14 outgroup species of the Campanulaceae family were included in the study. All taxonomic groups within the genus *Jasione* were sampled, including different populations in those taxa with wider distribution ranges. The sampling aimed to encompass all possible cryptic evolutionary lines to avoid a priori assumptions about the internal diversity of the genus. This approach is advisable in genus with high levels of morphological stasis in which potential cryptic species may be recognizable. Given taxonomic names, events at the variety rank, when supported by morphological and ecological significance, should be considered hypotheses to be tested and never ruled out. As an example, the Balkan and Anatolian populations treated taxonomically by Stojanov (1926) are included. Therefore, sampling was not guided by the overly synthetic treatments currently available (Sales & Hedge, 2001; Lammers, 2007a; Castroviejo *et al.*, 2010). Different populations within taxa were also included to reveal hidden variability. First, the approach intended to sample all populations with different ploidy levels within taxa in order to map the phylogeny polyploidization events. Second, in widespread taxa (e.g. *J. montana*) the number of sampled populations was larger. In these cases, the inclusion of different morphotypes was favoured.

The samples of other genera of the Campanulaceae represented the Lobelioideae subfamily (*Lobelia erinus* and *L. urens*), as well representatives of all major groups of the Campanuloideae subfamily. This includes representatives of the *Codonopsids* group (*Codonopsis* and *Platycodon*), the Wahlenbergeae tribe, the basal lineages of the Campanuleae and the three main clades of Campanuleae. The Campanulaceae data set was later complemented with six additional species of the Campanuleae tribe of the genera *Campanula*,

Hanabusaya and *Adenophora*, with the whole plastid genome available in GenBank. Samples information is provided via the shared link to the data set (see General Methods).

2.3. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from 15-20 mg of silica-gel dried leaves or herbarium specimens using either a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990) or with the commercial kit Nucleo Spin Plant II (Macherey-Nagel). In both cases, 0.6 ul of 2-mercaptoethanol was added during the first step. Foliar tissue was homogenised either with manual grinder or using a Mikro – dismembrator (Sartorius). DNA concentration and quality were assessed with Nanodrop 2000C (Thermo Scientific). Five regions of the chloroplast genome were amplified: the petD region, including the petB-petD intergenic spacer, the petD 5' exon and the petD intron; the rpl32-trnL(UAG) intergenic spacer; the psbA-trnH intergenic spacer; the 3'extreme of ndhF gene intron; and the trnL-trnF intergenic spacer. The primers used for each marker are listed in Table 3.1, including the relevant references. One internal primer, called "trnL(UAG)-Jasi" was designed in this study for the rpl32-trnL (UAG) intergenic spacer to enable amplification of the region in some *Jasione* accessions with a huge insertion in the 3' extreme of the region. PCR was conducted in 25 ul reactions with PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Sweden). PCR products were firstly assessed on 1.5 % agarose electrophoresis gels stained with RedSafe, Nucleic Acid Staining Solution (INtRON Biotechnology Inc., Korea) and purified with E.S.N.A. Cycle Pure Kit. (Omega). DNA Sanger sequencing was carried out by the Sequencing Service of the University of Santiago de Compostela (Spain) or STAB Vida (Portugal). The thermocycler profiles used for each region are listed in Table 2.

Table 3.1. List of primers used for each gene region

DNA Region	*Primer Name*	*Sequence*	*Reference*
petD	PlpetB1411F	5′-GCCGTMTTTATGTTAATGC 3′	Löhne & Borsch., 2005
	PlpetD738R	5′ AATTTAGCYCTTAATACAGG-3	
ndhF	5bF	5' GGAGCTACTTTAGCTCTTG 3'	Kallersjo *et al.*, 2000
	10bR	5' CCTACTCCATTTGGAATTCCATC 3'	
trnL-F	E	5' GGTTCAAGTCCCTCTATCCC 3'	Taberlet *et al.*,1991
	F	5' ATTTGAACTGGTGACACGAG 3'	
trnH-psbA	psbA-F	5' GTTATGCATGAACGTAATGCTC 3'	Sang *et al.*, 1997
	trnH-R	5' CGCGCATGGTGGATTCACAAATC 3'	
rpL32-trnL(UAG)	trnL (UAG)	5' CTGCTTCCTAAGAGCAGCGT 3'	Shaw *et al.*, 2007
	rpL32-F	5' CAGTTCCAAAAAAACGTACTTC 3'	
	trnL(UAG)-Jasi	5' GGTCGTAAGAGCAGCCAAGA 3'	Designed here.

Table 3.2. PCR thermocycler profiles used to amplify each gene region.

PCR programmes	**Region**
94 ºC/1 min – 35 cycles: 94 ºC/45 s; Tª a/45 s; 72ºC/9 s – 72 ºC/7 min	ndhF
94 ºC/4 min – 40 cycles: 94 ºC/1 min; 50 ºC /1 min; 72 ºC/1 min – 72 ºC/7 min	ndhF
95 ºC/3 min – 34 cycles: 94 ºC/30 s; 54 ºC /30 S; 72 ºC/2 min – 72 ºC/10 min	ndhF pedtD rpL32-trnL(UAG) trnL-F
95 ºC/2 min – 31 cycles: 94 ºC/30 s; 48-50 ºC /1 min; 71 ºC/2 min 10 s – 71 ºC/8 min	psbA-trnH
94 ºC/3 min – 46 ºC/1 min; 71 ºC/1 min 20 s – 40 cycles: 94 ºC/1 min; 50 ºC /1 min; 71 ºC/1 min 20 s – 71 ºC/7 min	ndHF trnL-trnF
95 ºC/2 min—33 cycles: 94 ºC/30 s; 45 ºC/30 s; 70 ºC/1 min 40 s – 70 ºC/8 min	ndHF

Sequences were initially aligned in BioEdit version 7.2 (Hall, 2011) with the MUSCLE algorithm (Edgar, 2004), with posterior manual editing.

2.4. Phylogenetic analyses

Several phylogenetic approaches were applied to the concatenated cpDNA data set to generate a phylogeny. These included two Maximum-Likelihod methods, applied using the software IQ-TREE (Nguyen *et al.*, 2015) and RAxML (Stamatakis, 2014), and two Bayesian approaches, applied using the software MrBayes (Ronquist *et al.*, 2012) and BEAST2 (Bouckaert *et al.*, 2019), the latter of which was used for lineage dating and time divergence analyses.

Maximum-likelihood analyses were first performed with IQ-TREE version 1.6.12 in W-IQ-TREE (Trifinopoulos, *et al.*, 2016). Best-fit substitution models and partition schemes for the IQ-TREE analysis were inferred with ModelFinder (Kalyaanamoorthy *et al.*, 2017) in W-IQ-TREE, with three information criteria: the BIC or Bayesian Information Criterion (Schwarz, 1978), the AIC or Akaike information criterion (AIC) (Akaike, 1974) and the AICc or corrected Akaike information criterion (Hurvich & Tsai, 1989). BIC and AIC criteria selected the TVM+F+G4 model (Transversion model with AG=CT unequal base frequencies, F as empirical base frequencies and G as discrete Gamma model with four rate categories). AIC selected the same model for the petD and psbA-trnH regions, but selected the model GTR+F+G4 (General time reversible model with unequal rates and unequal base frequencies, empirical base frequencies, and G as a discrete Gamma model with four categories) for the rpl32-trnL (UAG), ndhF and trnL-trnF regions. ModelFinder uses BIC by default as the optimality criterion as it has been observed that AIC and AICc in this software are slightly biased toward more parameter-rich models (Kalyaanamoorthy *et al.*, 2017). Consequently, all loci were treated as unique partitions and a partitioned scheme with TVM+F+G4 for all regions was chosen for the analysis. ModelFinder also indicated that the partitioned model yielded a poorer fit than a single model. Therefore,

another maximum-likelihood analysis was performed without data set partition and allowing IQ-TREE to auto-select the substitution model. In this case, AIC, AICc and BIC consistently selected TVM+F+G4 as the best-fit model. Branch support was assessed using the ultrafast bootstrap (BS) approximation (Hoang *et al.*, 2018). Log-likelihood of the tree was better and with fewer parameters in the unpartitioned analysis than in the partitioned analysis, although the tree from the latter analysis yielded better (lower) BIC values, but not better AIC and AICc values (Table 3.3).

Table 3.3. Maximum-likelihood values of the trees generated with IQ-TREE 1.6.12 for the partitioned and unpartitioned analyses with the TVM+F+G4 substitution model.

Maximum-likelihood tree scores	Five partition analysis	Unpartitioned analysis
Log-likelihood of the tree	-24805.4689	-25179.58
AIC	50096.93	50773.17
AICc	50124.95	50793.34
BIC	51653.71	52099.3141
Number of free parameters	243	207

RAxMl analyses were performed in the CIPRES Science Gateway portal v3.3 (Miller *et al.* 2010) with RAxML-HPC2 on XSEDE v. 8.212. The best-fit partitioning scheme for the RAxML analysis was selected with PartitionFinder2 v2.1.1 (Lanfear *et al.*, 2016) with the greedy algorithm (Lanfear *et al.*, 2012) and PhyML (Guindon *et al.*, 2010). One subset was defined for each gene region by the BIC, AiC and AICc criteria. PartitionFinder2, considering only substitutions models allowed by RAxML, determined GTR+G as the best-fit model of nucleotide evolution for all subsets. Therefore, RAxML analyses applied the GTRGAMMA model to each subset, and nodes support was quantified by RAxML rapid bootstrap with 1,000 replicates (Stamatakis, 2006).

Bayesian analyses were carried out with MrBayes version 3.2 (Ronquist *et al.*, 2012). MrBayes only allows a relatively a small

collection of models, so PartitionFinder2 v2.1.1 was used to test only these models. Each gene region was treated as unique and unalike partitions with all allowed to evolve at different rates. The model selected with the greedy algorithm and BIC criterion was GTR with gamma-distributed rate variation across sites and a proportion of invariable sites. Four runs with 10 million generations and four Monte Carlo Markov chains (MCMC) were carried out, sampling every 1,000 generations. Convergence in all parameters was evaluated with Tracer v1.7 (Rambaut *et al.*, 2018) and burn-in was set to discard the first 10% trees. Support for internal nodes was quantified by Bayesian posterior probabilities.

Five different aspects of evolutionary significance were mapped onto the phylogeny, including the average number of hydathodes, life history habit, mountain or lowland habitat, ploidy level and signs of introgression. The number of hydathodes was based on average measurements of the current author in rosette and stem leaves of the taxa or putative Evolutionary Significant Units represented by the terminals. Within clade variation was represented by a range. Life history habit was represented by annual or perennial habit. Habitat in relation to elevation was represented by lowland habitats (0-500 m a.s.l.), mid-elevation mountain habitats (500-1,600 m a.s.l) and high elevation alpine or oro-Mediterranean habitat (1,600-3,500 m a.s.l.). Ploidy levels were mainly obtained by the current author following the procedures detailed in the General Methods section. Bibliographic information was used in some cases (e.g.,Lammers, 2007a). Signatures of introgression were identified when a clear reticulate pattern was suggested by the phylogenetic position of a terminal and by discordance between morphology and plastid nucleotide sequences.

2.5. Time divergence estimations and calibration of the Campanulaceae tree

Bayesian analysis was carried out in BEAST2 (Bouckaert *et al.*, 2019) to simultaneously infer the phylogeny and divergence times. A relaxed lognormal clock model was assigned to each partition of the

concatenated plastid data set. The prior rate of the branches was drawn from a parametric distribution that has as mean a function of the rate of the parent branch (Drummond *et al.*, 2006). Prior distributions of ages are set as calibration points of certain nodes to estimate the time of divergence of the lineages.

Different fossil information was used to specify priors and calibrate the phylogeny. However, dating the tree of the Campanulaceae is controversial. The fossil record in the Campanulaceae is particularly poor (Lammers, 2007b), although some reliable fossils are available. Two seed types assigned to *Campanula* from Poland (Nowy Sącz basin) have been dated from the Middle Miocene (Upper Carpathian, 16.0-17.5 Mya) (Lancucka-Srodoniowa, 1977; Lancucka-Srodoniowa, 1979). Pollen grains of the Campanulaceae have been dated to the Miocene epoch (c. 23 Mya) (Mildenhall, 1980; Benton, 1993). More strikingly, pollen grains have been attributed to the Campanulaceae in a Late Cretaceous (Maastrichtian, 72.1-66 Mya) fluvio-lacustrine formation from Inner Mongolia (Van Itterbeeck *et al.*, 2007). These authors offer no indication about the morphological traits of the grains or whether they would belong to the Campanuloideae subfamily or the whole Campanulaceae s.l., i.e. including the Lobelioideae subfamily. In any case, the fossil remains include eight Campanulaceae pollen grains, all from a plant community dominated by gymnosperms and ferns as is typical in plant formations prior to the Cretaceous-Paleogene extinction event, which adds reliability to this early dating (Van Itterbeeck *et al.*, 2007). The Maastrichtian lies temporally far before the inferred split between the Campanuloideae and the Lobelioideae, which has been dated at around 45.6 Mya by Magallón *et al.* (2015) and at around 52 Mya by Wikström *et al.* (2003). Nevertheless, it is posterior to the inferred age of the most recent common ancestor between the Roussaceae family and the long lineage leading to the Campanulaceae s.l., a split inferred between 76.1 Mya (Magallón *et al.* 2015) and 80 Mya (Wikström *et al.*, 2003. Bell *et al.*, 2010). All of these fossils, except the pollen grains from the late Cretaceous, have been used for node calibration in lineage dated phylogenies. Controversially, they have not been used to date the same nodes in the Campanulaceae phylogeny, leading to conflicting results (see Cano-Maqueda *et al.*,

2008; Frajman & Schneeweiss, 2009; Roquet *et al.*, 2009; Cellinese *et al.*, 2009; Mansion *et al.*, 2012; Crowl *et al.*, 2016). The two types of seeds assigned to the genus *Campanula* from the Upper Carpathian are notably different. In one case, they are regular Campanuloid seeds with a smooth seed coat (Lancucka-Srodoniowa, 1977). The other type shows a reticulate coat formed by cells divided by thin walls. The latter resembles the seeds of the extant *Campanula pyramidalis* and is in fact the type specimen of the extinct species *Campanula paleopyramidalis*, interpreted as an immediate ancestor of *C. pyramidalis* (Lancucka-Srodoniowa, 1979). The fossil seeds occurred in a warm and wet environment dominated by peat bogs and swampy forests of tropical-subtropical vegetal communities linked to extant south-eastern Asian flora (Lancucka-Srodoniowa, 1979). However, *C. pyramidalis* and its more closely related species are Italian-Balkan plants occurring in limestone crevices or rocky-garrigue Mediterranean dry habitats (Lakušić *et al.*, 2013). *C. carpatica* is in the same clade as the *C. pyramidalis* complex (Mansion *et al.*, 2012), from the Polish Carpathians, it has reticulate seeds (Olesen *et al.*, 2012), although its habitat is upper mountain limestone cliffs. Moreover, reticulate seed ornamentation is not restricted to the *C. pyramidalis* group in the Campanulaceae. For example, it is not rare within the tribe Wahlenbergeae in the genus *Wahlenbergia* (Plunkett *et al.*, 2009; Cupido *et al.*, 2011). The tribe Wahlenbergeae is mostly distributed in the southern hemisphere and is almost absent in Europe, although the situation may have been different in the paratropical conditions of the European Miocene. However, the absence of past or current evidence of the Wahlenbergeae in Central Europe makes assignment of the fossil seeds to the genus *Campanula* more likely. Several works have used the Nowy Sącz fossil seeds to calibrate phylogenies, although with different perspectives. Roquet *et al.* (2009) and Olesen *et al.* (2012) set a calibration point of 16 Mya at a node ancestral to the tribes Campanuleae, Wahlebergeae, plus *Jasione* and its allied lineages. Cellinese *et al.* (2009) used the same age, but for an much more derived node, the stem node between *C. pyramidalis* and *C. carpatica*, arguing that these are the only two species with a seed similar to *C. paleopyramidalis*. This point was contested by Olesen *et al.*, (2012),

who stated that a reticulate coat is found in other Campanula species, such as the American *C. scouleri*. Mansion *et al.* (2012) followed the approach of Cellinese *et al.* (2009). Although in all of these cases, other calibration points were used (e.g. indirect dating of the root or other basal nodes from published angiosperm phylogenies), which nuanced the effects of the fossil seed calibration, the results from the two approaches are very different. For example, with the former approach, the divergence of two core *Campanul*a clades (*Campanula* s. str. and "Rapunculus") was estimated between 20 Mya (Roquet *et al.*, 2009) and 13.5 Mya (Olesen *et al.*, 2012), while with the latter approach it was estimated between 60 Mya (Cellinese *et al.*, 2009) and 39 Mya (Mansion *et al.*, 2012). Later, in a work focused on a group of eastern Mediterranean *Campanula* species, Crowl *et al.* (2015) still used the Nowy Sącz seeds to calibrate the stem node between *C. pyramidalis* and *C. carpatica*. These researchers used the 16 Mya calibration point as an older offset for a lognormal distribution of time with mean around 5 Mya. The approach used in the present work is different from those used in the previously cited studies. Although the literature dealing with the seed morphology of the Campanulaceae is not very extensive, it is known that seeds with reticulate coats appear in more groups than the *C. pyramidalis* clade (Belyaev, 1984; Shetler & Morin, 1986; Cupido *et al.*, 2011). Within the genus Campanula, reticulate seed coat morphology can be found in either Eurasian species, such as the Anatolian *C. olympica* (Akcin, 2009), or American species, such as *C. divaricata* or *C. prenanthoides*, among other species (Shetler & Morin, 1986), and none of these belong to the *C. pyramidalis*-*C. carpatica* clade. Nevertheless, the position of all these species is nested in all published phylogenies within the "Rapunculus" clade of *Campanula* (Wendling *et al.*, 2011; Mansion *et al.*, 2012).

The approach applied in the present work uses all this evidence and the fossilized seeds of *Campanula* sp. and *Campanula paleopyramidalis* from the Nowy Sącz Miocene to establish the calibration point within the Rapunculus clade, between the early divergence (in the current author's data set) that led to the genera *Hanabusaya* and *Adenophora* and the lineage that led to other Rapunculus groups (represented in the current author's data set) by *C.*

pyramidalis and *Campanula* gr. *rotundifolia*. A late Burdigalian (around 16 Mya) Miocene scenario with the Rapunculus clade already diversified is plausible and supported by *C. paleopyramidalis* seeds. However, the scattered occurrence of reticulate seeds in the Rapunculus clade suggests that using this trait to calibrate the derived clade of *C. pyramidalis*-*C. carpatica* may be somewhat adventurous. Conversely, placing this calibration point at the base of the Campanuleae as in Roquet *et al.* (2009) seems too conservative. Other works, apparently unaware of the existence of the Nowy Sącz fossil seeds, and using other lines of evidence, have dated the early divergence of the Rapunculus clade in the late Burdigalian (16.5 Mya). Park *et al.* (2006) used divergence times inferred in the angiosperm phylogeny of Wikström *et al.* (2003). This approach was also used by Cano-Maqueda *et al*. (2008), who established a maximum age of 23 Mya to the Rapunculus clade based on the occurrence of Miocene Campanulaceae pollen records (Benton, 1993). In the current study, a lognormal prior distribution with mean 16 Mya and median 15.3 Mya and a standard deviation of 0.3 to the stem node was established for the node subtending *C. rotundifolia* and *C. pyramidalis* lineages. The root was also calibrated, as deep calibration points are needed to capture a larger proportion of the overall genetic variation (Duchêne *et al.*, 2014). The root node represents the divergence between the subfamily Lobelioideae and the lineage leading to the Campanuloideae. Wikström *et al.* (2003) and Magallón *et al.* (2015) calculated mean ages for this node of 52 Mya and 46.5 Mya, respectively. Mansion et al. (2015) inferred a mean age of 76.5 Mya, although this inference is strongly influenced by the use of the *C. paleopyramidalis* seeds to date the split between *C. pyramidalis* and *C. carpatica*. Nevertheless, the existence of unassigned Campanulaceae pollen in the Late Cretaceous (72.1-66 Mya, Van Itterbeeck *et al.*, 2007) is not very consistent with the inferred divergence between the Campanulacee and the Rousseaceae calculated to be about the same age by Magallón *et al.* (2015) and slightly earlier, about 80 Mya, by Wikström *et al.* (2003). Consequently, the inferred ages for the split between the Lobeliodieae and Campanuloideae calculated by these authors seem excessively recent. Therefore, in this study an age older than the mean age provided by these studies was used

to calibrate this node and using instead their older boundaries. A normal prior distribution was established for the root, with mean in 56 Mya, standard deviation of 1 and 95% of the distribution of ages between 54 Mya and 58 Mya. The prior substitution model was established as TVM+G4, with a concatenated five partition scheme as the best-fit model selected by ModelFinder. Although BEAST2 does not include TVM as default model, the Site Model tab of BEAUTI2 can be established by selecting the GTR model and setting the AG rate parameter at 1.0. The analysis was preliminarily run with two Tree Models, a Yule model and Birth-Death model. The second would be more realistic when many terminals are absent, as in a data set with only a few representatives of each Campanulaceae group included. On the other hand, the Yule model demands fewer parameters. Nevertheless, no significant differences between the results of both models were observed. Final analyses were carried out with a birth-death tree prior. Five independent runs, each with four Monte Carlo Markov chains (MCMC) and 10 million generations, were carried out, sampling every 1,000 generations. Convergence as effective sample sizes (ESS) values in all parameters was evaluated with Tracer v1.7 (Rambaut *et al.*, 2018) and burn-in was set to discard the first 25% trees, with the conserved trees combined in LogCombiner and summarized in TreeAnnotator (Drummond & Rambaut, 2007). Support for internal nodes was quantified by Bayesian posterior probabilities and node ages were set to median ages.

2.6. Diversification analyses

The maximum-credibility clade tree from the BEAST2 time calibrated Bayesian analysis and a random subsample of 750 trees from the posterior distribution of the Bayesian analysis were used to create lineage-through time (LTT) plots to study diversification in *Jasione*. After the set of randomly sampled trees from the posterior distribution was plotted, a Confidence Interval (CI) was calculated for each tree (CI = (1- α)-percent; α = 0.05) on the number of lineages given times, and plotted on the median LTT (Revell, 2012). The trees were cropped to only include *Jasione* terminals and pruned to conserve differentiated

lineages. The LTT representations of the diversification and statistical tests were generated and performed in R v.4.1 (R Core Team, 2021) with the packages ape (Paradis *et al.*, 2004) and phytools (Revell, 2012). The net rate of diversification under a birth-death model ($r = \lambda - \mu$) was calculated on the LTT from the maximum-clade credibility tree, where λ is the rate at which new species are added through speciation and μ is the rate at which species are lost by extinction. The net rate of diversification and extinction fraction ($\varepsilon = \mu/\lambda$) are key parameters to determine the mean clade diversification over time (Rabosky & Benson, 2021). Pybus and Harvey γ constant-rates tests were performed on the LTT from the maximum-clade credibility tree to detect deviations from a pure birth Yule model, with two-tailed p-value for the γ-test (Pybus & Harvey, 2000). Accounting for unsampled lineages is considered essential, as missing terminals can cause the spurious detection of declines in diversification rates (Pybus & Harvey 2000; Morlon *et al.*, 2012). Incomplete lineage sampling could favour the hypothesis of deceleration of diversification rates, leading to early burst interpretations (Fordyce, 2010). To avoid the effects of incomplete lineage sampling, a Monte Carlo constant rate test was performed, where the 0.05 p-value used to reject constant rate was calculated from a distribution of γ for simulated trees including incomplete lineage sampling comparable to the studied tree (Pybus & Harvey, 2000). Complementarily, any important departures in the obtained significance of the γ statistics between the standard Pybus and Harvey γ-test and the Monte Carlo constant rate γ-test will provide information about the completeness (or otherwise) of sampled lineages in the data set. A plot representing the computed Pybus and Harvey γ statistic through time is generated by slicing the tree in 100 points, with the function gtt in phytools, to facilitate the identification of the most influential evolutionary periods in the measured value of γ.

2.7. Biogeographical analysis

The ancestral area reconstruction and biogeographical analyses were performed on the cropped and pruned Maximum-Credibility Clade tree from the Bayesian posterior distribution. Two analyses were

conducted, one retaining *Hesperocodon* as an outgroup and other "only *Jasione*". As no major difference on the estimation of the ancestral area was detected, only the results of the latter analysis are shown. Six areas were considered: (A) north-western Africa; (B) the southern Iberian Peninsula; (C) northern and Central Iberian Peninsula, including the Pyrenees; (D) intermediate Europe including Italy and all of central and northern Europe; (E) the Balkan Peninsula; and (F) the Anatolian Peninsula (Figure 3.2). The biogeographical analysis was performed in R v.4 (R Core Team, 2021) with the package BioGeoBEARS (Matzke, 2013).

This R-package implements six biogeographic models in a common likelihood framework: a likelihood version of Dispersal-Vicariance analysis (DIVALIKE; Ronquist, 1997), LAGRANGE Dispersal and Extinction Cladogensis (DEC model, Ree *et al.*, 2005; Ree & Smith, 2008), a likelihood version of BayArea (Landis *et al.*, 2013), and an alternative version for each of the models that includes founder-event speciation (+J). BioGeoBEARS has two primary advantages over other biogeographical programs: (1) the best model is selected with likelihood ratio tests, and (2) founder-event speciation is included, a process ignored by most other methods.

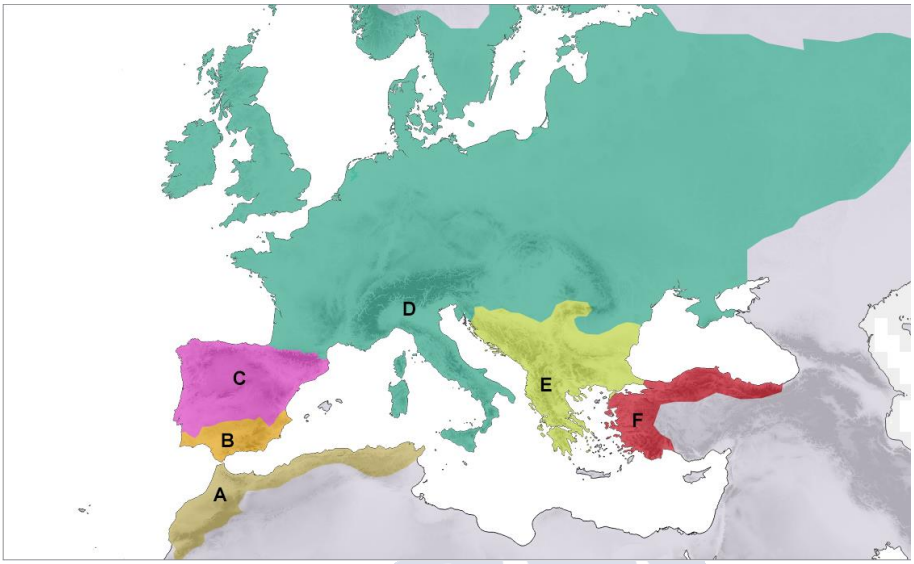


Figure 3.2. The six areas defined for the biogeographical analyses: A, north-western Africa; B, the southern Iberian Peninsula; C, Anatolia; D, intermediate and northern Europe; E, the Balkan Peninsula; and F, the Anatolia Peninsula.

2.8. Niche modelling

Species distribution modelling (SDM) was performed to compare niche suitability inferred from the potential distribution of the western and eastern groups under current climate conditions. Only taxa diverged from the common ancestor immediately older than the initial divergence leading to the E-W disjunction were included in the analysis. The approach used here pooled the occurrence data into two sets, the eastern and western groups. These groups are phylogenetically and geographically defined. Pooling of closely related species is one of the options to consider when most of the taxa are narrow endemics with small sample sizes (Ahmadi *et al*., 2021). A number of taxa of the eastern and western groups have < 5 occurrences, once the data are thinned to avoid spatial autocorrelation. To diminish excessive contribution of those taxa with more occurrences (e.g. *Jasione sessiliflora* in the west or *Jasione orbiculata* var. *bosniaca* in the east) no taxon had more than 6 occurrences in the pooled data. Nevertheless,

the results should be interpreted with caution as pooling can lead to inflation of the predicted niches in each group (Hernández *et al*., 2006). The "eastern group" included the Balkan-southern Italian *Jasione orbiculata* complex, the Anatolian *Jasione supina* complex and those populations of the Balkan-Anatolian *Jasione heldreichii* complex, including populations assigned to *Jasione jankae* that were recovered in the previous clade (see Results). The purely annual populations of *J. heldreichii* recovered in a different clade are not included in any of the groups. The "western group" included the northern African *Jasione cedretorum* and the populations assigned to *Jasione crispa* subsp. *lanuginella* and *Jasione crispa* subsp. *mesatlantica* and all the south-western European groups of *Jasione* sect. *Jasione*, and it excluded the annual-biennial *Jasione montana* and *Jasione maritima*, diploid and tetraploid lineages as forms related to the recently evolved *J. montana* lineage. The entropy algorithm implemented in MAXENT v3.3 (Phillips *et al.*, 2006) was used for suitability modelling. The area of study includes Europe and the Mediterranean region. The occurrence was thinned to 5 km to prevent spatial autocorrelation. Nineteen bioclimatic variables for the area of study with current climatic conditions were downloaded from the WorldClim repository (Hijmans *et al.*, 2005). Seven uncorrelated variables were selected: Bio1 (annual mean temperature), Bio3 (isothermality), Bio4 (temperature seasonality), Bio7 (temperature annual range), Bio9 (mean temperature of the driest quarter), Bio12 (annual precipitation) and Bio 19 (precipitation of the coldest quarter). These variables were used as model calibration predictors in MAXEN. Randomly selected occurrences (75%) from each geographic group were used as training data and the remaining occurrences (25%) were used as test data.

3. RESULTS

3.1. Phylogenetic analyses

The concatenated data set had a total length of aligned sequences of 4,476 nucleotide sites, with 58.6% of constant sites and 1,205 parsimonially informative sites.

Analysis of the concatenated data set was mostly congruent between the Maximum Likelihood Analyses (IQ-TREE and RAxML) and the Bayesian analyses (MrBayes and BEAST2) in the recovered tree topologies. Interestingly, the main differences were not related to the method used (maximum-likelihood or Bayesian), but to the software used to generate the phylogeny. IQ-TREE (Figures 3.3 and 3.4) and BEAST2 phylogenies are essentially identical, while the RAxML (Figure 3.5) and MrBayes (Figure 3.6) trees show some differences in the topological position of some *Jasione* internal clades. The results may reflect the influence of the substitution model used. IQ-TREE and BEAST2 (Figure 3.7) used TVM+G4 as a substitution model, the best-fit model selected by ModelFinder. However, neither MrBayes nor RAxML allow use of this model, so that the analyses in these programs were carried out with the GTR+G model, which was previously identified as suboptimal in the model selection analyses. The different node supports obtained by each method are indicated as IQ-BS (UltraFast-bootstrap in IQ-TREE), RA-BS (bootstrap in RAxML), MrB-PP (posterior Probability in MrBayes) and BE-PP (posterior Bayesian probability in BEAST2).

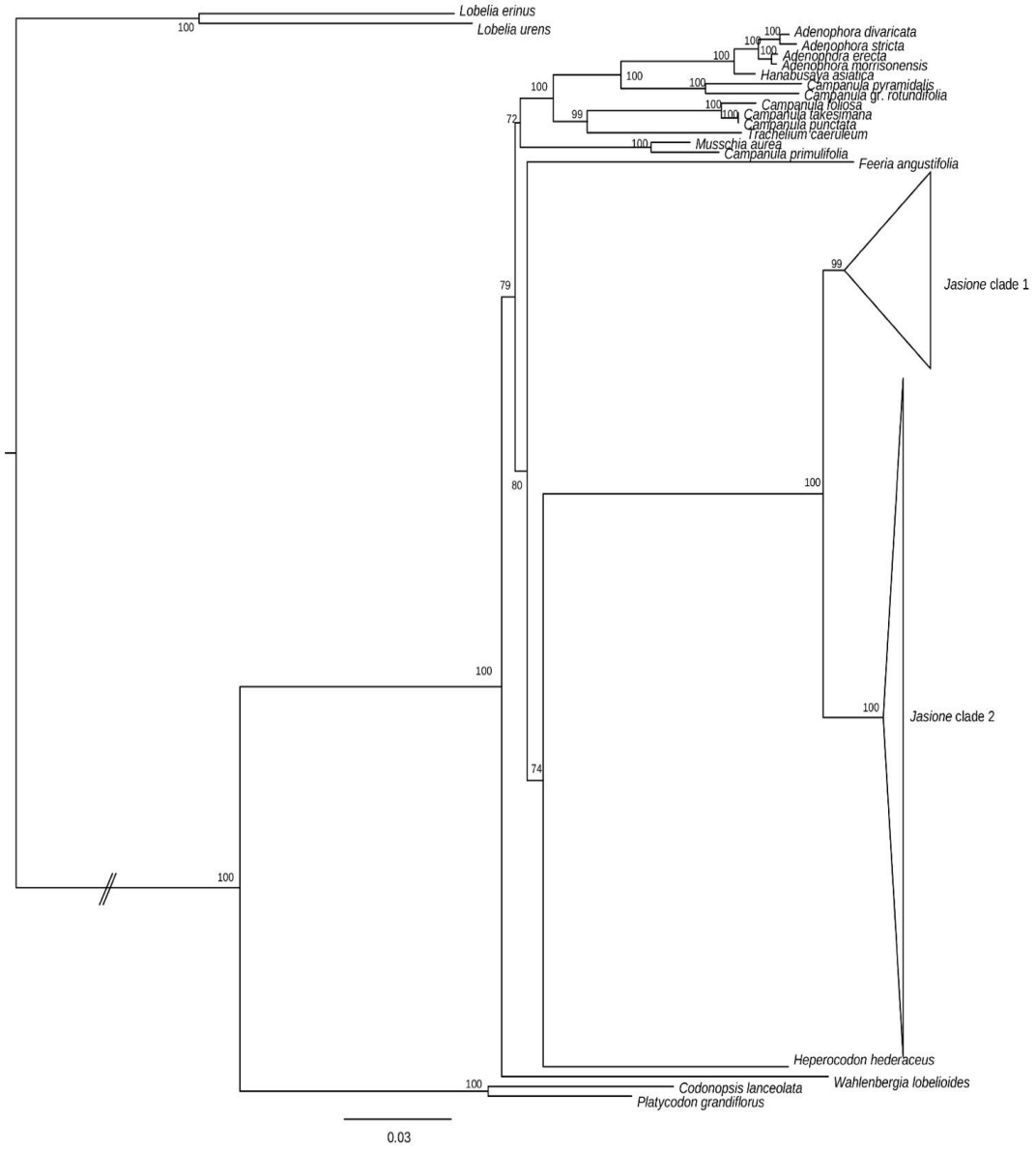
In all analyses, *Jasione* was recovered as a monophyletic lineage (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1). Regarding the relationships of the other "transitional groups". The clade Cam01 (sensu Mansion *et al*., 2012) was recovered as sister to a clade grouping the *Campanula* s.str. and Rapunculus clades, although with low or not significant support (IQ-BS = 72%, RA-BS = < 50%, MB-PP = 0.55, BE-PP = 0.72*). Wahlenbergia hederacea/Heperocodon, Feeria,* and *Jasione* were grouped in a clade with moderate or good support, except in the RAxML analysis, in which the support was low (IQ-BS = 80%, RA-BS = 55%, MB-PP = 0.96, BE-PP = 0.98). Sister relationships between Jasione and *Wahlenbergia hederacea/Heperocodon* were recovered in all analysis, with low support (IQ-BS = 74%, RA-BS = 57%, MB-PP =0.77, BE-PP = 0.72).

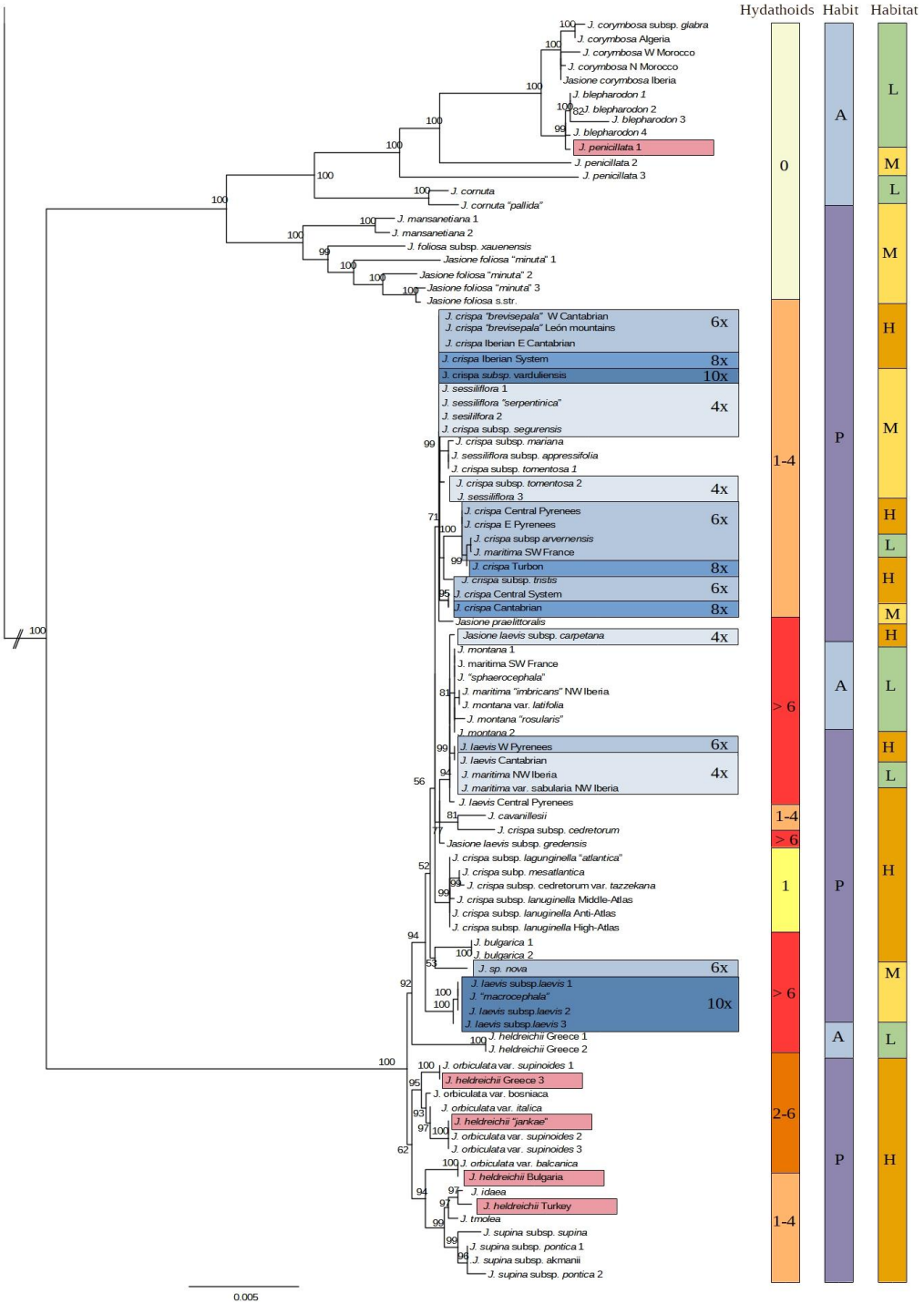
Regarding the conflicting issues of *Hesperocodon* and *Feeria* our results suggest that they are probably due to misidentification of the original samples used in different works. The current author

downloaded the petD sequences used by Mansion *et al.* (2012) and the position of the species was recovered among the *Wahlebergieae* although not within *Wahlenbergia*, but closer to other taxa *as Prismatocarpus* and *Merciera*. This would keep the *Hesperocodon* name legitimate, remaining only the issue of the phylogenetic position of the lineage. Nevertheless, the results suggest that Mansion *et al*., (2012) and Crowl *et al*. (2016) probably used a different species, or suffered a contamination from DNA of a plant different the European endemic *H. hederaceous*. Regarding *Feeria angustifolia*, the results of the repsent work disagree with Eddie *et al.* (2003), Haberle *et al.* (2009) and Xu & Hong (2021). After analysis the sequences used by these authors, they match with sequences of the North African Campanula group of *C. filicaulis* (M. Serrano, own data), suggesting that conflict was based either in a confusion or DNA contamination.

Figure 3.3. First figure below. Maximum-Likelihood phylogram from IQ-TREE of Jasione and other representatives of the Campanulaceae. Ultrafast-Bootstrap values values ≥ 50% are indicated. Double slashes in the lineage leading to the Campanuloideae indicate that the branch has been shortened. The two main clades of *Jasione* appear collapsed to facilitate visualization of the outgroup lineages.

Figuure 3.4. Second figure below. Maximum-Likelihood phylogram from IQ-TREE of *Jasione*. Ultrafast-Bootstrap values ≥ 50% are indicated. Ploidy over diploid level are highlighted in each terminal by blue rectangles, the deeper the tone the higher the ploidy level. Potential introgression is indicated by pink rectangles. Columns on the right of the tree indicate: 1- Average hydathoid number in the encompassed terminals. The only terminals not accurately represented by the bar are those of *J. sessiliflora*, with a greater average (around 5.5) number than the 1-4 range indicated by the bar. 2- Annual (a) or perennial (P) habit. The annual category includes terminals with both annual and biennial habit. Habitat in relation to mountain elevation. Lowland (L) habitats (0-500 m a.s.l.), mid-elevation mountain (M) habitats (500-1,600 m a.s.l.) and high mountain (H) habitats (1,600-3,500 a.s.l.).


Lobelia erinus
Lobelia urens
Adenophora divaricata
Adenophora stricta
Adenophora erecta
Adenophora morrisonensis
Hanabusaya asiatica
Campanula pyramidalis
Campanula gr. rotundifolia
Campanula foliosa
Campanula takesimana
Campanula punctata
Trachelium caeruleum
Musschia aurea
Campanula primulifolia
Feeria angustifolia
Jasione clade 1
Jasione clade 2
Heperocodon hederaceus
Wahlenbergia lobelioides
Codonopsis lanceolata
Platycodon grandiflorus
0.03


Hydathoids
Habit
Habitat
J. corymbosa subsp. glabra
J. corymbosa Algeria
J. corymbosa W Morocco
J. corymbosa N Morocco
Jasione corymbosa Iberia
J. blepharodon 1
J. blepharodon 2
J. blepharodon 3
J. blepharodon 4
J. penicillata 1
J. penicillata 2
J. penicillata 3
J. cornuta
J. cornuta "pallida"
J. mansanetiana 1
J. mansanetiana 2
J. foliosa subsp. xauenensis
Jasione foliosa "minuta" 1
Jasione foliosa "minuta" 2
Jasione foliosa "minuta" 3
Jasione foliosa s.str.
J. crispa "brevisepala" W Cantabrian
J. crispa "brevisepala" León mountains
J. crispa Iberian E Cantabrian
6x
J. crispa Iberian System
8x
J. crispa subsp. varduliensis
10x
J. sessiliflora 1
J. sessiliflora "serpentinica"
J. sesilifora 2
J. crispa subsp. segurensis
4x
J. crispa subsp. mariana
J. sessiliflora subsp. appressifolia
J. crispa subsp. tomentosa 1
J. crispa subsp. tomentosa 2
J. sessiliflora 3
4x
J. crispa Central Pyrenees
J. crispa E Pyrenees
J. crispa subsp arvernensis
J. maritima SW France
6x
J. crispa Turbon
8x
J. crispa subsp. tristis
J. crispa Central System
6x
J. crispa Cantabrian
8x
Jasione praelittoralis
Jasione laevis subsp. carpetana
4x
J. montana 1
J. maritima SW France
J. "sphaerocephala"
J. maritima "imbricans" NW Iberia
J. montana var. latifolia
J. montana "rosularis"
J. montana 2
J. laevis W Pyrenees
6x
J. laevis Cantabrian
J. maritima NW Iberia
J. maritima var. sabularia NW Iberia
4x
J. laevis Central Pyrenees
J. cavanillesii
J. crispa subsp. cedretorum
Jasione laevis subsp. gredensis
J. crispa subsp. lagunginella "atlantica"
J. crispa subp. mesatlantica
J. crispa subsp. cedretorum var. tazzekana
J. crispa subsp. lanuginella Middle-Atlas
J. crispa subsp. lanuginella Anti-Atlas
J. crispa subsp. lanuginella High-Atlas
J. bulgarica 1
J. bulgarica 2
J. sp. nova
6x
J. laevis subsp.laevis 1
J. "macrocephala"
J. laevis subsp.laevis 2
J. laevis subsp.laevis 3
10x
J. heldreichii Greece 1
J. heldreichii Greece 2
J. orbiculata var. supinoides 1
J. heldreichii Greece 3
J. orbiculata var. bosniaca
J. orbiculata var. italica
J. heldreichii "jankae"
J. orbiculata var. supinoides 2
J. orbiculata var. supinoides 3
J. orbiculata var. balcanica
J. heldreichii Bulgaria
J. idaea
J. heldreichii Turkey
J. tmolea
J. supina subsp. supina
J. supina subsp. pontica 1
J. supina subsp. akmanii
J. supina subsp. pontica 2
0.005
0
1-4
> 6
1-4
> 6
1
> 6
2-6
1-4
A
P
A
P
A
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H

Two main *Jasione* sister clades with full support are identified in all analyses, Clade 1 and Clade 2 (Fig.3.3). Clade 1 (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1) is composed of taxa from southern Iberian Peninsula and northern Africa, without hydathoids in all cases. Within Clade 1, two main sister clades are recoved with full support. One is composed by *Jasione foliosa* and *J. mansanetiana*, both species separated in two clades with full support. In the *J. foliosa* clade, the African subspecies *J. foliosa* subsp. *xauenensis* is sister of the all the other populations, suggesting its validiy as separate taxon. Conversely, samples of *J. foliosa* subsp. *minuta* from southern Iberian Peninsula do not form a separate group of *J. foliosa* subsp. *foliosa* from the eastern Iberian Peninsula. The remaining clade groups annual taxa from northern Africa and southern Iberian Peninsula. North-African *J. cornuta* group is recovered as sister to all other groups (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1), including *J. penicillata, J. blepharodon and* the *J. corymbosa* group. Interestingly, *J. blepharodon* and *J. corymbosa* are recovered as sister clades, but nested within *J. penicillata.* Thus, one sample of *J. penicillata* is sister (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1) to a clade with sister relationships between another sample of *J. penicillata* and the lineage leading to *J. blepharodon* and *J. penicillata*. It suggests than an important amount of biodiversity could be hidden within *J. penicillata* populations. Within the *J. blepharodon* clade (IQ-BS = 100%, RA-BS = 96%, MB-PP = 0.96, BE-PP = 1) one population of *J. penicillata* is recovered nested, being the most plausible explanation an introgression event from the former species after secondary contact. This population has the same plastid sequence found in a *J.blepharodon* population from a close location at lower elevation, in the same mountain range, the Sierra Blanca de Ojén (Málaga, Spain). Within the trans-Mediterranean *J. corymbosa* clade (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1), the Iberian population is nested among the African populations. *J. corymbosa* subsp. *glabra* is recovered in a clade with one Algerian population of *J. corymbosa.* Clade 2 (IQ-BS = 100%, RA-BS = 100%, MB-PP = 1, BE-PP = 1) is larger and composed by all remaining taxa, therefore including eastern and western Mediterranean species, in all cases with hydathoids. The different analysis obtained

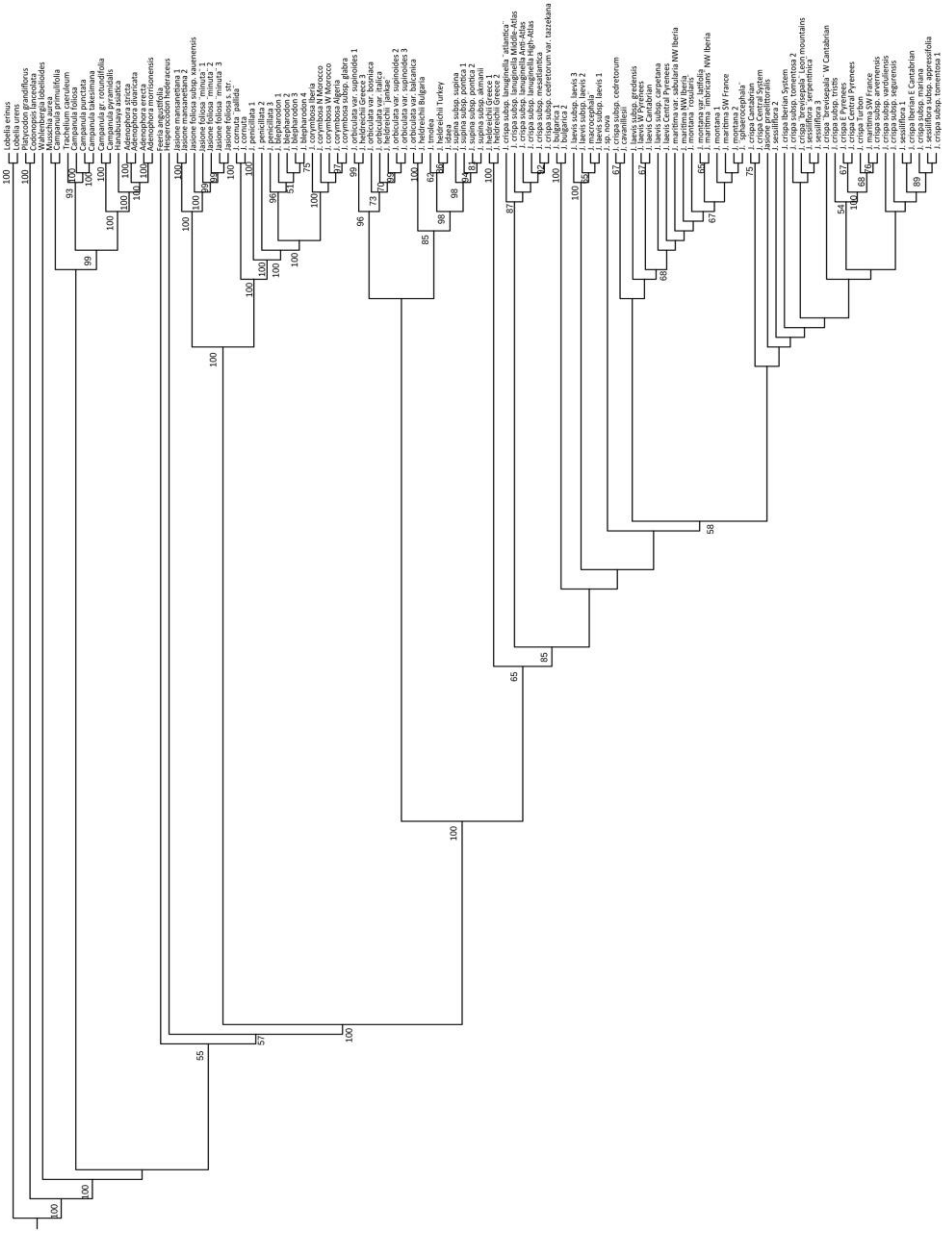

Figure 3.5 Best Maximul-Likelihood tree for *Jasione* produced with RAxML. Bootstrap support values ≥ 50% are indicated on the nodes.

somewhat incongruent phylogenetic relationships. Thus, a sister relationship between a clade grouping, among others, the *J. supina* and the *J. orbiculate* clades was recovered by the BEAST2 and IQ-TREE analyses, although with low support (IQ-BS = 62 %, BE-PP = 0,87). The clade is recovered in the maximum-likelihood RAxML analysis, although without statistical support (Figure 4.5). The MrBayes consensus tree recovers a first split of the *J. supina* clade and then a clade (without statistical support, BA-PP = 0.54%) with the *J. orbiculata* as sister to the remaining groups (Figure 3.6). The *J. supina* clade is well supported (IQ-BS = 94%, RA-BS = 85%, MB-PP = 0.98, BE-PP = 1) recovering unexpected relationships, with a clade sister to all the Anatolian samples including the Balkan taxon *J. orbiculata* var. *balcanica* and a Bulgarian population of *J. heldreichii*, suggesting introgression and chloroplast genome capture. Within the Anatolian subclade (IQ-BS = 99%, RA-BS = 98%, MB-PP = 1, BE-PP = 1), *J. idaea* and *J. tmolea* are recovered in a sister position (IQ-BS = 97%, RA-BS = 62%, MB-PP = 0.98, BE-PP = 1), supporting the recognition of *J. tmolea* as species independent of *J. tmolea*. One Turkish population is grouped with *J. idaea*, as another example of introgression. The lineage *J. supina* s. str. is recovered in another subclade subclade (IQ-BS = 99%, RA-BS = 98%, MB-PP = 1, BE-PP = 1) sister to a clade grouping *J. supina* subsp. *pontica* and *J. supina* subsp. *akmanii*. The population from Kartal Kaya mountains, assigned by Damboldt (1978) to *J. supina* subsp. *pontica* is recovered with *J. supina* subsp. *akmanii* from Köröglu mountains, in accordance with geographic distance and morphological features (M. Serrano, own data). The *J. orbiculata* clade (IQ-BS = 99%, RA-BS = 98%, MB-PP = 1, BE-PP = 1) recovers one lineage leading to a weel supported clade with a population of *J. orbiculate* var. *supinoides* from northern Greece plus a geographically close population of *J. heldreichii*, suggesting introgression. The remaining terminals of the *J. orbiculata* group are revovered in a clade (IQ-BS = 93%, RA-BS = 73%, MB-PP = 0.99, BE-PP = 1) with *J. orbiculata* var. *bosniaca* as sister of the others, including *J. orbiculata* var. *italica* and *J. orbiculata* var. *supinoides* from western Greece. *J. jankae*, a perennial member of the group *J. heldreichii* group, is nested among the populations of *J. orbiculata* var. *supinoides* from

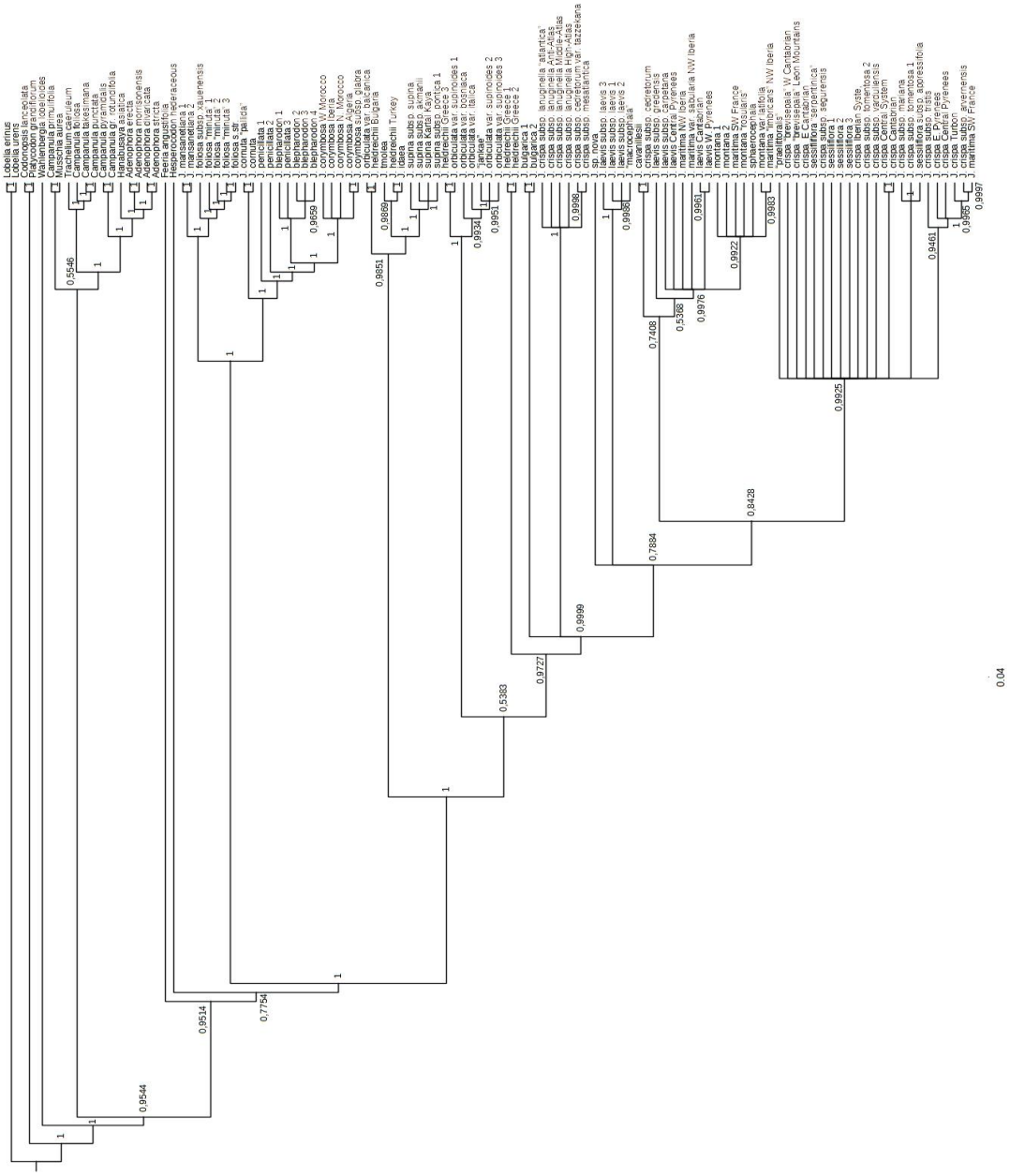

Figure 3.6. Bayesian consensus tree for *Jasione* produced with MrBayes. Bayesian posterior probabilities are represented close to the nodes. Nodes with support values below 0.5 are collapsed.

the same region in Pindos Mountain range. The remaining groups of *Jasione* are grouped in a clade (IQ-BS = 92%, RA-BS = 65%, MB-PP = 0.97, BE-PP = 0.90), itself split between a lineage leading to typical annual forms of *J. heldreichii* from Greece and a well-supported clade (IQ-BS = 92%, RA-BS = 65%, MB-PP = 0.97, BE-PP = 1). Within this clade there are some discordances between the analyses, there are several strongly supported clades, but their mutual relationships are poorly supported, as evidence of a fast and simultaneous radiation. IQ-TREE and BEAST2 analyses recover a basal lineage leading to fully supported decaploid (2n=60) clade encompassing the different populations of *Jasione laevis* subsp. *laevis*, although this sister relationship lacks statistical support (IQ-BS = 52%, BE-PP = 0.7). A sister relationship between the Balkan *J. bulgarica* and an undescribed hexaploid species from the Eastern Pyrenees is suggested by some analysis (IQ-TREE and BEAST2) but without significant support. The remaining species group in clade, within which several well-supported clades are identified. The North African lineage from the Atlas mountains of *Jasione crispa* subsp. *lanuginella* and *J. crispa* subsp. *mesatlantica* (IQ-BS = 92%, RA-BS = 87%, MB-PP = 1, BE-PP = 1). Interestingly, it does not include the Riffian taxon *J. crispa* subsp. *cedretorum* s.str., wich is grouped in another clade with the Cantabrian *J. cavanillesii*. This latter relationship seems a by-product of homoplasy since both taxa share a poly-adenine insertion. Nevertheless, other analyses deleting the insertion region do not relate the lineage "*cedretorum*" to the Atlas Mountain group (M. Serrano, own data). Within the Atlas clade it is nested the population from Mount Tazekka, questioning its inclusion under the subps. *cedretorum* (Dobignard, 1997). Two well-supported remaining clades are recovered, the "*montana*" clade (IQ-BS = 94%, RA-BS = 68%, MB-PP = 0.99, BE-PP = 0.97) and the "*crispa*" clade (IQ-BS = 99%, MB-PP = 0.99, BE-PP = 0.99). *Jasione cavanillesii* and *J. crispa* subsp. *cedretorum* are recovered in several analysis as sister to the "montana", lade, with low support. The "*montana*" clade encompasses several sublineages, with the lineage of the diploid Iberian endemic from the western Central System Mountain Range *J. laevis* subsp. g*redensis* recovered as basal.

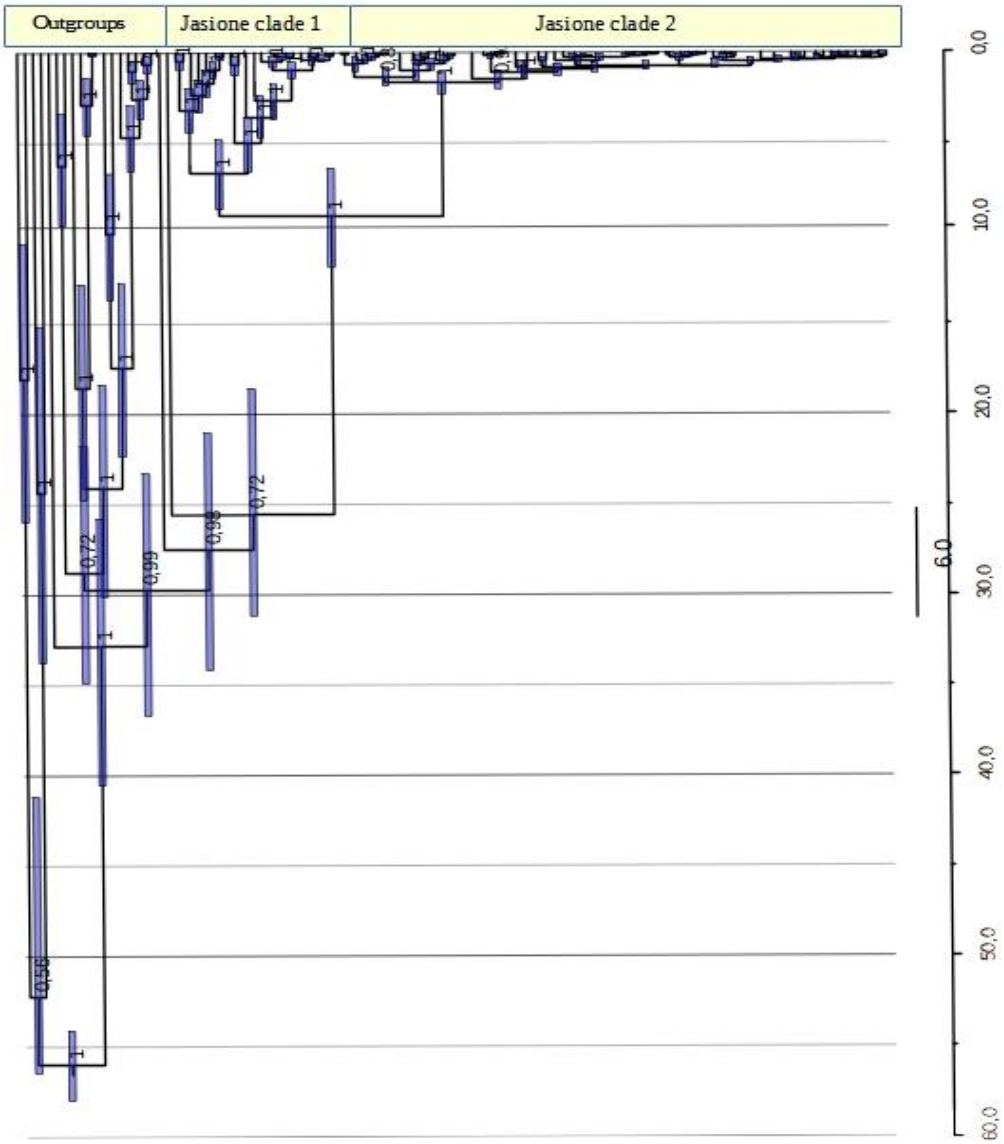

Figure 3.7: Maximum Clade Credibility tree generated by a relaxed molecular clock in BEAST2 for *Jasione* and other representatives of the Campanulaceae. Support values of Bayesian posterior probability are indicated on the nodes. Blue node bars indicate 95% highest posterior density time intervals of divergence of the lineages.

Other populations of *J. laevis* are scattered within the clade, with typical tetraploid populations of J. laevis subsp. carpetana separated from the other lineages. The hexaploidy ploidy level in J. laevis is reported for the first time in this complex, in population from western Pyrenees related to Cantabrian tetraploids from oceanic environments. Diploid populations of *J. montana* are grouped in a well-supported clade with diploid populations of "*J. maritima*" either form NW Iberia or SW France, while tetraploid populations of J. maritima from NW Iberian are not grouped with them. The southern Italian endemic J. sphaerocephala is recovered nested within J. montana, suggesting that this taxon is only an ecotype within the variability of J. montana. The "crispa" clade encompasses several Iberian-French subclades with different ploidy levels, suggesting independent polyploidization paths between the hexaploids from the Pyrennes and French Massif Central, and the hexaploids from other Iberian Mountain Ranges. Two diploid lineages are identified, one basal leading to "*praelittoralis*" populations from Prades mountais in Catalonia, other and unrelated, leading to a clade with J. crispa subsp. tomentosa, *J. crispa* subsp. *mariana* and J. sessiliflora subsp. appressifolia. Hexaploid populations of *J. maritima* from SW France are recovered in a well-supported clade with *J. crispa* subsp. *arvernensis*, within the J. crispa s.str. clade from the Pyrenees. Interesting relationships are suggested between the hexaploids from the Central System and octaploids from the Cantabrian mountains, but not with hexaploids from the Cantabrian Mountains or hexaploids and octaploids from the Iberian System Mountain range. *J. crispa* subsp. *varduliensis*, from limestone Basque-Navarrese mountains is identified as a decaploid (2n=60), a ploidy level reported for the first time in the *J. crispa* group.

3.2. Time divergence and diversification analyses.

Jasione diverged from *Hesperocodon* in the Oligocene (Figure 3.7, 25.5 Mya,18.7-31.1 Mya 95% HPD). This lineage diverged in from a common ancestor with the *Feeria* lineage also in the Oligocene (27.1 Mya, 21.4 -34.2 Mya 95% HPD), diverging in turn from the common ancestor of the *Campanula* groups in the lower Oligocene (29.9 Mya, 23.7-37.2 Mya 95% HPD). The first divergence within the clades 1 and 2 of *Jasione* was inferred much later, for the second half of the Miocene (Figure 3.8, 8.9 Mya, 7.1-10.2 Mya 95% HPD). The first cladogenesis in the Clade 1 between the perennials of the *J. foliosa* group and its sister clade of annual taxa occurred in the late Miocene (7.0 Mya, 6.2-7.9 Mya 95% HPD). Within the clade of annual taxa, the African group of *J. cornuta* diverged from its sister mostly Iberian clade shortly after the boundary between the Miocene and the Pliocene (5.2 Mya, 4.0-6.4 Mya 95% HPD) marked by the opening of the Gibraltar strait after the MSC event. Cladogenesis in the Clade 2 between the clade of *supina-orbiculata* groups and the remaining *Jasione* is dated to the first moments of the Pleistocene (2 Mya 1.6-2.6 Mya 95% HPD), with the striking diversification in the clade occurring during this period of progressive cooling (Figure 3.8).

Speciation rate (lambda) under a pure-Birth (Yule) model was 0.62241, with standard error of 0.0972 and a maximum-likelihood estimate of the speciation rate of 57.3319, obtained by the ratio of the number of speciation events on the cumulative number of lineages through time. The estimation of speciation and extinction rates under a

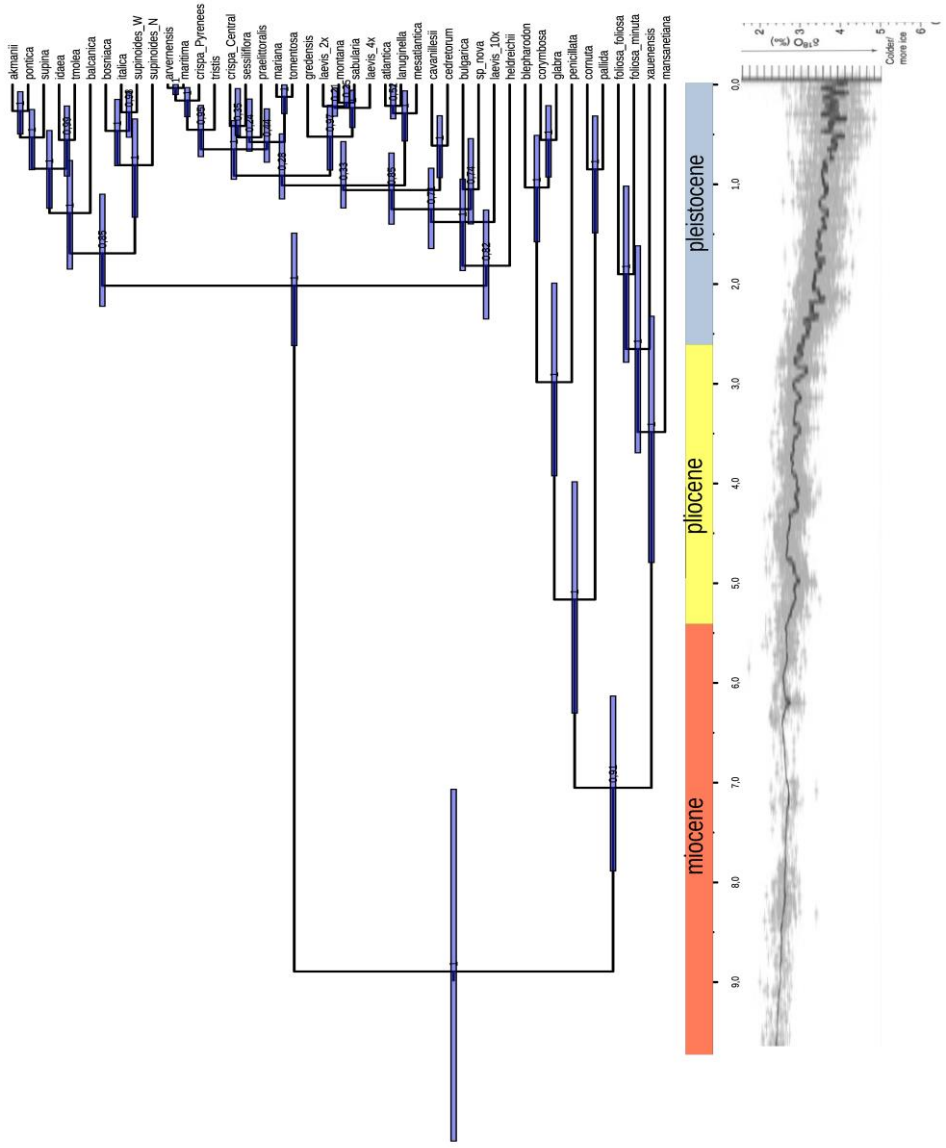

Figure 3.8 Maximum Clade Credibility tree (pruned to only one population of *Jasione* and one population generated by BEAST2. Bayesian posterior probability is indicated on the nodes. On the right side, a graphic showing the progressive cooling of global temperatures inferred form values of δ18O values (modified from wikisource).

Birth-Death model allowed the calculation of a net rate of diversification r = 0.1949678 with a relatively high value of extinction fraction ε = 0.82927 on the LTT from the maximum credibility tree cropped to only have *Jasione* terminal and pruned to only have one terminal per population (Figure 3.8) The LTT of the random samples is represented in Figure 3.9, showing greater internode distances in the older parts of the tree and a striking increasing in speciation during the Pleistocene.

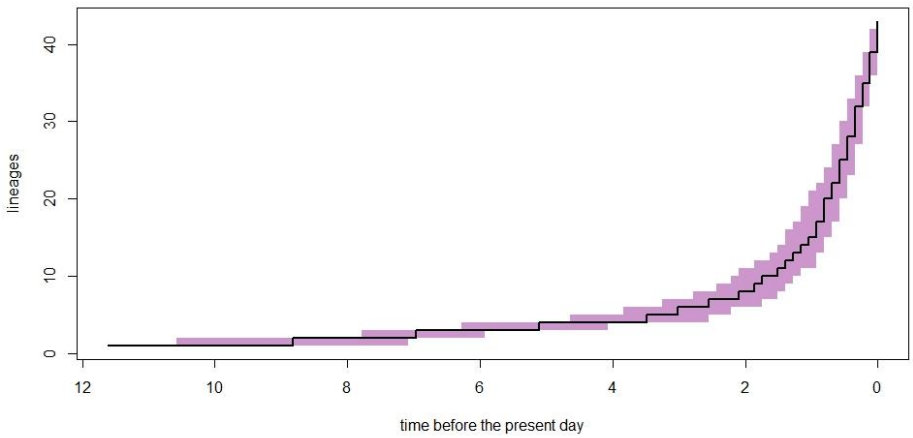

Figure 3.9. Lineage-through-time plot of 750 randomly sampled trees from a Bayesian posterior distribution of trees of the time-calibrated phylogeny of *Jasione*. The shaded area represents the Confidence Interval of the trees, calculated as CI = (1-α)-percent, with α = 0.05.

Pybus & Harvey γ-test resulted in γ = 2.5675, where positive values can be related to the overall importance of extinction in the evolutionary history of *Jasione*. The estimated value of γ in the Pybus & Harvey test was significant at the 0.05 level, with p-value = 0.0102. The Monte Carlo γ-test was also significant with a very similar result, with p-value = 0.0098. The results of the simulated distribution of the Monte Carlo γ-test can be seen in Figure 3.10. The results of both tests

allow the detection of a deviation from a pure birth-model. Secondarily, similar results of significance values in both tests indirectly inform about the satisfactory completeness of the sampled lineages in the dataset of the study.

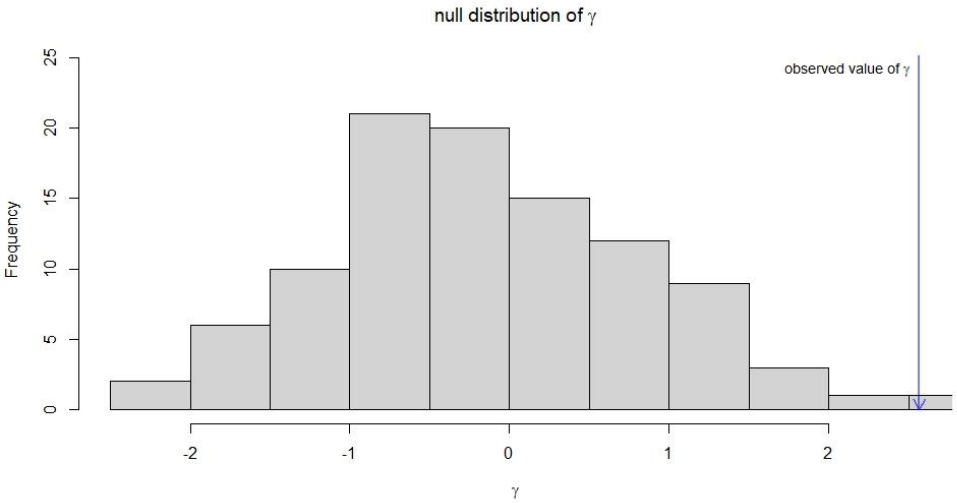

Figure 3.10. Distribution of 100 simulated trees under the Monte Carlo constant rates γ-test showing the location of the observed value of γ (p= 0.0098, significant).

The computation of γ values through time (Figure 3.11) identifies the repetitive decays in the diversification during the first six million years of evolution of the genus, followed by different bursts of diversification during the Pleistocene. The calculation of γ assumes a pure-birth process of lineage accumulation through time, what could be identified in the Pleistocene period analysing the LTT in Figure 3.9. It could be interpreted as an apparent "pull of the present" effect, although it would imply γ values close to zero in the most recent slices of time in Figure 3.11. However, γ calculation through time identifies a recent decrease in diversification rates in the last 90,000 years (Figure 3.11). in fact, γ statistic has a great power to detect decreases in recent diversification (Fordyce, 2010).

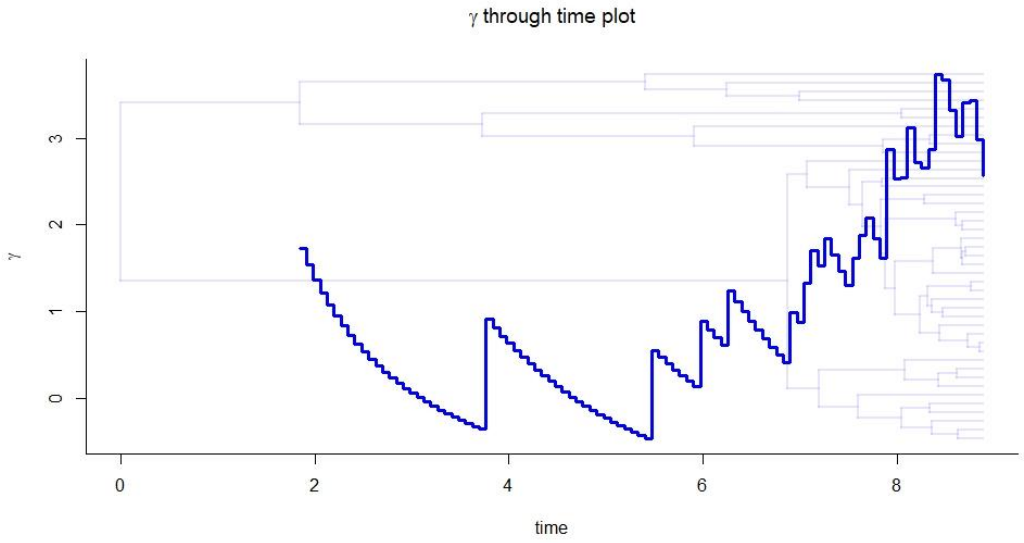

Figure 3.11. Pybus and Harvey γ statistic through time calculated in 100 time intervals across the evolutionary tree of *Jasione*.

3.3. Biogeographic analyses

The reconstruction of ancestral area for *Jasione* inferred many areas with similar probabilities, the majority showing disjunct pattern involving one or several western Mediterranean areas plus the Balkan peninsula. Among them, the combination with greater probability (5.8%) was southern Iberian and Balkan peninsulas (Figure 3.12). This type of pattern is not seen in any extant species of Jasione. The next more probable ancestral area was a western origin inferred by one or a combination of western areas (27%). For this node either dispersal or vicariance was inferred. The ancestor of the Clade 1 most probably originated in the southern Iberian Peninsula (B = 54.7%). The results point to a dispersal event from Iberia to Africa, but the high probability of a combination of a northern African origin (A = 24.7%) or a southern Iberian plus north African origin (AB = 20.5%) suggests that an ancestor species originated in the terrain that united the A and B areas

in the late Miocene could not be ruled out. A vicariant Betic-Riffian process was inferred (AB = 34.8%) to explain the divergence in the subclade of annual taxa, occurring in temporal coincidence with the aperture of the Strait of Gibraltrar, that separated the ancestor of the African *J. cornuta* grup from the lineage leading to the Iberian annuals (*J. blepharodon* and *J. penicillata*) plus *J. corymbosa*. This clade remained and diverged in southern Iberian Peninsula (B = 69.8%) between *J. penicillata* and the ancestor of the "*J. blepharodon*" lineage. The ancestor of the latter would have dispersed into southern Iberia and northern Africa simultaneously, latter originating the trans-Mediterranean coastal lineage of *J. corymbosa* (AB = 69.3%). The alternative of J. corymbosa having evolved only in Africa and then returned to the Iberian Peninsula is inferred with lower but important value (A = 30.6%). The ancestor of the perennial group of Clade 1 diverged in the southern Iberian Peninsula (B = 51.2%) and then one lienage dispersed to the mountain ranges of the Northern Valencian Area as the ancestor of *J. mansanetiana*. The other lineage became J. foliosa, with the divergence between the African J. foliosa subsp. xaunensis and the Iberian populations inferred as a vicariant event (AB 59.5 %). Nevertheless, as it occurred after the aperture of the Gibraltar Strait, an oversea dispersal from the Iberian peninsula is more plausible (B = 40.5%).

The ancestor of the Clade 2 of taxa with hydathoids is recovered as having an original disjunction between the Iberian and the Balkan Peninsulas with the highest probability (CE = 34.9 %). However, an eastern origin, either only in the Balkan peninsular or including Anatolia would sum a greater probability (45.8%). A trans-European widespread ancestor is also recovered with lower but not negligible probability (CDE = 11.3%). Overall, the analysis recovers as the most probable hypothesis an ancestor with eastern-western distribution but originated most probably in the western region. The ancestor of the "*orbiculata-supina*" clade evolved in the Balkan region (E = 73.1%), dispersing in the Balkan and the Anatolia Peninsulas, diverging between the "*orbiculata*" and the "*supina*" clade. The former orbiculate group reached southern Italian Peninsula during the Pleistocene in which a vicariant event is inferred (DE = 100%) as the origin of *J.*

orbiculata var. *italica*. The split between J. orbiculata var. balcanica and the Anatolian lineage is inferred as a vicariant event (EF = 87.62%). In the other main clade of the Clade 2 of *Jasione* the oldest divergences leading to the split of the eastern lineage of *J. heldreichii* (CE = 44.1%, E = 42.8%, CDE = 13.1%), the western-intermediate lineage of *J. laevis* subsp. *laevis* (CE = 54.5%, C = 33.0%, CD = 12.5%) and the eastern lineage of *J. bulgarica* CE = 55.8%, C = 44.2%) are inferred as diverging from ancestors with a disjunct eastern-western range. The analysis recovers not negligible values for widespread European ancestors, except in the case of the lineage leading to *J. bulgarica* and the remaining groups. Within this latter clade, two independent dispersal events from the Iberian Peninsula to northern Africa are identified, one leading to *J. crispa* subsp. *cedretorum* and other to the group "lanuginella-mesatlantica". The latter is inferred as an initial dispersal with the ancestor occupying both northern Iberian and north Africa, followed by a vicariant event and the extinction of the lineage in the Iberian Peninsula (AC = 70.1%). The remaining groups evolved with the northern Iberian Peninsula and dispersed from this area either to southern Iberia (e.g., *J. crispa* subsp. *tomentosa* and allied diploids) or intermediate areas of Europe (e.g., *J. montana* C = 100%).

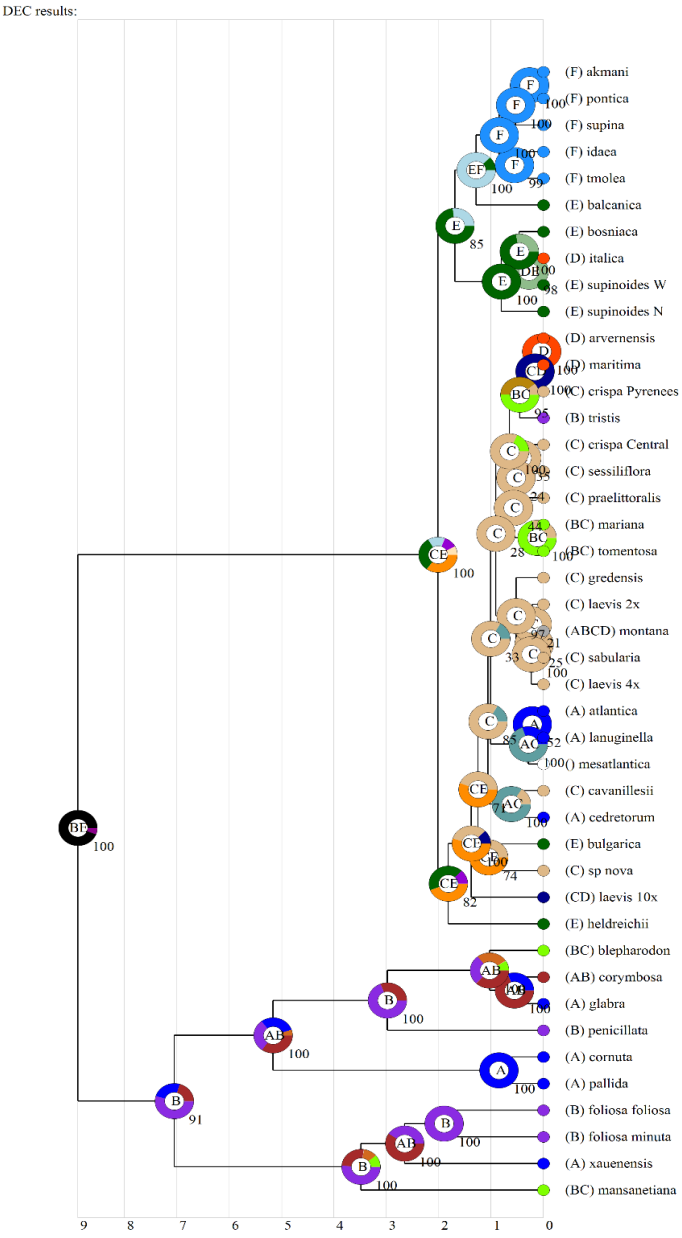

Figure 3.12- BIOGEOBEARs ancestral aeras reconstruction with DEC+J model, with node piecharts indicating marginal probabilities of alternative ancestral areas.

3.4. Niche modelling

The results of distribution models under current conditions are strikingly different between the western group (Figure 3.13A) and the eastern group (Figure 3.13B). In both cases the mean area under the receiver-operating curve (ROC) for the testing data was high, 0.985 in the western group and 0.987 in the eastern group, indicating good model fitting and predictive power of the model. The western group model encompasses all used occurrences except some populations of *Jasione laevis* subsp. *laevis* in the Vosges area (France). Interestingly, relatively good suitability (values > 50) is predicted in most of the areas with populations of the eastern group. The eastern group model predicts suitability in the areas used for training the model, although very narrowly around mountain ranges of the Anatolian species *Jasione tmolea* and *Jasione idaea*. Lowland and mid-mountain areas in these regions are not recovered with significant suitability, despite populations of *Jasione heldreichii* are known in the area. Conversely, the western group model predicts better suitability in western Anatolia that the own eastern group model. The jacknife tests indicated the two temperature variables, Bio4 (temperature seasonality) and Bio3 (isothermality) and one precipitation variable Bio19 (precipitation of the coldest quarter) as the most informative variables.

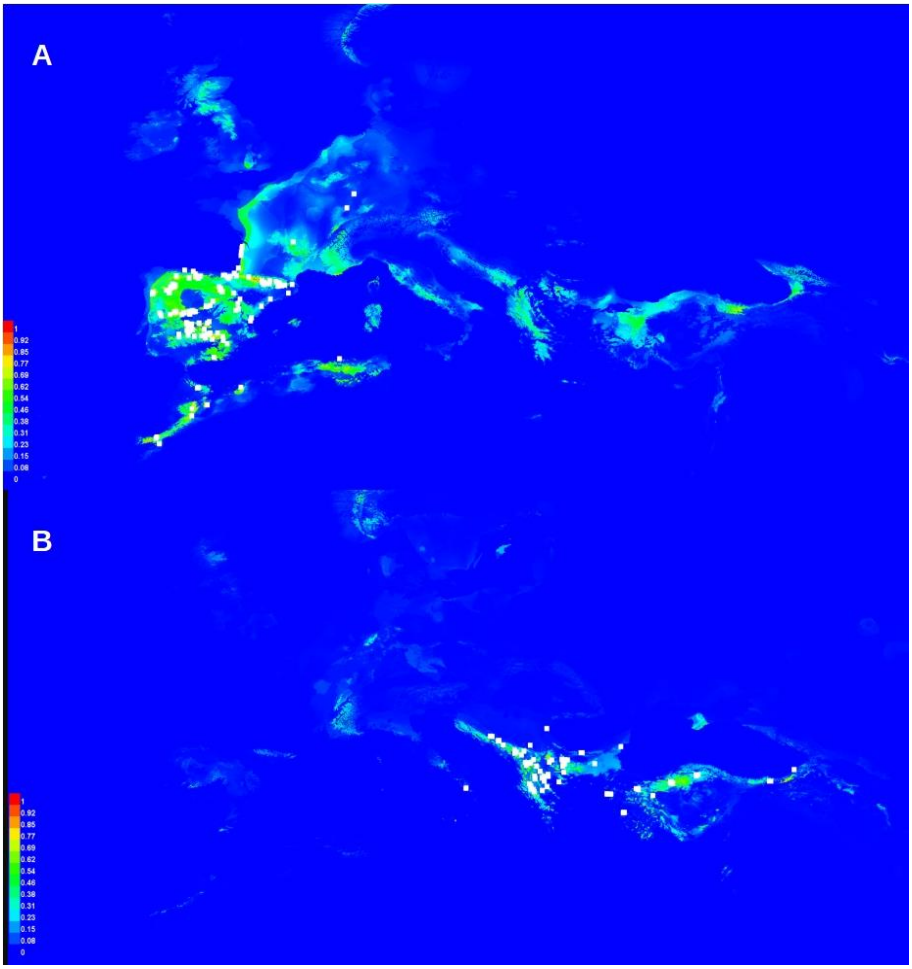

Figure 3.13 MAXENT distribution models of the eastern and western groups of the Clade 2 of genus *Jasione*. Western group of taxa model (A) and eastern group of taxa model (B). *Jasione montana* and diploid populations assigned to *Jasione maritima* are excluded from the analysis.

4. DISCUSSION

The origin of the Campanulaceae has been placed in the southern hemisphere, in agreement with the hypothesis of southern hemisphere origin of the Asterales order (Beaulieu *et al.*, 2013; Crowl *et al.*, 2016).

Africa is the best candidate continent for the origin of the family, as is suggested by diverse early-diverging African lineages in the Lobelioideae, Cyphioideae and Campanuloideae. Crowl *et al.* (2016) analyses of inference of ancestral area supported the African origin in line with the general trend exposed by Linder (2014) by which plant lineages originated in Africa diversifed within Africa, instead of having migrated from other areas. The results of the present work confirm that Wahlenbergioids is an African lineage, and definitely ruled out the doubts about the position of *Hesperocodon hederaceus.*

The early splitting of the Campanuleae led to four extant lineages (*Campanula*, *Feeria*, *Hesperocodon* and *Jasione*). This process occurred in a relatively short period of time (between 29.5 Mya and 25.5 Mya) in the Oligocene. The first divergence led to the Campanula clade, which shortly after (28.7 Mya) split between the ancestors of the E-W Mediterranean disjunct *C. peregrina* group and the clade with the bulk of *Campanula*. The ancestor of *Feeria* split from *Hesperocodon* and *Jasione* about the same time (27.5 Mya) with the final split between *Hesperocodon* and *Jasione* shortly after (25 Mya). The accumulation of these divergence events is followed by a devastating picture of long branches in the three clades. Only *Jasione*, much later (c. 9 Mya), could diverge in lineages that reached the present time with more than one extant species. However, *Feeria* and *Hesperocon* lineages had a poorer fate. This pattern of accumulated splits and short internode branching followed by long branches has been interpreted as a signature of severe extinction after a period of cladogenesis. Either the stepwise temperature decline of the Oligocene that allowed to the emergence of new habitats, like the expansion of grasslands (Miller *et al.*, 2008), with extreme fluctuations at the end of period, or the tectonic activity in the western Tethys region that involved the displacement of microplates in the space between Africa and Europe (Meulekamp *et al.*, 2000), could have favoured the concentration of divergence events resulting in the extant five early-diverging lineages of the Campanuleae. The apparent continued extinction affecting these lineages, only with later diversification in *Jasione,* identified by the diversification analysis during a large part of the earlier history of the genus in the Miocene, could be explained by maladaptation to the progressive cooler and dried

conditions already during the Oligocene or later extinction events. There have been post-Oligocene several phases critical that could have been produced the present day observed pattern, as the constant cooling and drying of climatic conditions characterized the Neogene after the Miocene Climatic Optimum, between 17-15 Mya (Zachos *et al.*, 2008; Methner *et al.*, 2020). But the general pattern shows that, except in one case in each lineage, none of the speciation events that could have occurred within the *Feeria* and *Hesperocodon* lineages in such long period of times was able to adapt. Even the extant species shows signatures of niche conservatism of Tertiary environmental conditions. *Feeria angustifolia* is a chasmophyte Moroccan endemic (Rankou *et al.*, 2013) restricted to the Moroccan mountains, an ecological refuge for some lineages of the Tertiary flora, where the general Mediterranean drought of the regions is alleviated by water condensation in elevation and extreme cold by the oceanic influence and southern latitute (Médail & Quézel, 1999). *Hesperocodon hederaceous* has a wider distribution, stretching from the Iberian Peninsula to the British Isles and NW Germany. Although this range reveals successful adaptation to colder conditions, the species is restricted to the mild climatic conditions of the European Atlantic region. The species occupy heathland areas with frequent and constant rain precipitation, otherwise it behaves as a hygrophilous species in bogs, stream borders or seeping slopes, needing a constant source of humidity. Its distribution has been defined as Oceanic southern-temperate (Eddie & Cupido, 2014).

The origin of *Jasione* as an old lineage originated in the Oligocene that only diversified in extant lineages in the late Miocene suggest low historical adaptability and/or high extinction rate, probably due to niche conservatism. The analysis of diversification (γ values through time) revealed that low diversification/extinction accompanied characterized the history of *Jasione* during its first six million years throughout the late Miocene and early Pliocene, with recurrent diversification decays (Figure 3.11). Of course, this pattern can be produced for a late (e.g., Pleistocene) extinction of unknown lineages. The area of origin of the most recent common ancestor of extant *Jasione* is most probably located in the Western Mediterranean region. The alternative inference of an already disjunct East-West ancestor (in sum less probable) could

mean that the ancestor was widespread in Europe in the Miocene. Unrealistic inferences of disjunct ancestral areas in basal divergences have described in other Mediterranean plant lineages (Carnicero *et al*., 2017).

The results suggest that the Betic-Riffian arch was fundamental in the evolutionary history of *Jasione*, being the ancestral area of *Jasione* Clade 1 in the Miocene. This landmass plus the kabylies were joined to the Balearic and the Tyrrhenian islands in the early Tertiary, but in the Oligocene displaced with SW direction fragmenting during this process. By the late Oligocene the Betic-Riff-Kabylies terrain had already separated from the Mediterranean islands (Rosenbaum *et al.*, 2002). The arrival and diversification of the ancestor of *Jasione* and its allied lineages *Hesperocodon* and *Feeria* could have occurred in this landmass after its breaking-up, as is suggested by the divergence-time results. It fits with the absence of these lineages from this ancient diversification in the Tyrrhenian or the Balearic Islands, where only the young (Pleistocene) *J. montana* lineage can be found indicating a recent arrival. The Betic terrain in the eastern Iberian Peninsula had a latter reconnection with part of the Balearic Islands, during the sea regression of the Mindel glaciation (around 0.4 Mya) that promoted biotic exchanges between the Dianic sector and the island of Ibiza (Garnatje *et al.*, 2013). *Jasione foliosa* is a characteristic plant of the coastal massifs of the Dianic area, but either could not take advantage of the Mindel land reconnection or at least not endure under Holocene climatic conditions, given its narrow environmental requirements. The lineage is restricted to limestone summits with significant amounts of fog condensation and horizontal precipitation (a climatological phenomenon that typically happens between 600 and 1,200 m a.s.l. in the area, while the highest mountain in Ibiza does not reach 500 a.s.l).

The origin of the East-West disjunction originates later in the evolution of the genus, shortly after beginning of the Pleistocene, with the ancestral area of the Clade 2 most probably located in the Balkan Peninsula. The pattern of Balkan as source of lineages functioned at least two times, at the first split of Clade 2 (c. 2 Mya) and shorty after (c. 1.8 Mya) at the divergence between *J. heldreichii* and the remaining groups. However, the somewhat more diverged lineage of the Balkan *J. bulgarica* could have originated in western (Iberian-European) ancestor that later adopted a disjunct vicariant distribution. Thus, the disjunct pattern in *Jasione* seems result of a two-direction process of range expansion and contraction of different lineages. The opposite sides of the Mediterranean basin would have allowed enduring places and "roundtrip" biogeographic histories, increasing the chances of the lineages of *Jasione* to survive in at least one of the two areas in periods of environmental niche shrinking and subsequent lineage extirpation in intermediate areas. In that way, it would be enough that the lineage could endure in one of the two refugia regardless of whether it originated in situ or elsewhere, the chances of range expansion in favourable periods and reaching at least one of the sides with suitable conditions for periods of range contraction are therefore doubled.

Jasione have mountainous environmental preferences (Figure 3.4), what probably stems from an ancestral preadaptation to mid-elevation mountain Ranges. This environmental preference characterizes the perennial lineage of Clade 1 and some sub-lineages of Clade 2, in which adaptation to high-elevation mountain habitats developed largely. Environmental niche analyses in the disjunct Clade 2 showed that suitable conditions for the perennial taxa are generally absent in the intermediate temperate regions of Europe, including the Alps and most of the mountainous Apennine Peninsula. These results suggest that Clade 2 *Jasione* fundamental niche of perennial species is mostly restricted to sub-Mediterranean and Mediterranean mountain ranges that concentrates in the extremes of the Mediterranean basin. This variant of the East-West disjunct pattern restricted to alpine sub-Mediterranean and oro-Mediterranean habitats has been identified in

insects (Varga & Schmitt, 2008). It seems that *Jasione* achieved a balance between niche conservatism and mountain adaptation in the last part Miocene Peninsula that still accounts for its distribution. A historical explanation could parallel the fate of Oligocene vegetal formations in the increasingly drier Miocene in the Iberian Peninsula. Previously widespread laurel-forest and other mesic formations were forced to retreat the Iberian Mountain ranges to avoid the general aridity of the lowlands and forming ecotone communities where enough humidity was provided by orographic factors (Barrón et al., 2010). In this sense, the habitat of the *J. foliosa* group, the extant perennial members of Clade 1, is constrained by the occurrence of summer orographic fogs (M. Serrano, own data). Also, in Clade 2 diverse lineages occur in mid-elevation mountains with frequent orographic fog formation as *J. "praelittoralis"* or *J. sessiliflora* subsp. *appressifolia*, in the Mediterranean siliceous mountains (Gutiérrez, 1988). The distribution of mountain habitat and life history habit (annual or perennial) across the phylogeny of *Jasione* points to a general evolutionary pattern. *Jasione* is mostly restricted to mountain areas except when a lineage is able to develop annual habit. As the closest lineages of *Jasione* (i.e., *Feeria* and *Hesperocodon*) are both perennial, perenniality is probably the ancestral state. Therefore, annuality seems to have evolved independently three times in the genus, characterizing the annual lineage (*J. cornuta, J. blepharodon*, etc ...) of Clade 1, the lineage of *J. heldreichii*, and the lineage of *J. montana*. Annuality evolves to maximize the number of individuals achieving maturity when adult survival rates are very low (Fox, 1990). Shift in habit trait probably allowed *Jasione* to escape from wetter but narrower mountain areas and expand to much wider lowland areas, environmentally forbidden to most perennial lineages. Indeed, the most widespread taxa in Clade 1 and Clade 2 are annual or annual-biennial lineages, *J. blepharodon* and *J. montana*, respectively.

Clade 2 is characterized by foliar hydathoids, a trait unique among the Campanulaceae. The evolutionary significance of the trait is still under study, but the analysis of its distribution across the phylogeny of *Jasione* and the average number of hydathoids compared with environmental conditions points to a relevant role shaping the current

distribution of Clade 2 lineages (Figure 3.4). The trait is completely absent in Clade 1, in which undifferentiated regular hydathodes are present, as in the other groups of the family. The general tendency in Clade 2 is that lineages occurring in mesic environments, as those from wetter regions or higher latitudes with less Mediterranean influence, would have greater average number of hydathoids. As an example, average numbers over 6 hydathoids per leaf can be found in mesic groups as *J. bulgarica* or *J. laevis* subsp. *laevis*, among others. Conversely, lineages from dried regions in the Iberian Peninsula, North Afirca or Anatolia would have lower numbers, frequently with most leaves bearing only one apical hydathoid. Although water uptake through hydathoids could be the evolutionary role of the trait (M. Serrano, currently under study), the structure also allows water loss (see General Introduction), what seems to explain the geographical distribution of the average number within the clade. Again, annual lineages deviate from the general pattern. Annual-biennial lineages as *J. heldreichii* and *J. montana* have high numbers of hydathoids even in comparatively dry environments. These results suggest that maximizing annual reproduction over individual survival allows annuals lineages of *Jasione* to relatively detach from the environmental hydric constraints probably imposed by hydathoids.

Polyploidy had an important role in the Pleistocene evolution of Iberian lineages of Clade 2. Polyploidy occurred in three independent lineages. In two of them, the "*montana*" clade and the "*crispa*" clade recurrent and independent events of polyploidization seems to have enhanced the environmental tolerances of these groups. Nevertheless, environmental niche studies in diploid-tetraploid systems in *Jasione* have showed that polyploidization can lead to either to niche statism, as in *J. montana* (Castro et al., 2019) or very moderate niche shift, as in Iberian "*J. maritima*" (Castro et al., 2020), but also to considerable niche expansion as in *J. sessiliflora* (see Chapter 4). The results of niche analysis in Clade 2 (Figure 3.13) suggest greater niche breath for combined perennial western lineages than for the eastern lineages. Distribution models from western lineages predict habitat suitability in the areas occupied by the eastern lineages, and scarcely elsewhere. Therefore, as western groups of Clade 2 most probably originated from

an eastern stock the environmental results point to some degree of niche conservatism behind the disjunct pattern. Conversely, eastern lineages do not predict western niche occupancies, probably indicating narrower niche breath. Recurrent polyploidy was an Iberian process, with polyploids occurring in mainland Europe explained by well-supported "out-of-Iberia" dispersal events (i.e., hexaploid *J. maritima* and *J. crispa* subsp. arvernensis, decaploid *J. laevis* subsp. *laevis*). On this regard, the results confirm the hypothesis of the Iberian origin of the polyploid French populations suggested by Küpfer (1974, 1981). Overall, polyploidy seems to have enhanced the environmental opportunities in the Iberian groups, allowing a radiation coping with relatively novel environments, and therefore adaptive speciation (Barraclough, 2019). As examples, hexaploid *J. maritima* conquered sand dunes from a mountain lineage occurring the Pyrennes and the French Massif Central, and decaploid *J. crispa* subsp. *varduliensis* adapted to limestone Basque-Navarrese mountains from a calcifuge lineage of siliceous Cantabrian mountains. Nevertheless, the extent of niche expansion in western Clade 2 representatives should be relativized as it still preferentially happens within the extent of a sub-Mediterranean climatic framework, evincing phylogenetic niche inertia. For example, hexaploid *J. maritima* has populations in SW French coast, but it range is restricted to areas with sub-Mediterranean climate with a climatic north boundary in the Charente region (Defaut, 2016). This pattern precisely matches distributions of other species tightly linked to Mediterranean environmental influences in Temperate SW France, as the lizard *Timon lepidus* (Jorcin *et al.*, 2019).

The remaining group that underwent polyploidization shows relict characteristics instead of niche expansion. It is one relativelly isolated lineage ("*Jasione* sp. nova" in Figure 3.4 and other figures), represented by a still undescribed hexaploid species, that will probably be named "*J. indiketica*" (M. Serrano, in prep.). This lineage occurs in relatively thermic and humid and very narrow areas with maritime influence in mid-elevation eastern Pyrenees. The results suggest a possible phylogenetic relationship with the Balkan endemic *J. bulgarica*, although without significant statistical support.

Secondary contact and introgression have been detected in *Jasione*. It cannot be ruled out that some polyploid lineages could have originated as allopolyploids after secondary contact between diverged taxa, as suggested by study of nuclear molecular markers (M. Serrano, in prep.). Signatures of introgression are detected in the present work both in Clade 1 and Clade 2, and probably involve altitudinal range shifts in different mountain ranges during the Pleistocene climatic oscillations, a well know pattern in refuge areas (Nieto-Feliner, 2014). In Clade 1, plastid capture in one population of *J. penicillata* from close, lower altitude, populations of *J. blepharodon* was identified (Figure 3.4). More striking is the recurrent introgressive process in Clade 2 affecting at least four Balkan and Anatolian populations of the *J. heldreichii* group (Figure 3.4). All these populations are located at basal or mid-elevation mountains belts and have plastid genomes similar, although not always identical to the populations of the *J. orbiculata* and *J. supina* groups occurring in the summits of close mountain areas, although considerable geographic distances exist in some cases. While there is no current range overlap, the situation could have been different in the Pleistocene, when glaciers occupied the highest elevations and "*supina*" and "*orbiculata*" had to retreat downwards. Interestingly, introgressed populations of the *J. heldreichii* group have partially or completely lost the annual-biennial habit characteristic of the species. Perennial *J. heldreichii* have been considered and independent species, *J. jankae* by some authors what raised a long-lasting taxonomic discussion (see Chapter 1). These results give an evolutionary basis to explain the nature of *J. jankae*, although more detailed studies are needed in such an interesting and probably multi-originated micro-specific system.

Adaptation to mountain habitats probably as evolutionary refuge against late the tertiary aridification that intensified after the onset of Mediterranean climate around 3 Mya (Suc 1984, Barrón *et al.*, 2010),66 explains the bust of diversification in Clade 2. The initial diversification in this group is inferred after the beginning of the Pleistocene (c. 2 Mya), contradicting the general pattern that explains the East-West disjunction as result of glacial vicariance in Pliocene lienages (Rosselló

et al., 2007; Casimiro-Soriguer *et al.*, 2010). Moreover, at least two more pulses of range expansion and connection between Balkan and Iberia and subsequent vicariance occurred during later Pleistocene periods. Short branches and low resolution in several basal nodes of Clade 2 coupled by enhanced divergence after a long period of recurrent decays in diversification (indicated by Pybus and Harvey γ test, Figures 3.10 and 3.11) suggest that contrary to previous periods, the first part of the Pleistocene was a period of fast diversification rate in Clade 2. Low resolution at basal nodes have been identified as periods of rapid diversification in other groups (Riina *et al*., 2013; Carnicero *et al*., 2017). Although apparent recent bursts in diversification have been related to the "pull of the present" artifact (Fordyce, 2010), *Jasione* clearly deviates from constant birth-death rates during its earlier history. Moreover, although the LTT plot (Figure 3.9) suggest constant diversification in the Pleistocene, the more powerful technique of computation of γ values through time (Figure 3.11) detect a marked decrease in diversification since the Last Interglacial Period that already initiated 5,000.000 years ago. Overall, adaptation to sub-Mediterranean mountains had favoured range expansion in glacial periods and range contraction in interglacial, with vicariant events leading to the current pattern of many mountain narrow endemics. Nevertheless, the comparatively more intense cold of the last glacial cycles (Rull, 2020) would have been less favourable for *Jasione* linages (Figure 3.8).

Vicariance between Iberian an African lineages can be attributed to maritime separation only in the case of the split between the African lineage of *J. cornuta* and its sister Iberian clade, dated c. 5.2 Mya and therefore shortly after the definitive aperture of the Strait of Gibraltar (Hsü, 1972). Other dispersal events from Iberian lineages in Clade 1 occurred over the Mediterranean Sea, as in *J. foliosa*. This event was dated c. 2.7 Mya, around the boundary between the Pliocene and the Pleistocene. Other cases of transference over the Gibraltar Strait in plant groups without adaptation to dispersal have been described (Fernández-Mazuecos & Vargas, 2011). The arising of the coastal lineage of *J. corymbosa* c. 1 Mya ago from a common ancestor with the inland Iberian species *J. blepharodon*, involved adaptation to maritime

environments. Late Pleistoce recurrent dispersal between Iberian and North African shore are more easily explained in this linage than in *J. foliosa,* enhanced permeability to dispersal during glaciation resulting from drop in sea level. Exposed continental shelves created a string of small sandy islands (Gràcia *et al.*, 2008), a favourable habitat for J. *corymbosa*. Pleistoce oversea range expansion into Africa from Iberian lineages occurred at least two independent times in Clade 2, between 1.5 and 1 Mya, giving place to the mesic-relict Riffian lineage of *J. crispa* subsp. *cedretorum* and to the more drought-adapted lineage of *J. crispa* subsp. *lanuginella* and *J. crispa* subsp. *mesatlantica,* that diverged internally in the Atlas massifs. The results support the elevation of these taxa to independent species unrelated to the *J. crispa* group, in other striking case of morphological stasis/convergence in the genus.

Biogeographic relationships between Balkan and Anatolian peninsulas are consistently inferred as expansion from the former to the latter area, contradicting other cases of study. Anatolia has been suggested as a main glacial refugium playing an important role in the recolonization of Europe during the Pleistocene cycles (Hewitt, 2011), with diverse study cases in different organisms supporting this hypothesis (Korkmaz *et al.*, 2014; Ali *et al.*, 2016) and encompassing diverse refugia within the peninsula acting as independent sources for different lineages (Bilgin, 2011), fitting in the so-called refugia within refugia pattern initially described in the Iberian Peninsula (Gómez & Lunt, 2007; Nieto Feliner, 2014). Özödugru & Mummenhoff (2020) described in Bornmuellera (Brassicaceae) two independent mountain paths by which the refuged biota in Anatolia would have use to endure and then colonize the Balkan peninsula, the southern Taurus mountains way in norther Anatolian Mountains way, supported by the basal position of Anatolian lineages with respect to Balkan ones. The hypothesis of "out-of-Anatolia" through dispersal mountains corridors followed by vicariance has been suggested in also in insects (Kaya & Çıplak, 2017). The results inf *Jasione* depicts an opposite scenario, suggesting a Balkan origin for the different Anatolian lineages, given the basal position in the "*supina*" clade of *Jasione orbiculata* var.

balcanica. Nevertheless, they give some support to the norther Antolian mountain corridor, although in this case working in a different direction. Precisely, the more derived clades occur in northern Anatolian Pontic mountains, as a very recent Late Pleistocene divergence and expansion to the East of *J. supina* subsp. *pontica*. This eastwards expansion could be still ongoing and could eventually reach environmentally suitable areas in the Caucasus Mountain region.

The results confirm the relative irrelevant role of the Apennine Peninsula in the evolutionary history of *Jasione.* Similar biogegraphic scenarios have been inferred in beetles of genus *Deronectes* although the pattern was explained by dispersal opportunities (García-Vázquez *et al.,* 2016). The absence of ancient lineages of *Jasione* or recent lineages with important diversification in the region could be explained by the apparent low amount of suitable habitat for the genus. High values of suitability are only inferred in the Southern Apennines. The Southern Apennines are an eco-tonal area between the Central European and the Mediterranean biomes in terms of their vegetation communities (Passalaqua, 1998). Within this range, the Sirino-Papa summits are covered by typically oro-Mediterranean thorny-cushion coomunities with scattered temperate grasslands mostly on northern faces. The thorny-cushion vegetation is considered of eastern origin and its presence is explained by the position of the massif, isolated and exposed to humid winds blowing from the sea (Pignati *et al.*, 1980). Because of that, this massif has been considered unique along the whole range of the Apennines (Tomaselli *et al.,* 2007). Although the areas with sub-Mediterranean conditions have been estimated to cover the 24% of the Apennine Peninsula, they represent conditions closer to the Temperate than to the Mediterranean biome (Pesaresi *et al.*, 2014). Current distribution of *Jasione* in Italy seems determined by a combination of environmental constrains and historical factors, like dispersal opportunities. Niche analyses suggest the Alps as an important barrier to dispersal, as has been proposed in many animal and plant groups (reviewed in Nieto-Feliner, 2014). Th Balkan lineage of *J. orbiculata* probably crossed the Adriatic Sea in a Glaciation period with drop in sea level, c. 500,000 ya. Instead of undergoing range expansion

through the Peninsula, the lineage (*J. orbiculata* var. *italica*) could be constrained to thrive in oro-Mediterranean environments just in the interface with the temperate formations.

As a final remark, the phylogenetic results suggest the need of a taxonomic reorganization of the genus and the recognition of diverse evolutionary significant units of diversity as species or subspecies.

5. CONCLUSIONS

The reconstruction of the biogeographical and evolutionary history of *Jasione* evinces how its diversification has been configured since the inception of the Miocene by climatic changes and geological events by recurrent range expansions and subsequent range fragmentations, with long Miocene-Pliocene periods of diversification decay of the extant leinages, and a Pleistocene burst of diversification. A western origin of the genus is suggested, with eastern colonization occurring by only one of the two main lineages. Within this clade, several range expansions can be inferred, giving a major role to the eastern area of the Mediterranean as source of lineages, even if higher diversification rates can be found in the western part. This roundtrip model between the west and east sides of the Mediterranean basin would have enhanced the survival opportunities of a lineage with access to two refuge harbours where to endure and, in some cases, to evolve and to adapt to changing conditions. This pattern is responsible for the extant taxonomic richness of *Jasione* in the Mediterranean basin and if made extensible to other groups it could be and important contributor to the overall richness of the region. The north side of the sea was the only scenario of the East-west biogeographic events in *Jasione*. Different groups of the clade that arrived the East can be found in North Africa, but these lineages are result of separate dispersal events from the Iberian Peninsula and African did not play a role in the colonization of the East. Pre-adaptation to mountain habitats could have offered a refuge against late Neogene general aridification, but also imposed environmental constrains that only are escaped by lineages evolving annual habit.

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CHAPTER 4.
Ecological niche conservation and niche shifts in a diploid/tetraploid group of taxa of the genus *Jasione* L. (Campanulaceae) endemic to the Iberian Peninsula

ABSTRACT

There is a general tendency for niche conservatism among closely related taxa, although in polyploid complexes niche divergence may enable polyploids to become established outside the range of distribution of their diploid relatives. Polyploidization leads to the immediate emergence of evolutionary barriers and speciation. However, the new polyploid appears within diploid populations and is thus exposed to the effects of competition and frequency-dependent processes that ultimately lead to reproductive exclusion of the minority cytotype. Ecological divergence would enable polyploids to avoid these processes, thus becoming a key mechanism in shaping geographical distribution of cytotypes in polyploid complexes. However, evidence supporting this hypothesis is still needed, as many contradictory findings have been reported. Moreover, it remains to be determined whether polyploids diverge ecologically to a greater degree than diploids. Study of polyploid complexes encompassing both polyploids and closely related diploid taxa provides an opportunity to compare polyploid niche shift or conservatism in relation to niche shift within an appropriate evolutionary framework. The niche shift hypothesis is evaluated here in a diploid-tetraploid system of closely related taxa of the genus *Jasione* L. (Campanulaceae) belonging to the *Jasione crispa* (Pourr.) Samp group. In two previous studies, the hypothesized niche

shift in other model polyploid groups of the genus *Jasione* was either rejected or not fully supported by the results, and current distribution ranges of those polyploid complexes must be explained by other factors. Here, the fine scale distribution of cytotypes and evolutionary significant taxonomic groups was determined by chromosome counting, flow-cytometry on silica-gel dried material and molecular markers of the plastid genome. Environmental modelling techniques were applied and species distribution models were developed to examine whether environmental niche shift or conservatism occurs between three diploid taxa and between these and a presumably descendent tetraploid taxon. The modelling process used climatic and edaphic variables, comparing species distribution models with and without edaphic variables. The inclusion of edaphic variables in modelling this polyploid complex provided a more accurate description of the ecogeographic reality of the taxa under study. In addition, the models demonstrated that the tetraploid group underwent a significant change and niche expansion relative to the diploid close relatives, in which niche conservatism is detected. Niche expansion towards tolerance of colder environments has shaped the current distribution of tetraploid *J. sessiliflora* while niche conservatism restricts the diploids to comparatively thermic and sub-humid areas in the central south-western and Mediterranean Iberian Peninsula. Diploid niche conservatism seems to reveal some residual inertia in the ancestral niches of the lineage. In summary, and conversely to other *Jasione* model systems, polyploidization probably caused successful environmental innovation in *Jasione sessiliflora*.

1. INTRODUCTION

It is generally assumed that organisms display evolutionary inertia that limits ecological niche shifts between closely related species and results in niche conservatism, with differentiation in geographic space preferentially operating between sister taxa in the first instance and differentiation in the environmental niche only occurring later, in evolutionary terms (Peterson *et al.*, 1999, Wiens & Graham, 2005). Nevertheless, this general tendency is characteristic of allopatric

speciation types and obviously is not followed when speciation is triggered by ecological adaptation to different niches (Schlutter, 2009; Nosil, 2012). Although ecological speciation is a result of adaptation to new niches and can occur in sympatry, it is also promoted in many cases by allopatry. It enables a reduction in gene flow favouring a positive feedback between environmental filtering and geographic distance that eventually leads to isolation and divergence (Barraclough, 2019). Nevertheless, sympatric speciation occurs frequently and is extensively detected in angiosperms: speciation derived from whole genome duplication (i.e. polyploidization), a mechanism that creates immediate barriers to gene flow between a newly arisen polyploid and the diploid or lower ploidy parental populations in which it originates (Otto & Whitton, 2000; Wood *et al*., 2009; Soltis *et al.*, 2010; Husband *et al*., 2013). The extensive reorganization of the genome that occurs after a polyploidization event could lead to substantial changes in the phenotype of the new polyploid relative to the parental organisms, both in the case of autopolyploids (i.e. polyploids with multiple chromosome sets from a single taxon) and allopolyploids (i.e. polyploids derived from two or more diverged taxa), although morphological inter-cytotype homogeneity is frequent, particularly in autopolyploids (Castro *et al.*, 2019). Polyploids can spread within the parental populations, but given their initial scarcity within the diploid/lower ploidy populations, their establishment is negatively affected by frequency-dependent processes, namely minority-cytotype exclusion (Levin, 1975; Husband & Schemske, 2000). Polyploids can evolve traits to increase the likelihood of thriving in sympatry with the parental groups, such as perenniality and increased rates of autogamy (Rodríguez, 1996; Husband & Schemske, 2000). In the absence of these traits, polyploids could use other strategies to outcompete the progenitors or to escape from minority-cytotype exclusion by occupying areas beyond the range of the parental groups. Indeed, different distribution ranges are frequently found in polyploids and their diploid counterparts (Balao *et al*., 2009; Casazza *et al*., 2017; Castro *et al*., 2020). Although historical factors, among others, may be involved in explaining these differences in distribution, the development by polyploids of different environmental tolerances (i.e., shifts in

ecological niche) is frequently cited as the main causative factor (Levin, 2004; Husband & Schemske, 2000, Adams & Wendel, 2005). Therefore, through genomic rearrangements caused by polyploidization and affecting gene expression and phenotype, the new polyploid could overcome the phylogenetic inertia that tends to cause niche conservatism, thus enabling colonization of new environments and areas. Precise investigation of the geographic distribution of cytotypes and environmental modelling of abiotic variables (Broennimann *et al.*, 2012) have been used to test the niche shift hypothesis in plant polyploid groups (reviewed in Castro *et al*., 2019), although more evidence from diploid-polyploid species pairs in which differences in environmental tolerances clearly account for geographical ranges are still needed (Castro *et al*., 2020). Characterization of the environmental envelope of different cytotypes determines the ecological constraints shaping current distributions. Nevertheless, several studies have suggested that different cytotypic geographical distributions (either parapatric or allopatric) cannot be explained by shifts in environmental tolerances, enabling researchers to suggest other causal factors, such as historical processes and interactions between cytotypes (Godsoe *et al*., 2013; Laport *et al*., 2013; Laport *et al*., 2016). The genus *Jasione* presents several poplyploid complexes in the Iberian Peninsula, and two of these complexes have already been used as model groups to address the question of niche evolution between cytotypes (Castro *et al*., 2019; Castro *et al*, 2020). Despite the initial assumption of inter-cytotype niche evolution, the results suggested that the current geographic patterns may be explained by other factors. Castro *et al*. (2019) examined the mosaic, non-overlapping distribution of diploid and tetraploid populations of *Jasione montana* L. in the Iberian Peninsula and found that the tetraploid abiotic niche was a subset of the diploid niche. Castro *et al*. (2020) analysed the cytogeographic pattern of the coastal *Jasione maritima* (Duby) Merino, with a linear parapatric diploid-tetraploid distribution in Galicia and northern Portugal, observing high degree of niche overlap between cytotypes and little support of variation in environmental tolerances in one of the tetraploid varieties (occurring in Portugal). Thus, these previous studies supported

niche conservatism in polyploid complexes, despite clear differentiation in the geographic pattern of the different cytotypes.

This study is focused on a polyploid complex of *Jasione* including diploids and tetraploids and also different closely related diploid taxa, to establish a cyto-phylogenetic framework for comparison. The model polyploid complex encompasses a set of Iberian endemic taxa related to the wider *Jasione crispa* (Pourr.) Samp group (Sales & Hedge, 2001; Mateo Sanz & Crespo, 2008). The group includes three diploid taxa, *J. crispa* subsp. *tomentosa* (A. DC.) Rivas Mart., *J. crispa* subsp. *mariana* (Willk.) Rivas Mart. and *J. sessiliflora* Boiss. & Reut. subsp. *appressifolia* (Pau) M.B. Crespo & Mateo, and a tetraploid taxon, *J. sessiliflora* subsp. *sessiliflora*. The distribution of the taxa is illustrated in Figure 1. *J. crispa* subsp. *mariana* and *J. crispa* subsp. *tomentosa* occupy numerous quartzite mountain systems in the southwestern central Iberian quadrant around the Tagus and Guadiana basins (Sales & Hedge, 2001), with a largely overlapping distribution. A few outpost populations of *J. crispa* subsp. *tomentosa* are present in granitic areas in the Central System, although these are considered "unassigned diploids" (Chapter 2). *Jasione sessiliflora* subsp. *appressifolia* is restricted to the Valencian and Castellón mountain ranges of Pina, Calderona and Espadán (Mateo Sanz & Crespo, 2008). The tetraploid *Jasione sessiliflora* subsp. *sessiliflora* is more widely distributed, occupying areas mainly in the northern half of the peninsula (Sales & Hedge, 2001). The areas inhabited by diploids are mainly located in the Luso-Extremadura and Valencian biogeographic sub-provinces, characterized by a Mediterranean climate with summer drought, although in the mid-elevation mountains where the plants occur drought is slightly nuanced by submediterranean thermic influences, in a subhumid and relatively warm mesomediterranean bioclimatic belt (Espírito-Santo *et al.*, 2017; Soriano & Costa, 2017). *Jasione sessiliflora* subsp. *sessiliflora*, on the other hand, occupies cooler, submediterranean areas, sometimes with subcontinental influences, both in the central and Iberian systems and in other mountain ranges around the northern Iberian plateau. *J. crispa* subsp. *tomentosa* and both subspecies of *J. sessiliflora* have numerous morphological similarities that reveal their evolutionary affinity, indicating the parental

relationship between some of these diploids and the tetraploid *J. sessiflora* subsp. *sessiliflora*. The *J. crispa* subsp. *mariana* taxon is closely related to *J. crispa* subsp. *tomentosa* (Sales & Hedge, 2001). The affinity between these taxa has also been revealed by examination of molecular markers (M. Serrano, Chapter 2 and 3). Although it is not clear whether *J. sessiliflora* subsp. *sessiliflora* is an autotetraploid or product of an allopolyploidization event, the slight morphological differences between the tetraploid and the polyploid suggest the second possibility.

Precise identification of the geographic distribution of the cytotypes and environmental modelling is used to analyse niche evolution within diploids and between diploids and tetraploids. The environmental variables most commonly used in environmental modelling (EM) and projection of species distribution models (SDM) are bioclimatic layers, which include temperature and precipitation data, and composite indices including these variables (Figueiredo *et al.*, 2018). However, edaphic-type variables are very rarely used (Figueiredo *et al.*, 2018; but see Castro *et al.*, 2019), despite their a priori fundamental importance in enabling the establishment of plant populations in an area, as e.g. in the numerous species that are not indifferent to the substrate pH. In this respect, the group of species analysed in this work belongs to an acidophilic, calcifuge lineage (M. Serrano, own data).

The distribution of the tetraploid *J. sessiliflora* s. str. seldom overlaps with the diploid distributions, with a reduced parapatric contact zone with the diploid *J. crispa* subsp. *tomentosa*. Moreover, in order to increase the span of geographic patterns to be tested for niche conservatism/shift between the diverged diploids, this study includes sympatric diploid taxa whose ranges partially overlap (*J. crispa* subsp. *tomentosa* and *J. crispa* subsp. *mariana*) and one diploid taxon with an allopatric distribution in a relatively distant geographic area (*J. sessiliflora* subsp. *appressifolia*). The objective of this study was to test whether any ecological niche shift has occurred during the emergence and divergence of the tetraploid *J. sessiliflora* subsp. *sessiliflora* from closely related diploid taxa or, alternatively, whether niche

conservatism prevails, as would be indicated by similar or lower niche differentiation than that existing between the diverged diploids. In addition, the accuracy of SDMs generated only with climatic variables compared with SDMs generated by climatic and edaphic variables was addressed.

2. MATERIAL AND METHODS

The geographical occurrences are taken from the author's own database, prepared from his own collections and by analysis of herbarium sheets and bibliography. The use of public databases was ruled out, given the high rate of misclassifications in groups with no clear morphological boundaries such as in the genus *Jasione*. For example, in gbid.org the percentage of misidentifications regarding *J. sessiliflora* was estimated to account for around 60% of the observations (M. Serrano, own data). Four localities with diploid plants are considered "unassigned diploids" in Chapter 2 due to subtle morphological differences with *J. crispa* subsp. *tomentosa*, although they are molecularly indistinguishable from *J. crispa* subsp. *tomentosa* or *J. sessiliflora* subsp. *appressifolia*. The low number of occurrences does not allow statistically significant SDMs and consequently cannot be included in the analysis as a separate diploid group. They are included in the analysis of *J. crispa* subsp. *tomentosa* environmental variability.

The use of molecular markers and ploidy level determination for the studied populations is described in the General Methods section and in Chapter 2.

For EM, 19 layers of WorldClim bioclimatic variables (WorldClim website) were used, in addition to five edaphic layers (Table 4.1) reported in the public database ISRIC world soil information (Hengl *et al.*, 2017), because of the importance of the soil factor in the distribution of plant species belonging to acidophilic lineages. For example, soil pH is used as a proxy for the presence of cations in the region, although the spatial pattern of this variable in the environmental layer used is greatly

influenced by precipitation (Hengl *et al.*, 2017). In theory, this does not detract from the ecological significance of the variable, as precipitation tends to facilitate the creation of organic soils and leaching of basic cations. However, the soil pH in water is measured at 100-200 cm depth, relatively close to the bedrock, and is indicative of the nature of the substrate. The apparent density provides information about the water storage capacity of a soil, as well as about the structural conditions and degree of compaction (Mukhopadhyay *et al.*, 2019).

Table 4.1: ISRIC edaphic variables evaluated.

Variable	Description	Units
ACDWRB	Degree to which a sub-soil is acid, e.g. of pH < 5 and low BS, grade	Grade
BLDFIE	Apparent density (fine earth)	Kg m^{-3}
ORCDRC	Soil organic carbon content	Per mile
PHIHOX	pH index measured in aqueous solution	pH
SNDPPT	Weight percentage of sand particles (0.05 mm -2 mm)	Percentage

The resolution of the climatic and edaphic environmental layers is different (i.e. the pixel size of the data is different), being around 1 km (30" of arc) in the first case and 250 m^2 in the second. In order to combine environmental raster layers in ecological modelling processes, they must have the same resolution, as well as the same geographic extension and cartographic projection. The edaphic layers were adjusted to the resolution of the environmental layer with the R package "raster". The variables finally chosen were selected for their ecological significance in relation to the taxa and because of the low collinearity between them, as indicated by Pearson's correlation coefficients < 0.8. For this purpose, a correlation matrix was generated in R (R Core Team, 2020) using the "gridExtra", "rasterVis" and "reshape2" packages. Two

sets of variables, i.e. climatic only and climatic plus edaphic variables, were used

The "spThin" package (Aiello-Lammens *et al.*, 2015) reduces the number of presences until there is only one registered point per information pixel, thus eliminating those points for which the distance was less than 5 km in *J. crispa* subsp. *mariana*, *J. sessiliflora* subsp. *sessiliflora*, and *J. crispa* subsp. *tomentosa*. By contrast, in *J. sessiliflora* subsp. *appressifolia*, the minimum distance between presences was established at 1 km due to the low number of initial data points. One hundred repetitions were generated for each species, to check whether the number of records remained stable.

The models were constructed with the R Biomod2 package and using Generalized Linear Model (GLM), Generalized Additive Models (GAM), MAXENT and Random Forest (RF), thus enabling the species records to be related to climatic variables. For this purpose, 4 validations and 3 replications of 5000 pseudo-absences per algorithm were run, creating a total of 48 models for each species. GLM and GAM are both regression methods that use the presence and absence of data. However, GAM, unlike GLM, can generate a non-linear model. MAXENT and RF are machine learning methods based on the presence of data on a background. MAXENT is based on the principle of maximum entropy for those models with a more uniform distribution (Philips & Dudik, 2008). The models created with the different algorithms for the taxa under study were evaluated with 20% of the data, and the remaining 80% of the data was used for calibration (Guisan & Zimmerman, 2000). The models were evaluated by threshold-independent statistical analysis: true skill statistic (TSS) and receiver operating characteristic (ROC). Models with TSS and ROC values > 0.7 were accepted, as this indicates that the predictions are more realistic than would result by chance. The different individual models were coupled creating consensus models. This assembly technique uses the weighted average and average statistical values to select the prediction that is closest to the actual observed data (Araújo & New, 2007). The coefficient of variation of the ensemble models was also calculated.

For the model projections, the program QGIS 3.14 (QGIS Development Team, 2020) was used by loading the raster environmental maps, the study environment and the author's own data. SDM maps with inferred grading of presence-absence optics were thus created.

Niches of the four taxa were visualized in the environmental space using environmental PCAs (PCAenv), an ordination approach calibrated with environmental values of the area of study (Di Cola *et al.*, 2017) using the R package "ecospat" (Broennimann *et al.*, 2012). The PCA computes the occurrence density and environmental factor density for each pixel along environmental axes, maximizing the environmental variance of the distribution ranges of each taxon. PCA scores of the occurrences were projected onto a grid of cells bounded by the maximum and minimum PCA scores, allowing the comparison of niches in terms of breadth and location within the environmental space of the area of study (Di Cola *et al.*, 2017).

As a measure of niche overlap, the D value of Schoener (1970) was selected as a metric that varies between 0 (indicating no overlap between niches) and 1 (indicating complete overlap). The D values observed between pairs of species were used to test niche similarity (Warren *et al.*, 2008; Broennimann *et al.*, 2012). The niche similarity test determines whether the observed overlap value (D) differs from the overlap between the observed niche in the distribution of one species and randomly selected niches in the distribution of the other species. In this case, the centre of the mesh density is randomly selected from the available environmental space in the opposite distribution (Broennimann *et al.*, 2012). The significance value was evaluated using 100 randomizations ($\alpha = 0.05$). Niche similarity was tested for all pairs of taxa with the ecospat package. PCAenv and similarity tests were performed only with climatic variables to avoid enlarging the niche breadth visualization with factors such as pH with low variation across recent temporal scales.

3. RESULTS

Once the number of records was reduced to a single point per pixel, 45 points were obtained for *J. crispa* subsp. *tomentosa*, 33 for *J. crispa* subsp. *mariana*, 17 for *J. sessiliflora* subsp. *appressifolia* and 203 for *J. sessiliflora* subsp. *sessiliflora*. Maps of 5000 pseudo-absences were created from these data by using the "dplyr" package (Figure 4.1), where pseudo-absences are represented in black and the distribution of each taxon is indicated. The maps show that *J. crispa* subsp. *mariana* and *tomentosa* have a similar geographic distribution, while *J. sessiliflora* subsp. *sessiliflora* and *appressifolia* occupy different geographical spaces.

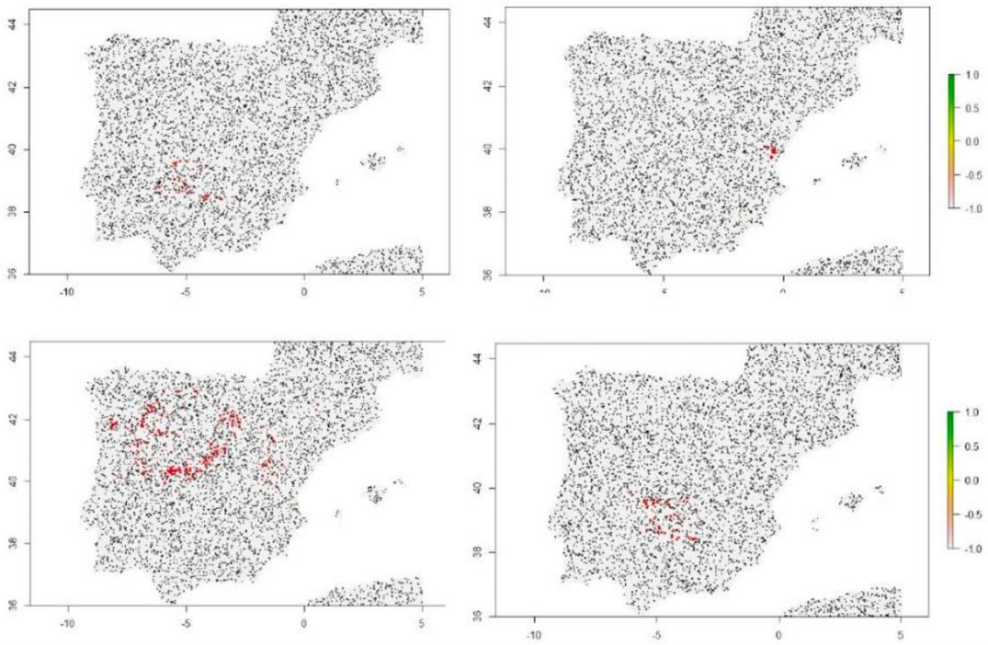

Figure 4.1: Distribution and pseudo-absence maps for *J. crispa* subsp. *mariana* (top left), *J. sessiliflora* subsp. *appressifolia* (top right), *J. sessiliflora* subsp. *sessiliflora* (bottom left) and *J. crispa* subsp. *tomentosa* (bottom right).

The models created for each taxon with the GLM, MAXENT, RF and GAM algorithms in the subset of climatic variables finally selected (Bio3, Bio7, Bio8, Bio15, Bio18, and Bio19) reflect that the most important variable in the distribution is the annual temperature range, i.e. the difference between the maximum temperature of the warmest month and the minimum temperature of the coldest month (Table 4.2). However, in *J. sessiliflora* subsp. *appressifolia*, precipitation during the coldest quarter of the year is of similar importance.

In the EM with climatic and edaphic variables (Bio6, Bio12, Bio14, pH, % sand and apparent density), the pH of the soil at a depth of 100-200 cm and the percentage of sand are particularly important.

The SDM projections obtained enable visualization of the potential distribution area of each species, estimated from climatic variables (Figure 4.2) and climatic plus edaphic variables (Figure 4.3). In addition, the real presences are observed in Figure 4.2, after elimination of the points between which the distance was less than that established as the threshold (1 km or 5 km), thus confirming the goodness of fit of the models to the known distributions of the species. The potential distribution area in the Iberian Peninsula is represented on the maps by shading, where more intense shading indicates a greater probability of finding a specimen of the species of interest.

Table 4.2: Importance of the bioclimatic variables in the models created for *J. sessiliflora* subsp. *sessiliflora*, *J. crispa* subsp. *tomentosa*, *J. crispa* subsp. *mariana* and *J. sessiliflora* subsp. *appressifolia* using the GLM, MAXENT, RF and GAM algorithms.

		GLM	MAXENT	RF	GAM
sessiliflora	Bio 3	0.259	0.004	0.081	0.286
	Bio 7	0.706	0.867	0.771	0.821
	Bio 8	0.290	0.254	0.334	0.343
	Bio 15	0.645	0.041	0.148	0.652
	Bio 18	0.683	0.321	0.263	0.510
	Bio 19	0.515	0.241	0.188	0.493
tomentosa	Bio 3	0.220	0.033	0.075	0.286
	Bio 7	0.744	0.862	0.759	0.832
	Bio 8	0.299	0.254	0.334	0.343
	Bio 15	0.477	0.046	0.141	0.592
	Bio 18	0.639	0.351	0.239	0.499
	Bio 19	0.601	0.280	0.188	0.477
mariana	Bio 3	0.178	0.004	0.047	0.512
	Bio 7	0.866	0.806	0.702	0.772
	Bio 8	0.299	0.254	0.334	0.343
	Bio 15	0.683	0.024	0.165	0.737
	Bio 18	0.477	0.336	0.313	0.602
	Bio 19	0.440	0.309	0.194	0.512
appressifolia	Bio 3	0.151	0.004	0.030	0.625
	Bio 7	0.780	0.849	0.491	0.901
	Bio 8	0.299	0.254	0.334	0.343
	Bio 15	0.645	0.041	0.148	0.652
	Bio 18	0.643	0.321	0.263	0.510
	Bio 19	0.852	0.910	0.638	0.821

The potential area of distribution of *J. sessiliflora* subsp. *sessiliflora* covers a large part of the interior area of the northern half of the peninsula south of the Ebro river, while *J. crispa* subsp. *mariana* and *J. crispa* subsp. *tomentosa* share areas in the southwestern part of the peninsula. The potential area of distribution of *J. sessiliflora* subsp. *appressifolia* is slightly further away, in the region of Valencia and in the province of Teruel. The areas identified as optimal in the SDM with climatic and edaphic variables are more restricted; the presence points have been deleted to facilitate visualization of these optimal areas (Figure 4.3).

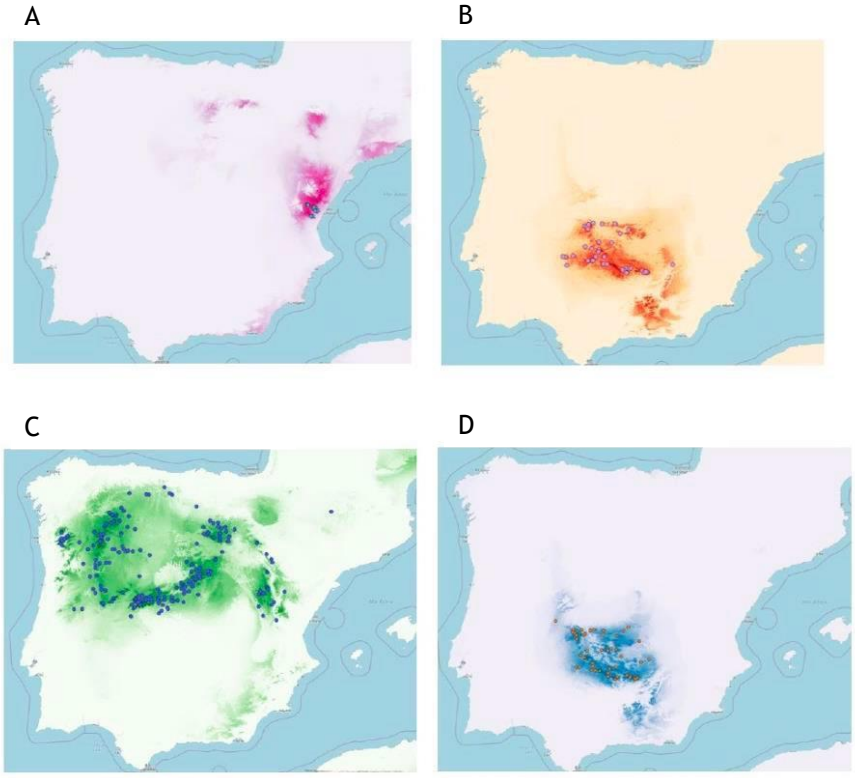

Figure 4.2: Potential distribution areas of (A) *J. sessiliflora* subsp. *appressifolia*, (B) *J. crispa* subsp. *mariana*, (C) *J. sessiliflora* subsp. *sessiliflora* and (D) *J. crispa* subsp. *tomentosa*, modelled using climatic variables.

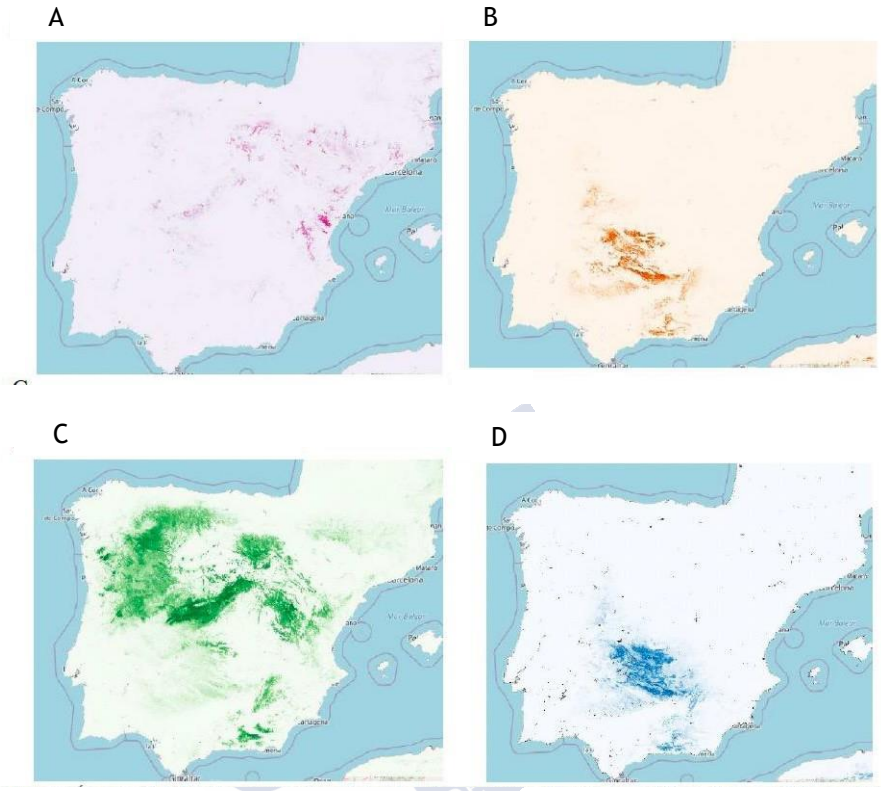

Figure 4.3: Potential distribution areas of (A) *J. sessiliflora* subsp. *appressifolia*, (B) *J. crispa* subsp. *mariana*, (C) *J. sessiliflora* subsp. *sessiliflora* and (D) *J. crispa* subsp. *tomentosa*, modelled using climatic and edaphic variables.

The ecological niche of the taxa is represented in the ecological space in the environmental PCAs (Figure 4.4). The first two axes explained 64.6% of the environmental (climatic) variance, with 34.7% and 29.9% explained by axis 1 and axis 2, respectively. The environmental PCA shows that the niche of *J. sessiliflora* subsp. *appressifolia* forms a subset of the multidimensional environmental space occupied by *J. sessiliflora* subsp. *sessiliflora.* However, the environmental conditions of *J. sessiliflora* subsp. *appressifolia* forms part of the environmental distribution of the tetraploid although there is no overlap in the geographic distributions or in the SDM projection (Figure 4.3). This observation may be explained by the marginal

position within the large environmental space occupied by *J. sessiliflora* subsp. *sessiliflora*.

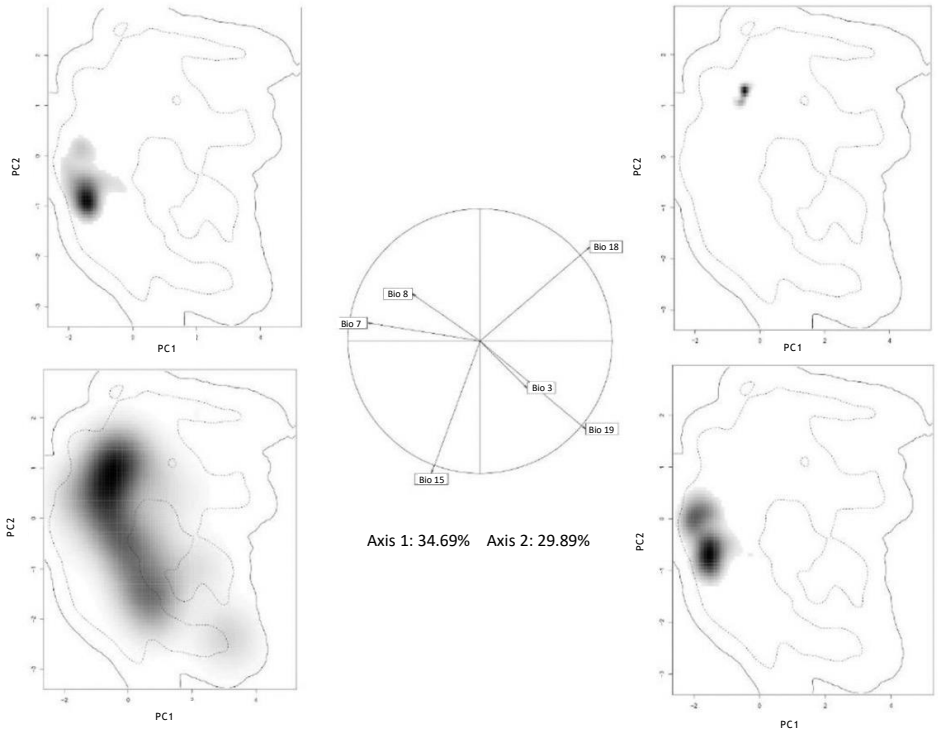

Figure 4.4: Environmental PCA of the species *J. crispa* subsp. *mariana* (top left), *J. sessiliflora* subsp. *appressifolia* (top right), *J. sessiliflora* subsp. *sessiliflora* (bottom left) and *J. crispa* subsp. *tomentosa* (bottom right).

The diploids *J. crispa* subsp. *tomentosa* and *J. crispa* subsp. *mariana* share a large part of their area of distribution. Both have similar environmental requirements for establishment. These results are correlated with the geographic space projected in the SDM, as confirmed by the results of similarity test performed for this pair of taxa (Figure 4.5). Nevertheless, *J. crispa* subsp. *mariana* would tend to require milder environments on average, i.e. slightly warmer and more humid than *J. crispa* subsp. *tomentosa*.

The similarity tests indicated no significant difference in the simulated niches between the observed overlap D between the tetraploid and any of the diploids (Table 4.3). Among the diploids, only the observed overlap D in environmental niches of *J. crispa* subsp. *mariana* and *J. crispa* subsp. *tomentosa* was significantly different (greater) than the simulated shifted values. Nevertheless, the test revealed markedly lower values (i.e. more similar niches) for all diploid pairs than for any pair of a diploid with the tetraploid. However, no evident overlap between either *J. crispa* subsp. *mariana* or *J. crispa* subsp. *tomentosa* and *J. sessiflora* subsp. appressifolia can be seen in Figure 4.4 (shaded areas). These relatively low values in the similarity test could indicate that some marginal overlap values exist and could be explained by other unrepresented axes. Relative similarity between all diploids is expected given their proximity in the environmental space, with all diploids occupying the warmer part of the space in an area positively influenced by variables Bio7 (temperature annual range) and Bio 8 (mean temperature of the wettest quarter).

Table 4.3: Significance values for similarity tests between pairs of taxa. Significant similarity in relation to a distribution of D values obtained from randomly assigned environmental values from the occupied areas is indicated with an asterisk.

	sessiliflora	*tomentosa*	*mariana*	*appressifolia*
sessiliflora	-	0.2079	0.3528	0.2901
tomentosa		-	0.0090 *	0.0967
mariana			-	0.0826
appressifolia				-

Although *J. crispa* subsp. *tomentosa* and *J. sessiliflora* subsp. *sessiliflora* are not geographically distantly separated in some areas, and there even seems to be a small contact zone, they have a low niche overlap. The similarity test indicates that the niche overlap is less than would be produced by chance (Table 4.3; Figure 4.5). A similar situation was found for *J. crispa* susp. *appressifolia* (Table 4.3).

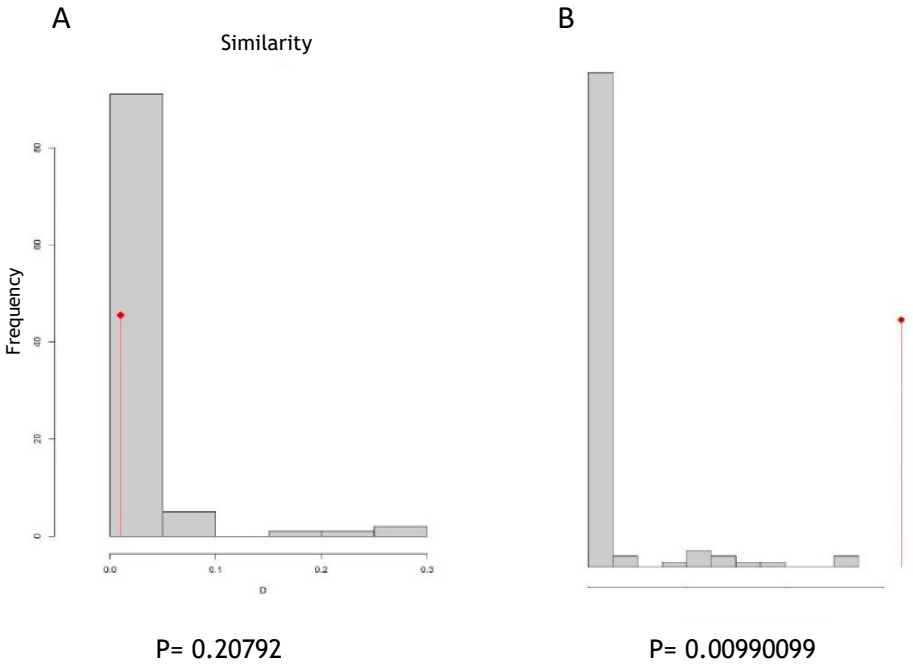

Figure 4.5: Test of similarity between two pairs of taxa: (A) *J. crispa* subsp. *tomentosa* (2x) - *J. sessiliflora* subsp. *sessiliflora* (4x), and (B) *J. crispa* subsp. *tomentosa* (2x) - *J.crispa* subsp. *mariana* (2x).

4. DISCUSSION

This study found strong evidence of niche shift in the polyploid complex of *Jasione sessiliflora*, with the tetraploid *J. sessiflora* subsp. *sessiliflora* showing a wide expansion in niche breadth beyond the narrow niches of its diploid relatives. Moreover, niche differentiation between the diploids is markedly lower, despite greater geographic distance (e.g. *J. sessifliflora* subsp. *appressifolia* and *J. crispa* subsp. *tomentosa*) or greater morphological divergence (e.g. *J. crispa* subsp. *tomentosa* and *J. crispa* subsp. *mariana*).

Within each taxon studied, the realized niche, expressed by the current distribution range, was very similar to the fundamental niche (Hutchinson, 1957), inferred by projected SDM (Figures 4.2 and 4.3). Moreover, the match between realized and fundamental niches was especially good when edaphic variables were included in the model (Figure 4.3). These results strongly suggest that current distributions of the studied taxa are related to physiological and ecological tolerances constrained by the geographical occurrence of suitable values of abiotic environmental factors (Guisan & Thuiller, 2005; López-Jurado *et al*. 2019), rather than being the result of dispersal opportunities/limitations or historical processes (Lobo *et al*., 2010; Glennon *et al.*, 2014). The results thus add confidence to the following discussion on the niche dynamics inferred from geographical occurrences.

Different hypotheses have been proposed to explain the effect that polyploidization has on the ecological niche, so that it can expand, move, remain static or contract (Parisod & Broennimann, 2016). Changes in environmental tolerances associated with polyploidization have been suggested as essential to promote geographical segregation and establishment of polyploid populations (Husband & Schemske, 2000; Ramsey 2011). By this means, polyploids could avoid the negative effects of frequency-selection mechanisms (Levin, 1975). Nevertheless, evidence reported in the scientific literature is conflicting and has been considered inconsistent (Castro *et al*., 2019). While different studies have identified different degrees of niche shift in diploid-polyploid systems (e.g. Thompson *et al*., 2014; Visger *et al*., 2016, Muñoz-Pajares *et al*., 2018, López-Jurado *et al.*, 2019), other studies have not observed the acquisition of tolerance to novel environmental conditions after polyploidization (e.g. Spoelhof *et al*., 2017; Castro et al., 2019; Castro et al., 2020). Glennon *et al*. (2014) specifically addressed the question by comparing 20 diploid-polyploid pairs and found that niche conservatism prevailed in most cases. Two studies used other polyploid complexes of the genus *Jasione* to examine niche evolution after polyploidization (Castro *et al.*, 2019, Castro *et al*., 2020). Although the aim of these studies was to obtain support for the niche shift hypothesis, the results suggested that other factors should be considered in explaining the differences in the distribution of these

diploid-tetraploid systems. Castro *et al*. (2019) found that the environmental niche of tetraploid *J. montana* was a subset of its diploid progenitor's niche, and no niche novelty evolved after polyploidization. Castro *et al*. (2020) focused on populations of *J. maritima* in NW Iberian Peninsula, where diploids are found in the northern-central Galician Atlantic coast and tetraploids in the southern Galician coast (as var. *maritima*) and on the northern coast of Portugal (as var. *sabularia*). Strong evidence of niche conservatism was inferred between the diploids and the tetraploid *J. maritima* var. *maritima*, while the southern populations of the tetraploid *J. maritima* var. *sabularia* showed environmental tolerance beyond the diploid niche. Nevertheless, niche differences restricted to one of the two tetraploid varieties are stronger indications of internal divergence within the tetraploid lineage than niche evolution associated with the process of polyploid establishment enabling diploid-tetraploid spatial segregation. Conversely to this previous evidence, the study case of the diploid-tetraploid complex of *J. sessiliflora* showed not only that niche change played an important role in shaping the realized distribution of the tetraploid, but also that the polyploid niche evolved much faster than the niche of their diploid relatives, even between those with greater morphological divergence. Faster niche evolution in polyploids than in related diploids was also found in a recent study of three polyploid groups (Baniaga *et al*., 2020).

More detailed analysis of niche evolution in the diploid-tetraploid *J. sessililfora*, along with the results of the niche similarity tests, as well as the environmental PCAs (PCAenv) of the diploids and of the tetraploids showed varying degrees of divergence regarding both the position in the environmental space and the breadth of the projected niches. The ecological niches of *J. crispa* subsp. *tomentosa* and *J. crispa* subsp. *mariana* were found to be very similar (Figure 4.5, similarity test) and essentially different from those of the tetraploid *Jasione sessiliflora*. The niche of *Jasione crispa* subsp. *mariana* tends to occur in locations with a lower annual temperature range. This tendency is manifested in the geographical space as a preference for areas in the Oretano-Marianic system with a more nuanced climate (Villuercas, Montes de Toledo, Sierra Morena), while *J. tomentosa* also

occupies areas of this mountainous system towards the edge of La Mancha climatic affinity (e.g. south of Toledo, in the region of Calatrava) with a tendency towards a slightly more continental climate. However, in general, the similarity in the ecological preferences of both diploids validates the general hypothesis of niche conservatism between sister and/or close species (Peterson *et al.*, 1999). These diploid taxa diverged at some point in the Pleistocene (see Chapter 3) and although they can be found in strict sympatry in many areas, they retain different morphological traits and diverging microecology. Indeed, they are sympatric but not syntopic, as in shared localities *J. crispa* subsp. *mariana* occupies crevices on north-facing ridges and cliffs, while *J. crispa* subsp. *tomentosa* preferentially occupies south-facing positions. Nevertheless, current barriers to gene flow are not completely efficient, as secondary contact and chloroplast capture were detected in several populations of *J. crispa* subsp. *mariana*. It is not known where the initial divergence from their common ancestor took place, but given the relative weakness of barriers to gene flow and the high degree of niche conservatism it probably occurred in refugial areas in different geographical locations. The ecological niche of *J. crispa* subsp. *appressifolia* appears in the environmental PCA somewhat displaced relative to that of the other two diploids. It is mainly due to the influence of lower values in precipitation in the coldest quarter of the year. The other diploids occur in the central SW Iberian Peninsula where rainfall relies on Atlantic depressions and western winds, with higher precipitation values concentrated in the coldest quarter. However, this influence is strongly attenuated in the Mediterranean mountains occupied by *J. sessiliflora* subsp. *appresifolia*. The niche position of this taxon in the environmental space is also influenced by higher values of precipitation in the warmest period of the year. This taxon will thus occupy ecological areas with slightly less seasonality than the southwestern diploids, which can be explained by the high rainfall that occurs in torrential form in the Mediterranean mountain systems in the late summer. Nevertheless, the niche is in the thermic range of the environmental space, not far from the other two diploids. The morphological features of this taxon are intermediate between the diploid *J. crispa* subsp. *tomentosa* and the tetraploid *J. sessiliflora* s.

str. Molecularly, it cannot be distinguished from the former with the plastid markers used in this work (Chapter 2). Morphological and molecular similarities, as well as the not very differentiated environmental niche, suggest that *J. crispa* subsp. *tomentosa* and *J. sessiliflora* subsp. *appressifolia* are sister taxa resulting after a vicariant event in a formerly more continuous population, fragmented precisely due to the relative niche conservatism after change in climatic conditions.

Overall, the niche of the three diploids, as explained by the PCAenv results, can be considered narrow and relatively marginal subsets of the large niche breath of the tetraploid *J. sessiliflora* subsp. *sessiliflo*ra. The latter displays niche preferences characterized by colder annual temperatures, less seasonality of precipitation and higher summer rainfall, and its realized range covers areas with characteristic submediterranean conditions. Nevertheless, *J. sessiliflora* subsp. *sessiliflora* has developed tolerances to a very wide range of environmental conditions, including temperate conditions beyond the Mediterranean climatic area (Chapter 2). This finding is consistent with the geographic breadth inferred by the SDM. The inclusion of edaphic variables in the SDM is more consistent with the known (realized) distribution of the species, which is adapted not only to siliceous crevices but to a sandy habitat. Interestingly, the banks of large rivers of the northern plateau, such as the Duero/Douro River, are recovered by the model as optimal habitat. These results are validated by some geographical presences of ecological significance (rivers facilitate the deposition of sand and other acidic materials even in basic environments), supporting the previously observed importance of including edaphic variables in the EM of plant species to generate SDMs (Figueiredo *et al.*, 2018), although these variables are rarely considered. However, the use of sandy habitats could be interpreted as a pre-adaptation prior to polyploidization, rather than a signature of niche shift, as the diploid *J. sessiliflora* subsp. *appressifoli*a can occur in both crevices and in sandy habitats derived from disintegrated sandstones. Focusing on climatic variables, the results of the study show that during or after the polyploidization process, *J. sessiliflora* subsp. *sessiliflora* has undergone a niche shift from warmer zones to colder,

more continental areas. The niche displacement of tetraploids relative to their diploid progenitors suggests a survival strategy to enable establishment of the new polyploid, avoiding low reproductive efficiency due to frequency selection effects of minority cytotype exclusion processes (Thompson & Lumaret, 1992). This expansive shift of the ecological niche is also reflected in the occupation of a geographic space inaccessible to diploids, although the distributions of diploids and tetraploids also seem to be mutually exclusive, even though the diploid niche is a marginal subset of the tetraploid niche. This relative marginality implies that tetraploid niche expansion occurred along with niche displacement, as reflected in the environmental PCA (Figure 4.4). However, since diploid niches are marginally nested within the tetraploid environmental tolerances, more contact zones could be expected. Thus, inter-cytotype competitive factors cannot be completely ruled out. However, interestingly, climatic SDM (Figure 4.2) do no predict significant contact zones.

Niche expansion has been considered an evolutionary response of increasing environmental tolerance in species under extreme conditions (Decanter *et al.*, 2020). Although polyploids arise within lower ploidy populations, i.e. under the same environmental conditions, temporal coincidence of polyploidization with a harshening of environmental conditions could cause a reduction in the diploid progenitors and appearance of novel vacant niches, triggering environmental adaptation, with auto or allopolyploid genomic rearrangements probably helping the species to take advantage of the new ecological opportunity. López-Jurado *et al.* (2019) found that tetraploid *Dianthus broteri* underwent a considerable expansion in environmental niche relative to their diploid relatives. These authors suggested that tetraploids would be characterized by large niche expansions when niche shift occurs and correlated this with the recurrent origins of the tetraploids. Therefore, tetraploid success in coping with new environments by adaptation may be related to increased genetic variability and subsequent adaptability caused by a number of independent origins (López-Jurado *et al.*, 2019). However, this does not seem to be the case with the tetraploid *J. sessiliflora*. Maternally inherited plastid molecular markers (Chapter 2) suggest that probably only one polyploidization event made a significant contribution

to the polyploid populations. Only four, little-differentiated haplotypes were found across all tetraploid populations, all diverging from a common ancestor, while no current diploid haplotypes (which may suggest recurrent formation) have been found in tetraploid populations

Overall, the niches of the diploid taxa of the studied complex, in addition to signatures of niche conservatism, may indicate the relict nature of these lineages. Compared to the tetraploid niche, all are narrow, or extremely narrow in the case of *J. sessiliflora* subps. *appressifolia*. Narrow niches have been related to reduced geographic distributions (Castro *et al.*, 2019). This explanation may apply to the narrow endemic *Jasione sessiliflora* subsp. *appresssifolia* but not to *J. crispa* subsp. *mariana* or *J. crispa* subsp. *tomentosa*. Both latter taxa are relatively widely distributed throughout most of the quartzite mountain ranges within or surrounding the Tagus and Guadiana basins. Relationships between narrow and broad niches in closely related species in plants and animals (Knouft *et al.*, 2006; Vamosi *et al.*, 2014) have been considered an indication of environmental specialization for the narrower niche. The same interpretation has been suggested for poplyploid complexes with a pattern of broad and narrow niche different cytotypes (Parisod & Broennimann, 2016). This would imply that the species and cytotypes with broader environmental tolerances would fill most of the potential niche, forcing those with narrow niches to specialize in a subset of the existing environmental conditions in which their optimal performance would outcompete other cytotypes. However, the results of this study do not suggest environmental assortment conditioned by specialization between cytotypes in the *J. sessiliflora* complex. Instead, the narrowness and ecologically closeness of niches in the environmental space of the three diploid taxa occupying mild ecological conditions suggest evolutionary inertia and a relict condition. Thus, the diploids would have remained in warm and subhumid enclaves under Mediterranean climatic conditions. *Jasione crispa* subsp. *mariana* and *J. crispa* subsp. *tomentosa* would benefit from the maritime Atlantic influence that penetrates deeply in inner south-central Iberian Peninsula. This large environmental area has been biogeographically defined as the Luso-Extremadurense subprovince (Rivas-Martínez *et al.*, 2017, Chapter 2) and harbours important populations of other species considered relict from preglacial

periods in the same mountain ranges occupied by the diploids, such as *Prunus lusitanica* L. (Calleja *et al.*, 2009). Indeed, the Luso-Extremadurense quartzite mountain ranges are rich in relict and endemic species, with *Jasione crispa* subsp. *mariana* and *J. crispa* subsp. *tomentosa* considered among the most characteristic taxa of this biogeographical area (Pérez-Chiscano, 1987). The remaining diploid, *J. sessiliflora* subsp. *appressifolia*, occurs in sandstone mountain ranges never further than 40 km from the Mediterranean Sea. Although this area of the Valencia-Castellón coast is characterized by dry conditions in summer, the modest altitudes of the mountain ranges (typically below 1.100 m.a.s.l.) are sufficient to allow retention of clouds formed over the Mediterranean Sea and cause frequent summer orographic fog formation that compensates for the general aridity, even enabling the occurrence of some broad-leaved tree formations (Vilches *et al.*, 2013). Indeed, the amounts of water collected from orographic fog during summer on the mountains close to the Valencian coast can reach more than 4.5 l/m^2/day, comparable to values found in relict fog-habitats in the Canary Islands (Estrela *et al.*, 2008). Warmer and subhumid conditions characterize narrow endemic rich communities in these mountains (Merlé Farinós & Ferriol Molina, 2008; Vilches *et al.*, 2013) supporting the relict condition of the environmental niche of the diploid *J. sessiliflora* subsp. *appressifolia*. In summary, the study of environmental constraints of the diploids suggests that niche conservatism has favoured the retention of relict environmental characteristics, leading to the current pattern of vicariance in the central SW Iberian Peninsula and the coastal Valencian mountains. The evidence of niche conservatism between the studied diploids underpins the suggested hypothesis that niche conservatism is one of the main drivers of current east-west Mediterranean disjunct biogeographic pattern that characterizes the genus *Jasione* (Chapter 3).

The tetraploid *Jasione sessiliflora* subps. *sessiliflora* underwent a striking niche enlargement that enabled the polyploid lineage to expand to colder regions and occupy a wide area with conditions ranging from sub-Atlantic climate in temperate NW Iberian Peninsula to continental climatic tendencies in the Iberian System Mountain range. Conversely to findings in other polyploid complexes in *Jasione* (Castro *et al.*, 2019,

2020), the case of *J. sessiliflora* complex indicates rapid niche shift and expansion in a polyploid. These results stress the utility of comparing niche dynamics of different close diploid relatives in order to properly characterize the magnitude of polyploid niche evolution, within a phylogenetic framework including more than one diploid lineage. This approach and the results are similar to the findings of the recent study of Baniaga *et al.* (2020). The breaking-up of phylogenetic inertia and niche conservatism after polyploidization in *J. sessiliflora* through cold-adaptation may have made an important contribution to establishing the genomic and phenotypic bases that enabled higher ploidy levels of the wider polyploid group of *J. crispa* to occupy colder high mountain environments and expand latitudinally beyond the Iberian Peninsula, as hexaploid populations of *J. crispa* reach the French Central massif (Küpfer, 1981; M. Serrano, own data). Niche expansion in the tetraploid may have been an immediate effect of the novel genomic make-up at the appearance of the polyploid (either auto or allopolyploid) or a result of later divergence processes involving adaptation under environmental pressures and the need to overcome the negative effects of minority cytotype exclusion. The tetraploid lineage most probably originated in the second half of the Pleistocene (Chapters 2 and 3). The climatic turmoil of Pleistocene glaciations repeatedly harshened environmental conditions at a fast rate in the Iberian Peninsula (Rull, 2020), providing vacant cold niches in the areas occupied by the diploids. It would be interesting to project the environmental niches to glacial period conditions in a future study. The response could help to explain the beginning of the evolutionary history of the polyploid lineage and the niche differentiation that has enabled the shift in environmental space relative to the relict diploids. It would also be interesting to conduct a thorough review of cases to ascertain whether niche shift in polyploid is more likely when the diploid relatives are characterized by slow niche dynamics.

Finally, differences in environmental envelopes support the recognition of functional biodiversity units and taxonomic reorganization of the complex, as suggested in the Species Delimitation analyses in Chapter 2. In the diploid-tetraploid system under study there are scarce differences in niche occupancy and geographical space, particularly

when edaphic variables are included in the SDMs. Consequently, there are no significant absences of any taxa of the complex in geographic areas with optimal prediction. This excludes the need to use competitive abilities to explain the pattern (Laport *et al*., 2013). Each cytotype probably has a unique performance in the different environments and should be considered distinct units of biodiversity (Laport & Ng, 2017). However, internal systematic organization within the diploids should be established using other lines of evidence as niche conservatism may blur the recognition of significant units of biodiversity from environmental information alone. Nevertheless, the relict nature and the narrow optimal conditions of the environmental niches of the diploids suggest the need for detailed study of the internal variability within the diploid group and the predicted responses under the potential threat of global climate change.

5. CONCLUSIONS

Polyploid niche shift in the tetraploid *J. sessiliflora* subsp. *Sessiliflora* is strongly indicated, with a remarkable expansion to colder and more continental submediterranean conditions than the thermic subhumid Mediterranean environments of the diploid relatives. Examination of three closely related diploid taxa provided a phylogenetic framework for comparison to confidently determine the magnitude and dynamics of the polyploid niche shift. Conversely, niche conservatism, niche narrowness and relict characteristics were identified in the environmental envelope of the diploid taxa. Narrow niches are not necessarily related to narrow distribution ranges, as two of the diploids are relatively widespread in the mountain ranges of the southern Iberian central plateau. Species Distribution Models including climatic and edaphic variables better define the fundamental niche of plant species, at least in calcifuge lineages such as the studied polyploid complex. Overall, this research contributes to accumulating evidence supporting the hypothesis that niche shift is an important mechanism in polyploids, at least when no differential reproductive traits (i.e. perenniality, autogamy) have developed to cope with minority cytotype exclusion processes. In summary, the findings suggest that niche shift

is significantly faster in polyploids than in closely related diploids, at least when the latter show relict characteristics.

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CHAPTER 5.
The edaphic factor in plant speciation: The case of the calcifugue complex *Jasione* gr. *crispa* (Pourr.) Samp. (Campanulaceae)

ABSTRACT

Soil variability is an important factor promoting microevolution in plants that can lead to the formation of new species in the long term. *Jasione crispa* (Campanulaceae) is a group of cespitose, mostly orophytes, practically restricted to acid soils. The identification of an endangered and isolated population of *Jasione* in the calcareous massif of Mont Caro (Tarragona, Spain) allows the study of new environmental pressures in an acidophile lineage and the initial steps of the speciation path. This population has been included in the variability *J. sessiliflora*, a tetraploid species. The ploidy level of the population is determined as hexaploid, evincing closer relationships with *Jasione crispa* subsp *crispa*. Reduced gene flow by geographic isolation is identified by cloning procedures of the ITS marker from the nrDNA, in a phylogenetic and phylogeographic framework including populations from all mountain ranges occupied by the taxon. Gene flow interruption and exposure to new environmental constraints are main promoters of adaptive speciation. To assess whether Mont Caro population is undergoing a process of ecological speciation, plants from Mont Caro and from two populations from siliceous mountain areas (Central System and Pyrenees) and different phylogenetic proximity to Mont Caro were cultivated on three soil types. The non-destructive reflectance colour measurements, using the chromatic parameters: a* (associated with changes in redness-greenness) and b* (associated with changes in yellowness-blueness) from the CIELAB colour system, proved relevant for evaluating these different behaviours and, thereby

are a good proxy variable for plant speciation studies in this model group. Biomass and Al, Ca, Fe and Mg concentrations were estimated at harvest. Mont Caro population showed better responses to basic soils (i.e., higher biomass production, low Mg concentration, equilibrium in root and leaf Ca concentrations) than the other two populations although worse performance on acidic soils and poor aluminium management at root level, what seems to be a negative evolutionary trade-off. These results suggest the occurrence of an on-ongoing ecological speciation process in *J. crispa* from Mont Caro, which could contribute to the description of this population as a new taxon.

1. INTRODUCTION

The importance of soils as promoters of plant speciation has been increasingly recognized in recent decades (Rajakaruna, 2004), although most of the studies have focused on soils with extreme characteristics and very restrictive for plants, such as hypersaline or those developed on streamers or plasters. Evolutionary divergence linked to environmental variation in soil pH values have boosted recent diversification of the plant endemic component in the Iberian Peninsula, albeit its importance has been greater in the eudicots than in the monocots (Buira *et al.*, 2020). Soils with extreme conditions and low extension across earth surface (e.g., dolomitic soils, gypsum soils, serpentines, etc.) work as ecological islands that cause reproductive isolation while promoting the appearance of adaptive mechanisms by natural selection (Rajakaruna, 2004). Less dramatic soil differences, as those between calcareous and siliceous soils also impose unequal environmental constraints that can promote different physiological strategies and divergent adaptations. Plants, as all organisms, must face with fluctuating environmental conditions over time and geography, with those following generalist strategies (e.g., soil indifferent plant species) needing to develop plastic genotypes that maximized fitness across variable environments (Lande, 2014). Conversely, specialist lineages adapt themselves to particular subsets of the environmental variability in order to cope better with the associated evolutive constraints. Plants groups with strong acidophilic adaptation like those

only thriving on soils of siliceous nature are hampered to successfully develop on calcareous habitats since under competition generalist and basophile species will be favoured. Therefore, dispersal to limestone areas will rarely cause the establishment of a population. However, successful establishment can trigger evolutionary shifts of edaphic association (Dillenberger & Kadereit, 2013). Environmental windows of opportunity could enhance the chances of endurance in sub-optimal environments, for instance when variation in other environmental factors could have extirpated competitive elements of the native community and closer to the optimal range of the newcomer. One example can be the effects of Quaternary glaciations causing dramatic turnover in plant assemblages and the geographic expansion in lowlands of temperature conditions suitable for cold adapted mountain plants. If the newcomers could endure for a long enough period of time in the new habitat, they will become a lineage under different selective edaphic pressures compared to the siliceous source population. When new selective pressures are sustained in time can drive speciation by divergent selection (Schlutter, 2009; Nosil, 2012). Diminished or absent gene flow form the original populations prevent genetic backward, what would have deleterious effects blurring the new adapted genetic landscape by loss of the acquired advantages. Although edaphic evolutionary divergence in mountain areas with heterogeneous calcareous and siliceous substrates has been proposed, the role of geographic interruption to gene flow seems of paramount importance. In this sense, phylogenetic studies in Alpine calcicole and calcifuge species of genus Minuartia, which previously have been an example of regional edaphic adaptation to limestone soils and evolutionary divergence, have conversely shown that calcicolous adaptation was not acquired in situ, but respond to a biogeographic history involving dispersal from distant calcareous areas in the Bakan Peninsula and pre-adapted lineages to calcareous conditions (Moore & Kadereit, 2013).

The group of taxa that make up the *Jasione* gr. *crispa* (Campanulaceae) occurs in southwestern Europe with environmental preference for mountain and alpine habitats This lineage is characterized by markedly siliceous edaphic preferences and strong calcifuge condition. However, there are a few populations capable of occupying

media of a basic nature with an important presence of calcium and magnesium. These have led, up to now, to the recognition of two endemic taxa of reduced area (*Jasione crispa* subsp. *varduliensis* and *Jasione crispa* subsp. *segurensis*), which suggests that the interaction with soils derived from calcareous or dolomitic substrates, normally negative and restrictive for the individuals of the *J. crispa* group, can favour adaptive processes in a situation of evolutionary isolation determined by edaphic factors. Nevertheless, none of these populations belong to *Jasione crispa* s. str. and polyploid evolution seems to have played a major role in the expansion to new edaphic niches in these taxa (M. Serrano, own data). In the context of the *J. crispa* group, there is a population that occurs on limestone substrate in the South of Tarragona, on the mid elevation (c. 1,450 m.o.s.l) summit of Mont Caro, a peak in the northeastern extreme of the Iberian System Mountain range. This population is currently being investigated, given that its scarcity in individuals (18 specimens in total) and the ability to grow on calcareous substrates, could suggest that it may be an endemism not yet described. For that reason, the basophilic population of *Jasione* gr. *crispa* from Mont Caro has been legally protected by the Catalonian regional government (Saéz *et al.*, 2010). Due to this, the optimal in vitro propagation protocol for obtaining plants has been established (M. Serrano, own data). Therefore, the hypothesis that this population could represent the first steps of an independent evolutionary path within the group needs to be formally evaluated. The population from Mont Caro has been assigned to *Jasione sessiflora* (Saéz *et al.*, 2010), a Iberian tretraploid endemic species of the *J. crispa* s.l.. Although it is mostly distributed in the NW quarter of the Iberian Peninsula, its range also encompasses siliceous outcrops in the Iberian System Mountain range, with relatively close population in the provinces of Teruel and Castelló. Despite that, morphological features of the Mont Caro individuals (e.g., leaves with only apical hydathodes or linear-lanceolate calyx teeth in Mont Caro plants instead of apical and several marginal leaf hydathodes or subulate calyx teeth in *J. sessiliflora*) would tell apart this population from *J. sessiliflora* and place it closer to the hexaplois/ octoploid complex of *Jasione crispa* subsp. *crispa*. This latter taxon has its closer population more than 200 km far, either in the Montseny mountain range, between Barcelona and Girona provinces, or

in the Moncayo mountain range, between Soria and Zaragoza provinces. Mont Caro elevation fits better with the average elevation range of *J. sessiliflora* than with the one of *J. crispa* sub. *crispa* and alpine or orophytic taxon from the highest mountains of central and northern Iberian Peninsula (Sales & Hedge, 2001). Ploidy level first, and phylogeographic affinities subsequently, need to be determined to ascertain the evolutionary affinities of the population. Geographic structure among populations and evidence of geographically related genetic isolation or, at least, scarcity of secondary contacts, affecting the Mont Caro populations would be favorable scenarios to the speciation process (Barraclough, 2019). The putative isolation of a population can be assessed by observing populational variation of molecular marker in phylogeography framework (Avise, 2000). Nuclear ribosomal DNA Internal Transcribed Spacer (ITS) can be used for this purpose since the ITS is an appropriate marker for screening population variability in different clades of genus *Jasione* (M. Serrano, own data). This gene region has been widely used in phylogenetic and populational studies in plants especially due to the relatively high evolution rates, amplification ease, and its characteristic genomic process of concerted evolution that makes it frequently works as a single-copy gene (Álvarez & Wendel, 2005; Kaveh *et al.*, 2019). Concerted evolution homogenizes the different nrDNA transcriptional copies existing within the genome by different genomic mechanisms (Dover, 1994; Li, 1997). Nevertheless, many studies have shown that complete concerted evolution is far from being universal (Roselló *et al.*, 2007; Holmes *et al.*, 2014; Morales-Briones & Tank, 2019). The existence of divergent intragenomic ITS copies, called ribotypes, has been interpreted as the intragenomic co-existence of paralogues, some of these could evolve without selective constraints what allows the accumulation of polymorphisms (Rosselló *et al.*, 2007). Either if the origin of the ribotypes within a set of gene copies found in a sample of individuals from different populations is paralogic or orthologic, they can be used as a proxy to the genetic (ribotypic) variability within and among the populations and to assess phylogenetic relationships between ribotypes geographic affinities among populations. In this work, the intrapopulation homogeneity is interpreted as the result of both concerted evolution and genetic isolation from other population

with different pool of ribotypes. Reduced effective population sizes would accelerate the intrapopulation impact of concerted evolution so founder effects or population decline could be related with low copy variability. Intragenomic ITS variability can be detected by direct sequencing by analyzing multiple peak patterns in electropherograms. Nevertheless, the occurrence of indels among copies can severely difficult the identification of the different copies. Moreover, copies with relative lower frequency can easily remain unnoticed leading to variability underestimation. Therefore, only after cloning procedures can the ribotypic population be confidently characterized (Rosselló *et al.*, 2007).

Although long-term geographic structure is a speciation factor, ecological divergence strength the effects of reduced or absent gene flow, accelerating the process of divergence (Barraclough, 2019). The aim of this work is to combine phylogeographic information with experimental measurements that could inform of quantifiable effects of novel edaphic selective pressures in a plant population from a calcifuge lineage. Thus, soil: plant relationships and plant performance under different conditions were analysed. The objective is to use the Mont Caro population as a model system to unveil signatures of initial steps of adaptation after edaphic niche shift. Alternatively, it could be that a mere case of temporary survival of a marginal population in sub-optimal habitat conditions was observed. In the former case, they are expectable soil-associated differential performances during the experiment in phenotypic expression and in functional traits assessed after the experiment, as well as in the management of critical soil elements, as a proxy of differential abilities to regulate cellular concentrations of limiting elements for calcifuge species. An informed phylogenetic context for the experiment to account for phylogenetic charge and to rule out the influence of pre-adaptive mechanisms. Whether signatures of geographic isolation and selective pressures can be identified, the Mont Caro population would be a case of in course edaphic speciation, probably deserving the current legal protection and supporting its formal consideration as an evolutionary significant entity by taxonomic recognition.

2. MATERIAL AND METHODS

2.1. Plant material

This work uses two sources of plant material from Mont Caro population, encompassing material from four individuals in total. It was the know population, although thorough sampling works in the area have elevated to 18 exemplars the number of known effectives. Fresh sample of two individuals were collected, preserved in cool conditions and within two days prepared for their use in *in vitro* micropropagation. Successful micropropagation protocols were developed (M. Serrano, own data), allowing the obtention of living plants. Cloned plants from these two lines were acclimated to outdoor conditions and cultivated on ground, with successful establishments and root developments. These two cloned lines were the source material for chromosome counting and plant: soil experiments. Leave material sampled in field and immediately dried in silica gel from two additional individuals were used in the genetic analyses.

2.2. Chromosome counting

Root tips measuring c. 5 mm were excised from *in vitro* plants cultivated more than two months in ground pot. All plants were acclimated to outdoor conditions and were directly moved from temperatures around 15ºC or lower to the laboratory to conduct immediate root excision, always at first hours in the morning (before 9:00 a.m.) to avoid high laboratory room temperatures. Root tips were washed in distillate water a pre-treated in 2 MM 8-hydroxyquinoleine for 3 hours, followed by a rinsing step in distillate water and then a fixation step in freshly prepared 3:1 96% ethanol - 1 glacial acetic acid solution for 24 hours. Samples were thoroughly rinsed in 70% ethanol to remove glacial acetic acid, since it has been described to interfere with later chromosome staining (Norman *et al.*, 2012). Samples were either stored in 70% ethanol at 4ºC for further use or directly used in the following steps. Root tips were rinsed in distilled water for 5 minutes, followed by an additional 5 minutes rinsing step in fresh

distilled water. Samples were hydrolised by plunging the root tips in and Eppendorf tube with HCL 1 N for 6-9 minutes in a water bath at 60ºC. The tubes were pre-heated to bath temperature before root tip plunging. Two rinsing steps 5 minutes each in distillate water followed to eliminate all possible HCL remains. For mitotic analysis, root tips were stained in Schiff's reagent for 12 hours in dark conditions. Stained root apices were excised under binocular magnifying glass, and immediately squashed on a slide. Cells were observed in a Leica TCS-SP2 confocal laser microscope (LEICA Microsystems Heidelberg GmbH, Mannheim, Germany) under a HCX PL APO CS 63.0x1.40 OIL objective, 580nm-725nm emission band and excitation wavelength of 561 nm with DPS (561 nm) laser diode.

2.3. Phylogenetic and phylogeographic relationships of the Mont Caro population

Plant material from Mont Caro and representative material from hexaploid *Jasione crispa* subsp. *crispa* populations covering its whole distribution range were sampled to assess nr DNA ITS variation (Table 5.1). Total genomic DNA was mainly extracted from field sampled and immediately silica gel dried leaves. In few cases, herbarium specimens were used for DNA extraction. DNA extraction was performed using either a modified cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1990) or a commercial kit Nucleo Spin Plant II (Macherey-Nagel). In both cases 0.6 ul of 2-Mercaptoethanol was added during the first liquid step. Foliar tissue was homogenised either with manual grinder or using Mikro – dismembrator (Sartorius). DNA concentration and quality was assessed with Nanodrop 2000C (Thermo Scientific).

The complete ITS region (ITS-1, 5.8S gene, and ITS2) was amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). PCR reactions were carried out in 25 ul with PuReTaq Ready-To-Go PCR BEADS (GE Healthcare) and 80-150 ng of genomic DNA. Thermocycler was programmed for amplification of the ITS region with an initial denaturation step of 95ºC for 2 min, then followed by 31

cycles of 1 min at 94ºC, 1 min at 46-48ºC and 2 min 10 s at 72ºC, with a final extension step of 8 min at 72ºC. PCR products were firstly assessed on 1.5 % agarose electrophoresis gels stained with the nucleic acid staining solution RedSafe (iNtRON biotechnology) and purified with E.S.N.A. Cycle Pure Kit. (Omega).

PCR products from between two and four individuals from each population were used for cloning procedures, except in some cases when the source material was an herbarium specimen and only one individual was used. PCR amplification products were ligated into the pCR2.1-TOPO vector and cloned according to instructions of the TOPO-TA cloning kit (Invitrogen, Carlsbad, Germany). Plasmid DNA from 10 separated competent cells colonies from each original PCR product was isolated using the S.N.A.P MiniPrep kit (Invitrogen, Carlsbad, Germany). DNA Sanger sequencing was carried out in at least one direct PCR product from almost all populations and in the then isolated DNAs from each population, with an 8-capillary 3500 analyzer (Applied Biosystems) by the DNA sequencing service of the University of Santiago de Compostela.

Sequences were initially aligned in BioEdit using the MUSCLE algorithm (Edgar, 2004), although manual editing performed equally well. Pseudogenes chimeras and recombinant sequences were exclude from the analysis. They were easily identified by comparison with those sequences obtained by direct PCR sequencing. GC content, extreme divergence, within single sequence mix of ribotypes and rarity where used criteria. Only those combinations of polymorphism occurring in at least three cloned sequences, or two cloned sequences plus one direct PCR product, even in the form of double peak, were considered true ribotypes and included in the analyses.

2.4. Phylogenetic and phylogeographic analyses

Phylogenetic reconstruction was conducted by a median-jonining network in PopART v.1.7 (Population Analysis with Reticulate Trees) considering the six main Iberian Mountain ranges occupied by *Jasione crispa* subsp. *crispa*, to simultaneously represent phylogenetic

relationships, ribotypic frequencies and geographic distribution. The considered six main mountain ranges are the Cantabrian-Leonese Mountain range, the Central System, the Moncayo in the Iberian System, the Pyrennes, the Montseny Mountain Range and Mont Caro.

DnaSP v6.1 (Rozas *et al.*, 2017) was used to calculate Haplotypic diveristies (Hd), interpreted as ribotypic diversity, and Pi, nucleotide diversity. An Analisis of Molecular Variance (AMOVA) was performed to estimate the relative contribution to genetic variances of different sets of samples (Excoffier *et al.*, 1992). The AMOVA was performed in R version 4.1.0 (R Core Team, 2021) with the packages apex, adegenet, pegas, mmod, poppr, and ade4. The analysis was set to consider differences between regions, that is, the six main mountain ranges, between populations within mountain ranges and within populations, following a stratified approach. Randomization Monte-Carlo tests with nreps = 1000 were performed in the R package ade4 to assess the significance of the AMOVA results revealed for the different levels of strata (Grünwald & Hoheseil, 2006).

Pairwise genetic differentiation coefficients (GammaSt) were calculated in DnaSP v6.1 to estimate the relative importance of gene flow among the six Mountain ranges. Gene flow was calculated as Nm by the equation Nm = (1/GammaSt−1)/2 (Zhu *et al.*, 2019).

The Nst nucleotide-based statistics (Lynch &Crease, 1990) and the net number of nucleotide substitution per site (Da) were calculated in DnaSP v6.1 between every population and the remaining set of populations, to estimate the frequency of gene flow affecting each population.

2.5. Soil: plant experiments

Jasione crispa subsp. *crispa* plants from two Mountain ranges, Pyrenees and Central System, were micropropagated *in vitro* and outdoor acclimated simultaneously with the Mont Caro plants (M. Serrano, own data), in order to have completely comparable individuals.

Pyrenees and Central System are representative of siliceous populations and Mont Caro of the new calcicole population. A total of 126 plants were used, 42 individuals representing each Mountain Range, were cultivated for five months on three substrates with different chemical characteristics. The three substrates used were, (1) a soil of basic nature from the limestone outcrops of the Serra do Courel (Lugo), here called "Courel", (2) a soil formed on a granite substrate from a natural environment in Santiago de Compostela, here called "Pedroso", and (3) a commercial COMPO SANA® substrate, composed of peat, perlite, an amorphous silicate fertilizer enriched with phosphorus, lime and some nutrient elements, to which an extra calcium and magnesium amount was added in the experiment, here called "Composana".

Natural soils from the original population to avoid adaptation and performances related to factors other than soil acid/basic chemical nature, like soil biota or compounds of organic origin from the surrounding plant communities.

Plant phenotypical performance was measured as segmented (root, stem and leaves) final biomass and colour changes measured on leaves of living plants with a colorimetric technique adapted for plants with small leaves developed using *Jasione* as model group (Sanmartín *et al.*, 2020). Soil analyses consisted of measuring pH in water, organic matter, total elements, exchangeable cations and effective CEC. In plants, the total elements in root, stem and leaves were measured, fractions that were separated after determining the total biomass per surviving individual. All measurements were performed in triplicate.

Soils were air dried and passed through a 2-mm sieve before analysis. Soil pH was determined in water and 1M KCl suspensions with 1:2.5 sample: solution ratios. Soil total organic matter was determined by weight loss on ignition of dried ground soil at 450 °C. Total elements from soils and plants were extracted after digestion of dried ground sample with aqua regia (HCl and HNO3 in 3:1 ratio) and the elements were analyzed in the extracts by Inductively Coupled Plasma Spectrometry, ICP-MS (Varian 820-MS), equipped with

collision reaction interface (CRI) technology to reduce polyatomic interferences. In soils, exchangeable Al was determined after extraction with 2 N KCl (Bertsch & Bloom, 1996). Cation exchange capacity (CEC) and exchangeable bases were determined after saturation of the soil with 1 N ammonium acetate and washing with 1 N KCl (Pansu & Gautheyrou, 2006). Exchangeable Ca, Mg, Na and K were measured in the ammonium acetate extracts by flame atomic absorption/emission spectrophotometry (Varian FS220).

Contact-type colour measurements were made on living leaves using a portable spectrophotometer (Konica Minolta CM-700d) equipped with CMS100w (SpectraMagicTM NX) software. The measurements were taken with the specular component included (SCI) mode using a CIE standard daylight illuminant D65, a small area view (SAV) of 3 mm and an observer angle of 2°. A total of 3 measurements per sample were made following Sanmartín *et al.* (2020). Colour measurements were analysed using the CIELAB colour system (CIE S014-4/E: 2007) which represents each colour by means of three scalar parameters or Cartesian coordinates: L*, lightness, which varies from 0 (absolute black) to 100 (absolute white); a*, associated with changes in redness-greenness (positive a* is red and negative a* is green); and b*, associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue).

3. RESULTS

3.1. Chromosome counting

The number of chromosomes (2n=36) observed in the mitotic metaphases of root tips from plants of the Mont Caro population consistently assign these plants to the hexaploidy level (Figure 5.1).

These results definitively separate this population from *Jasione sessiliflora*, an exclusively tetraploid species. Ploidy level matches information from morphological traits indicating that the Mont Caro population more probably belong to the *Jasione crispa* subsp. *crispa*

group of populations (M. Serrano, own data). Under this assumption, following phylogenetic and phylogeographic studies are conducted with the remaining hexaploidy populations of this taxon.

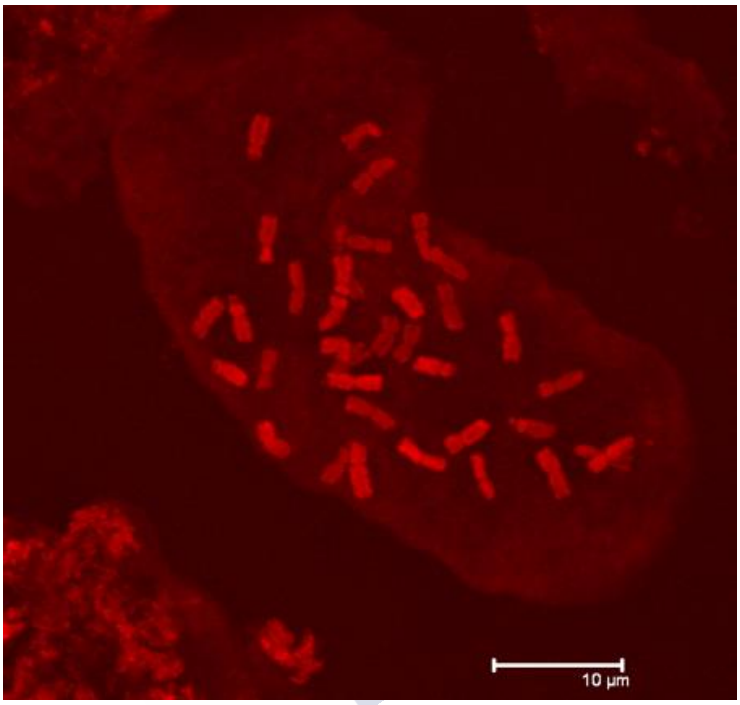


Figure 5.1. Mitotic metaphase of *Jasione crispa* from Mont Caro with 2n=36 chromosomes.

3.2. Genetic diversity and structure, phylogeographic relationships and comparative gene flow

Total length of the aligned ITS region was 710 bp with 27 variable and parsimony informative sites and two indels After discarding recombinant chimeras, pseudogenes or unsuccessful/dirty sequencing a total of 228 sequences were included in the analysis, with 11 different ribotypes confidently identified. Total Ribotype diversity (Rd) was 0.802 and nucleotide diversity (Pi) was 0.015. Regarding Mountain range genetic diversity, Rd ranged from 0.000 to 0.671, and nucleotide diversity ranged from 0.000 to 0.00898 (Table 5.1). The Cantabrian-

Leonese has the highest genetic diversity, while there is no genetic diversity in the three ranges where only one haplotype was detected, that is Montseny, Moncayo and Mont Caro.

Table 5.1. Mountain ranges genetic diversity and ribotype distribution.

Mountain range	Rd	Pi	Ribotypes
Cantabrian-Leonese	0.671	0.00898	R1, R2, R4, R6, R10, R11
Pyrenees	0.639	0.00355	R2, R3, R5, R6
Central System	0.520	0.00530	R2, R6, R7, R8, R9, R10
Moncayo	0.000	0.000	R2
Montseny	0.000	0.000	R2
Mont Caro	0.000	0.000	R7

The Analysis of molecular variance (AMOVA) stratified by Mountain ranges and populations identified relatively high differentiation level when all populations are considered (Phi = 0.67214), with somewhat lower values of differentiation for populations within Mountain ranges (Phi = 0.55227) and low differentiation level comparing regions. The main part of the genetic variance is explained by variations between populations within the mountain ranges (40.4 %), followed by variations within the populations (32.8 %), with the lesser part of the variance is explained by variations between mountain ranges (Table 5.2).

Table 5.2. AMOVA based on ITS sequences of *Jasione crispa* subsp. *crispa*

	Σ	%	Phi	Significance
Variations between Mountain ranges	0.2363637	26.8	0.2677348	0.001
Variations between populations within Mountain ranges	0.3570251	40.4	0.5522737	0.001
Variations within populations	0.2894389	32.8	0.6721456	0.001
Total	0.8828278	100	NA	NA

Randomization tests revealed that the values of variance observed for the three levels of analysis were significantly different than random distributions from the data matrices, meaning that there is significant population structure for the three levels of stratification. (Figure 5.2 and Table 5.2). Therefore, there is clear evidence that these groups are significantly differentiated at the three stratification levels, although the variation between regions is comparatively the less important.

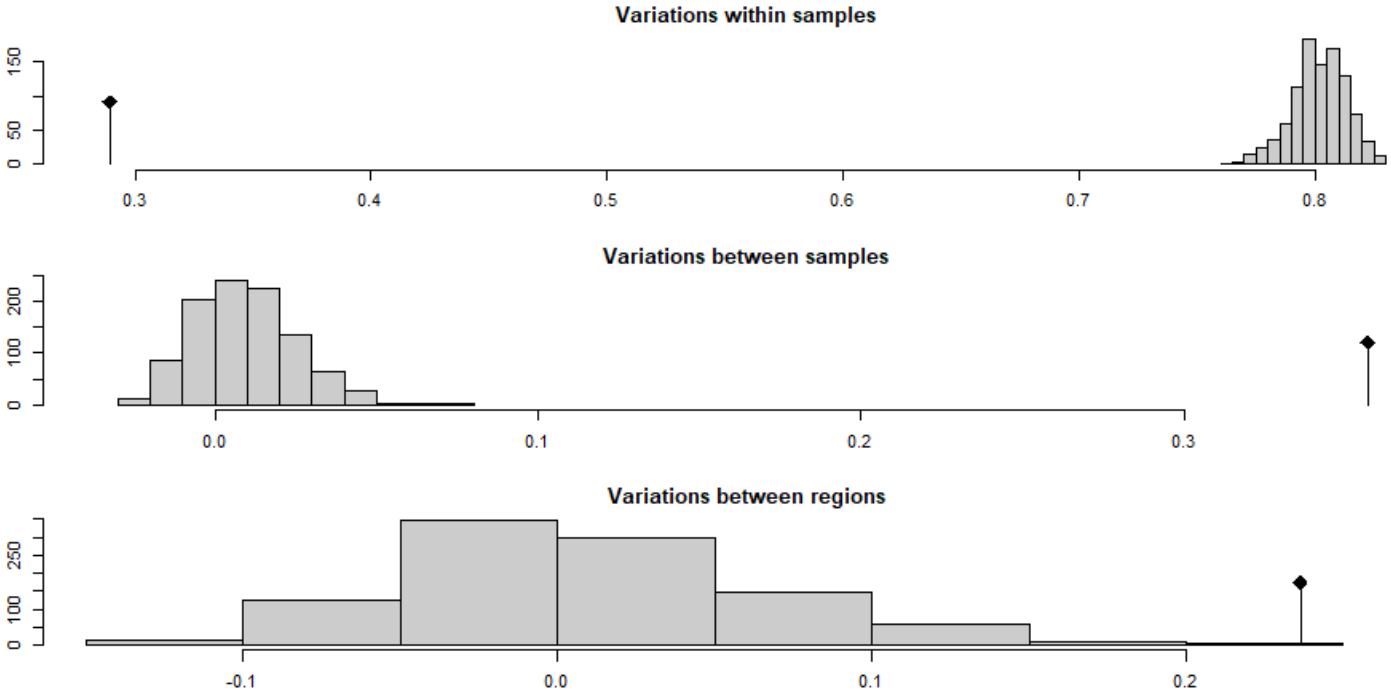


Figure 5.2. Histograms of the distribution of randomized values of variance for each level of the stratified AMOVA. The black lines represent the observed values in the AMOVA. Samples = populations, regions = Mountain ranges.

Coefficients of genetic differentiation (GammaSt) and gene flow values (Nm) averaged per each Mountain range are showed in Table 5.3. Gene flow values ranged from 1.783 for the Cantabrian-Leonese mountain range to 0.309 for the Mont Caro population, the latter having the highest differentiation and the lowest gene flow.

Table 5.3. Coefficients of genetic differentiation (GammaSt) and gene flow (Nm) values averaged per each Mountain range.

Mountain range	Averaged GammaSt	Averaged Nm (Gene flow)
Moncayo	0.322	1.052
Cantabrian – Leonese	0.219	1.783
Mont Caro	0.618	0.309
Central System	0.452	0.606
Montseny	0.311	1.107
Pyrenees	0.271	1.345

Differences in the Nst nucleotide-based statistics (Lynch & Crease, 1990) and the net number of nucleotide substitution per site (Da) of each population with respect to all other populations, as a measurement of gene flow for each population. Higher differentiation values and lower values are observed in the Mont Caro population (Figures 5.3 and 5.4).

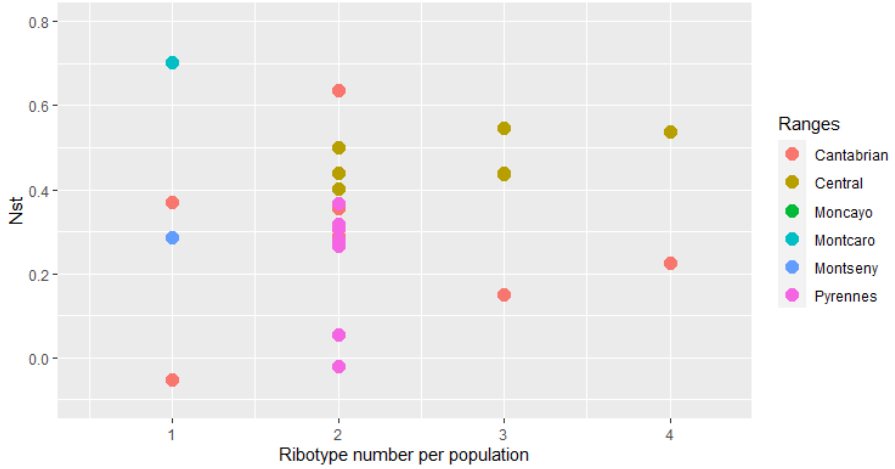


Figure 5.3. Nst nucleotide-based statistics between each population and all the other populations. Mountain ranges are identified by colours. Populations are separated by their number of ribotypes. Note that some populations are unrepresented because of overlapping values.

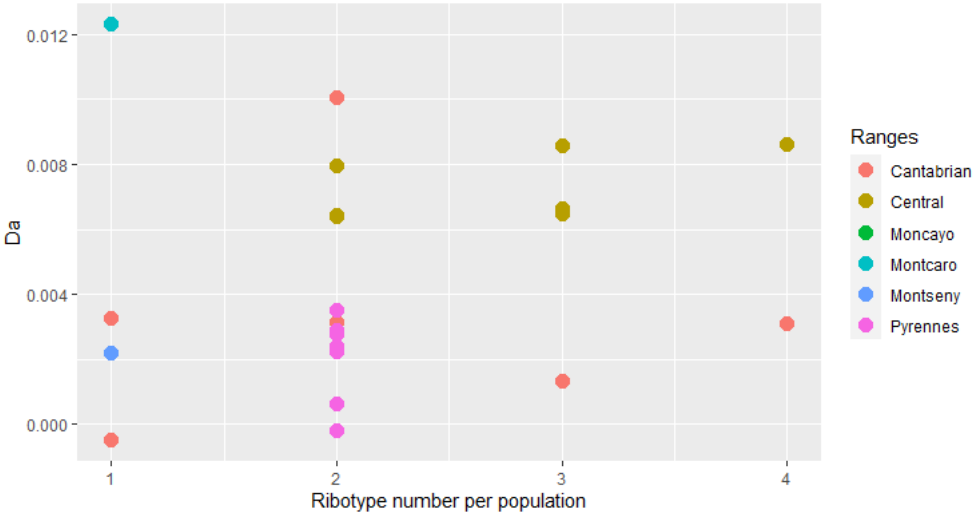
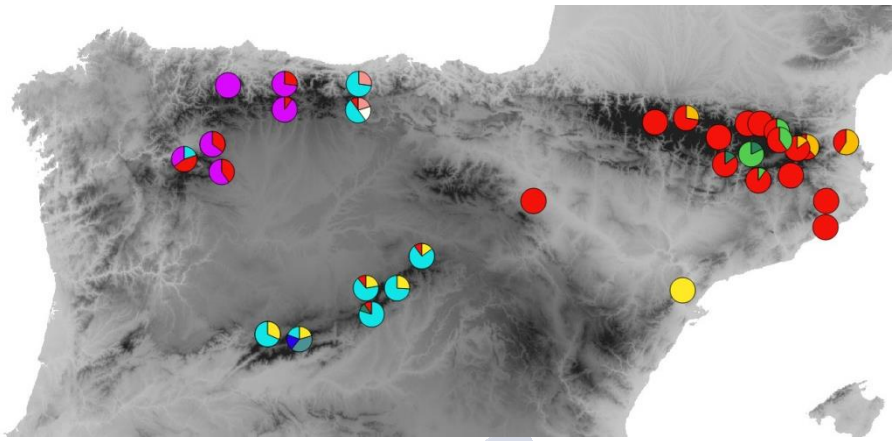


Figure 5.4. Net number of nucleotide substitution per site (Da) statistics between each population and all the other populations. Mountain ranges are identified by colours. Populations are separated by their number of ribotypes. Note that some populations are unrepresented because of overlapping values.

3.3 Phylogenetic relationships and geographic pattern of the ribotypes

Eleven ribotypes were identified of which three are unique of one population (two in two populations of the Central System and one in one population of the Cantabrian-Leonese range). Geographical ribotype distribution is showed in Figure 5.5. A medium-joining phylogenetic network is showed in Figure 5.6.



R1
R2
R5
R3
R6
R10
R8
R9
R7
R4
R11

Figure 5.5. Ribotype geographical distribution in hexaploid populations of *Jasione crispa* subsp. *crispa*. The geographic position of the pie charts is slightly displaced withs respect to the actual locations to reduce overlapping.

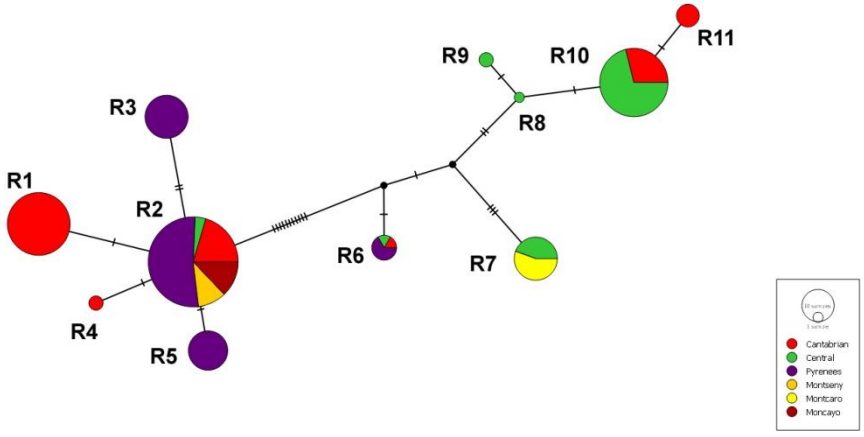


Figure 5.6. Median-joining phylogenetic network of ribotypes. Each circle represents a ribotype with sizes proportional to the number of copies detected. Missing ribotypes are drawn as small black circles and mutational steps by hatch marks. Colours indicate each of the six main mountain ranges and do not correspond to ribotype colours in Figure 5.5.

3.4. Plant: soil analyses

The analysis of soils showed that Composana and Courel were slightly basic and Pedroso very acidic (Table 5.4). All three were rich in organic matter, especially the Composana substrate. Pedroso presented limitations due to low effective exchange capacity (CECe) and potential aluminum toxicity, as 90% of the exchange positions were occupied by this cation. In Courel and Pedroso, the low magnesium concentration was limiting (less than 0.4 cmol kg^{-1}) and also in Courel there was a strong imbalance with respect to calcium (Ca / Mg = 122).

The biomass produced by the three populations was different in each soils (Figure 5.7). Mont Caro's behavior was different from that of Central System and Pyrenees. Mont Caro's highest productivity was observed in Courel soils (38%), while the highest biomasses in Central System and Pyrenees were obtained in Pedroso (60% and 49%, respectively). Mont Caro biomass from the acidic substrate only represented 31% of the total harvested amount. In Composana, it was again Mont Caro that presented the highest relative biomass values.

Table 5.4. Results of the soil analyses.

	Soil		
	Composana	Courel	Pedroso
pH (H_2O)	7.4	7.7	4.1
Organic matter (%)	76.2	12.3	10.9
Total elements (g kg^{-1})			
Al	2.8	27.7	18.6
Fe	2.6	46.3	13.5
K	1.2	5.6	3.0
Ca	49.7	218	0.2
Mg	2.9	3.5	1.7
P	0.9	1.0	0.3
Ca/Mg	17.1	62.3	0.1
Interchangeable elements ($cmol_c$ kg^{-1})			
Al	0.0	0.0	5.1
Ca	79.6	36.6	0.2
K	0.7	0.1	0.2
Mg	6.3	0.3	0.1
Na	0.3	0.1	0.1
Ca/Mg	12.6	122	2.0
CICe	84.9	37.1	5.7

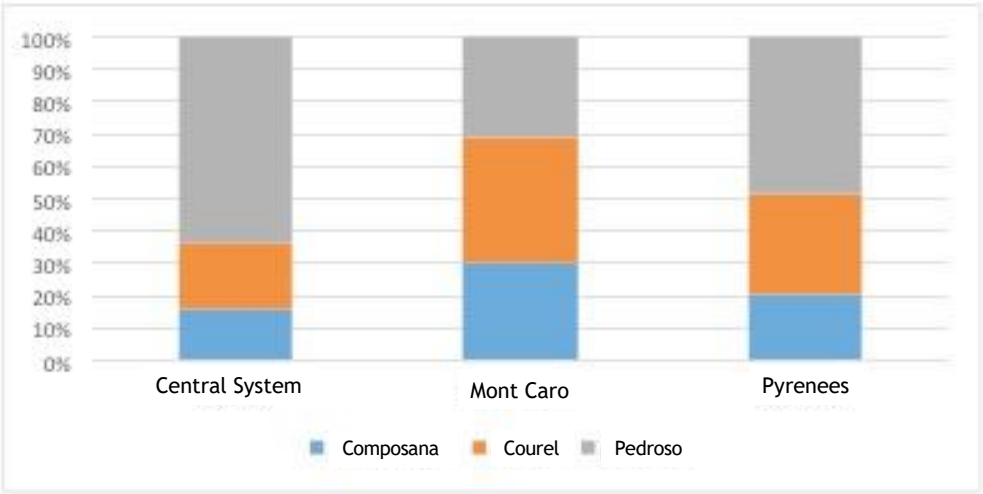


Figure 5.7. Percentages of total biomass (sum of leaf, stem and root) produced in each substrate by the three populations.

The presence of elements in root, stem and leaves of the three populations in the different soils was also different (Table 5.5). In Pedroso, the amount of magnesium and aluminum (this element was only present in the change complex of this soil) in Mont Caro leaves doubled the values in Central System and Pyrenees. The same behavior was observed relative to iron, both in root and stem, as well as in leaves, with a notably greater iron presence in Mont Caro on acid soil. The Ca/Mg ratio in this combination (Mont Caro in acidic soil) was also notably higher in leaves and stem. These results seem to indicate that Mont Caro is undergoing a process leading to lower efficiencies to management of disposable elements in acid soils and loose of adaptation to acidic edaphic environments.

Table 5.5. Results of the analysis of the total elements (g kg^{-1}) in the leaves (H), stem (T) and root (R) of the populations of *Jasione crispa* from the Central System (C), Mont Caro (M) and Pyrenees (P) mountain ranges.

Soil		Composana			Courel			Pedroso		
		C	M	P	C	M	P	C	M	P
Ca	H	23.0	12.6	14.0	61.3	20.0	21.6	9.5	9.6	10.8
	T	6.3	8.6	7.3	13.5	12.3	10.1	4.3	6.1	5.7
	R	7.6	7.6	6.8	12.7	23.8	11.7	5.7	4.3	4.9
Mg	H	3.2	1.2	2.3	2.4	3.9	4.6	2.6	4.3	2.0
	T	1.6	1.0	1.4	1.1	3.1	2.9	1.9	3.8	0.8
	R	3.7	1.3	2.9	1.6	12.0	8.6	4.8	9.1	5.0
Al	H	0.2	0.5	0.2	12.6	3.9	4.6	2.6	4.3	2.0
	T	0.5	0.8	0.3	3.4	3.1	2.9	1.9	3.8	0.8
	R	0.4	0.3	0.2	6.4	12.1	8.6	4.8	9.1	5.0
Fe	H	0.1	0.4	0.2	1.1	4.8	3.9	1.8	2.7	1.2
	T	0.4	0.5	0.2	3.0	3.0	1.9	1.2	2.3	0.6
	R	0.3	0.3	0.2	6.0	10.4	6.7	3.2	5.6	2.7
Ca/Mg	H	7.0	9.7	6.0	25.5	15.9	11.0	5.2	7.5	4.6
	T	3.7	8.3	4.9	12.1	14.2	8.6	3.8	7.0	5.5
	R	2	5.8	2.3	7.8	13.2	6.0	4.2	3.7	3.5

In the basic Courel soil, with a high presence of calcium and where the Ca / Mg ratio in the CECe presents extreme values (viz., 122), the amount of calcium in Mont Caro roots doubles the amount of this element present in the roots of Central System and Pyrenees. However, the leaf: root ratio in Mont Caro remains around the unit, while it is two times and six timed greater in Pyrenees and Central System, respectively (Table 5.5). Composana soils is characterized by the greater amount of magnesium in the exchange complex, 6.3 cmol kg^{-1} compared with 0.3 cmol kg^{-1} and 0.1 cmol kg^{-1} in Pedroso and Courel soils, respectively (Table 5.4), The presence of this element in plants cultivated in Composana soils is lower in plants from Mont Caro than in plants from the other two populations, with these consistently observed in leaf, stem and root (Table 5.5). Moreover, plants cultivated in Composana soil reveal differences in Ca/ Mg ratio between Mont

Caro on one side and plants from Pyrenees and Central System on the other, with much greater Ca/Mg ratio values in all three tissues for the Mont Caro.

Regarding the chromatic coordinates (a* and b*) it is observed a differential behaviour of Mont Caro in relation to the silicicole populations, particularly in the siliceous Pedroso soil. Interestingly, the colour values of Mont Caro in Pedroso soil are located over the a* axis, evincing achromatic response in this parameter. Mont Caro values in Pedroso are lower in the b* axis compared to Mont Caro in Courel and Composana soils, around 10 CIELAB units towards blue colour. Conversely, Mont Caro in Courel and Composana shift to more extreme values in both axis, either green or red, but in any case escaping from the achromatic axis. Central System and Pyrenees seems to behave similarly, despite their greater phylogenetic distance. Moreover, under the basic conditions of the calcareous Courel soil, Central system tends to adopt closer or over the achromatic axis (Figure 5.8).

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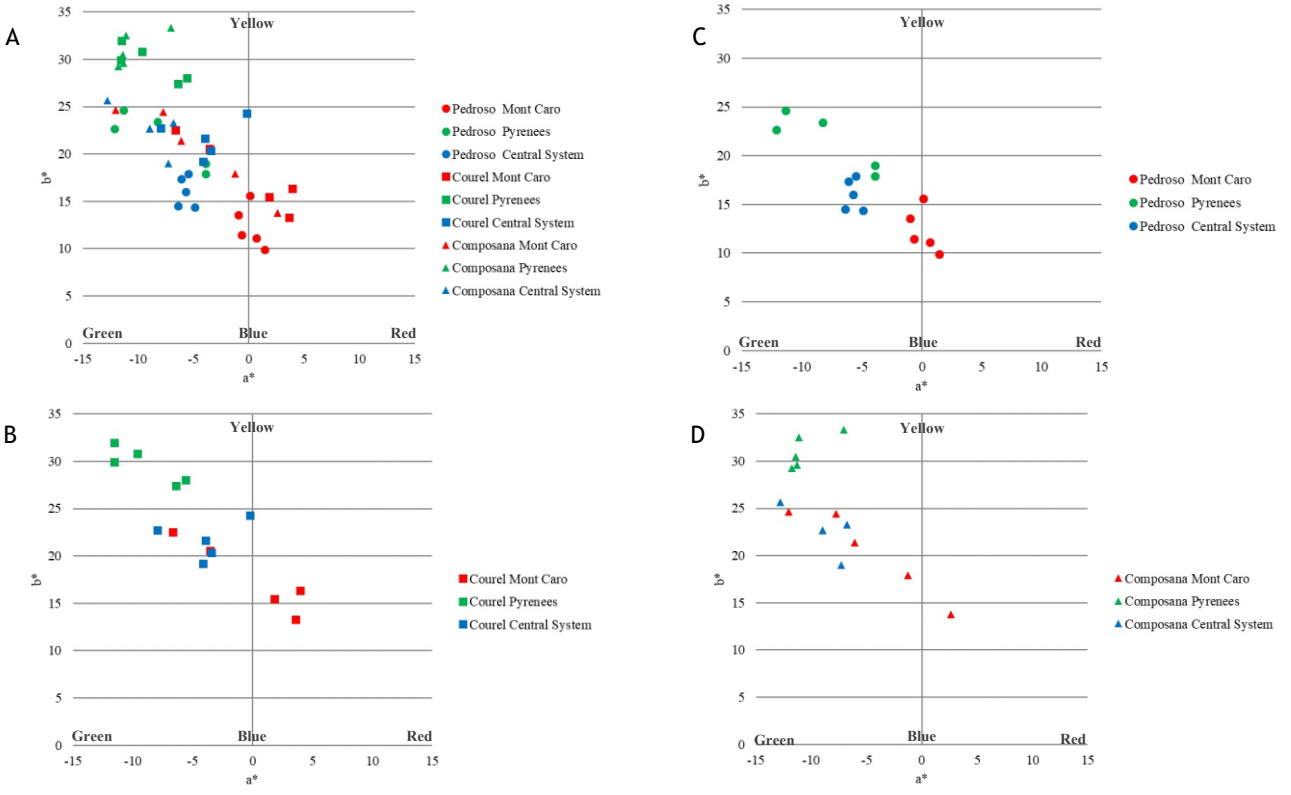

Figure 5.8. Changes in the green-red colour component (a*) and blue-yellow colour component (b*) in leaves. All data (A), Courel soil (B), Pedroso soil (C), and Composana soil (D).

4. DISCUSSION

The expansion of a lineage to a new environment is a requisite for ecological speciation. However, this evolutionary process can be only apparent, and the patterns observed in nature can better respond to long-term pre-adaptations than to novel adaptations (Moore & Kadereit, 2013). The identification of features of ecological speciation needs to verify phylogenetic and biogeographic hypothesis by which the origin of the population undergoing novel adaptations under new environmental constraints can be tracked to a lineage not pre-adapted to these putatively new ecological pressures. In this work, the initial steps of ecological speciation are shown in a robust phylogeographic framework and signatures of adaptation to calcareous edaphic niche in a population from an otherwise calcifuge lineage.

The singularity of the calcicolous population from Mont Caro is brought to light in this study, as well as its phylogenetic affinities and biogeographical relationships. It is identified for the first time as an hexaploid population. This ploidy level rule out its inclusion withing the variability of *Jasione sessiliflora*, a tretraploid species. Hexaploids orophile populations of the species are considered *Jasione crispa* subsp, *crispa* (Sales & Hedge, 2001). Although morphological floral characters like calyx teeth shape or leave traits as number and location of hydathodes support the inclusion of the Mont Caro population within the variability *Jasione crispa* subsp, *crispa*, environmental factors like low elevation of Mont Caro population (c. 1,400 m) compared with other populations of the species, or the calcareous nature of the Mont Caro massif *while J. crispa* subsp. *crispa* is calcifugue, could raise doubts about the assignation of Mont Caro populations to this subspecies. The genus *Jasione* is characterized by pervasive morphological stasis and scarce morphological diagnostic characters to confidently differentiate even distantly related species. For this reason, relying on morphological characters can be deceiving in *Jasione*, as has been exposed in other parts of this thesis. As a matter of fact, different evolutionary approaches of the current author are in conflict with previous taxonomic treatments of different *Jasione* groups.

Ploidy level and phylogenetic analysis reliably place this population within *J. crispa* subsp. *crispa*. Ribotypic distribution of *Jasione crispa* subsp *crispa* unveils the phylogeographic history in *Jasione crispa* subsp. *crispa*, that would be analysed in more detail elsewhere. As a summary, there is a relatively high ribotypic diversity within *J. crispa* subsp. *crispa* (Hd = 0.802), and significant genetic structuring between populations, withing populations of the Iberian Mountain ranges and between the different mountain ranges. However, the latter level of differentiation (i.e., between mountain ranges) is less marked than in the two former levels. Nevertheless, all three main Mountain ranges, the Central System, the Pyrenees and the Cantabrian-Leonese Mountain range, harbour diverse unique ribotypes. This pattern can be explained by a recurrent history of cycles of vicariance and within Mountain range differentiation followed by expansion periods and secondary contact, fitting in the model of refugia within refugia proposed for the Iberian Peninsula by Nieto-Feliner (2014). Pleistocene stadial and interstadial cycles would range allow expansion in colder periods and range shrinking in hotter ones, with these fluctuant biogeographic model fitiing well in cold-adapted lineages (Rull, 2020). Three narrow range Mountain massifs with isolated populations of hexaploid of *Jasione crispa* subs. *crispa* have been included in the study. All three shows low levels of internal genetic variability, with only one ribotype identified in each massif. This low variability agrees with out hypothesis that in small sized and isolated populations the mechanisms of concerted evolution would be more effective homogenizing the pool of ribotypic copies occurring within the mountain range, while the dynamics of ribotypic variability will be more complex in Mountain areas with larger population sizes, where random direction of concerted evolution can be different in each subpopulation, keeping the pool of copies and increasing the variability after gene flow among populations within each mountain range. Interestingly, the inferred amounts of gene flow are different for Moncayo and Montseny on one side, and Mont Caro in the other, with the latter population being outlined as the one with lower values of gene flow of all populations in the Iberian Peninsula (Table 5.3 and Figures 5.3 and 5.4). It is caused for the information given by ribotype fixed in

each of the three populations and its distribution across other populations in all Mountain ranges. The only ribotype identified in Moncayo and Montsenty (R2) is the most frequent in the taxon and the only one present in all mountain ranges but in Mont Caro. Conversely, the ribotype fixed in Mont Caro population (R7) has its only other occurrence as a minority ribotype in some populations of the Central System. Interestingly, this pattern suggests a surprising history of relationships between Mont Caro and the distant populations of the Central System. Therefore, there is no molecular evidence that the much closer populations of Montseny and Moncayo could have been the source for Mont Caro populations. It could be that the Ebro river valley could have been an effective barrier to dispersal. Ebro river is between Mont Caro and Motseny. Moncayo is South of the Ebro, but distantly upriver and separated from Mont Caro by the lowlands of the Ebro valley where environmental conditions suitable for the orophyte *J. crispa* subsp. *crispa* could be absent. In any case, the current author data suggest for Mont Caro an evolutionary history of belonging to a calcifuge lineage only occurring in the Central System what makes unlikely pre-adaptation to calcicole edaphic niche. This pattern is opposite to those recovered in other model groups, like the Alpine calcicole lineages of *Minuartia* (Moore & Kadereit, 2013). Low variability and ribotype fixation can be a signature not only of absent gene flow and founder effect, as would be plausible given the long distances to other populations, but as well as by environmental filtering to dispersal by the regionally predominant calcareous substrates.

Mont Caro shows genetic signatures of increased isolation to gene flow from other populations in a relatively structured phylogeographic context. Gene flow interruption related to marked geographic structure is combined in Mont Caro with environmental (edaphic) distinct conditions, setting one scenario suitable for ecological divergence (Nosil, 2012; Barraclough, 2019). Gene flow, otherwise, would promote genetic exchanges from other populations decreasing genetic differentiation (Slatkin, 1987), and threatening the preservation of acquired abilities to cope with new environmental constraints. Interestingly, the experimental part of this work points to the existence of these acquired abilities, and signatures of novel adaptation to

calcareous environment are identified. Nevertheless, it cannot be ruled out that adaptation cold be working in other environmental dimensions, as environmental consequences of the elevation range in Mont Caro (around 1,400 m.o.s.l), lower than the elevation range of other populations. For example, Central System populations are most frequently found around 2,000 m.o.s.l.

In summary, there exists in the plants from Mont Caro a greater productivity when are cultivated in calcareous or basic soils than when are cultivated in acid siliceous soils. Conversely, better performances in calcareous soils are not registered in plants from the silicicole population of Central System and the Pyrenees. The performances of the plants from the Central System are more similar to the ones from the Pyrenees, despite Mont Caro and Central System plants are more closely related. Phenotypic colour analysis agrees with the biomass results in a way that colour expression in the different soils works differently in Mont Caro when compared to the silicicole populations. Distinct management of calcium and magnesium, and in aluminium accumulation, have been pointed as the main differences between calcicole and calcifuge plants (Jansen *et al.*, 2002). These differences are here identified between Mont Caro and the s silicicole populations of the Central System and the Pyrenees. These results suggest that the ability to thrive in calcareous environments is newly acquired in process of ecological divergence. It is coupled with an apparent loss of efficacy to cope with conditions occurring in acid substrates, particularly the relationship with toxic disposable elements as aluminium. Therefore, not only new pressures modify the genetic makeup of the new population at its phenotypic expression to cope with them, but also the absence of previous ecological pressures in which the lineage evolved before the expansion to the new environment caused the rapid loss of their associated adaptations as an interesting evolutionary trade-off. Whether if all these abilities rely in the in genetic changes or they can be explained by or in combination with other mechanism, like the epigenetic make up, remains open to new studies. Nevertheless, the results suggest that natural selection is likely shaping the evolutionary fate of the Mont Caro population, suggesting an ongoing speciation process by ecological divergence in condition of gene flow isolation.

5. CONCLUSIONS

The hexaploid populations of the calcifuge taxon Jasine *crispa* subsp. *crispa* occurring in the Mountain ranges of the northern half of the Iberian Peninsula shows high global genetic variability and geographic structuring of ITS ribotypes, both across populations and within Mountain ranges. The only calcicole population, located in the coastal Mont Caro massif in Tarragona, is related to the distant populations of the Central System. Mont Caro shows the lower levels of gene flow among all studied populations, evincing clear geographic isolation.

This population has signatures of ecological divergence under calcareous edaphic conditions.

Experiments in siliceous and calcareous soils evince differential phenotypic performances and biomass production between Mont Caro populations and two silicicole populations. Mont Caro performed clearly better in calcareous soils, conversely to the silicicole populations. The management of cations was also different, with Mont Caro accumulating a notably greater amount of aluminum, iron and magnesium in strongly acidic soil, while in basic substrates (despite the abundance of calcium and magnesium), Mont Caro accumulated significantly less amounts of magnesium and showed a balanced relationship of calcium between root and leaf. These results suggest the existence of an adaptive ecological speciation process in *Jasione crispa* from Mont Caro, which would contribute to support its differentiated taxonomic recognition.

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CHAPTER 6.
Presence and conservation status of *Jasione corymbosa* Poir. ex. Schult. (Campanulaceae) in the Iberian Peninsula

1. INTRODUCTION

Jasione corymbosa Poir. ex Schult., interpreted in a broad sense, is an annual polymorphic endemic to the Betic-Mauritanian area, whose preferred habitat are dunes and coastal sands of southern the Iberian Peninsula and the Maghreb, from the western Algeria up to the Atlantic coast of Morocco (Quézel, 1953; Sales & Hedge, 2001; Peris *et al.*, 2002).

Records of the species presence in southern Spain are quite rare. There are bibliographic references (Fernández López *et al.*, 1989) and herbarium sheets, cited in Bokhari & Sales (2001) and Sales & Hedge (2001), which locate the species in the province of Malaga, between Estepona and Fuengirola, as well as in Torrox. It is also known a sheet collected in 1984 in Calahonda, Granada province. This would be the last citation found so far, since the most recent record from Malaga is from the beginning of the 20th century. References to Portugal (Pereira Coutinho, 1939; Parnell, 1987) are confusions either with *Jasione montana* L. or with *Jasione blepharodon* Boiss. & Reut. *Jasione corymbosa* is closely related to the later species, as has been shown I other parts of this thesis.

The scarcity of records in the Iberian Peninsula makes it presumable a concerning state of conservation for this species. However, this aspect has not received attention until relatively recent times. Its first inclusion in a regional Andalusian red list was in

Cabezudo & Talavera (2005), where it is classified under the IUCN category of Data Deficient (DD). This category conveys that there is inadequate information of the risk of extinction of the taxon based on the current knowledge of its distribution and/or population status. (IUCN, 2001). Although it is not a category of threat its inclusion under the DD category in a list is based on the plausibility that the understudied taxon could be threatened. The authors of the Andalusian red list (Cabezudo & Talavera, 2005) searched intensely the species in its historical range without positive results, both before and after the publication of the red list (Cabezudo, pers, comm.). This is the reason that motivated its later inclusion as an extinct species at the Iberian regional level (EX, RE) in the Spanis Red List of the Vascular Flora (Moreno, 2008). The current author had also looked for the species several times, always unsuccessful. However, it remained the suspicion that there could be some remnant population, more likely in Malaga given the broader presence of suitable habitat in this province. It led to get in contact with the technical staff the department of flora conservation of the Andalusian regional government, to establish an official searching protocol. After thorough analysis of suitable habitat across the coast of Malaga and sunsequent intense searching, *J. corymbosa* could be located by M. Becerra and J. Pereña, in a single locality, in the municipality of Manilva, in the southwestern coast of Malaga (Pereña Ortiz *et al.*, 2008; Pereña Ortiz *et al.*, 2009).

Numerous individuals, in bloom and early fruiting, settled in the best preserved area of the dunes of Manilva. The data of the collection, deposited in the Herbaria of the University of Malaga (MGC) and University of Santiago de Compostela (SANT), are: MALAGA. Manilva. Coastal sandbanks, 2 m.s.n.m. 06/13/2008, M. Becerra & J. Pereña. MGC 67726 and SANT 59905; idem: 05.25.2009, B. Cabezudo, A.V. Pérez Latorre & F. Casimiro-Soriguer. MGC 70.000 (Figure 6.1) and MA s / n.

This area is located in the Tingitano-Onubo-Algarbiense chorological province, Aljíbico Sector (Galán de Mera *et al.*, 2003), and extends species range in the Iberian Peninsula in its southwestern boundary, bringing it closer to the border with the province of Cadiz.

The complexity of the genus *Jasione* and the scarcity of discrimination characters between species, plus the significant intraspecific variability of *J. corymbosa* in the populations of Morocco, led to some taxonomist at the time of location about the correct assignment of the Manilva plants to this species (B. Cabezudo, pers. com.).

Although the current author was sure that the assignation of the Manilva population to *Jasione corymbosa* was correct, it was necessary to support it with molecular evidence. The material was studied from the molecular and morphological perspectives in the Department of Botany of the University of Santiago de Compostela.

DNA from three individuals or Manilva and diverse individuals from North African populations was extracted. The ITS region of the ribosomal nuclear DNA, and the psbA-trnH spacer and the ndhF region, that is, the 3' extrem of the F subunit of the NADH dehydrogenate gene of the Choroplast genome, were PCR amplified. These markers have proven to be informative to discriminate between North African populations of the species, as well as between J. *corymbosa* and close taxa.

Figure 6.1. *Jasione corymbosa* Poir. ex Schult. Malaga: Manilva (MGC 70,000).

The results of various phylogenetic analyses (Maximum Likelihood, Bayesian), in the framework of a comprehensive study covering the whole genre, revealed that the amplified DNA sequences from of Manilva are part of a clade also encompassing all samples from North-African populations of *J. corymbosa*. The inferred relationships had a strong statistical support and suggested that *J. corymbosa* is the sister group of another species of the southern half of the Iberian Peninsula, *J. blepharodon* Boiss. & Reut. This phylogenetic affinity between the two taxa had already been pointed out by Pérez-Espona *et al.* (2005).

Due to their morphology, the specimens of *J. corymbosa* collected in Andalusia can be clearly differentiated from some North African populations, mainly from those of Western Rif. However, studies more detailed are needed to future evaluation of a possible differentiated

entity differentiated, given the wide range of variation existing within the species.

2. Discussion. Considerations on the state of conservation (Serrano et al., 2009)

The species occupies in the Manilva dunes around 1.5 ha. The regional government surrounded the occupied are by a perimetral ditch to avoid cars entry. It allowed an increasing in the number of exemplars from 2,000 individuals in 2010 to 6.000 individuals in 2012. The restrictedness of its Iberian distribution, the apparent disappearance of the remaining known localities, as well as the strong regression suffered by the dune communities occupied by *J. corymbosa* throughout the Mediterranean Andalusian coast, threatened by tourism intensification, suggested the proposal for the Iberian Peninsula population the threat category of Critically Endangered (CR), according to the IUCN classification (2001).

According to that, the proposal of the 2008 Red List of the Spanish Vascular Flora (Moreno, 2008) should be changed from "EX, RE" to: CR B1ab (i, ii, iii) +2 ab (i, ii, iii).

Besides the *in situ* conservation measures, germplasm collected in the Manilva population is currently conserved in three germplasm facilities, the Córdoba Botanical Garden (BGVA) seeed bank, the Polytechnic University of Madrid seed bank, and the University of Santiago de Compostela seed bank. The discovery of this plant in Manilva arise the possibility that the species may continue to exist in some other locations, what makes it advisable to keep looking for it in the Andalusian coast.

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CHAPTER 7.
A simple, reliable, and inexpensive solution for contact colour measurements in small plant samples, assessed in plants of the genera *Jasione* L. and *Campanula* L. (Campanulaceae)

ABSTRACT

Correct color measurement by contact-type colour measuring devices requires that the sample surface fully covers the head of the device, so their use on small samples remains a challenge. Here, cardboard adaptors on the two aperture masks (3 and 8 mm diameter measuring area) of a broadly used portable spectrophotometer are proposed to use. Adaptors in black and white to reduce the measuring area by 50% and 70% were applied in this study. Representatives of the family Campanulaceae have been used to test the methodology, given the occurrence of small-leaved. The results of the current author show that, following colorimetric criteria, the only setting providing indistinguishable colours according to the perception of the human eye is the use of a 50%-reducing adaptor on the 3-mm aperture. In addition, statistical analysis suggests the use of the white adaptor. The contribution of the current author offers a sound measurement technique to gather ecological information from the colour of leaves, petals and other small samples.

1. INTRODUCTION

The colour of plant organs, particularly of leaves and flowers, is a phenotypic trait traditionally used as a visual indicator of the plant

physiological status. Indeed, environmental stress regarding light quantity and quality, nutrients, temperature and drought result in a change of plant colour (Rahaman *et al.*, 2015). These correlations are so informative that colour is one of the phenotypic traits for monitoring plant growth in high-throughput classification approaches (Chen *et al.*, 2014). The colour of leaves and flowers fulfill also an ecological role in plants, since bright colours attract pollinators and seed dispersers (Kevan, 1983). It has been also hypothesized that insects could interpret the colour intensity of plant leaves as a signal of the strength of the plant, thus being attracted or repelled (Archetti, 2000; Yamazaki, 2008). Leaves colour reflects also the accumulation of secondary metabolites with important chromatic components, which can have a great commercial and therapeutic value. For example, the purple colour of bud and leaves in tea cultivars (e.g., Benibana-cha, Sunrouge tea, Zijuan tea and Ziyan tea) is associated with the accumulation of anthocyanins, that have been shown to have a therapeutic role against colorectal carcinoma cells (Hsu *et al.*, 2012) and in reinforcing the brain's antioxidant capacity in mice (Rashid *et al.*, 2014). In addition, since these metabolites accumulate in response to the plant interaction with soil, the leaves and petals colour is an indicator of the characteristics of the soil. Well known examples are the overexpression of carotenoids and anthocyanins in plants growing in soil with high salt concentration (Borghesi *et al.*, 2011) or the changes in colour of flowers in Hortensia (*Hydrangea macrophylla*), which varies from blue to pink, according to the soil acidity. Besides, colour transitions and pigment variation in plants also inform about genetic variability within an among populations and taxa, allowing the formulation of evolutionary hypothesis in a phylogenetic framework (Abdelaziz *et al.*, 2014; Valenta *et al.*, 2018). Changes in colour, both in vegetative and reproductive organs (Ougham,*et al.*, 2008), tell about important evolutionary processes like pollinator-mediated reproductive isolation (Yuan *et al.*, 2013). Finally, plant disease, including fungal and bacterial parasites, cause lesions and chromatic changes of leaves and flowers (Barbedo, 2013; Matsunaga *et al.*, 2017). Their early perception is fundamental to act on time, to eradicate the disease and to limit economic loss, or to monitor the interaction of plants with damaging

organisms or environmental factors. Given the intimate connection of flower and leaf colour with the physiology of the plant, its quantification by human eye has been traditionally used to guide breeding experiments to accumulate metabolites, but also as a proxy for soil parameters and detection of disease.

Unfortunately, colour detection and classification by human eye are extremely unreliable, because of their dependency from the experience and the capacity of the observer. The choice of a colour by a living organism is based on psychological and evolutionary or survival criteria. For example, among the colours distinguished by the human eye, green is perceived more readily and with the largest shift in their hue than any other colour, because of the combined perception of rods and cones (Gordon & Abramov, 1977). It is hypothesized that this could represent an adaptation to the environment of primates in search for food (Nerger *et al.*, 1995).

The colour can be described in an objective and precise way through the use of spectrophotometers, colorimeters or chroma-meters that describe the reflected colour in the standardized CIELAB colour system (see e.g., CIE 1986; Fairchild, 1998; Hunt & Pointer, 2011). They work by contact on a surface, like other contact-type spectrometer devices (Lu *et al.*, 2019), and they average the light reflected from an aperture on the head of the device. The aperture is circular and has a diameter between 3 mm and 60 mm (see e.g., Fairchild, 1998; Hunt & Pointer, 2011; Prieto *et al.*, 2010). Surfaces with an area in a different shape or smaller than the aperture of the device do not reflect entirely the emitted light, thus leading to a consistent loss of light and to unreliable results. To perform the colour measurement correctly, the sample surface must cover entirely the aperture of the device to prevent leakage of light or interference from external light. For this reason, the colour cannot be measured when the target area is smaller than 3 mm and/or not circular.

To address this issue, it is proposed to reduce by 50% and 70% the aperture area of a portable spectrophotometer with cardboard adaptors in white and black colours, in order to describe the colour in the

CIELAB space of small biological samples, such as leaves and petals. Thus, the current author further enhances the usability of the sensor device by extending its application to smaller samples than those for which it was designed. This unlocks previously unexplored ways to tackle current issues in the environmental monitoring field, such as the biodiversity response to climate change. In this frame, the colour of leaves from species of the genus of *Jasione* and other of the Campanulaceae family is measured, thus validating this methodology within a broad phylogenetic group with species of different ecology and frequently with small-sized leaves (Bokhari & Sales, 2001). This group encompasses a number of alpine species, with a habit of dense rosettes with small leaves, being this a characteristic plant adaptation to alpine environments (Nagy & Grabherr, 2009). In the present work, the use of leaves big enough to be measured with the large colorimeter aperture guaranteed the reliability of this methodology also for leaves where only the smallest aperture of the colorimeter aperture can be used, as is the case of many the alpine species. In addition, herbarium samples, where dry plant tissues are conserved as valuable ecological, systematic and historical evidences of plant biodiversity, are included. By extending the present methodology to conserved dry samples, it is proposed the use of colorimetry as a tool to describe the colour of leaves and petals after some time and monitoring the conservation conditions of these specimens.

2. MATERIAL AND METHODS

2.1. Plant materials

The genus *Jasione* has many representatives occurring in mountain environments (Sales & Hedge, 2001), having spread around the Mediterranean basin with a number of species endemic of the alpine ranges of the region (Pérez-Espona *et al.*, 2005). They belong to the alpine flora: the mountain zone located above tree line and below the permanent snow line and considered a conservation target (Byers, 2005). Alpine plant communities are in one of the most threatened ecosystems in the world by climate change (Rixen *et al.*, 2014). Thus,

the alpine flora is currently subject of a number of international monitoring projects to study the response of plants to global temperature increase and other environmental stressors (GLORIA, 2019). Colour variation in the leaves of *Jasione* could be used as ecological indicator in future monitoring studies on alpine plant species. Nevertheless, the peculiar growth of many alpine plant taxa, forming a cushion of dense rosettes of small leaves as a common evolutionary adaptation (Nagy & Grabherr, 2009), represents a challenge for the use of colour as a proxy of physiological status.

For these reasons, the proposed methodology has been tested on six fresh plants (*Jasione montana*, L. *Trachelium caeruleum* L., *Campanula rotundifolia* L., *Campanula isophylla* Moretti, *Hesperodocon hederaceus* (L.) Eddie & Cupido., and *Jasione laevis Lam*, Figure 7.1) and six plants from herbarium (*Campanula adsurgens* Levier & Leresche, *Campanula latifolia* L., *Campanula mollis* L., *Campanula glomerata* L., *Campanula trachelium* L., and *Campanula versicolor* Andrews, Figure 7.2), all belonging to the family of Campanulaceae. The two species of *Jasione* were selected from populations with wide leaves in order to satisfy the conditions required by the study (i.e. 11 mm of diameter in MAV without adaptors, see Section 2.2.). These plants belong to populations of *Jasione laevis* subsp. laevis and *Jasione montana* var. *latifolia* Pugsley.

The herbarium record of University of Santiago de Compostela, Spain (SANT) was accessed to obtain the specimens. Each herbarium specimen was checked for possible misidentification, and the herbarium code, name of the specie, sampling location, collector and year were recorded (Table 7.1).

Figure 7.1. Pictures of fresh plant specimens used in the study. From left to right and top to bottom: (a) *Hesperocodon hederaceus* (L.) Eddie & Cupido, (b) *Jasione laevis* Lam, (c) *Campanula rotundifolia* L., (d) *Campanula isophylla* Moretti, (e) *Trachelium caeruleum* L., and, (f) *Jasione montana* L. Scale bars are 2 cm.

Figure 7.2. Pictures of herbarium plant specimens used in the study. From left to right and top to bottom: (a) *Campanula latifolia* L., (b) *Campanula adsurgens* Levier & Leresche, (c) *Campanula mollis* L., (d) *Campanula trachelium* L., (e) *Campanula glomerata* L., and (f) *Campanula versicolor* Andrews. Scale bars are 2 cm.

Table 7.1. Data of the six herbarium samples belonging to the Campanulaceae family and used in this study.

Herbarium Code [1]	Species	Location [2]	Collector	Year
SANT 210	*Campanula latifolia* L.	ES. Peña Oroel, Jaca	Bellot, Vieitez	1947
SANT 232	*Campanula trachelium* L.	ES. Province of Barcelona	Marcos	1944
SANT 21287	*Campanula adsurgens* Levier & Leresche	ES. Triacastela, Lugo	J. Amigo, J. Giménez de Azcárate	1990
SANT 29493	*Campanula versicolor* Andrews	IT. Santa Cesarea, Lecce	J. Izco	1994
SANT 51406	*Campanula glomerata* L.	ES. Penedo de Oulego, Ourense	J. Amigo	2004
SANT 62512	*Campanula mollis* L.	ES. Sierra de Grazalema, Cádiz	J. Izco	1976

[1] SANT: Herbarium of the University of Santiago de Compostela; [2] ES: Spain; IT: Italy.

2.2. Colour measurements with and without the adaptors

The colour was measured with a portable spectrophotometer Konica Minolta CM-700d, a contact-type colour measuring device, commonly used in scientific works. A spectrophotometer is a specific type of spectrometer, designed to measure light over the visible and near-visible portion of the electromagnetic spectrum, i.e. from 360 nm to 740 nm. It uses an optic set up of diffuse illumination geometry (recommended by the CIE, "International Commission on Illumination") accomplished by an integrating sphere covered with a white reflective coating and with 8°/diffuse illumination/viewing geometry (Figure 7.3). The sphere acts both as a means of producing a diffuse light source through a series of reflections off the white sphere wall, and for collection of light from the sample surface, placed in the entrance or sample port. The spectrophotometric method utilizes multiple sensors to measure the spectral reflectance of the sample. The sensor's electronics then calculate the tristimulus values from the

spectral reflectance data using integration. The device is equipped with the CM- S100w (SpectraMagicTM NX) software.

The measurements were made in the specular component included (SCI) mode, in which the gloss trap of the spectrophotometer is closed, includes the total reflectance (considering both specular and diffuse reflections), with illuminant D65, observer 2°, and both circular aperture masks: an aperture for medium area view (MAV) with a diameter of 11 mm, measuring a circular area of 8 mm of diameter, and an aperture for small area view (SAV) with a diameter of 6 mm, measuring a circular area of 3 mm of diameter. In addition, eight types of adaptors, four for each aperture mask, have been designed and realized in cardboard: in black or white colour, and decreasing the tested surface by 50% (hereinafter called "black 50%" and "white 50%", respectively) or 70% (hereinafter called "black 70%" and "white 70%", respectively) (Figure 7.4). By employing the adaptors, the sample port opening (hole) is reduced to 50% or to 70% by the template, which expand the sphere wall (Figures 7.3 and 7.4).

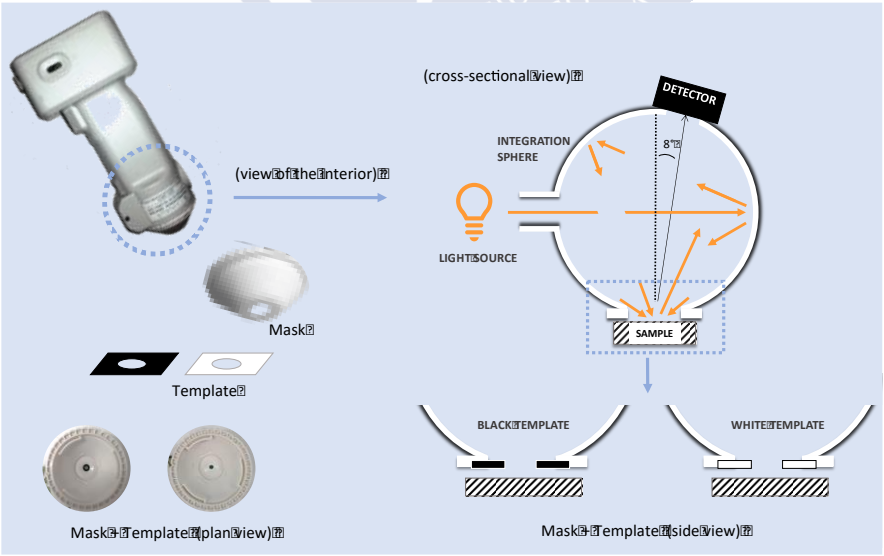

Figure 7.3. Schematic diagram showing the custom-designed set up for colour measurement provided by the portable spectrophotometer. The template partially plugs the sample port that becomes 50% or 70% smaller and, in turn, expanding the sphere wall.

Colour measurements were analyzed by considering the CIELAB colour system (CIE 1986), the most widely accepted by both industry and scientific community. It represents each colour by means of three scalar parameters or the Cartesian coordinates: L*, lightness or luminosity of colour, which varies from 0 (absolute black) to 100 (absolute white); a*, associated with changes in redness- greenness (positive a* is red and negative a* is green); and b*, associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue).

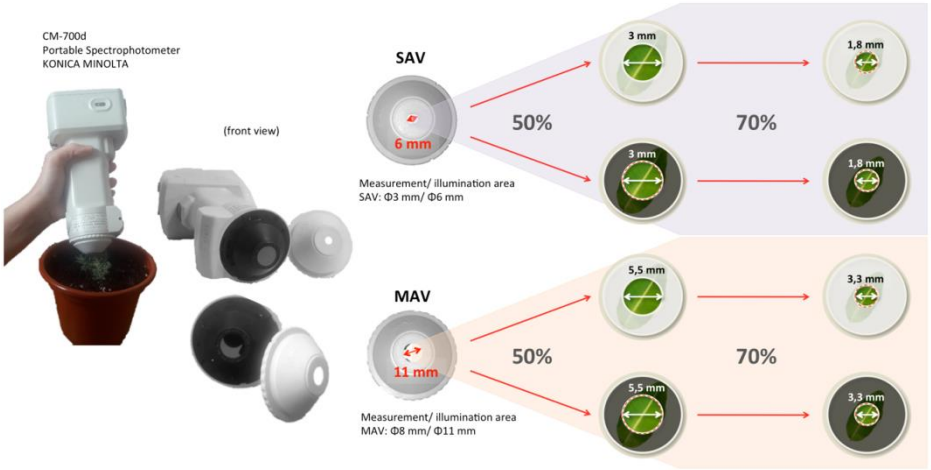

Figure 7.4. Explanatory diagram of the reduction of the measurement area of the device by means of adaptors in black and white.

Five healthy leaves from each species of Campanulaceae have been randomly picked and, on the surface of each of them, five points have been randomly selected to measure the colour, following previous works (Sanmartín *et al*., 2011a, 2011b). Although the aim of the work is not to define the species colour variability, the number of small leaves and of measurements in each leaf was high enough to average the colour variation in small leaves of the Campanulaceae species, in agreement with previous works that analyzed cumulative averages for parameters L*, a* and b* on heterogeneous surfaces (Prieto *et al.*, 2010; Sanmartín

et al., 2011a, 2011b). Five different set-up for each of the two aperture masks, MAV and SAV, were used to perform the colour record: no adaptor, black 50%, white 50%, black 70% and white 70%. The colour values obtained from the reduced area have been compared with the recorded colour without adaptor, using colorimetric criteria and statistical tools.

Because the dishomogeneus pattern of the vein network, the convexity of the leaf surface, and the intrinsic features of each leaf in each plant may affect the determination of colour, it should be established if the new method developed and here proposed can be extended to other plant samples. In order to address this concern, four patches homogenously coloured in green (green, foliage, yellow green and bluish green) from the color-checker colour rendition chart with 24 colour patches by Gretag Macbeth (McCamy *et al.*, 1976; Sanmartín *et al.*, 2014) were measured with the same protocol used for the leaves, as described in the previous paragraph.

2.3. Colorimetric analysis

Partial (ΔL^*, Δa^*, Δb^*) and total (ΔE^*_{ab}) colour differences have been calculated in absolute values with the following equations (1-4):

$$\Delta L^* = L^*_i - L^*_x \quad (1)$$

$$\Delta a^* = a^*_i - a^*_x \quad (2)$$

$$\Delta b^* = b^*_i - b^*_x \quad (3)$$

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (4)$$

where L^*_i, a^*_i, and b^*_i are the colour parameters by using the adaptor and L^*_x, a^*_x, and b^*_x are the same parameters by using no adaptor. Here, the threshold of 3 CIELAB units has been adopted as limit of rigorous colour tolerance or noticeable by an observer with normal colour vision, as in several other studies (Catrysse *et al.*, 1999; Martínez *et al.*, 2001; Molada Tebar *et al.*, 2018; Sanmartín *et al.*, 2012, 2014; Vrhel *et al.*, 1994).

2.4. Statistical analysis

For a robust evaluation of the effect of the adaptors on the colour measurements, the collected data have been analyzed with three different multivariate approaches: Principal Coordinates Analysis (PCoA), Quadratic Discriminant Analysis of multiple groups (QDA) and a combined approach based on k-means and agglomerative clustering analyses. PCoA was also applied to the measurements obtained from the four patches (green, foliage, yellow green and bluish green) of the Gretag Macbeth color-checker colour rendition chart.

With the PCoA analysis the contribution from each adaptor type (black or white, 70% or 50% reduction, or no adaptor) on both MAV and SAV to the colorimetric information (L*, a* and b*) was assessed. For each species, data were standardized by subtracting the mean of measurements with no adaptor to preserve the units of deviation from the actual colour and lay out all measurements in the same multivariate space. The measurements were distributed in a 2-dimensional Euclidean ordination space using ggplot2 and Stat R packages (Wickham, 2009).

To assess the similarity of the measurements obtained with and without the different tested adaptors, a QDA was performed. The individual measurements were assigned to predefined or actual groups (SAV and MAV with different adaptors or without adaptors), and the adaptor type most alike to SAV and MAV (i.e., those whose observations are significantly misclassified as observations obtained from SAV and MAV with no adaptors) were quantitatively identified from the results of the QDA confusion matrix. The analyses were run separately for SAV and MAV measurements and, in order to compare all species together, data were standardized by subtracting the mean of measurements, respectively from SAV or MAV with no adaptor. A Leave-Out-Out cross-validation was performed after the analysis to validate the QDA. Multivariate normality was assessed with the MVN R package (Korkmaz *et al.*, 2014) and the MASS R package was used for the remaining analysis (Venables & Ripley, 2002).

To verify the results pointed out by the QDA, an approach combining k-means and agglomerative clustering was performed on the same data from SAV, treated as for QDA. By k-means cluster analysis, similar colour measurements were categorized by defining clusters to minimize the total intra-cluster variation. The optimal number of k-clusters in each species was determined using 30 indices provided by the R package NbClust (Charrad *et al.*, 2014). The agglomerative clustering was conducted by computing a dissimilarity matrix based on Euclidean distances and the Ward.D2 agglomeration method in the Stats R package to produce a hierarchical tree for each species (R Core Team, 2020). The identified k-means groups were then superimposed on the dendrogram and, by means of the pvclust R package (Suzuki *et al.*, 2006), the statistical support of all clusters was calculated with approximately unbiased (AU) *p*-values by multiscaling bootstrap resampling. Clusters with AU *p*-value equal to or greater than 95% were considered strongly supported by data.

3. RESULTS

A colorimetric analysis of the gathered measurement from each species of Campanulaceae, as well as the green patches from the Gretag Macbeth color-checker colour rendition chart, was performed. Partial (ΔL^*, Δa^*, Δb^*) and total (ΔE^*_{ab}) colour differences using one of the four adaptors and no adaptor were calculated. The latter are summarized in Table 7.2. The colour parameters most widely varying with the use of the adaptors in plant samples were L* (lightness of colour) and b* (associated with changes in yellowness-blueness). L* varied to a larger extent with the use of the white adaptor, increasing the lightness in the registered colour (data not shown), and a similar observation can be made for the parameter b*: when the black adaptor is used, the blue component increases in the registered colour (data not shown). As expected, widening the diameter of the adaptor from 50% to 70% increases the differences in colour calculated with respect to the measurement without adaptor (Table 7.2). In the green patches similar results were obtained. Furthermore, for the green patch, the parameter a* (associated with changes in redness-greenness) changed the most,

closely followed by b*. For the foliage and yellow green patches, L* and b* varied the most, and for bluish green patch, it was L* and a* to be affected, while the change in b* was practically negligible.

As in other studies, the threshold of 3 CIELAB units as limit noticeable by an observer with normal colour vision (see Section 2.3.) is adopted. With the SAV aperture and the 50% white or black adaptors, none of the twelve studied plant species or of the four Gretag Macbeth patches crossed that threshold in the total difference of colour (ΔE^*_{ab}), being 2.9 CIELAB units the highest difference observed (Table 7.2). Remarkably, the 50% white adaptor on the SAV aperture performed slightly better than the black adaptor on specific plant samples (Table 7.2). With all other settings, instead, the differences of colour spanned from 3.8 to 48.3 CIELAB units (mostly between 20-30 CIELAB units) in plant samples and from 6.6 to 57.2 CIELAB units in Gretag Macbeth patches, corresponding to a very noticeable difference respect to the colour measured without adaptor. In the case of plant samples, the impact on colour measurements is extreme if the white 50% adaptor is used on the MAV aperture. In the homogeneous patches (except in bluish green), this occurs when employing the white 70% adaptor on the MAV aperture, and to a minor extent on the SAV aperture. The adaptors did not perform better on fresh plants than on their counterpart from the herbarium. It was on two fresh plants, *C. isophylla* and *H. hederaceus*, that measurements with adaptors not only lay within the non-noticeable colour differences (< 3 CIELAB units), but they even come close to 1 CIELAB unit.

The contribution from the adaptor type (black or white, 70% or 50%, or no adaptor) on both MAV and SAV to the colorimetric information (L*, a* and b*) was analysed by distributing the measurements in a 2-dimensional Euclidean ordination space with a Principal Coordinates Analysis (PCoA) The colour measurements obtained with an adaptor (black or white) reducing 50% of the SAV opening overlaps accurately with the measurements without adaptors. The first two axes explain the major part of the data variability, both in plants (84.38% PC1, 11.68% PC2) (Figure 7.5) and in the four green Gretag Macbeth patches (59.28 % PC1, 31,81% PC2) (Figure 7.6).

Between the two, the 50% white shows a slightly more accurate approximation than black 50%, as visible from the distance centroids-ellipse. In the case of the MAV, the best adaptor is the black 50%, but overall it is evident that the measurements obtained by using the remaining adaptors are very far from the real colour.

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Table 7.2. Colorimetric analysis (total colour differences, ΔE^*_{ab}) of the measurements with black 50%, white 50%, black 70% and white 70% adaptors, compared to those obtained with no adaptor. ΔE^*_{ab} below the threshold of perception (< 3.0 CIELAB units) are in bold and underlined. Plants marked with an asterisk (*) are dry material from herbarium specimens.

		Jasione montana	*Trachelium caeruleum*	*Campanula rotundifolia*	*Campanula isophylla*	*Hesperocodon hederaceus*	*Jasione laevis*
MAV	50% black	9.5	7.4	4.5	3.8	5.6	5.3
	50% white	25.0	26.1	24.1	24.5	22.8	23.9
	70% black	22.1	19.1	15.0	12.4	17.4	12.3
	70% white	46.4	48.3	46.8	47.8	45.1	47.1
SAV	50% black	**2.5**	**2.4**	**2.0**	**0.8**	**0.7**	**1.2**
	50% white	**2.1**	**2.7**	**1.8**	**1.1**	**1.1**	**1.4**
	70% black	21.1	19.6	15.9	11.7	16.1	11.0
	70% white	34.4	36.1	34.4	35.9	34.6	34.9

		*C. adsurgens**	*C. latifolia**	*C. mollis**	*C. glomerata**	*C. trachelium**	*C. vesicular**
MAV	50% black	7.4	8.5	4.1	6.5	9.7	5.4
	50% white	14.8	18.6	15.9	20.4	17.1	19.1
	70% black	19.9	18.1	17.4	21.0	21.1	19.8
	70% white	34.5	40.1	35.2	41.8	37.5	39.0
SAV	50% black	**2.1**	**2.9**	**1.5**	**2.4**	**2.9**	**2.1**
	50% white	**1.1**	**2.4**	**2.8**	**2.4**	**2.0**	**0.7**
	70% black	18.9	17.6	17.3	18.4	19.5	18.9
	70% white	30.6	35.1	26.4	37.2	34.0	33.4

		GREEN	FOLIAGE	YELLOW GREEN	BLUISH GREEN
MAV	50% black	10.7	6.6	12.7	7.9
	50% white	27.1	22.3	26.3	13.1
	70% black	34.5	20.4	42.4	29.7
	70% white	54.2	45.6	57.2	31.4
SAV	50% black	**2.8**	**1.9**	**2.9**	**2.5**
	50% white	**1.3**	**1.4**	**2.6**	**0.5**
	70% black	29.7	16.5	36.6	23.9
	70% white	47.2	38.7	50.9	25.2

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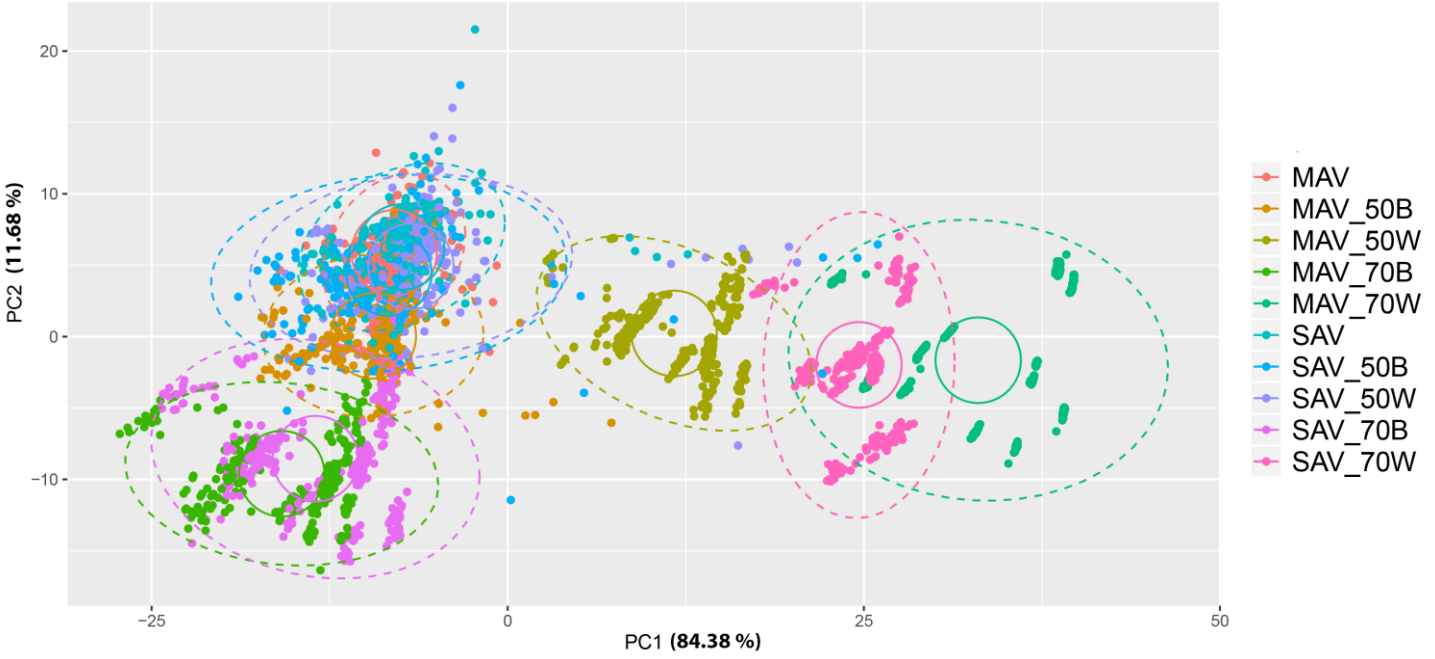

Figure 7.5. PCoA representing all the colour measurements of the plants with and without adaptor. Ellipses around each type of adaptor indicate the dispersion of the measurements and circumferences encircle the centroids of the distributions of measurements of each adaptor type. Samples were coloured according to the used adaptor. MAV, medium area view; SAV, small area view; 50B, 50% reduction with black adaptor; 50W, 50% reduction with white adaptor; 70B, 70% reduction with black adaptor; 70W, 70% reduction with white adaptor.

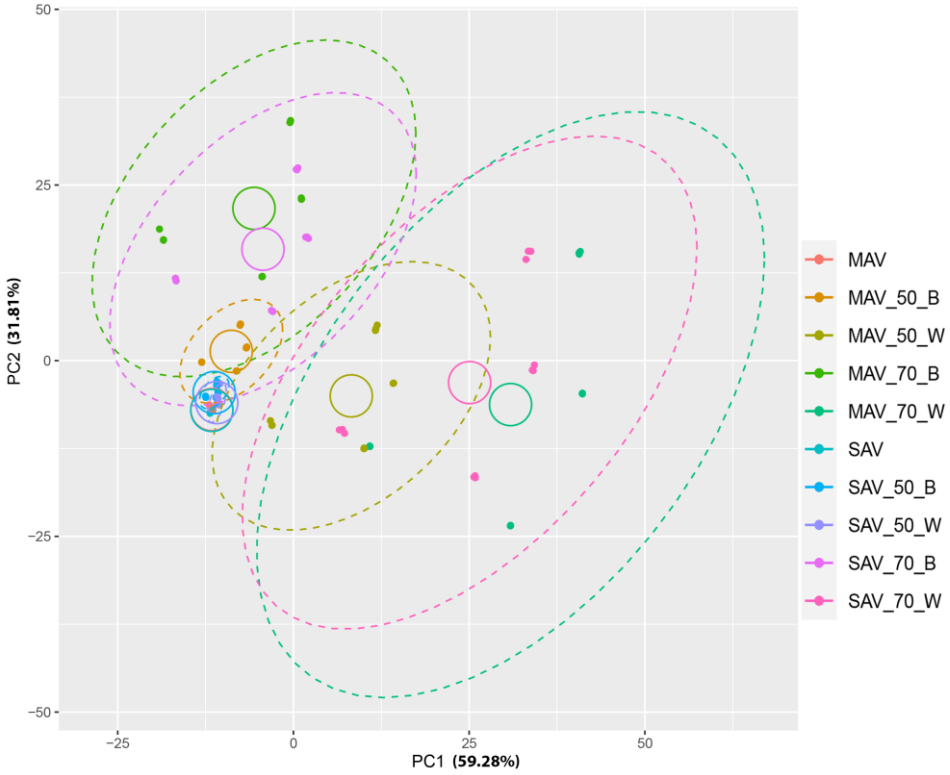

Figure 7.6. PCoA representing all the colour measurements of green patches of the Gretag Macbeth chart with and without adaptor. Ellipses around each type of adaptor indicate the dispersion of the measurements and circumferences encircle the centroids of the distributions of measurements of each adaptor type. Samples were coloured according to the used adaptor. MAV, medium area view; SAV, small area view; 50B, 50% reduction with black adaptor; 50W, 50% reduction with white adaptor; 70B, 70% reduction with black adaptor; 70W, 70% reduction with white adaptor.

Figure 7.7. Percentage of the measurements that have been assigned to a predicted group after the use of the adaptors on (a) MAV and (b) SAV. Samples were coloured according to the used adaptor. MAV, medium area view; SAV, small area view; 50B, 50% reduction with black adaptor; 50W, 50% reduction with white adaptor; 70B, 70% reduction with black adaptor; 70W, 70% reduction with white adaptor.

To assess the similarity of the measurements obtained with and without the different adaptors, a Quadratic Discriminant Analysis (QDA) was performed. The mean squared error (MSE) was 0.338 in the SAV analysis and 0.046 in the MAV analysis. The cross-validation of QDA classifications left misclassification rates almost identical to the original analyses, with 0.317 and 0.047 MSE in SAV and MAV, respectively, validating the model in both analyses. The data obtained with SAV reveal a relatively high misclassification rate (33.8 %) in the confusion matrix that can be used to identify the adaptors with the minor impact on the real colour measured with SAV. The major part of measurements from SAV with white (57%) and black (53%) 50% adaptors are better classified as obtained with SAV without adaptor than within their predefined group. Thus, to measure the colour with these adaptors is as reliable as to measure it without (Figure 7.7). In addition, some mutual misclassification is detected between the measurements obtained with white and black 50% reducing adaptors: 17% of the measurements obtained with the white adaptor are classified as obtained with the black and 16% of the ones obtained with the black are classified as obtained with the white. Very few measurements from the 50% reducing adaptors were assigned to the 70% reducing adaptors (2%) and no measurement from white or black 70% adaptors was misclassified, indicating that measurements from these adaptors are very dissimilar to those obtained without adaptor. On the contrary to SAV, the misclassification rate in the QDA of the data collected with MAV was very low (0.046 %). The only significant misclassification in the analysis, showing some similarity between measurements obtained with any adaptor and without adaptor, is also explaining most of the total misclassification related to MAV: 10% of data collected with the black 50% reducing adaptor to the group of MAV without adaptor. Nevertheless, the percentage obtained with MAV is far from the values obtained with the SAV 50% reducing adaptor, thus suggesting it as the most appropriate adaptor for measuring colour in small biological samples.

An approach combining k-means and agglomerative clustering was performed on the data from SAV only, as MAV adaptors has been repeatedly identified as highly dissimilar to the colour measured

without adaptor by the previous analyses. Table 7.3 summarizes the clustering of measurements, with the number of k-clusters and the AU *p*-values of each k-clusters. Except for few data from *C. glomerata* (4% of SAV 50% black and 4% of SAV 50% white) and *C. latifolia* (8% of SAV 50% black), all the measurements obtained with the 50% adaptors fall in the same clusters as with no adaptor. Thus, this approach strongly supports the results of the previous analyses, by establishing that the 50% adaptor is the best solution to obtain a reliable measurement on small samples. Between the two 50% adaptors, the only observable difference is in the case of *C. latifolia*, where the white adaptor performs better than the black.

4. DISCUSSION

Trait-based approaches are increasingly widespread, because of the important role of colour. The use of spectrophotometers, colorimeters or chroma-meters to measure the colour of leaves and flowers as a proxy is a valuable strategy to gather information about the eco-physiological status of plants and the expressed genetic variability within and among populations or taxa. The former is particularly relevant in a world affected by global warming to detect anomalies in crops due to environmental stressors, nutrient deficiencies, contaminants or diseases (Hang *et al.*, 2019). In particular, the colour of leaves is considered as a key feature for the plant functional traits, since leaves are primary drivers of the capture of light and photosynthesis processes (Chianucci *et al.*, 2019). For instance, the called "winter-red-leaf syndrome" (Kytridis *et al.*, 2008; Nikiforou *et al.*, 2011) has been correlated with a nitrogen deficiency, low carboxylation efficiency and high risk of photoinhibition.

By using a portable version of these devices, colour can be measured on-site, thus preserving the integrity of the plant and protecting species that may be at risk of extinction (Cordlandwehr *et al.*, 2013). In addition, a portable device can be useful for botanists, ecophysiologists or ecologists to save specimens for future analysis and compare the colour after periods of time (Carranza-Rojas *et al.*, 2017).

Regular colour measurement can be also planned from time to time to monitor the conservation conditions of herbaria: the correct temperature, humidity and light should be constantly maintained to avoid degradation and the specimens' colour is highly indicative of their conservation state (Bedford, 1999).

For this study, leaves from 12 different species of the Campanulaceae family, coming from different habitat types and conservation conditions, from living plants to herbarium specimens, and therefore encompassing a wide range of colour variations were chosen. They belong to a group of plants with a number of representatives from threatened alpine environments. Within them, *Jasione* is a genus in evolutionary radiation with numerous endemics of small area, many from alpine habitats, and different edaphic restrictions and ecological preferences (Parnell, 1987; Serrano *et al.*, 2009), which makes it suitable to test plant response to edaphic stress. Indeed, plants from alpine ecosystems are also ideal indicators of environmental variations related to global climate change (GLORIA, 2019). Despite their importance, the small size of the leaves gathered in rosettes of many alpine plants (Nagy, 2009) offered many challenges to the researchers. Leaves of Campanulaceae, though, are not the only ones offering a similar challenge.

To perform unbiased free detections of colour measurement of small plant leaves, a simple method based on narrowing the field of view of the device is outlined and proposed here. To assess the reliability of the method development outputs, both the perception limit of the human eye (colorimetric criteria) and multivariate statistic approaches have been chosen. It has been demonstrated that 3 mm aperture with an adaptor reducing 50% of the area allows a sound colour assessment In addition, cardboard adaptors in white performed better than their counterpart in black. This conclusion is reached both in the samples of plant leaves and in the homogeneously coloured Gretag Macbeth patches. Since it is proved that the method works for both sample types, it is possible to extend it to other types of plant samples, regardless of their specific characteristics (e.g. concavity, intrinsic colorimetric heterogeneity, small defects).

Table 7.3. Results of the k-means cluster analysis on SAV measurements with the different adaptors compared to no adaptor. The number of clusters formed (k) and the related approximately unbiased (AU) p-values are indicated for each k-cluster. Clusters with AU p-value equal to or greater than 95% are considered strongly supported by data. The clusters where the SAV measurements with no adaptor (real colour) fall are indicated in bold.

Fresh material				**Herbarium material**			
		AU *p*-value	**k**			**AU *p*-value**	**k**
Jasione laevis	**A= {100% SAV, 100% SAV50W, 100% SAV50B, 100% SAV70W}**	**97**	2	*Campanula adsurgens*	**A= {100% SAV, 100% SAV50W, 92% SAV50B}**	**99**	3
	B= {100% SAV70B}	97			B= {100% SAV70W, 8% SAV70B}	97	
					C= {10% SAV70B}	97	
Jasione montana	**A= {68% SAV, 100% SAV50W, 100% SAV50B}**	**86**	4	*Campanula glomerata*	**A= {100% SAV, 96% SAV50W, 96% SAV50B}**	**89**	3
	B= {32% SAV}	**100**			B= {100% SAV70W, 4% SAV50W}	97	
	C= {100% SAV70W}	100			C= {100% SAV70B, 4% SAV50B}	99	
	D= {100% SAV70B}	100					
Campanula isophylla	**A= {100% SAV, 100% SAV50W, 100% SAV50B, 100% SAV70W}**	**97**	2	*Campanula latifolia*	**A= {100% SAV, 100% SAV50W, 92% SAV50B}**	**99**	3
	B= {100% SAV70B}	97			B= {100% SAV70W, 8% SAV50B}	97	
					C= {100% SAV70B}	97	

Fresh material				Herbarium material			
		AU *p*-value	k			AU *p*-value	k
Campanula rotundifolia	**A= {56% SAV, 56% SAV50B, 60% SAV50W}**	**88**	4	*Campanula mollis*	**A= {84% SAV, 72% SAV50W, 88% SAV50B}**	**89**	3
	B= {44% SAV, 44% SAV50B, 40% SAV50W}	**88**			**B= {16%SAV, 28% SAV50W, 12% SAV50B}**	**86**	
	C= {100% SAV70B}	100			C= {100% SAV70B}	99	
	D= {100% SAV70W}	100					
Trachelium caeruleum	**A= {100% SAV, 100% SAV50W, 100% SAV50B}**	**100**	3	*Campanula trachelium*	**A= {100% SAV, 100% SAV50W, 100% SAV50B}**	**100**	3
	B= {100% SAV70W}	97			B= {100% SAV70W}	99	
	C= {100% SAV70B}	97			C= {100% SAV70B}	97	
Hesperocodon hederaceus	**A= {100% SAV, 100% SAV50B, 100% SAV50W}**	**100**	3	*Campanula versicolor*	**A= {100% SAV, 100% SAV50W, 100% SAV50B}**	**99**	3
	B= {100% SAV70B}	100			B= {100% SAV70W}	97	
	C= {100% SAV70W}	100			C= {100% SAV70B}	97	

Furthermore, it seems to indicate that the effectiveness of the proposed method is independent of the sample surface texture, defined as the visual characteristic and tactile quality of the surface of a material. This may be largely due to having included the specular component in the measurements (i.e. SCI mode). While the Specular Component Excluded (SCE) mode magnifies the colour differences due to the surface of the material, the SCI mode is adequate for analyzing the intrinsic colour of objects and minimizes the colour differences caused by the texture or surface finish (Sanmartín *et al.*, 2011c).

On the other hand, the better performance of the white template could be related to the optic set up of diffuse illumination geometry, accomplished by an integrating spherical device coated with a white material such as barium sulphate (see Section 2.2.). The white adaptor may form a continuum with the sphere, with a material as white as the sphere, thus amplifying the effect of the sphere and providing accurate measurements on SAV.

Also, there are results showing a different behavior of the colour parameters (L*, a* or b*) when recovered with the use of the templates, varying widely both within and among the plant species and colour patches. This difference in performance seems to follow a clear pattern: the parameters with a higher value (stronger component colour) are those most influenced by the templates. For example, for bluish green patch, with achromatic values in the yellow-blue component around 1 CIELAB unit (data not shown), the impact on b* parameter is practically negligible when the adapter is used.

5. CONCLUSIONS

An affordable and reliable methodology to measure the colour in samples that do not fully cover the aperture of a spectrophotometer is proposed. Following colorimetric and statistical criteria, the only setting providing indistinguishable colours from the real one (measured with no adaptor) is the 50%-reducing adaptor on the 3-mm aperture (SAV). In addition, statistical analyses suggest the choice of a white

adaptor. This research demonstrates that, by using the proposed methodology, it is possible to gather ecological information in a sound, inexpensive and non-destructive way from the colour of leaves, petals and other small samples.

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General Conclusions

Jasione is an ancient lineage originated in the Oligocene but relatively recent diversification of extant groups, c. 9 Mya. Significant morphological stasis and recent radiation, with a Pleistocene burst of diversification, make *Jasione* one of the most enigmatic and taxonomically difficult genera of the Campanulaceae.

Jasione shows one of the most remarkable biogeographic patterns of the Mediterranean basin, the east-west disjunction. Although the genus originated in western Mediterranean, the history of vicariant and dispersal events that lead to extant lineages of *Jasione* was as a dynamic roundtrip of lineages between both sides of the Mediterranean. Niche conservatism and adaptation to ecotonal sub-Mediterranean mountain shape the disjunct pattern, as intermediate European and Alpine regions are environmentally unsuitable regions. Several processes have relatively enhanced the niche breath of different groups, as recurrent polyploidization in different Iberian lineages, or evolution of annual habit, that appeared independently three times during the evolution of the genus and allowed widespread expansion in lowland areas, with *J. montana* as the most striking case. Hydathoid is a charismatic trait exclusive of one of the two main clades of *Jasione*. Apparently related with the hydric status of the plant, its occurrence seems to impose physiological constraints, and reduction in hydathoid number is positively correlated with habitat aridity.

Environmental niche analyses, phylogeographic approaches and integrative taxonomic studies from a combination of lines of evidence in different Iberian taxonomic complexes, revealed the importance of detailed studies to unveil hidden biodiversity and its underlying processes, as niche shift associated to ploidy changes or niche

expansion to new edaphic environments after adaptation, that eventually lead to speciation

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