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Genetic structure and geographic variation in ecophysiological traits of *Arbutus unedo* L.

Memoria que presenta
Xabier Santiso Carral
para optar ao Grao de Doutor en Bioloxía

Fdo: **Xabier Santiso Carral**
Santiago de Compostela, Maio 2015


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CERTIFICAN:

Que a presente memoria titulada "Genetic structure and geographic variation in ecophysiological traits of *Arbutus unedo* L." presentada por D. **Xabier Santiso Carral** para optar ao **Grao de Doutor en Bioloxía** foi levada a cabo baixo a nosa dirección.

Considerando que dito traballo está en condicións de ser defendido como Tese Doutoral, autorizamos a súa presentación ante o Tribunal correspondente.

E para que así conste, firmamos a presente en Santiago de Compostela a ____ de Maio de 2015.

Fdo: J.C. Rubén Retuerto Franco   Fdo: Rodolfo Barreiro Lozano


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O que a eiruga interpreta coma a fin do mundo é
o que o mestre chama bolboreta.
Richard Bach

A todas esas persoas que sempre creron neste día...


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General Introduction


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Intraspecific variation

During the last few years, the importance of intraspecific variation has attracted increasing attention in the field of Ecology. Traditionally, many models of interaction between species assume that all conspecific individuals are effectively interchangeable, as ecologically equivalent (Bolnick *et al.*, 2003; Bolnick *et al.*, 2011). This simplification is only justified if inter-individual niche variation is rare, weak, or has a trivial effect on ecological processes (Bolnick *et al.*, 2003). However, the most common situations are those where the intraspecific trait variation alters ecological dynamics. In fact, a review of Bolnick *et al.* (2011) summarized several general mechanisms by which intraspecific variation changes the outcome of ecological interactions. Some of them are: when ecological parameters are nonlinear functions of the traits, when trait variance affects niche width, in cases of distinct phenotypes which exhibit asynchronous fluctuations in density over time, or when trait variation is stochastically sampled in small populations.

Many studies have analysed the effect of intraspecific variation showing that it can account for differentiation in anti-predator defences, parasite resistance, abiotic tolerances, use of resources, competitive ability, survival, and breeding probabilities (Cam *et al.*, 2002; Bolnick *et al.*, 2011). Furthermore, many studies showed a tight relationship between functional diversity and species richness, and some models determined that the existence of intraspecific variability facilitates species coexistence (Lichstein *et al.*, 2007; Cianciaruso *et al.*, 2009; Yamauchi & Miki, 2009). Likewise, Albert *et al.* (2010a) demonstrated the important role of intraspecific variation for several plant species, using many different traits and sampling populations from a wide range of environmental conditions. In another experiment, Albert *et al.* (2010b) showed that variability between populations was partly explained by environmental gradients. Consequently, individual or population variation has important ecological, evolutionary, and conservation implications (Bolnick *et al.*, 2003; Bolnick *et al.*, 2011), and the recognition of intraspecific variation opens new opportunities to understand and predict ecological patterns in a changing environment (Cianciaruso *et al.*, 2009; Albert *et al.*, 2010b).

Recent developments in trait-based community ecology have underlined the need to integrate variation at intraspecific and interspecific levels which, combined with traditional analytical tools, can provide unique insights to understand the primary forces that structure communities (Violle *et al.*, 2012). Despite the importance of integrating both levels, there is still a lack of studies incorporating intraspecific variation. There is, therefore, a need to carry out experiments testing within species variation and here we contribute to this aim by showing a range of analyses at intraspecific level in the widespread shrub *Arbutus unedo* L. In particular, we studied the phylogeography of this plant, determined its genetic diversity and ecophysiological variance under key environmental factors such as water and nutrient availability. In addition, we followed an integrative approach (Q_{ST}-F_{ST}) where quantitative traits and genetic information were analysed jointly to increase our understanding of the adaptive and evolutionary strategies of the species.

Arbutus unedo L.

Description

Arbutus unedo L. (strawberry tree) is a small tree or shrub described by Carl Linnaeus in 1753. According to Andrés Laguna (a Spanish botanist, physician and humanist of the XVI century), the name *unedo* has its origin in the Latin numeral *unus* (one) combined with the verb *edo* (to eat) to mean “to eat just one (fruit)”, suggesting that it is advisable a moderate consumption of its fruits because their alcoholic content can produce headaches. The name of the genus *Arbutus* refers to its condition of “small tree”. Also related to the fruit is the name that *A. unedo* receives in some areas of Ireland, “Cain´s apple tree”, which can be the origin of another quite common name given to the species “Cane apple”. “Cain´s apple tree” would refer to the biblical character Cain, who killed his brother Abel with a branch of *A. unedo*, being the red fruits an evocation of the blood (García M. & Fernández Z., 2011).

Arbutus unedo is an evergreen tree usually rather small (6-8 meters tall), sometimes referred to as shrub, that occasionally reaches 12-14 meters tall in competition for light, as it cannot tolerate shading (Fig. 1), and with a crown up to 8 meter wide (Sealy, 1949; Sealy & Webb, 1950; Mitchell, 1993). The highest

Fig. 1. *Arbutus unedo* in Ireland.

strawberry tree in Spain can be found at the Palace of Aranjuez (13 meters tall) (García M. & Fernández Z., 2011). The stem and branches are covered by a reddish brown bark that peels off into small strips (Fig. 2); the degree of development of this red pigment is directly dependent on the intensity of illumination received. Leaves are leathery (laurel-like) with a finely serrated margin, while mycorrhizas are common in the root system (Sealy & Webb, 1950; García M. & Fernández Z., 2011). Flowering avoids summer droughts and ranges from late September to December when nights are lengthening (Cox & Moore, 2005). Pollination is entomophilous and pollen is neither abundant nor well dispersed (Mitchell, 1993). Typical pollinators are bees and bumblebees (Fig. 3); its flowers are considered of special relevance for the ecosystem as they are a source of nectar and pollen for *Bombus terrestris*, which develops huge autumn populations feeding exclusively on this species (Soro & Paxton 1999; Rasmont *et al.*, 2005). Accordingly, cross-pollination probably takes place

Fig. 2. Typical bark of *Arbutus unedo*.

Fig. 3. Flowers and pollinator (bumblebee) of the strawberry tree.

via insects, but self-pollination is probably frequent as well (Sealy 1949; Sealy & Webb, 1950; Mitchell 1993). Fruits, 20-25 mm warty red berries (Fig. 4) with many small seeds, ripen 12-14 months later, when plants flower again, and are dispersed by animals. Some of the birds that eat these fruits are *Turdus philomelos* Turt, *Turdus merula* L., *Turdus pilaris* L., *Columba palumbus* L., *Garrulus glandarius* L., *Sylvia atricapilla* L. or *Erithacus rubecula* L. (Sealy 1949; Sealy & Webb, 1950; Cox & Moore, 2005; García M. & Fernández Z., 2011). Likewise, besides the association of

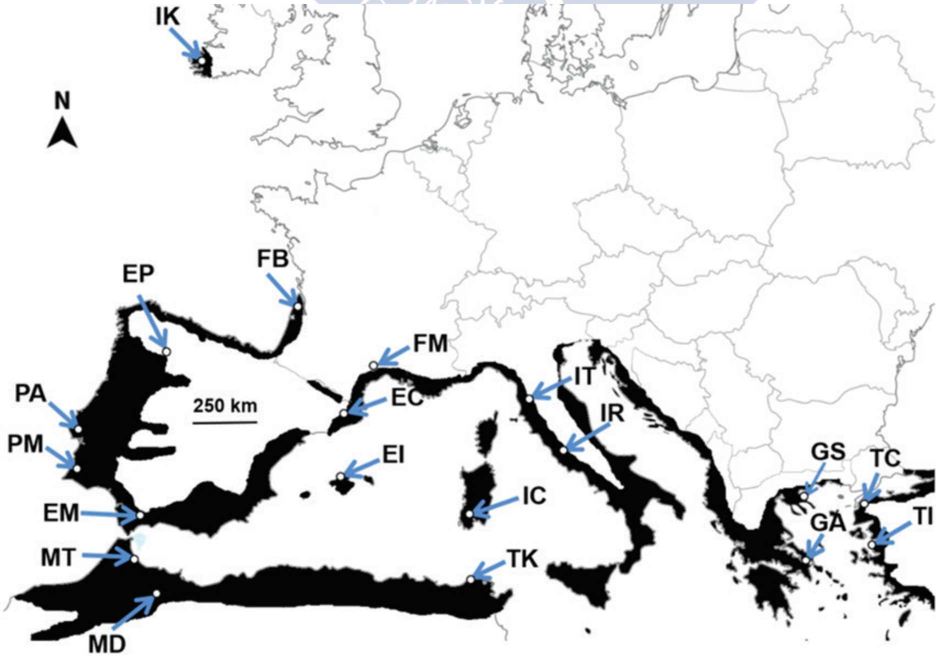
Fig. 4. Strawberry tree fruits.

the strawberry tree with the mentioned bees, bumblebees and birds, the butterfly *Charaxes jasius* also shows a strong larvae-dependence on *Arbutus unedo* (Verdasca *et al.*, 2012).

Finally, it seems important to highlight the phenological particularities of the Irish populations. In Ireland, winter is unfavourable for plant growth and *A. unedo* passes it in a dormant state. Together the lack of pollinating agents during the flowering period, these harsh climatic conditions explain the low fruit production of the Irish populations (Sealy 1949; Cox & Moore, 2005).

The strawberry tree grows from sea level up to 1200 m, mostly on cliffs, broken rocky slopes, and rock outcrops, which are edaphically unsuitable for woodland and where the vegetation is restricted to shrub communities, conditions under which *Arbutus* can compete successfully with other plants (Sealy, 1949). The species prefers a Mediterranean climate with an oceanic component, inhabiting mainly coastal and inland areas with benign climates where frosts are unusual and summer dryness is weak (Torres *et al.*, 2002). Regarding soil conditions, the strawberry tree prefers siliceous or decarbonated substrata, although it also occurs on formations of carbonated substrata, on heavy clay soils, sandstone and limestone, with pH ranging from 4 to 7 (Sealy, 1949; Torres et al. 2002; Özcan & Haciseferoğullari, 2007; Takrouni & Boussari, 2010). The distribution of the species is limited by a mean January temperature of 40° F (4.4°C) (Sealy 1949), probably due to the effects of low temperatures on seedlings because adult trees tolerate quite intense frosts without any obvious damage, although recurrent temperatures of -10°C can kill them (Sealy & Webb, 1950). As a result of these preferences and, of course, by the effects of historical and geographical circumstances that may have modulated its spread, the current distribution range of *A.*

Fig. 5. Distribution range of *Arbutus unedo* (black area) and populations sampled for this study (Ireland Killarney-IK; France Bordeaux-FB; Spain Ponferrada-EP; Portugal Arrabida-PA; Portugal Monchique-PM; Spain Málaga-EM; Morocco Tanger-MT; Morocco Debdou-MD; Spain Mallorca Island-EI; Spain Catalonia-EC; France Montpellier-FM; Italy Cagliari-IC; Tunisia Kroumerie-TK; Italy Rome-IR; Italy Toscana-IT; Greece Sithonia-GS; Greece Athens-GA; Turkey Izmir-TI; Turkey Çannakale-TC), marked with the small white dot that the arrowheads denote.



unedo L. (Fig. 5) is mainly circum-Mediterranean (it belongs to the Mediterranean maquis), from Morocco to Tunisia and from Spain to Turkey. However, the strawberry tree also inhabits the Atlantic facade of Europe along the coast of the Iberian Peninsula, spreading along the west coast of France and even reaching Ireland where it occurs mainly in the South West (Sealey 1949; Webb 1983). In some other countries, such as Tunisia, the populations of *A. unedo* have been severely depleted by deforestation (Takrouni *et al.*, 2012; personal observations).

Germination and propagation

According to the literature, a large fruit of the strawberry tree may contain up to 40 seeds, although the average number of seeds is about 22; fruit weight ranges 1-11 g and seed viability is around 55% (Sealy 1949; Sealy & Webb, 1950; Özcan & Haciseferoğullari, 2007; Celikel *et al.*, 2008). Seed germination is strongly influenced by temperature, with an optimum at 20°C (germination rate of 19.2 % after seven weeks); beyond this temperature, germination success decreases sharply (Hammami *et al.*, 2005). Interestingly, sulphuric acid did not affect germination capacity nor velocity, suggesting an effective dispersal by endozoochory (Narbona *et al.*, 2003). In comparison, seeds included in the fruit pulp have a very low rate of germination (< 4.2 %) because of the presence of certain inhibitory substances (Hammami *et al.*, 2005; Narbona *et al.*, 2003). After germination, seedling mortality has been reported to be >60 % (Sealy 1949).

We have some additional (unpublished) information on germination as well as on seed and fruit characteristics from 19 populations of *A. unedo*. Seed viability (%) was determined using the tetrazolium test (Rubio-Casal *et al.*, 2003; Sawma & Mohler, 2002), and seed germination rate (%) was assessed in peat (census after three months under natural environmental conditions) and petri plates (census after three months in phytotron at 20°C) (Table 1). The lowest mean fruit weight was 1.46 g in population IK, almost four times lower than the maximum mean fruit weight of 4.75 g recorded in GS (Fig. 6). The average weight of seeds ranged from 0.94 mg in MT to 2.27 mg in MD (Fig. 7) while the mean number of seeds per fruit ranged from 11 in FB to 31 in MD,

Table 1. Seed viability and germination rates in peat and Petri plate assays for each provenance (Ireland Killarney-IK; France Bordeaux-FB; Spain Ponferrada-EP; Portugal Arrabida-PA; Portugal Monchique-PM; Spain Málaga-EM; Morocco Tanger-MT; Morocco Debdou-MD; Spain Mallorca Island-EI; Spain Catalonia-EC; France Montpellier-FM; Italy Cagliari-IC; Tunisia Kroumerie-TK; Italy Rome-IR; Italy Toscana-IT; Greece Sithonia-GS; Greece Athens-GA; Turkey Izmir-TI; Turkey Çannakale-TC) of *Arbutus unedo*.

Provenance	Seeds viability (%)	Germination rate (peat)	Germination rate (Petri)
IK	69%	5%	28%
FB	72%	2%	54%
EP	93%	9%	84%
PA	81%	11%	38%
PM	83%	12%	50%
EM	100%	12%	88%
MT	60%	6%	46%
MD	100%	28%	86%
EI	22%	16%	18%
EC	93%	18%	58%
FM	97%	25%	72%
IC	55%	43%	44%
TK	86%	34%	78%
IR	86%	14%	34%
IT	76%	24%	46%
GS	36%	8%	22%
GA	56%	2%	6%
TI	83%	20%	74%
TC	93%	6%	76%

Fig. 6. Average fruit weight (g) (± standard error) for each provenance (Ireland Killarney-IK; France Bordeaux-FB; Spain Ponferrada-EP; Portugal Arrabida-PA; Portugal Monchique-PM; Spain Málaga-EM; Morocco Tanger-MT; Morocco Debdou-MD; Spain Mallorca Island-EI; Spain Catalonia-EC; France Montpellier-FM; Italy Cagliari-IC; Tunisia Kroumerie-TK; Italy Rome-IR; Italy Toscana-IT; Greece Sithonia-GS; Greece Athens-GA; Turkey Izmir-TI; Turkey Çannakale-TC) of *Arbutus unedo*. The highest and lowest values are indicated.

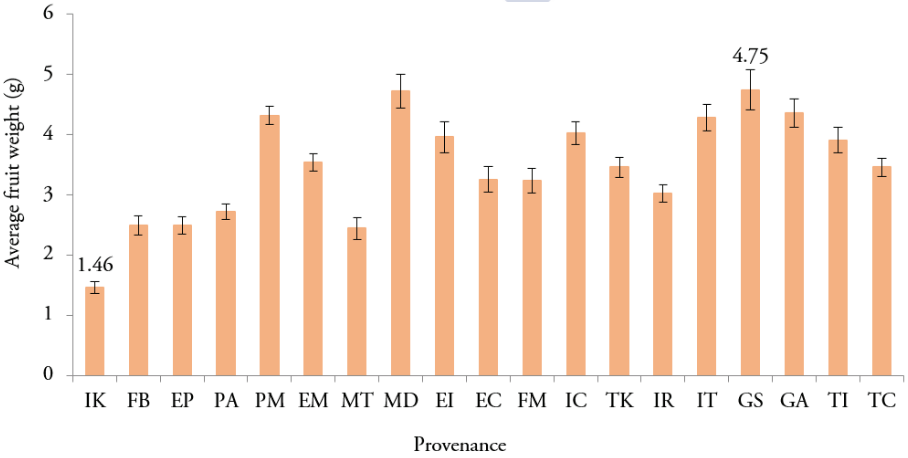


Fig. 7. Average seed weight (mg) (± standard error) for each provenance (Ireland Killarney-IK; France Bordeaux-FB; Spain Ponferrada-EP; Portugal Arrabida-PA; Portugal Monchique-PM; Spain Málaga-EM; Morocco Tanger-MT; Morocco Debdou-MD; Spain Mallorca Island-EI; Spain Catalonia-EC; France Montpellier-FM; Italy Cagliari-IC; Tunisia Kroumerie-TK; Italy Rome-IR; Italy Toscana-IT; Greece Sithonia-GS; Greece Athens-GA; Turkey Izmir-TI; Turkey Çannakale-TC) of *Arbutus unedo*. The highest and lowest values are indicated.

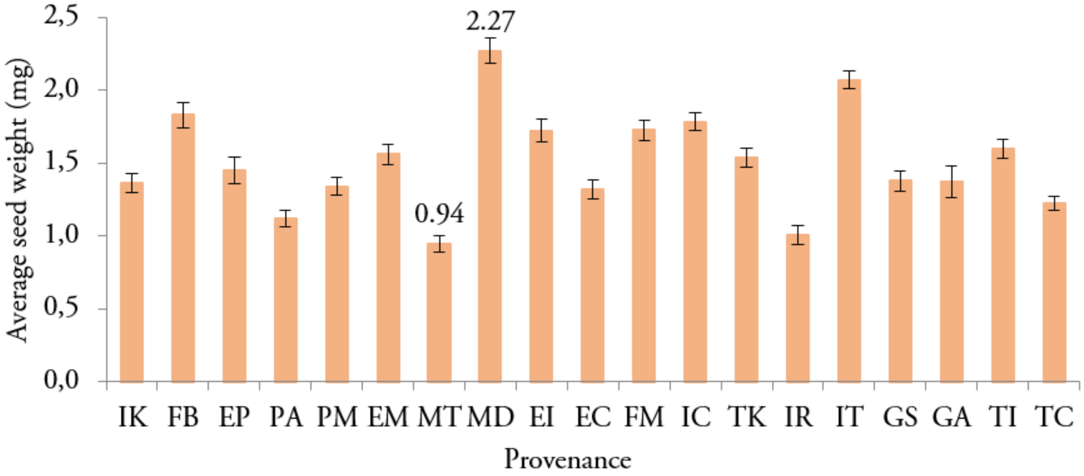
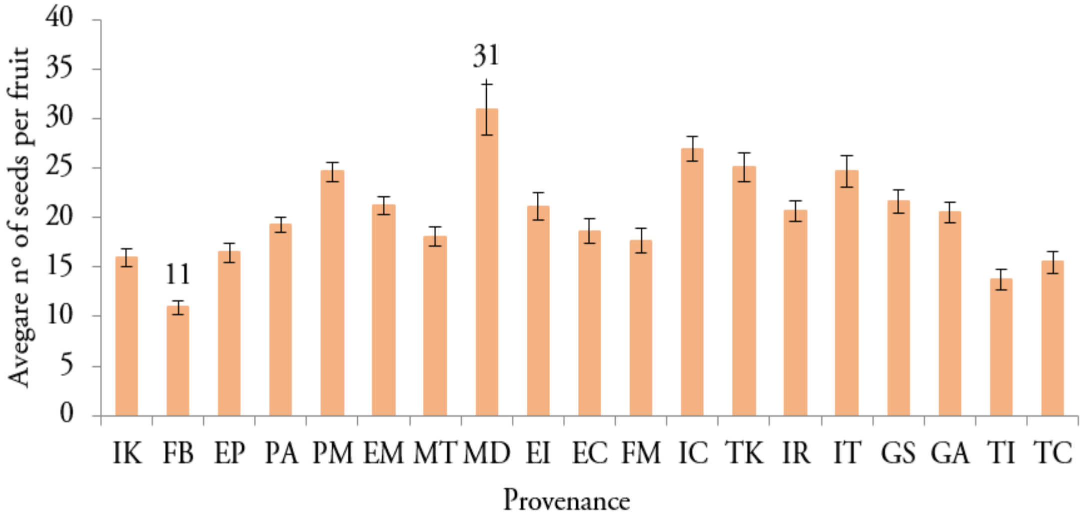


Fig. 8. Average number of seeds per fruits (± standard error) for each provenance (Ireland Killarney-IK; France Bordeaux-FB; Spain Ponferrada-EP; Portugal Arrabida-PA; Portugal Monchique-PM; Spain Málaga-EM; Morocco Tanger-MT; Morocco Debdou-MD; Spain Mallorca Island-EI; Spain Catalonia-EC; France Montpellier-FM; Italy Cagliari-IC; Tunisia Kroumerie-TK; Italy Rome-IR; Italy Toscana-IT; Greece Sithonia-GS; Greece Athens-GA; Turkey Izmir-TI; Turkey Çannakale-TC) of *Arbutus unedo*. The highest and lowest values are indicated.



almost three times higher (Fig. 8). Seed viability was consistently high, ranging from 56% in GA to 100% in MD and EM. Lastly, we observed the highest variation in the seed germination rate, varying from 2% in FB and GA to 43% in IC in peat, and from 6% in GA to 86% in MD in Petri plate (Table 1). Of note, germination rate was always lower in peat (typically below 50%) than in Petri plate. Altogether, the high intraspecific variation revealed by these values, especially in germination, support the need of an appropriate assessment of the ecophysiological and genetic intraspecific variation in *A. unedo*.

While asexual micropropagation of *A. unedo* in the laboratory has been shown using woody plant medium (WPM) containing peat and perlite (Mereti *et al.*, 2002), vegetative reproduction has been rarely described under field conditions (Sealy, 1949; Sealy & Webb, 1950). Nonetheless, the plants show strong resistance to hard environments and they are extremely tenacious under natural conditions. Accordingly, they have a natural ability for regenerate after cutting, and they survive damages to the main stem by sending new shoots from the base to replace the damaged stem (Sealy, 1949; Sealy & Webb, 1950; Özcan & Haciseferoğullari, 2007).

Medicinal properties and uses of the strawberry tree

The strawberry tree is a species of especial interest due to its medical applications. The leaves of *A. unedo* are used as a source of natural antioxidants given their phenolic content in tannins and flavonoids (Maleš et al., 2006; Oliveira et al., 2009); they also have diuretic, antiseptic, antidiarrheal, astringent, depurative and anti-inflammatory uses (Kivcak *et al.*, 2001; Afkir et al., 2008; Oliveira et al., 2009). In Morocco, *A. unedo* is one of the plants used as therapy for hypertension and diabetes (Ziyyat et al., 1997). The fruits of the strawberry tree are also a good source of antioxidants because they have a high flavonoid content, but also vitamins C and E, carotenoids, organic acids, sugars, potassium and dietary fibre (Maleš et al., 2006; Pallauf et al., 2008; Ruiz-Rodríguez et al., 2011). Nonetheless, flavonoid content is lower in fruits (0.10–0.29%) than in leaves (0.52–2.00%) (Maleš et al., 2006). Furthermore, extracts of *A. unedo* show antiplatelet properties in humans, so they might be used for the treatment and/or prevention of cardiovascular diseases (El Haouari et al., 2007). Similarly, *A. unedo* extracts reduce the development of hypertension, ameliorate cardiovascular and renal functions in rats (Afkir et al., 2008), and showed a potential cancer preventive character (Carcache-Blanco et al., 2006).

The wild berries of the strawberry tree are highly appreciated in the Mediterranean region where they have been traditionally collected and transformed into jams, marmalades and liquors that contribute to the local economy of the region (Alarcão-E-Silva et al., 2001; Ruiz-Rodríguez et al., 2011). For example, in Portugal berries are typically used to make spirits and liquors (Pallauf et al., 2008). Honey from the strawberry tree, mainly from Sardinia, is also very valued and its characteristic bitter taste makes this honey the most typical of the Mediterranean (Tuberoso et al., 2009). Other parts of the tree are likewise valuable. The bark is used for tanning leather given its high tannins content (García M. & Fernández Z., 2011), the branches have been used as decoration on festive days (Sealy 1949) while the wood, besides its use for charcoal, has served for ornamental articles and in carpentry due to its hardness and strength (Sealy & Webb, 1950; García M. & Fernández Z., 2011). Additionally, *A. unedo* is often cultivated as ornamental tree.

Finally, it is noteworthy to mention the existence of a genotype selection program for the strawberry tree that highlights its relevance. In this program, high quality phenotypes from the Central Black Sea are selected according to fruit weight, vitamin C content, acidity, fruit taste, juiciness or phenology (Celikel *et al.*, 2008). Furthermore, the berries of *A. unedo* have been proposed as an alternative to other fruits currently available in markets (as already happens in certain countries, such as Morocco), and they have been considered a source of bioactive compounds for dietary supplements or functional foods (Ruiz-Rodríguez et al., 2011).

Phylogeography

Phylogeography is a relatively new discipline, coined by Avise *et al.* (1987), that deals with the spatial arrangements of genetic lineages, especially within and among closely related species. Phylogeographic perspectives have consistently challenged conventional genetic and evolutionary paradigms, and they have forged empirical and conceptual bridges between the formerly separate disciplines of population genetics (microevolutionary analysis) and phylogenetic biology (in macroevolution) (Avise, 2009). An understanding of the phylogeographic history is crucial to addressing a range of evolutionary and ecological questions, from understanding the source of adaptive divergence to determining the factors structuring ecological communities (Knowles, 2009). Phylogeography is useful to detect the most recent common ancestry, to recognize the presence of admixed individuals or recent immigrants, to estimate the demographic history of a population (by determining the time of past bottlenecks or growth events) or to find the geographical location of refuge areas (Excoffier, 2004). Phylogeographic analyses also allow the identification of historically and evolutionary independent regions and to understand historical responses to changes in the landscape (Bermingham & Moritz, 1998), the exploration of phylogenetic relationships in small areas (Valcárcel *et al.*, 2006), and even the discovery of cryptic species (Shaw, 2000).

When undertaking a phylogeographic study, it is imperative to adhere to the principles of genealogical concordance: I) concordance within-locus; II) multi-locus; III) multi-species; IV) among multiple lines of empirical evidence (Avise, 2009). This means that results must be reinforced by observation from independent sources of evidence. Accordingly, to analyse the phylogeography of a Mediterranean species such as *A. unedo*, in addition to use several markers, we must take into account the patterns observed for other species in this area. Of note, however, the Mediterranean basin largely lacks the common phylogeographic patterns across species seen in other regions such as the Alps or North America. Factors contributing to this scarcity of common patterns could be the less drastic effects of Pleistocene glaciations, environmental heterogeneity, and the blurring of genetic footprints via admixing or by the accumulation of responses to paleoclimatic changes

(Nieto Feliner, 2014). Nonetheless, some commonalities can be still found in the region (see below), and potential drivers of the phylogeographic patterns have been described, such as vicariance events, gradual range expansion, long-distance dispersal or radiations (Nieto Feliner, 2014).

It is largely acknowledged that the current genetic structure of populations, species and communities in the Mediterranean area mostly formed during Quaternary ice ages, when most of the living organisms suffered great changes in their distribution (Hewitt, 2000). A total of 52 refugia (climatically stable area), where species were able to survive glacial periods, have been identified in the Mediterranean bioclimatic region (Médail & Diadema, 2009). Many of them (up to 25) are located in three major Mediterranean peninsulas (Iberia, Italy, and the Balkans), which contain high levels of genetic and species diversity ('phylogeographical hotspots'), probably because their mountainous topography helped to the survival of species through the ice ages (Médail & Diadema, 2009; Hewitt, 2011). For instance, postglacial colonization from three distinct, southerly refugia located in each of these three peninsulas has been reported (Lumaret *et al.*, 2002), and it has also been described that northern regions were colonized from Iberian and Italian refugia (Taberlet *et al.*, 1998). These events lead to a decrease in genetic diversity towards higher latitudes in Europe (Hewitt, 2011). However, further considerations must be taken into account when analysing the role of Quaternary peninsular refugia in the Mediterranean. Firstly, the long-term persistence of isolated populations seems to have been common in the Mediterranean and, consequently, in many cases the current genetic structure probably reflects pre-quaternary divergence not related to a particular cold period (Taberlet *et al.*, 1998; Petit *et al.*, 2005). For example, Tertiary migrations of some tree taxa seem to have left their footprints in the current genetic structure (Petit *et al.*, 2005), it is suggested that some species may have persisted in Tertiary refugia (Lumaret *et al.*, 2002). Additionally, we must consider the likely existence of 'refugia-within-refugia', multiple and independent refugia inside what had been previously thought as a single refugium (typically the Peninsulas). The unaccounted existence of ‘refugia-within-refugia’ may confound the interpretation of phylogeographic patterns of European species (Gómez & Lunt, 2007).

A west-east increase in genetic diversity is another relatively common pattern in the Mediterranean (Conord *et al.*, 2012). This pattern suggests the important role that two processes might have played in shaping the genetic diversity during and after the cold periods of the Pleistocene: an east-west recolonization during the Holocene, and a population size contraction under local Last Glacial Maximum climate in resident western Mediterranean populations (Conord *et al.*, 2012). On this regard, Lumaret *et al.* (2004) reported substantial genetic differentiation between eastern and western Mediterranean populations in many species. On the other hand, the consequences of phylogeographic barriers, with a focus in the role of straits (Gibraltar, Sicily, Otranto, Dardanelles-Bosphorous), have also been explored (Hewitt, 2000). Some species show evidence of multiple colonization events across these barriers (Fernández-Mazuecos & Vargas, 2010) whereas an ancient isolation between African and European populations has been deducted for other species where the straits acted as barriers, despite recent events of long-distance dispersal over them (Fernández-Mazuecos & Vargas, 2011). Therefore, it is currently acknowledged that the role of the straits varied depending on the species and historical context. Besides, the ecological conditions (precipitation, temperature, soils) can also be determinants for the distribution of the species (Fernández-Mazuecos & Vargas, 2010).

The known history of the strawberry tree

Arbutus is one of the six genera of the Arbutoideae subfamily (other genera are *Arctostaphyllos, Arctous, Comarostaphylis, Ornithostaphylos* and *Xiloccocus*). Subfamily Arbutoideae includes evergreen, shrub-like woody taxa with laurel-like and sclerophyllous leaves of the family *Ericaceae*. The origin of the Arbutoideae were the broadleaved evergreen sclerophyllous taxa which occupied a subhumid belt across North America-Eurasia by the middle Eocene (45 Million years ago, Mya); these taxa originated from alliances in older laurophyllous forests that adapted to dry climate. Numerous taxa of these sclerophyllous vegetation were linked to the Madrean (western North America) Tethyan (Eurasia) regions during the Tertiary and until the end of the Oligocene (25 Mya) (Axelrod, 1975). Following Hileman *et al.* (2001), the subfamily Arbutoideae diverged earlier than 21.2 Mya. In fact, genus *Arbutus* appeared in the Mediterranean during the Oligocene (25-30 Mya) when the link with North America still existed (Palamarev, 1989). Accordingly, *Arbutus* includes seven extant species in America (*A. xalapensis*, *A. texana*, *A. penninsularis*, *A. tessellata*, *A. arizonica*, *A. madrensis* and *A. meziessi*) and four in Europe (*A. andrachne* L. in eastern Mediterranean, *A. pavarii* Pampanini in the coasts of Libya, *A. canariensis* Veill. in the Canary Island, and *A. unedo* L.) (Torres *et al.*, 2002; García M. & Fernández Z., 2011). *A. andrachne* is the closest relative to *A. unedo* (Hileman *et al.*, 2001). Of note, the Mediterranean species of *Arbutus* originated in restricted and discontinuous areas; in particular, *Arbutus praeunedo* (the ancestor of *A. unedo*), was described for the Hungary-Poland-Bulgary area (Palamarev, 1989).

Until now, no study has investigated the phylogeography of *A. unedo* and, to the best of our knowledge, there is just one published work on the phylogeny of Arbutoidae subfamily (Hileman et al. 2001). Accordingly, reconstructing the Mediterranean phylogeography of this plant seems necessary. Additionally, there has been an intense debate on the origin of the Irish population, because the strawberry tree belongs to the intriguing "Lusitanian element" of British flora, i.e. species that, like *A. unedo*, inhabit the Iberian Peninsula and western Ireland but they are absent from England (Sealy, 1949; Sealy & Webb, 1950; Webb, 1983; Mitchell, 1993; Beatty & Provan, 2013; Beatty & Provan, 2014). Initially, Sealy (1949) hypothesized four possible alternatives to explain the distribution of the Lusitanian flora: 1) relicts of the Tertiary flora of the British Isles which survived the glacial epoch; 2) post-glacial human-mediated introduction; 3) post-glacial bird-mediated seed dispersal; 4) post-glacial invasion of the British Isles from the continent followed by a brake up of the originally continuous distribution (Sealy, 1949).

The idea that *A. unedo* may have persisted the Quaternary glacial period in Ireland, or on land now submerged off its west coast, seems unlikely and has been discarded given the biological requirements of the species (Sealy, 1949). On the other hand, pollen records (Mitchell, 1993) and radiocarbon test (Van Rijn, 2004) dated the occurrence of the strawberry tree in Ireland to 4000 and 3700 years ago, respectively, and suggest that it was more abundant in the past than today (Mitchell, 1988; Mitchell, 1993). Recent work with other Lusitanian species also indicates their persistence throughout the Last Glacial Maximum in southern refugia and its arrival to Ireland via northward dispersal after the retreat of the ice sheets (Beatty & Provan, 2013; Beatty & Provan, 2014). Therefore, it seems likely that *A. unedo* migrated northwards in post-glacial times (Sealy & Webb, 1950). Migration could have happened during the Xerothermic period (8000-4000 B.P.), when shrubs were greatly favoured in areas previously covered with sclerophyllous woodland (Axelrod, 1975) through the Atlantic coast of France, arriving later to Ireland. However, the question is whether the warmer period was long enough to allow *A. unedo* to reach Ireland from the South (>1000km), or whether the origin of the Irish population could be different, such as a long-range dispersal (Webb, 1983) from Iberia. After the arrival, the climate became colder but *A. unedo* managed to survive. This survival could be partially a result of the warm Gulf Stream, which gives to Western Ireland a wet and relatively mild and frost-free climate (Cox & Moore, 2005).

Genetics for ecology

The use of molecular tools allows the analysis of individual genomes without the restrictions of physiological, morphological or phenological traits ("actual" traits), which are typically influenced by the environmental conditions experienced by each individual. The molecular approach is commonly used to assess the genetic diversity, which is the basis for selection and, therefore, for plant survival (Ramanatha Rao & Hodgkin, 2002). Indeed, genetic diversity affects ecological processes such as primary productivity, population recovery from disturbance, or fluxes of energy and nutrients (Hughes *et al.*, 2008). Likewise, genetic diversity can have important ecological consequences at population, community and ecosystem levels and, in some cases, its effects are comparable in magnitude to the effects of species diversity (Hughes *et al.*, 2008). As the most genetically diverse populations persist longer and are more stable (Agashe, 2009), small changes in the amount of adaptive genetic variance can radically alter eco-evolutionary dynamics (Becks *et al.*, 2010). Furthermore, genetic diversity allows species to respond to selection, and this ability promotes the coexistence of species (Vellend, 2006). Therefore, genetic variation in one species can shape the ecological properties of entire communities (Johnson & Stinchcombe, 2007). At this point, it is remarkable that much of the genetic diversity is sometimes found within individual populations while in other cases it is partitioned among a number of different populations (Ramanatha Rao & Hodgkin, 2002). Consequently, when we focus the attention to one species, it always is advisable to investigate a set of populations throughout its distribution range.

While molecular studies have largely focused on assessing the amount of genetic diversity, it is likewise essential to identify the underlying mechanisms (Hughes *et al.*, 2008). For example, the appropriate management of environmental problems (physiological effects of environmental pollutants, or a rapid spread of diseases) requires an understanding of the interplay between molecular mechanisms and the biology of organisms and ecosystems (Jackson *et al.*, 2002). Genomic techniques provide tools to study the molecular basis of physiological changes in response to environmental changes (Thomas & Klaper, 2004). Thus, to understand how global change affects the genetic patterns, it is important to know how the spatial patterns of landscape and environmental factors modulate the genetic variation (Manel *et al.*, 2003; Manel & Holderegger, 2013). A common genetic spatial pattern is the positive correlation between the geographical distance between populations and among-population diversity, the well-known "isolation by distance" (IBD) model (Nybom, 2004). Geographic isolation is a primary force in driving speciation as result of the gradual increase in among-populations differentiation; nonetheless, we can also find cases where speciation takes place in a continuous population (Beebee & Rowe 2004). IBD and/or the occurrence of fragmented populations can lead to inbreeding depression, i.e. a loss of genetic diversity with a consequent increase in extinction risk. The latter is especially in outbreeding species (Frankham, 2005), which can reduce their fecundity due to pollen limitation (Ghazoul, 2005). Another type of genetic spatial differentiation typically occurs between core and peripheral populations. Effective population sizes (Ne) can be 2 to 30 times lower near the range edge than near the core, leading to genetic impoverishment by genetic drift in the edge. However, peripheral populations sometimes have even higher genetic diversity than those from the core (Vucetich & Waite, 2003). Thus, Hampe & Petit (2005) suggested that the response of a species to changing environments is largely determined by the genetic pool of marginal populations. Also, Petit *et al.* (2003) showed that the most genetically diverse populations can be located at intermediate latitudes rather than in the south (the location of glacial refugia), probably as a result of admixture among divergent lineages from separate refugia. Altogether, these studies suggest that a knowledge of the population genetic structure can be fundamental to delineate appropriate conservation strategies (Ramanatha Rao & Hodgkin, 2002; Crutsinger *et al.*, 2006).

The genetics of *Arbutus*

To date, few studies have addressed the genetic structure of *Arbutus*. Of note, the work done with a close relative, *A. menziesii* (Beland *et al.*, 2005), and the remarkable work of Sealy & Webb (1950) with *A. unedo,* that established the diploid nature of this plant (2n = 26, x = 13). More recently, several one-country studies have been conducted with the strawberry tree in Portugal (Lopes *et al.*, 2012; Gomes *et al.*, 2013) and Tunisia (Takrouni & Boussaid, 2010; Takrouni *et al.*, 2012). Nevertheless, these studies often led to inconsistent conclusions. Thus, genetic diversity within populations was found to be low (Takrouni & Boussaid, 2010), moderate (Lopes *et al.*, 2012) or high (Takrouni *et al.*, 2012) while genetic variation among populations has been described as moderate (Takrouni & Boussaid, 2010) or low (Lopes *et al.*, 2012; Takrouni *et al.*, 2012). While these inconsistencies could be partly attributed to the use of different molecular markers (RAPDs in (Takrouni & Boussaid, 2010; Lopes *et al.*, 2012; Gomes *et al.*, 2013), ISSR in (Lopes *et al.*, 2012; Gomes *et al.*, 2013), isozymes in (Takrouni *et al.*, 2012), it seems likewise likely that the pattern of genetic variation may vary from one set of populations to another.

The aforementioned inconsistencies highlight the need of a comprehensive, range-wide investigation of the genetic structure of *A. unedo*. This study seem particularly necessary for a better assessment of the conservation status of this tree, and/or to evaluate any management plan. As already mentioned, *A. unedo* is an animal-pollinated (outbreeding) plant (Sealy & Webb, 1950), with scarce and poorly dispersed pollen (Mitchell 1993). Furthermore, its populations live under very different environmental conditions throughout the species range. This variation could limit the gene flow across the range and, in turn, it may enable the genetic differentiation of some populations. The latter might be particularly relevant in the case of geographically isolated populations such as those currently located in Ireland. The available regional studies found high gene flow among populations (Lopes *et al.*, 2012; Takrouni *et al.*, 2012) and no evidence of correlation between genetic and geographic distances (Takrouni *et al.*, 2012; Gomes *et al.*, 2013). However, these conclusions might not be applicable to the entire distribution range where both spatial scale and environmental variability are notably larger.

Ecophysiology

The study of the physiological response of plants to different environmental factors is topic of great relevance given the ample evidence about how global change alters their performance. It has been predicted that global change will modify temperature, precipitation, and soil nutrient availability; these changes can modify the physiology and phenology of plants and promote shifts in their distribution (Aerts *et al.*, 2006). For instance, Parmesan

& Yohe (2003) described poleward range shifts averaging 6.1 km per decade and an advancement of spring events by 2.3 days per decade. Similarly, Aitken *et al.* (2008) observed moderate to strong clines in the phenology and growth of trees along temperature gradients. Similarly, range shift in herbs and shrubs towards northern regions has been shown to have consequences for the structure of ecosystems (Aerts *et al.*, 2006). The pattern of redistribution depends on factors such as the strength of selection, tree fecundity, the capabilities to induce phenotypic variation, interspecific competition, and other biotic interactions (Aitken *et al.*, 2008). Likewise, the impact on biodiversity will depend on the interaction between climatic and no climatic drivers such as land-use change, which will probably have a strong effect in the communities (Sala *et al.*, 2000). Accordingly, the study of the physiological adaptation to these climatic and non-climatic changes seems imperative to understand how communities will evolve in future scenarios.

With an exceptional total number of endemic plants of 13.000 (4.3% of all endemic plants), the Mediterranean basin—our area of study—is notably relevant because it is regarded as one of the 25 hotspots of biodiversity on Earth (Myers *et al.*, 2000). Accordingly, this region seems essential for the conservation of Earth's biodiversity. Moreover, this remarkable reservoir of biodiversity is expected to experience dramatic changes in a future because the Mediterranean basin will be more strongly affected by global change than other regions of the Earth (Sala *et al.*, 2000; Petit *et al.*, 2005; Giorgi & Lionello, 2008). The predicted climate change over the Mediterranean will cause a pronounced decrease in precipitation and an increase in warming and drought, especially in summer (Giorgi & Lionello, 2008). Therefore, investigating the physiology of *A. unedo*, a plant with populations all around the Mediterranean basin under contrasted climatic conditions, seems particularly pertinent because it will enable us to gain a better understanding of how global change may affect the future of this representative member of the Mediterranean flora. Here, we chose to analyse the response of the strawberry tree to changes in the availability of water and nutrients because

(i) they are two of the environmental factors that most strongly constrain terrestrial productivity (Lambers *et al.*, 2008), and (ii) because their availability will be strongly affected by the ongoing global change.

Nutrients

The productivity of virtually every natural ecosystem, even arid ecosystems, responds to the addition of one or more nutrients, indicating the key ecological relevance of nutrient limitation (Lambers *et al.*, 2008). Interestingly, the lack of nutrients sometimes is the effective factor of mortality without being the immediate cause of death (Grime & Curtis, 1976). Therefore, the predicted changes on nutrient cycles that may result from modifications in rainfall regimes (higher rainfall enhances nutrient cycling whereas less rain increases the risk of loss by erosion) will have important implications on plant fitness and ecosystem functioning (IPCC 2013; Sardans & Peñuelas, 2007; Matías *et al.*, 2011).Moreover, soil processes can be affected by changes in land-use, nitrogen deposition, biotic exchange, or elevated carbon dioxide concentration (Sala et al., 2000). For example, warming for three years in tundra increased net N-mineralization by 70%, and this higher nutrient availability affected the species composition (Aerts *et al.*, 2006).

Root architecture and biomass allocation are determinants for the plastic response of plants to soil heterogeneity (Fitter, 1994). Plants exploiting infertile soils show low relative growth and low morphological plasticity because the enhancement of nutrient uptake does not compensate the expenditure of construction and relocation of the root system (Sibly & Grime, 1986; Crick & Grime, 1987; Fitter & Stickland, 1991). However, soil is a heterogeneous environment in terms of nutrient availability and variation between species in the ability to exploit this heterogeneity can affect their distribution (Farley & Fitter, 1999; Lambers *et al.*, 2008). Below species level, it seems likewise reasonable to assume that a similar variation in the ability to use soil resources might be found among conspecifics from areas with very different environmental conditions, as in the Mediterranean. In this regard, nutrient availability has been commonly investigated in the Mediterranean basin. For instance, Mayor & Rodà (1992) analysed the role of nutrients as limiting resources for primary production while Hanley & Fenner (1997) determined the importance of internal nutrient supplies and the external nutrient environment in seed germination and seedling establishment. In addition, Monokrousos *et al.* (2004) showed significant temporal variation in nutrients availability. Here, we address a new question and investigate whether plants from different provenances differ in their responses to variations in nutrient availability.

Water

Water availability probably is the most determinant environmental resource for plant growth as plants are commonly subjected to periods of soil and atmospheric water deficit during their life cycle (Chaves *et al.*, 2002). Accordingly, the adverse effect of drought has been widely reported (Pigott & Pigott, 1993; Chaves *et al.*, 2002; Chaves *et al.*, 2003). Pigott & Pigott (1993) showed the primary role of water supply in controlling small and large scale patterns of species distribution in Europe while Galmés *et al.* (2007) described a general pattern of decrease in stomatal conductance in response to water stress that strongly affects photosynthetic performance and growth. In addition, there is a growing interest on understanding the mechanisms of plant resistance to drought stress to improve crop management (Chaves *et al.*, 2003).

Plants under water limitation reprogram their metabolism and growth. Particularly interesting are the changes in the balance between growth and survival (Claeys & Inzé, 2013). Thus, the mechanisms that deal with low water availability can be divided into three major categories: drought evasion, desiccation avoidance and desiccation tolerance. Specifically, evasion tends to minimize the biological activity during water-deficit period; avoidance aims to elude low water potentials and dehydration by accumulating solutes and by modifying the properties of cell walls; lastly, tolerance intends to prevent cell damage by specific mechanism such as the use of protective proteins (Larcher, 2003; Verslues et al., 2006; Lawlor, 2013). For example, some reported responses to water stress are changes in phenology, root size and depth, root: shoot ratio, hydraulic conductivity or temporary accumulation of reserves in the stem (Chaves *et al.*, 2002; Chaves *et al.*, 2003). Other important component of plant resistance to drought is the ability to get rid of excess radiation at leaf level (Chaves *et al.*, 2002; Chaves *et al.*, 2003). However, some of the differences among species are due to different capacities for water acquisition rather than to metabolic differences at a given water status (Chaves *et al.*, 2002). It is clear that plant responses to water scarcity are complex and, under field conditions, these responses can be synergistically or antagonistically modified by the superimposition of other stresses (Chaves *et al.*, 2002).

The warm and dry summers typical of the Mediterranean climate imply that water availability is generally adverse for most plants. Furthermore, the region seems particularly sensitive to future increase of temperature and drought conditions (Luterbacher *et al.*, 2004; Giorgi & Lionello, 2008; Petit *et al.*, 2005; IPPC 2013). Finally, an aggravating factor is the predicted increase in fire frequency, a phenomenon that reduces ecosystem resilience and enhances the adverse effects of drought (Díaz-Delgado *et al.*, 2002; Pausas *et al.*, 2008). Consequently, the Mediterranean basin seems particularly interesting to study plant responses to water shortage, especially when climatically contrasted provenances are considered such as in the case of our work with the strawberry tree.

Arbutus unedo ecophysiology

The responses of single populations of *Arbutus unedo* to the main environmental resources and conditions have been largely studied. For example, under water shortage, *A. unedo* has been reported to delay its phenophases while maintaining a higher proportion of individuals with flower and fruits than other species (Ogaya & Peñuelas, 2004). From an ecophysiological point of view, leaf water potential, stomatal conductance, chlorophyll content, net CO_2 assimilation, photochemical efficiency and photosynthetic activity of *A. unedo* have been shown to decrease under water deficit (Gratani & Ghia, 2002; Baraldi *et al.*, 2008). However, Castell & Terradas (1994) pointed out that the responses depend on the developmental stage, with resprouts showing a less conservative use of water (i.e, higher water potential, stomatal conductance and transpiration) than mature plants. In addition, Munné-Bosch & Peñuelas (2004) described differentiated responses depending on the degree of drought: under moderate stress, the levels of photosynthetic pigments and ascorbate were kept constant, but under severe stress the pigments decreased and zeaxanthin and ascorbate concentrations increased, increasing the redox state. Thus, the antioxidative protection provided by the xanthophyll cycle is an efficient mechanism to prevent irreversible damages to the photosynthetic machinery in situations of drought-induced oxidative stress in *A. unedo*, which has been also pointed out by Baraldi *et al.* (2008). Gratani & Ghia (2002) described an increase of leaf area, improving the capacity of light interception, as another mechanism to prevent photoinhibition in water-stressed leaves. Hence, according to its abilities to deal with drought, *A. unedo* has been considered in the borderline between drought semi-deciduous species and evergreen sclerophyllous species (Gratani & Ghia, 2002). As for nutrients, the other factor that we are going to analyse, there is one single study that considered its effect, together with water regime variations (Castell & Terradas, 1994). They found that the presence of nutrients increased leaf nutrient content, but they concluded that nutrients were less limiting than water stress.

Recovering after fire in *A. unedo* has also been a subject of interest. Fire can enhance the growth of the *A. unedo* resprouts, with pre-fire plant size affecting post-fire growth (Konstantinidis *et al.*, 2006). Interestingly, after-fire resprouts show some juvenile characteristics, such as higher morphological and physiological plasticity, compared to new sprouts from unburned plants (Savé & Terradas i Serra, 1993). This after-fire resprouter ability together with its low to intermediate flammability (Liodakis and Kakardakis, 2008) make the strawberry tree an ideal plant for restoration in the fire-prone Mediterranean area (Vallejo et al. 2012).

The response to gases has also been investigated in the strawberry tree. Jones *et al.* (1995) verified that *A. unedo* leaves formed at elevated CO_2 had lower stomatal conductance, lower stomatal density and higher specific leaf area than leaves formed at ambient CO_2, increasing their instantaneous water use efficiency. Thus, we can expect that *A. unedo* will have higher growth rates if the CO_2 concentrations increase. Jones *et al.* (1995) also noted that the chlorophyll fluorescence measurements suggested that elevated CO_2 provides some protection against photoinhibition in mid-summer. Finally, some studies investigated the response to other gases, such as the ozone, which slowed the stomatal response (stomatal closure more than aperture) to abrupt reductions in light intensity and increasing water stress (Paoletti, 2005), and sulphur dioxide. In this latter case, a long-term exposure to sulphur dioxide did not produce visible symptoms on leaves, but photosynthetic activity and growth was markedly reduced (Panicucci *et al.*, 1998).

In relation to the physiology of *A. unedo*, there is a noteworthy study that characterized the intra-annual density fluctuations (IADFs) (Battipaglia *et al.*, 2010) showing that IADFs can provide information about the relationship between environment and growth. Finally, we would like to highlight a couple of studies on plant-fungi interactions. *A. unedo* maquis offers suitable conditions for the development of lignicolous fungi and supports a high fungal diversity. Thus, fifty-two species representing 32 aphyllophoraceous genera were found growing on *A. unedo* and at least one, *Antrodia sandaliae*, is expected to be exclusive of *A. unedo* (Perez Gorjon *et al.*, 2006). On the other hand, Moralejo *et al.* (2008) described many other fungi that live in *A. unedo*, but focusing in diseases such as *Phytophthora ramorum*, *P. syringae*, *P. citrophthora*, *P. ramorum*, *P. tropicalis* or *P. nicotianae*.

Altogether, this review of the literature on the biological responses of *A. unedo* reveals that no study has covered the complete distribution range of the species. Therefore, while these studies can be useful to establish some aspects of the behaviour of this plant, it is not possible to forecast whether the strawberry tree shows different strategies depending on its provenance and/or how this variation could affect its potential to adapt and evolve.

Q_{ST}-F_{ST} comparisons

In the context of the ongoing environmental changes, plant adaptation strategies is a critical issue in biology. In this regard, the comparison between quantitative and genetic variation is one of the most powerful tools. Differentiation among populations for quantitative traits has been quantified as Q_{ST} (Spitze 1993), and its calculation for any trait requires two quantities, the additive genetic variance of the trait within a population ($V_{A,within}$) and the genetic variance among populations ($V_{G,among}$). For diploids, Q_{ST} is calculated as: $Q_{ST} = V_{G,among} / V_{G,among} + 2V_{A,within}$ (Spitze, 1993; Whitlock, 2008). Q_{ST} has been so defined to be compared to neutral genetic variation estimated as F_{ST} (Wright, 1951), because both Q_{ST} and F_{ST} vary between 0 and 1. The Q_{ST} of a trait, or suite of traits, can only be compared to F_{ST} estimates derived from a set of neutral loci (not subjected to selection) that quantifies the amount of differentiation due to neutral processes such as mutation, migration and genetic drift (Whitlock, 2008). Accordingly, Q_{ST} values greater than the mean F_{ST} value suggest that populations experience geographically variable selection, and the trait has diversified more than would be expected by migration and genetic drift. Consequently, we can infer the existence of local adaptation (directional selection). Conversely, if Q_{ST} is significantly lower than F_{ST}, we would have evidence that the trait was under a stabilizing selection, with a similar performance in every population, even in the face of drift. Finally, when Q_{ST} values are approximately the same as F_{ST}, we have little evidence that selection acted in a spatially heterogeneous way (Whitlock, 2008).

The performance of Q_{ST}-F_{ST} comparisons has some important considerations that we must take into account. On the one hand, the values of F_{ST} for neutral loci and Q_{ST} for neutral quantitative traits are expected to be extremely variable and, therefore, it is not enough to show that a given Q_{ST} value is greater than the mean F_{ST}. Any Q_{ST} value must be compared to the distribution of Q_{ST} neutral values, check the position on this distribution and test the statistical probabilities of having higher or lower value than expected. The latter happens when the value is at the tail of the distribution, that is, the 2.5% of the lowest values or the 2.5% of the highest values (Whitlock, 2008; Whitlock & Guillaume, 2009). The Q_{ST} neutral distribution is obtained through simulation, because it has been demonstrated that this distribution can be inferred from the empirical mean F_{ST} value (Whitlock & Guillaume, 2009). On the other hand, we must keep in mind that Q_{ST} must be estimated from a breeding design, what enables to obtain the additive genetic variance of the traits within population ($V_{A,within}$) required to calculate F_{ST} and Q_{ST} (Whitlock & Guillaume, 2009). It is likewise essential that the studied organism may grow in a common garden to avoid mistaking phenotypic plasticity with local adaptation (Kawecki & Ebert, 2004; Pujol *et al.*, 2008; Whitlock & Guillaume, 2009), and it is advisable to use a large number of populations (>10) to increase statistical power (Whitlock & Guillaume, 2009).

The use of Q_{ST}–F_{ST} comparisons for addressing a wide range of relevant questions for evolutionary and ecological genetics has increased rapidly in recent years (Merilä & Crnokrak, 2001; Edelaar & Björklund, 2011; Kort *et al.*, 2013; Leinonen *et al.*, 2013). An important result derived from these studies is that Q_{ST} typically exceeds F_{ST}, suggesting a prominent role of local adaptation among contemporary populations (Merilä & Crnokrak, 2001; Leinonen et al. 2008; Kort *et al.*, 2013). Some of these studies found Q_{ST} - F_{ST} correlations (Merilä & Crnokrak, 2001; Leinonen *et al.*, 2008), and that Q_{ST}–F_{ST} values increased with the distance between populations, indicating the common occurrence of isolation by distance (Kort *et al.*, 2013). It is likewise remarkable that some studies analyse Q_{ST}–F_{ST} values taking into account the kind of trait. Thus, Kort *et al.* (2013) found that traits related to plant physiology and reproduction had lower Q_{ST} values than traits related to biomass and phenology.

Some circumstances can lead to biases, especially about the role of local adaptation. Firstly, the comparison Q_{ST}-F_{ST} is valid whenever the mutation rates of the neutral markers used to obtain F_{ST} are similar to those of the quantitative traits used to estimate Q_{ST}. Therefore, the use of highly variable neutral markers such as microsatellites can lead to serious biases (F_{ST} decreases fictitiously), resulting in overestimated inferences about the role of divergent selection over the studied populations (Edelaar & Björklund, 2011). Furthermore, researchers may deliberately choose populations with *a priori* knowledge about their phenotypic divergence, inducing a publication bias in favour of local adaptation (Kort *et al.*, 2013). Finally, empirical and theoretical studies might have focused on the $Q_{ST} > F_{ST}$ outcome because of the absence of a straightforward biological interpretation of a $Q_{ST} < F_{ST}$ pattern (Lamy *et al.*, 2012). Indeed, Lamy *et al.* (2012) supported a meaningful interpretation of $Q_{ST} < F_{ST}$ as either a canalization phenomenon or a consequence of uniform selection. Accordingly, canalization could explain the constancy of a trait among species or populations within a species, meaning that the genetic background of the species share the same genetic constraints rather than the same selective optima. In the case of *A. unedo*, the lack of knowledge about its adaptive capabilities suggests that Q_{ST}–F_{ST} comparisons in a framework of various traits and provenances may be highly pertinent.

Plasticity and evolutionary adaptation

Q_{ST}-F_{ST} comparisons provide evidence on the adaptive process of a species. Here, we briefly review the main strategies followed by plants to survive when their environments are altered. According to Williams *et al.* (2008), all organisms have some intrinsic capacity to adapt to changing conditions, via ecological plasticity (physiological and/or behavioural) or by evolutionary adaptation. Additionally or complementarily to these mechanisms, extinction can also be avoided if populations move to favourable habitats (Hoffmann & Sgrò, 2011). Evolutionary adaptation can be rapid and help the species to overcome stressful conditions, or even to realize ecological opportunities arising from climate change (Hoffmann & Sgrò, 2011). Thus, it has been described that range shifts may be driven by phenotypes previously established at the centre of the distribution that were adapted to conditions now prevalent in new areas (Atkins & Travis, 2010). Biological invasions are a typical example of rapid evolutionary changes because the adaptation of invaders to new environments can occur at very short (≤ 10 year) timescale (Whitney & Gabler, 2008). Likewise, natural selection leading to local adaptations has also been described for native Mediterranean species (Ramírez-Valiente *et al.*, 2011). Moreover, the analysis of evolutionary processes should be incorporated into management programmes designed to minimize biodiversity loss under the ongoing global change (Hoffmann & Sgrò, 2011). The study of adaptive responses is a valuable tool in conservation biology because identifies populations unable to evolve at sufficiently high rates and identifies possible donor populations for genetic rescue (Hansen *et al.*, 2012). However, since evolutionary rescue from extinction usually requires abundant genetic variation or a high mutation rate (Bell & Collins, 2008), other adaptive processes are commonly required for plant survival.

Phenotypic plasticity is the capacity of a single genotype to produce different phenotypes across a range of environments (Sultan, 2000). It reflects the direct influence of the environment on the development of individual phenotypes, and it is increasingly considered an important component of phenotypic change in the wild (Chevin *et al.*, 2010). Many responses perceived as evolutionary adaptations could be, indeed, plastic responses (Gienapp *et al.*, 2008). Plasticity in plants is widespread and occurs in many ecologically important traits, ranging from several aspects of morphology and physiology to anatomy, development, reproduction, breeding system and offspring developmental patterns (Sultan, 2000). For example, a plastic delay in phenology may enable survival in unpredictable environments as the Mediterranean (Mutke *et al.*, 2010). Plasticity can also be useful in many natural populations where fluctuating selection has been described (Bell & Collins, 2008). Phenotypic plasticity seems especially indicated whenever environmental change can revert because it can be reversed faster and easier than genetic change (Hansen *et al.*, 2012). Moreover, plasticity increases the chances of persistence of populations under environmental change by approaching the phenotypes to the new optimum (Chevin *et al.*, 2010). It can also be relevant for the establishment and persistence of populations at the colonizing edge of climatically induced range shifts (Matesanz *et al.*, 2010). Some simulation studies demonstrated that a high plasticity among populations reduces the area lost by species (Valladares *et al.*, 2014). Besides, phenotypic plasticity can promoting coexistence and diversity when it favours an adjustments to the community composition (Callaway *et al.*, 2003). Accordingly, phenotypic plasticity should always be incorporated into projections of the persistence of populations (Chevin *et al.*, 2010); not only for the species as a whole but for different populations as the latter can vary in their degree of plasticity (Valladares *et al.*, 2014).

A realistic analysis must take into account the combined action of plasticity and evolutionary adaptation as the effect of just one single mechanism seems unlikely. In this regard, some studies have been able to separate the effects of genetic adaptation and plasticity finding that the latter seems to be more important than the former (Mutke *et al.*, 2010). The incidence of each mechanism will depend on factors such as the intensity and direction of environmental change, life-history traits, amount of genetic variation, and interactions among coexisting species (Matesanz *et al.*, 2010). The first consequence of a combined action is that the plasticity of certain functional traits may be beneficial and selected (Matesanz *et al.*, 2010). Plasticity may have an additive genetic component and, when plastic responses are favoured by selection, these responses may become genetically fixed in the populations (Williams *et al.*, 2008). Furthermore, plasticity can play a role both in the short-term response of plant populations to global change as well as in their long-term fate through the maintenance of genetic variation (Matesanz *et al.*, 2010). Moderate levels of phenotypic plasticity are optimal in permitting population survival in a new environment and, at the same time, they may facilitate evolution (Price *et al.*, 2003). According to Lande (2009), plasticity causes the phenotype to vary towards the new optimum of the new environment and, subsequently, the phenotype suffers a slow genetic assimilation where the reduction in plasticity is compensated by genetic evolution of the reaction norm. However, high levels of plasticity may increase the probability of population persistence but reduce the likelihood of genetic change (Price *et al.*, 2003). Hence, a probable optimal strategy would be a balanced presence of both adaptive procedures.

Canalization

Canalization is an adaptive mechanism less known than plasticity or evolutionary adaptation. Nonetheless, its ecological role seems likewise essential. Canalization is a mechanism of genetic buffering that evolved under natural selection to reduce the expression of genetic variation at the level of the phenotype (Wagner et al., 1997). In other words, canalization reduces the genetic response to selection and stabilizes the phenotypic performance (Gibson & Wagner, 2000; Kawecki, 2000), being an event that can happen at many different levels, either within individuals, populations or species (Kalisz & Kramer, 2008). More specifically, canalization can be understood in terms of transmission patterns such as epistasis or pleiotropy, and genotype by environment interactions (Flatt, 2005). Likewise, the chaperone protein HSP90, in plants, has been described as a key element linked to canalization, given its involvement in the regulation of the phenotypic plasticity, developmental stability, and buffering of genetic variation (Sangster & Queitsch, 2005). Besides, genetic canalization may result from interactions involving quantitative trait locus (QTLs). Lee *et al.* (2014) identified the nFT as a QTL that affects to the existence of canalization, although the magnitude and direction of this canalization also depends on the environment.

Canalization reduces the phenotypic impact of mutations, resulting in the appearance of hidden genetic variation invisible to selection that allows an accumulation of genetic differences under apparent stasis (Gibson & Wagner, 2000). However, this phenotypically cryptic variation can be released after a decanalizing event (e.g. some environmental perturbations) leading to changes in previously stable traits (Gibson & Wagner, 2000; Flatt, 2005). Consequently, the accumulation of cryptic genetic variation by canalization may increase evolvability at population level as a result of the potential phenotypic diversification subsequent to a decanalizing event (Flatt, 2005). It seems logical that the most favourable conditions for canalization are those of stabilizing selection where genes that decrease environmental variance are clearly favoured (Wagner *et al.*, 1997; Le Rouzic *et al.*, 2013). However, it has been demonstrated that canalization can also happen under fluctuating selection (Le Rouzic *et al.*, 2013). In fact, if the direction of selection changes from generation to generation, the ability to respond to selection is maladaptive because the response to selection in one generation leads to reduced fitness in the next. Indeed, the existence of weak to moderately strong fluctuating selection favours genetic canalization (Kawecki, 2000). For example, Valladares *et al.* (2002) described a high degree of genetic canalization in the Mediterranean ecosystems, what is consistent with the environmental unpredictability of this area. Likewise, the existence of canalization in the Mediterranean region has also been shown in some other studies (Quero *et al.*, 2008; Lamy *et al.*, 2011). Therefore, in our study about the strawberry tree, a species mainly circum-Mediterranean, canalization is a mechanism to keep in mind when interpreting our results.

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General Objectives


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General Objectives

This thesis is arranged into five chapters. In the first and the second chapter we employed DNA markers to unravel the phylogeography and the molecular structure and diversity of *Arbutus unedo*. The third and fourth chapters focused in analysing the ecophysiological response of the species to variations in nutrients and water availability. Finally, in the fifth and last chapter we compared the ecophysiological and molecular variance by using the Q_{ST}-F_{ST} approach.

Specifically, our objectives were:

1. To disentangle the colonization routes of *A. unedo* along the Mediterranean basin and the Atlantic façade, with special interest in the estimation of the time to the most recent common ancestor (TMRCA) of the species. *Conducted in Chapter I, by using chloroplastidial (cpDNA) markers.*

2. To determine the concordance between the phylogeographic patterns observed in other Mediterranean plants (longitudinal/latitudinal clines of diversity, location of glacial refugia, and role of the straits as barriers to dispersal) and those that we observed in the strawberry tree. *Conducted in Chapter I, by using cpDNA markers.*

3. To analyse wether the largely one-dimensional distribution of the strawberry tree led to the existence of an isolation by distance (IBD) pattern, either around the Mediterranean basin or along the Atlantic coast. *Carried out in Chapter II, by using amplified fragment length polymorphism (AFLP) markers.*

4. To stablish whether the contrasted climatic conditions found throughout the distribution range of the species have resulted in gene flow restrictions and/or differentiation between regions. *Carried out in Chapter II, by using AFLP markers.*

5. To contribute to the Lusitanian debate by determining the most likely origin for the Irish population of *A. unedo*. *Carried out in Chapter I and II by using cpDNA and AFLP markers.*

6. To describe differences in the plasticity of structural and physiological traits in plants of strawberry tree subjected to variations in nutrients and water availability, what will increase our ability to predict how this species will respond to ongoing global changes. *Performed in Chapter III (nutrients) and IV (water), by using the phenotypic plasticity index.*

7. To elucidate whether the plants of different provenances of *A. unedo* differed in the level of plasticity in response to variations in nutrients and water availability, what may be important for determining how the distribution of this species could be affected in future environmental scenarios. *Performed in Chapter III (nutrients) and IV (water), by using the phenotypic plasticity index.*

8. To model how nutrient levels affected photosynthetic responses of *A. unedo*, given that plant responses to light are influenced by nutrient availability. *Performed in Chapter III, by using light response curves.*

9. To define the role of natural selection and local adaptation in determining the current genetic structure of *A. unedo*. *Performed in Chapter V by using the* Q_{ST}-F_{ST} *approach.*

10. To estimate the additive genetic variance, heritability and evolvability in a set of morphological and physiological traits of the strawberry tree, in order to know how this could affect to its evolution. *Performed in Chapter V by calculations associated with the* Q_{ST}-F_{ST} *comparison.*

Chapter I

West to East migration along the Mediterranean basin: the case of *Arbutus unedo* L.


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ABSTRACT.

The Mediterranean basin is an important biogeographical region that resists the description of concordant phylogeographic patterns. Species with linear distribution ranges such as the strawberry tree (*Arbutus unedo* L.), a circum-Mediterranean shrub that also occurs along the Atlantic coast, may be particularly useful to unravel these patterns. Here, we describe its phylogeographic structure and check for concordance with patterns seen in other Mediterranean plants: longitudinal/latitudinal clines of diversity, evidence for glacial refugia and/or 'refugia-within-refugia', and the role of the straits in the spread of the species. Furthermore, we also aim to identify the most likely source for the disjunct Irish population. To this aim we sampled 23 populations from the whole distribution range of the species and sequenced four chloroplast non-coding fragments (1.6% of the chloroplast genome) in a total of 207 individuals to determine genetic diversity, population structure, haplotype genealogy, and Time to the Most Common Ancestor (TMRCA). The genealogy revealed two clades that separated during the last 700 ky but before the LGM. One clade occupies Atlantic Iberia and North Africa while the other occurs in the Western Mediterranean (Eastern Iberia included). The Eastern Mediterranean is inhabited by newer haplotypes derived from both clades while the Irish population is closely related to Iberian demes. The straits of Sicily and Gibraltar partially restricted the historical gene flow. We concluded that a vicariance event during the Late Quaternary in the Western end of the species' range followed by eastward migration seems a likely explanation for the phylogeographic pattern of the strawberry tree. The role of straits indicate an occasional communication between Europe and North Africa, suggesting that the latter was a novel refuge. The East-West genetic divide in Iberia is consistent with the refugia-within-refugia model. Finally, the strawberry tree possibly reached Ireland from Iberia by crossing the Celtic Sea rather than throughout the maritime fringe of France as previously thought.

Keywords: *Arbutus unedo*, cpDNA, haplotype, vicariance, clades, strait, refugee, Lusitanian, Mediterranean, Atlantic, Ireland.

INTRODUCTION

Phylogeography uses molecular information to infer the historical forces that have shaped the genealogical architecture of populations and closely related species (Avise, 2009). Appropriate inference involves distinguishing historically deep population subdivisions from younger population structures. The typical way to do so is by reference to the framework of genealogical concordance (Avise & Ball, 1990) that includes four main aspects: concordance within-locus, among-loci, across co-distributed species, and between genealogical and other biogeographic evidence. In particular, the concept of multi-species concordance has stimulated a number of comparative phylogeographic surveys for faunas and floras with mixed success (Avise, 2004).

One region that has resisted the description of multi-species concordant patterns is the Mediterranean basin, one of the earth's 25 biodiversity hot-spots (4.3% of the world's 300,000 plant species) (Myers *et al.*, 2000). The basin serves as a contact-zone for three continents separated by a tortuous sea, resulting in a very heterogeneous region in terms of climate and geography (Quézel & Médail, 2003) which is mirrored by an intricate phylogeography. Thus, a recent review of the patterns and processes in plant phylogeography concluded the no existence of largely common phylogeographic patterns across species in the Mediterranean, compared to the Alps o North America (Nieto Feliner, 2014). In addition to the environmental heterogeneity, the phylogeographic complexity was also attributed to a blurring of genetic footprints. Thus, the less drastic effects of Pleistocene glaciations in the Mediterranean may have facilitated an accumulation of species responses to successive palaeoenvironmental changes (Migliori *et al.*, 2012).

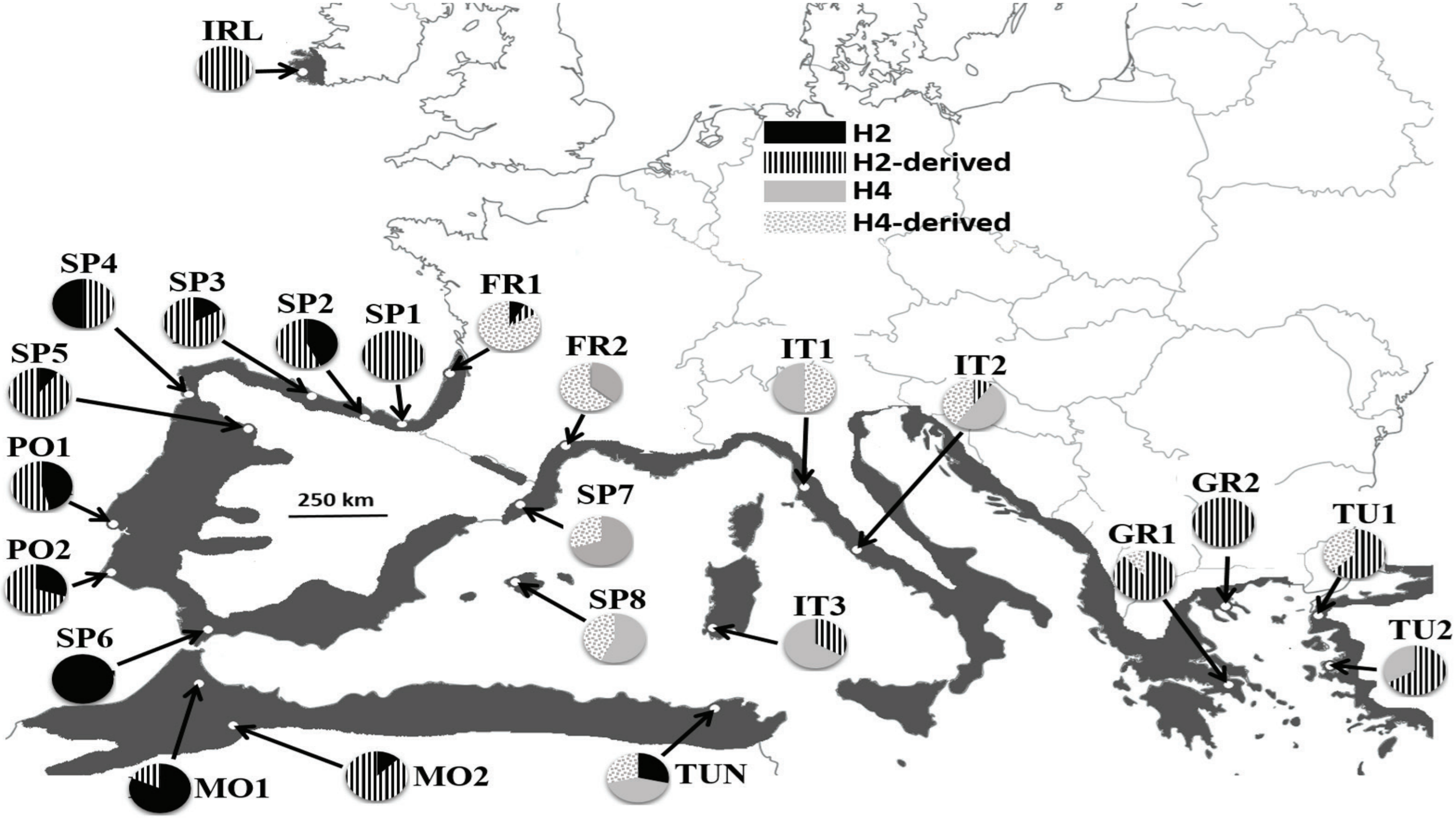
Despite the above, commonalities still exist, although they frequently show some inconsistencies. Like other areas of the world, there is a south-north decrease in genetic diversity that is more evident in the northern edge of the Mediterranean basin. This latitudinal cline must have been caused by a leading-edge expansion from southern refugia (Hewitt, 2000), and many studies have corroborated the role of the three large Mediterranean peninsulas (Iberia, Italy, Balkans) as refugia for the survival of species and engines of speciation (Hewitt, 2000, 2011). Moreover, it is now widely acknowledged that the 'refugia-within-refugia' model (Gómez & Lunt, 2007) explains the phylogeographic breaks identified within these peninsulas, particularly in Iberia (López de Heredia *et al.*, 2007; Rodríguez-Sánchez *et al.*, 2010). However, refugia were not confined to major peninsulas as many of them were identified in areas that had previously been attributed a lesser role (large islands, North Africa, Turkey, Catalonia-Provence). Again this complex arrangement of locations possibly evidences the cumulative effects of historical and environmental factors that occurred since the Tertiary (Médail & Diadema, 2009).

Another interesting pattern is the finding that the genetic diversity increases from west to east along the Mediterranean basin in many species (Conord *et al.*, 2012). Several Mediterranean trees even show a clear divide between the eastern and the western ends of the Mediterranean, with eastern lineages pre-dating those found in the west (Lumaret *et al.*, 2002; Rodríguez-Sánchez *et al.*, 2010). These longitudinal patterns have been attributed to relatively recent processes such as stronger demographic impacts under local LGM climate in west populations and an east-west recolonization during the Holocene (Conord *et al.*, 2012). Alternatively, the east–west vicariance has also been interpreted as a genetic footprint of pre-Quaternary range dynamics (Petit *et al.*, 2005; Médail & Diadema, 2009; Rodríguez-Sánchez *et al.*, 2009; Rodríguez-Sánchez *et al.*, 2010). Finally, the effectiveness of the sea straits (Gibraltar, Sicily, Otranto, Dardanelles-Bosphorous) as phylogeographic barriers has varied over time given the eustatic sea-level shifts during the Pleistocene (Hewitt, 2000). However, even the same strait (e.g. Gibraltar) served as barrier or not depending on the species (Fernández-Mazuecos & Vargas, 2010; Hewitt, 2011), and no obvious connection between dispersal ability and genetic exchange across this strait has been identified (Nieto Feliner, 2014).

Given its phylogeographic complexity, the study of simplified systems such as species with linear distribution ranges may help in the search for congruence in the Mediterranean basin (Clausing *et al.*, 2000; Nieto Feliner, 2014). This is the case of *Arbutus unedo* L. (strawberry tree), a shrub with a neat circum-Mediterranean distribution (Sealy, 1949; Torres *et al.*, 2002) that occupies a narrow coastal fringe from Tunisia to Morocco and from Spain to Turkey (Fig. 1). From an historical perspective, Axelrod (1975) cited the genus *Arbutus* as a component of the Madrean-Tethyan flora that existed between western North America and Eurasia until the end of the Oligocene (25 Mya). The divergence between Mediterranean and North American *Arbutus* was dated in at least 21.2 Mya (Hileman *et al.*, 2001), and the ancestral *A. unedo* remained in the area currently occupied by Hungary-Poland-Bulgary until the Messinian (Palamarev, 1989) with *Arbutus andrachne* as its closest relative (Hileman *et al.*, 2001). Interestingly, the strawberry tree also occurs outside the Mediterranean bioclimatic region, being found along the Atlantic coasts of Morocco, Iberian Peninsula, France and South-West Ireland. Hence, it belongs to the "Lusitanian flora", a group of species that largely puzzled biogeographers because they occur in Ireland and northern Iberia while they are largely absent from intervening countries (Sealy, 1949; Beatty & Provan, 2013). Early interpretations viewed the Lusitanian species as relicts of the Tertiary flora that survived through the glacial period (Sealy, 1949; Webb, 1983) but a post-glacial colonization of Ireland from southern refugia is currently considered a more likely alternative, although the precise location of the putative refugia remains elusive (Beatty & Provan, 2013, 2014). Some Lusitanian species may even have recolonized Ireland with human help rather than naturally (Foss *et al.*, 1987; Smith & Waldren, 2010). In the particular case of *A. unedo,* its presence in Ireland since at least 4000 years ago has been confirmed by both pollen (Mitchell, 1993) and wood remains (Van Rijn, 2004). Thus, the arrival to Ireland could have happened during the mid-Holocene thermal optimum, from 9000 to 6000 years before present (Davis *et al.*, 2003; Peyron *et al.*, 2012).

In this study we sequenced four chloroplastidial non-coding fragments to address a number of specific issues: (i) to estimate the

Figure 1. Population frequencies of the cpDNA lineages across the range (greyed) of *Arbutus unedo*. See Table 1 for population codes.



time to the most recent common ancestor (TMRCA) of *A. unedo* and to infer possible colonization routes along the Mediterranean and the Atlantic façade; (ii) to test whether the patterns detected in other Mediterranean plants are also applicable to the strawberry tree (longitudinal/latitudinal clines of diversity, location of glacial refugia, role of the straits as barriers to dispersal); (iii) to identify the most likely source for the disjunct Irish population. To our knowledge, this is the first range-wide study of the phylogeographic structure of *A. unedo.*

MATERIALS AND METHODS

The species

Arbutus unedo is a shrub from the family *Ericaceae,* subfamily *Arbutoideae*. Genus *Arbutus* includes several members in America and four in Europe: *A. andrachne* L., *A. pavarii* Pampanini, *A. canariensis* Veill. and *A. unedo* L. (Torres *et al.*, 2002). Flowering spans from September to December and fecundation is entomophilus (Mitchell, 1993). Fruits take 12 months to ripe and seeds are dispersed mainly by birds (Sealy, 1949).

Sample collection and DNA extraction

The sampling plan covered the whole range of *Arbutus unedo*, including leaf samples from 23 sites in nine countries (Table 1). We also collected samples from two individuals of *Arbutus andrachne*, the closest relative of *A. unedo*, from Çanakkale (Turkey). At each population, leaves collected from 12 haphazardly selected trees were individually wrapped in Kimtech Science wipes (Kimberly-Clark Europe Ltd, United Kingdom) and stored in silica gel until DNA extraction. DNA was extracted with the Realpure Genomic DNA extraction kit (REAL) according to manufacturer´s instructions except that we extended the incubation times for cell lysis (2 hours at 55ºC) and protein precipitation (20 minutes at -20ºC). Extractions were conducted in batches of 23 samples plus a negative control; each batch combined individuals from at least five populations. The quality of extracted DNA and negative controls was checked on 1.5% agarose gels.

Sequencing

After reviewing previous phylogeographic studies, initial trials were conducted with five non-coding cpDNA regions commonly used with trees and, specifically, several close species: trnS(GCU)-trnG(UUC), trnH(GUG)-psbA, trnL(UAG)-rpl32, trnT(UGU)-trnL(UAA) and Intrón L (Taberlet *et al.*, 1991; Shaw *et al.*, 2005; Shaw *et al.*, 2007). These initial trials revealed a widespread occurrence of illegible sequences due to long poly-A fragments in non-coding regions (polymerase slippage). Sequencing problems were solved for four out of the five non-coding regions, all except trnS(GCU)-trnG(UUC), by using a high fidelity polymerase (Phusion High-Fidelity ADN Polymerase, Thermo Scientific) (Fazekas *et al.*, 2010). PCR reactions were performed using 10µL of 2x Phusion Master Mix (with 1U of Phusion High-Fidelity ADN Polymerase), 0.5 µmol/L of each primer, and 1 µL of DNA (diluted 1:10). Amplification conditions were 30 s at 98 ºC for DNA denaturation; 30 cycles of 10 s at 98 ºC, 20 s at the specific annealing temperature of each primer, and 30 s at 72ºC; and a final extension of 7 min at 72 ºC. Annealing temperatures were 60.8ºC (trnT-trnL), 59.7ºC (trnL-rpl32), 61.8ºC (Intron L), and 66.8ºC (trnH-psbA). Before sequencing, PCR products were checked on 1.5% agarosa gels and purified with 1 µL of Exonuclease I (20 U/µL) and 2 µL of FastAP (10 U/µL) (Fermentas, Waltham, Madison, USA). Purified PCR products were sequenced under BigDye Terminator cycling conditions on an Automatic Sequencer 3730XL (Applied Biosystems, USA). The program Geneious R6 v.6.1.4 was used to check the quality of the chromatograms and to perform alignments. Singleton polymorphisms and unique haplotypes (i.e. detected in one single individual) were corroborated with new PCR and sequencing reactions to discard sequencing artefacts. As the non-recombinant nature of cpDNA makes it equivalent to a single-locus marker, sequences from the four fragments were combined into a single haplotype for every individual. Still, the identity of each fragment was kept when we calculated the TMRCA to allow for differences in the mutation rate of each region.

Data analysis

Haplotypes were identified with DnaSP v.5 (Librado & Rozas, 2009) which also provided estimates for haplotype diversity (Hd) (Nei, 1987). Genetic diversity was evaluated as nucleotide diversity (π) using Arlequin 3.5 (Excoffier & Lischer, 2010). Arlequin was also used to carry out an analysis of molecular variance (AMOVA) to assess population structure and to calculate the Fu's F_S test to detect any evidence of demographic expansion (Fu, 1997). Population structure was further investigated by defining clusters of genetically similar populations with a spatial analysis of molecular variance (SAMOVA)(Dupanloup *et al.*, 2002). SAMOVA groups populations into a user-defined number of groups (K) using a simulated annealing procedure to maximize the variance (F_{CT}) among those groups. The analysis was performed in SAMOVA 2.0 (Dupanloup *et al.*, 2002) running 10,000 iterations from 100 initial conditions, testing K = 2-9 with and without constraint for the geographic composition of the groups. The final number of groups was chosen for which F_{CT} began to plateau. In addition, Permut 1.0 was used to estimate population differentiation by calculating G_{ST} and N_{ST}, under the assumption that a significantly higher N_{ST} value suggests the existence of phylogeographic structure (Pons & Petit, 1996).

The genealogy of the haplotypes was inferred with the median-joining network algorithm and MP (*maximum parsimony*) calculation (which deletes non-MP links from the network) implemented in Network 4.6 (Bandelt *et al.*, 1999; Polzin & Daneshmand, 2003). We run the program with epsilon = 0 and mutations weighted following recommended guidelines: 10 as a default value, 5 for very common mutations (where the various alternative stages occurred in similar proportions), and 20 for indels.

Estimates of the TMRCA were conducted with the 207 *A. unedo* sequences in BEAST 2.1 (Bouckaert *et al.*, 2014) by combining two searches with 50 million Markov chain Monte Carlo (MCMC) each and a sample frequency of 1000. The first 5 million generations (10%) were discarded as a burn-in. We used jModelTest 2.1 (Darriba *et al.*, 2012) to determine with

Akaike and the Bayesian Information Criteria the simplest model of sequence evolution that best fitted our data. The four cpDNA fragments were tested both separately and concatenated into a single sequence. As F81 and HKY were consistently identified as best models, we used the model HKY available in BEAST. A Coalescent Constant Population was employed as tree prior after checking that a Coalescent Exponential Population prior was unsuitable for our data set. We implemented the strict molecular clock recommended for intra-species analysis (no expected rate variation among branches). Given the wide variation of substitution rates seen among flowering plants (Smith & Donoghue, 2008; Huang *et al.*, 2012), we intentionally specified a broad prior using an uniform distribution with bounds 1.0^{-10} and 1.0^{-8} (substitutions/ site/ year) based on the literature for the cpDNA fragments used in our study (Huang *et al.*, 2012). The four cpDNA fragments were designed as unlinked partitions to allow for variable mutation rates. Chain convergence was assessed with Tracer 1.6 (available in the BEAST2 package at http://beast.bio.ed.ac.uk/) and by checking the effective sampling size (ESS) values. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 2.1 and visualized in FigTree 1.4.1 (both also available in the BEAST2 package).

RESULTS

Genetic diversity and population structure

We produced sequences for 207 individuals of *A. unedo* (Table 1) and for two individuals of *A. andrachne*. The four non-coding fragments revealed polymorphisms along the range of *A. unedo;* therefore, they were retained for the phylogenetic analysis. Once concatenated, and depending on the occurrence of indels, the four fragments resulted in sequences 2401-2449 bp long, equivalent to 1.6% of the chloroplast genome of *A. unedo* (Martínez-Alberola et al., 2013). The data set of *A. unedo* contained 16 polymorphic sites that included 7 point mutations and 9 indels. Three indels were 1 bp long, two indels were 9 bp long, and the remaining four indels were 10, 11, 35 and 36 bp long, respectively. A total of 28 haplotypes were found (see supplementary material, Fig. S1), half of them (14) of them were private (Hp); interestingly, five out of the 14 Hp were detected in North Africa (Morocco). Only one haplotype was detected in *A. andrachne* that differed for the consensus sequence of *A. unedo* by 16 new point mutations. Total haplotype diversity (Hd) in *A. unedo* was 0.901 (± 0.01) (standard error) while total nucleotide diversity (π)(x 10^2) was 1.238 (±0.60). Diversity was unevenly partitioned among populations; thus, Hd and π ranged 0.000-0.810 and 0.000-0.934, respectively (Table 1). The highest π values were consistently recorded in populations that contained a mixture of haplotypes separated by long indels (35 or 36 bp). Total gene diversity (ht) was 0.919 (±0.02) while the average within-population gene diversity (hs) was 0.585 (±0.04). We also calculated the diversity at a regional scale by partitioning the populations into three groups suggested by the phylogeographic analyses (see below): Eastern Mediterranean (GR1, GR2, TU1, TU2), Western Mediterranean (FR1, FR2, SP7, SP8, IT1-IT3, TUN), and Atlantic coast (IRL, SP1-SP6, PO1, PO2, MO1, MO2). Diversity at regional scale increased from the Eastern (Ht = 7, Hd = 0.693 +/- 0.076, $\prod$ x 10^2 = 0.58 +/- 0.30) to the Western Mediterranean (Ht = 15, Hd = 0.762 +/- 0.044, $\prod$ x 10^2 = 0.63 +/- 0.32), and to the Atlantic coast (Ht = 13, Hd = 0.787 +/- 0.029, $\prod$ x 10^2 = 0.75 +/- 0.38).

The AMOVA revealed that 38.8% of the genetic variation was due to differences between populations, and the resulting F_{ST} value was highly significant ($p < 0.001$). N_{ST} (0.645 ± 0.067) was significantly higher than G_{ST} (0.363 ± 0.040) ($p < 0.001$) indicating the existence of a strong phylogeographic structure (Pons & Petit, 1996). Besides, SAMOVA showed that F_{CT} began to plateau at 0.697 for three groups of populations regardless of the alternative used (with or without constraint for the geographic composition of the groups) (Table 2). One group was geographically homogeneous and corresponded to the populations sampled in the Eastern Mediterranean plus Bordeaux (FR1) on Atlantic France. Another group was split in two sets of non-adjacent populations: populations from Atlantic Iberia-North Africa were grouped with populations from the Western Mediterranean. Finally, a third smaller group clustered the Irish

Table 1. *Arbutus unedo* sampling localities, UTM coordinates, sample size (n), number of segregating sites (S), number of total (Ht) and private (Hp) haplotypes, haplotype (hd) and nucleotide (π* 100) diversities (with SD) obtained based on the analysis of sequences with non-coding cpDNA fragments. I indicates co-occurrence of haplotypes separated one large indel (36 or 37 bp), respectively.

Population	**Country**	**ID**	**Position**	**N**	**S**	**Ht**	**Hp**	**H_d**	**$\pi*10^2$**
Killarney	Ireland	IRL	29U 465211 5763297	12	1	2	0	0.530 (0.077)	0.022 (0.02)
Bordeaux	France	FR1	30T 643276 4939413	12	6	4	1	0.455 (0.170)	**0.631 (0.34)**^{I}
Montpellier		FR2	31T 531757 4829416	11	3	4	1	0.764 (0.083)	0.047 (0.04)
Botaleku	Spain	SP1	30T 573213 4792097	6	2	3	0	0.733 (0.155)	**0.812 (0.49)**^{I}
Balmaseda		SP2	30T 485165 4778410	7	3	4	0	0.810 (0.130)	**0.457 (0.27)**^{I}
La Franca		SP3	30T 372037 4805531	6	2	3	1	0.600 (0.215)	0.172 (0.11)
Tomiño		SP4	29T 515032 4641671	6	2	2	0	0.600 (0.129)	0.049 (0.42)
Ponferrada		SP5	29T 705440 4722721	10	2	3	0	0.378 (0.181)	**0.303 (0.18)**^{I}
Malaga		SP6	30S 376766 4076446	12	0	1	0	0.000 (0.000)	0.000 (0.00)
Montseny		SP7	31T 454080 4620578	10	3	3	0	0.511 (0.164)	0.170 (0.11)
Esporles		SP8	31S 461243 4392754	7	3	4	1	0.714 (0.181)	**0.934 (0.54)**^{I}
Arrabida	Portugal	PO1	29S 497741 4257559	11	3	4	2	0.709 (0.099)	**0.358 (0.20)**^{I}
Monchique		PO2	29S 538873 4126983	10	1	2	0	0.467 (0.132)	**0.689 (0.38)**^{I}
Tanger	Morocco	MO1	30S 267001 3940810	11	2	2	1	0.327 (0.153)	0.134 (0.08)
Debdou		MO2	30S 492658 3759875	8	4	5	4	0.786 (0.151)	0.196 (0.12)
Orciatico	Italy	IT1	32T 637192 4811775	8	2	2	0	0.571 (0.094)	**0.867 (0.49)**^{I}
Roma		IT2	33T 279753 4619932	10	3	3	1	0.644 (0.101)	0.039 (0.03)
Cagliari		IT3	32S 491121 4333556	9	6	3	0	0.556 (0.165)	**0.850 (0.47)**^{I}
Kroumerie	Tunisia	TUN	32S 470929 4061547	7	6	4	0	0.810 (0.130)	**0.816 (0.47)**^{I}
Atenas	Greece	GR1	34S 746586 4206313	9	5	3	0	0.639 (0.126)	**0.387 (0.22)**^{I}
Sithonia		GR2	34T 739962 4452294	8	3	3	1	0.464 (0.200)	0.031 (0.03)
Cannakale	Turkey	TU1	35T 459279 4441142	8	5	3	1	0.750 (0.097)	**0.901 (0.51)**^{I}
Izmir		TU2	35S 458841 4254352	9	6	3	0	0.639 (0.126)	**0.850 (0.47)**^{I}
Total				207	16	28	14	0.901 (0.01)	1.238 (0.60)

Table 2. Results of the SAMOVA analysis for the number of groups (K = 3) in *Arbutus unedo* for which F_{CT} reached a plateau. *** p <0.001.
Group composition 1: FR1, FR2, SP7, SP8, IT1, IT2, IT3, TUN.
2: SP1, SP2, SP3, SP4, SP6, PO1, MO1, MO2, GR1, GR2, TU1, TU2
3: IRL, SP5, PO2. Population codes as in Fig. 1

Source of variation	d.f.	MS	Percentage of variation	Fixation index
Among groups	2	1891.7	69.73	$F_{CT} = 0.697^{***}$
Among populations	20	373.3	7.42	$F_{SC} = 0.245^{***}$
Within populations	184	883.6	22.85	$F_{ST} = 0.771^{***}$

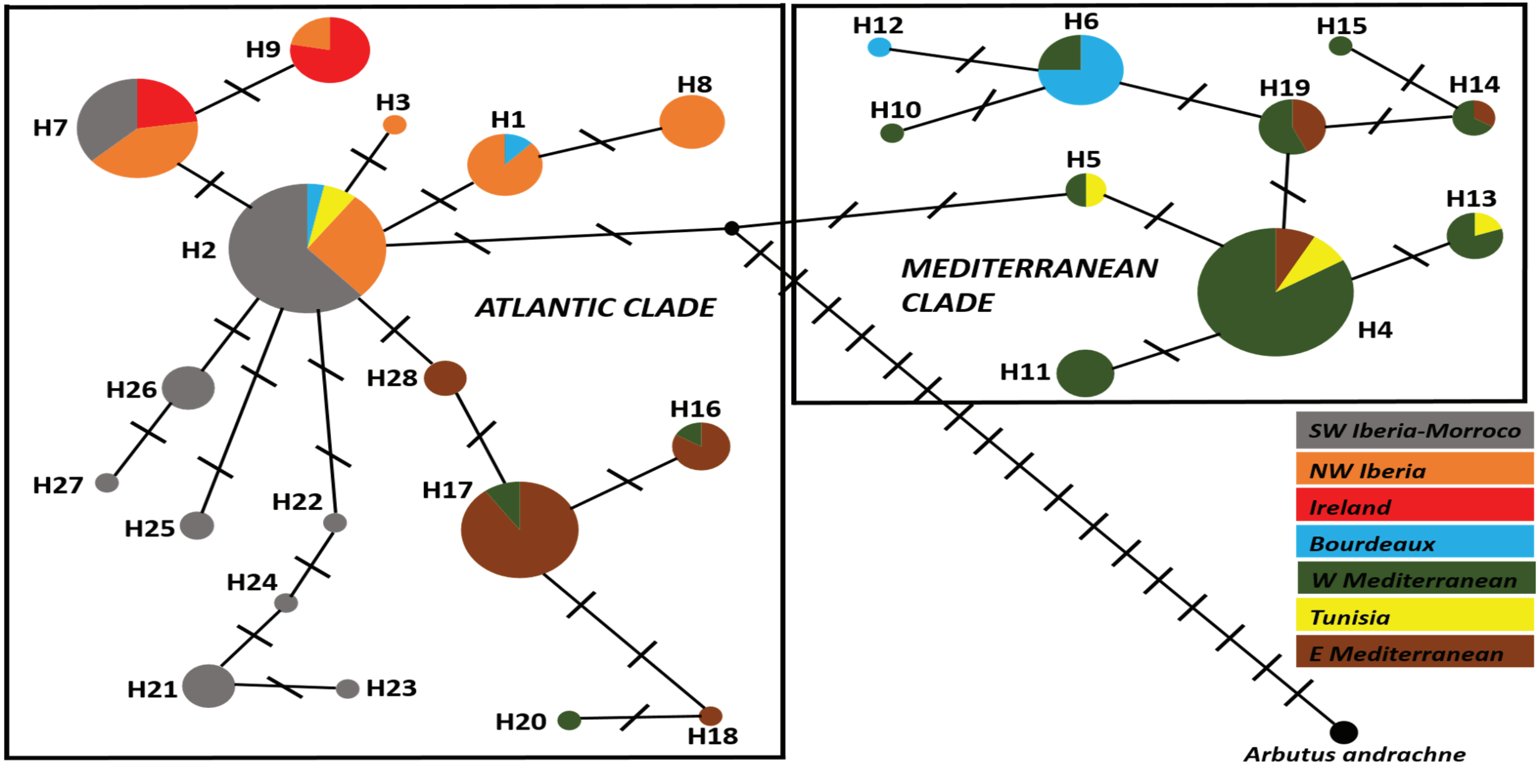
population with two locations in Atlantic Iberia. Less than 8% of the variation was among populations within groups and nearly 23% was within populations.

Haplotype genealogy and distribution.

Haplotypes H2 and H4 dominated the data set (37%) whereas 12 haplotypes were very rare as they were detected in just one (10 unique haplotypes) or two individuals (2 haplotypes) (see supplementary material, Table S1). The median-joining network (Fig. 2) yielded a genealogy where many haplotypes were just one mutational step away from their closest relative. The topology of the network revealed two clades stemming from the ancestral node inferred using *Arbutus ancrachne.* Each clade was separated from the ancestral node by two mutational steps. One clade was dominated by H2 at the center of 17 haplotypes arranged in a star-like pattern (henceforth H2-derived); H4 dominated the other clade, again surrounded by 9 less-common haplotypes (H4-derived).

The partition of haplotypes in two large lineages was remarkably consistent with their geographical arrangement and with the clusters of populations identified by SAMOVA (Fig. 2). The dominant haplotypes H2 and H4 were regionally segregated and their ranges barely overlapped: H4 was detected only in the Mediterranean while plants assigned to H2 came from sites along the Atlantic coast and North Africa. Kroumerie (TUN) in

Figure 2. Median-joining haplotype network for 18 *Arbutus unedo* non-coding cpDNA haplotypes with Arbutus andrachne as external rooting. Each circle represents a haplotype (name outside) while colours indicate sampling regions (SW Iberia-Morocco= PO1, PO2, SP6, MO1, MO2; NW Iberia= SP1-SP5; Ireland= IRL; E France= FR1; W Mediterranean= FR2, SP7, SP8, IT1-IT3; Tunisia= TUN; W Mediterranean= GR1, GR2, TU1, TU2). Circle size is proportional to haplotype frequency. Thick solid lines delineate two main clades (Atlantic and Mediterranean) separated by the inferred ancestral node (root; solid black dot). Short transversal lines are mutational steps.



North Africa was the only population where H2 and H4 lived in sympatry. Like H2, most of the H2-derived haplotypes occurred in Atlantic populations from Ireland to Morocco. In fact, H2 and H2-derived sequences (henceforth the Atlantic clade) were the only components of the populations sampled along the Atlantic shoreline except FR1 (see below). Still, a subset of five closely related H2-derived sequences had a non-Atlantic provenance as they belonged to plants sampled at Sardinia (IT3), Rome (IT2) and, particularly, the Eastern Mediterranean (Greece and Turkey). In comparison, no haplotype from the Atlantic clade was found in the NW Mediterranean (NE Spain, W France and N Italy). The H4-derived haplotypes (Mediterranean clade) also resembled the geographical distribution seen in their ancestral haplotype H4, as they were largely confined to the Mediterranean basin. The exception were two H4-derived haplotypes (H6 and H12) that eventually reached a small section of the Atlantic species' range in W France (FR1), where this haplotype lives with congeners from the Atlantic phylogroup. Interestingly, the ancestral haplotypes H2 and H4 were largely absent from the populations sampled at the northern (Ireland) and eastern (Greece, Turkey) edges of the species' range, only H4 was detected in three individuals from Turkey (population TU2).

Timing of the diversification

Initial trials in BEAST were run using exponential growth as the coalescent tree prior. Regardless of the data set tested (complete data set, Atlantic populations, Mediterranean populations, individuals from the Atlantic clade or individuals from the Mediterranean clade), the posterior distribution of the growth rate (95% HPD) always included zero indicating that we could not reject a constant population based on our data. The absence of demographic expansion was further confirmed by Fu's F_S tests run for the complete data set and for each genetic clade treated separately (p-value always >0.65). Therefore, final estimates of TMRCA relied on a constant growth model. The Bayesian tree inferred by BEAST reproduced the neat divide in two clades (Atlantic and Mediterranean) found in the network analysis. The BEAST inference suggested that the split between Atlantic and

Mediterranean lineages must have occurred before the LGM, around the Middle/Late Pleistocene boundary. Thus, the TMRCA for *A. unedo* was 365 ky (95% HPD: 48-859 ky; effective sample size, ESS = 1295) while the MRCAs for the Atlantic and the Mediterranean lineages were dated 222 ky (95% HPD: 24-527 ky) and 172 ky (95% HPD: 9-432 ky) before present, respectively.

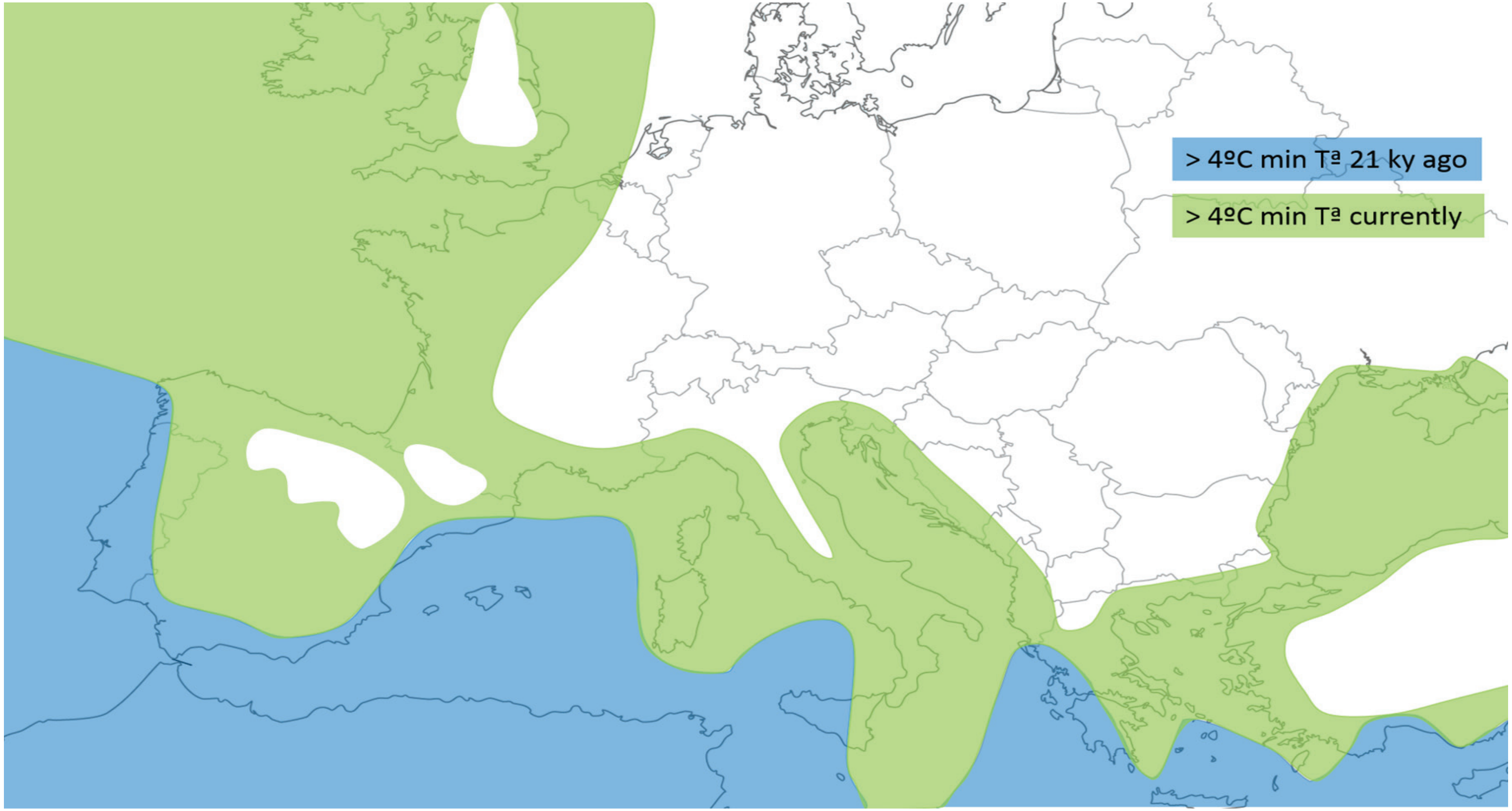
DISCUSSION

The most conspicuous attribute in our data set is the separation of the strawberry tree in two neat matrilineal lineages with longitude. East-west phylogeographic breaks are common in the Mediterranean, and extreme cases include plants with disjunct populations on both ends of the Mediterranean (Kadereit & Yaprak, 2008; Casimiro-Soriguer *et al.*, 2010; Nieto Feliner, 2014). In plants, however, there are more examples of species with a continuous range that still show a phylogeographic divide between the Eastern and the Western Mediterranean (Lumaret *et al.*, 2002; Hampe *et al.*, 2003; Rodríguez-Sánchez *et al.*, 2009; Escudero *et al.*, 2010). The peculiarity in the strawberry tree is that both lineages appear to have originated in the western Mediterranean: one in the Atlantic coasts of Iberia and North Africa, the other in the western subbasin of the Mediterranean itself. The presence of several genetic clades in the western Mediterranean has been observed in other plants and linked to range contractions during the Quaternary glaciations (Kadereit *et al.*, 2005; Lumaret *et al.*, 2005). Our inference suggests that the diversification of *A. unedo* in two lineages also occurred in the late Pleistocene but before the LGM, concurring with the hardest glaciations (Médail & Diadema, 2009; Stewart *et al.*, 2010). Thus, *A. unedo* may have survived in some of the glacial refugia detected in the western Mediterranean, where the ancestral haplotypes are commonly present. In particular, some areas with a previously neglected role as refugia such as North Africa seem likely candidates for the glacial refugia of *A. unedo* (Médail & Diadema 2009).

The more recent colonization of the Eastern Mediterranean by the strawberry tree contrasts with most studies of plants where eastern lineages typically are more ancient (Lumaret *et al.*, 2002; Kadereit & Yaprak, 2008; Escudero *et al.*, 2010; Conord *et al.*, 2012; Migliori *et al.*, 2012). In trees, the predominance of westward migrations has been attributed to divergence that predates the Pleistocene (Lumaret *et al.*, 2002; Lumaret *et al.*, 2005; Petit *et al.*, 2005). In other cases, however, genetic and fossil evidence linked the East-West break to range fragmentations and to more severe climate in the west during the Pleistocene (Escudero *et al.*, 2010; Migliori *et al.*, 2012). In comparison, there are few examples of colonisation in the opposite direction. Still, North African populations were the likely candidates for the ancestral pool of *Europhaca* (Casimiro-Soriguer *et al.*, 2010), while the submediterranean alpine *Anthyllis montana* also migrated eastward from Iberia up to the Balkans along the northern edge of the Mediterranean (Kropf *et al.*, 2002). Similarly, *Pinus pinaster* (Bucci *et al.*, 2007) and *Erica arborea* (Désamoré *et al.*, 2011) migrated eastwards along North Africa to Europe. Nonetheless, the example that seems closer to strawberry tree is *Myrtus communis*, another circum-Mediterranean shrub with fruits dispersed by birds. Following differentiation events during the Pleistocene, some matrilineal lineages of *M. communis* spread from west to east; in particular, to the Balkan Peninsula where they met with older lineages (Migliori *et al.*, 2012). In *A. unedo,* the eastward colonization would be consistent with the unsuitability of the West Mediterranean for this plant during glacial maxima. Thus, paleoclimatic reconstructions of the LGM suggest that minimum monthly temperatures in the Aegean-Anatolia area were below the 4°C limit required by the strawberry tree for survival (Sealy, 1949) (Fig. 3).

The likely route for the movement of the Atlantic lineage towards the Eastern Mediterranean was through North Africa to mainland Italy and the Balkans by crossing the Strait of Sicily. The latter has been involved in biogeographical connections for many taxa. In the particular case of trees, a crossing by matrilineal lineages from North Africa has been inferred for some species (Lumaret *et al.*, 2005; Désamoré *et al.*, 2011; Migliori *et al.*, 2012) but not for others (Hewitt, 1999; Lumaret *et al.*, 2002). Nonetheless, the

Figure 3. Areas with minimum monthly temperatures suitable for *Arbutus unedo* (>4ºC) during the LGM (21 kya, blue) and in modern times (green) according climate simulation models (Van Andel, 2002) (adapted from plots available in http://www.esc.cam.ac.uk/research/research-groups/oistage3/stage-three-project-simulations).



strait still had an impact on the migration of the Atlantic clade of *A. unedo* because only a few H2-derived haplotypes managed to reach the eastern Mediterranean, contrarily to other trees where several haplotypes were common to both sides of the strait (Lumaret *et al.*, 2005; Migliori *et al.*, 2012). Our estimate of the TMRCA for the Atlantic clade suggests that Strait of Sicily was crossed in the last 200000 years but still before the LGM. The impact of the strait on the Mediterranean lineage was different because the same set of haplotypes occurs on both sides of the strait. However, the Mediterranean clade seemed unable to spread beyond Tunisia, a confinement that would be consistent with the 'high density blocking hypothesis' (Hewitt, 1999) if North Africa was already colonized by the Atlantic lineage before the arrival of the Mediterranean clade.

Gibraltar, the other large strait in the region, also had partial success in interrupting the gene flow of *A. unedo*. Only plants with Atlantic ancestry live on both sides of the strait, and the ancestral haplotype H2 is equally common to the north and to the south of Gibraltar. However, none of the H2-derived haplotypes recorded in Iberia occurs in North Africa and the other way around. Hence, the isolation of the populations on both sides of the strait must have lasted long enough to allow the diversification of distinct sets of haplotypes on each area, suggesting that North Africa may have acted as a refugium. Interestingly, Rodríguez-Sánchez *et al.* (2008) noted that colonization ability, rather than dispersal potential, is a better predictor of the genetic structure across the strait.

The phylogeographic arrangement of *A. unedo* does not show the west to east increase in genetic diversity seen in many other plants and animals (Conord *et al.*, 2012). Instead, genetic diversity within populations is notably variable and populations with high or low diversity are interspersed all along the species' range. If any, genetic diversity increases from East to West at regional level due to the dominance of plants with an Atlantic ancestry and the small number of haplotypes detected in this region.

The predominance of H4-derived haplotypes in FR1 reveals that the Mediterranean lineage reached the Atlantic coast. Interestingly, FR1 shares a private haplotype (H6) with north-western Iberia (SP7) and the Balearics (SP8). Indeed, north-western Iberia (SP7) is more closely related to French demes than to other Iberian populations. Thus, the strawberry tree adds to the many cases of trees with a clear-cut East-West divide in the Iberian Peninsula (Rodríguez-Sánchez *et al.*, 2010), and provides another example of the pattern typically attributed to the 'refugia-within-refugia' model (Gómez & Lunt, 2007). Since western and eastern Iberia are characterized by different soil types, it has been speculated that soil type might have promoted the isolation of lineages in the peninsula (Rodríguez-Sánchez *et al.*, 2010). However, the potential of ecological factors to determine the genetic structure of *A. unedo* is debatable since common garden experiments show the absence of local adaptation in this species (Santiso *et al.*, 2015).

Finally, our results discard a Tertiary origin for *A. unedo* in Ireland (Sealy, 1949). Instead, a relatively recent colonization of Ireland seems the most plausible alternative given (i) the absence of private haplotypes in Ireland, and (ii) the genetic proximity between Irish and Iberian population (SAMOVA results). The arrival of the strawberry tree to Ireland has been attributed to movements along northern Iberia and the maritime fringe of France (Sealy, 1949; Cox & Moore, 2005). Our results do not support this hypothesis either, because none of the haplotypes recorded in Ireland was detected in FR1, at the northern edge of the continuous range of *A. unedo* in the Atlantic. As noted above, FR1 is predominantly composed by haplotypes with a Mediterranean ancestry that are conspicuously absent from Ireland. Hence, our data support an Iberian origin for the Irish populations, and the genetic proximity to populations from northwest Spain is in agreement with an arrival to Ireland by crossing the Celtic Sea as it has been described for other tree species (Mitchell, 2006).

Conclusions

The view that species respond individualistically to climate change and create new community patterns has gained increasing support in the last decades (Rodríguez-Sánchez *et al.*, 2010). Here, we have shown concordances and discrepancies between the phylogeographic structure of the strawberry tree and that of other plants investigated in the Mediterranean basin. The partition of the strawberry tree in two clades could be attributed to vicariance events during the Quaternary glaciations of the last 700 ky, but before the LGM. Likely refugia are North Africa-Atlantic Iberia for the Atlantic clade, and the European rim and large islands of the Western Mediterranean subbassin for the Mediterranean clade. We also found evidence of occasional connections between Europe and North Africa through the straits of Gibraltar and Sicily, but North Africa still retains a distinctive genetic composition, which increase the evidence about its role as refugium. Further, the strawberry tree shows the East-West genetic divide found for other trees in the Iberian Peninsula, providing another example consistent with refugia-within-refugia model (Gómez & Lunt, 2007). Unlike many other species, the Eastern Mediterranean must have been colonized more recently, and its lower genetic diversity contrasts with the pattern more commonly seen in other plants. Our results discard a Tertiary origin for *A. unedo* in Ireland and do no support a continuous range along the maritime fringe of France. Instead, it may have arrived to Ireland on post-glacial times from N Iberia by crossing the Celtic Sea. Altogether, our results reveal a considerable ability to disperse for the strawberry tree, migrating over thousands of kilometres and even crossing stretches of sea (Mediterranean straits), which could be useful in a scenario of future changes.

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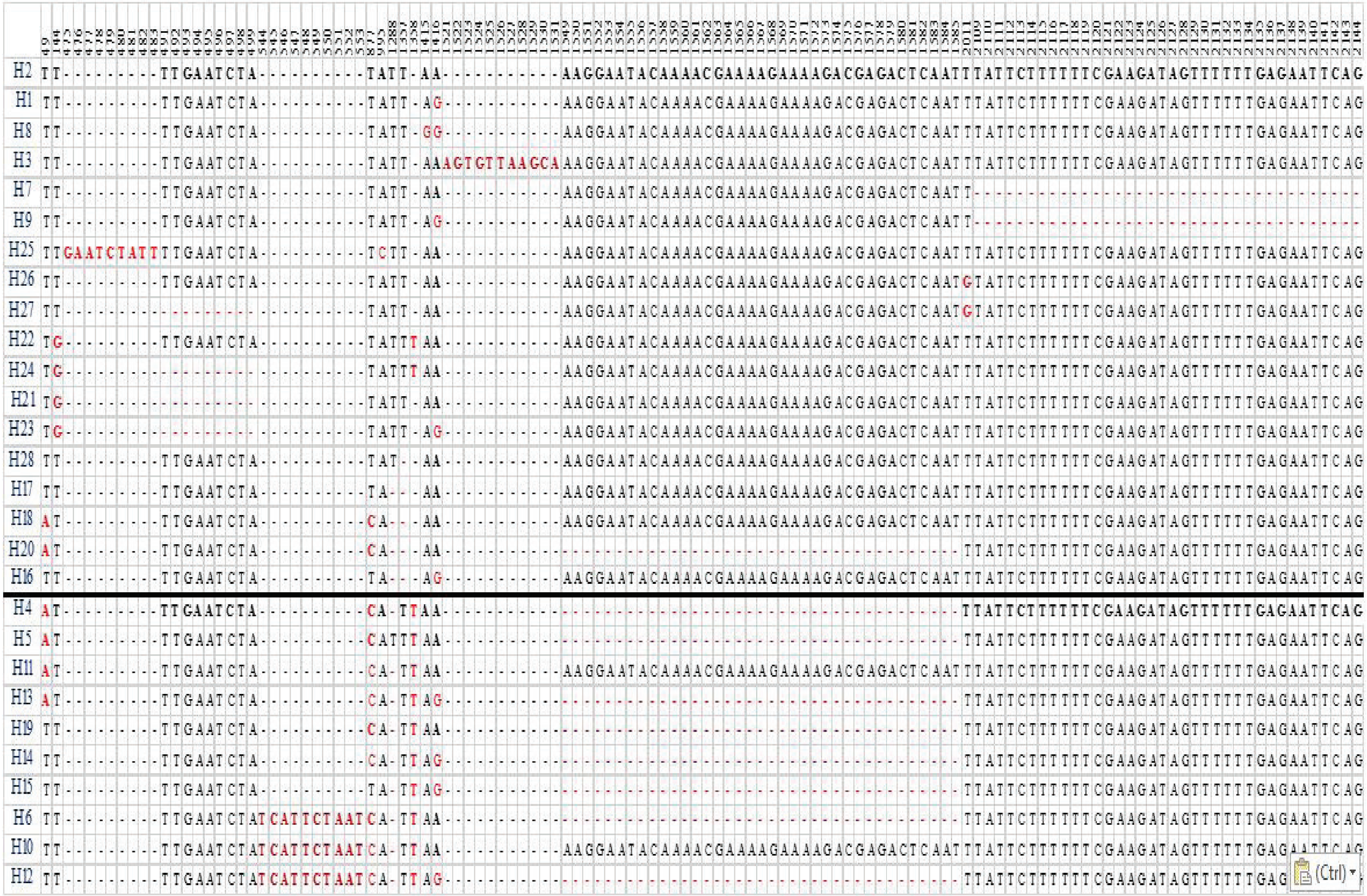
Supplementary Material

Table S1. Number of individuals per haplotype in 23 population of *Arbutus unedo*. Population codes as in Fig. 1

	Atlantic clade																		Mediterranean clade										
Pop	H2	H1	H8	H3	H7	H9	H25	H26	H27	H22	H24	H21	H23	H28	H17	H18	H20	H16	H4	H5	H11	H13	H19	H14	H15	H6	H10	H12	**Total**
IRL					5	7																							**12**
SP1		1	3			2																							**6**
SP2	3	2	1		1																								**7**
SP3	1	4		1																									**6**
SP4	3		3																										**6**
SP5	1		1		8																								**10**
PO1	5				1			4	1																				**11**
PO2	3				7																								**10**
SP6	12																												**12**
MO1	9						2																						**11**
MO2	1									1	1	4	1																**8**
TUN	2																		3	1		1							**7**
SP7																			7	1						2			**10**
SP8																			4		1					1	1		**7**
FR1	1	1																								9		1	**12**
FR2																			4			4		2	1				**11**
IT1																			4		4								**8**
IT2																	1		5				4						**10**
IT3															2			1	6										**9**
GR1															5			3						1					**9**
GR2															6	1		1											**8**
TU1														3	2								3						**8**
TU2															5			1	3										**9**
Total	**41**	**8**	**8**	**1**	**22**	**9**	**2**	**4**	**1**	**1**	**1**	**4**	**1**	**3**	**20**	**1**	**1**	**6**	**36**	**2**	**5**	**5**	**7**	**3**	**1**	**12**	**1**	**1**	**207**

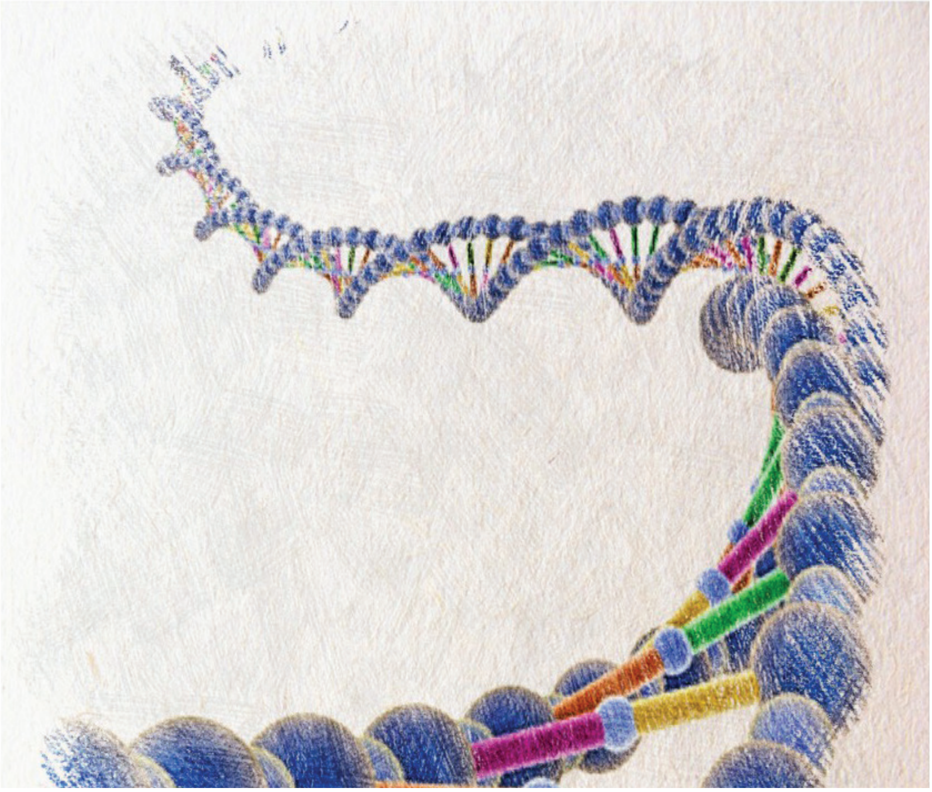
Figure S1. Variable positions in the 28 haplotypes detected in *Arbutus unedo*. The first 18 haplotypes belong to the Atlantic clade, the last 10 to the Mediterranean one.



H2	TT---------TTGAATCTA----------TATT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H1	TT---------TTGAATCTA----------TATT-AG-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H8	TT---------TTGAATCTA----------TATT-GG-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H3	TT---------TTGAATCTA----------TATT-AAAGTGTTAAGCAAAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H7	TT---------TTGAATCTA----------TATT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATT------------------------------------
H9	TT---------TTGAATCTA----------TATT-AG-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATT------------------------------------
H25	TTGAATCTATTTTGAATCTA----------TCTT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H26	TT---------TTGAATCTA----------TATT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATGTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H27	TT----------------------------TATT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATGTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H22	TG---------TTGAATCTA----------TATTTAA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H24	TG----------------------------TATTTAA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H21	TG----------------------------TATT-AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H23	TG----------------------------TATT-AG-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H28	TT---------TTGAATCTA----------TAT--AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H17	TT---------TTGAATCTA----------TA---AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H18	AT---------TTGAATCTA----------CA---AA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H20	AT---------TTGAATCTA----------CA---AA--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H16	TT---------TTGAATCTA----------TA---AG-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H4	AT---------TTGAATCTA----------CA-TTAA--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H5	AT---------TTGAATCTA----------CATTTAA--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H11	AT---------TTGAATCTA----------CA-TTAA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H13	AT---------TTGAATCTA----------CA-TTAG--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H19	TT---------TTGAATCTA----------CA-TTAA--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H14	TT---------TTGAATCTA----------CA-TTAG--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H15	TT---------TTGAATCTA----------TA-TTAG--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H6	TT---------TTGAATCTATCATTCTAATCA-TTAA--TTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H10	TT---------TTGAATCTATCATTCTAATCA-TTAA-----------AAGGAATACAAAACGAAAAGAAAAGACGAGACTCAATTTATTCTTTTTTCGAAGATAGTTTTTTGAGAATTCAG
H12	TT---------TTGAATCTATCATTCTAATCA-TTAG--TTATTCTTTTTTCGAAGATAGTTTTTTGAG

Chapter II

Molecular evidence reveals a strong Atlantic-Mediterranean divide and an unexpected Iberian-Irish link in the strawberry tree (*Arbutus unedo* L.)

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ABSTRACT

Arbutus unedo L. is a predominantly circum-Mediterranean plant that also reaches the Atlantic coasts of Morocco, Iberia, southern France, and, interestingly, Ireland, where there is a disjunct population. In this study, we assessed whether this extended and largely lineal range has affected its gene flow resulting in an isolation by distance (IBD) pattern; In addition, we have tried to elucidate the origin of the Irish population and to determine whether or not the broad and climatically contrasted range of the species may have lead to the existence of genetic structure. To fulfill these aims, we sampled the whole range of the species from Ireland to North Africa and Turkey and produced AFLP genotypes for 491 individuals from 19 populations. The geographical arrangement of genetic diversity was investigated with Nei's gene diversity index (Hj), F_{ST} statistics, and analysis of molecular variance (AMOVA) to assess the genetic differentiation between populations, and Mantel tests to check for IBD. The differentiation between populations was further checked with a spatial analysis of molecular variance (SAMOVA) while the population structure was also inferred with individual-based Bayesian clustering algorithms. We found evidence of a significant IBD pattern that was stronger, and F_{ST} increased faster with distance, along the Atlantic than around the Mediterranean. Genetic diversity was significantly lower in NW Iberia (11% lower) and Ireland (20%) than the typical values recorded in other populations and pairwise F_{ST} estimates showed that Ireland was genetically more similar to N Iberia than to the geographically closer W France. Finally, the Bayesian analysis revealed a clear-cut split between circum-Mediterranean and Atlantic facade populations. Our results are consistent with a scenario of independent glacial refugia in the Atlantic and the Mediterranean portions of the distribution range of *A. unedo*. Altogether, the IBD pattern, the northward decrease of within-population genetic diversity, and the arrangement of pairwise F_{ST} estimates, point to a postglacial stepping-stone colonization along the Atlantic façade and to an Iberian origin for the Irish population.

Keywords: AFLP, Atlantic-Mediterranean divide, *Arbutus unedo*, disjunct distribution, genetic diversity, genetic structure, Ireland.

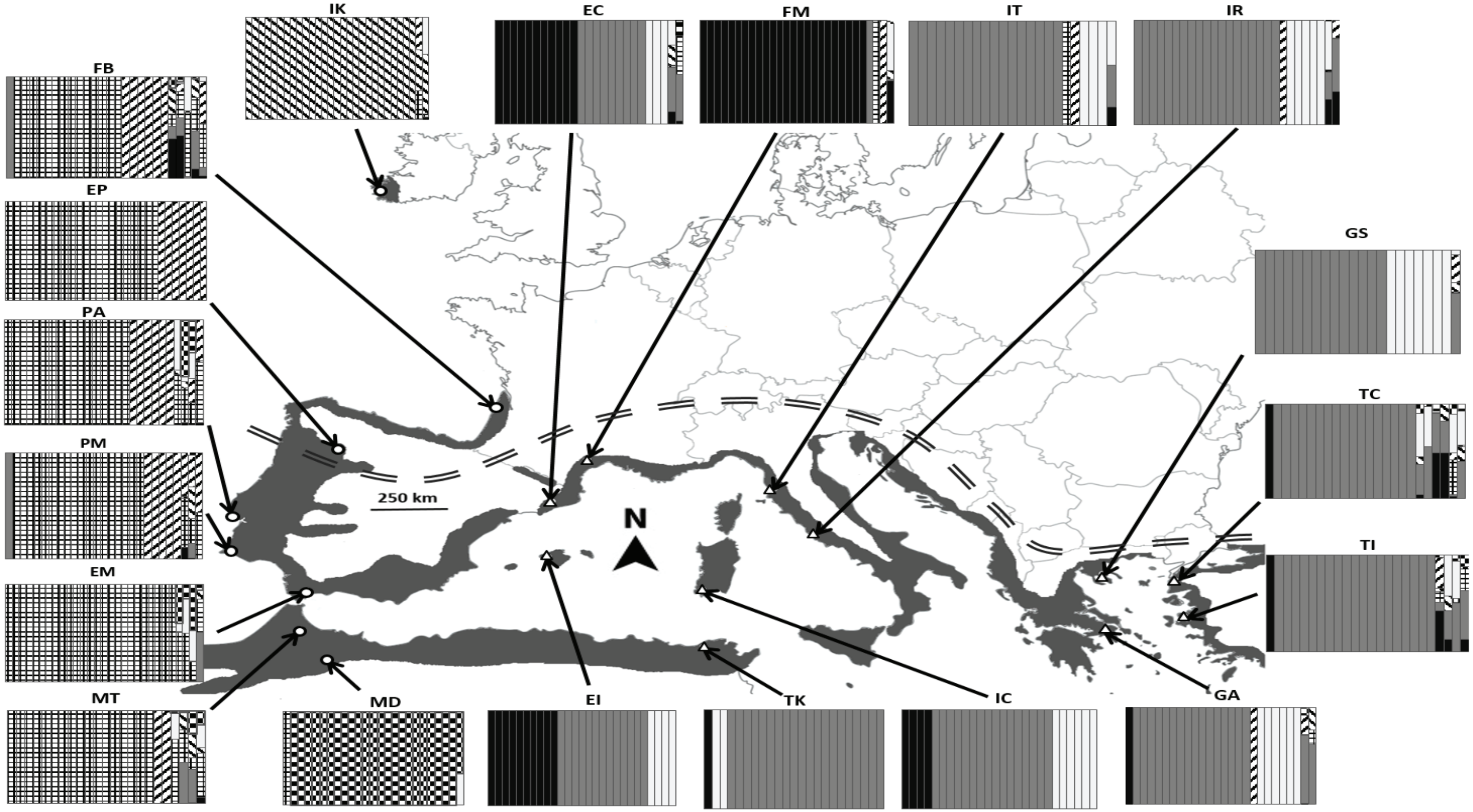
INTRODUCTION

The future of a species, in terms of adaptive ability and evolutionary potential, depends on its genetic diversity (Frankham *et al.*, 2002). The latter can be related to the pattern of spatial distribution of the species, as the latter influences the amount of gene flow among populations (Frankham *et al.*, 2002). In this regard, species with peculiar distributions offer interesting opportunities to investigate the resulting genetic structure and the processes that may have determined it. That is the case of *Arbutus unedo* L. (strawberry tree), an evergreen shrub with a predominantly Mediterranean distribution that also occurs along the Atlantic coast of Europe up to Ireland (Sealy, 1949; Webb, 1983; Cox & Moore, 2005).

The strawberry tree is a typical element of the Mediterranean biogeographical region where it shows a neat circum-Mediterranean distribution (Torres *et al.*, 2002) that evokes the one-dimensional spatial pattern typically found in ring species (Irwin *et al.*, 2001). This small tree occupies a narrow coastal fringe that spans from Tunisia to Morocco along the north of Africa, and from Spain to Turkey along southern Europe (Fig. 1). Nonetheless, this plant is also widely distributed in the area where the Mediterranean biogeographic region meets the Atlantic Ocean (Portugal and northern Morocco) and it even enters into the Euro-Siberian region. Thus, *A. unedo* ranges northwards along the coast of Atlantic Europe with populations in northern Iberia, western France, and south-western Ireland, concurring with the 4ºC limit for the mean temperature of January (Sealy, 1949).

The presence of *A. unedo* in Ireland but not in Britain means that this tree belongs to the puzzling Lusitanian flora: organisms that are found in southern and western Ireland and in northern Iberia but are largely absent from intervening countries (Sealy, 1949; Beatty & Provan, 2013). The origin and distribution of the Lusitanian biota has been the subject of considerable debate. Initially, two main hypotheses were proposed (Sealy, 1949): (i) that these species could be relicts of the Tertiary flora of the British Islands that survived through the glacial period (Forbes, 1846),

Fig. 1. Distribution range with the location of the 19 *Arbutus unedo* populations sampled in this study and their genetic structure according to an individual-based spatial analysis with BAPS. Histograms show the genetic cluster of each individual (vertical bars denote individuals), for the optimal number of seven genetic clusters (P = 0.9793) obtained after admixture with the spatial analysis option. Open triangles and circles are populations assigned to the Mediterranean and Atlantic genetic groups, respectively. Double-dashed line indicates the limits between the Euro-Siberian and Mediterranean biogeographical regions. See Material and Methods for population names.



or (ii) that they arrived to Ireland via a postglacial migration. However, Sealy and others already considered the glacial survival unlikely due to the biotic requirements of the species and, consequently, saw the postglacial migration as a more plausible alternative (Reid, 1913; Webb, 1983). Indeed, both palynological and genetic evidence support the postglacial migration of other Lusitanian elements (Mitchell, 2006; Beatty & Provan, 2013). The timing and route for these postglacial arrivals continue to be controversial. For example, the maritime fringe of Western Europe (Iberia-France-Ireland) was described as the likely route for the arrival of *A. unedo* to Ireland after the Last Glacial Maximum (LGM) (Sealy, 1949; Cox & Moore, 2005). Thus, the arrival may have taken place during the Xerothermic period (8000-4000 B.P.) described by Axelrod (1975) as the time when shrubs were greatly favoured in areas previously covered with sclerophyllous woodland. However, the French origin of the Irish populations is far from proved. Indeed, there is growing evidence that many tree species did not enter Ireland by crossing the Irish Sea from Britain but instead they migrated from the south via the Celtic Sea (Mitchell, 2006), what open the possibility of direct arrival from Iberia, as Reid (1913) had already suggested.

Despite its interesting distribution and the uncertainties around the origin of Irish populations, no study has investigated the genetic structure of *A. unedo* throughout its range. The only population studies available so far are country-specific. Thus, there are a couple of studies in Tunisia which found low (Takrouni *et al.*, 2012) and moderate (Takrouni & Boussaid, 2010) genetic differentiation between populations, while in Portugal low-moderate differentiation was recorded among populations (Lopes *et al.*, 2012). Further, more genetic diversity within populations was determined to be low (Takrouni & Boussaid, 2010), moderate (Lopes *et al.*, 2012) or high (Takrouni *et al.*, 2012) depending on the set of sampled populations. Moreover, since these studies covered only a very small portion of the species range, their results cannot contribute to elucidate whether the genetic structure of the strawberry tree shows any imprint left by its peculiar and largely one-dimensional distribution. Thus, there is no information on whether the populations of *A. unedo* located in the Eurosiberian portion of the species range may show any evidence of reduced gene flow and genetic differentiation from those from the main Mediterranean range. Similarly, since *A. unedo* occupies most of the shoreline around the Mediterranean but it is largely absent from Syria to Libya (> 2500 km), it could be speculated that the populations that encircle the Mediterranean might fit an isolation-by-distance (IBD) pattern if gene flow is restricted to movements along the shoreline. Additionally, an IBD pattern could also be observed along the Atlantic façade if the restriction to the gene flow occurred along this coast. Finally, it would be reasonable to anticipate that the Irish enclaves may show signs of genetic erosion given their peripheral and isolated position (Vucetich & Waite, 2003).

Molecular markers are the typicall tools used to obtain information that may disentangle the spatial patterns of genetic variability of species. An important limitation in non-model species is the need to develop molecular markers with appropriate resolving power for an organism with minimal, or none, sequence information. In these instances, amplified fragment length polymorphisms (AFLPs) (Vos *et al.*, 1995) have been widely used in plants because they do not require previous sequence information while providing high reproducibility and potential to comprehensively scan the genome (Mba & Tohme, 2005; Peters *et al.*, 2009; Lopez & Barreiro, 2013). Compared to other markers (e.g. SSR), AFLP bands are dominantly inherited and do not allow to detect heterozygotes. Moreover, dominant markers typically estimate lower levels of within population diversity and higher levels of among-population differentiation than their codominant counterparts (Nybom, 2004). However, AFLP loci have been shown to be more efficient that SSR loci in systems characterized by weak population structure (Campbell *et al.*, 2003). Here, we developed AFLP markers for *A. unedo* to address a number of specific issues: (i) whether the largely one-dimensional distribution of the strawberry tree can lead to an IBD pattern, in our particular case around the Mediterranean and/or Atlantic façade; (ii) whether the Irish population has a French or an

Iberian origin in an effort to contribute to the Lusitanian debate; and (iii) whether the climatic contrast between Eurosiberian and Mediterranean populations has resulted in some sign of reduced gene flow and/or genetic differentiation between them. To fulfil these aims, we conducted a range-wide survey of *A. unedo* from Turkey in the East to Iberia in the West, and from Morocco in the South to Ireland in the North.

MATERIALS AND METHODS

The species

Arbutus unedo is a diploid species (2n = 26, x = 13) (Sealy & Webb, 1950) belonging to the family *Ericaceae,* subfamily *Arbutoideae*, which includes evergreen, shrub-like woody taxa with sclerophyllous, laurel-like leaves (Torres *et al.*, 2002). Genus *Arbutus* has several members in America and four in Europe: *A. andrachne* L. (eastern Mediterranean), *A. pavarii* Pampanini (Libyan coasts), *A. canariensis* Veill. (Canary Islands) and *A. unedo* L. (Torres *et al.*, 2002). Flowering spans from September to December and fecundation is entomophilus (Mitchell, 1993); indeed, its nectar and pollen are the main food source for *Bombus terrestris* (Rasmont *et al.*, 2005). Fruits take 12 months to ripe, and seeds are dispersed by animals (mainly birds); seed viability was reported to be about 55% in favourable places while seedlings loss was found to exceed 60% (Sealy, 1949).

Sample collection and DNA extraction

Our sampling scheme covered the entire distribution range of the species around the Mediterranean Sea and Atlantic facade. Leaf samples were collected from 19 sites covering nine countries (Table 1 and Fig. 1): two sites in Turkey (Izmir-TI and Çannakale-TC), two in Greece (Athens-GA, Sithonia-GS), one in Tunisia (Kroumerie-TK), three in Italy (Rome-IR, Toscana-IT, Cagliari-IC), two in Morocco (Tanger-MT, Debdou-MD), two in Portugal (Arrabida-PA, Monchique-PM), four in Spain (Catalonia-EC, Mallorca Island-EI, Málaga-EM, Ponferrada-EP), two in France (Montpellier-FM, Bordeaux-FB), and one in Ireland (Killarney-IK). At each population, leaves were collected from 24-30

Table 1. Sampling sites and genetic diversity estimates based on 125 segregating loci (5% criterion) obtained with AFLPs for *Arbutus unedo*. *ATL* and *MED* are total values for populations assigned to Atlantic (8) and Mediterranean (11) genetic lineages by other analyses. See Material and Methods for population names. Pop, population; N, sample size (effective sample size after accounting for loci with missing values in some individuals); PL, number (and percentage) of polymorphic loci (5% criterion); PB, no. of private bands; *H*j: Nei's gene diversity (± standard error).

Pop	Coordinates (UTM)	N	PL	PB	*H*j (±S.E.)
IK	29U 465211 5763297	27 (29)	94 (75.2%)	0	0.26 (±0.016)
FB	30T 643276 4939413	24 (26)	103 (82.4%)	0	0.31 (±0.015)
EP	29T 705440 4722721	27 (29)	99 (79.2%)	0	0.28 (±0.016)
PA	29S 497741 4257559	24 (27)	108 (86.4%)	0	0.32 (±0.015)
PM	29S 538873 4126983	24 (27)	109 (87.2%)	0	0.30 (±0.015)
EM	30S 376766 4076446	26 (29)	103 (82.4%)	0	0.30 (±0.015)
MT	30S 267001 3940810	21 (23)	112 (89.6%)	0	0.32 (±0.014)
MD	30S 492658 3759875	23 (25)	108 (86.4%)	0	0.31 (±0.014)
ATL		*202 (215)*	*111 (88.8%)*	*0*	*0.31 (± 0.014)*
EC	31T 454080 4620578	24 (25)	108 (86.4%)	0	0.31 (±0.015)
FM	31T 531757 4829416	26 (28)	102 (81.6%)	0	0.30 (±0.015)
EI	31S 461243 4392754	25 (27)	104 (83.2%)	0	0.31 (±0.015)
IC	32S 491121 4333556	24 (26)	114 (91.2%)	0	0.34 (±0.014)
TK	32S 470929 4061547	21 (23)	110 (88.0%)	0	0.32 (±0.015)
IR	33T 279753 4619932	26 (27)	104 (83.2%)	0	0.32 (±0.015)
IT	32T 637192 4811775	21 (23)	109 (87.2%)	0	0.33 (±0.015)
GA	34S 746586 4206313	24 (26)	113 (90.4%)	0	0.34 (±0.014)
GS	34T 739962 4452294	20 (22)	108 (86.4%)	0	0.33 (±0.015)
TC	35T 459279 4441142	23 (24)	107 (85.6%)	0	0.31 (±0.016)
TI	35S 458841 4254352	24 (25)	109 (87.2%)	0	0.32 (±0.015)
MED		*263 (276)*	*113 (90.4%)*	*2*	*0.34 (±0.014)*

haphazardly selected trees. To minimize the risk of sampling half siblings, distance between sampled trees was >10 meters; in this regard, it is worth to note there is no true vegetative reproduction by suckers in *A. unedo* (Sealy, 1949). In the field, leaves were individually wrapped in Kimtech Science wipes (Kimberly-Clark Europe Ltd, United Kingdom) and stored in silica gel until DNA extraction.

DNA was extracted with the "Realpure Genomic DNA extraction from plants and fungi kit" (REAL, Durviz s.l.u., Spain) following manufacturer's instructions with minor modifications: incubation times at cell lysis and protein precipitation steps were extended up to 2 hours and up to 20 minutes, respectively. Samples were processed in batches of 23 samples (from at least five populations) plus 1-2 negative controls with no tissue. Besides, 10% of the samples were extracted twice on different batches. The quality of the extracted DNA and negative controls was checked on 1.5 % agarose gels.

AFLP analyses

Given that AFLP performance can be sensitive to reaction conditions (Bonin *et al.*, 2004), we used several control measures to guarantee the reproducibility of our set of markers. First, primer combinations were chosen after screening twelve pairs of primers, with three selective bases, on 20 individuals from five populations that covered the whole range of the strawberry tree (four individuals per population). The whole procedure was repeated with new, independent DNA extractions of the same 20 individuals to assess the reproducibility of each primer combination. Nine primer combinations that were highly reproducible and easy to score were selected (EcoRI/TruI): TGG/CAA TCA/CAT TAG/CAT TCA/CTG TAC/CTG TAG/CTT TGC/CAC TAC/CAA TAG/CTG. Second, DNA was re-extracted from approximately 10% of individuals (evenly distributed among the 19 sampling sites) and run in parallel with other DNA samples to check for reproducibility during the study. Samples and replicates were run in a blind-manner to avoid any bias during scoring. In addition, individuals from each one of the 19 sites were evenly partitioned among the 96-well plates used for

PCR; replicates and originals were always run on separate plates to avoid potential biases inherent to one particular plate. Third, each batch of DNA extractions (24 samples) included a negative control that went through the entire genotyping procedure (DNA extraction included). The estimated global genotyping error (1.5%) was consistent with results of reproducibility tests conducted for AFLP on both plants and animals (Bonin *et al.*, 2004); the maximum error rate for individual loci (5%) was well below the maximum recommendable for AFLP analyses (10%) (Bonin *et al.*, 2007).

AFLP analyses were performed following Vos et al. (1995) but nonradioactive fluorescent dye-labelled primers were used and fragments were separated on a DNA sequencer. Approximately 250 ng of genomic DNA were digested at 37° C for 3 h in a final volume of 20 µl with 1.25 units of EcoRI and TruI (Fermentas) and 2X Tango Buffer (Fermentas). Digested DNA was ligated for 3 h at 37° C to double-stranded adapters (50 pmols of adaptors E, 5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'; and M, 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') using 0.5 units of T4 DNA ligase (Fermentas). Then, 2 µl of the ligation product was pre-amplified with 0.3 µM of each single selective primer (EcoRI-T and TruI-C), 2.5 mM $MgCl_2$, PCR buffer 1X (Applied Biosystems), 0.8 µM dNTPs, 0.04 µg/µl BSA, Betaine 1 M and 0.4 U of Taq polymerase (Applied Biosystems) in a final volume of 20 µl. Amplification conditions were 2 min at 72 °C; 2 min at 94 °C; 20 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension of 30 min at 60 °C. Pre-amplification fragments were diluted 1:5 with Milli-Q water; 2.5 µl of the resulting solution were selectively amplified using 0.6 µM of the selective primers, 0.8 µM dNTPS, 2.5 mM $MgCl_2$, 0.04 µg/µl BSA, PCR Buffer 1X (Applied Biosystems) and 0.4 U of AmpliTaq Gold polymerase (Applied Biosystems) in a final volume of 10 µl. Selective amplification was performed as follows: 4 min at 95 °C; 12 of cycles of 30 s at 94 °C, 30 s at 65 °C (first cycle, then decreasing 0.7 °C for each one of the last 11 cycles), and 2 min at 72 °C; 29 cycles of 30 s at 94 °C, 30 s at

56 ºC, and 2 min at 72 ºC; and a final extension of 30 min at 72 ºC. Digestion, ligation, and PCR reactions were performed in a PxE thermal cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). Selective amplification products were electrophoresed on an ABI 3130xl automated DNA (Applied Biosystems) sequencer with HD-500 as size standard (Applied Biosystems). Fragments from 70 to 400 bp were manually scored for presence/absence at each selected locus with the help of GeneMarker v.1.70 (SoftGenetics LLC, State College, PA, USA) following common recommendations (Bonin *et al.*, 2005).

Data analysis

Sampling sites were regarded as separate populations and their allele frequencies were estimated using the Bayesian Method of Zhivotovsky (1999) implemented in AFLPsurv 1.0 (Vekemans *et al.*, 2002) with the option of non-uniform prior distributions of allele frequencies. Genetic diversity per population was estimated as the proportion of polymorphic loci (5% criterion), the number of private bands, and Nei's gene diversity (*H*j). Significant differences in gene diversity between populations were tested with the GT2-method for multiple unplanned comparisons among pairs of means based on unequal sample sizes (Sokal & Rohlf, 1995).

Allele frequencies were also employed to estimate genetic differentiation between samples as F_{ST} values following Lynch and Milligan (1994). The significant of pairwise F_{ST} values was tested by resampling the statistics with 10000 permutations. Pairwise F_{ST} values were also used to scale the populations with a non-metric multidimensional scaling (nMDS) (Cox & Cox, 1994) with the help of the statistical package Primer v6 (PRIMER-E, United Kingdom) (Clarke & Gorley, 2006). To facilitate comparison with other studies, we also estimated the differentiation between populations with an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) that calculates Φ_{PT} (an analogue of F_{ST}), using the squared Euclidean distance between AFLP phenotypes. The significance of the Φ_{PT} values was estimated after 9999 random permutations of individuals among populations performed with GenAlex 6.5 (Peakall & Smouse, 2006). The pattern of genetic differentiation was further investigated with a spatial analysis of molecular variance (SAMOVA) that defines groups of populations that are geographically homogeneous and maximally differentiated from each other. SAMOVA was conducted with the software SAMOVA 1.0 (Dupanloup *et al.*, 2002) running 100 simulated annealing process for each configuration of K groups, with K ranging from 2 to 17, and searching for the configuration that maximize F_{CT} (the proportion of total genetic variance due to differences among groups of populations).

The correlation between genetic using the pairwise F_{ST} values and geographic distance (Km) among populations was tested with the Mantel test implemented in the Isolation by Distance Web Service 3.23 (Jensen *et al.*, 2005); significance was tested with 10 000 bootstrap randomizations. Tests for isolation by distance (IBD) were repeated for straight-line distances and for a matrix of geographic distances estimated avoiding conspicuous barriers to dispersal (high mountains and long sea stretches). We repeat these analysis using the Nei´s genetic distance between populations to further confirmation of the results.

An alternative view of the population structure was obtained with the individual-based Bayesian clustering algorithms implemented in BAPS 6.0. Initially, genetic mixture analyses were done using both the spatial (Corander *et al.*, 2008) and the non-spatial model (Corander *et al.*, 2006). However, since both clustering models yielded very similar partitions, only the results of the spatial model are shown here. With the complete data set (19 populations), BAPS was run three times for each K from 2 until 25. Later, separate analyses were run for the 11 Mediterranean populations and for the 8 Atlantic populations with K from 2 to 15. The optimal partition determined by the program was used to estimate the levels of genetic admixture of individuals with 200 reference individuals simulated for each genetic group and each original individual analysed 20 times. To assess the strength of the signal in our data, the population structure was confirmed by using the alternative Bayesian approach implemented in STRUCTURE v.2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2003). STRUCTURE was run assuming correlated allele frequencies, with a burn-in period of 150 000 replications and a run length of 1 000 000 Markov chain Monte Carlo (MCMC) steps. Four iterations per K were performed for a number of clusters ranging from $K = 1$ to $K = 24$ (complete data set of 19 populations), and six iterations per K were performed for K ranging from $K = 1$ to $K = 15$ (separate analyses of the Mediterranean and Atlantic groups). The value of K that captured most of the structure in our data was determined using the approach originally proposed by Pritchard *et al.* (2000), with further guidance derived from the procedure of Evanno *et al.* (2005). Runs of selected K were averaged with the CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007).

Finally, the occurrence of fine-scale spatial genetic structure (SGS) was investigated with GenAlex 6.5 (Peakall & Smouse, 2006). This procedure calculates an autocorrelation coefficient (r) that is closely related to Moran's I (Peakall *et al.*, 2003) and measures the genetic similarity between pairs of individuals whose geographic separation falls within a set of specified distance classes. In our study, distance class size was variable to get a similar number of individuals within each distance class (distance class limits: 15, 25, 40, 70, 100, 150, 250, and 500 m). The occurrence of spatial autocorrelation on each site was assessed with a multiclass test criterion (ω) with null hypothesis of $r = 0$ (Smouse *et al.*, 2008). The significance of ω was estimated with 1000 random permutations and significance was declared when $P < 0.01$ following Banks and Peakall (2012). In addition, to assess whether the various populations showed differences in their fine-scale SGS, an overall test of heterogeneity was calculated and its significance assessed with 1000 bootstrap resamples.

RESULTS

Nine primer combinations produced a total of 226 reproducible AFLP loci, 125 (55.3%) of which were polymorphic for the whole data set (5% criterion) and were kept for further analyses. The number of polymorphic loci per population ranged from 94 (IK in the northern Atlantic edge of the distribution range) to 113 (IC in the Mediterranean) (Table 1). Likewise, Nei's gene diversity ranged from 0.26±0.016 in IK to 0.34±0.014 in IC and GA (two populations in the Mediterranean). The GT2-method showed that Nei's diversity in two Atlantic sites (IK and EP) was significantly lower than in any other population (P <0.05). Moreover, IK was significantly less diverse than EP (0.28±0.016). No population showed private bands.

Since the analyses of population structure revealed the occurrence of two clearly separated sets of populations (see below), separate estimates of diversity were obtained for each group. The Mediterranean group (EC, FM, EI, IC, TK, IR, IT, GA, GS, TC, TI) had slightly more polymorphic loci (113 *vs.* 111) and significantly higher gene diversity (0.34±0.014 *vs.* 0.31±0.014, P < 0.001, t-test) than the Atlantic group (IK, FB, EP, PA, PM, EM, MT, MD). Besides, two private bands were detected in some, but not all, Mediterranean populations.

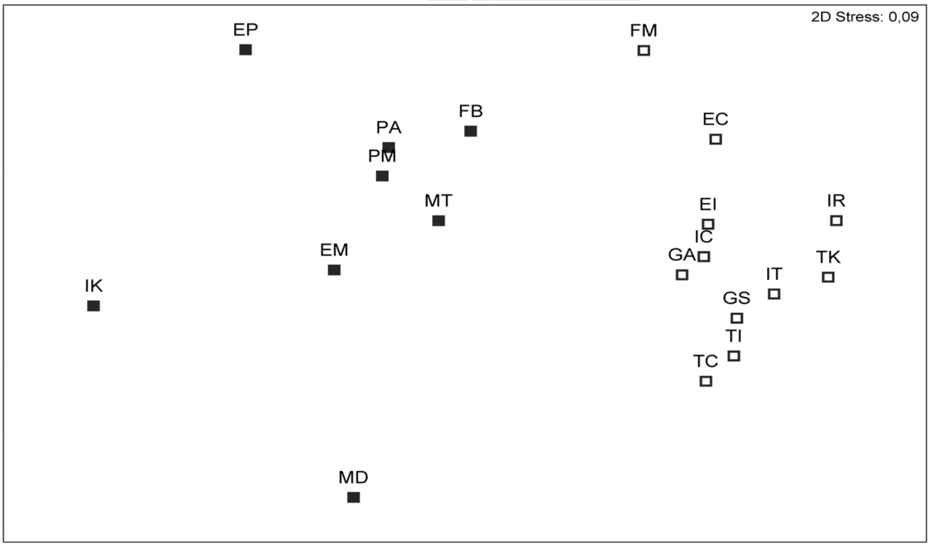
Both the global F_{ST} (0.083) and Φ_{PT} statistics among populations (0.105) revealed the occurrence of highly significant genetic differences among populations (P < 0.001), although the main variation occurred within populations (90%) (Table 2). Similarly, most of the pairwise Φ_{PT} estimates were significant (P < 0.05)

Table 2. Analysis of molecular variance (AMOVA) of based on 125 AFLPs segregating loci obtained for 491 individuals from 19 populations of *Arbutus unedo* sampled throughout its whole distribution range. Φ_{PT}= differentiation among populations; Φ_{RT}= differentiation among regions Φ_{PR}= differentiation among populations inside the regions.

Source of variation	d.f.	MS	Variance components	P-value	Φ statistics
Global (19 populations)					
Among Populations	18	82.882	2.412 (10%)	<0.0001	Φ_{PT} = 0.105
Within Populations	472	20.583	20.583 (90%)		
Regions (2 regions)					
Among regions	1	555.995	2.060 (10%)	<0.001	Φ_{RT} = 0.103
Among populations	17	57.409	1.594 (8%)	<0.001	Φ_{PR} = 0.089
Within populations	472	16.262	16.262 (82%)	<0.001	Φ_{PT} = 0.183

except for comparisons involving pairs of populations separated by < 500 km and some comparisons between populations located in Italy and Greece (1000-1300 km apart). The pattern of pairwise F_{ST} values depicted by the nMDS revealed two groupings of populations: the set of circum-Mediterranean sites and the set of demes sampled along the Atlantic façade (Fig. 2). The stress coefficient (0.09) indicated that the 2-dimensional plot was a good representation of F_{ST} values. A more detailed examination of Fig. 2 showed that the Mediterranean group was more compact than the Atlantic one, suggesting a lower differentiation among the Mediterranean populations. Indeed, only FM showed signs of moderate genetic differentiation and its average pairwise F_{ST} value (0.069) clearly surpassed the values registered in other Mediterranean sites (average pairwise F_{ST} values ranged from 0.028 to 0.049). In comparison, three Atlantic populations visibly separated from the Atlantic set. Interestingly, the three divergent populations included the two sites sampled at the northern (IK)

Fig. 2. Non-metric multidimensional scaling (nMDS) showing genetic distances between 19 populations of *Arbutus unedo.* Genetic distances were estimated as pairwise F_{ST} values obtained after inferring allele frequencies with the Bayesian method of Zhivotovsky (1999) implemented in AFLP-SURV. Open symbols are populations assigned to the Mediterranean genetic group, closed symbols are populations assigned to the Atlantic group. See Material and Methods for population names.



and southern (MD) edges of the distribution range of *A. unedo* along the Atlantic and the site sampled on the northwest corner of the Iberian Peninsula (EP). Average pairwise F_{ST} values for these three populations ranged from 0.075 to 0.103, clearly exceeding the estimates recorded for other Atlantic sites (0.045 - 0.062).

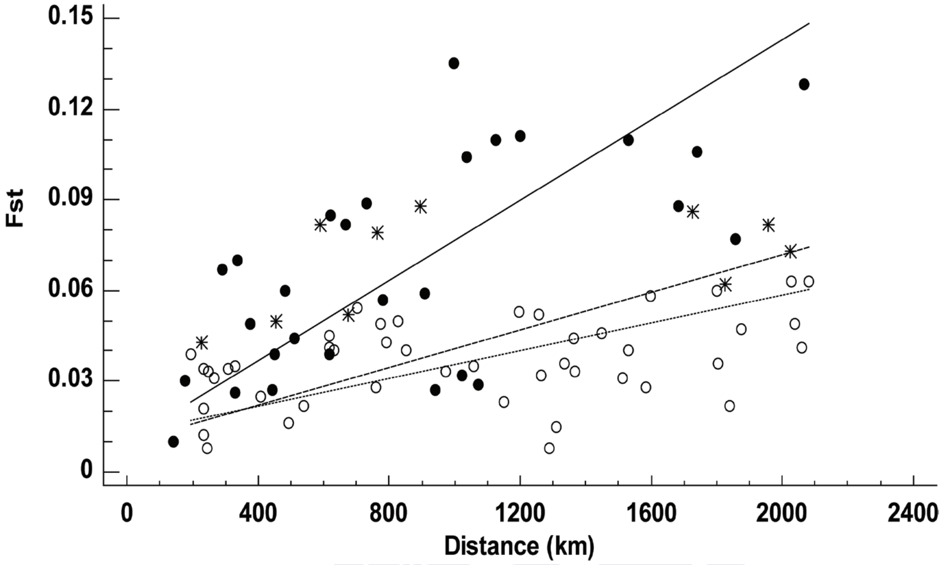
SAMOVA results corroborated the partitioning of populations in two sets as well as the presence of a few genetically differentiated populations (Table 3). As expected, F_{CT} estimates increased with larger K values but reached a plateau for $K = 4$ ($F_{CT} = 0.125$, $P < 0.001$). Thus, the optimal partition separated the circum-Mediterranean set from the samples collected along the Atlantic. Most of the Atlantic populations fell together in a single group but IK (northern edge) and MD (southern edge) were held separately. EP separated from other populations for $K = 5$, but the F_{CT} estimate of this structure (0.124, $P < 0.001$) indicates that keeping EP as a separate entity does not improve the proportion of genetic variance attributed to differences between groups of populations. Unlike nMDS, SAMOVA provided not support to the hypothesis that FM should be considered as particularly differentiated from other Mediterranean populations.

The Mantel test revealed the existence of IBD throughout the entire range of *A. unedo* (19 populations), but the correlation with genetic differentiation (as F_{ST}) was slightly tighter when the geographic distance was measured as a straight-line ($r = 0.57$, $P < 0.001$) rather than avoiding conspicuous barriers to dispersal ($r = 0.48$, $P < 0.001$). Separate analyses for each set of populations (straight-line geographic distances only) revealed that both the slope and the strength of the relationship changed from one area to another (Fig. 3). The correlation was tighter ($r = 0.64$, $P = 0.010$) and steeper (slope = $6.65\ 10^{-5} \pm 1.00\ 10^{-5}$) in the Atlantic than in the Mediterranean ($r = 0.36$, $P = 0.009$, slope = $3.13\ 10^{-5} \pm 4.01\ 10^{-6}$). Since the highest F_{ST} estimates recorded within the Mediterranean were consistently produced by comparison with a single population (FM), Mantel calculations were repeated with FM excluded from the Mediterranean group (Fig. 3). Excluding FM improved the correlation ($r = 0.43$, $P < 0.001$) and yielded a flatter relationship (slope = $2.30\ 10^{-5} \pm 3.17\ 10^{-6}$). Similar

Table 3. Fixation indices corresponding to the groups of populations (19 in total) of *Arbutus unedo* inferred by spatial analysis of molecular variance (SAMOVA) algorithms (*** = P < 0.001). F_{SC}= genetic differentiation between populations within groups; F_{ST}= genetic differentiation between samples; F_{CT}= genetic variance due to differences among groups of populations.

	Groups composition	F_{SC}	F_{ST}	F_{CT}
Two groups	1. EM, EP,FB,IK, MD, MT, PA, PM (Atlantic group)	0.091***	0.191***	0.111***
	2. EC, FM, EI, IC, IR, IT, TK, GS, GA,TC, TI (Mediterranean group)			
Three groups	1. IK	0.081***	0.189***	0.118***
	2. EM, EP,FB, MD, MT, PA, PM (Atlantic group)			
	3. EC, FM, EI, IC, IR, IT, TK, GS, GA,TC, TI (Mediterranean group)			
Four groups	1. IK	0.0725***	0.188***	0.125***
	2. MD			
	3. EM, EP,FB, MT, PA, PM (Atlantic group)			
	4. EC, FM, EI, IC, IR, IT, TK, GS, GA,TC, TI (Mediterranean group)			
Five groups	1. IK	0.069***	0.184***	0.124***
	2. MD			
	3. EP			
	4. EM,FB, MT, PA, PM (Atlantic group)			
	5. EC, FM, EI, IC, IR, IT, TK, GS, GA,TC, TI (Mediterranean group)			

Fig 3. Relationship between geographic and genetic (F_{ST}) distances among the 19 populations of *Arbutus unedo*. Separated analyses were conducted for Atlantic (solid circles) and Mediterranean (asterisks and open circles) populations; asterisks denote pairwise comparisons with location FM. Lines are reduced major axis regressions for the Atlantic group (solid line, r = 0.64, P = 0.990), the Mediterranean group (dashed line, r = 0.36, P = 0.991), and the Mediterranean group with FM excluded (dotted line, r = 0.43, P = 1.000).



results were obtained when using Nei´s genetic distance between populations instead of F_{ST}.

The spatial analysis of BAPS identified that the optimal number of genetic clusters (*K*) for the 19 populations was 7 (log-likelihood = -25960.681, probability for 7 clusters = 0.9793); most plants (91.4%) were assigned to a single genetic cluster. The admixture clustering graphs reinforced the divide between Atlantic and Mediterranean because most of the genetic clusters were largely region-specific (Fig. 1). Likewise, plants from IK and MD were consistently assigned to separate lineages. The non-spatial analysis with BAPS yielded highly similar results, suggesting that the use of a geographical prior did not compromised the partition. Results from STRUCTURE (not shown) revealed a similar divide between the Mediterranean and the Atlantic and corroborated the peculiar genetic composition of IK and MD.

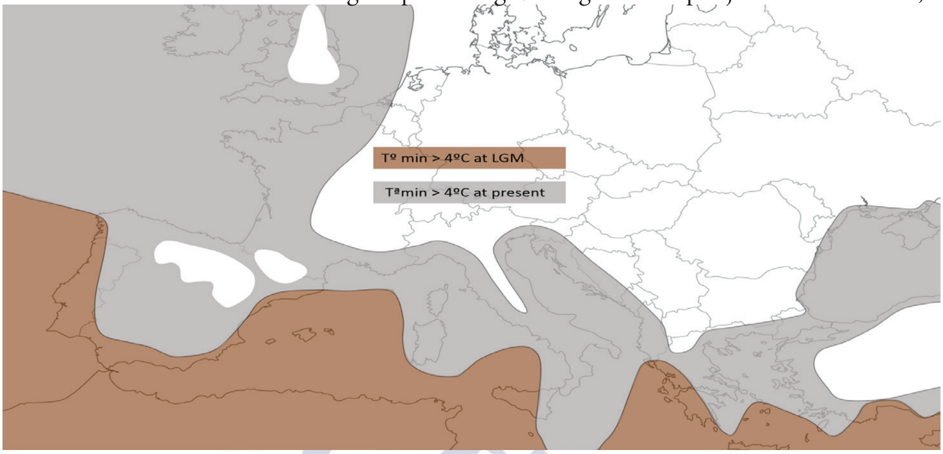
Finally, the spatial autocorrelation analyses failed to detect any evidence of fine-scale SGS. The test of heterogeneity among populations indicated that the 19 sites displayed a similar lack of spatial pattern (total $\omega = 102.867$, $P = 1.000$). The combined autocorrelogram was non-significant (multiclass test $\omega = 23.441$, $P = 0.073$) and showed a very flat profile around the null hypothesis of $r = 0$.

DISCUSSION

At the onset of this study, we speculated that the circum-Mediterranean range of *A. unedo* evokes the distribution pattern of a ring species (Irwin *et al.*, 2001) and may thus fit a pattern of IBD. Effectively, IBD seems doubtless but we also found that F_{ST} was likewise related to geographic distances estimated as straight-lines (i.e. across the Mediterranean) or avoiding barriers (i.e. around the Mediterranean). Consequently, the circum-Mediterranean distribution does not seem to be a determinant of the restricted gene flow suggested by the IBD pattern. It is likewise noteworthy that the genetic differentiation was generally lower among circum-Mediterranean populations than among Atlantic populations separated by similar geographic distances, suggesting that gene flow around the Mediterranean, although restricted, still is higher than between Atlantic demes (Templeton, 2006). The stepper IBD and higher F_{ST} estimates recorded along the Atlantic may also be partially attributed to historical events. Ramachandran et al. (2005) showed that a stepping stone colonization model can generate a gradual increase in genetic differentiation with increasing distance from the initial source population. Accordingly, the colonization of the Atlantic façade by a gradual northward expansion following a serial-founder model may have favoured a non-equilibrium explanation for the IBD pattern (Kimura & Weiss, 1964). Indeed, a detailed examination of Fig. 3 shows that equilibrium expectations (IBD) in the Atlantic were met for populations separated by less than 1500 km, but non-equilibrium conditions (no IBD) appeared at larger distances. Distances >1500 km always involved comparisons with the Irish deme and, in these cases, differentiation no longer depends on the distance; instead, it possibly reflects the effects of a reduced gene flow and larger drift induced by a founder effect (Hutchison & Templeton, 1999). Further support to the distinctive nature of the Irish population is derived from the individual-based analysis with BAPS, as it assigns the Irish population a to unique genetic cluster (Fig. 1). Similarly, the genetic differentiation detected in the Moroccan population MD, at the southern edge of the species range, could be a consequence of fragmentation followed by a reduction in population size (Frankham, 2005). The presence of *A. unedo* in Ireland as a consequence of a recent founder effect it is also supported by pollen and charcoal analyses indicate its presence in Ireland since at least 4000 years BP, and that the strawberry tree was more abundant and widespread just in a recent past (Mitchell, 1993; Van Rijn, 2004). Moreover, it has been determined that the earliest plant colonists arrived to Ireland around 13000 BP and, with the exception of *Betula*, no trees arrived until 9600 BP (Mitchell, 2006). These evidences agree with recent studies that used Ecological Niche Model (ENM) reconstructions for other Lusitanian elements, that concluded that Ireland underwent a postglacial colonization from southern refugees (Beatty and Provan 2013, 2014). In this regard, a postglacial arrival of *A. unedo* to Ireland is likewise supported by paleoclimatic reconstructions that show that winter temperature in Ireland during the LGM was clearly below the 4ºC limit required for the survival of the specie (Sealy, 1949)(Fig. 4).

In general, within-population diversity in most of our populations was similar to the estimates reported in previous, smaller-scale studies of *A. unedo* or another *Ericaceae* (Zawko *et al.*, 2001; Takrouni & Boussaid, 2010; Lopes *et al.*, 2012), higher than in the North American member of genus *Arbutus* (Beland *et al.*, 2005), and in agreement with the estimates reported for late-successional perennials with outcrossing breeding system (Nybom, 2004). Nonetheless, we found a significant evidence of reduced genetic variation in NW Iberia (11%) and Ireland (20%), that agrees with a stepping-stone colonization from S Iberia to N Iberia and, subsequently, to Ireland. The pairwise F_{ST} values also support a connection between Iberia and Ireland because the Irish deme is genetically closer to those from Iberia than to the deme

Fig. 4. Area of Western Mediterranean and Atlantic facade where the minimum monthly temperatures were >4ºC (suitable for the *A. unedo* survival) during the LGM (21 kya, brown) and in modern times (grey)according climate simulation models (Van Andel, 2002) (adapted from plots available in http://www.esc.cam.ac.uk/research/research-groups/oistage3/stage-three-project-simulations).



sampled in the French Atlantic. Consequently, the arrival of *A. unedo* to Ireland through France seems unlikely, pointing to the arrival to Ireland via the Celtic Sea as Mitchell (2006) showed for many tree species. In this regard, the ENM reconstructions of Beatty and Provan (2013, 2014) showed the presence of refugia in N Iberia for another Lusitanian species. Therefore, this postglacial connection Ireland-NW Iberia seems a plausible hypothesis and should be taken into consideration when interpreting possible human introductions to Ireland.

Unlike our expectations of a genetic divide between the Euro-Siberian populations (IK, FB, EP) and those located in the Mediterranean biogeographic region, our results consistently revealed a sharp separation between the circum-Mediterranean and the Atlantic façade. This clear-cut divide was evident despite the moderate global level of genetic differentiation across the species range. The genetic divide detected in the strawberry tree involves two private AFLP bands in the circum-Mediterranean group, suggesting that the split has been in place by some time (Vilatersana *et al.*, 2007). In addition, the circum-Mediterranean group had slightly, but significantly, higher levels of within-population genetic variation. The latter may suggest a more ancient condition for Mediterranean populations, as already observed in other sclerophyllous evergreen trees such as *Olea europaea* (Lumaret *et al.*, 2004) or *Laurus nobilis* (Rodríguez-Sánchez *et al.*, 2009) or maybe the conditions for the gene flow were more favourable in the Mediterranean basin.

The divide between Mediterranean and Atlantic involves the Iberian Peninsula, where demes from each group are separated by relatively short distances (<750 km) while each genetic group includes populations up to 2000 km apart. However, many other widespread Iberian trees show a deep and often remarkably clear-cut divide between populations from the Mediterranean and from the Atlantic regions of the Iberian Peninsula. The genealogical concordance between multiple co-distributed species is often interpreted as evidence that the responsible evolutionary forces must have had widespread effects at the level of biotic communities and ecosystems (Avise, 2009). In the particular case of the Iberian trees, the clear-cut genetic divide has been interpreted as evidence of the existence of mutually isolated glacial refugees located near each of the two coasts, followed by a subsequent expansion inland after the LGM (Benito Garzón *et al.*, 2007; López de Heredia *et al.*, 2007; Médail & Diadema, 2009; Rodríguez-Sánchez *et al.*, 2010). Moreover, a recent review of phylogeographic studies with trees concluded that this divide could even antedates the LGM, arising when species entered Iberia from the south and expanded independently along both coastlines (Rodríguez-Sánchez *et al.*, 2010), as could have happened with *A. unedo*.

In conclusion, we observed that *A. unedo* showed a significant IBD pattern that is stronger in the Atlantic than around the Mediterranean and may be interpreted as evidence of a stepping-stone colonization along the Atlantic façade. The latter would also explain the gradual decrease in within-population genetic diversity detected in NW Iberia and, specially, Ireland. Our results point to the Iberian Peninsula as the origin for the Irish populations rather than the previously suggested migration through the maritime fringe of Western Europe. Furthermore, there is a strong genetic structure that clearly divides the populations of *Arbutus unedo* in two groups: Atlantic and circum-Mediterranean. The latter showed higher gene flow. The divide is concordant with what has been observed in other widespread trees and could be attributed to the existence of separated and independent LGM refugia.

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Chapter III

Structural and functional plasticity in response to nutrients in seven provenances of *Arbutus unedo* L.


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ABSTRACT

The Mediterranean region is an area of special interest for conservation and the projected global change has been widely reported. One of the factors predicted to change is soil-nutrient availability, an essential factor for plant growth. Thus, study of the effects of variation in this parameter is especially relevant in species with a circum-Mediterranean distribution, such as *A. unedo*, in which the different provenances grow under different nutritional conditions. We aimed to determine the effect of the provenance on plasticity, to establish whether structural and morphological traits differ in the level of plasticity, and to assess how nutrients affect the photosynthetic light response. For this purpose, we studied 7 provenances representing the circum-Mediterranean range of *A. unedo*. We obtained 20 plants per provenance and assigned 10 to each treatment (low and high nutrients availability). We measured 14 plant traits (10 physiological and 4 structural) and determined the phenotypic plasticity index to quantify the level of plasticity. Radiation effects were tested by construction and analysis (ANOVAR) of light response curves. Provenance did not explain a significant amount of variance, and the plasticity was higher for the structural traits than for the physiological traits. Finally, we established that the availability of nutrients improves the plant response under increasing radiation. Therefore, the plasticity to nutrient availability will not favour or prevent the expansion or contraction of the range of any particular provenance of *A. unedo*. Furthermore, the structural plasticity demonstrated the ability of the strawberry tree to optimize resource allocation, whereas the physiology remained stable, thus avoiding extra expenditure. The study findings also suggest that increased availability of nutrients would improve the performance of the species during the Mediterranean summer, characterized by high irradiance. These abilities will be key to the survival of the species under the future scenario of changes in nutrient availability.

Keywords: *Arbutus unedo*, nutrients, plasticity, phenotypic plasticity index, global change, adaptation, canalization.

INTRODUCTION

As a result of their high biological diversity, Mediterranean ecosystems have been recognized as biodiversity hotspots and a prime target for conservation efforts (Myers *et al.*, 2000). Unfortunately, the prevalence of multiple drivers of global change in the Mediterranean region, including climate, atmospheric and land use changes, is expected to affect Mediterranean ecosystems at very different levels (Sala *et al.*, 2000). Changes in atmospheric deposition of nitrogen, with the consequent effects on ecosystem productivity and biogeochemistry, have been identified as one of the main drivers of global change in Mediterranean-type ecosystems (Moreno & Oechel 1995).

Plants can respond to global change, which creates new environmental scenarios and selective pressures, by changes in phenology, physiology or reproduction (Matesanz *et al.*, 2010); they can even migrate to areas with more favourable environmental conditions, potentially over long distances (Hampe & Petit, 2005; Williams *et al.*, 2008; Hansen *et al.*, 2012). Non-migratory changes can lead to adaptation, mainly via genetic evolution (local adaptation) through natural selection (Kandemir *et al.*, 2010; Hoffmann & Sgrò, 2011; Ramírez-Valiente *et al.*, 2011) or via phenotypic plasticity (Callaway *et al.*, 2003; Williams *et al.*, 2008; Lande, 2009), although adaptation to stationary fluctuations sometimes occurs via canalization (Kawecki, 2000; Le Rouzic *et al.*, 2013). The mechanisms of adaptation that operate will depend on factors such as intensity and direction of the environmental change, life-history traits, standing genetic variation and interactions between coexisting species (Matesanz *et al.*, 2010). Evolutionary adaptation can occur rapidly and potentially help species to counteract stressful conditions or to take ecological opportunities arising from global changes (Hoffmann & Sgrò, 2011). However, Atkins and Travis (2010) used a simulation model to show that evolutionary adaptation may not be sufficient by itself to enable a species to survive a period of continuous changes. Phenotypic plasticity, or the capacity of a given genotype to render different phenotypes under different environmental conditions, may assist species, particularly sessile organisms, to cope with environmental heterogeneity (Sultan, 2000).

In the short term, species can acclimate to shifting environmental conditions or adjust to the composition of their communities via phenotypic plasticity, developing and expressing particular traits in response to local environmental conditions, buffering the environmental change and increasing their tolerance to stress (Callaway *et al.*, 2003; Lande, 2009; Matesanz *et al.*, 2010; Hansen *et al.*, 2012). Indeed, forecasted changes in species range indicate less lost area and extinction probabilities in more adaptable species (Chevin *et al.*, 2010; Valladares *et al.*, 2014). Additionally, when environmental stresses are reversed, plastic phenotypic changes will also rapidly reverse, in contrast to genetic changes, which are more difficult to reverse (Hansen *et al.*, 2012). Thus, in the short term, adaptability may be important for the persistence of species under continuous global change. In addition, separation of genetic and plastic contributions has shown that the latter often seem more important (Mutke *et al.*, 2010; Hoffmann & Sgrò, 2011). However, rather than being exclusive, plasticity and evolutionary adaptation may be complementary, because if plants maintain or achieve greater fitness in a stressful environment as a consequence result of their ability to adapt, this will prevent the plant provenances from losing genetic variability (Matesanz *et al.*, 2010), which may later promote further genetic adaptation, evolution and long-term survival (Lande, 2009). Therefore, plasticity, acting alongside evolutionary change, is crucial for increasing survival under rapidly changing environmental conditions (Hoffmann & Sgrò, 2011; Matesanz & Valladares, 2014). However, in situations where the environmental conditions change periodically, canalization may be an alternative to buffering changes and avoiding maladaptation, i.e. adaptation to one situation that may lead to reduced fitness when the environment changes (Kawecki, 2000).

Species habitats are expected to be modified as result of the ongoing environmental changes and this will particularly affect soil systems and nutrient availability (IPCC, 2013). Soil-nutrient availability is one of the most important limiting factors affecting plant growth (Lambers *et al.*, 2008), and it has been shown to be a fundamental abiotic factor in the structure and distribution of Mediterranean ecosystems (Mayor & Rodà, 1992; Hanley & Fenner, 1997). Soil-nutrient availability is highly heterogeneous, showing a strong spatial and temporal variation that is frequently associated with seasonal and climatic variations, as shown in a Mediterranean shrubland (Monokrousos *et al.*, 2004). It is increasingly clear that changes in temperature or precipitation provoked by climate change will alter nutrient cycles and ecosystems (Sardans & Peñuelas, 2007; Matías *et al.*, 2011). Furthermore, soil processes can be affected by land-use change, nitrogen deposition, biotic exchange and elevated carbon dioxide concentration (Sala *et al.*, 2000).

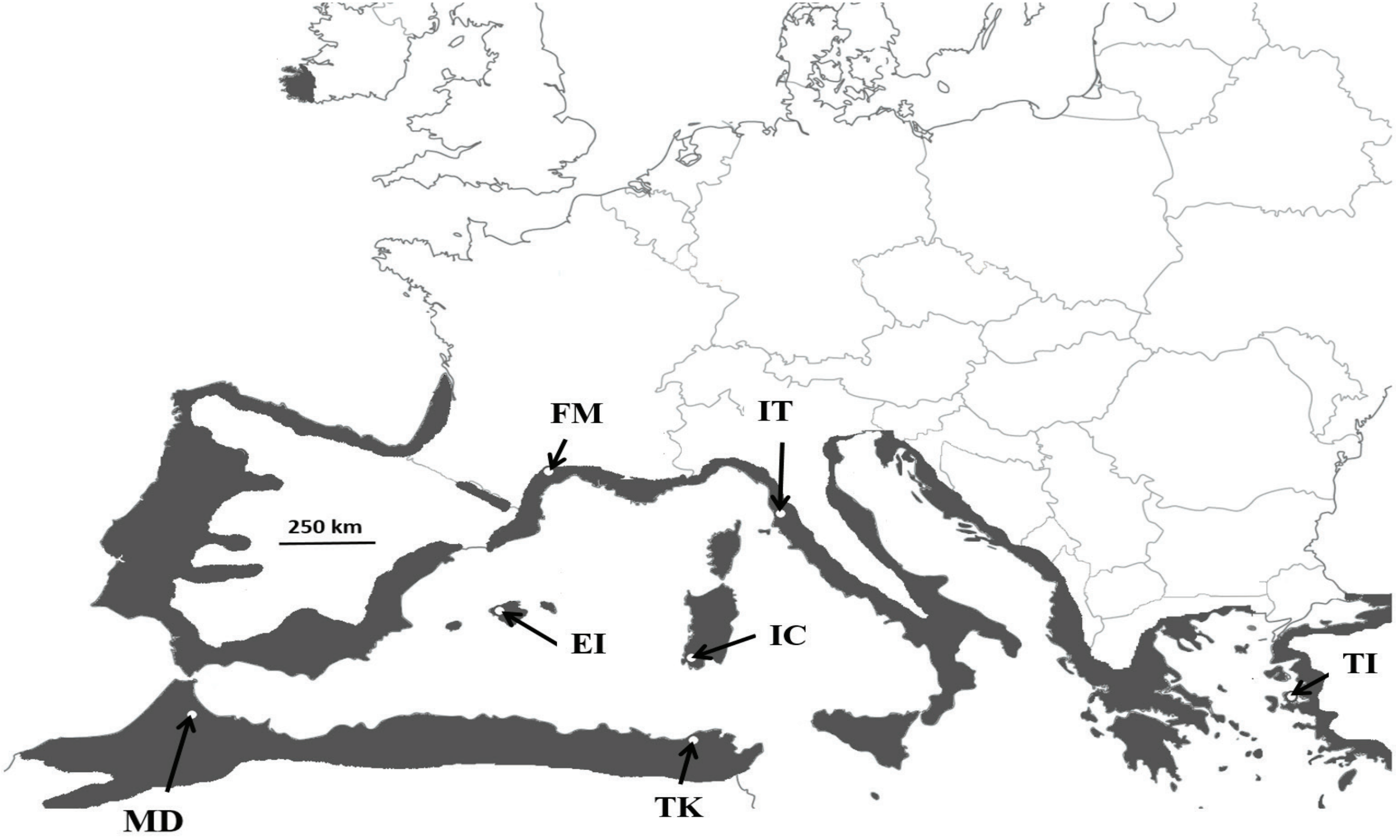
Some studies of nutrient-related issues in Mediterranean forests have shown correlations between soil nutrient availability and plant growth, plant anatomy and physiological traits (Valladares *et al.*, 2002; Sardans *et al.*, 2005, 2006b; Sardans *et al.*, 2006a; Pulido *et al.*, 2014). These studies provide evidence of the capacity of Mediterranean plants to adjust their morphology and physiology in response to variations in nutrient availability. Mediterranean plants are also able to respond to the environmental unpredictability of their ecosystems (Valladares *et al.*, 2002) and are well suited to adapt to the ongoing changes they are facing (Sardans *et al.*, 2006b). The degree of plasticity differs depending on the species, provenance and traits considered (Matesanz & Valladares, 2014). Previous studies have shown differences in plasticity between physiological and structural traits (Mou *et al.*, 2013) and that the capacity to exhibit structural and physiological plasticity may be complementary and, thus, negatively correlated (Derner & Briske, 1999). Therefore, knowledge about the degree of plasticity of both types of traits is also necessary to determine the evolutionary strategy of a species.

The study of the degree of plasticity in widely distributed plants, living under different environments, will provide the opportunity to assess the role of plasticity in the evolution of plant distribution ranges. The circum-Mediterranean distribution of *Arbutus unedo*

L. (strawberry tree) is of a special interest (Torres *et al.*, 2002), occupying a narrow coastal fringe from Tunisia to Morocco along the north of Africa, and from Spain to Turkey along southern Europe (Fig. 1). Along the Atlantic Ocean, it is distributed between northern Morocco and the Iberian Peninsula and western France, with outliers (disjunct provenances) in south-western Ireland, where the limit of distribution concurs with the isotherm of 4ºC for the mean temperature of January (Sealy, 1949). Specifically, in relation to nutrient supply, a different degree of plastic response is expected due to the different soil nutritional conditions under which each provenance grows (Crick & Grime, 1987; Fitter & Stickland, 1991).

Here, we applied a robust, the simple and widely used phenotypic plasticity index (PPI) (Valladares *et al.*, 2000; Balaguer *et al.*, 2001; Valladares *et al.*, 2002; Gratani *et al.*, 2003; Gratani *et al.*, 2006; Valladares *et al.*, 2006; Zhao *et al.*, 2010) to determine the level of plasticity in response to nutrient availability in 10 physiological and 4 structural traits in plants of 7 provenances distributed throughout the distribution range of *A. unedo*. More specifically, we aimed to ascertain a) whether plants of the different provenances differ in the level of plasticity to nutrients and b) whether structural and physiological traits differ in the level of plasticity. Identification of differences in the level of plasticity between provenances may be important for determining how the distribution of this species may be affected by global changes. Likewise, knowledge of the levels of plasticity in structural and functional traits will increase our ability to predict how this species will respond to ongoing global changes. In addition, given that plant responses to light constitute a key component of the survival strategies of species in Mediterranean ecosystems and potentially able them to be influenced by nutrient availability (Demmig-Adams & Adams, 1992; Bermúdez & Retuerto, 2014), we modelled how nutrient levels affect photosynthetic responses.

Figure 1. Distribution range of *Arbutus unedo* (grey area) and location of the seven provenances studied.



MATERIALS AND METHODS

The species

Arbutus unedo belongs to the family *Ericaceae,* subfamily *Arbutoideae*, which includes evergreen, shrub-like woody taxa with sclerophyllous, laurel-like leaves (Torres *et al.*, 2002). The genus *Arbutus* has several members in America and four in Europe: *A. andrachne* L. (eastern Mediterranean), *A. pavarii* Pampanini (Libyan coast), *A. canariensis* Veill. (Canary Islands) and *A. unedo* L. (Torres *et al.*, 2002). Flowering spans from September to December and fecundation is entomophilous (Mitchell, 1993); indeed, the nectar and pollen are the main food source for *Bombus terrestris* (Rasmont *et al.*, 2005). Fruits take 12 months to ripe, and seeds are dispersed by animals (mainly birds); seed viability has been reported to be about 55% in favourable places while seedlings loss was found to exceed 60% (Sealy, 1949).

Sample collection and experimental design

Between September and December 2010, we obtained samples from 7 provenances of *A. unedo* L. growing in 6 countries: France (FM) Spain (EI), Morocco (MD), Italy (IC and IT), Tunisia (TK) and Turkey (TC), i.e. throughout the circum-Mediterranean range of the species (Fig. 1). We collected several fruits per tree and stored them at 4°C. We extracted the seeds and kept them in a dry atmosphere before sowing in forestry trays with fifteen 1 litre cavities, each filled with Tref substrate (Tref Substrates BV, Moerdijk, The Netherlands), using a 3:1 mixture of this potting compost and perlite. To minimize the possible influence of maternal effects, we selected seeds of similar weight for each provenance and maintained the growing plants in the same environment (an outdoor plot) for 6 months (March to September 2012). We then selected twenty plants per provenance at random and transferred them to 7 litres pots filled with the previously above-mentioned substrate. Ten plants of each provenance were randomly assigned to the low nutrient availability (LN) treatment (no nutrients added) and ten plants were assigned to the high nutrient availability (HN) treatment (supplied with 6 gr/l of nutrient: 16-8-16 NPK, Osmocote, Serrablen). The plants were watered as required, to avoid stress, and were maintained for another 9 months, until June 2012. All of the traits described below were measured in each plant and all plants were then harvested to determine the biomass.

Physiological traits

Leaf spectral reflectance

Measurements were made on all plants on the adaxial side of one leaf per plant, with a portable spectrometer (Unispec, PP Systems, Haverhill, MA, USA), which registers the leaf reflectance spectrum. The chlorophyll index (CHL), calculated as $R_{750}:R_{550}$, where R_x represents the reflectance at x nm, is positively correlated with the chlorophyll content of leaves (Lichtenthaler *et al.*, 1996). The normalised difference vegetation index (NDVI), computed as $(R_{800}-R_{680})/(R_{800}+R_{680})$, has been related to the 'greenness' or general vigour of vegetation because it is correlated with leaf chlorophyll content, photosynthetic efficiency, foliar nitrogen, phosphorus and potassium content (Gamon *et al.*, 1995). The water index (WI), calculated as $R_{900}:R_{970}$, is correlated with the plant water content, which is crucial for leaf expansion, photosynthesis and growth (Peñuelas *et al.*, 1997). The photochemical reflectance index (PRI), determined as $(R_{531}-R_{570})/(R_{570}+R_{531})$, is inversely correlated with the dissipation of excess radiation energy as heat (Peñuelas *et al.*, 1995) and is indicative of a photoprotective mechanism that dissipates excess excitation energy. These traits, related to foliar physiology constitute the main determinants of plant growth and survival (Chaerle & Van Der Straeten, 2000).

Chlorophyll fluorescence

Measurements were taken on every plant on the adaxial side of one leaf per plant, with a portable pulse amplitude modulated fluorometer (Mini-PAM; Heinz Walz, GmbH, Effeltrich, Germany). The effective quantum yield of Photosystem II (PSII) (ΦPSII), was calculated as $(F_m'-F_t)/F_m'$, where F_m' is the maximum fluorescence emitted by an illuminated leaf after a saturating pulse and F_t is the basal fluorescence emission. The ΦPSII provides information about the proportion of the light absorbed by the chlorophyll that is used photochemically, i.e.

the radiation use efficiency (RUE), a main determinant of the net primary productivity (Genty *et al.*, 1989). This parameter was also used to construct light response curves. The fluorometer recorded the ΦPSII after successive exposure of three minutes to increasing photosynthetic photon flux densities (PPFD): 10, 75, 120, 180, 250, 375, 500, 800 and 1200 µmol m^{-2} s^{1}. These measurements were made in five plants per treatment.

The maximum quantum yield of PSII (F_v/F_m) was determined as $(F_m - F_0)/F_m$, where F_m and F_0 are the maximum and the basal fluorescence emission respectively in dark adapted samples, when all reaction centres of PSII were fully open. Leaves were dark adapted by using dark leaf clips (Heinz Walz, GmbH) for 30 min before the measurement. F_v/F_m estimates the efficiency of capture of the excitation energy by open photosystem II reaction centres, and represents the fraction of incident photon energy that is processed photochemically (Krause & Weis, 1991).

Infrared carbon dioxide analyzer

The rate of photosynthesis (µmol CO_2 m^{-2} leaf area s^{-1}), intercellular CO_2 concentration (Ci) (µmol CO_2 mol^{-1}), conductance to H_2O (mol H_2O m^{-2} s^{-1}) and transpiration rate (mmol H2O m^{-2} s^{-1}) were measured in all plants, on one leaf per plant, by gas exchange, with a portable carbon dioxide analyzer (LI-6400XT; Li-Cor, Lincoln, NE, USA), at a leaf temperature of 32.5° C (±0.2)(±standard error, S.E.) and chamber radiation of 500 µmol m^2s^1. These measurements related to plant water use efficiency (WUE) and photosynthetic efficiency are the main determinants of the tolerance to water stress, fecundity and plant survivorship (Arntz *et al.*, 2000).

Structural traits

Biomass

Plant biomass was separated into roots, stems, and leaves. After washing the roots, all plant material was oven-dried to constant weight at 40° C. Each part was weighed separately to the nearest 0.0001g (Mettler AJ100, Switzerland). Root biomass, shoot biomass (stem + leaf biomass) and root/shoot biomass were used in the analyses. The root and shoot biomass perform essential functions for the plants, including absorption, structural support, storage and reproduction (Mokany *et al.*, 2006) and the root: shoot ratio is related to precipitation, temperature and forest stand height (Mokany *et al.*, 2006).

Specific leaf area

The specific leaf area (SLA) (cm^2 leaf area/ mg leaf dry mass) was calculated as the average value for two leaves per tree. The area was measured using a scanner (CanoScan LiDE 50; Canon, Tokyo) and an image analysis programme (ImageJ software; National Institute of Health). SLA has been associated with photosynthetic capacity and tolerance to drought (Sánchez-Vilas & Retuerto, 2007).

Data analysis

For each variable and provenance, plasticity was determined by the phenotypic plasticity index (PPI) (Valladares *et al.*, 2002). The index (the values of which range from zero to one) was calculated as **(maximum - minimum mean values) / maximum mean values**, where the numerator is the difference between the mean values for the two nutrient treatments and the denominator is the mean value for the highest of the two nutrient treatments. We used a two-way ANOVA to test for effects of nutrient treatments and plant provenances on growth and physiological traits after log transformation of the data to meet the required assumptions of normality and homogeneity of variance. We also used a one-way ANOVA to check the effect of provenance on the PPI. We analysed the ten physiological traits and the four structural traits separately. To determine the effects of the light curves, we used a two-way analysis of variance with repeated measures (ANOVAR), where "provenance" and "nutrient availability" were considered for the between-subject and "light" (the different measures at each light level as a repetition) was the within-subject effect.

Before carrying out the tests, we checked that dependent variables were normally distributed (Shapiro-Wilk test) and that variances of the differences between related groups were homogeneous (Mauchly's test of sphericity). When sphericity was violated, we used the Greenhouse-Geisser procedure to correct the degrees of freedom of the F-distribution. All analyses were carried out using IBM SPSS Statistics V.22 (Armonk, NY, USA), and differences were considered significant at P< 0.05, after sequential Bonferroni correction.

RESULTS

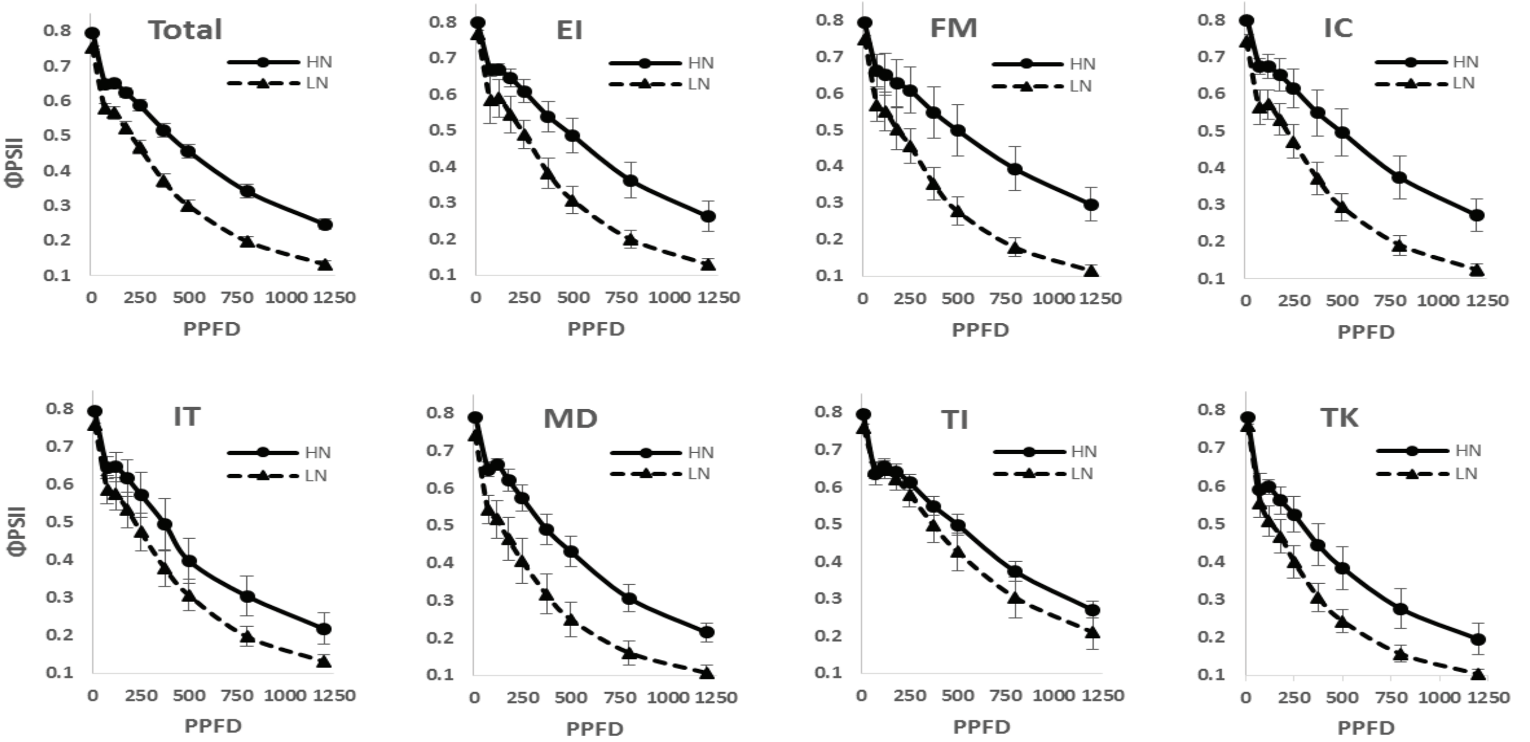
Light response curves

The decreasing effect of light on the effective quantum yield of PSII was significant throughout the experiment, but was also significantly higher for plants grown under low nutrient availability than in those grown under high nutrient availability (Table 1; Fig. 2). Irrespective of the nutrient status, the decreasing effect of light on the effective quantum yield did not differ significantly between the plants of the different provenances.

Table 1. Results of two-way repeated-measures analysis of variance used to assess the between-subject effects of 'provenance' and 'nutrient availability' and the within-subject effect of light on the effective quantum yield of photosystem II (ΦPSII) in *Arbutus unedo* L. plants. Significant effects ($P < 0.05$) after sequential Bonferroni correction are shown in bold type. The Greenhouse-Geisser procedure was used to correct the degrees of freedom of the F-distribution.

Source	MS	Df	F	p-value
Between-subject effect				
Provenance	0.598	6	1.765	0.123
Nutrient availability	1.843	1	32.637	**<0.001**
Provenance X nutrient	0.203	6	0.601	0.729
Error	3.162	56		
Within-subject effect				
Light	18.974	2.265	1131.230	**<0.001**
Light*Provenance	0.120	13.589	1.192	0.291
Light*Treatment	0.206	2.265	12.283	**<0.001**
Light*Provenance*Treatment	0.054	13.589	0.540	0.901
Error	0.939	448		

Figure 2. Light-response curves representing the effective quantum yield of Photosystem II (ΦPSII) as a function of the photosynthetic photon flux density (PPFD) for plants of each of the seven provenances (EI: Spain Balearic Islands; FM: France Montpellier; IC: Italy Cagliari; IT: Italy Tuscany; MD: Morocco Debdou; TI: Turkey Izmir; TK: Tunisia Kroumerie) of *Arbutus unedo* grown under conditions of high (HN) and low (LN) nutrient availability. The light response curve for the average values of all provenances is also shown. Symbols represent mean values of ΦPSII (± 1 standard error).



Physiological and structural traits

Plants of the different provenances did not differ significantly in any traits (Table 2, Fig. 3). Furthermore, the level of plasticity across provenances (both in morphological traits and physiological traits) did not vary depending on the provenance (Fig. 4). This result was confirmed by ANOVA (data not shown). Regardless of plant provenance, the addition of nutrients significantly increased the structural traits root and shoot dry weights, but decreased root/shoot ratios (Table 2; Fig. 3). Regarding the physiological traits, and also irrespective of the plant provenance, the availability of nutrients significantly increased the leaf reflectance parameters CHL and PRI, and the effective quantum yield of PSII, but decreased transpiration and Ci (Table 2; Fig. 3).

According to the phenotypic plasticity index (Table 3), the structural traits were more plastic than the physiological traits (mean value 0.553 *vs* 0.125, respectively; Fig. 4). Considering the structural traits, the plasticity index for SLA (0.101) was more than five times lower than the values for root, shoot and root: shoot biomass ratio (0.688, 0.849 and 0.574, respectively), which explains the high mean value. On the other hand, for the physiological traits, the plasticity values for NVDI and WI were the lowest (0.011 and 0.010, respectively), and those for PRI was the highest (0.280).

DISCUSSION

One of the greatest challenges for plants today is coping with environmental changes, and soil nutrient availability is one of the key environmental factors that it is expected to be modified by global change (IPCC, 2013). By expanding ranges of physiological tolerance, phenotypic plasticity may allow certain genotypes to buffer environmental change, thus increasing their tolerance to stress and subsequent survival (Sultan, 2000; Matesanz *et al.*, 2010), as well as preventing a reduction in the area of distribution under changing environmental conditions (Valladares *et al.*, 2014). Study of the level of plasticity in response to changes in nutrient availability in *Arbutus unedo*, an emblematic and widely

Table 2. Results of factorial ANOVA for physiological traits (CHL: chlorophyll content index; NDVI: Normalised Difference Vegetation Index; WI: Water index; PRI: photochemical reflectance index; effective (ΦPSII) and maximum (Fv/Fm) quantum yield of photosystem II; Photosynthesis: photosynthesis rate; Transpiration: transpiration rate; Ci: intercellular CO2 concentration; Conductance: conductance to water) and structural traits (Root: root biomass; Shoot: shoot biomass; Root: Shoot: root biomass/ shoot biomass; SLA: specific leaf area) in seven provenances of *Arbutus unedo* L. grown under two different nutrient levels. Significant effects ($P < 0.05$) after sequential Bonferroni correction are shown in bold type.

Trait	Provenance				Nutrient availability				Provenance*Nutrient			
	Df	MS	F	p-value	df	MS	F	p-value	df	MS	F	p-value
CHL	6	0.010	1.411	0.217	1	0.034	28.469	0.000	6	0.011	1.551	0.169
NDVI	6	0.000	0.445	0.847	1	3.04 E-5	0.583	0.447	6	0.000	0.325	0.922
WI	6	5.89 E-5	0.209	0.973	1	1.59 E-5	0.338	0.562	6	0.000	0.505	0.803
PRI	6	0.000	1.487	0.190	1	0.000	15.846	0.000	6	0.000	0.878	0.514
ΦPSII	6	0.02	0.371	0.896	1	0.011	13.898	0.000	6	0.003	0.615	0.718
FvFm	6	0.00	0.330	0.920	1	6.92 E-6	0.057	0.811	6	0.001	1.712	0.126
Photosynthesis	6	0.228	2.054	0.65	1	1.61 E-5	0.001	0.976	6	0.022	0.200	0.976
Transpiration	6	0.212	3.337	0.05	1	0.089	8.414	0.005	6	0.032	0.505	0.803
Ci	6	0.004	0.595	0.733	1	0.015	12.720	0.001	6	0.009	1.280	0.273
Conductance	6	0.008	2.315	0.039	1	0.002	2.918	0.091	6	0.003	0.746	0.614
Root	6	0.465	1.387	0.227	1	7.529	134.673	0.000	6	0.259	0.773	0.592
Shoot	6	0.287	0.751	0.610	1	22.510	353.220	0.000	6	0.377	0.985	0.439
Root:shoot	6	0.049	0.400	0.878	1	4.028	198.430	0.000	6	0.174	1.432	0.209
SLA	6	0.046	0.722	0.633	1	0.044	4.140	0.044	6	0.066	1.041	0.403

Figure 3. Mean values ± 1 standard error for physiological traits (CHL: chlorophyll content index; NDVI: Normalised Difference Vegetation Index; WI: Water index; PRI: photochemical reflectance index; effective (ΦPSII) and maximum (Fv/Fm) quantum yield of photosystem II; Photosynthesis: photosynthesis rate; Transpiration: transpiration rate; Ci: intercellular CO2 concentration; Conductance: conductance to water) and structural traits (Root biomass; Shoot biomass; Root: Shoot: root biomass/ shoot biomass; SLA: specific leaf area) in *Arbutus unedo* plants of seven provenances grown under conditions of high (HN) and low (LN) nutrient availability.

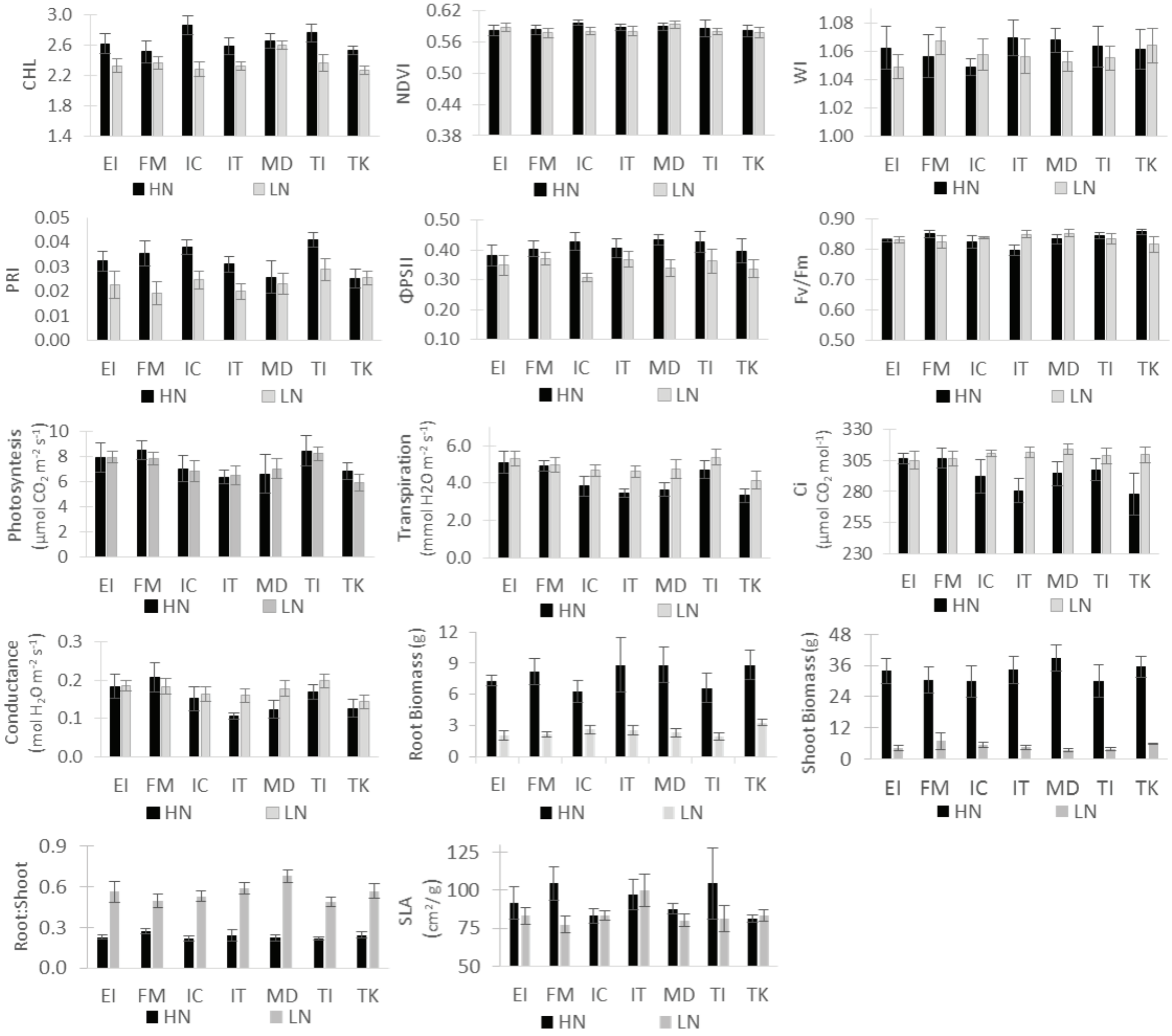
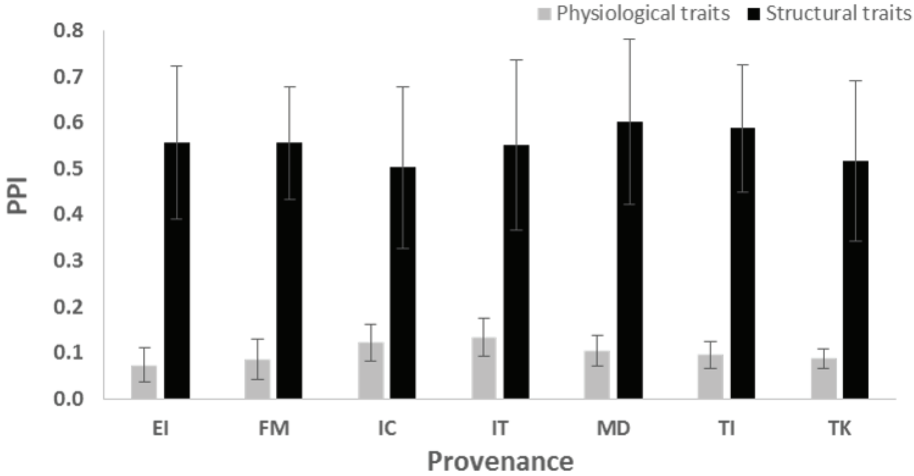


Table 3. Index of phenotypic plasticity, (maximum mean - minimum mean)/ maximum mean, in response to nutrient availability for physiological traits (CHL: chlorophyll content index; NDVI: Normalised Difference Vegetation Index; WI: Water index; PRI: photochemical reflectance index; effective (ΦPSII) and maximum (Fv/Fm) quantum yield of photosystem II; Photo: photosynthesis rate; Transp: transpiration rate; Ci: intercellular CO2 concentration; Cond: conductance to water) and structural traits (Root: root biomass; Shoot: shoot biomass; Root:Shoot: root biomass/ shoot biomass; SLA: specific leaf area) in plants of seven provenances (EI: Spain Balearic Islands; FM: France Montpellier; IC: Italy Cagliari; IT: Italy Tuscany; MD: Morocco Debdou; TI: Turkey Izmir; TK: Tunisia Kroumerie) of *Arbutus unedo* L.

Provenance	**Physiological traits**										**Structural traits**			
	CHL	**NDVI**	**WI**	**PRI**	**ΦPSII**	**Fv/Fm**	**Photo**	**Transp**	**Ci**	**Cond**	**Root**	**Shoot**	**Root:shoot**	**SLA**
EI	0.133	0.006	0.016	0.375	0.141	0.000	0.026	0.037	0.003	0.004	0.692	0.858	0.589	0.089
FM	0.059	0.010	0.010	0.458	0.082	0.030	0.079	0.009	0.001	0.121	0.740	0.774	0.450	0.259
IC	0.203	0.026	0.009	0.351	0.281	0.015	0.028	0.179	0.061	0.068	0.587	0.822	0.595	0.004
IT	0.103	0.012	0.012	0.359	0.088	0.064	0.021	0.251	0.099	0.335	0.717	0.872	0.591	0.027
MD	0.021	0.006	0.014	0.108	0.214	0.023	0.058	0.233	0.063	0.307	0.739	0.911	0.671	0.086
TI	0.143	0.011	0.008	0.295	0.151	0.013	0.029	0.123	0.036	0.147	0.710	0.872	0.547	0.220
TK	0.102	0.005	0.003	0.010	0.151	0.047	0.130	0.196	0.103	0.128	0.630	0.837	0.571	0.025
Mean value per trait	**0.109**	**0.011**	**0.010**	**0.280**	**0.158**	**0.027**	**0.053**	**0.147**	**0.052**	**0.159**	**0.688**	**0.849**	**0.574**	**0.101**

Figure 4. Mean values (± 1 standard error) for the index of phenotypic plasticity in response to nutrients for ten physiological traits (shaded bars) and four structural traits (unshaded bars) in *Arbutus unedo* plants of seven provenances.



distributed Mediterranean species, deserves great attention in order to improve our ability to predict plant responses to the new scenarios that are arising.

Our results clearly show that *A. unedo* responds to the addition of nutrients, as expected by the widely recognized pivotal role that nutrient availability plays in determining plant growth (Lambers *et al.*, 2008). The effect of high nutrient availability in increasing CHL, PRI and ΦPSII was expected as high nutrient content has been associated with higher chlorophyll contents and light use efficiency (Tissue *et al.*, 1993; Gamon *et al.*, 1997). The effect of high nutrient availability in decreasing transpiration may be indirect. High nutrient availability also reduced root/shoot allocation. Lower root allocation implies less water within the plant and a smoother plant-to-atmosphere vapour pressure gradient and less water to transpire (Ma *et al.*, 2010), which in turn would cause the observed decreased in Ci.

Do provenances differ in their plasticity to nutrient availability?

Contrary to our expectations, the effect of nutrient availability on structural and physiological plant traits did not differ between the seven provenances studied, all of which showed very similar levels of phenotypic plasticity in response to nutrient availability. Thus, we conclude that provenance does not explain a significant amount of the variance in the response to nutrient availability. Likewise, analysis of the levels of plasticity of the provenances, both in structural and physiological traits, enabled us to conclude that all provenances exhibit a common level of response to nutrient availability. Therefore, under scenarios of global change, any provenance would be equally susceptible to being favoured or hindered on the basis of its ability to respond to nutrients. These findings contrast somewhat with those of previous studies showing that the extent of phenotypic plasticity of different plant species in response to nutrients depended on the temporal nutrient availability in the original habitats (Fitter & Stickland, 1991). Therefore, taking into account the diverse provenances of *A. unedo*, we would expect to find differences between them. However, the observation of very similar levels of plasticity among provenances suggests the existence in *A. unedo* of some degree of canalization in the response to nutrients. According to Wagner et al. (1997), the canalization decreases the amount of genetic variation that is expressed by the phenotypes. This is related to a reduction in genetic variance (a decrease in the number of alleles) induced by natural selection when the stabilization of the phenotypes is advantageous (Gibson & Wagner, 2000). Thus, when the direction of the natural selection changes frequently within short periods, it would be very expensive to undergo continuous adaptations and therefore canalization is favoured (Kawecki, 2000; Le Rouzic et al., 2013). Examples of canalization in situations of seasonal environmental fluctuations have been described (Kawecki, 2000; Picotte *et al., 2*009; Le Rouzic *et al.*, 2013) and it has been reported as a common strategy in the Mediterranean (Valladares *et al.*, 2002). The existence of canalization in the strawberry tree would therefore genetically constrain the variability of the provenances and explain the homogeneous responses.

Do structural and physiological traits differ in plasticity to nutrient availability?

Knowledge of how traits of different nature, such as structural and functional traits, respond to nutrients is important for predicting how a species may evolve in the face of rapid environmental changes. Thus, the variation in plasticity between traits and the relationship between the plasticity in physiological and structural traits has previously been considered (Derner & Briske, 1999; Mou *et al.*, 2013; Matesanz & Valladares, 2014). In the strawberry tree, we observed that both types of traits clearly differed in their plasticity, with higher values of PPI for structural traits (especially for the biomass traits) than for physiological traits. This is consistent with previous findings showing that the plasticity was higher for the structural traits (also mainly for dry weight traits) when the nutrient availability was constant over a long period of time (Mou *et al.*, 2013). By way of explanation for this observation, Hodge (2006) has suggested that the construction cost of new biomass could be buffered over time, and, at the same time, the high cost of increasing the uptake rate under physiological plasticity could be avoided. Furthermore, other studies have reported that structural and physiological plasticity were negatively correlated due to their complementary nature (Derner & Briske, 1999), as we also observed. The higher values of plasticity in structural traits in response to nutrients are also explained by the key role that biomass allocation plays in resource acquisition, whereas physiological plasticity would be favoured in response to other factors such as light (Gratani *et al.*, 2006). Consequently, our results suggest that in a future scenario of increased nutrient availability, structural plasticity will determine the plant response. On the other hand, the lower plasticity in the physiological traits may be explained by stabilizing selection, which has been reported for *A. unedo* (Santiso et al., 2015). This behaviour leads to a strategy where trait variation is constricted, avoiding the cost associated with continuous responses (Kawecki, 2000). Therefore, we conclude that *A. unedo* is able to adjust its biomass allocation for a more appropriate and efficient distribution of the nutrients, but without spending resources to modify its physiology.

Beyond the plastic response to nutrient availability, we must take into account that the simultaneous occurrence of several global change drivers in the Mediterranean area may limit plasticity if the environmental cues that elicit this response are not always reliable. This could occur because of the action of another factors and would produce costs in maintaining an induced phenotype (cost of plasticity) (Valladares *et al.*, 2007; Matesanz *et al.*, 2010). A reduction in root/shoot allocation under conditions of high availability of nutrients may have an important negative impact on the water status of the plant, which could limit plant growth in areas where increased intensity and frequency of drought are predicted (IPCC, 2013). Additionally, the behaviour of other species in the community may also modify the response of *A. unedo* (Jones *et al.*, 1996). Therefore, exploration of the interactions between nutrients and water or between species would be a promising area for future studies.

Another well-known route to adaptation is by genetic selection and evolutionary adaptation (Hoffmann & Sgrò, 2011; Hansen et al., 2012), as reported for another Mediterranean species (Kandemir et al., 2010; Ramírez-Valiente et al., 2011). Regarding *A. unedo*, it has been shown that most of the traits evaluated, especially structural traits, have significant additive genetic variance and therefore the species has significant potential to undergo genetic evolution and adaptation (Santiso et al., 2015). Thus, this ability to evolve, acting together with the observed plasticity, will enhance the adaptability and survival of the strawberry tree under future scenarios of changes in the unpredictable environment of the Mediterranean ecosystems (Valladares et al., 2002; Hoffmann & Sgrò, 2011; IPCC, 2013; Matesanz & Valladares, 2014)

Are light responses curves modified by nutrient availability?

It is well known that nutrient availability can modify plant responses to light (Demmig-Adams & Adams, 1992; Bermúdez & Retuerto, 2014), an essential factor determining plant growth. Furthermore, it has been widely demonstrated that light can typically produce plant stress in the Mediterranean, especially during the summer (Faria *et al.*, 1998; Werner *et al.*, 1999). Therefore, study of the interaction between nutrients and light is of great interest. Here, we observed that plant performance, as estimated by photochemical quantum yield, improved with nutrient supply under increasing radiation. This positive correlation has been observed in some studies (Fetcher *et al.*, 1996; Martin & Jokela, 2004), in which the interaction was key to improving plant growth, but not in another (Lawler *et al.*, 1996), in which the high light counteracted the effects of nutrients. Consequently, under a scenario of increased nutrient availability, photoinhibition would be decreased in *A. unedo*, thus improving growth and survival of the plant.

In conclusion, considering the uniform plastic capabilities observed for *Arbutus unedo* across provenances, none of the provenances seem to be more capable of colonizing or more susceptible to becoming extinct than others as a consequence of predicted changes in nutrient availability. This is consistent with the existence of some degree of canalization, which constrains the variations in provenances. However, the response to nutrients depends on the type of trait and is considerably more marked for the redistribution of the biomass (structural traits) than for physiological traits, indicating that the strategy of the strawberry tree is to optimize resource allocation by maintaining the physiology quite stable. This low physiological variation is consistent with the demonstrated stabilizing selection for the species. Stabilizing selection and canalization are both evolutionary strategies that reduce the variability in the responses and, consequently, reduce the costs of allowing unnecessary changes. Finally, we also observed that supply of nutrients improved the performance of *A. unedo* under high radiation conditions. Thus, the responses to addition of nutrients observed in the strawberry tree are associated with the capacity of the tree to modify the biomass structure and with the economy of expenditure provided by stabilizing selection and possible canalization. This, together with the demonstrated ability of the strawberry tree to evolve, explains how this species has been able to inhabit such a broad distribution range and may also be determinant in enabling the future survival of the species.

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Chapter IV

Master of one trade: *Arbutus unedo* relies on plasticity to persist in habitats differing in water availability


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ABSTRACT

The limited water supply in the Mediterranean Basin may be exacerbated in the immediate future as result of the reduced precipitation, rising air temperatures and increased intensity and frequency of severe drought events predicted under global climate change scenarios. Thus, the study of the intraspecific variability in emblematic Mediterranean plant species is important as such variability modulates species' responses to environmental changes. Here, we aimed to analyse the variability in plasticity of physiological and structural traits of *Arbutus unedo* L. in response to water availability. We also aimed to determine whether levels of phenotypic plasticity varied in *A. unedo* plants of different provenances. To achieve these aims, we measured a set of structural and physiological traits in *A. unedo* plants of different provenances grown under two different conditions of water availability. Biomass, effective quantum yield of Photosystem II and all gas exchange traits, except water use efficiency, increased under well-watered conditions. Plasticity differed significantly between groups of traits, with structural and gas exchange parameters displaying higher plasticity than fluorescence and reflectance traits. The level of variability in biomass and physiological traits between provenances was low, and provenance did not account for significant variation in plasticity. We concluded that *A. unedo* has evolved a unique "good saver" strategy, involving decreasing growth and water use -when this resource is unavailable- and also dissipation of excess of radiation that the plant is unable to process in such circumstances. This unique strategy may be the result of a canalization event in *A. unedo*. The strategy, based on a high degree of plasticity, is probably a determining factor in the success of the species over the wide range of environmental conditions where it occurs and will be critical in determining the capacity of the species to withstand the ongoing environmental changes. We therefore expect that this species will continue to play an important role in the Mediterranean plant communities. Finally, we conclude that it is not possible to reach general conclusions about the level of plasticity of traits of different nature as this depends on the environmental factors that induce the variability.

Keywords: Adaptation, canalization, drought, global change, phenotypic plasticity.

INTRODUCTION

The Mediterranean Basin has been designated for priority conservation because is one of the 25 world's biodiversity hotspots, harbouring more than 25 000 plant species, 13 000 of which are endemic to this region (Myers *et al.*, 2000). The Mediterranean climate is characterized by a rather wet and mild winter and a dry and warm to hot summer, with high solar intensity due to cloudless skies and low humidity, and with at least 65% of annual rainfall falling in the winter (Aschmann, 1973). The summer conditions create very high evapotranspiration rates that impose severe restrictions for plant water availability. Such conditions are predicted to become more extreme in the immediate future, as the Mediterranean Basin will be more affected than other areas by reduced precipitation, rising air temperatures and increased intensity and frequency of severe drought events (Luterbacher *et al.*, 2004; Petit *et al.*, 2005; Giorgi & Lionello, 2008; IPCC, 2013). Thus, areas subject to heatwaves and prolonged drought, where even sclerophyllous species suffer severe photoinhibition and photosynthetic limitations, will increase (Bussotti *et al.*, 2014). Furthermore, human-induced land degradation and desertification will also intensify in the Mediterranean area, thus, exacerbating the consequences of the water deficit (Díaz-Delgado *et al.*, 2002; Pausas *et al.*, 2008).

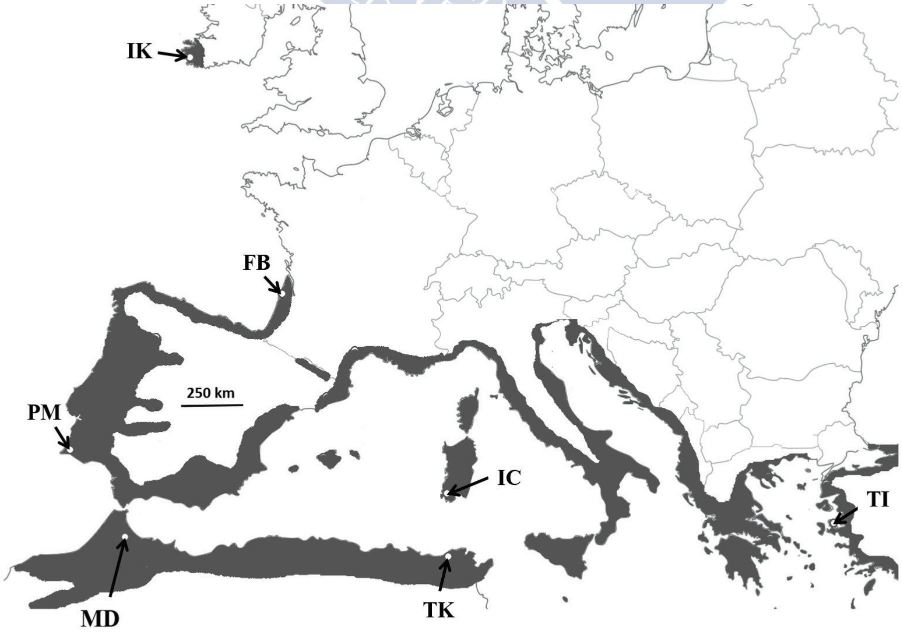
Water availability, together with high temperatures and radiation, is one of the most important environmental constraints for plant recruitment, growth and persistence in Mediterranean ecosystems, especially at early growth stages when the small root system of juvenile plants makes them highly vulnerable to water deficits (Pigott & Pigott, 1993; Chaves *et al.*, 2003). Thus, the study of the impact of drought in a key ecosystems such as the Mediterranean deserves great attention (Tenhunen *et al.*, 1982; Werner *et al.*, 1999; Manes *et al.*, 2006; Quero *et al.*, 2006; Baraldi *et al.*, 2008). Many traits that configure the strategies for plant adaptation to drought are constitutive rather than induced by stress (Chaves *et al.*, 2003). To face water stress, plants can follow three main strategies: drought evasion (the highest biological activity does not coincide with the water-deficit period), desiccation tolerance (the plant resists water deficit) and desiccation avoidance (plant tissues prevent or minimize water stress by maximizing water capture or minimizing water loss) (Larcher, 2003). Physiological mechanisms are critical components in the strategies of response to water stress. Modulation of gas exchange and stomatal conductance as well as a conservative resource-use strategy enable achievement of an adequate water balance (Tenhunen *et al.*, 1982; Manes *et al.*, 2006; Quero *et al.*, 2006). The ability to dissipate excess radiation through the xanthophyll cycle, which prevents damage to photosynthetic machinery, is also an efficient mechanism for plant resistance to drought (Thenot *et al.*, 2002; Chaves *et al.*, 2003; Baraldi *et al.*, 2008). On the other hand, structural traits such as biomass allocation, which determines how plants capture resources, or the type of leaves (the leaves of evergreen plants are more costly than those of deciduous plants, but this cost can be repaid by increased longevity) are also crucial determinants of the strategies of responses to water stress (Ludlow, 1989; Werner *et al.*, 1999; Poorter *et al.*, 2012). Nevertheless, the relative role that each type of trait plays depends on the species and conditions. Thus, several studies have shown that the phenotypic plasticity of physiological traits is higher than that of structural traits, in response to drought (Gratani *et al.*, 2003; Quero *et al.*, 2006; Zunzunegui *et al.*, 2009). However, other studies have indicated that structural mechanisms, focused on minimizing the demand (such as increased root-mass fraction), may play a more important role than physiological adaptations (Sack *et al.*, 2003; Aranda *et al.*, 2005). Finally, another study noted that structural traits may be better indicators of differences in drought adaptation between provenances (Vasques *et al.*, 2013).

The adaptive response of plants mainly occurs via genetic evolution (evolutionary adaptation) (Kandemir *et al.*, 2010; Hoffmann & Sgrò, 2011; Ramírez-Valiente *et al.*, 2011) or via phenotypic plasticity (Callaway *et al.*, 2003; Williams *et al.*, 2008; Lande, 2009). Thus, evolutionary adaptation may be rapid and help the species to respond to environmental changes (Hoffmann & Sgrò, 2011). However, simulation models show that evolutionary adaptation by itself may not be sufficient to warrant the survival of species during periods of continuous changes (Atkins & Travis, 2010). On the other hand, phenotypic plasticity, which allows a genotype to exhibit different phenotypes under different environmental conditions, is a very useful strategy, especially for sessile organisms (Sultan, 2000). Furthermore, by increasing physiological tolerance, phenotypic plasticity allows species to buffer environmental change, thus preventing area loss and reducing extinction probabilities (Callaway *et al.*, 2003; Lande, 2009; Matesanz *et al.*, 2010; Hansen *et al.*, 2012). Moreover, plasticity allows plants to improve their fitness without losing genetic variability (Matesanz *et al.*, 2010), which enables posterior genetic evolution (Lande, 2009). Both processes may be complementary and act together to increase survival under the rapid global changes (Hoffmann & Sgrò, 2011; Matesanz & Valladares, 2014). In situations of drought, it has been observed that plasticity prevails over evolutionary adaptation (Nicotra *et al.*, 2007; Gimeno *et al.*, 2009), even blurring ecotypic divergence (Baquedano *et al.*, 2008), but in some situations evolutionary adaptation and phenotypic plasticity operate together (Ramírez-Valiente *et al.*, 2010). Furthermore, beyond plasticity and evolutionary adaptation, some studies have reported that different conditions do not produce phenotypic differentiation, which can be attributed to the existence of canalization (Valladares *et al.*, 2002; Quero *et al.*, 2008; Lamy *et al.*, 2011). Therefore, it seems logical to focus on phenotypic plasticity when studying plant responses to water stress, while bearing in mind the possible existence of genetic adaptation and/or a possible role of canalization.

Several studies have demonstrated that patterns of plasticity in the response to water stress vary between species, provenances or sites (Nicotra *et al.*, 2007; Sánchez-Gómez *et al.*, 2008; Zunzunegui *et al.*, 2011). Thus, populations from warm sites may benefit more from wet conditions than populations from cool sites (Ramírez-Valiente *et al.*, 2010). Consequently, changes in the length and intensity of drought events may favour one species or provenance to the detriment of another, which can lead to changes in community structure (Lázaro-Nogal *et al.*, 2013). Therefore, the study of species with a broad range of distribution across different climate condition, such as *Arbutus unedo* L. (strawberry tree), will enable researchers to determine whether different provenances respond differently to water stress, and whether this affects the evolution of their distribution.

The distribution of the strawberry tree is limited by the 4ºC isotherm for the mean temperature of January (Sealy, 1949). It shows a predominant circum-Mediterranean distribution ranging from Morocco to Tunisia and from Spain to Turkey, but also inhabits the Atlantic coast from Morocco to Ireland (Sealy, 1949; Torres *et al.*, 2002; Cox & Moore, 2005) (Fig. 1), where its presence is especially intriguing due to the colder and wetter climate than in the Mediterranean sites. The contrasting conditions in Mediterranean areas (severe and prolonged summer drought stress) and Atlantic areas (scarce water deficit throughout the year) have induced ecological differentiation among provenances in other species (De la Mata *et al.*, 2014). So far, many studies have

Fig. 1. Map showing the distribution range of *Arbutus unedo* L. (grey area) and location of the seven provenances studied (white dots indicated with arrowheads).



evaluated physiological and morphological strategies of drought adaptation in one or a few provenances of *A. unedo* (Castell & Terradas, 1994; Gratani & Ghia, 2002; Thenot *et al.*, 2002; Ogaya *et al.*, 2003; Gratani & Varone, 2004; Munné-Bosch & Peñuelas, 2004); however, no studies have compared the drought response of provenances throughout the whole distribution range, which is crucial to predict the possible evolution of the species.

In this study, we aimed to analyse the variability in the response to water availability in a set of physiological and structural plant traits, by using the Phenotypic Plasticity Index (PPI) (Valladares *et al.*, 2000). The PPI is a robust simple index that is widely used to determine the plastic response to water stress (Gratani *et al.*, 2003; Quero *et al.*, 2006; Sánchez-Coronado *et al.*, 2007; Zunzunegui *et al.*, 2009; Zunzunegui *et al.*, 2011; Catoni *et al.*, 2012). We also aimed to determine whether levels of phenotypic plasticity differ in *A. unedo* plants of different provenances.

MATERIAL AND METHODS

The species

Arbutus unedo is a species belonging to the family *Ericaceae,* subfamily *Arbutoideae*, which includes evergreen, shrub-like woody taxa with sclerophyllous, laurel-like leaves (Torres *et al.*, 2002). The genus *Arbutus* has several members in America and four in Europe: *A. andrachne* L. (eastern Mediterranean), *A. pavarii* Pampanini (Libyan coast), *A. canariensis* Veill. (Canary Islands) and *A. unedo* L. (Torres *et al.*, 2002). Flowering spans from September to December and fecundation is entomophilous (Mitchell, 1993). The fruits take 12 months to ripen and seeds are dispersed by animals (mainly birds). Seed viability was reported to be about 55% in favourable places while seedlings loss was found to exceed 60% (Sealy, 1949).

Sample collection and experimental conditions

Between September and December 2010, we sampled seven populations of *A. unedo* in seven countries (Ireland, IK; France, FB; Portugal, PM; Morocco, MD; Tunisia, TK; Italy, IC; and Turkey, TI), which represent the entire distribution range of *A.*

unedo L. (Fig.1). At each site, we collected several fruits from each of 30 trees, and stored the samples at 4°C. We later extracted the seeds and stored them in a dry atmosphere until germination. In the laboratory, we placed the seeds (homogeneous in size and appearance, to minimize maternal effects) on filter paper wetted with double-distilled water, in Petri dishes. Immediately after the seeds germinated, we placed the seedlings in forestry trays each with fifteen 1 litre cavities filled with a 3:1 mixture of potting compost and perlite (Tref Substrates BV, Moerdijk, The Netherlands). To reduce maternal effects, we kept the plants for 1 year, between March 2012 and March 2013, in a common environment (an outdoor plot at the University of Santiago de Compostela). We then took 40 similarly sized plants of each provenance and transferred them to 7 litre pots containing the previously mentioned substrate. Twenty randomly selected plants per provenance were kept in well-watered conditions (High Water treatment, HW), by irrigating to field capacity every two days. The other 20 plants per provenance were kept under water stress conditions (Low Water treatment, LW), by irrigating to field capacity every 10 days. The soil water contents in the pots were monitored with a moisture sensor (ML3 - ThetaProbe Soil Moisture Sensor, Delta-T Devices, Cambridge, UK) to check the differences between treatments. The plants were kept under the experimental conditions for 4 months, before being harvested.

Physiological traits

The physiological traits were measured twice, on June and July 2013, as follows:

Leaf spectral reflectance

Leaf reflectance spectra, ranging from 300 to 1100 nm, were recorded on the adaxial side of one leaf per plant with a portable spectroradiometer (Unispec, PP Systems, Haverhill, MA, USA). Leaf reflectance was calculated by dividing the spectral radiance of the leaf by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere, North Sutton, NH, USA). Reflectance data were processed to calculate the following indices related to functional processes. The chlorophyll index

(CHL), calculated as R_{750}:R_{550} (R_n indicates reflectance at n nanometres), is very sensitive to changes in chlorophyll content (Lichtenthaler et al., 1996). The photochemical reflectance index (PRI), determined as $(R_{531} - R_{570})/(R_{570}+R_{531})$, is associated with zeaxanthin-antheraxanthin-violaxanthin interconversion and displays a functional relationship with the photosynthetic radiation-use efficiency and the dissipation of excess radiation energy as heat (Peñuelas et al., 1995).The normalised difference vegetation index (NDVI), computed as $(R_{900}-R_{680})/(R_{900}+R_{680})$, has been related to the 'greenness' or general vigour of vegetation due to its correlation with leaf chlorophyll content, foliar nitrogen, phosphorus and potassium contents (Gamon et al., 1995; Peñuelas & Inoue, 1999). The water index (WI), calculated as R_{900}:R_{970}, although correlated with the plant water content and crucial for leaf expansion, photosynthesis and growth, was used to calculate the WI/NDVI, which is a better indicator of hydric status (Peñuelas et al., 1997; Peñuelas & Inoue, 1999). All these physiological traits, related to the foliar physiology, are main determinants of the growth and survival of plants (Chaerle & Van Der Straeten, 2000)

Chlorophyll fluorescence

These measurements were taken on the adaxial side of the same leaves used to record leaf reflectance sprectra, with a portable pulse amplitude modulated fluorometer (Mini-PAM; Heinz Walz, GmbH, Effeltrich, Germany). The effective quantum yield of Photosystem II (PSII) (ΦPSII), was calculated as $(F_m' - F_t)/F_m'$, where F_m' is the maximum fluorescence emitted by an illuminated leaf after a saturating pulse and F_t is the basal fluorescence emission at a given photosynthetic photon flux density. This parameter measures the proportion of the light absorbed by the chlorophylls that is used in photochemistry (Genty *et al.*, 1989). ΦPSII therefore provides information about the efficiency of excitation energy capture by PSII reaction centres and it has been found to be directly correlated with radiation use efficiency (Maxwell & Johnson, 2000; Roháček, 2002).The maximum quantum yield of PSII (F_v/F_m) was determined as $(F_m - F_0)/F_m$, where F_m and F_0 are respectively the maximum and the basal fluorescence emission in dark adapted samples (i.e. in which all PSII reaction centres are fully open). Leaves were dark adapted for 30 min before the Fv/Fm was measured by using dark leaf clips (Heinz Walz, GmbH). F_v/F_m was found to be linearly correlated with the photosynthetic activity of thylakoids, as it estimates the efficiency of capture of the excitation energy by open PSII reaction centres (Krause & Weis, 1991). Therefore, F_v/F_m represents the maximum potential efficiency (after dark adaptation) in the photochemical use of the photon energy.

Gas exchange parameters

Photosynthesis rate (μmol CO_2 m^{-2} leaf area s^{-1}), intercellular CO_2 concentration (Ci) (μmol CO_2 mol^{-1}), hydraulic conductance (mmol m^{-2} s^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}) were measured in one leaf per plant with the aid of a portable infrared carbon dioxide analyzer (LI-6400XT; Li-Cor, Lincoln, NE, USA). The intrinsic water use efficiency of photosynthesis (WUE) (mmol CO_2/ mol de H_2O) was obtained by calculating the ratio photosynthesis rate/ transpiration rate. Light intensity in the chamber was fixed at 500 μmol $m^{-2}s^{-1}$, and the CO_2 concentration in the air entering the cuvette was maintained at 400 mmol L^{-1}. All of parameters are closely affected by plant water status (Lambers *et al.*, 1998) and have also been related to plant fecundity and survival (Arntz *et al.*, 2000).

Structural traits

All plants were harvested after four months growth under the experimental water treatments. At harvest, each plant was separated into roots, stems and leaves. The roots were washed to remove the soil, and the roots, stems and leaves were then dried in an oven at 40° C to constant weight. Each plant part was then weighed separately to the nearest 0.0001 g (Mettler AJ100, Switzerland). As root and shoot biomass perform essential functions for the plants, including absorption, structural support, storage and reproduction (Mokany *et al.*, 2006), we assessed biomass allocation patterns by calculating root/shoot ratios, where shoot is stem + leaf dry weight. Root/shoot ratios have been found to respond to variations in precipitation, temperature and forest stand height (Mokany *et al.*, 2006).

Data analysis

The effects of provenance and water treatments (within-subject effects) and time (between-subject effect) on physiological plant traits were assessed by two way repeated-measure analysis of variance (ANOVAR). According to Mauchly´s test, the data did not satisfy the assumption of sphericity and therefore the Greenhouse-Geisser procedure was used to correct the degrees of freedom of the F values.

A two-way ANOVA was used to analyse the effects of provenance (seven levels) and water availability (high vs low irrigation) on plant dry weights and root/shoot ratio. In order to satisfy the conditions of normality and homoscedasticity, root and shoot dry weights were transformed using the hyperbolic sine transformation or the logarithmic transformation (for root/shoot ratio).

The Phenotypic Plasticity Index (PPI) (Valladares *et al.*, 2000) was used to determine the degree of phenotypic plasticity for each trait and provenance. The PPI was calculated as (maximum mean - minimum mean)/ maximum mean, where the numerator is the difference between the mean values of the trait for the two water treatments and the denominator is the mean value of the trait for the higher of the two water treatments. For the physiological traits, we averaged the PPIs for the two dates. To estimate differences in the plasticity between provenances, we calculated the PPI per provenance by averaging the 14 PPI values of each provenance. Finally, we calculated the PPI for each of the four groups of traits considered: (I) Reflectance traits (CHL, PRI, NDVI, WI/NDVI) (II) Fluorescence traits (Fv/Fm and ΦPSII_2); (II) Gas exchange parameters (Photosynthetic rate, Stomatal Conductance, Ci, Transpiration and WUE) and (III) Structural traits (root and shoot dry weights and root/shoot ratio). Differences in phenotypic plasticity between provenances and between types of traits were analysed by one way analysis of variance. All statistics analyses were carried out with the IBM SPSS Statistics V.22 package (Armonk, NY, USA).

RESULTS

Physiological traits

Provenance significantly affected NDVI and WINDVI (Table 1, Fig. 2). The effective quantum yield of Photosystem II (ΦPSII) (Table 1, Fig. 3) and all the gas exchange traits (Table 2, Fig. 4) increased under well-watered conditions. Differences between water treatments depended on time for photosynthesis, C_i and transpiration. The chlorophyll content (expressed as CHL) and the dissipation of excess of radiation (determined as PRI) were also significantly higher under water stress conditions (Table 1, Fig. 2), as was the water use efficiency (Table 2, Fig. 4). All physiological traits varied significantly over time.

Fig. 2. Mean values (± one standard error) for each provenance (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and reflectance trait (CHL: chlorophyll content index; PRI: photochemical reflectance index; NDVI: Normalised Difference Vegetation Index; WI/NDVI: Water index /NDVI) in *Arbutus unedo* L. plants under high (HW) and low water (LW) availability on the two dates (1: first date; 2: second date).

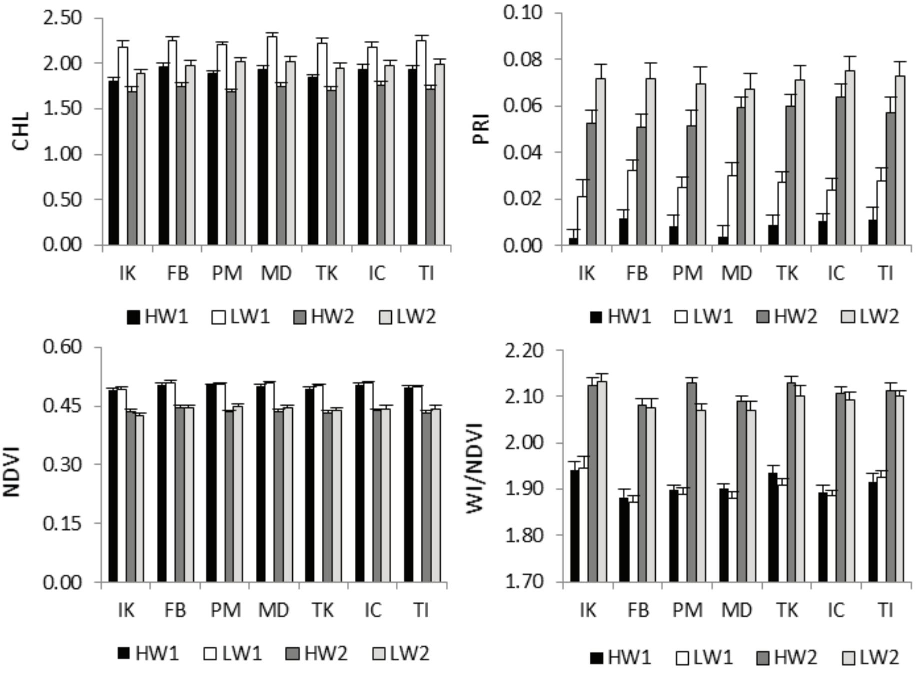
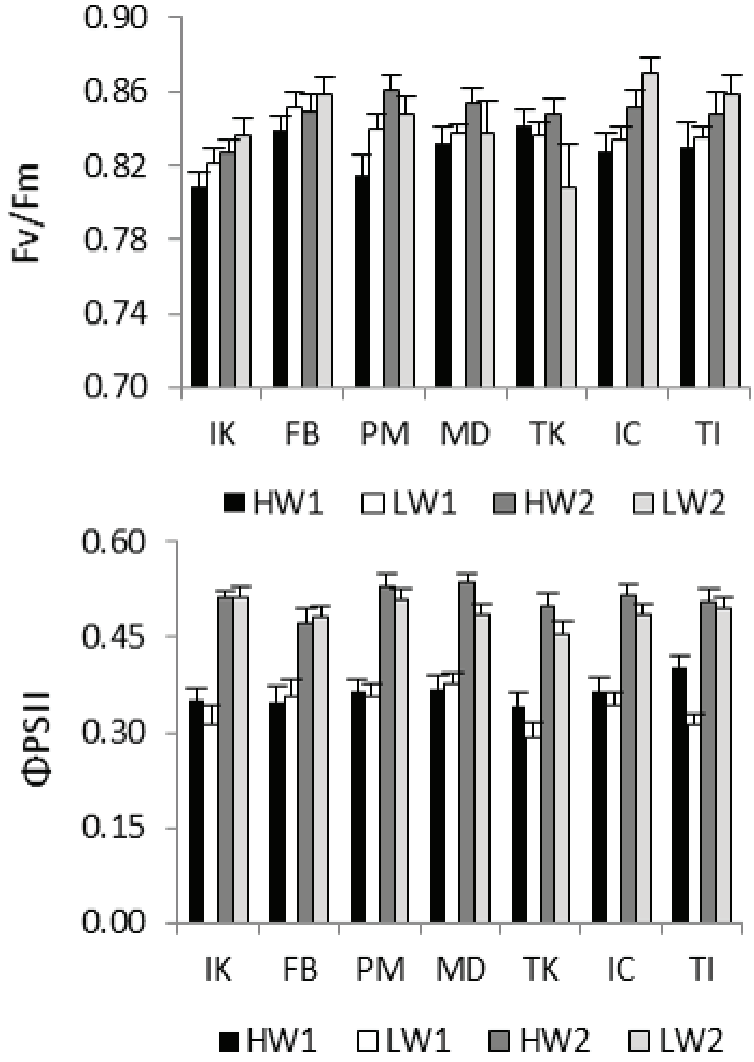


Table 1. Results of the two-way repeated-measure analysis of variance, using Greenhouse-Geisser correction, for differences in reflectance (CHL: chlorophyll content index; PRI: photochemical reflectance index; NDVI: Normalised Difference Vegetation Index; WI/NDVI: Water index /NDVI), and chlorophyll fluorescence (maximum (Fv/Fm) and effective (ΦPSII) quantum yield of photosystem II) traits in seven provenances of *Arbutus unedo* L. We tested "provenance" and "water availability" as the between-subject effects and "time" as the within-subject effect. Notes: Significant P values (<0.05), after sequential Bonferroni correction are shown in bold type; # denotes higher values for low water treatment.

Trait	*Df*	**CHL**		**PRI**		**NDVI**		**WI/NDVI**		**Fv/Fm**		**ΦPSII**	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Between-subject effect													
Provenance	6	2.220	0.042	0.624	0.711	3.157	**0.005**	5.048	**<0.001**	2.812	0.011	2.536	0.021
Water	1	226.101	**<0.001**#	48.694	**<0.001**#	4.222	0.041	4.852	0.029	0.766	0.382	9.486	**0.002**
Provenance X water	6	0.577	0.749	0.183	0.981	0.601	0.729	0.732	0.624	1.586	0.152	1.198	0.308
Error	252												
Within-subject effect													
Time	1	134.588	**<0.001**	632.746	**<0.001**	816.115	**<0.001**	1249.231	**<0.001**	17.618	**<0.001**	424.252	**<0.001**
Provenance X time	6	0.282	0.945	0.808	0.565	0.043	1.000	0.265	0.953	2.100	0.054	1.094	0.367
Water X time	1	4.132	0.043	0.451	0.503	0.0001	0.989	0.194	0.660	2.518	0.114	0.193	0.661
Provenance X water X time	6	0.483	0.820	0.615	0.718	0.429	0.859	0.597	0.732	1.022	0.411	1.015	0.416
Error	252												

Fig. 3. Mean values (± one standard error) for each provenance (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and chlorophyll fluorescence trait (maximum (Fv/Fm) and effective (ΦPSII) quantum yield of photosystem II) in *Arbutus unedo* L. plants under high (HW) and low water (LW) availability on the two dates (1: first date; 2: second date).



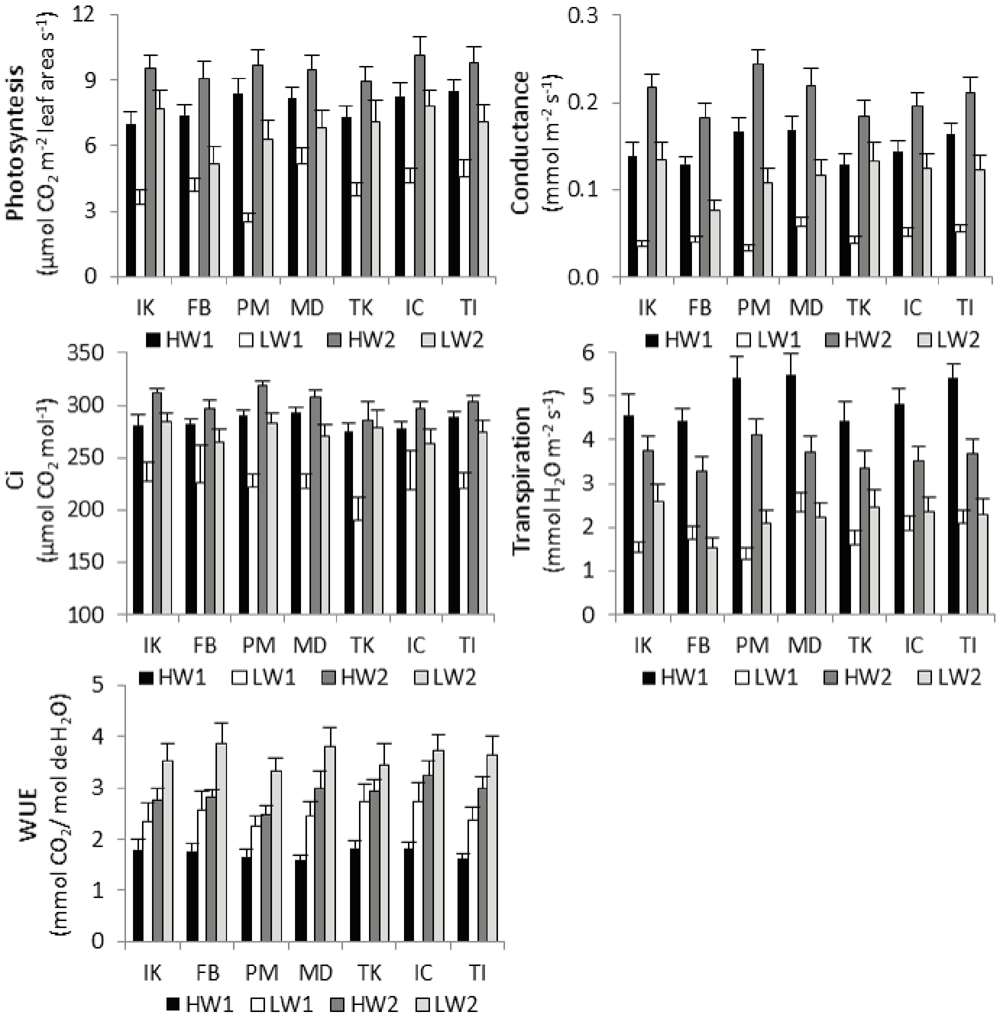
Structural traits

Root biomass was the only structural trait affected by provenance (Table 3). On the other hand, root, shoot and root: shoot biomass were significantly higher in well-watered plants (Table 3, Fig. 5).

Table 2. Results of the two-way repeated-measure analysis of variance, using Greenhouse-Geisser correction, for differences in gas exchange traits (Photo: photosynthesis rate; Cond: hydraulic conductance; Ci: intercellular CO2 concentration; Transp: transpiration rate; WUE: water use efficiency of photosynthesis) in seven provenances of *Arbutus unedo* L. The "provenance" and "water availability" were tested as the between-subject effect and "time" as the within-subject effect. Note: significant P values (<0.05), after sequential Bonferroni correction, are shown in bold type; # denotes higher values for low water treatment.

Trait	*Df*	**Photo**		**Cond**		**Ci**		**Transp**		**WUE**	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
Between-subject effect											
Provenance	6	1.374	0.226	2.078	0.056	0.915	0.485	1.254	0.279	0.925	0.477
Water	1	124.195	**<0.001**	241.419	**<0.001**	69.364	**<0.001**	201.360	**<0.001**	41.154	**<0.001#**
Provenance X water	6	0.758	0.604	1.437	0.201	0.078	0.998	0.816	0.559	0.257	0.956
Error	252										
Within-subject effect											
Time	1	92.895	**<0.001**	189.403	**<0.001**	189.403	**<0.001**	20.419	**<0.001**	143.302	**<0.001**
Provenance X time	6	1.296	0.259	1.474	0.187	0.245	0.961	1.959	0.072	0.509	0.801
Water X time	1	7.136	**0.008**	2.229	0.137	11.972	**<0.001**	75.212	**<0.001**	0.001	0.978
Provenance X water X time	6	0.825	0.552	0.451	0.844	0.307	0.933	0.646	0.693	0.167	0.985
Error	252										

Fig. 4. Mean values (± one standard error) for each provenance (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and gas exchange trait (Photosynthesis: photosynthesis rate; Conductance: hydraulic conductance; Ci: intercellular CO2 concentration; Transpiration: transpiration rate; WUE: water use efficiency of photosynthesis) in *Arbutus unedo* L. plants under high (HW) and low water (LW) availability on the two dates (1: first date; 2: second date).



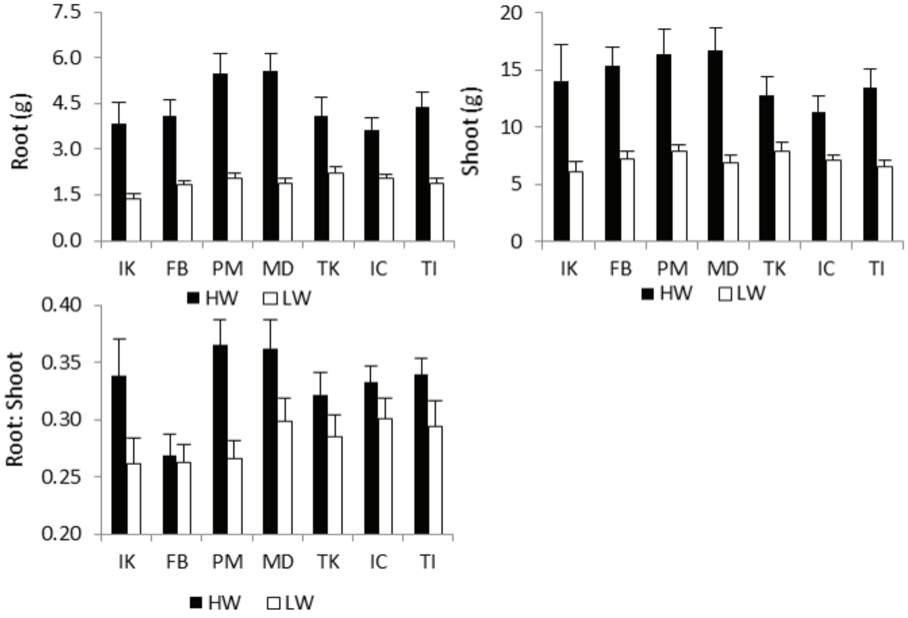
Plasticity

Structural traits, with values for the phenotypic plasticity index ranging from 0.149 to 0.565 (mean = 0.403) (Table 4, Fig. 6), showed similar levels of plasticity as gas exchange parameters, for which the PPI values ranged from 0.163 to 0.574 (mean = 0.379). These two groups of traits exhibited substantially higher levels of

Table 3. Results of factorial ANOVA test for differences in structural traits (Root: root biomass; Shoot: shoot biomass; Root: Shoot: root biomass/ shoot biomass) in plants of seven provenances of *Arbutus unedo* L. The effects of "provenance" and "water availability" were tested. Note: significant P values (<0.05), after sequential Bonferroni correction, are shown in bold type.

	Df	Root		Shoot		Root: Shoot	
		F	***P***	***F***	***P***	***F***	***P***
Provenance	6	3.189	**0.005**	2.194	0.044	1.905	0.081
Water	1	140.946	**<0.001**	69.717	**<0.001**	21.819	**<0.001**
Provenance X water	6	1.502	0.178	0.882	0.509	1.189	0.313
Error	239						

Fig. 5. Mean values (± one standard error) for each provenance (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and structural trait (root: root biomass; shoot: shoot biomass; root: shoot: root biomass/ shoot biomass) in *Arbutus unedo* plants under high (HW) and low water (LW) availability on the two dates (1: first date; 2: second date).

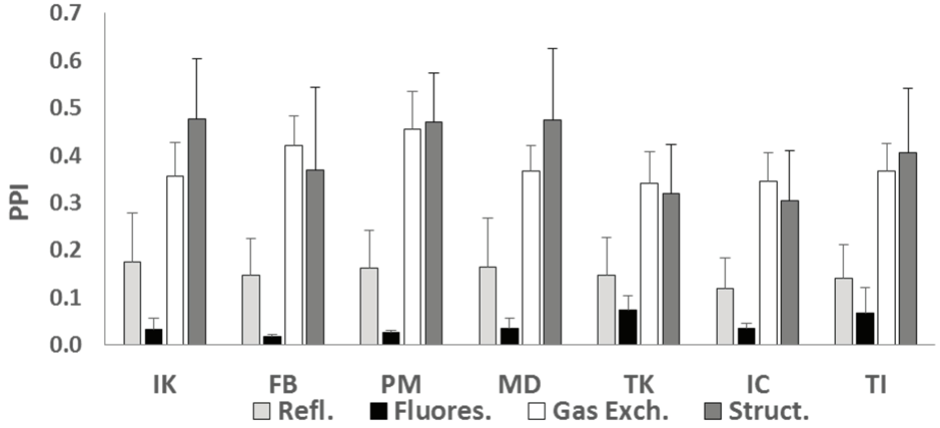


plasticity than the fluorescence traits, with values ranging from 0.008 to 0.449 (mean = 0.115) and reflectance traits, with values of between 0.016 and 0.067 (mean = 0.042). The PPI values were low for the root: shoot ratio (of the dry weight traits) and Ci (of

Table 4. Phenotypic plasticity index (maximum mean - minimum mean)/ maximum mean, in *Arbutus unedo* L. for each provenance (prov.) (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and for each reflectance trait (CHL: chlorophyll content index; PRI: photochemical reflectance index; NDVI: Normalised Difference Vegetation Index; WI/NDVI: Water index /NDVI), chlorophyll fluorescence trait (maximum (Fv/Fm) and effective (ΦPSII) quantum yield of photosystem II), gas exchange trait (Photo: photosynthesis rate; Cond: hydraulic conductance; Ci: intercellular CO2 concentration; Trans: transpiration rate; WUE: water use efficiency of photosynthesis), and structural trait (Root: root biomass; Shoot: shoot biomass; Root: Shoot: root biomass/ shoot biomass) traits. The mean values per trait and per provenance are also indicated.

Prov.	**Reflectance**				**Fluorescence**		**Gas exchange traits**					**Structural traits**			**Total mean**
	CHL	**PRI**	**NDVI**	**WI NDVI**	**FvFm**	**ΦPSII**	**Photo**	**Cond**	**Ci**	**Trans**	**WUE**	**Root**	**Shoot**	**Root: Shoot**	**All traits**
IK	0.136	0.554	0.013	0.003	0.014	0.054	0.361	0.564	0.139	0.499	0.223	0.638	0.566	0.226	**0.285**
FB	0.122	0.460	0.005	0.004	0.013	0.025	0.452	0.638	0.152	0.574	0.291	0.556	0.529	0.021	**0.275**
PM	0.155	0.461	0.017	0.017	0.022	0.030	0.525	0.691	0.173	0.631	0.255	0.623	0.517	0.273	**0.314**
MD	0.144	0.493	0.015	0.010	0.012	0.059	0.322	0.562	0.183	0.489	0.284	0.660	0.587	0.176	**0.285**
TK	0.146	0.415	0.017	0.013	0.026	0.120	0.349	0.493	0.167	0.454	0.243	0.461	0.380	0.115	**0.243**
IC	0.111	0.352	0.010	0.005	0.015	0.055	0.357	0.519	0.159	0.463	0.234	0.441	0.375	0.095	**0.228**
TI	0.140	0.408	0.013	0.005	0.010	0.126	0.369	0.554	0.165	0.498	0.249	0.573	0.509	0.135	**0.268**
Mean per trait	**0.137**	**0.449**	**0.013**	**0.008**	**0.016**	**0.067**	**0.391**	**0.574**	**0.163**	**0.516**	**0.254**	**0.565**	**0.495**	**0.149**	

Fig. 6. Mean phenotypic plasticity index (± one standard error) in response to water treatments in *Arbutus unedo* L. The mean values for each provenance (IK: Ireland Killarney; FB: France Bordeaux; PM: Portugal Monchique; MD: Morocco Debdou; TK: Tunisia Kroumerie; IC: Italy Cagliari; TI: Turkey Izmir) and for each group of traits (Refl: reflectance traits; Fluores: chlorophyll fluorescence traits; Gas Exch: gas exchange traits; Struct: structural traits) are show.



the gas exchange parameters) and the value of the PRI index was high for the reflectance parameters. Results of analysis of variance showed that provenance did not account for any significant variation in PPI values. However, this analysis reveals significant differences in plasticity between groups of traits ($p< 0.001$; data not shown), with biomass and gas exchange traits showing higher PPI values than reflectance and fluorescence traits (Fig. 6).

DISCUSSION

The role of water as an essential factor for plant growth and survival in the Mediterranean biodiversity hotspot is matter of special interest due to the decreased rainfall and increased temperatures that are predicted for this area (Luterbacher *et al.*, 2004; Petit *et al.*, 2005; Giorgi & Lionello, 2008; IPCC, 2013). So far, some studies have described the consequences of drought in plants in terms of phenotypic plasticity, evolutionary adaptation and genetic canalization (Gimeno *et al.*, 2009; Ramírez-Valiente *et al.*, 2010; Lamy *et al.*, 2011), and some have evaluated plant responses based on physiological (Tenhunen *et al.*, 1982; Manes *et al.*, 2006; Quero *et al.*, 2006) and/or structural traits (Werner *et al.*, 1999; Poorter *et al.*, 2012). However, none of these studies have considered species over a wide distribution range in which different climatic conditions may have led to different adaptive responses of plants, depending on their provenance (Ramírez-Valiente *et al.*, 2010; De la Mata *et al.*, 2014). This, in turn, could affect the evolution of the distribution range of the species or the community structure (Lázaro-Nogal *et al.*, 2013).

Variability in structural and physiological traits in response to water availability

The observed effects of low water availability in decreasing root and shoot dry weights were expected, considering the well–known effect of water on plant growth in general (Pigott & Pigott, 1993) and in *A. unedo* in particular (Ogaya *et al.*, 2003). Although increased root/shoot ratios have been interpreted as a mechanism for avoiding drought by maximizing water capture by roots relative to water loss through transpiration (Ludlow, 1989), we found the opposite, indicating non-optimal adaptation of the strawberry tree to drought. However, this poor adaptation may be compensated by conservative physiological behaviour. In fact, the ΦPSII and all gas exchange traits, except WUE, were adversely affected by low water availability, which is consistent with previous studies showing that the strawberry tree, under drought conditions, exhibit a conservative water use strategy, with control of the photosynthetic rate, conductance and stomata regulation (Tenhunen *et al.*, 1982; Castell & Terradas, 1994; Gratani & Ghia, 2002; Gratani & Varone, 2004). The observed increased WUE in plants with low water availability confirms the conservative use of water in *A. unedo* plants in response to drought, as previously reported (Gratani & Ghia, 2002). In relation to the reflectance traits, we obtained some remarkable results. First, we observed a substantial increase in the PRI under LW conditions, which also showed one of the highest PPI among the physiological traits. This is consistent with previous results showing that dissipation of excess radiation through the xanthophyll cycle is an efficient mechanism to avoid photoinhibition during conditions of drought (Chaves *et al.*, 2003). Similar results have been observed in *A. unedo* (Munné-Bosch & Peñuelas, 2004; Baraldi *et al.*, 2008), and the PRI has been proposed as a reliable water-stress index for the strawberry tree (Thenot *et al.*, 2002). Likewise, we also observed the highest concentration of chlorophyll, as estimated by the CHL index, in plants with low availability of water, which is not consistent with previous observations of a decrease in chlorophyll concentration under severe water stress (Munné-Bosch & Peñuelas, 2004). However, Munné-Bosch and Peñuelas (2004) also showed that a moderate water stress, as in the present study, did not affect chlorophyll concentration. Other authors have also reported that high chlorophyll concentration could be explained as an osmotic response to water deficit (García-Valenzuela *et al.*, 2005; Gupta *et al.*, 2010).

The PPI values were much higher for the structural and gas exchange parameters than for the reflectance and fluorescence traits. Previous studies have reported higher levels of plasticity in structural than in physiological traits and suggested that structural and physiological plasticity are negatively correlated due to their complementarity (Derner & Briske, 1999). In support of these observations, Hodge (2006) suggested that the investment made in increasing biomass can be more easily compensated over time than the cost of modifying the physiological traits. However, somewhat in contrast with these findings, we found that some physiological traits, such as the gas-exchange parameters, displayed similar or even higher levels of plasticity than the structural traits. In a previous study in which we examined levels of plasticity in *A. unedo* in response to nutrient availability (Santiso & Retuerto, unpublished results), we found that the same gas exchange parameters displayed much lower levels of plasticity than structural traits. This clearly shows that it is not possible to reach general conclusions about the plasticity of traits of different nature as the level of plasticity depends on the environmental factor that induces the variability. For example, Gratani *et al.* (2006) have shown a high level of physiological plasticity in response to light.

Provenance variability

The *A. unedo* plants of different provenances did not differ in their strategies to response to drought. Thus, the predicted changes in water availability will not increase or decrease the survival possibilities of any particular provenance of this species. The common strategy is contrary to that suggested in some previous studies, which showed, under different climatic conditions, significant variation between different provenances of *A. unedo* (Vasques *et al.*, 2013; De la Mata *et al.*, 2014). In fact, Vasques *et al.* (2013) reported that structural traits were better indicators of differences in drought adaptations between provenances of *A. unedo* than physiological variables, and that the interpretation of physiological results should be assisted by morphological traits. Although we observed high PPI for the morphological traits, the provenance did not account for a significant amount of variance. On the other hand, other studies also reported low differentiation between sites in Mediterranean scrub communities (Zunzunegui *et al.*, 2009; Zunzunegui *et al.*, 2011). Other evidence supporting the low variation between provenances in *A. unedo* is the demonstrated role of the stabilizing selection, both in morphological and physiological traits, during the evolution of the strawberry tree (Santiso *et al.*, 2015).

The existence of canalization is also consistent with the low variation between provenances and represents a possible mechanism for the observed stabilizing selection. Canalization is a reduced expression of genetic variation at the level of phenotype (Wagner *et al.*, 1997), whose origin is a constraint on the genetic variance that could be originated under natural selection in order to stabilize the phenotype (Gibson & Wagner, 2000). In situations where the direction of the selection changes quickly, canalization may be favoured as it avoids the cost of continuous adaptations (Kawecki, 2000; Le Rouzic *et al.*, 2013). In fact, several studies have highlighted the role of genetic canalization in the strategy of Mediterranean species (Valladares *et al.*, 2002; Quero *et al.*, 2008; Lamy *et al.*, 2011). In the particular case of *A. unedo*, canalization would enable the different provenances to have a homogeneous plastic response to water availability oscillations, thus avoiding the cost of adaptation to each local condition. However, despite the possible role of canalization and the demonstrated stabilizing selection, in *A. unedo*, most of the analysed traits, and especially structural traits, possess significant additive genetic variance, which is the raw material for genetic evolution (Santiso *et al.*, 2015). Consequently, the strawberry tree has significant potential to undergo genetic adaptation, which will be also determinant in the current scenario of climate change.

In conclusion, the high degree of plasticity in structural and in some important physiological traits of the strawberry tree in response to water availability strongly suggest that the species shows a high capacity to reduce water use and growth under conditions of drought and also possesses an effective mechanism for dissipating excess radiation. Surprisingly, although the provenances studied were growing far apart from each other under rather different climatic conditions, they consistently showed very similar levels of phenotypic plasticity and responses to water availability. This suggests that *A. unedo* has evolved a unique "good saver" strategy, based on a high degree of plasticity. This plasticity is likely to be critical for the persistence of the species and a main determinant of its success over the wide range of environmental conditions in which it occurs. Our findings are consistent with the previously reported stabilizing selection in the strawberry tree and this strategy may be the result of a canalization event in *A. unedo*. However, according to previous studies carried out by our research group, the reduction in the genetic variance (canalization) does not exclude a significant potential for genetic adaptation in *A. unedo*. Thus, *A. unedo* shows a conservative strategy in regard to water use, considerable phenotypic plasticity in structural and physiological traits and a significant evolutionary potential. All of these factors may be critical in determining the capacity of the species to withstand the ongoing environmental changes and to persist in the new arising scenarios. We therefore expect that this species will continue to play an important role in Mediterranean plant communities. Finally, we conclude that it is not possible to reach general conclusions about the plasticity of traits of different nature as the level of plasticity depends on the environmental factors that induce the variability.

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Chapter V

Patterns of genetic variation within and among populations in *Arbutus unedo* and its relation with selection and evolvability.

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ABSTRACT

The study of plant adaptation is especially relevant in the context of expected global change, which will modify key environmental factors, such as temperature and precipitation. Here, we study the strawberry tree (*Arbutus unedo* L.) throughout its climatically variable range: predominantly circum-Mediterranean, but also along the Atlantic coasts from Morocco to Ireland. In a common garden experiment, we studied 12 plant traits to assess the hypothetical effects of natural selection on the population genetic structure. These traits were selected because it is well known that they are highly affected by climatic factors such as temperature and precipitation. To this aim, we used the Q_{ST}-F_{ST} approach. Firstly we calculated the additive genetic variance (V_A), heritability (h^2) and evolvability. Then, we obtain Q_{ST}, which is a standardized measure of genetic differentiation among populations displayed by quantitative traits, and compare it with F_{ST}, a measure of the genetic differentiation of putatively neutral loci. Our results showed that Q_{ST} was significantly lower than F_{ST} in 6 of the 12 traits, and 11 of the traits had demonstrable V_A and evolvability. Low Q_{ST} values are typically explained by stabilizing selection. We propose that stabilizing selection may select for a norm of reaction that produces a similar phenotype even from diverse genotypes from different localities. Further, the evolvability that we found in key morpho-structural traits predicts that *Arbutus unedo* retains genetic variation that will allow it to continue to adapt to the diverse environments throughout its species range.

Keywords: *Arbutus unedo*; environmental changes; Q_{ST} – F_{ST}; additive genetic variance; evolvability; plasticity.

INTRODUCTION

One of the main goals in evolutionary biology is the study of variation, as well as to find the reason for its origin and preservation. Heritable phenotypic variation is the raw material for evolutionary change, and its maintenance is crucial for the adaptability and evolutionary potential of a population or species (Frankham, 2005). Related to that, the study of plant adaptation has recently gained importance in the context of on-going global change (Aitken et al., 2008). It is known that the Earth's climate has warmed by approximately 0.6 ºC over the past century, a rate of warming greater than at any other time during the past 1000 years (IPCC 2013). The new environmental conditions arising as result of climate warming are leading to changes in plant species, such as climate-induced movement towards higher latitudes and/or altitudes (Parmesan and Yohe, 2003), shifts that may have consequences for the structure and dynamics of the ecosystems (Aerts et al., 2006). In this context, the study of widespread species, living under a wide range of climatic conditions is relevant to understand environmental effects on their genetic population structure. This is because populations existing under different environmental conditions are especially likely to experience different processes of local selection.

Arbutus unedo L. (strawberry tree) is a diploid species (2n=26), belonging to the *Ericaceae* family, *Arbutoideae* subfamily (Torres et al., 2002). Its main distribution is around the Mediterranean Sea, but, interestingly, it has populations along the Atlantic coast of the Iberian Peninsula, France, and south western Ireland (Figure 1), where its distribution limit coincides with the 4ºC isotherm for the January mean temperature (Sealey, 1949). *A. unedo* is one of the species belonging to the "Lusitanian group of flora". These are species present in the Iberian Peninsula and in Western Ireland, but absent in Britain (Sealey, 1949). A suggested explanation for this presence in Ireland is arrival via a postglacial migration (Sealey, 1949; Webb, 1983). It is intriguing how the Irish populations can survive in climate conditions so different to those of the Mediterranean populations. Therefore, the study of this disjunct and wide range distribution pattern (more than 2000 km latitude) may be of special interest (Cox and Moore, 2005). Specifically, the study of their genetic differentiation is relevant to understand the effect of the Atlanto-Mediterranean contrast. Likewise, the knowledge of its genetic diversity is crucial to predict the evolvability and, therefore, the hypothetical survival of the species. This knowledge is especially relevant for traits with proven responses to changes in light, temperature and precipitation, which have been predicted to vary as a result of the ongoing climate change (IPCC, 2013).

Frequently, the influence of selection on population divergence has been estimated by comparing the genetic differentiation in quantitative traits, Q_{ST} (Spitze, 1993), with neutral marker differentiation, F_{ST} (Wright, 1951). Populations can diverge genetically without selection due to migration and drift, and this variation is estimated using F_{ST} from neutral markers. Therefore, if $Q_{ST} = F_{ST}$, genetic drift is enough to explain the genetic structure in the population. However, if $Q_{ST} > F_{ST}$, the differences in quantitative traits are higher than expected due only to drift, which is interpreted as evidence of spatially divergent selection on the trait. In the opposite situation, $Q_{ST} < F_{ST}$, the existence of convergent (stabilizing) selection is inferred, because the differences in quantitative traits are lower than expected (Whitlock, 2008). However, some criticisms about the use of Q_{ST} must be taken into account. First, the use of quantitative trait measures taken from individuals grown in diverse localities is not appropriate, because differences in environmental effects on development could mask (or exaggerate) the real genotypic differentiation among populations (Pujol et al 2008). Second, the use of highly variable neutral markers such as microsatellites to calculate F_{ST} produces biases and must be avoided (Edelaar and Björklud 2011, Edelaar et al 2011). Third, the use of > 10 populations is highly advisable to increase the reliability of the Q_{ST} F_{ST} comparison (Whitlock and Guillaume 2009). Consequently, we decided to use a common garden design and the application of AFLP neutral markers (not highly variable), and we obtain samples from 19 populations (further details follow) to mitigate these difficulties.

Q_{ST} has been frequently used to test for the effects of spatially divergent selection, as has been shown in several reviews (Merilä and Crnokrak, 2001; Leinonen et al., 2008; Kort et al., 2013). These reviews found positive correlations between Q_{ST} and F_{ST}. They found that, on average, Q_{ST} was higher than F_{ST}, revealing the main role of divergent selection for quantitative traits. Therefore, natural selection, including indirect selection through correlated characters, is a powerful force in the evolution of plant phenotypes for adaptations to local environments and the evolution of functional traits (Linhart and Grant, 1996; Geber and Griffen, 2003). However, reports of $Q_{ST} > F_{ST}$ potentially contain several biases: trait selection bias, due to the deliberate selection of populations from contrasting environments, and publication bias, favouring studies reporting the outcome $Q_{ST} > F_{ST}$ (Leinonen et al., 2008). Likewise, there is a bias in the calculation of F_{ST} due to the use of highly variable neutral markers, such as microsatellites, whose mutation rate is higher than the genome on average (thus higher than in quantitative traits). This kind of marker leads to a higher within-population heterozygosity (H_e) that produces an F_{ST} value lower than for low-mutation loci or traits, causing a bias in Q_{ST}-F_{ST} comparisons (Edelaar and Björklund, 2011). Because our study includes a wide range of climatic conditions, we could expect *a priori* a result of $Q_{ST} > F_{ST}$. However, if $Q_{ST} < F_{ST}$, an alternative and reasonable explanation for plant adaptation, especially for plants living under such contrasted climatic condition as *A. unedo*, is the existence of adaptive plasticity that would cause diverse genotypes to produce similar phenotypes in a common garden (Price et al., 2003; Kawecki and Ebert, 2004).

The study of the amount of genetic and epigenetic variability in ecologically important traits is crucial to predict the ability of a species to respond to environmental change. If ecologically important traits lack genetic variation, they cannot respond and adapt to future challenges. Previous work has used the Q_{ST}-F_{ST} approach to examine climatic effects on physiological traits in Mediterranean species (Ramírez-Valiente et al., 2009), and even some work has combined the Q_{ST}-F_{ST} approach with the study of

V_A (Navarro et al., 2005). In our study with *A. unedo* we sampled 19 populations from throughout its range. We used AFLP markers to obtain F_{ST} estimates of the pattern of population structure, and we measured the Q_{ST} for 12 traits related to plant physiology and biomass. In addition, we assessed the additive genetic variance (V_A), related with the evolvability, and the narrow sense heritability (h^2) in traits related to the performance of plants, such as water and light use efficiency, photosynthesis, and growth. All of these traits, which have been related to plant fitness (Arntz et al., 2000), are sensitive to environmental conditions that are susceptible to be modified by global change. Further, their evolutionary potential has been studied in some reviews (Geber and Griffen, 2003).

Our objectives are:

1. To determine the degree of population differentiation in neutral genetic markers and quantitative traits through the Q_{ST}-F_{ST} approach. With these results, we will infer the role of natural selection and local adaptation in the population genetic structure of the species.

2. To estimate additive genetic variance, heritability and evolvability, in order to know the evolutionary potential of the studied traits and how this could affect the evolution of the species.

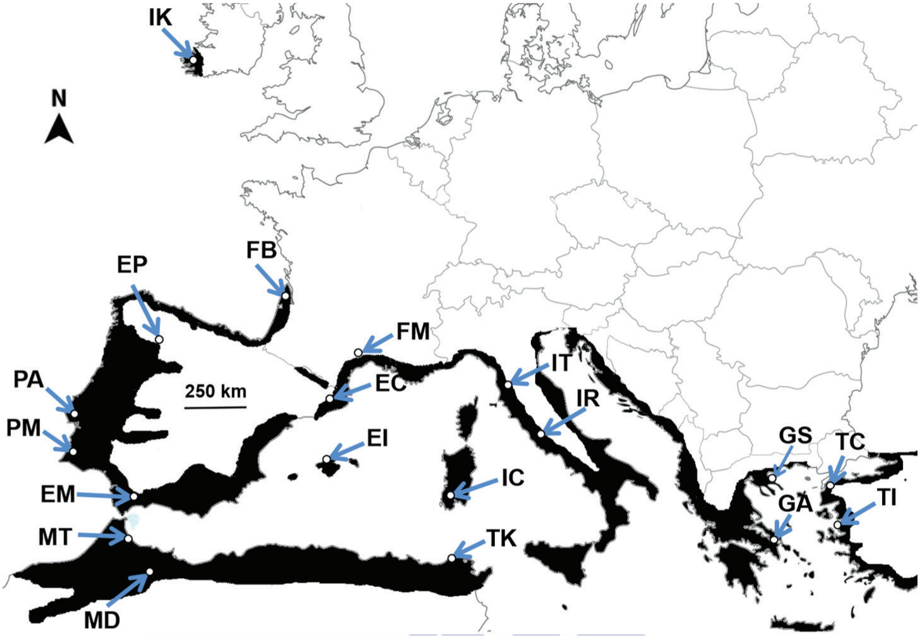
MATERIALS AND METHODS

Sample collection and experimental design

We took samples from 19 different populations from 9 countries over the entire distributional range of *A. unedo* L. from September to December 2010 (Fig. 1, Table 1). We collected fruits (~10) and leaves (~12) of at least 24 trees per population, with sampled trees separated by at least 15 meters to minimize the possibility of taking siblings. The leaves were dried in silica gel and stored until DNA extraction. Fruits were preserved at 4°C and processed to extract the seed just after collection.

Q_{ST} measurement requires plants with known familial relationship and grown in a common garden. To this aim, we extracted seeds

Figure 1. European map showing the distribution range of *Arbutus unedo* and the location of the 19 sampled populations.



from the fruits and kept them in a dry atmosphere. Then, we sowed the seeds in forestry trays with fifteen 1 litre cavities each, filled with Tref substrate (Tref Substrates BV, Moerdijk, The Netherlands), a 3:1 mixture of potting compost and perlite. We took 15 trees from each of the 19 populations and 30 seeds from each tree (family). Seeds were selected to obtain a similar mean weight to minimize maternal effects. In each cavity we put 5 seeds of the same family, so each family was represented in 6 different cavities, although no more than one seedling was maintained per cavity. However, due to the unexpectedly low germination rate, we did not obtain seedlings in all cavities. Both populations and families were sown randomly in the trays, and this random order was followed during the posterior measurements to avoid biases. We obtained a total of 740 seedlings, with 5 to 15 (mean = 11.6 ± 0.8 standard error) families per population and 1 to 6 (3.4 ± 0.1) offspring per family (details per population are given in Table 1). We kept the plants in an outdoor common garden in Santiago de Compostela (Spain) over an 18 month period from March 2011 until September 2012. All the plants were fertilised and watered

Table 1. Name and geographic position of the 19 populations of *Arbutus unedo,* the AFLP sample size, number of families (Nº Fam.) per population and mean number of offspring per family (± S.E.).

Population	Coordinates (UTM)	AFLP sample	Nº Fam.	Offspring per family (±S.E.)
Ireland Killarney (IK)	29U 465211 5763297	27	8	2.25 (0.62)
France Bordeaux (FB)	30T 643276 4939413	24	5	1.60 (0.40)
Spain Ponferrada (EP)	29T 705440 4722721	27	11	3.00 (0.36)
Portugal Arrabida (PA)	29S 497741 4257559	24	14	2.64 (0.37)
Portugal Monchique (PM)	29S 538873 4126983	24	14	2.93 (0.35)
Spain Malaga (EM)	30S 376766 4076446	26	14	3.07 (0.51)
Morocco Tanger (MT)	30S 267001 3940810	21	10	2.10 (0.46)
Morocco Debdou (MD)	30S 492658 3759875	23	15	4.47 (0.42)
Spain Cataluña (EC)	31T 454080 4620578	24	14	3.93 (0.44)
France Montpellier (FM)	31T 531757 4829416	26	13	4.15 (0.49)
Spain Mallorca Island (EI)	31S 461243 4392754	25	10	3.70 (0.50)
Italy Cagliari (IC)	32S 491121 4333556	24	14	4.93 (0.32)
Tunisia Kroumerie (TK)	32S 470929 4061547	21	15	4.27 (0.34)
Italy Rome (IR)	33T 279753 4619932	26	10	3.70 (0.58)
Italy Toscana (IT)	32T 637192 4811775	21	15	3.87 (0.38)
Greece Athens (GA)	34S 746586 4206313	24	5	1.60 (0.24)
Greece Sithonia (GS)	34T 739962 4452294	20	7	3.00 (0.76)
Turkey Cannakale (TC)	35T 459279 4441142	23	11	1.82 (0.30)
Turkey Izmir (TI)	35S 458841 4254352	24	15	3.27 (0.42)

as necessary to avoid stress and minimize maternal effects. Each individual was then measured for all the traits described below.

Genetic markers

F_{ST} was calculated based on AFLP genetic markers. DNA was extracted from the sampled leaves from the wild populations using the REAL "Realpure Genomic DNA extraction from plants and fungi" kit. We prepared the AFLP using nine selective primer combinations, (EcoRI/TruI): TGG/CAA TCA/CAT TAG/CAT TCA/CTG TAC/CTG TAG/CTT TGC/CAC TAC/CAA TAG/CTG. AFLP analyses were performed based on Vos et al. (1995) with the following specifications. Approximately 250 ng of genomic DNA were digested at 37° C for 3 h in a final volume of 20 µl with 1.25 units of EcoRI and TruI (Fermentas) and 2× Tango Buffer (Fermentas). Digested DNA was ligated for 3 h at 37° C to double-stranded adapters (50 pmols of adaptors E, 5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'; and M, 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') using 0.5 units of T4 DNA ligase (Fermentas). Then, 2 µl of the ligation product was pre-amplified with 0.3 µM of each single selective primer (EcoRI-T and TruI-C), 2.5 mM $MgCl_2$, PCR buffer 1X (Applied Biosystems), 0.8 µM dNTPs, 0.04 µg/µl BSA, Betaine 1 M and 0.4 U of Taq polymerase (Applied Biosystems) in a final volume of 20 µl. Amplification conditions were 2 min at 72 °C; 2 min at 94 °C; 20 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension of 30 min at 60 °C. Pre-amplification fragments were diluted 1:5 with Milli-Q water; 2.5 µl of the resulting solution were selectively amplified using 0.6 µM of the selective primers (listed above), 0.8 µM dNTPS, 2.5 mM $MgCl_2$, 0.04 µg/µl BSA, PCR Buffer 1× (Applied Biosystems) and 0.4 U of AmpliTaq Gold polymerase (Applied Biosystems) in a final volume of 10 µl. Selective amplification was performed as follows: 4 min at 95 °C; 12 of cycles of 30 s at 94 °C, 30 s at 65 °C (first cycle, then decreasing 0.7 °C for each one of the last 11 cycles), and 2 min at 72 °C; 29 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension of 30 min at 72 °C. Digestion, ligation, and PCR reactions were performed in a PxE thermal cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). Selective amplification products were electrophoresed on an ABI 3130xl automated DNA (Applied Biosystems) sequencer with HD-500 as size standard (Applied Biosystems) and the obtained fragments were manually scored at each locus with GeneMarker v.1.70 (SoftGenetics LLC, State College, PA, USA). Individuals from each of the 19 sites were evenly partitioned among the 96-well plates used for PCR. Further, we also ran replicates, 10% evenly distributed among the 19 sampling sites. Replicates and originals were extracted independently and always run on separate plates to avoid potential biases. In addition, each batch of 24 samples included a negative control that went through the entire genotyping procedure (including DNA extraction). The estimated global genotyping error was 1.5%, and the maximum error rate for individual loci (5%) was well below the maximum recommendable for AFLP analyses (10%) (Bonin et al. 2007).

This resulted in 491 samples (at least 20 per population) with 125 polymorphic markers used to calculate F_{ST}. Here we report the F_{ST} over all populations as the basis of neutral comparison for the Q_{ST} calculations (below).

Phenotypic traits

On each individual in the breeding experiment, 12 phenotypic traits were measured, as follows:

Leaf spectral reflectance (CHL, NVDI, WI, PRI)

These measurements were made on the adaxial side of one leaf per plant using a portable spectrometer (Unispec, PP Systems, Haverhill, MA, USA), which registers the leaf reflectance spectrum. All of these traits, related with the foliar physiology, are main determinants of the growth and survival of the plants (Peñuelas and Filella, 1998; Chaerle and Van der Straeten 2000).

The chlorophyll index (CHL) was calculated as the R_{750}:R_{550} ratio (Lichtenthaler et al., 1996), where R_x represents the reflectance at x nm. R_{750} is relatively insensitive to variations in the chlorophyll content of leaves (Lichtenthaler et al., 1996), whereas R_{550} is sensitive to that variation. Consequently, CHL is positively correlated with the chlorophyll content of leaves.

The normalised difference vegetation index (NDVI) was calculated as $(R_{800}–R_{680}) / (R_{800}+R_{680})$ (Gamon et al., 1995). Reflectance at 680 nm is sensitive to variations in chlorophyll concentration, whereas reflectance at 800 nm is sensitive to leaf water content. NDVI has been related to the 'greenness' or general vigour of vegetation due to its correlation with leaf chlorophyll content, photosynthetic efficiency, foliar nitrogen, phosphorous and potassium content (Gamon et al., 1995).

The water index (WI) was computed as the R_{900}:R_{970} ratio (Peñuelas et al., 1997). The reflectance at 970 nm indicates variations in leaf water content, and the reflectance at 900 nm is used as a reference. WI is correlated with the plant water content

(Peñuelas et al., 1997), a crucial element for leaf expansion, photosynthesis and growth.

The photochemical reflectance index (PRI), was calculated as $(R_{531} - R_{570})/(R_{570}+R_{531})$ (Peñuelas et al., 1995). R_{531} is sensitive to changes in the epoxidation state (EPS) of the xanthophyll cycle, transthylakoid pH gradient formation and conformational changes in chloroplasts, whereas R_{570} is used as a reference. PRI is inversely correlated with the dissipation of excess radiation energy as heat (Peñuelas et al., 1995); being indicative of a photoprotective mechanism that dissipates excess of excitation energy.

Chlorophyll fluorescence

The effective quantum yield of Photosystem II (PSII) (ΦPSII) was measured on the adaxial side of one leaf per plant, with a portable pulse amplitude modulated fluorometer (Mini-PAM; Heinz Walz, GmbH, Effeltrich, Germany). ΦPSII was calculated as $(F_m' - F_t)/F_m'$, where F_m' is the maximum fluorescence emitted by an illuminated leaf after a saturating pulse and F_t is the basal fluorescence emission at a given photosynthetic photon flux density (Genty et al., 1989). This trait provides information about the proportion of the light absorbed by the chlorophyll used in photochemistry, (the radiation use efficiency, RUE), a main determinant of the net primary productivity (Genty et al., 1989).

Photosynthesis

Photosynthesis rate (µmol CO_2 m^{-2} leaf area s^{-1}) was measured in one leaf per plant by gas exchange, using a portable carbon dioxide analyzer (LI-6400XT; Li-Cor, Lincoln, NE, USA), at a leaf temperature of 26.3º C (±3.4) and the chamber photosynthetically active radiation fixed at 500 µmol m^{-2} s^{-1}. This measurement gives some relevant information on physiology of the species that add to the information provided by the other physiological traits, and it has been related with fecundity and survivorship (Arntz et al., 2000).

Biomass

At harvest, after 18 months growing in the common garden, plants were separated into roots, stems, and leaves. We washed the roots, and thereafter all plant material was oven dried to constant weight at 40° C. Each part was weighed separately to the nearest 0.0001 g (Mettler AJ100, Switzerland). Root, Shoot (stems biomass + leaf biomass), and Root/Shoot biomass were used in the analyses.

The root biomass performs a range of functions, including structural support, absorption and storage. Shoot biomass also functions in support, transport, storage, photosynthesis, and reproduction (Mokany et al., 2006). Therefore, their variation is clearly linked to environmental variation (higher root biomass in water stress conditions, higher shoot biomass in shade conditions). The root: shoot ratio is related to precipitation, temperature, forest stand age, and forest stand height (Mokany et al., 2006).

To calculate the specific leaf area (SLA) (cm^2 leaf area/ mg leaf dry mass), we used two leaves per tree and weighed them individually. To determine the area, we obtained their projected image with a scanner (CanoScan LiDE 50; Canon, Tokyo), which is processed with an analysis program (ImageJ software; National Institute of Health). SLA has been associated with the photosynthetic capacity and tolerance to drought (Sánchez-Vilas and Retuerto, 2007).

Stomatal density

Stomatal Density ($number/mm^2$) was obtained from leaf imprints, applying a thin layer of clear nail varnish on the abaxial side (there are no stomata on the adaxial side in *Arbutus unedo*). After the imprints dried, they were removed from the leaves and placed on microscope slides. We took 2 pictures (Olympus SC30 camera coupled to an Olympus CX41 microscope) of 300x300 µm, where we manually counted the number of stomata. Stomatal density by its contribution to stomatal conductance regulates CO2 diffusion during photosynthesis, transpiration, water use efficiency (WUE), and consequently growth (Hetherington and Woodward, 2003; Wang et al., 2007).

$\delta^{13}C$ isotopic composition

The $^{13}C/^{12}C$ composition ($\delta^{13}C$) was measured in dried leaf tissue, by grinding each sample with a ball mill (Retsch Mixer Mill MM 400) to a homogenized fine powder. An isotope ratio mass spectrometer (Deltaplus, Finnigan MAT, San Jose, CA, USA) was used to determined $\delta^{13}C$ value. The $\delta^{13}C$ value is related to the WUE and responds to variations in light, water supply (Retuerto et al., 2000), and photosynthetic rate (Farquhar et al., 1989). Therefore, the $\delta^{13}C$ is an interesting trait for its integrative value.

Statistical analysis of variance components, Q_{ST} and F_{ST}

F_{ST} was calculated following the procedures of Lynch and Milligan (1994), as implemented in the R package QstFstComp (Gilbert and Whitlock, 2014) using R v3.1.0. (R Core Team 2013). Input values of and variances were obtained from AFLP-SURV (Vekemans et al., 2002).

Quantitative genetic parameters were estimated from the breeding design, assuming that the offspring of each dam were sired by different pollen parents (in other words, assuming that the offspring of each dam were half-siblings). A nested ANOVA approach, accounting for the unbalanced data available after mortality, was used to estimate the genetic variance among populations and the additive genetic variance within populations for each trait. Additive genetic variance (V_A) was estimated by four times the variance component among dams, and the phenotypic variance (V_P) was estimated by the residual variance plus the dam variance (Lynch and Walsh, 1998). Narrow sense heritability (h^2) was calculated as the ratio of V_A / V_P.

These calculations have been made assuming that each offspring of a dam has a different sire. If multiple ovules from the same plant are effectively pollinated by the same pollen donor, then the relatedness of the offspring from the same dam would be greater than is assumed here, and the estimates of additive genetic variance would be upwardly biased. In the extreme case where all offspring of a given plant are full siblings, the estimates of V_A given here would be double their true values. We also perform the analysis under this less probable assumption.

In order to compare qualitatively the genetic variability of different traits, it is necessary to use a standardized scale. We use CV_A and I_A, which are measures of genetic variation standardized by the mean. Hansen and Houle (2008) have argued that such mean-standardization is preferable to variance standardization for appropriate comparisons. The coefficient of additive genetic variance was calculated by (Houle, 1992) for traits that had values consistently on one side of zero. The distributions for both NDVI and PRI straddled both sides of zero, and therefore the CV_A is not informative for these traits. $\delta^{13}C$ is consistently negative, so the CV_A for this trait was calculated for $-\delta^{13}C$. $I_{A,}$ the mean-scaled additive variance (Houle, 1992) was calculated as: $I_A = V_A / (\text{mean})^2$, whereas $I_A * 100$ can be interpreted as the expected proportional change under a unit strength of selection (Hansen et al., 2003; Hansen et al., 2011) and is recommended as a measure of evolvability (Hansen et al., 2011).

Confidence intervals for the genetic parameters were generated using a parametric bootstrap approach (O'Hara and Merilä, 2005). In brief, mean squares from the ANOVA for each trait were parametrically resampled assuming normality of variance components, using the rchisq function in R. Both V_A and Q_{ST} were estimated from the bootstrap replicate mean squares following the same formulae used to calculate the point estimates from the original data. For each estimate, we used 1000 bootstrap replicates, and the confidence intervals for V_A and Q_{ST} were estimated from the 2.5% and 97.5% quantiles of the distributions of bootstrap estimates. Bootstrap confidence intervals for CV_A were generated in a similar way, with each bootstrap replicate estimate of divided by the observed mean (not the bootstrap mean) of that trait (R code for these calculations is implemented in the package QstFstComp, available at https://github.com/kjgilbert/QstFstComp. See Gilbert and Whitlock (2014) for more details.

Hypothesis testing for the difference between Q_{ST} and F_{ST} was done following the method by Whitlock and Guillaume (2009) who assumed that additive genetic variance was estimated by a half-sib model with several dams per sire with a balanced design. Gilbert and Whitlock (2014) modified this method to account for the plant model used here and for the unbalanced design using mean squares based on the ANOVA model described above. In brief, this method simulates the sampling distribution for Q_{ST} assuming the null hypothesis ($Q_{ST}= F_{ST}$) as true, and resamples mean F_{ST} and Q_{ST} for each trait. The rejection region for the null hypothesis $Q_{ST} = F_{ST}$ is calculated from the bootstrapped distribution of this difference (Whitlock and Guillaume, 2009). We report 2-sided *P*-values.

RESULTS

Genetic variance within populations

All the measured traits, with the exception of ΦPSII, have demonstrable additive genetic variance (entire confidence interval above zero) and CV_As (Table 2). For these 11 traits, V_A was significantly greater than zero (Figure 2), showing that

Table 2. Mean value, phenotypic variance (V_P) and coefficient of phenotypic variance (CV_P) ($CV_P= \sqrt{V_P}$ / mean) of each studied trait (^{P} indicating whether population has a significant effect), estimates of additive variance (V_A) with their 95% CI and coefficients of additive variance (CV_A) ($CV_A= \sqrt{(V_A)}$/mean), percentage mean-scaled additive variance (I_A), and narrow sense heritability (h^2). CV_P, CV_A and I_A are not interpretable for NDVI and PRI, because these traits have distributions of their measured values that span zero, and so they are not reported here for these two traits.

Trait	Mean	V_P	CV_P	V_A	95% CI for V_A	CV_A	I_A	h^2
CHL	2.000^P	0.111	16.6	0.062	(0.025,0.100)	12.5	1.55	0.560
NDVI	0.577	0.010	-	0.01	(0.006,0.014)	-	-	0.961
WI	1.050	0.004	5.7	0.0023	(0.001,0.004)	4.6	0.21	0.641
PRI	0.020	0.001	-	0.00071	(0.0004,0.001)	-	-	0.800
ΦPSII	0.503	0.016	25.5	0.0035	(0,0.008)	11.8	1.39	0.212
Photosyntesis	6.902	8.657	42.6	5.04	(2.31,8.10)	32.5	10.58	0.582
Root Biomass	3.008	6.322	83.6	2.56	(0.58,4.69)	53.2	28.29	0.405
Shoot Biomass	4.527	18.932	96.1	6.23	(0.60,11.87)	55.1	30.40	0.329
Root/ Shoot	0.727	0.042	28.3	0.023	(0.009,0.038)	20.4	4.16	0.519
SLA	6.228	3.378	29.5	1.13	(0.10,2.19)	17.1	2.91	0.335
Stomatal Density	270.201^P	3583.933	22.2	2168	(949,3464)	17.2	2.97	0.605
$\delta^{13}C$	-30.547^P	0.645	2.6	0.458	(0.245,0.680)	2.2	0.05	0.710

Figure 2. Coefficients of additive genetic variation (CV_A) with 95% confidence interval (CI) for most traits. CV_A is not interpretable for NDVI of PRI, because these traits have distributions of their measured values that span zero, and so CV_A for these two traits is not reported here. For the remaining ten traits, all but one (ΦPSII) show significant values (entire CI above zero) of CV_A within these populations.

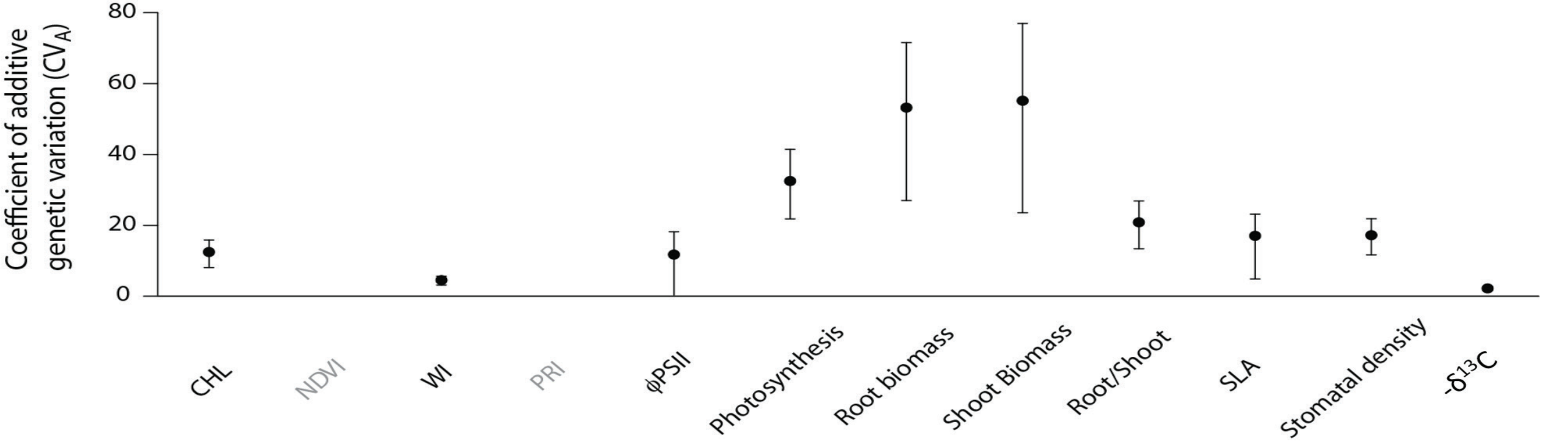


Figure 3. $Q_{ST} - F_{ST}$ for each trait, shown with the regions (in grey) consistent with the null hypothesis that $Q_{ST} = F_{ST}$. All traits showed $Q_{ST} - F_{ST} < 0$; for six traits (shown with black solid dots) Q_{ST} was significantly smaller than F_{ST}. Traits for which Q_{ST} was not significantly different from F_{ST} are shown with white circles.

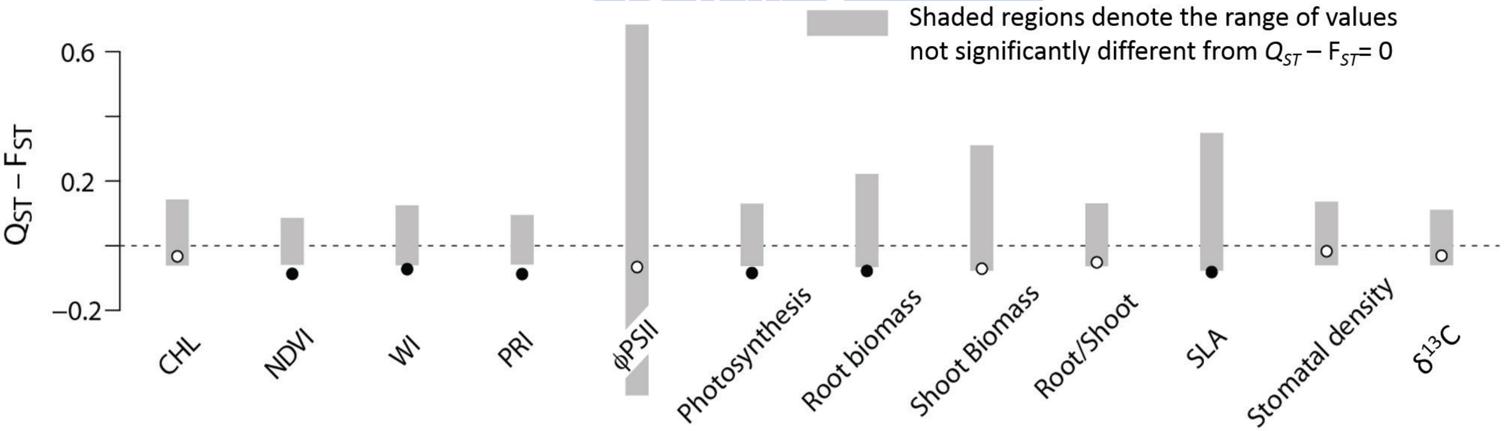


Table 3. Estimates of Q_{ST} of each trait and the P-value (the significant ones in bold) for the null hypothesis $Q_{ST} - F_{ST} = 0$, assuming that all offspring of a dam are half-siblings (indicated HS) and also assuming that they are full-siblings (indicated FS). For the HS assumption we also indicate the 95% CI for Q_{ST} and the $Q_{ST} - F_{ST}$ values. ^{p} means a significant effect of the population in the mean value.

Trait	Q_{ST} HS	95% CI for Q_{ST} HS	$Q_{ST} - F_{ST}$ HS	*P*-value HS	Q_{ST} FS	*P*-value FS
CHL	0.05^{p}	(0.008,0.14)	-0.033	0.39	0.095	0.83
NDVI	-0.0042	(-0.014,0.012)	-0.087	**<0.001**	-0.008	**0.004**
WI	0.01	(-0.010,0.050)	-0.073	**0.016**	0.021	0.15
PRI	-0.005	(-0.017,0.013)	-0.088	**<0.001**	-0.0096	**0.012**
ΦPSII	0.017	(-0.14,0.27)	-0.066	0.27	0.033	0.67
Photos	-0.0015	(-0.019,0.030)	-0.0846	**0.004**	-0.003	**0.02**
Root Biomass	0.0048	(-0.022,0.062)	-0.0783	**0.016**	0.010	0.11
Shoot Biomass	0.012	(-0.034,0.113)	-0.0716	0.082	0.023	0.32
Root/ Shoot	0.031	(-0.004,0.098)	-0.053	0.176	0.061	0.74
SLA	0.0015	(-0.037,0.088)	-0.082	**0.044**	0.0029	0.12
Stomatal Density	0.066^{p}	(0.014,0.177)	-0.017	0.74	0.124	0.47
$\delta^{13}C$	0.052^{p}	(0.009,0.136)	-0.03	0.40	0.10	0.71

these important traits have the potential to evolve in response to environmental change. The range of variation of CV_A was wide, from 2.2% ($\delta^{13}C$) to 55.5% (Shoot Biomass). For evolvability (I_A), the variation was from 0.05% ($\delta^{13}C$) to 30.40% (Shoot Biomass). The results for h^2 show a general high value for all the traits, varying from 0.212 (ΦPSII) to 0.961 (NVDI), as could be expected in a common garden experiment (Houle, 1992).

Genetic differentiation among populations

We observed that the mean F_{ST} value (0.083) was always higher than the Q_{ST} value for all the traits, leading to negative values for $Q_{ST} - F_{ST}$ (Table 3). It is noteworthy that some Q_{ST} values are negative (NDVI, PRI, Photosynthesis), which is consequence from the statistic calculus when the Q_{ST} is close to 0. These negative value does not have biological meaning (must be considered like zero), but we kept them because if we shift them to zero, any meta-analysis that compares these values with other values obtained by the same method would be biased.

The test of the null hypothesis $Q_{ST} - F_{ST} = 0$, produces *P*-values which vary among traits (Table 3, Figure 3). The traits with Q_{ST} significantly lower than F_{ST} (*P*-values below 0.05, when we reject the H_0, $Q_{ST} - F_{ST} = 0$) were PRI, NVDI, WI, Photosynthesis, SLA and Root Biomass. Therefore, for these traits, the populations are more homogeneous than expected by a neutral model of drift and migration without selection.

In the analysis in the previous paragraph, we assumed that each pollen source is independent, i.e. that the offspring of a given mother are half-sibs. Since we have no information on the pollen sources for seeds of the same plant, in order to be conservative in the conclusion that Q_{ST} was often significantly lower than F_{ST}, we also calculated Q_{ST} and related quantities under the less realistic assumption that all offspring of a given mother are full-siblings (i.e. that they all share the same pollen parent). These results are also shown in Table 3. Three of the traits, NDVI, PRI, and Photosynthesis, show significantly lower Q_{ST} than F_{ST}, even under this very conservative assumption.

DISCUSSION

One of the major goals of evolutionary biology is to understand the mechanisms for the maintenance of genetic variation, and spatially heterogeneous selection may be a major mechanism for this process. The $Q_{ST}- F_{ST}$ approach is useful to investigate that process. In this study, we observed, under common garden conditions, that the Q_{ST} for ecologically important traits in *A. unedo* are always lower than F_{ST}, sometimes significantly, suggesting convergent selection across the range of the species for similar values in these traits. Compared with recent meta-analyses (Merilä and Crnokrak, 2001; Leinonen et al., 2008), our Q_{ST} values were lower than the vast majority of Q_{ST} reported in these reviews, both for life history and morphological traits, although physiological traits were underrepresented. Furthermore, in contrast to the general trend in these reviews, Q_{ST} for our measured traits was always lower than F_{ST}. According to previous knowledge, this result would imply stabilizing selection for these traits. However, some studies have reported Q_{ST} values significantly lower than F_{ST}

even in changing environments (Navarro et al., 2005; Yoshida et al., 2008; Lamy et al., 2011), and it is possible that low Q_{ST} values were underreported (Leinonen et al., 2008; Edelaar et al., 2011). In our situation, we should consider that the low Q_{ST} values could be due to the small sample size in some populations. However, reanalysis of data after removing FB and GA (populations with the poorest representation) provided very similar results. Further, we must consider that these results are obtained in the existing common garden conditions, which are within the environmental range for all the populations of the strawberry tree. However, it would be interesting to establish a new common garden with new conditions (with modifications on water regime or light conditions) to see whether Q_{ST} is modified in extreme conditions.

In situations of low Q_{ST}, adaptive plasticity may be seen as an evolutionary alternative to local adaptation (Price et al., 2003; Kawecki and Ebert, 2004). The traits measured in this study could have responded similarly to the environmental conditions in our common garden because of their plasticity. This plasticity also could have led to adaptive response across the environments that the populations experience, even without underlying genetic changes (Kawecki and Ebert, 2004). In this regard, it has been reported that individuals with plastic response can have higher fitness than those that do not, and the moderate levels of phenotypic plasticity favor the population survival in a new environment (Price et al., 2003). With migration between populations, plasticity may be favored over local specialization in a broad range of conditions (Sultan and Spencer, 2002). The preliminary results of one factorial experiment that we ran (work in preparation), where two equivalent sets of plants of the same populations showed marked phenotypic change when grown in contrasting environments, also point to the existence of plasticity. Thus, a combination of stabilizing selection and plasticity may explain the low Q_{ST} in *A. unedo*; the traits could have been selected around an optima, but with plasticity allowing phenotypic variation among different environments. This could explain the survival of the strawberry tree in the different climatic scenarios of its distribution range and the similar phenotypic responses in a common garden (low observed Q_{ST}).

Our results also support the existence of evolutionary potential for *A. unedo* in many traits. In fact, except ΦPSII, all the traits had significant CV_A. Along this line, it is especially interesting to compare our CV_A with those obtained in a review from Garcia-Gonzalez et al. (2012). They obtained the CV_A from 38 papers, and showed a mean value of 16.01 (±2.38). In our study, the physiological traits CHL, $\delta^{13}C$, WI and ΦPSII had lower CV_A than this mean, and we show these have low potential to evolve, especially for $\delta^{13}C$ and WI. These results are also observed with the I_A index. On the other hand, the photosynthesis, and, interestingly, the morpho-structural traits (root and shoot biomass, root: shoot ratio, SLA and stomatal density) showed higher evolutionary potential than the mentioned mean (16.01) and also higher I_A. There are also noticeably high CV_A values for Root Biomass and Shoot Biomass, especially taking into account the lower value for the Root:Shoot ratio. That means that the evolution of Root Biomass and Shoot Biomass is interdependent in a certain degree, producing lower evolutionary potential in their ratio. However, despite this interdependence, this Root:Shoot ratio still shows a CV_A higher than 16.01 (average of the Garcia-Gonzalez review) and, therefore, this ratio has potential to adapt to future environmental variation.

The evolutionary potential of these traits is especially relevant if we notice that they are related with key attributes for the survival of the species, such as the efficiency in the photosynthetic process (measured as photosynthesis), the regulation of the hydric status and response to water stress (SLA and stomatal density), and especially biomass production and the ability to vary allocation depending on the environmental conditions. Therefore, we observed that the species maintains the ability to evolve despite low genetic differentiation and stabilizing selection, and independent of any plasticity. Further, this evolutionary potential has been observed in traits related with changeable environmental conditions such as temperature and precipitation (IPCC 2013) and, consequently, can determine the survival of *A. unedo*.

It would be interesting to study other species to determine whether or not some relation exists between the nature of traits (physiological or morpho-structural) and their evolvability. Such research will be relevant because the traits that show higher evolvabilities will determine the evolutionary path followed.

Conclusions

Our results for Q_{ST}–F_{ST} and the analysis of V_A show genetic uniformity among populations and high evolvability within populations for several ecologically important physiological traits. These attributes will allow the adaptation and successful evolution of *Arbutus unedo* under changing climatic scenarios. These capabilities are especially relevant because we observe them mainly in traits which are related with the response to environmental conditions such as light, temperature and water. Because all these conditions are susceptible to variation in the context of global change during the next decades, these attributes of *A. unedo* may be the key to its survival.

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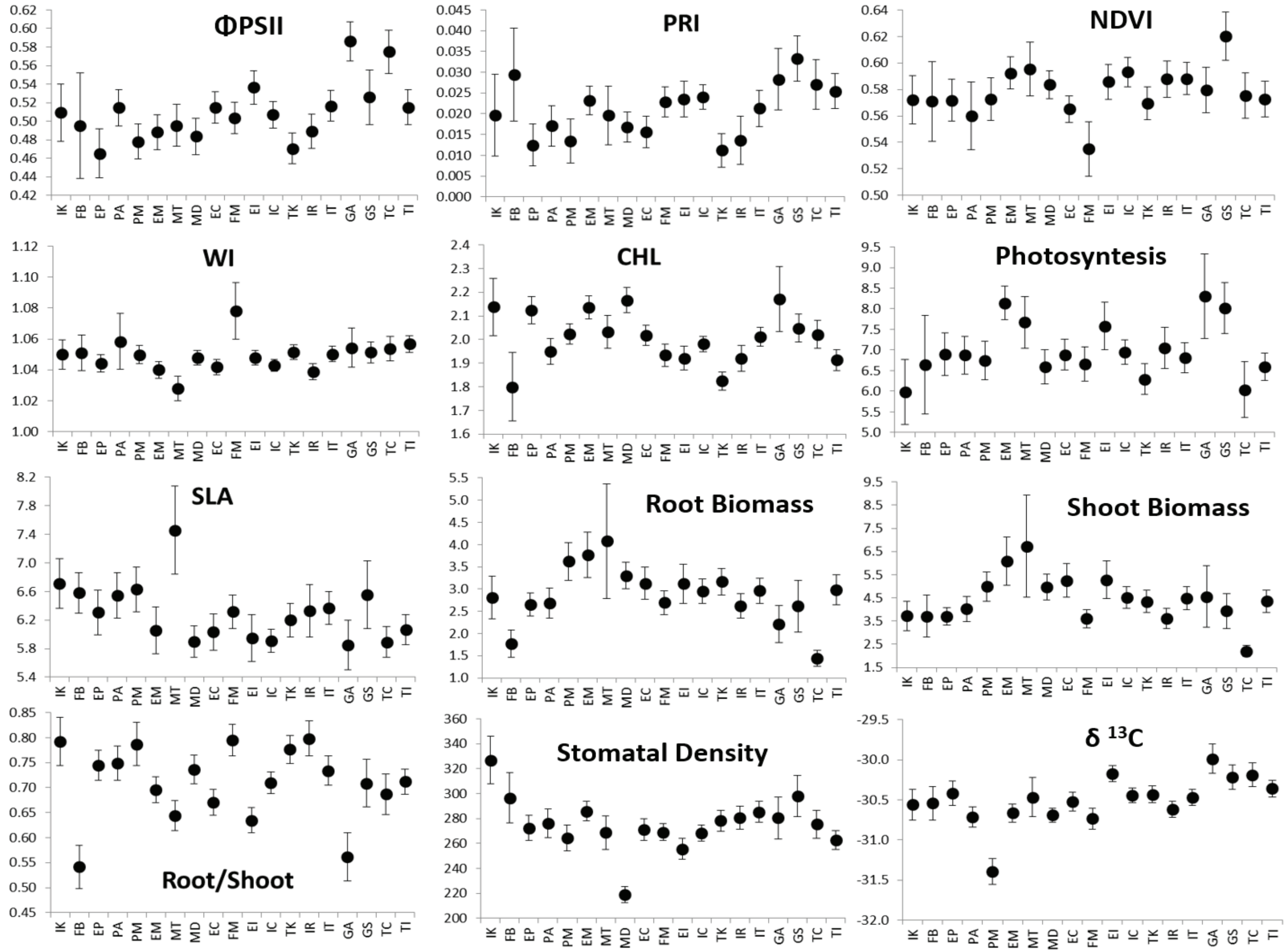
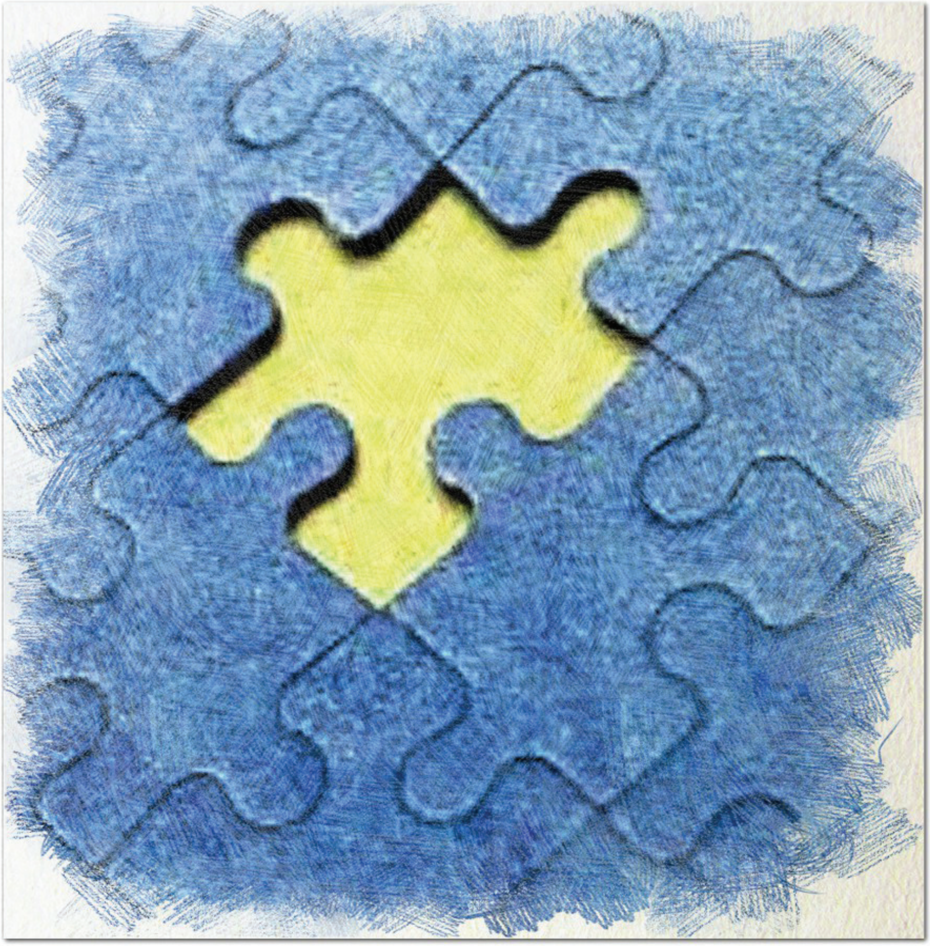
Supplementary Material.

Figure S1. Raw data per population of the 12 traits used in the study with *Arbutus unedo*. It is represented the mean value ± Standard Error.



General Conclusions

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General Conclusions

Throughout the five chapters of this thesis we analysed the strawberry tree from the point of view of molecular ecology and ecophysiology, as well as from the integration of both perspectives in the Q_{ST}-F_{ST} approach. Considering the results obtained we have reached the following conclusions (C.), which respond to the general objectives:

C. 1. The strawberry tree showed a partition in two chloroplastidial clades, one Atlantic and another Mediterranean, as result of possible vicariance events during the Quaternary glaciations of the last 700 ky but before the last glacial maximum (20 ky ago). North Africa-Atlantic Iberia seems the likely refugium for the Atlantic clade, whereas the European rim and/or large islands of Mediterranean would be the refugia for the Mediterranean clade. Both clades underwent a subsequent westward expansion, the Atlantic also moved northwards. *Conclusion based on results from chapter I.*

C. 2. The strawberry tree showed occasional connections between Europe and North Africa through the straits of Gibraltar and Sicily, but North Africa still retains a distinctive genetic composition. The species showed the East-West genetic divide found for other trees in the Iberian Peninsula, consistent with the refugia-within-refugia model. In comparison the Eastern Mediterranean showed lower genetic diversity and may have been more recently colonized, in contrasts with the pattern commonly seen in other plants. *Conclusion based on results from chapter I.*

C. 3. *Arbutus unedo* showed an isolation by distance pattern that is stronger along the Atlantic than around the Mediterranean basin, and could be interpreted as evidence of a stepping-stone colonization along the Atlantic façade. The latter would also explain the gradual decrease in within-population genetic diversity detected in NW Iberia and, specially, Ireland. *Conclusion based on results from chapter II.*

C. 4. The genetic structure derived from neutral genomic markers shows a clear divide of the strawberry tree in two groups, one Atlantic and another circum-Mediterranean. This separation fits the pattern observed in other widespread trees and could be attributed to the existence of separated and independent Last Glacial Maximum refugees. *Conclusion based on results from chapter II.*

C. 5. Our results discard the existence of a continuous range of *A. unedo* along the maritime fringe of France. Instead, the species possibly arrived to Ireland on post-glacial times from N Iberia. *Conclusion based on results from chapter I and II.*

C. 6. The response to low nutrient availability of *A. unedo* was more marked for structural traits (reduction and redistribution of biomass) than for physiological traits, indicating that the strategy is to optimize resource allocation but maintain the physiology stable. However, in response to water shortage, besides the high plasticity in structural traits that slows down the growth, we also observed notable plasticity in some physiological traits, which results in a high capacity to reduce water use and dissipate excess of radiation. We also conclude that it is not possible to generalize about the plasticity of traits of different nature as the level of plasticity depends on the environmental factors that induce variability. *Conclusion based on results from chapter III (nutrients) and IV (water).*

C. 7. We observed similar levels of plasticity for *Arbutus unedo* provenances in response to variations both in nutrients and water availability. Therefore, none of the provenances seems to be more able of colonizing or more susceptible to become extinct than others as a consequence of changes in the availability of these resources. *Conclusion based on results from chapter III (nutrients) and IV (water).*

C. 8. We observed that the supply of nutrients improved the performance of *A. unedo* under high radiation conditions, what could be particularly advantageous under the typical high irradiance of the Mediterranean summer, given the predicted increase in nutrient deposition. *Conclusion based on results from chapter III.*

C. 9. *Arbutus unedo* showed lower Q_{ST} than F_{ST} in many traits, which means that stabilizing selection may select for a norm of reaction that produces a similar phenotype even from diverse genotypes from different localities. *Conclusion based on results from chapter V.*

C. 10. The evolvability that we found in key morpho-structural traits predicts that *Arbutus unedo* retains genetic variation that will allow its genetic adaptation to future changeable environments. *Conclusion based on results from chapter V.*

Taking into account these conclusions, we can formulate some remarkable and general conclusions (G.C.):

G. C. 1. The strawberry tree possess a considerable ability to disperse, as it seems to have been able to migrate over thousands of kilometres and crossing stretches of sea as the Mediterranean straits. Surprisingly, this ability does not erase the genetic split between East and West Iberia. This ability to disperse could be useful in a scenario of future changes that creates favourable conditions for this species. *Conclusion based on C. 1, C. 2, C.4, C. 5.*

G. C. 2. The low variation between provenances of *A. unedo* in response to variations in the nutrients and water availability is consistent with the demonstrated stabilizing selection for the species, what could be partially explained by an event of canalization. The stabilizing selection and canalization reduce the variability in the responses, and, consequently, reduce the costs associated with changes. *Conclusion based on C. 6, C. 9.*

G. C. 3. *Arbutus unedo* has evolved a conservative strategy in the use of nutrients and water when they are scarce. This strategy based on a high degree of plasticity on key traits along with a relevant degree of evolvability. Both, plasticity and evolvability, explain the current persistence of the species throughout its distribution range and will be determinant to cope with future changes of the environmental conditions. Therefore, it seems likely that the strawberry tree will continue to play an important role in the Mediterranean plant communities. *Conclusion based on C. 7, C. 10.*

Resumo


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Introdución. A variación interespecífica

A variación interespecífica (dentro dunha mesma especie) é un elemento de variabilidade que non sempre foi considerado en Ecoloxía. De feito, moitos modelos de interacción entre especies teñen asumido que todos os individuos da mesma son intercambiables, isto é, ecoloxicamente equivalentes (Bolnick *et al.*, 2003; Bolnick *et al.*, 2011). Porén, esta simplificación é xeralmente inadmisible, pois demostrouse que a variación interespecífica pode afectar á dinámica dos ecosistemas en diversas circunstancias. Por exemplo, se os parámetros ecolóxicos non son funcións lineais das respostas individuais, se a varianza individual afecta á amplitude do nicho ecolóxico ou se os diferentes fenotipos amosan flutuacións non sincrónicas na súa densidade (Bolnick *et al.*, 2011). Deste xeito, tense amosado que a integración da variación interespecífica con técnicas tradicionais de cuantificación da variación pode dar un punto de vista excelente para comprender a estrutura das comunidades (Violle *et al.*, 2012). Nesta situación faise evidente a necesidade de estudar máis en profundidade a variación dentro das especies, que será o noso obxectivo para *Arbutus unedo* L. (érbedo), unha emblemática e amplamente distribuída especie do mediterráneo.

Introdución. A especie: *Arbutus unedo* L. (érbedo)

Arbutus unedo é unha especie que foi descrita en 1753 por Carl Linnaeus. O nome do xénero, *Arbutus*, alude á condición arbustiva, mentres que e o nome da especie, *unedo*, descomponse en dúas partes: un-, que fai referencia ao numeral un, e –edo, que significa comer. Así, pode traducirse como "comer só un", pois o contido alcohólico dos froitos pode producir dor de cabeza. O érbedo é un arbusto perenne que tipicamente non supera os 8 metros pero pode chegar a acadar os 12-14 metros cando compite pola luz (Sealy, 1949; Sealy e Webb, 1950). O tronco recóbrese por unha cortiza avermellada que se desprende en forma de tiras e as follas son coriáceas (tipo loureiro) e con bordo en forma de serra (Sealy e Webb, 1950). A floración dáse entre setembro e decembro e a fecundación é entomófila (Mitchell, 1993), sendo os polinizadores máis habituais as abellas e os abellóns.

O érbedo habita de forma primaria zonas de comunidades arbustivas, nas cales compite con éxito con outras plantas (Sealy, 1949). Esta especie ten como hábitat preferente as zonas costeiras, onde as xeadas e as secas non son moi intensas, aínda que pode medrar ata altitudes de 1200 metros sobre o nivel do mar (Torres *et al.*, 2002). En canto aos solos, prefire os silíceos, pero pode tolerar pH edáficos desde 4 a 7 (Sealy e Webb, 1950; Torres *et al.*, 2002; Takrouni e Boussaid, 2010). Finalmente, no tocante á temperatura, a súa distribución vese limitada pola isoterma de 40° F (4.4°C) de temperatura media en xaneiro (Sealy 1949). Deste xeito, o rango de distribución da especie é principalmente arredor do Mediterráneo, desde Marrocos a Turquía e desde Túnez ata España. Ademais, o érbedo esténdese pola costa Atlántica de Europa desde a Península Ibérica a Francia e Irlanda (Sealy e Webb, 1950; Webb, 1983).

No referente á reprodución, viuse que o érbedo non presenta propagación asexual, aínda que ten unha elevada capacidade para rebrotar e rexenerarse (Sealy, 1949; Sealy e Webb, 1950). En canto á reprodución sexual tense demostrado que a xerminación das sementes está fortemente influída pola temperatura, describíndose unha xerminación en torno a un 20% na temperatura óptima de 20°C (Hammami *et al.*, 2005). Emporiso, nós puidemos comprobar como esta taxa variou entre o 2% e o 43% cando se xerminou en turba, mentres que variou entre o 6% e o 86% cando se xerminou en placa Petri. Por outra banda observamos que o peso medio dos froitos variou entre 1.46 e 4.75 gramos, o das sementes entre 0.94 e 2.27 gramos, o número medio de sementes por froito entre 11 e 31 e a viabilidade das sementes entre o 56% e o 100%. Todos estes datos poñen de manifesto o elevado grao de variación interespecífica na especie e a necesidade de que sexa estudada.

Para esta tese doutoral recollemos mostras en 19 poboacións en 9 países ao longo de toda a súa distribución do érbedo: 1 en Irlanda, 2 en Francia, 3 na España peninsular e 1 nas Illas Baleares, 2 en Portugal, 2 en Marrocos, 2 na Italia peninsular e 1 en Cerdeña, 1 en Túnez, 2 en Grecia e 2 en Turquía. A partir das follas recollidas extraemos o ADN para as análises xenéticas, e partires das sementes obtidas dos froitos obtivemos as plantas coas que realizamos os experimentos de fisioloxía. Cabe destacar que para o experimento de filoxeografía conseguimos 4 poboacións máis (en Galicia, Cantabria e País Vasco) e que nos experimentos de auga e nutrientes empregamos 7 poboacións representativas de todo o rango de distribución.

Capítulo 1. Filoxeografía

A filoxeografía é unha disciplina descrita por Avise *et al.* (1987), que trata do estudo da distribución espacial das linaxes xenéticas que se dan, principalmente, dentro dunha especie ou de especies próximas. Un dos principios básicos desta rama da ciencia é que a reconstrución da historia evolutiva debe manter a referencia aos principios de concordancia entre múltiples liñas de evidencia científica (Avise, 2009). Isto é, as conclusións deben basearse en varias fontes independentes.

Polo tanto, tendo en conta que no noso estudo analizaremos a filoxeografía do érbedo no seu rango de distribución, principalmente Mediterráneo, debemos considerar os patróns previamente descritos nesta área. En primeiro lugar, hai que considerar que a conca mediterránea presenta unha escaseza de patróns comparada con outras zonas coma os Alpes ou os Andes (Nieto Feliner, 2014). Malia isto, é posible describir a existencia dalgunhas pautas comúns. A primeira delas é a que a estrutura xenética de moitas poboacións e especies actuais xurdiron durante as glaciacións do Cuaternario, cando moitos organismos sobreviviron en refuxios (Hewitt, 2000), tipicamente situados nas tres grandes penínsulas do Mediterráneo (Ibérica, Itálica e Balcáns) (Médail e Diadema, 2009), aínda que nalgúns casos os efectos sobre a estrutura xenética poidan ser anteriores ao Cuaternario (Petit *et al.*, 2005). Así mesmo, tense observado que dentro dun mesmo refuxio pode existir múltiples refuxios independentes (Gómez e Lunt, 2007). Outro patrón xeral que se observa no Mediterráneo é o incremento de diversidade xenética desde o Oeste cara ao Leste (Conord *et al.*, 2012). Tamén é moi común o estudo dos efectos que teñen os estreitos, como o de

Xibraltar ou o de Sicilia, na dispersión das especies, véndose que en ocasións son eficientes barreiras xeográficas, mentres que noutras son superados con facilidade (Fernández-Mazuecos e Vargas, 2011).

Ademais do interese que suscitan os patróns filoxeográficos no Mediterráneo, o érbedo tamén presenta especial relevancia por pertencer á denominada "flora Lusitánica", especies que habitan a Península Ibérica e Irlanda, pero ausentes en países intermedios como Inglaterra (Sealy, 1949; Sealy e Webb, 1950; Webb, 1983). Isto deu lugar a un debate acerca de cando (antes ou despois da época glacial) e como (dispersión natural ou introdución) chegaron estas especies a Irlanda e cal podería ser a súa orixe. Actualmente a posible colonización pre-glacial descártase no caso do érbedo debido a que foi datado na illa hai 4000 anos e non antes (Mitchell, 1993; Van Rijn, 2004), e a hipótese que se manexa é que chegou a través da costa Atlántica de Francia despois de ter rematada a última glaciación, fai 10.000 anos (Cox e Moore, 2005).

Neste estudo filoxeográfico co *A. unedo*, no cal empregamos catro xenes do cloroplasto, plantexamos os seguintes obxectivos:

1. Coñecer as rutas de colonización do érbedo ao longo do Mediterráneo e da costa Atlántica, con especial interese en descubrir o tempo no que se produciu a diverxencia da especie (tempo ata o antecesor común máis recente).

2. Determinar a existencia de concordancias entre os patróns observados no érbedo e aqueles que se dan noutras especies do Mediterráneo (patróns de diversidade, localización de refuxios glaciares, análise do papel dos estreitos).

3. Determinar cal é a orixe máis probable da poboación irlandesa do érbedo.

Ao respecto destes obxectivos, chegamos ás seguintes conclusións:

1. O érbedo, segundo a información obtida co cloroplasto, divídese en dous grupos xenéticos, un principalmente Atlántico e outro Mediterráneo, que se separaron nalgún momento durante os últimos 700 mil anos, pero antes do último máximo glacial

(hai 20 mil anos). O grupo Atlántico tivo o seu refuxio na zona do Norte de Africa-Península Ibérica, mentres que o do grupo Mediterráneo se localizou no Oeste do Mediterráneo. Ambos grupos expandíronse posteriormente cara ao Leste, e o grupo Atlántico tamén cara ao Norte.

2. Viuse que o érbedo amosou unha conexión ocasional entre Europa e o Norte de África a través dos estreitos de Xibraltar e de Sicilia, dando lugar a que o Norte de África manteña unha composición xenética particular. Así mesmo observamos que dentro da Península Ibérica existe unha división xénica entre ambas costas, o cal constitúe un exemplo de refuxio dentro de refuxio. Por último, o Leste do Mediterráneo, colonizado máis recentemente, amosa menos diversidade que o Oeste, o cal contrasta co patrón máis común visto noutras plantas.

3. Os nosos resultados apuntan a que a orixe da poboación irlandesa é a Península Ibérica e non a costa atlántica de Francia, como xa se suxeriu.

Capítulo 2. Análise da diversidade xenética

A diversidade xenética existente nunha poboación é un factor determinante para a capacidade adaptativa da especie e a súa supervivencia (Frankham *et al.*, 2002), e incluso pode ter efectos a nivel de poboación, comunidade e ecosistema (Hughes *et al.*, 2008). Un elemento clave na determinación da diversidade dunha especie é a distribución espacial de mesma. Deste xeito, un dos patróns espaciais xenéticos máis descritos é a correlación positiva que existe entre a distancia xeográfica e a xenética, trátase do chamado illamento por distancia (Nybom, 2004). Como consecuencia do mesmo prodúcese un aumento na diferenciación entre poboacións, sendo polo tanto unha das forzas primarias para a especiación. A existencia deste fenómeno, xunto coa fragmentación das poboacións, pode producir depresión por endogamia (especialmente en especies exogámicas) que induciría a perda de diversidade xenética e incrementaría o risco de extinción (Frankham, 2005). Outro dos efectos derivados da distribución espacial é o chamado efecto centro-periferia, segundo o cal as poboacións dos extremos posúen menor tamaño efectivo e maiores efectos da deriva xenética, co cal tenden a empobrecerse, aínda que non sempre sucede (Vucetich e Waite, 2003).

En canto ao érbedo, cómpre resaltar que a súa estrutura xenética foi escasamente estudada ata agora. Poden mencionarse algunhos traballos feitos en Portugal (Lopes *et al.*, 2012; Gomes *et al.*, 2013) e en Túnez (Takrouni e Boussaid, 2010; Takrouni *et al.*, 2012), pero ademais de incluír poboacións dun único país, os seus resultados son inconsistentes, xa que a diversidade xenética foi determinada como baixa (Takrouni e Boussaid, 2010), moderada (Lopes *et al.*, 2012) e alta (Takrouni *et al.*, 2012) e a diferenciación entre poboacións como moderada (Takrouni e Boussaid, 2010) e baixa (Lopes *et al.*, 2012; Takrouni *et al.*, 2012). Polo tanto, queda patente a necesidade de levar a cabo un estudo da estrutura xenética da especie que cubra todo o seu rango de distribución. Isto é o que efectuamos neste capítulo, mediante o emprego dos marcadores moleculares AFLPs, cos seguintes obxectivos:

1. Analizar se a distribución do érbedo, cun carácter principalmente unidimensional (arredor do Mediterráneo e ao longo da costa Atlántica), conduciu á existencia dun patrón de illamento por distancia.

2. Determinar se as condicións climáticas contrastadas que existen ao longo do rango de distribución do érbedo puideron inducir algún tipo de restrición ao fluxo xénico e/ou diferenciación entre rexións.

3. Comprobar a condición xenética da poboación irlandesa en relación coas demais como evidencia para determinar a súa orixe.

Tras a análise dos patróns de diversidade e estrutura xenética obtidos para o érbedo, as respostas que atopamos a estas cuestións foron:

1. O érbedo amosa un patrón de illamento por distancia máis forte ao longo da costa Atlántica que arredor do Mediterráneo. Isto pode evidenciar unha colonización por etapas ao longo da costa Atlántica de Europa, situación que tamén explica o gradual descenso da diversidade xenética cuantificado no noroeste da

Península Ibérica e especialmente en Irlanda. Este empobrecemento detectado en Irlanda concorda co esperado en función da súa condición de poboación periférica.

2. A estrutura xenética da especie amosa unha clara división en dous grupos, un Atlántico e outro Mediterráneo, semellando este último máis antigo. Esta división concorda co advertido noutras árbores e podería ser o resultado da existencia de refuxios independentes durante a época glacial.

3. Os resultados apoian unha orixe ibérica da poboación irlandesa, que foi a etapa de colonización previa á chegada a Irlanda.

Capítulos 3 e 4. Respostas ecofisiolóxicas a variacións na dispoñibilidade de nutrientes (3) e auga (4)

O estudo das respostas ecofisiolóxicas a factores ambientais resulta fundamental no contexto actual no que se teñen predito cambios ambientais que modificarán as condicións actuais de temperatura, precipitación ou dispoñibilidade de nutrientes (IPCC, 2013). Todo isto pode inducir alteracións na fisioloxía e fenoloxía das plantas, así como no seu rango de distribución (Aerts *et al.*, 2006). Neste senso, as predicións amosan que a nosa área de estudo, a conca do Mediterráneo, será especialmente afectada por estes cambios ambientais, véndose reducida a precipitación e incrementadas as temperaturas e as secas (Giorgi e Lionello, 2008). Tendo isto en conta, decidimos analizar a resposta do érbedo a dous dos recursos ambientais con maiores previsións de cambio e de maior importancia para a supervivencia das plantas, os nutrientes e a auga.

Respecto dos nutrientes, tense demostrado que se trata dun elemento fundamental para incrementar a produtividade de practicamente todos os ecosistemas do mundo, manifestándose deste a relevancia ecolóxica da súa limitación (Lambers *et al.*, 2008). Prevese que os devanditos cambios ambientais teñan como consecuencia a alteración dos ciclos dos nutrientes. Así a redución das choivas incrementará as perdas por erosión, podendo causar importantes modificacións no funcionamento dos ecosistemas

(Matías *et al.*, 2011). Alén disto, os procesos edáficos poden verse afectados por cambios no uso do solo, deposición de nitróxeno atmosférico ou incremento na concentración de CO_2 (Sala *et al.*, 2000). Non obstante, cando falamos dos efectos dos nutrientes, debemos considerar que os solos son espazos heteroxéneos espacial e temporalmente, como ben demostraron Monokrousos *et al.* (2004) no Mediterráneo. Por outra banda, as diferenzas entre especies na capacidade para explotar estes nutrientes pode chegar a afectar á súa distribución (Lambers *et al.*, 2008). Isto tamén se pode aplicar a diferenzas entre poboacións dunha mesma especie, como pretendemos comprobar no érbedo. O único estudo feito ata o momento con esta especie (Castell e Terradas, 1994) analizou tan só unha poboación, corroborando que a presenza de nutrientes ten un efecto positivo nas súas follas.

En canto á auga, un dos recursos ambientais máis limitantes para o crecemento das plantas, verase especialmente afectado polo cambio global (IPCC 2013). Os efectos nocivos da seca son ben coñecidos (Chaves *et al.*, 2002), e tamén se ten demostrado que os recursos hídricos poden determinar o patrón de distribución das especies (Pigott e Pigott, 1993). En resposta á escaseza de auga, as plantas poden amosar cambios na fenoloxía, modificación do tamaño e profundidade das raíces ou na proporción raíz/ parte aérea, cambios na condutividade hidráulica ou acumulación de reservas (Chaves *et al.*, 2002; Chaves *et al.*, 2003). Ata o día de hoxe lévanse feito diversos estudos, cunha ou poucas poboacións, sobre a resposta do érbedo á restrición hídrica. Nestas condicións redúcese a súa capacidade fotosintética e de crecemento (Gratani e Ghia, 2002). Tamén se determinou que o ciclo das xantofilas é un mecanismo eficaz para a disipación do exceso de enerxía luminosa, o cal evita danos maiores nos orgánulos celulares (Munné-Bosch e Peñuelas, 2004). Así e todo, quedan por analizar as diferenzas entre as diversas poboacións.

Os obxectivos plantexados nestes dous traballos, nos que analizaremos a resposta plástica mediante o uso do índice de plasticidade fenotípica, foron:

1. Describir a resposta estrutural e fisiolóxica do érbedo cando se somete a variacións na dispoñibilidade de nutrientes e auga,

o cal se relaciona coa súa estratexia de resposta ante os cambios ambientais.

2. Determinar se as diversas poboacións de érbedo amosan diferenzas no nivel de plasticidade en resposta á dispoñibilidade de nutrientes e auga. Isto determinará se a distribución da especie resultará afectada por futuros cambios nos mesmos.

3. Estudar como os niveis de nutrientes poden afectar á resposta fotosintética do érbedo, posto que se sabe que a resposta á luz está ben influenciada pola dispoñibilidade de nutrientes (exclusivo do capítulo III).

Despois da análise das respostas observadas, chegamos ás seguintes conclusións:

1. A resposta á dispoñibilidade de nutrientes foi máis marcada no caso dos caracteres estruturais (redución e redistribución de biomasa) que nos fisiolóxicos, indicando que a estratexia é optimizar os recursos mantendo a fisioloxía estable. Con todo, en resposta á seca, ademais da plasticidade estrutural (redúcese o crecemento), existe unha notable plasticidade en caracteres fisiolóxicos relacionados cunha maior eficiencia no uso da auga. Así mesmo, obsérvase unha elevada capacidade para disipar o exceso de radiación. En consecuencia, os niveis de plasticidade dependen do factor ambiental que induce a variación.

2. A capacidade plástica en resposta a variacións na dispoñibilidade de nutrientes e auga é uniforme entre as poboacións do érbedo. Polo tanto, ningunha delas será especialmente hábil para colonizar ou susceptible de extinguirse como consecuencia de cambios nestes recursos.

3. Os nutrientes melloran a resposta do érbedo en condicións de elevada radiación, resultando de gran utilidade durante o típico verán mediterráneo, especialmente nun futuro escenario de incremento na deposición atmosférica.

Capítulo 5. Comparación Q_{ST}-F_{ST}.

Como sinalamos con anterioridade, os efectos previstos no contexto do cambio climático tornan en fundamentais os estudos que analizan a capacidade de resposta das plantas. Neste senso, resulta clave analizar o potencial adaptativo das mesmas. Unha das ferramentas máis poderosas que se desenvolveron ao respecto é a comparación da variación en caracteres cuantitativos, Q_{ST} (Spitze, 1993), coa variación xenética neutral, F_{ST} (Wright, 1951). Ambos valores varían entre 0 (sen diferenciación) e 1 (diferenciación máxima). Deste xeito, se o Q_{ST} é maior que o F_{ST} dedúcese que as poboacións sofren selección diverxente (adaptación local), os caracteres cuantitativos afastáronse máis que a variación xenética neutral, non sometida a selección. Se o Q_{ST} é menor que o F_{ST}, conclúese que existe selección converxente, pois a diferenciación entre poboacións é menor da esperada. No caso de que ambos valores sexan similares non hai evidencias de ningún tipo de selección (Whitlock, 2008).

No cálculo do Q_{ST} é fundamental o deseño dun xardín común para evitar que se confunda a plasticidade fenotípica coa adaptación local. Así mesmo, precísase un deseño que identifique proxenitores e descendentes, para así poder cuantificar correctamente a varianza xenética aditiva (aquela que é herdable), valor necesario nos cálculos do F_{ST} e Q_{ST} (Pujol *et al.*, 2008; Whitlock e Guillaume, 2009). Actualmente, o uso da comparación F_{ST}-Q_{ST} é moi común, polo cal poden extraerse certas tendencias xerais. Así, vese que o Q_{ST} é xeralmente maior que F_{ST}, indicando un papel predominante da adaptación local nas especies contemporáneas (Merilä e Crnokrak, 2001; Leinonen *et al.*, 2008). Cómpre sinalar que estes resultados poden non ser realistas, pois vese como moitos destes estudos poden estar preconcebidos en poboacións que se sabe que diverxen (Kort *et al.*, 2013). Ademais, aqueles traballos nos que se detecta un valor de Q_{ST} menor que o de F_{ST} poderían tender a non publicarse debido a que a interpretación biolóxica non é tan interesante (Lamy *et al.*, 2012).

Neste capítulo, co uso da comparación Q_{ST}-F_{ST}, plantexámonos os seguintes obxectivos:

1. Definir o rol da selección natural na determinación da estrutura xenética e historia evolutiva do érbedo.
2. Coñecer a varianza xenética aditiva e o potencial evolutivo

que existe no conxunto de caracteres estruturais e fisiolóxicos que foron analizados na especie.

Coa información obtida tras os análises podemos concluír que:

1. O Q_{ST} foi menor que o F_{ST} para moitos dos caracteres do érbedo, indicando isto que a selección estabilizante produciu que existan fenotipos similares nas diferentes localidades. A especie non se adapta xeneticamente ás condicións climáticas de cada localidade.

2. Existe un potencial evolutivo notable en caracteres morfolóxicos e estruturais do érbedo. Polo tanto, a especie posúe un grao de variación xenética que permitirá unha boa capacidade de adaptación aos diversos ambientes futuros.

Conclusión xerais

Considerando de forma conxunta as conclusións extraídas ao longo da tese, podemos dicir, con carácter xeral:

1. O érbedo posúe unha considerable habilidade para dispersarse, pois é capaz de migrar ao longo de milleiros de quilómetros. Tamén é quen de cruzar rexións mariñas coma os estreitos de Xibraltar e de Sicilia. Sorprendentemente, esta capacidade non logrou a eliminación da división xenética existente entre ambas costas da Península Ibérica.

2. Malia a diversa orixe dos érbedos, as respostas a modificacións na dispoñibilidade de nutrientes e auga presentan baixa variabilidade. En consecuencia, ningunha poboación se verá especialmente favorecida nin prexudicada nun escenario futuro de alteración destes recursos. Esta baixa variabilidade entre procedencias é coherente coa tamén demostrada selección estabilizante. Desta maneira, conséguese unha redución na variabilidade das respostas, minimizando os custos evolutivos inherentes aos cambios.

3. O érbedo desenvolveu unha estratexia xeral de diminución de gastos fisiolóxicos cando os recursos (nutrientes e auga) son escasos, pero amosando un alto grao de plasticidade en caracteres clave e mantendo un elevado potencial evolutivo. Conxuntamente, a plasticidade e o potencial evolutivo explican a persistencia da

especie ao longo do seu rango de distribución. Ambos serán determinantes para afrontar con garantías os futuros cambios ambientais. Polo tanto, semella que o érbedo pode chegar a xogar un papel importante nas futuras comunidades vexetais do Mediterráneo.

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Agradecementos


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Cantas veces temos oído iso de "non por ir de último é o menos importante". Dende logo, estou totalmente de acordo. Tanto que estiven a piques de comezar así este texto. Pero eu teño a sensación de que iso de repetir xera unha sensación como de rutina, como de que o dis porque toca dicilo. E, dende logo, non é este o caso. Porque si, todos sabemos que iso de agradecer é de boa educación, que nunca debemos esquecernos de facelo e que, por suposto, sería dunha prepotencia insólita que faltasen os agradecementos nun traballo de tal magnitude coma unha tese doutoral. Mais non quería que todo iso que se dá por feito fixera que estar escribindo aquí e agora se convertese nunha especie de trámite. Porque foron moitos os acontecementos, momentos e sentimentos que fixeron deste camiño unha aventura incrible e maxistral. Pero foron aínda máis os que fan profundamente inesquecible.

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