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Phylogeography and genetic variability of the *Arnica montana* chemotypes in NW Iberian Peninsula

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Abstract

The threatened European species *Arnica montana* L. is a plant much appreciated in the pharmaceutical industry, Galicia (North-Western Spain) being one of the main supply regions. Two chemotypes based on the content of sesquiterpene lactones have been found in this area: a chemotype dominated by helenalins, common in Central Europe, and another dominated by dihydrohelenalins, only found currently in Galicia. The aim of this work was to analyse the phylogenetic relationships and genetic diversity of *Arnica montana* in Galicia and their concordance with previous biochemical and environmental information. Two polymorphic chloroplast markers were sequenced in individuals from populations showing significant biochemical differences and situated in different environments (heathland, hay-meadow and peatland). Three haplotypes, different from those previously described in Europe, were found in Galicia and our results suggest that one of them could be ancestral inside the species. Significant population differentiation was detected in the studied area but genetic diversity within populations was low, only showing variability in heathland populations. This study is the first one describing the genetic diversity of *Arnica montana* in the extreme SW of Europe. The results suggested the presence of two different genetic groups (one of them ancestral) and were congruent with the two chemotypes described. Plants from heathlands displayed the highest variability because the two chemotypes were present in them. Future design of conservation and economical management plans for this threatened species should take into account this genetic variability and prioritize further genetic and chemical characterizations across the distribution range of the species.

Key words: Arnica montana, genetic variability, chloroplast DNA, sesquiterpene lactones, phylogeography, conservation.

Introduction

European arnica (*Arnica montana* L., Asteraceae) is a plant with traditional medicinal use. In spite of its current classification as “Least Concern” by the European Red List of vascular plants, the monitoring of population is recommended due its strong regional decline (IUCN, 2014). Major threats are pharmaceutical industry exploitation, habitat loss partly due to agriculture, abandonment of land, and reforestation (LUIJTEN *et al.*, 2000; IUCN, 2014). As a consequence, several European countries have included *A. montana* in national red lists or approved laws for protection of its wild resources. The Galician region (NW Spain) is one of the main suppliers of *A. montana* for the European pharmaceutical industries but, unfortunately, no legislation on its exploitation is currently applied. In recent decades a significant decline in the populations of arnica in Galicia has been documented (LANGE, 1998; ROMERO *et al.*, 2011).

Due to the morphological variability of *A. montana*, some subspecies and varieties have been described with-

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in its distribution area (TUTIN *et al.*, 1980). BOLOS y VAYREDA (1945) described the subspecies *A. montana* ssp. *atlantica* from individuals collected in the Southwest of France, North of Spain, and Portugal. Its presence has never been reported outside of this area. This author considered that smaller size, thinner floral stems, lanceolate leaves, and smaller flower heads with less bracts were the morphological characters differentiating this subspecies from *A. montana* ssp. *montana*. In addition, their habitat preferences would also be distinct, with *A. m. atlantica* growing between 0–400 m (maximum 1000 m) in areas of oceanic climate and *A. m. montana* growing between 1000–2800 m in areas under a more continental climate. The distribution map included in BOLOS y VAYREDA (1945) suggests that *A. m. atlantica* would be widespread in Galicia whereas *A. m. montana* would be present only in mountain ranges near the eastern limit of the region.

However, a recent report of biometrical data from 310 individuals of *A. montana* collected in 37 populations in Galicia (ROMERO *et al.*, 2011) questioned the validity of the subspecies proposed by BOLOS y VAYREDA (1945). None of the individuals fulfilled all the required characteristics of each subspecies and most characteristics varied continuously without clearly defining two groups of individuals. These results were in agreement with the morphological variability of *A. montana* observed by other authors in some areas of NW Iberia next to the Galician border (Asturias region: LAÍNZ, 1982; Castile-León region: NIETO FELINER, 1985; GONZÁLEZ DE PAZ 2012). Nevertheless, ROMERO *et al.* (2011) reported that *A. montana* plants in Galicia are smaller when growing in grazed or mowed meadows and peatlands, and larger when growing in mountain (>1200 m) heathlands. The data also showed that the altitude factor was not the cause of these differences and the authors proposed that they might be due to grazing or mowing pressures associated with the different habitats.

Nowadays *A. montana* is a plant with a high demand for the pharmaceutical industry, which is interested in the secondary metabolite extracts from the flowering heads. Sesquiterpene lactones (SL) are the main compounds responsible for the anti-inflammatory properties of *A. montana* extracts (WILLUHN, 2004). Helenalin esters (H) showed more active anti-inflammatory properties than dihydrohelenalin esters (DH) (KLAAS *et al.*, 2002) while DH are less allergenic than H (LASS *et al.*, 2008). Studies on the SL in extracts of flowers collected in different arnica populations of Galicia have revealed the existence of two different chemotypes in this area (PERRY *et al.*, 2009). The first one is characterized by the predominance of H, similar to the chemotype common in

Table 1. – Environmental, phytochemical and molecular information for each individual plant analysed in the study. Helenalin (H) and dihidrohelenalin (DH) contents and cpDNA haplotypes for each individual and haplotype diversity (h) and nucleotide diversity (π) for each sampling site are included.

Sampling site	Individual	LUGO Herbarium Voucher code	H (mg/g plant dry weight)	DH (mg/g plant dry weight)	Chemotype	rps16 intron	ycf4 cemA spacer	combined
Ponte Pedrido	PPED1	1406	1.76^{a}	8.30^{a}	Iberian	Hap1	Hap1	Hap1
505 m	PPED2	1407	1.22^{a}	15.73^{a}	Iberian	Hap1	Hap1	Hap1
Peatland	PPED3	1408	0.89^{a}	10.12^{a}	Iberian	Hap1	Hap1	Hap1
PPED	PPED4	1409	0.87^{a}	13.70^{a}	Iberian	Hap1	Hap1	Hap1
01° 55' 00'' W	PPED5	1410	0.68^{a}	10.26^{a}	Iberian	Hap1	Hap1	Hap1
43° 17' 10'' N	PPED6	1411	0.27^{a}	6.18^{a}	Iberian	Hap1	Hap1	Hap1
	PPED7	1412	0.46^{a}	6.96^{a}	Iberian	Hap1	Hap1	Hap1
	PPED8	1413	0.79^{a}	10.27^{a}	Iberian	Hap1	Hap1	Hap1
	PPED9	1414	1.00^{a}	11.80^{a}	Iberian	Hap1	NA	NA
	h					0.0000	0.0000	0.0000
	π					0.000000	0.000000	0.000000
Reibocha	REIB1	1416	0.42^{a}	10.08^{a}	Iberian	Hap1	Hap1	Hap1
479 m	REIB2	1417	1.06^{a}	12.73^{a}	Iberian	Hap1	Hap1	Hap1
Hay meadow	REIB3	1418	0.59^{a}	9.28^{a}	Iberian	Hap1	Hap1	Hap1
REIB	REIB4	1419	0.76^{a}	8.70^{a}	Iberian	Hap1	Hap1	Hap1
01° 48' 35'' W	REIB5	1420	0.90^{a}	9.61^{a}	Iberian	Hap1	Hap1	Hap1
43° 16' 55'' N	REIB6	1421	1.58^{a}	12.68^{a}	Iberian	Hap1	Hap1	Hap1
	REIB7	1422	1.27^{a}	9.50^{a}	Iberian	Hap1	Hap1	Hap1
	REIB8	1423	0.54^{a}	6.34^{a}	Iberian	Hap1	Hap1	Hap1
	REIB9	1424	1.19^{a}	7.83^{a}	Iberian	Hap1	NA	NA
	REIB10	1425	1.96^{a}	7.57^{a}	Iberian	Hap1	Hap1	Hap1
	h					0.0000	0.0000	0.0000
	π					0.000000	0.000000	0.000000
Alto do Couto	COUT1	1376	3.92^{a}	2.46^{a}	Central-European	Hap2	Hap2	Hap2
1330 m	COUT2	1377	10.84^{a}	3.96^{a}	Central-European	Hap2	Hap2	Hap2
Heathland	COUT3	1378	6.16^{a}	3.69^{a}	Central European	Hap2	Hap2	Hap2
COUT	COUT4	1379	10.23^{a}	1.63^{a}	Central-European	Hap1	Hap1	Hap1
01° 05' 37'' W	COUT5	1380	3.38^{a}	5.99^{a}	Iberian	Hap2	Hap2	Hap2
42° 36' 54'' N	COUT6	1381	8.67^{a}	2.07^{a}	Central European	Hap2	Hap2	Hap2
	COUT8	1383	4.51^{a}	5.19^{a}	Iberian	Hap2	Hap2	Hap2
	COUT10	1385	12.26^{a}	2.68^{a}	Central-European	Hap2	Hap2	Hap2
	h					0.2500	0.2500	0.2500
	π					0.000369	0.000498	0.000446
Pico Formigueiros	PFOR1	1628	7.77	3.05	Central-European	Hap2	Hap2	Hap2
1635 m	PFOR2	1629	10.25	3.64	Central-European	Hap2	Hap2	Hap2
Heathland	PFOR4	1631	8.72	2.34	Central European	Hap2	Hap2	Hap2
PFOR	PFOR5	1632	7.40	2.48	Central-European	Hap2	Hap2	Hap2
01° 05' 59'' W	PFOR7	1634	7.78	1.84	Central-European	Hap1	Hap2	Hap3
42° 36' 22'' N	PFOR8	1635	7.17	2.12	Central European	Hap1	Hap2	Hap3
	PFOR10	1637	6.59	3.67	Central European	Hap1	NA	NA
	h					0.5714	0.0000	0.5333
	π					0.000843	0.000000	0.000317

[a] Data from PERRY et al. (2009). NA: Not Analysed.

Central Europe and only detected in arnica populations living in heathlands of the eastern mountains of Galicia. The second chemotype is characterized by a dominance of DH compounds. It was found in individuals growing in peatlands and hay-meadows of a large range of altitudes (from 420 to 1460 m) and it was not described in the rest of Europe (PERRY *et al*., 2009). Hereinafter, they will be indicated as Central-European and Iberian chemotype, respectively.

Although the taxonomic status of the *A. montana* subspecies is doubtful in the study area (ROMERO et al., 2011), the morphological and chemotypical variability described above suggests the possible existence of genetic differences among arnica populations in this area. Genetic studies on this species are scarce (KAHMEN and POSCHLOD, 2000; LUIJTEN *et al*., 2000; EKENÄS *et al*., 2007, 2009) despite the economical and conservation interests of the species. Therefore the main objective of this study was to investigate for the first time the phylogeography and genetic variation associated with the two previously described chemotypes for *A. montana* in order to consider this information for the conservation strategy of the species in Galicia and provide more effective management plans.

Materials and Methods

Sampling strategy

The genetic analyses were performed on herbarium material collected from previous studies on ecology and chemistry of *A. montana* in Galicia (PERRY *et al*., 2009) and stored at the LUGO Herbarium, Universidade de Santiago de Compostela (*Table 1*). The selected material came from four locations showing the highest SL content differentiation in the region: the individuals from Reibocha (REIB) and Ponte Pedrido (PPED) corresponded to the Iberian chemotype (dihydrohelenalin dominated) and those from Pico Formigueiros (PFOR) corresponded to the Central-European chemotype (helenalin dominated). In the fourth location, Alto do Couto (COUT), most individuals showed the Central-European chemotype but two belonged to the Iberian chemotype (*Table 1*, *Fig. 1*). These locations also represent the different habitats of the species in this zone: high-altitude heathlands (COUT, PFOR), peatlands (PPED) and hay-meadows (REIB). Collected plants in each location were selected well apart to ensure sampling of different individuals under vegetative reproduction (KAHMEN and POSCHLOD, 2000).

Helenalin (H) and dihydrohelenalin (DH) content

SLs data (H and DH content) from individuals of REIB, PPED, and COUT sites were previously published (PERRY *et al*., 2009) whereas individuals of PFOR were analysed in this study at the first time. The methods for purification, identification, analytical extraction, and reversed-phase liquid chromatography (RPLC) analyses for SLs were done according to DOUGLAS *et al*. (2004) and PERRY *et al*. (2009).

DNA extraction, PCR amplification and sequencing

Approximately 3 mg of dried leaf were used to extract genomic DNA for each individual using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

Available resources on public DNA databases are very scarce. In order to select the most informative DNA

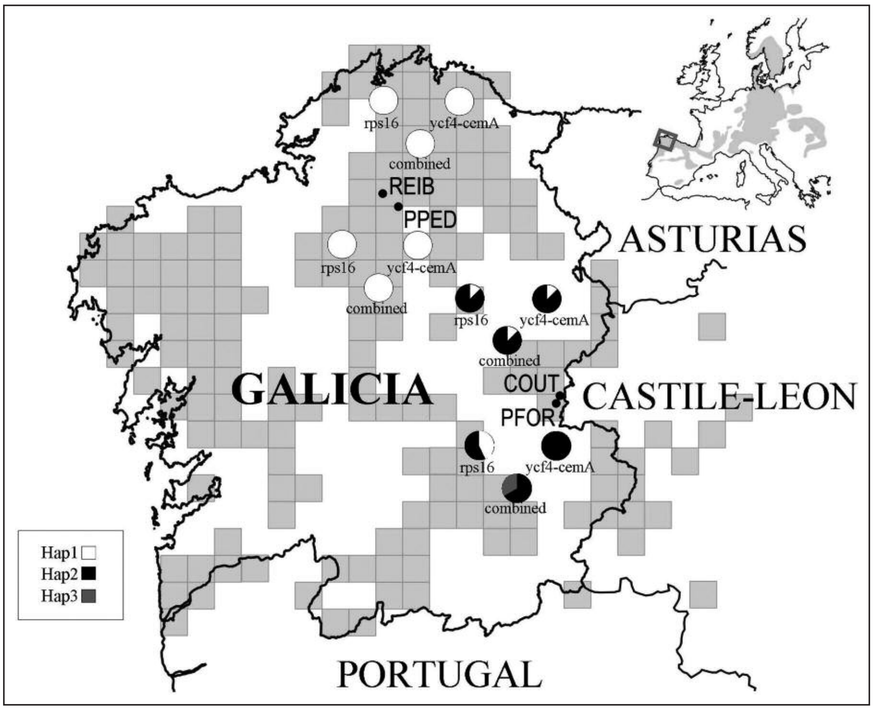

Figure 1. – Distribution of the studied locations. Sampling sites and haplotype distribution in the studied area for rps16 intron (rps16), ycf4-cemA spacer (ycf4-cemA) and both combined markers (combined). Shady areas show the distribution of *A. montana* in Europe (in grey) and in Galicia (grey squares).

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markers, available GenBank sequences for the species were downloaded. These sequences came from two voucher individuals, one of them from Sweden (MO-1 Sweden, *Andreasen* 304 (UPS)) and another from France (herb. France, s.c. 500084 (DS)). The alignments were performed using ClustalX 2.0 (THOMPSON *et al.*, 1997) for five non-coding chloroplast DNA (cpDNA) regions (*rpl16* intron (Swedish voucher specimen: AM690527, French voucher specimen: AM690540), *rps16* intron (AM690611, AM690625), *trnT-L* spacer (AM690489, AM690504), *psbA-trnH* spacer (AM690567, AM690582), and *ycf4-cemA* spacer (AM690643, AM690654)) and two nuclear regions (*ITS 18S-26S* (AM690714, AM690729) and *ETS* (AM690673, AM690688)) previously analysed in the phylogeny of the genus Arnica (EKENÄS *et al.*, 2007). The most variable DNA regions, *rps16* intron and the *ycf4-cemA* spacer, were selected for further amplification in this study, using the following primers: i) rpsf (5'-GTGGTAGAAAGCAACGTGCGACTT-3') and rps16r2 (5'-TCGGGATCGAACATCAATTGCAAC-3') for *rps16* intron (OXELMAN *et al.*, 1997), ii) rbcL/cemA-F5 (5'-CTCCGATTTCTTATAAAAGATATTCAG-3') and cemA-R (5'-AGTATTCCACCAAGTAGTAACCCAAG-3') for *ycf4-cemA* spacer (provided by B. OXELMAN). PCR reaction mixture (50 µL) contained 100–300 ng of template DNA, 1X PCR Gold Buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 10 pmol of each primer, and 2.5 units of AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR were carried out in a PTC-100™ thermocycler (MJ Research) as follows: initial denaturation at 95 °C for 10 min; 34 cycles of 95 °C for 30 s, annealing at 50 °C for 1 min and 72 °C for 2 min; and final extension at 72 °C for 10 min. PCR products were purified using the Spinclean PCR Purification Kit (MBiotech, Seoul, South Korea) and sequenced following the ABI Prism BigDye™ Terminator v3.1 Cycle Sequencing Kit protocol on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

Analysis

Sequence alignment was carried out using the program ClustalX 2.0. The retrieved GenBank sequences AM690611 and AM690643 (belonging to the Swedish voucher specimen) were used as reference sequences to align the *rps16* intron and *ycf4-cemA* spacer markers, respectively. Variable sites for both markers were checked by hand using SEQSCAPE 2.5 (Applied Biosystems, Foster City, CA) and haplotypes were identified using Mega 5.05 (TAMURA *et al.*, 2011).

To determine the phylogenetic relationships of the detected *A. montana* haplotypes, Median-Joining networks (BANDELT *et al.*, 1999) using molecular markers individually and concatenated were constructed with NETWORK version 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm). Available GenBank sequences from different *Arnica* taxa phylogenetically related with *A. montana* were included (EKENÄS *et al.*, 2007, see *Appendix 1* for species and accession numbers). Gaps (including one or more indels) among aligned sequences were considered as a single mutational step. The networks were rooted using the sequences of *A. unalaschcensis* as outgroup due its basal position in the *Arnica* genus (EKENÄS *et al.*, 2007).

Genetic variability within locations was estimated by haplotype (h) and nucleotide (π) diversity estimators (NEI and TAJIMA, 1981) using Arlequin 3.1 (EXCOFFIER *et al.*, 2005). Population differentiation estimates taking into account the nucleotide diversity (Φ_{ST}) were also calculated. The nucleotide substitution models applied for each marker were estimated using the "best DNA model" option implemented in Mega 5.05: Tamura 3-parameter model (TAMURA, 1992) with gamma value of 0.09 or without gamma value for *rps16* intron and *ycf4-cemA* spacer, respectively. Analysis of the molecular variance (AMOVA) was performed to study the distribution of genetic variation within and among location groups based on the chemotypes (H vs DH dominance). The significance of population differentiation was tested performing 1,000 permutations. These analyses were carried out using Arlequin 3.1.

Results

All individuals were amplified for *rps16* intron and *ycf4-cemA* spacer markers except three individuals

Table 2. – Variable sites for *A. montana* in the *rps16* intron and *ycf4-cemA* spacer haplotypes detected in this study. Each column indicates one variable site.

	rps16 intron variable sites							*ycf4-cemA* spacer variable sites						Combined markers
Haplotypes	52	56	74	95	222	651	Haplotypes	35	713	807	818	947	948	
AM690611	T	G	A	T	-	A	AM690643	C	G	G	-	T	C	*A. montana* Sweden
AM690625	C	-	-	T	G	?	AM690654	A	A	T	T	G	T	*A. montana* France
Hap1	T	G	A	T	-	-	Hap1	A	G	G	-	G	T	Hap1
Hap2	T	G	A	G	-	-	Hap2	A	G	G	-	T	C	Hap2
Hap1	T	G	A	T	-	-	Hap2	A	G	G	-	T	C	Hap3

"?" indicates no position present in the sequence, and "–" indicates indel.

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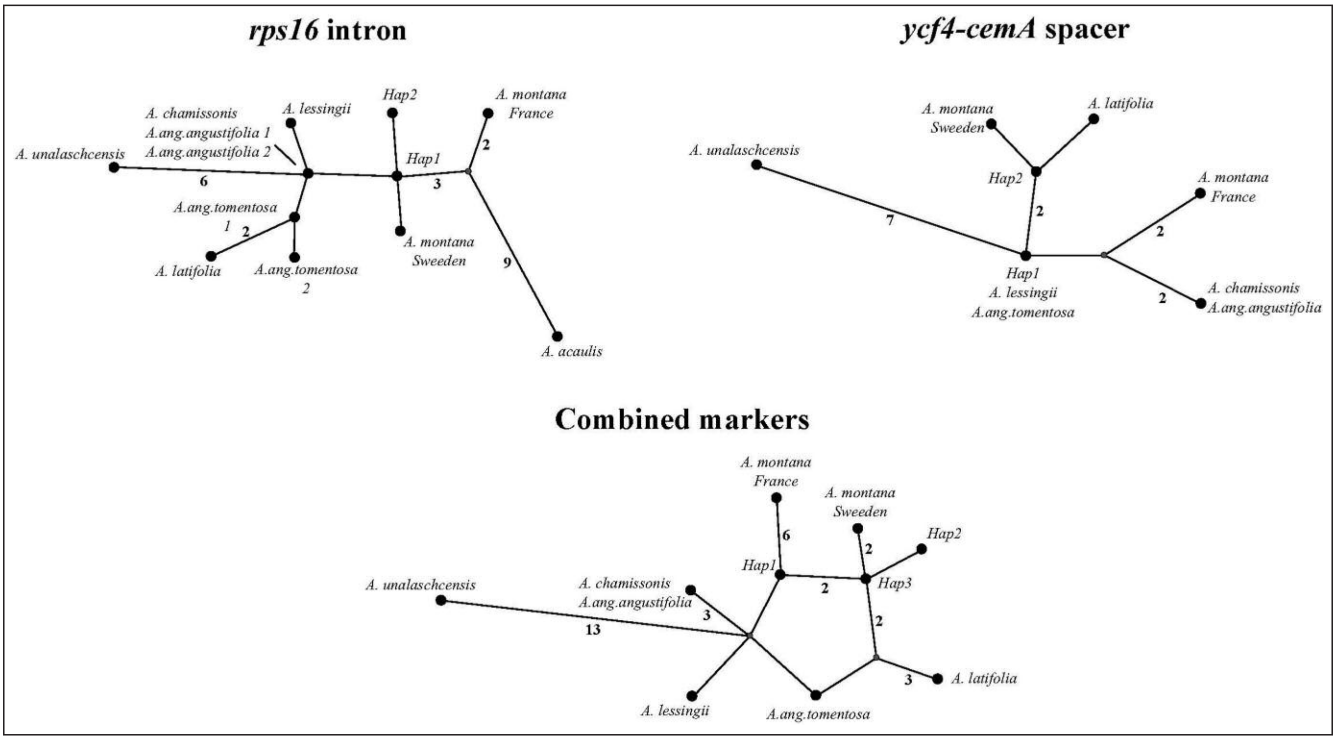

Figure 2. – Median-Joining networks of *Arnica* sp. haplotypes. Grey circles represent median vectors needed to connect all the observed haplotypes. Bold numbers indicate the mutational steps in lines where more than one mutational step is involved.

for *ycf4-cemA* spacer (PPED9, REIB9 and PFOR10; *Table 1*).

The total length of aligned *A. montana* sequences was 680 base pairs (bp) for *rps16* intron and 1005 bp for *ycf4-cemA* spacer. Two different haplotypes were detected for the two markers. The *rps16* intron haplotypes only showed one variable site, whereas the *ycf4-cemA* spacer haplotypes showed two variable sites in the zone under study (*Table 2*). Galician haplotypes for both markers were different from GenBank sequences from Sweden and France voucher specimens (*Table 2*). All novel haplotypes were submitted to GenBank (Accession numbers: KF679679 and KF679680 for *rps16* intron; KF679681 and KF679682 for *ycf4-cemA* spacer).

There was congruence between haplotypes of the two cpDNA regions (*Table 2*). Thus, when an individual displays Hap1 in the *rps16* region, Hap1 in *ycf4-cemA* spacer was found (combined Hap1). Just the same, individuals with Hap2 in the *rps16* intron displayed Hap2 in the *ycf4-cemA* spacer (combined Hap2, *Tables 1* and *2*). There were only two exceptions (PFOR7 and PFOR8) where a third combined Hap3 was detected (*rps16* intron Hap1 and *ycf4-cemA* spacer Hap2; *Tables 1* and *2*).

The maximum number of mutation events inside *Arnica* genus was 19 (between *A. unalaschcensis* and *A. acaulis*), 10 (*A. unalaschcensis* versus *A. montana*, *A. latifolia*, *A. angustifolia* ssp. *angustifolia*, *A. chamisonis*), and 20 (between *A. unalaschcensis* and *A. montana*) for *rps16* intron (alignment length = 688 bp), *ycf4-cemA* spacer (alignment length = 1026 bp) and combined markers (alignment length = 1711 bp), respectively (*Fig. 2*). Some species showed the same haplotype for the molecular markers. Thus, *A. angustifolia* ssp. *angustifolia* and *A. chamisonis* had identical haplotypes in both markers, while the *ycf4-cemA* spacer haplotypes for *A. angustifolia* ssp. *tomentosa*, *A. lessingii* and Hap1 were identical. Inside the species, the different networks grouped all the *A. montana* haplotypes. The most diverse sequences were always associated to the French voucher specimen. The phylogenetic relationships showed that the Hap 1 (either at individual or combined markers) was the most internal (*Fig. 2*). This internal position indicated the ancestrality of Hap1 inside *A. montana*.

The H content was higher than DH content for all PFOR individuals. Therefore, all of them were assigned to the Central-European chemotype (*Table 1*, *Fig. 3*). Genetic diversity was very low inside locations, with only H-dominated chemotype locations (heathlands) showing variability (*Table 1*). The Φ_{ST} values were high and significant (0.67731, 0.89781 and 0.84017 for *rps16* intron, *ycf4-cemA* spacer and combined markers, respectively; $P < 0.001$), indicating genetic differentiation among sampling sites studied. AMOVA assigned the highest percentage of variability to differences among groups when sampling sites were grouped according to chemotype present in the population (*rps16* intron: 72.84%; *ycf4-cemA* spacer: 93.13%; combined markers: 88.97%, see *Table 1* for chemotype assignment of the analysed populations) and very low fraction to differences among locations within groups (<3.00% for all analyses performed).

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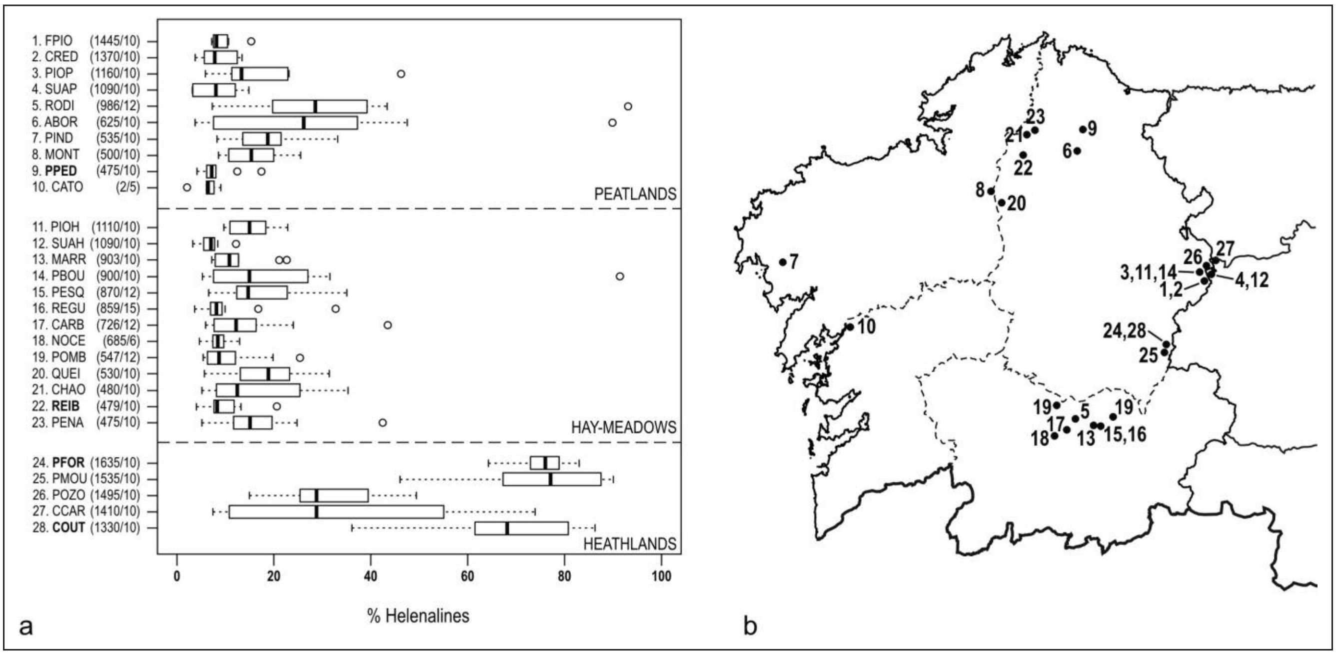

Figure 3. – (a) Boxplot of the percentage of helenalines in the populations studied in Galicia grouped by their habitat. The altitude and number of specimens analyzed of each population is indicated in parentheses beside their code name. The four populations selected for the genetic analysis have their code names in bold face. (b) Locations of the studied populations. Numeric codes as in (a).

Discussion

Genetic variability

This is the first study describing the genetic diversity of *Arnica montana* in the extreme SW of Europe. The number of mutational events among A. *montana* haplotypes were similar to the interspecific differences within the *Arnica* genus. Despite the low levels of variability detected in the DNA regions analysed, cpDNA haplotypes were congruent with chemotypes and geographical variation found in Galicia. Thus, although analysed data was limited, the presence of two different divergent chloroplast lineages cannot be ruled out. Significant population differentiation was detected in the studied zone, mostly assigned to differences among groups, suggesting the presence of two genetically divergent population groups. Moreover, one of these groups is different from the European *A. montana* reported to date, based on the phylogenetic and chemotypic data (EKENAS *et al.*, 2007; PERRY *et al.*, 2009), highlighting its conservation interest. Posterior studies on a broader area across the distribution range of the species, including more sampling sites, would help to corroborate the distribution of the suggested groups. Moreover, cpDNA inheritance (mainly maternal) can limit the definition of population ranges and evolutionary forces depicting the current distribution of genetic variability. Thus, the use of highly variable and codominant molecular markers (i.e. microsatellite loci, not available to date) will also allow us to refine these limits.

Phylogenetic networks grouped all the *A. montana* haplotypes and related them with *A. angustifolia*, the other European species (*Fig. 2*). Networks also showed that the Hap1 haplotypes (either individually or combined) were ancestral inside *A. montana*. These haplotypes were fixed in locations with Iberian chemotype. Thus, these Iberian locations may represent the source for post-glacial North-European colonisations (GOMEZ and LUND, 2006), being the Southernmost limit in Europe. Moreover, the presence of one haplotype combining Hap1 for *rps16* intron and Hap2 for *ycf4-cemA* spacer (combined Hap3) was a surprising result. EKENÄS *et al.* (2007) suggested disrupted concerted evolution, hybridization processes and homoplasy to explain conflicting positions for different taxa using cpDNA and nuclear ribosomal markers. Presence of shared haplotypes suggests hybridization processes among species and complex phylogenetic relationships. Recurrent mutation produced in mutational “hotspots” leads to homoplasy among sequences. The genetic diversity was low, being the *rps16* intron haplotypes only differed by one nucleotide site. Thus, recurrent mutation is the most plausible explanation for the combined Hap3 appearance, although cpDNA recombination cannot be ruled out (HUANG *et al.*, 2001; MARSHALL *et al.*, 2001).

Environmental, chemical and molecular information

Recent phylogenetic analyses showed congruence between molecular data and SL contents in the genus *Arnica* (EKENÄS *et al.*, 2009). In addition, our study suggests the existence of relationships among cpDNA haplotypes, SL contents and habitat of the population. All individuals, except one, collected in hay meadow (REIB) or peatland (PPED) locations had combined Hap1 and all of them had Iberian chemotype. On the other hand, the individuals collected in heathland locations (COUT and PFOR) were more variable. Most of them (82%) had

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Central-European chemotype and combined Hap2, but two individuals from COUT with Iberian chemotype had also combined Hap2, and two individuals from PFOR had Central-European chemotype and combined Hap3 (see *Table 1*). Moreover, one individual from COUT (COUT4), with Central-European chemotype, showed the combined haplotype Hap1, which was fixed in locations of Iberian chemotype. Thus, these incongruences suggest the presence of gene flow in the zone. Gene flow has been proposed for the maintenance of gene diversity and connectivity in fragmented *A. montana* populations (KAHMEN and POSCHLOD, 2000). A possible explanation could be the transportation of seeds by wind or animals (including man).

These results cannot be extended to the whole Galician area because we selected populations with very different chemotypes in order to maximize the chance of finding genetic differences. The reduced number of sampling sites used in this study also limited the generalization/extrapolation of present results. For example, *figure 3* shows the variability of the proportion of helenalines for the whole set of Galician populations studied so far (PERRY *et al*., 2009, unpublished data. Mean number of individuals analyzed per population= 10.1 ± 1.7, see *Fig. 3*) and information about altitude and habitat type. The chemotypes of REIB and PPED populations were clearly Iberian and showed small variability. The individuals from COUT and PFOR were more variable but the Central-European chemotype was the most abundant there. Other populations (e.g. RODI, ABOR, POZO, CCAR) showed high variability in SL composition and a mixture of both chemotypes, which suggests that these populations could also be genetically variable. Thus, further genetic analyses should be extended to other populations showing biochemical and/or habitats differences both in the studied region and in other European populations to generalize and support our observations (i.e. what the relationship between chemotype information and genetic information is).

On the conservation and sustainable exploitation of this species

Despite the fact that biometric data suggest that *A. montana* ssp. *atlantica* is not a good taxon, the results presented here showed that there is genetic and biochemical variability in the Galician populations that could be associated to habitat differences (including grazing pressure). Knowledge about the relationships between the genetic and chemical variability and the habitats where the species lives is a key point for the design of management plans that warrant the persistence of the species while allowing for its exploitation (ANDROSIUK *et al*., 2013). Not in vain the Annex V of the European Council Directive 92/43/EEC includes *A. montana* as a "plant species of community interest whose taking in the wild and exploitation may be subject to management measures". Maintenance of genetic diversity of socio-economically valuable species is one of the main targets of the Global Strategy for Plant Conservation 2011–2020 (CDB 2011–2020).

At present, there is no data about the distribution of the Iberian chemotype and the haplotypes found in this study outside Galicia. In case their distributions were restricted to this or nearby areas the risk of loss of genetic, pharmaceutical, and economic resources is high. Therefore, further genetic analyses must been performed to correlate biochemical and genetic information in order to define the proper conservation units (e.g. Iberian group vs Central-European group). Finally, management strategies should also prioritize habitat maintenance and exploitation regulation in order to allow the preservation of arnica diversity in the zone.

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Appendix 1.

Species and their GenBank accession numbers (among brackets) used in this study for the *Arnica* genus.

For *rps16* intron: *A. acaulis* (AM690603), *A. angustifolia* ssp. *angustifolia* (AM690615, AM690629), *A. angustifolia* ssp. *tomentosa* (AM690630, AM690631), *A. chamissonis* (AM690616), *A. latifolia* (AM690618), *A. lessingii* (AM690619), *A. montana* (AM690611, AM690625), *A. unalaschcensis* (AM690614).

For *ycf4-cemA* spacer: *A. angustifolia* ssp. *angustifolia* (AM690647), *A. angustifolia* ssp. *tomentosa* (AM690656), *A. chamissonis* (AM690648), *A. latifolia* (AM690649), *A. lessingii* (AM690650), *A. montana* (AM690643, AM690654), *A. unalaschcensis* (AM690646).

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