Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe

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A preliminary synthesis of diversification patterns of alpine plants in the Mediterranean region of Europe is presented based on seven plant groups displaying morphological differentiation and infraspecific taxa. Both previous and new phylogenetic results from ITS sequences and fingerprinting data suggest different colonization routes and modes of speciation in Androsace vitaliana (recent differentiation in the Iberian Peninsula), Anthyllis montana (west-to-east colonization and differentiation in Europe), Arenaria tetraquetra (colonization and differentiation from SE Iberian mountains to the Pyrenees; increasing number of chromosome comple-ments), Saxifraga oppositifolia (colonization from the Arctic to the Iberian Peninsula), Saxifraga pentadactylis (differentiation in Mediterranean and Eurosiberian mountains by geographic isolation), and Soldanella alpina (differentiation and colonization from northern Iberia to the Alps, and then to the Pyrenees and the Balkan Peninsula). Relative static diversification of Juniperus communis var. saxatilis in Europe, based on identity of chloroplast trnL-F sequences, is also described. Most morphological variation, expressed by number of subspecies recognized in previous taxonomic treatments of the seven plant groups, appears to have occurred during the Pleistocene (< 1.75 Myr). Recurrent change of Quaternary climatic conditions in the Mediterranean Basin, coupled with geographic characteristics, life cycle, dispersal mechanisms, and pre-Holocene genetic structure are not convincing factors to account for all the observed diversification. Additionally, stochastic processes are also considered for evaluating present-day distributions and processes of speciation.

KEYWORDS: Androsace, Anthyllis, Arenaria, colonization patterns, Juniperus, molecular diversification, Saxifraga, Soldanella, subspecific differentiation.

INTRODUCTION

Southern Europe encompasses elevated mountains that harbour alpine plants in the Mediterranean region. The highest European mountains, which are still in an uplifting process, are the result of the onset of the Alpine orogeny in Mesozoic-Cenozoic times (c. 65 Myr). Plate tectonic activity of microplates generated main movements during the latest uplifting periods in Mediterranean Europe: Betic range (Jurassic-Miocene), Cantabrian range and the Pyrenees (Paleocene-Eocene), Jura (Miocene-Pliocene), the Alps (Oligocene-Miocene), Apennines (Paleocene-Miocene), and the Balkan Peninsula (Paleocene-Pleistocene) (Ager, 1975). Important is that the present configuration of these mountains was established far earlier than differentiation of alpine species in Europe.

Distribution and genetic structure of alpine plants have been ultimately molded by the geologic complexity of the European continent, coupled with Tertiary and Quaternary climatic episodes. The occurrence of the Mediterranean climate in the Pliocene (c. 3.2 Myr) caused seasonal rhythm and summer drought that became stable approximately 2.8 Myr ago (Suc, 1984). Accordingly, floras of mountain ranges may have also evolved during the Pliocene and Pleistocene (Stebbins, 1984), including surviving floras in high altitudes encircled by Mediterranean conditions. Glacial-interglacial cycles in the Pleistocene promoted isolation of alpine plants in high-altitude mountains of the Mediterranean Basin during warm (interglacial) periods. Conversely, plant populations from isolated mountain ranges were connected during long glacial stages. Most alpine plants that we find in southern European mountains are the result of migrations from lower altitudes after glacial periods or descendants of colonists from northern Europe (Stebbins, 1984).

Molecular phylogenetics help infer historical biogeography and evolutionary patterns. Relationships among living populations of alpine plants in a phylogeographic context seems to be the most appropriate approach to address diversification patterns of alpine plants in Europe (Avise & al., 1987; Comes & Kadereit, 1998; Avise, 2000). Intraspecific phylogeography is concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species (Avise, 2000). This approach has been successfully
implemented to describe postglacial colonization routes of trees in central Europe (Demesure & al., 1996; Dumolin-Lapegue & al., 1997; King & Ferris, 1998; Raspé & al., 2000; Petit & al., 2002) and to test the hypothesis of nunatak areas for in situ glacial survival in arctic and alpine plants (Brochmann & al., 1996; Gabrielsen & al., 1997; Stehlik & al., 2002; Schönswetter & al., 2002). Quaternary glaciations may not have dramatically affected the survival of plants in southern Europe, in contrast with total extinction of angiosperms in northern Europe due to long-term establishment of ice sheets. In fact, three main refugia in the Iberian, Italian, and Balkan peninsulas have been proposed, from which re-colonization occurred (Taborlet & al., 1998; Hewitt, 2000). Genetic structure and speciation patterns of alpine floras in southern Europe are more complex due to a continuous presence of plants in Mediterranean mountains along fluctuating vegetation belts.

This paper is a preliminary synthesis of results from seven plant groups using molecular markers: Androsace vitaliana, Anthyllis montana, Arenaria tetraquetra, Juniperus communis var. saxatilis, Saxifraga oppositifolia, Saxifraga pentadactylis, Soldanella alpina (Vargas, 2002). Our working hypothesis is that geographic characteristics, recent climatic events (glaciations), life cycles, dispersal mechanisms, and pre-Holocene genetic structures are mostly responsible for present distribution and differentiation within alpine plants. Subspecific taxa represent morphological differentiation of each species in an evolutionary context. The main objective is to test this phylogeographic hypothesis based on variation of molecular markers, phylogenetic reconstructions, and geographic arrangement of mountains that have led to present distributions and modes of speciation in Europe.

**MATERIALS AND METHODS**

**Species selection.** — Alpine plants are typically defined as those obtaining optimal habitat conditions over 2300 m in the Mediterranean region (Körner, 1999). Unfortunately, we have found few examples in the literature of alpine vascular plants in southern Europe containing subspecific taxa, which also have been analyzed using phylogeographic data. Seven plant groups have been considered in this paper thanks to previous publications from several authors on Anthyllis montana (Kropf & al., 2002a), Saxifraga oppositifolia (Abbott & al., 2000; Holderegger & al., 2002), and Soldanella alpina (Zhang & al., 2001). Additionally, we have obtained and analyzed new data from Androsace vitaliana, Arenaria tetraquetra, Juniperus communis var. saxatilis, and Saxifraga oppositifolia, and extended the sample of Saxifraga pentadactylis (Vargas, 2001). Some other studies have not been included due to insufficient number of populations, low morphological variation or uninformative molecular results.

**Molecular markers.** — Molecular variation within the same species is the basic requirement to infer relationships among populations. Fingerprinting techniques screening the nuclear genome, such as RAPDs, ISSRs, AFLPs, and microsatellites, ensure a large number of data to be analysed by phylogenetic methodologies (Hillis & al., 1996a). In some cases, sequences of nuclear ribosomal DNA (ITS, ETS) display a minimal number of phylogenetically informative characters among populations. Recombination of biparental nuclear genomes may obscure historical relationships based on hierarchical genealogies, particularly in cases of reticulation. Uniparental haplotypes either from the mitochondria or chloroplast alleviate this problem because they are haploid and non-recombinant macromolecules (Avise & al., 1987; Comes & Kadereit, 1998). However, low levels of genetic variation in organelle genomes, together with phylogenetic reconstructions based on genes inherited by only one parental organism (typically maternal inheritance in angiosperms; Mogensen, 1996), are disadvantages for the use of these markers in phylogeography of plants and inference of morphological character evolution.

**Phylogeographic reconstructions.** — Traditional biogeography considers two major possibilities to account for the origin of present distributions of populations and species of alpine plants: dispersal and vicariance (Ronquist, 1997). The vicariance hypothesis argues for isolation into island-like areas of populations on sun-dered mountains from a continuous area separated during interglacial periods. In contrast, under dispersal, distribution of populations on elevated mountains is the result of colonization from one or few individuals. We abandon any inference based on area cladograms because our phylogenetic reconstructions are analysed at the populational level, and distributions of alpine plants are profoundly disparate. Genealogies of neutral genes are used to evaluate colonization patterns and microevolutionary processes (Schaal & Olsen, 2000). The use of nuclear markers link morphological differentiation and phylogeography because most genes responsible for character evolution are contained in the nuclear genome. Neighbor-joining and parsimony-based analyses of nuclear fingerprinting and ribosomal ITS sequences are implemented.

The internal transcribed spacer (ITS) region of nuclear 18S-26S ribosomal DNA has proven to be informative for both biogeographic studies and inferences of character evolution at inter- and intraspecific levels (Baldwin & al., 1995). Availability of ITS sequences in GenBank is the result of ease of sequencing the ITS region coupled with an acceptable number of
phylogenetically informative characters in some infraspecific studies (Baldwin 1993; Vargas & al., 1999a). Despite intrinsic problems in reconstruction of molecular phylogenies by using multi-copy genes, such as the ITS region affected by concerted evolution (Fuertes & al., 1999), reliable phylogenetic signal at the infraspecific level has been corroborated using different molecular markers (Kropf & al., 2002a; Zhang & al., 2001). Additionally, the body of ITS sequence analyses allow comparison across angiosperms and the use of this molecule to estimate maximum age of diversification through molecular clocks (Richardson & al., 2001). To obtain ITS and trnL-trnF sequences we used a sequencing strategy as described in Vargas & al. (1999a) and Taberlet & al. (1991).

**Molecular clocks.** — To include a time frame for most molecular and morphological differentiation of each species, we searched for maximum ages of diversification. Diversification times were calculated from both previous estimates (Zhang & al., 2001; Kropf & al., 2002a) and new ones based on the logic of Baldwin & Sanderson (1998) and Sanderson (2002). Calibration points depend on the geological events and the plant group (e.g., origin of Madeira island and split of the Strait of Gibraltar in Saxifraga; Vargas & al., 1999a) and the fossil record (Dorofeev, 1963, for Primulaceae). A calibrated rate of Saxifraga in Richardson & al. (2001) is also considered, even though it is clearly divergent in comparison to other calibration points. Levels of diversification are also compared using average molecular variation of ITS sequences in a group of angiosperms (Richardson & al., 2001).

**RESULTS**

*Androsace vitaliana* (Primulaceae). — The former genus *Vitaliana* has been lately considered as a section (*Vitaliana*) of *Androsace* (Kress, 1997), a circumscription in agreement with an ITS phylogeny where accessions of *Androsace vitaliana* form part of the lineage of *Androsace* sect. *Aretia* (L.) W.D.J. Koch (P. Schönswetter, pers. comm.). This European endemic occurs in the Abruzzi, Alps, Pyrenees, and elevated mountains of the Iberian Peninsula, at an altitudinal range between 1500 and 3300 m. It is characterized by fruits in capsules with 2–3 seeds each and two ploidal levels (2n = 20, 40). The taxonomic treatment of Ferguson (1972) includes five infraspecific taxa under *Vitaliana primuliflora* Bertol.: subspp. *asoana* M. Laínz (S and E Iberia), *canescens* O. Schwarz (SW Alps and Pyrenees), *cinerea* (Sü nd.) I. K. Ferguson (C and SW Alps and E Pyrenees), *praetutiana* (Buser ex Sü nd.) I. K. Ferguson (Apennines), and *primuliflora* (SE Alps). A most recent taxonomic treatment also includes subssp. *flosjugorum* Kress (Cantabrian range) described, as the other subspecies, under *Androsace* (Kress, 1997) (Table 1). The ITS region is 604–606 bp in length: 222–223 bp in ITS-1; 164 bp in 5.8S; 219–220 bp in ITS-2 (Álvarez, Luceño & Vargas, unpubl.). Eight variable and five parsimony-informative characters were obtained and analysed. When using *Douglasia alaskana* (Coville & Standl. ex Hultén) S. Kelso, *Douglasia beringsensis* S. Kelso, *Androsace ciliata* DC., *A. sempervivoides* Jaquem. ex Duby, and *A. spinulifera* as the outgroup, the accessions of *A. vitalia* form a monophyletic group. A polytomy of three clades of *A. vitaliana* was retrieved containing three samples from the Alps and Mount Ventoux, eight accessions from the Iberian Peninsula, and one from the Apennines (Fig. 1). A large polytomy is the result of identity of ITS sequences from the entire Iberian Peninsula, except for two samples from the Cantabrian range (subsp. *flosjugorum*). Populations of subssp. *flosjugorum* have certain independence, as well as those of subssp. *cinerea* and *vitaliana* from the Alps and Mount Ventoux. A maximum age of 0.7 Myr for the split of populations of *Androsace vitaliana* from Iberia is estimated from the highest K2P (Kimura-2-Parameter) pairwise distance between the populations from Mount Ventoux and the Cantabrian range (1.16%). The populations from the Iberian Peninsula may have diverged in the last 0.2 Myr, as suggested by the highest K2P pairwise distance (0.33%) between accessions from the Cantabrian range and the rest of the Iberian Peninsula.

*Anthyllis montana* (Leguminosae). — Morphological differentiation is reflected by four subspecies considered in the last taxonomic treatments (Cullen, 1968; Greuter & al., 1989). They have two ploidal levels (2n = 14, 28), fruits typically with one seed, and occur between 200 and 2700 m. The ITS region is 605 bp in length: 231 bp in ITS-1; 162 bp in 5.8S; 212 bp in ITS-2 (Kropf & al., 2002a). The alignment of the nine accessions (Table 1) rendered six variable sites, of which three were parsimony-informative. When using *Hymenocarpos circinnatus* (L.) Savi, *Anthyllis vulneria* L., and four species of *A. sect. Oraenthyllis* Grisebach as the outgroup, the ITS tree was resolved into four lineages of *A. montana* in a pectinate topology: southern Spain, Algeria-French Alps, central Italy, and Austria-NE Italy-Slovenia-Croatia (Fig. 2). Average K2P pairwise distance of sequences among the four ITS lineages was 0.36 ± 0.16 %, which implies a maximum diversification time of 0.25–0.66 Myr (Kropf & al., 2002a). The AFLP tree is congruent with that of ITS and provides further evidence of population discontinuity into western and eastern groups. Interestingly, populations from central and southern Italy belong to two different groups, however weakly supported.

*Arenaria tetraquetra* (Caryophyllaceae). —
Six ploidal levels have been found in this plant, which is endemic to elevated mountains (between 1400 and 3400 m) of the Iberian Peninsula: subsp. *amabilis* (2x; Sierra Nevada); subsp. *murcica* (3x, 4x, 5x; Betic range); and subsp. *tetraquetra* (6x, Pyrenees; 7x, Sierra de la Pela) (López, 1990). A synthetic treatment including only two subspecies, *tetraquetra* and *amabilis*, is found in Chater & Halliday (1993). A characteristic cushion-like habit and capsules with seeds adapted to dispersal by rain drops (Goyder, 1987) seem to be mechanisms not favorable for long-distance dispersal.
most closely related species in the phylogeny of A. sect. Plinthine (Reichenb.) Pau. As a result, a pectinate topology of the ITS tree was obtained (Fig. 3), where populations from SE Iberia (Sierra Nevada and Betic range) are basal, and northern accessions (Sierra de la Pela and Pyrenees) are at terminal nodes. Populations of subsp. murcica are polyphyletic, accessions of tetraquetra are not fully resolved, and all accessions of subsp. amabilis form a monophyletic group. Considering molecular-clock evolution of ITS sequences and using variation average within angiosperms (Richardson & al., 2001), ITS sequence variation (0–1.6 % K2P pairwise distance) is high enough to hypothesize a maximum age < 1.92 Myr for present distribution and increment of chromo-

Fig. 1. Distribution (A) and phylogeographic hypothesis (B) for Androsace vitaliana (C, subsp. vitaliana; Spain, Pyrenees, Bonaigua pass) based on ITS sequences (Álvarez, Luceño & Vargas, unpubl.). Hypothetical barrier represented by a hatched bar.

Fig. 2. Distribution (A) and phylogeographic hypothesis (B) for Anthyllis montana (C, subsp. montana; Spain, Pyrenees, Sallent de Gallego) based on ITS sequences and AFLP variation. Hypothetical migration routes (arrows) inferred from Kropf & al. (2002a).
some complements to 7x.

**Juniperus communis** (Cupressaceae). — In Europe, two taxa have recently been recognized within this species: var. *communis* and var. *saxatilis* (Farjon, 2001). However, a different taxonomic treatment considered three subspecies (Franco, 1993): subspp. *communis*, *hemisphaerica* (J. & C. Presl) Nyman, and *alpina* (Suter) Celak. This juniper occurs primarily between 1900 and 2600 m in southern Europe, has a uniform chromosome number (2n = 22), and displays blue, fleshy cones. Six chlorotypes based on nucleotide substitutions and large indels in *trnL-trnF* sequences have been found within a Mediterranean juniper (*J. oxycedrus* L.; Martínez & Vargas, 2002). This species also belongs to sect. *Juniperus* and contains four subspecies. In contrast, *trnL-F* sequences of nine European populations of *J. communis* var. *saxatilis* (Table 1) display no molecular variation. Identical *trnL-trnF* sequences have also been obtained when sampling former infraspecific taxa considered within *J. communis* (subsp. *communis* var. *communis*, subsp. *hemisphaerica*). Distribution of *Juniperus communis* var. *saxatilis* in central and southern Europe and the sample of nine populations (numbered from 1 to 9) are shown in Figure 4.

**Saxifraga oppositifolia** (Saxifragaceae). — Synthetic taxonomic treatments of this species can be found in Webb & Gornall (1989) and Webb (1993). Seven subspecies are considered, of which five occur in Europe (subsp. *plepharophylla*, *oppositifolia*, *paradoxa*, *rudolphiana*, *speciosa*). The species occurs between 1700 and 3000 m in southern Europe. The most common chromosome number is 2n = 26, but 2n = 52 has also been reported from arctic areas. In *Saxifraga*, fruits are typically capsules with numerous seeds (Webb & Gornall, 1989). We have sequenced nine populations of subsp. *oppositifolia* and two of subsp. *paradoxa* (Table 1). The ITS region is 659 bp in length: 277 bp in ITS-1; 163 bp in 5.8S; 219 bp in ITS-2. Eleven nucleotide substitutions and five parsimony-informative characters were found. Chromatograms with nucleotide peaks overlapping at the same sites were observed in six populations after upstream and downstream sequencing reactions. IUPAC symbols were used for the analyses. The semistrict consensus ITS tree of 140 minimum-length Fitch parsimony trees yielded four major clades (Fig. 5) when using *S. spathularis* and *S. aizoides* as the outgroup. The first one supports monophyly of *S. oppositifolia* (95% bootstrap), a support value similar to that obtained when including four more species from sect. *Porphyron* (Conti & al., 1999; results not shown). A basal polytomy includes four accessions of *S. oppositifolia*, three of samples from southern Norway, Iceland, and Austrian Alps and one of the rest of the accessions (68% bootstrap; Fig. 5). This second clade is resolved, in part, by a sequential branching pattern, where one accession from the Central Pyrenees comes first, followed by a group (third clade, 72% bootstrap) of four accessions at a basal position [Dolomites, Cantabrian range (Palencia), Sierra de Urbión (La Rioja), and Sierra Nevada] and a fourth clade (45% bootstrap) containing one sample of subsp. *oppositifolia* from the Cantabrian range (Cantabria) and the two samples of subsp. *paradoxa*.
from the Pyrenees (Sierra del Cadí and Aran Valley). Four separated populations from the Alps (Dolomites), Sierra de Urbión (La Rioja), Cantabrian range (Palencia), and SE Iberia (Sierra Nevada) have the same ITS sequence (Fig. 5). The highest K2P pairwise distance within Iberian accessions (0.31%) is found between samples from Sierra de Urbión and Aran Valley. Maximum age of diversification of \( S. \) oppositifolia in Iberia is estimated between 0.88 and 0.30 Myr using different calibration events: unspecific calibration in \( Saxifraga \) (0.88 Myr, Richardson & al., 2001); origin of Madeira (0.60 Myr, Vargas & al., 1999a); split of the Strait of Gibraltar (0.43 Myr, Vargas & al., 1999a); estimation average in angiosperms (0.3 Myr, Richardson & al., 2001).

**Saxifraga pentadactylis** (Saxifragaceae). — This saxifrage is endemic to the northern half of the Iberian Peninsula and occurs between 1500 and 3000 m (Vargas, 1997). Neighborhood diffusion (Shigesada & al., 1995) seems to be a predominant dispersal mechanism of plants with capsules containing over 100 seeds, as are populations of the three endemic taxa of \( S. \) pentadactylis (Vargas & Nieto, 1996): subsp. pentadactylis (Pyrenees); subsp. willkommiana (central part of northern half of Iberia); subsp. almanzorii (part of Sierra de Gredos) (Fig. 6). A single chromosome number has been found (\( 2n = 32 \)). An extended sample (Table 1) with two more accessions from Sistema Central (Sierra de Urbión) and Cantabrian range (Peña Prieta) provided figures of ITS sequence variation within the range described in a previous publication (Vargas, 2001): 682–683 bp in length (281 bp in ITS-1; 168 bp in 5.8S; 233–234 bp in ITS-2); six variable sites; three parsimony-informative characters. Similar phylogenetic conditions were used as in Vargas (2001). The ITS consensus tree displays a biphyletic topology (Fig. 6) with one clade weakly supported (52% bootstrap) of samples from northern Iberia and a second one of two samples from central Iberia (70% bootstrap). Although a fully resolved tree was not obtained, this topology indicates that subsp. willkommiana is not monophyletic. The average K2P pairwise distance between ITS accessions was 0.58 %, being the highest distance of 1.21 % (between Sierra de Urbión and Sierra de Guadarrama). Separation times are suggested at a maximum between 3.55 and 1.21 Myr by the use of different calibration events: unspecific calibration in \( Saxifraga \) (3.55 Myr, Richardson & al., 2001); origin of Madeira (2.42 Myr, Vargas & al., 1999); split of the Strait of Gibraltar (1.72 Myr, Vargas & al., 1999a); estimation average in angiosperms (1.21 Myr, Richardson & al., 2001).

**Soldanella alpina** (Primulaceae). — Two subspecies are recognised (subsp. alpina and cantabrica) based on floral shapes and sizes (Kress, 1984; Zhang & al., 2001). Both of them are \( 2n = 40 \). The ITS region is 644–645 bp in length: 249 bp in ITS-1; 165 bp in 5.8S; 230–231 bp in ITS-2. Number of nucleotide substitutions within \( Soldanella \) was 30 (15 parsimony-informative characters) and only one within \( S. \) alpina. A polytomy included all accessions in the ITS tree. The AFLP reconstruction, using \( Primula latifolia \) and \( P. \) bulleyana as the outgroup, depicted a pectinate tree in which subsp. cantabrica is the basal-most lineage followed by populations within \( Soldanella \) at 30 (15 parsimony-informative characters) and only one within \( S. \) alpina.
DISCUSSION

Recent migration and differentiation of Androsace vitaliana in Iberia. — The branching pattern obtained from analyses of ITS sequences indicates isolation between populations from the Iberian Peninsula, Alps, and Apennines. Two polytomies prevent inferring patterns of migration within Europe and within the Iberian Peninsula (Fig. 1). A fragmentation process separating populations from Iberia and the Alps is interpreted from relatively significant DNA sequence divergence between them (1.16% K2P pairwise distance), but similar levels occur within the two groups (0.33 and 0.16%, respectively). Similarly, Alpine populations are isolated from that from the Apennines (0.66–8.3%). Separation times, based on the same logic as in Zhang & al. (2001), suggest split of the three lineages in the late Pleistocene (<0.7 Myr), and then differentiation in the Iberian Peninsula in the last 0.24 Myr. Lack of variation in ITS sequences indicates recent isolation in four mountain ranges of Iberia: Pyrenees, Sistema Iberico, Sistema Central, and Sierra Nevada (Fig. 1). None of the previous taxonomic treatments are in agreement with the ITS tree, where populations of subsp. vitaliana from the Alps and Pyrenees are not monophyletic (Fig. 1).

West-to-east differentiation of Anthyllis montana. — A pectinate branching pattern of the ITS and AFLP phylogenies is described (Kropf & al., 2002a), in which the Iberian populations are basal, followed by those from the western, southern, central and eastern Alps, and the Balkan Peninsula. These phylogenetic reconstructions suggest an eastward migration (Fig. 2). Tree resolution of European populations also revealed fragmentation into western and eastern areas corresponding approximately with subsp. hispanica/montana and jacquinii/atropurpurea, respectively. Character evolution of floral traits based on morphometric and AFLP analyses leads to the conclusion that A. montana contains only two infraspecific taxa (subsp. montana and jacquinii) as a result of vicariance in W-E Europe (Kropf & al., 2002b). Evidence of a secondary contact was observed in the Alps Maritimes/Liguria region. Estimation of a maximum age of 700,000 years indicates infraspecific divergence in Late Quaternary times (Kropf & al., 2002a).

South-to-north colonization and increment of polyploidy in Arenaria tetraquetra. — As part of a wider investigation in Arenaria sect. Plinthine (Valcárcel, Nieto & Vargas, unpubl.), preliminary data of ITS sequence evolution reveal that populations of subsp. tetraquetra may have colonized the Pyrenees from SE
Iberia (Fig. 3). Differentiation northwards from SE Iberian populations of subsp. *amabilis* (2x) and subsp. *murcica* (3x, 4x, 5x) is in agreement with a previous hypothesis of increment of chromosome complements (Nieto & Favarger, 1988). Assuming a clock-like evolution of the ITS region, this mode of speciation in a ploidal series is occurring since the onset of the Pleistocene (c. 1.75 Myr). The possibility of increment of chromosome complements via hybridisation (allopolyploidization) should be cautiously contemplated (Nieto & Favarger, 1988). Phylogenetic reconstructions based on chloroplast markers (already in progress) may shed further light on modes of speciation and colonization of *A. tetraquetra*.

**Static diversification in Juniperus communis.**—Lack of molecular variation, not only within var. *saxatilis* from Iceland to the Caucasus but also with respect to var. *communis*, suggests a single origin of this species and a relative static diversification pattern in Europe (Fig. 4). Higher levels of variation have been obtained by using AFLP data in *J. communis* (van der Merwe & al., 2000), suggesting multiple colonization patterns at a local scale (the British Islands). Identity of *trnL-F* sequences among populations separated over 3000 km, coupled with active dispersal of fleshy cones of *J. communis* (Jordano, 1993; Martínez & Vargas, 2002), lead us to interpret that relatively recent colonization may have occurred across Europe. A more variable molecular marker is necessary to test this interpretation.

**Colonization of Saxifraga oppositifolia in the Iberian Peninsula from the arctic.**—Survival of an angiosperm (*Saxifraga oppositifolia*) in the arctic and further circumpolar migration from Beringia has been recently documented (Abbott & al., 2000). It has also been hypothesized that lack of geographical genetic patterns in the Alps may be the result of both immigrations after glaciations and *in situ* survival (Holderegger & al., 2002). Phylogeographic studies including populations from Mediterranean Europe have not been addressed for this species. Our results based on ITS sequences (Fig. 5) are in agreement with those based on chloroplast variation in the EuroSiberian region (Abbott & al., 2000; Holderegger & al., 2002). Terminal placement of ITS accessions in a sequential branching pattern indicates that colonization in the Iberian Peninsula occurred from the north. Ancient arctic lineages may have been present across Europe pre-dating Quaternary episodes (Abbott & al., 2000). Maximum divergence times between 0.88 and 0.30 Myr is herein estimated for the Iberian populations. Interestingly, identity of ITS sequences in four separated
mountain ranges of Europe (Dolomites, Pyrenees, Cantabrian range, and Sierra Nevada) indicates genetic affinities (Fig. 5). Presence both of ancient and recent lineages depicts a complex genetic structure not only in the Eurosiberian region of Europe (Abbott & al., 2000), but also in the Mediterranean region. The finding of additive polymorphism in ITS sequences supports the likelihood of hybridization processes. Some genotypes surviving in Iberia after Quaternary climatic changes may have had secondary contacts, as in northern Europe (Gabrielsen & al., 1997) and the Alps (Holderegger & al., 2002). Further investigations using markers unaffected by concerted evolution (dissimilar to ITS sequences) are needed to evaluate the impact of secondary contacts on the genetic structure of southern European populations. Acquisition of alternate leaves in subsp. paradoxa (Pyrenees) seems to be derived from opposite leaves, a character described for all the other subspecies (Fig. 5). A larger sample of the entire subsection Oppositifoliae (already in progress by R. Holderegger, pers. comm.) may shed light on character evolution within S. oppositifolia.

**Differentiation of Eurosiberian and Mediterranean populations of Saxifraga pentalactylis.** — Our phylogenetic hypothesis (Fig. 6) is in agreement with previous results of genetic drift in mountains of the Iberian Peninsula (Vargas, 2001). Populations of subsp. wilkommiana do not form a natural group because morphological differentiation, as described in two taxonomic treatments (Webb & Gornall, 1989; Vargas 1997), do not reflect isolation between Eurosiberian (Cantabrian range, Pyrenees, Sierra de Urbión) and Mediterranean (Sierra de Guadarrama, Sierra de Gredos) mountains. In the same mountain range (Sistema Central), genetic isolation through predominant autogamy in subsp. almanzorii (Sierra de Gredos) from populations of subsp. wilkommiana has already been described (Vargas, 2001). Differentiation of leaf and flower morphologies is estimated to occur at most in the last 3.55 Myr, even though it is most plausible at the onset of the Pleistocene (c. 1.75 Myr).

**Differentiation from relict, Cantabrian populations of Soldanella alpina.** — Low levels of ITS sequence variation (one nucleotide substitution between subspp. alpina and cantabrica) were found (Zhang & al., 2001). The AFLP tree supports a basalmost placement of subsp. cantabrica, followed by populations of subsp. alpina in a sequential fashion, where populations from the Pyrenees and the Balkan Peninsula are included in groups of populations from the Alps (Fig. 7). As the Pyrenees are situated between the Alps and the Cantabrian range, two interpretations are considered for the occurrence of S. alpina in the Pyrenees: (1) colonization to the Pyrenees from the Alps after long-distance dispersal from the Cantabrian range to the Alps; and (2) early colonization from the Cantabrian range, extinction, and re-colonization from the Alps. Seed dispersal inferred by using chloroplast markers would help to test these hypotheses, particularly in this case because evidence of hybridisation and introgression based on morphological, nuclear DNA, and distributional patterns has been documented (Zhang & al., 2001). Using a conservative estimated rate, divergence between subspp. alpina and cantabrica may have taken place in the last 150,000 years, coinciding with the Late Penultimate Glacial (van Andel & Tzedakis, 1996).

To explore patterns of colonization and evolution of alpine plants, cladistic analyses based on DNA sequences help infer hierarchical relationships of populations. Because of limitations in cytotoxic characters, Stebbins (1984) recommended use of cladistics and sequences of mitochondrial, 

![Fig. 7. Distribution (A) and phylogeographic hypothesis (B) for Soldanella alpina (C, subsp. alpina; Spain, Pyrenees, Bonaigua Pass) based on ITS sequences and AFLP variation. Hypothetical migration routes (arrows) inferred from Zhang & al. (2001).](image-url)
chloroplast, and nuclear ribosomal DNA for comparisons between closely related species and races of the same species. This author warned that no absolute rules or generalizations could be made on migration patterns of arctic-alpine flora. The above-discussed plant groups occurring in southern Europe support this prediction also for alpine species in Mediterranean mountains. Some populations of the seven species studied in the present paper cluster together into monophyletic groups of subspecies. A search for naturalness in the evolution of alpine plants reveals that populations of *Androsace vitaliana* subsp. *flosjugorum* (Fig. 1), *Anthyllis montana* subsp. *hispanica* (Fig. 2), *Arenaria tetraquetra* subsp. *amabilis* (Fig. 3), and *Soldanella alpina* subsp. *alpina* (Fig. 7) form natural groups in the phylogenetic reconstructions. Lack of resolution in some reconstructions is the result of identity of sequences (*Juniperus communis*), low number of characters (*Androsace vitaliana*) or character additivity (*Saxifraga oppositifolia*). In contrast, some subspecies recognized in recent classifications are not supported by molecular evidence for monophyletic populations: *Anthyllis montana* subspp. *montana* and *jacquinii* (Fig. 2), *Arenaria tetraquetra* subsp. *tetraquetra* and *murcica* (Fig. 3), and *Saxifraga pentadactylis* subsp. *willkommiana* (Fig. 6). Island-like distribution of alpine plants in southern Europe suggests that allopatry appears to be the main force for divergence and isolation of subspecies. Intraspecific taxa are considered to be intermediate stages in the speciation process in a geographic context (Stuessy, 1990), and it is expected that the phylogenetic status will vary over time to achieve monophyly via sorting and extinction of lineages in a sequence of polyphyly → paraphyly → monophyly (Rieseberg & Brouillet, 1994).

Time of diversification and colonization was outlined by applying a conservative rate of ITS sequence evolution at the highest pairwise distances in all cases. As a result, we found that maximum age of morphological differentiation in southern Europe for six of the seven study cases, expressed by subspecific taxa, is significantly different for each plant group: *Androsace vitaliana* (< 0.66 Myr); *Anthyllis montana* (< 0.88 Myr); *Arenaria tetraquetra* (< 1.92 Myr); *Saxifraga oppositifolia* (< 1.37 Myr); *Saxifraga pentadactylis* (< 1.75 Myr); and *Soldanella alpina* (< 0.15 Myr). In any case, subspecific taxa appear to have differentiated in the Pleistocene (< 1.75 Myr, www.iugs.org/iugs/pubs/instratchart.htm) in most of the estimates using different calibration points. The above figures are higher than time of speciation calculated in oceanic islands (< 0.73 for silverswords alliance, Baldwin & Sanderson, 1998; < 0.79 for *Dendroseris*, Sang & al., 1994), but similar to diversification rates within herbaceous species from Mediterranean floristic regions of America (< 1.5 Myr for *Sanicula*; Vargas & al., 1999b). In contrast, speciation of a group of seven gentians (*Gentiana sect. Ciminalis*) has been hypothesized to have occurred in the Pleistocene (Hungerer & Kadereit, 1998). Interestingly, the estimates of infraspecific variation herein presented based on molecular data are mostly in consonance with previous estimates of speciation rates for herbs (1.15 Myr) based on chromosomal evolution (Levin & Wilson, 1976). Generalization of diversification times for angiosperms, herbaceous plants, members of the same family, or even species within the same genus should be cautiously contemplated because every plant group undergoes unique differentiation histories (Hillis & al., 1996b).

Diversification rates across taxa are the result of idiosyncratic characteristics of each species, extinction of their populations, and taxonomic artefacts (Barraclough & Nee, 2001). Morphological differentiation of the seven species has resulted in fluctuating taxonomic treatments at the infraspecific level. Intraspecific taxa have been long discussed and subjected to historical interpretations (Stuessy, 1990). Irrespective of taxonomic subjectivity, present populations of alpine European taxa display morphological differentiation as a result of sorting and extinction primarily in the Pleistocene. Further studies using taxonomic treatments of additional genera, morphological differentiation, and molecular divergence rates will serve to test the hypothesis that infraspecific variation does not predate the Quaternary climatic changes in a significant number of cases.

Three basic hypotheses on glacial survival are proposed: (1) total extinction through glaciations (*tabula rasa*), as considered for thermophilous plants in the arctic (Brochmann & al., 1996) and trees in central and northern Europe (Demesure & al., 1996; Dumolin-Lapegue & al., 1997; King & Ferris, 1998; Raspé & al., 2000; Petit & al., 2002); (2) *in situ* persistence of populations within glaciated regions in ice-free mountains (nunataks) (Steinhil, 2000; Gugerli & Holderegger, 2001); (3) glacial-induced migration to lowland and peripheral refugia, followed by recolonization of alpine habitats after ice-sheet retreat (altitudinal migration) (Schönswetter & al., 2002; Gutiérrez & al., 2002). Unlike extinction of most trees and arctic plants in central and northern Europe under the *tabula rasa* hypothesis, populations of alpine species in Mediterranean mountains may have primarily survived by altitudinal migrations due to absence of an ice sheet across lowlands in southern Europe (Van Andel & Tzedakis, 1996; Hewitt, 2000). The importance of re-colonization from nunatak areas should be analysed in future investigations. Therefore, recurrent altitudinal re-colonizations of neighbouring populations, as found in *Armeria* from Sierra Nevada (Gutiérrez & al., 2002), and migrations from northern regions, as inferred in *Saxifraga oppositifolia* from the
arctic (Holderegger & al., 2002; this paper), may account, in part, for present distribution and genetic structure of alpine plants in Mediterranean Europe. Additionally, colonization patterns in the four geographic directions (north-to-south, south-to-north, east-to-west, and west-to-east) are herein also documented.

Multiple diversification patterns are indeed promoted by disparate biological characteristics (population structure, phylogenetic relationships, breeding system, dispersal syndromes, and ecological requirements), together with geologic complexity of the European continent, recent climatic events (glaciations), and pre-Holocene genetic structure (Taberlet & al., 1998; Comes & Kadereit, 1998; Hewitt, 2000). Lack of parallelism in diversification patterns among our seven plant groups, which share similar phylogenetic relationships (Primulaceae, Saxifraga spp.), dispersal syndromes (neighborhood diffusion through seeds in capsules for five cases), habitat requirements (rocky slopes), breeding systems (predominant allogamy), and existence in the same continent during Quaternary episodes, indicates additional causes. Most alpine species are distributed independently of one another along mountain ranges in southern Europe, which implies per se different colonization and extinction histories. Recent studies by Taberlet (2002) and collaborators on phylogeography of six alpine species sharing similar distributions and habitats also illustrate that phylogeographic patterns are not concordant (see also Gugerli & Holderegger, 2001). Additional biotic and abiotic causes may be responsible for disparate evolutionary patterns of alpine plants, including random processes. Stochastic dispersal in Saxifraga, with a non-specific long-distance syndrome, appears to have been involved in colonization of Madeira from the continent (c. 1000 km), but isolation of Mediterranean populations of S. globulifera by the formation of the Strait of Gibraltar (separated by only c. 14 km) appears to have been successful (Vargas & al., 1999a). This surprising result is in agreement with a considerable high number (c. 29 %) of colonizers of Macaronesia displaying non-specific long-distance syndromes (Vargas, in press). Ecological facilitation (Callaway, 1995), in which positive interactions among plants facilitate establishment and survival of species, has also played an important role in stochastic processes for colonization. In this context, it has been recently demonstrated that interactions among alpine plants of physically harsh environments in mountains of America and Eurasia are predominantly positive (Callaway & al., 2002), including Juniperus communis, Saxifraga oppositifolia, and Soldanella alpina in the study. Comparative phylogeography of alpine plants lead us to conclude that we are not only far from compiling data from a significant number of species to infer common patterns, but also to comprehend all factors responsible for present distributions and predominant modes of speciation in Mediterranean Europe.

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**LITERATURE CITED**


