**Disjunct distribution of Hypericum nummularium L. (Hypericaceae): molecular data suggest bidirectional colonization from a single refugium rather than survival in distinct refugia**

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*Hypericum nummularium* has a strongly disjunct, bi-areal distribution in Europe: it is abundant in the Pyrenees and grows in a very restricted part of the Alps, more than 1000 km away. My aim was to estimate the genetic divergence between these areas and to identify the factors responsible for the disjunction: glacial relicts, bidirectional colonization from a common refugium, long-distance dispersal and/or human introduction? Internal transcribed spacers (ITS) sequencing (680 bp) and amplified fragment length polymorphism (AFLP) fingerprinting (104 polymorphic markers) showed very low differentiation between populations in the Alps and the Pyrenees, indicating that *H. nummularium* probably survived in a single refugium. Moreover, levels of genetic diversity were similar in the two areas, making human introduction and long-distance dispersal unlikely. Thus, the species probably survived in one refugium, subsequently colonizing both areas more or less simultaneously. The comparison of genetic and geographical distances suggested a step by step migration in the Alps (isolation by distance), whereas random dispersal events were more likely in the Pyrenees. Finally, I discuss possible causes for the restricted distribution area of *H. nummularium* in the Alps (e.g. unsuitable habitat, low dispersal capacities) and conclude that strong human disturbance is probably the major limit to the expansion of the species in this region. © 2006 The Linnean Society of London, Biological Journal of the Linnean Society, 2006, 87, 437–447.


**INTRODUCTION**

Understanding how species colonized their present distribution areas remains a major challenge of biogeography. This is important not only to improve basic scientific knowledge, but also to anticipate potential distribution changes (expansion, regression and shifts) that appear likely in the perspective of increasing anthropogenic disturbance and rapid climatic change. By merging phylogenetics and population genetics with biogeography, phylogeography (Avise, 2000) provides useful information for the delineation of evolutionarily significant units (ESU; Moritz, 1994) and the location of glacial refugia. Because ESU are, by definition, important components in the evolutionary history of a species, and glacial refugia are likely to represent hotspots of biodiversity (Taberlet & Cheddadi, 2002; but see Petit et al., 2003), these particular units and geographical areas should be protected first.

The present distributions of species are determined by many factors, such as the availability of suitable habitats (e.g. substrate specificity, light and water requirements) and the capacity to colonize (dispersal ability and presence of physical barriers). Phylogeog-
raphy has also revealed the dominant role of historical events (e.g. climate changes) in the geographical distribution and genetic structure of species. This is especially relevant for plants, which are sessile and cannot undergo rapid range expansion through migration. In the northern hemisphere, species have been strongly influenced by the long glacial episodes of the Pleistocene (with the last glacial maximum ending about 10 000 years ago), when suitable environment was much more restricted than it is in the present interglacial (Comes & Kadereit, 1998). At that time, species were restricted to ice-free, sometimes distant areas, from which they subsequently colonized their present-day distribution when glaciers retreated. There is an increasing amount of data about the distribution and recolonization history of plant species in the Alps (e.g. Holderegger, Stehlik & Abbott, 2002; Stehlik et al., 2002b; Stehlik, Schneller & Bachmann, 2002a; Tribisch, Schönswetter & Stuessy, 2002; Reisch, Poschlod & Wingender, 2003; Schönswetter et al., 2003, 2004). Within this mountain range, many taxa exhibit disjunct distributions that are often hypothesized to be climatic relics of the ice ages (Hewitt, 1996, 2000; Stehlik et al., 2000). The Alpine–Pyrenean disjunction has been much less investigated (but see Krof, Kadereit & Comes, 2002, 2003), but the influence of disjunction on diversification pathways of species and/or populations makes it very attractive to investigate in the field of evolutionary biology.

Advances in DNA technology have allowed the use of molecular tools to reconstruct the history of species. However, a major limitation in large-scale studies of plant population genetics is the availability of a suitable molecular marker, displaying an adequate level of polymorphism (reviewed in Karp, Seberg & Buiatti, 1996; Schaal et al., 1998). Chloroplast DNA presents the advantages of uniparental inheritance, lower effective population size than nuclear DNA and absence of recombination, but the level of polymorphism often proves too low to investigate intraspecific questions. Thus, many studies considered nuclear DNA. The relatively small and conserved size of internal transcribed spacers (ITS), their high mutation rates (Hillis & Dixon, 1991) and their easy amplification with universal primers (Baldwin et al., 1995) make them an attractive and extensively used tool for phylogenetic analysis (reviewed in Baldwin et al., 1995; Soltis & Soltis, 1998). Few attempts have been made to investigate ITS genetic diversity within species and within populations. While some studies have reported low ITS resolution (Soltis & Kuzoff, 1993; Givret & Petit, 2002; Krof et al., 2002; Holderegger & Abbott, 2003), others found substantial levels of variation (Brochmann et al., 1998; Vargas, Morton & Jury, 1999). The amplified fragment length polymorphism approach (AFLP; Vos et al., 1995) provides an alternative approach (Després et al., 2003). It has become a popular choice for studies of plant population genetics because it does not require any knowledge of the genome and quickly provides a large number of markers. Because AFLP markers are usually highly polymorphic, their use is limited to low taxonomic levels (within species or among closely related species; Mueller & Wolfenharger, 1999).

Hypericum nummularium L. (Hypericaceae) is a perennial herbaceous plant species with a bi-areal distribution range. It is very common throughout the Pyrenees (across France and Spain) but its distribution is restricted in the Alps, where it is only found in the 25 × 50-km Nature Reserve of Chartreuse near Grenoble, France, and in adjacent Southern Bauges (only one site). Since some natural sites located between the Alps and the Pyrenees do appear to meet the ecological requirements of the species (calcareous bedrock, sunny places), the main question is, which historical processes lead to the present distribution pattern? The bi-areal distribution of H. nummularium most likely reflects its survival and recolonization history during and after the last ice ages. Specifically, it could result from three different scenarios: (i) survival in two distinct and geographically distant refugia, with the expectation of two genetically well-differentiated groups; (ii) survival in a single refugium outside the present distribution area of the species from which it would have colonized both the Alps and the Pyrenees more or less simultaneously. This latter scenario should result in similar (rather low) levels of genetic diversity in the Pyrenees and the Alps, with no or only slight genetic differentiation between them. (iii) The third scenario involves survival in a single refugium (either in the Pyrenees or in the Alps) from which the species would have colonized the other vacant area, reaching it by long-distance dispersal between the two mountain ranges. As the species is much more abundant in the Pyrenees than in the Alps, colonization of the Alps from the Pyrenees seems more likely. In particular, the probable use of H. nummularium in a commercialized local liqueur renders human introduction possible. In the light of the third scenario, both areas should belong to one gene pool, but the source area should be genetically much more diverse than the sink area due to founder effects during colonization of the latter. To assess the levels of genetic diversity and differentiation within and between the Pyrenees and the Alps in H. nummularium, I included H. maculatum and H. richeri as reference groups. The genetic characterization of the isolated populations in the Alps is of particular importance, because in the case of their unique genetic setup, conservation measures should be recommended to protect them.
MATERIAL AND METHODS

PLANT MATERIAL

The Hypericum genus comprises about 20 species in France. H. nummularium usually grows between 500 and 2500 m high, in calcareous, sunny and rocky habitats. The aerial part of the plant is composed of one or several 10–30-cm high quadrangular stems, opposite orbiculate leaves, and 2–3-cm large flowers with yellow, finely black punctated petals. H. maculatum is common throughout Europe and Russia, whereas H. richeri is restricted to the Alps, Apennines and Pyrenees. These three species occur in both the Alps and the Pyrenees but their ecological requirements differ; they are taxonomically clearly distinguished and no hybrid is known. In the related species H. perforatum, the mode of reproduction is highly variable (vegetative propagation, sexual and asexual seed formation) and polyploidy occurs (Pank et al., 2003). No similar data are available on H. nummularium, H. richeri and H. maculatum.

Twelve populations of H. nummularium, four populations of H. richeri and four populations of H. maculatum were sampled. In each case, half the populations originated from the Alps and the other half from the Pyrenees. Each population was coded as follows: the first two letters represent the initials for genus and species (Hn, Hr and Hm), the third one stands for Alps or Pyrenees and the number identifies populations from the same geographical area (Fig. 1, Table 1). In each population, leaf material from eight individuals was collected, dried immediately in silica gel, and stored at room temperature. Because vegetative propagation was suspected, individuals were always sampled several metres from each other.

AFLP PROCEDURE

DNA extraction was performed with the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany), following the manufacturer’s protocol. The AFLP method was

Figure 1. Geographical location of the 12 studied populations of Hypericum nummularium (Hn) in the Alps (A) and in the Pyrenees (P). The numbers distinguish between populations from the same geographical area.

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carried out as described in Gaudeul, Taberlet & Till-Bottraud (2000): the EcoR1 adapter was 5′-CTCGTAGACTGCGTACC-3′ and Mse1 adapter was 5′-GACGATGAGTCCTGAG-3′. After total genomic digestion and ligation of double-stranded adapters, preselective amplification was performed with the following parameters: 2 min at 72 °C, then 25 cycles of 30 s denaturing at 94 °C, 30 s annealing at 56 °C, and 2 min extension at 72 °C, ending with 10 min at 72 °C to complete extension, using the EcoR1 primer E.A (5′-CTCGTAGACTGCGTACCA-3′) and Mse1 primer M.C (5′-GACGATGAGTCCTGAGC-3′). For selective amplification, the PCR parameters were: 10 min at 95 °C, then 36 cycles of 30 s denaturing at 94 °C, 1 min annealing and 1 min elongation at 72 °C, ending with 10 min at 72 °C to complete extension. Annealing was initiated at a temperature of 65 °C, which was then reduced by 0.7 °C for the next 12 cycles and maintained at 56 °C for the subsequent 23 cycles. Preliminary tests were performed on four samples (one sample of Alpine H. nummularium, one sample of Pyrenean H. nummularium, and two Alpine samples of H. richeri and H. maculatum), and two selective primer pairs (E.AGT/M.CTA and E.ATG/M.CAA) were chosen for the quality of the resulting AFLP patterns (i.e. even distribution of bands with relatively homogeneous intensity). Fragments were finally separated by electrophoresis for 6 h on a 5% polyacrylamide gel (automated sequencer ABI 377, Applied Biosystems). AFLP patterns were visualized with GeneScan Analysis 3.1 (Applied Biosystems) and a presence/absence (i.e. 0/1) matrix was constructed manually.

**ITS SEQUENCING**

PCR amplifications of the complete ITS region (ITS1, 5.8S and ITS2) were performed with primers ITS-4 (5′-TCTCCGCTTATATGCAGC-3′) and ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) of White et al. (1990) in a 24-µL volume containing 0.2 mM each dNTP (Applied Biosystems), 0.5 µM each primer (Genset Oligo), 2.5 mM MgCl₂ (Applied Biosystems), 1 U Taq polymerase (Applied Biosystems), and 1× Taq buffer (Applied Biosystems). The PCR parameters were: 10 min at 95 °C, then 40 cycles composed of 45 s denaturing at 95 °C, 1 min annealing and 1 min elongation at 72 °C, ending with 10 min at 72 °C to complete extension. Annealing was initiated at a temperature of 65 °C, which was then reduced by 0.7 °C for the next 12 cycles and maintained at 56 °C for the subsequent 23 cycles. Preliminary tests were performed on four samples (one sample of Alpine H. nummularium, one sample of Pyrenean H. nummularium, and two Alpine samples of H. richeri and H. maculatum), and two selective primer pairs (E.AGT/M.CTA and E.ATG/M.CAA) were chosen for the quality of the resulting AFLP patterns (i.e. even distribution of bands with relatively homogeneous intensity). Fragments were finally separated by electrophoresis for 6 h on a 5% polyacrylamide gel (automated sequencer ABI 377, Applied Biosystems). AFLP patterns were visualized with GeneScan Analysis 3.1 (Applied Biosystems) and a presence/absence (i.e. 0/1) matrix was constructed manually.

**Table 1. Characteristics and AFLP genetic diversity of the 12 studied populations of Hypericum nummularium**

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Altitude</th>
<th>Number of plants</th>
<th>Nei index</th>
<th>Percentage of polymorphic markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>HnP01</td>
<td>Couflens</td>
<td>1095</td>
<td>100–1 000</td>
<td>0.30</td>
<td>82.2</td>
</tr>
<tr>
<td>HnP03</td>
<td>Bagnères de Bigorre</td>
<td>1910</td>
<td>1 000–10 000</td>
<td>0.27</td>
<td>70.2</td>
</tr>
<tr>
<td>HnP04</td>
<td>Sengouaguet</td>
<td>1820</td>
<td>100–1 000</td>
<td>0.20</td>
<td>57.7</td>
</tr>
<tr>
<td>HnP05</td>
<td>Gourette</td>
<td>1745</td>
<td>100–1 000</td>
<td>0.22</td>
<td>60.6</td>
</tr>
<tr>
<td>HnP07</td>
<td>Bagnères de Bigorre</td>
<td>1770</td>
<td>100–1 000</td>
<td>0.28</td>
<td>72.1</td>
</tr>
<tr>
<td>HnP10</td>
<td>Asturies, Spain</td>
<td>800</td>
<td>10–100</td>
<td>0.30</td>
<td>82.2</td>
</tr>
<tr>
<td>Average (Pyrenees)</td>
<td></td>
<td></td>
<td></td>
<td>0.26 ± 0.04</td>
<td>70.8 ± 9.5</td>
</tr>
<tr>
<td>Overall (Pyrenees)</td>
<td></td>
<td></td>
<td></td>
<td>0.33</td>
<td>98.1</td>
</tr>
<tr>
<td>HnA01</td>
<td>Voreppe</td>
<td>520</td>
<td>&lt; 50</td>
<td>0.30</td>
<td>72.1</td>
</tr>
<tr>
<td>HnA02</td>
<td>Saint Jean de Chevelu</td>
<td>1300</td>
<td>100–1 000</td>
<td>0.21</td>
<td>52.9</td>
</tr>
<tr>
<td>HnA03</td>
<td>Saint Pierre de Chartreuse</td>
<td>1770</td>
<td>80</td>
<td>0.17</td>
<td>44.2</td>
</tr>
<tr>
<td>HnA04</td>
<td>Saint Pierre d’Entremont</td>
<td>1550–1650</td>
<td>&gt; 10 000</td>
<td>0.17</td>
<td>76.0</td>
</tr>
<tr>
<td>HnA05</td>
<td>Entremont le Vieux</td>
<td>1380</td>
<td>70</td>
<td>0.27</td>
<td>68.3</td>
</tr>
<tr>
<td>HnA06</td>
<td>Saint Pierre de Chartreuse</td>
<td>1330–1340</td>
<td>100–1 000</td>
<td>0.19</td>
<td>49.0</td>
</tr>
<tr>
<td>Average (Alps)</td>
<td></td>
<td></td>
<td></td>
<td>0.22 ± 0.05</td>
<td>60.4 ± 12.2</td>
</tr>
<tr>
<td>Overall (Alps)</td>
<td></td>
<td></td>
<td></td>
<td>0.34</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Data analysis

AFLP data obtained for *H. nummularium* (no data were produced for *H. richeri* and *H. maculatum*; see Results) were analysed with TFPGA 1.3 (Miller, 1997), assuming Hardy–Weinberg equilibrium within each population and taking only polymorphic markers into account. Genetic diversity within populations and within regions was quantified (i) by the percentage of polymorphic loci (out of all the polymorphic loci) and (ii) by Nei’s unbiased expected heterozygosity (Nei, 1987). A t-test was performed to determine if within-population diversities were higher in one region compared with the other, and the nonparametric correlation between the size of populations (approximate number of plants) and their genetic diversity was tested (Spearman’s rank correlation; MINITAB for Windows vers. 12.2, Minitab Inc.). Moreover, I checked the whole dataset for private alleles (unique to a population or region). An analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was carried out with GenAlEx 5.0 (Peakall & Smouse, 2001) to partition the total genetic variance into among-region, among-population and within-population components. The association between genetic distance (*Φ*st, derived from the AMOVA) and geographical distance (in km) was tested by a Mantel test in FSTAT 2.9.3 (Goudet, 1995; significance estimated by 5000 random permutations). A principal coordinate analysis (PCA) was performed in GenAlEx 5.0 and a neighbour-joining (NJ) tree was constructed with NJBS (J. M. Cornuet, pers. comm.) to visualize the genetic relationships (i) among the 96 individuals and (ii) among the 12 populations. Both PCA and NJ analyses were based on p-distances (proportion of differing sites).

For ITS sequence data, all three species – *H. nummularium*, *H. richeri* and *H. maculatum* – were analysed. Indels were considered as 1-bp substitutions and heterozygous sites were treated as undetermined. A NJ tree was constructed with MEGA 2.0 (Kumar et al., 2001), based on the percentage of site differences between all pairs of samples (p-distance; Kumar et al., 2001). Bootstrap values were obtained by running 1000 random replicates. Moreover, I calculated the average p-distance (i) between all pairs of species, (ii) within species, (iii) within species among regions, and (iv) within species within regions. When calculated between two groups (either species or regions), p-distances were corrected by subtracting half the sum of the average within-group distances. Finally, I used an AMOVA to partition the *H. nummularium* total genetic variance into among- and within-region components (among- and within-population components were not separated because of low sample sizes within populations).

Results

AFLP markers

Preliminary tests, performed with four samples and 16 primer pairs, showed that the AFLP patterns could not be compared among *H. nummularium*, *H. maculatum* and *H. richeri* because their divergence was too strong (no shared band). Therefore, the AFLP procedure was carried out on 12 × 8 = 96 samples of *H. nummularium* from the Alps and Pyrenees. The two primer pairs E.AGT/M.CTA and E.ATG/M.CAA allowed the identification of 53 and 51 polymorphic markers, respectively.

The overall Alpine and Pyrenean genetic diversities were very similar and, although indices were slightly lower in the Alpine populations compared with the Pyrenean ones, this difference was not significant (t-test, *P* > 0.05; Table 1). The correlation between population diversity and number of individuals was not significant (Spearman’s rank correlation, *P* > 0.05). No population exhibited any private alleles. However, six markers were observed in the Pyrenees but not in the Alps (or in only one individual out of the 60 Alpine samples). These markers were not rare in the Pyrenees, as all of them were found in at least three populations, with overall regional frequencies from 0.08 to 0.51.

The AMOVA showed that only 9% of the total genetic variance was found among regions, with 15% of the variance being among populations within region and as much as 76% being within populations. All these percentages were significantly different from zero (*P* < 0.001). When performed at the individual level, neither the PCA nor the NJ tree (not shown) clustered the plants into discrete populations, confirming the high proportion of genetic diversity found within populations compared with the among-population diversity. At the population level, the first two axes of the PCA explained 49.8% and 17.5% of the variance. The first axis separated the two geographical regions, whereas the second axis allowed a more or less clear distinction of the populations within each region (Fig. 2). The NJ tree (Fig. 3) showed a clear distinction (95% bootstrap support) between populations HnP03, HnP04, HnP05 and HnP07 on the one hand, and the Alpine populations that were grouped together with HnP01 and HnP10 on the other hand. All other branches were supported by low bootstrap values (below 80%). Within the Alps, the AMOVA detected 19% of the genetic variance among populations and the Mantel test between genetic and geographical distances was significant (*P* = 0.01, *r* = +0.65; Fig. 4). In spite of the much larger geographical area investigated, the among-population differentiation was lower in the Pyrenees (14% of the variance was found among populations) and the Mantel test was significantly nega-
positive ($P = 0.02, r = -0.58$; Fig. 4). This was due to the very low genetic divergence of the most distant populations, HnP01 and HnP10. When the HnP10 population was ignored, the test was not significant ($P = 0.68$).

**ITS SEQUENCING**

The ITS region was sequenced successfully in 66 samples (Table 2). The sequence was 680 bp long and 46 substitutions and five indels were identified in the overall dataset. Paralogous ITS loci have been reported in some vascular plant groups (Mayol & Rossello, 2001), caused by hybridization, introgression and/or polyploidization events, and leading to within-individual variability. Here, the high consistency of the results did not suggest the occurrence of pseudogenes and eight sites showing evidence of heterozygosity were discarded from the analysis. Only a few haplotypes were found, spanning from one to three depending on the species × region combination (Table 2). The NJ tree showed the three species to be clearly separated (Fig. 5) and, in *H. nummularium*, the two observed haplotypes were shared by the two regions. *H. nummularium* was the least diverse species, and it was also the most divergent among the three species investigated. *H. maculatum* and *H. richeri* had similar levels of diversity (Table 2). In *H. maculatum*, the average p-distance between Alps and Pyrenees was lower than was the average p-distance within the Pyrenees, due to two very divergent haplotypes in this region. Although this explanation does not hold true for *H. nummularium*, the same pattern was found (i.e. the average distance among regions was lower than within each region) and confirmed by AMOVA; only 23.7% of the genetic variance occurred among regions (not significant; 10 000 random permutations, $P = 0.064$), whereas 76.3% of the variance occurred within regions.
DISCUSSION

Both AFLP and ITS data provided evidence for the existence of a single *H. nummularium* lineage throughout the Alpine and Pyrenean mountain ranges: in contrast to the wide discrepancy of AFLP patterns among *H. nummularium*, *H. maculatum* and *H. richeri*, they were very consistent (with numerous shared bands) within *H. nummularium*, even among regions. The NJ tree constructed on ITS data further strengthened this result: haplotypes were clearly clustered into three groups, which corresponded to the three species under study.

Alpine and Pyrenean populations of *H. nummularium* appeared genetically close in spite of the large geographical distances between them (over 1000 km). This was especially striking when considering ITS nucleotide distances: in spite of the stronger sampling effort, the average genetic distance within *H. nummularium* was lower than within *H. richeri* and *H. maculatum* and, within *H. nummularium*, the average distance between Alpine and Pyrenean plants was lower than the average distance within a region. This pattern was also observed in *H. maculatum* but was due to the strong divergence between the two Pyrenean haplotypes. I also observed that only in *H. nummularium* were some haplotypes shared by Alpine and Pyrenean samples, leading to a nonsignificant among-region component of variance when the AMOVA was performed on the ITS dataset. The AMOVA carried out on the AFLP data showed a similar pattern, with only 9% of the genetic variance being among regions. Although statistically significant, this value was markedly lower compared with those obtained in two other AFLP studies investigating the Alpine–Pyrenean disjunction: the among-region component represents 14.4% and 13.1% of the total genetic variance in *Anthyllis montana* (Kropf et al., 2002) and *Pritzelago alpina* (Kropf et al., 2003), respectively. Moreover, even when studies are restricted to the Alps, the genetic variation among groups of populations often explains up to 30–40% of the total genetic variance (Schönswetter et al., 2002, 2003, 2004; Stehlik et al., 2002a; Reisch et al., 2003).

Therefore, all these results suggest that the disjunct distribution of *H. nummularium* did not lead to strong genetic differentiation between the two regions. Consequently, at the present time, conserving *H. nummularium* in the Alps is not really required to preserve the overall genetic background of the species. However, the geographical isolation, and, as a consequence, the reproductive isolation, will probably increase the genetic divergence between the two regions. This was already suggested by the significant

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**Figure 4.** Correlation of genetic ($\Phi_{st}$ calculated from AFLP data) and geographical distances between all pairs of *Hypericum nummularium* populations (A) in the Alps ($N=6$ populations) and (B) in the Pyrenees ($N=6$ populations).

**Table 2.** ITS haplotype diversity and $p$-distances within and among regions in *Hypericum nummularium*, *H. richeri* and *H. maculatum*. $N$, number of individual samples

<table>
<thead>
<tr>
<th>Haplotype number</th>
<th>Average net $p$-distance ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>within the species</td>
</tr>
<tr>
<td><em>H. maculatum</em></td>
<td>6.58</td>
</tr>
<tr>
<td><em>H. richeri</em></td>
<td>7.03</td>
</tr>
<tr>
<td><em>H. nummularium</em></td>
<td>1.83</td>
</tr>
</tbody>
</table>
among-region component of the AMOVA and the observation that although the divergence between the Alps and the Pyrenees was not very strong, the first axis of the PCA and the main branching pattern of the NJ tree clearly separated populations from the two geographical regions.

Several scenarios were proposed to explain the disjunct distribution of *Hypericum nummularium*. One scenario was of an introduction of plants from the Pyrenees to the Alps by monks. *H. nummularium* is very probably used in the preparation of a commercialized local liqueur (the recipe is secret) produced since the 18th century by monks whose monastery is located in Chartreuse. The monks were expelled from France and fled to Spain in 1903, before coming back to their Mother House of Chartreuse in 1930. Thus, they could have discovered *H. nummularium* during their stay in Spain and introduced the species in Chartreuse when they settled back in the region. However, this scenario seems very unlikely, because, despite the fact that both areas belonged to the same gene pool, the Alpine populations are not a depauperated subsample of the Pyrenean populations (the existence of alleles that are private to the Pyrenees might be explained by the much larger sampling scale in this region). For the same reason, the hypothesis of natural long-distance gene flow from the Pyrenees to the Alps (or vice versa) was excluded.

Figure 5. Unrooted neighbour-joining tree based on p-distances calculated from ITS sequencing data (680 bp) obtained from 66 samples of *Hypericum nummularium* (Hn), *H. richeri* (Hr) and *H. maculatum* (Hma). The number of samples (N) of each species × region combination is indicated in brackets. Bootstrap values are expressed as percentages and, when above 50%, are indicated next to the corresponding node.
The two alternative hypotheses included (i) survival in two geographically distinct refugia, and (ii) survival in a single refugium outside the species’s present distribution area, followed by a more or less simultaneous colonization of both present distribution areas. The main criterion for the recognition of a population group indicative of a glacial refugium is its genetic divergence. Here, the close genetic relationships observed between the Alps and the Pyrenees suggested the glacial survival of *H. nummularium* in a single refugium. Then, postglacial recolonization could have occurred either directly, from the refugium to the two distribution areas, or sequentially, from the refugium to one of the areas and later to the other area. Given that the levels of genetic diversity were similar in both regions, the colonization probably occurred directly and more or less simultaneously from the refugium to the two mountain ranges: if not, the signature of the founder effect (lower diversity, higher differentiation) would be stronger in the more recently colonized area. When the extension of glaciers was maximal, the whole Alpine range (except some peaks protruding from the ice sheet, called nunataks; Stehlik, 2003) and, to a lesser extent, the central part of the Pyrenees, were glaciated (Kaiser, 1969). By comparing patterns observed in both animal and plant species, phylogeography has identified three major glacial refugia in Europe, located in Portugal–Spain, in southern France–Italy and in the Balkans (Taberlet et al., 1998). Given that *H. nummularium* colonized both the Alps and the Pyrenees from the same refugium, it was probably located in southern France. This would be in agreement with Braun-Blanquet’s hypothesis (Carraz-Billat & Nétien, 1961), according to which the species survived glaciations in southern France and reached the Alps via a mountain range erected during the Eocene which later collapsed. Moreover, in the case of *Erinus alpinus*, Stehlik et al. (2002a) also found evidence for immigration from southern France to Switzerland (in addition to local survival on northern Alpine nunataks).

The AFLP NJ tree was not resolved within each geographical region, making it difficult to draw any firm conclusions about the relationships among populations and the precise colonization processes of *H. nummularium*. The pattern of isolation by distance was significant in the Alps but not in the Pyrenees. Moreover, among-population divergence was higher in the restricted distribution area of the species in the Alps than in the much larger Pyrenean one: it accounted for 19% and 14% of the regional genetic variance for average distances of 23 and 167 km between pairs of populations in the Alps and in the Pyrenees, respectively. These values of among-population differentiation showed that drift is more influential in the Alps than it is in the Pyrenees. Thus, it is very likely that the positive correlation between genetic and geographical distances observed in the Alps reflects the colonization process rather than recent gene exchanges. This pattern of isolation by distance suggested that the species expansion occurred along a migration wave that was followed by low gene exchange between populations. On the contrary, in the Pyrenees, the absence of isolation by distance and, more specifically, the close genetic relationships observed between the two geographically most distant populations (HnP01 and HnP10) suggest that colonization was mediated by random, long-distance dispersal events. Given that *H. nummularium* seeds are very small, birds could be involved in such events (e.g. *Trichodroma muraria*; Carraz-Billat & Nétien, 1961).

Why is *H. nummularium* not more widespread in the Alps? Given that ecologically suitable habitat seems available (e.g. the Vercors mountain range, close to Chartreuse), other factors must be invoked to explain the limited range of the species in this region. Similar levels of diversity in the Alps and the Pyrenees suggest that the colonization of *H. nummularium* occurred roughly simultaneously in both regions. Thus, the time scale is not likely to have been an important restriction to the expansion of the species. Moreover, the fact that the species colonized the whole Pyrenean range allowed us to exclude the hypothesis of low dispersal capacity. Finally, no major difference in the interspecific relationships of *H. nummularium* has been reported between the two geographical areas (e.g. presence of a competing species or absence of a dispersing species in the Alps compared with the Pyrenees). Therefore, human disturbance is probably the major factor impeding colonization of new sites in the Alps: the Chartreuse Nature Reserve, where *H. nummularium* grows, is characterized by high precipitation and calcareous substrate and is separated from similar ecological habitats by strongly anthropized areas. Human influence seems considerably lower in the Pyrenean region.

By using both ITS sequencing and AFLP markers, this study has shown that although disjunct distributions are often interpreted as the signature of multiple glacial refugia, they can also occur after survival in a single refugium. Further investigations of the biogeography of *H. nummularium* will require increased sampling designs to investigate the within-region patterns of genetic diversity. It is noteworthy that other plant species display peculiar distribution patterns, growing in very distant regions and being ubiquitous in one while quite rare in the other. This is the case in, for example, *Arenaria purpureascens*, which, like *H. nummularium*, is common in the Pyrenees but found only in the Chartreuse area within the Alps (R. Douzet, pers. comm.). Investigating the phylogeography of *A. purpureascens* and comparing the patterns

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obtained on both species could provide valuable information on the factors determining their present distribution area (although a shared pattern does not necessarily result from the same causes). Phylogeography is also faced more and more often with increasing and unpredictable human influence on the fate of species. Although the hypothesis of human transport was ruled out in the case of *H. nummularium*, this possibility (either inadvertent or on purpose) should not be underestimated, especially when a species occurs at a single, very remote, site from its main distribution area.

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REFERENCES


DISJUNCT DISTRIBUTION OF HYPERICUM NUMMULARIUM  


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