American Journal of Botany 95(5): 577–587. 2008.

GENETIC STRUCTURE AND DIVERSITY IN *RAMONDA MYCONI* (GESNERIACEAE): EFFECTS OF HISTORICAL CLIMATE CHANGE ON A PREGLACIAL RELICT SPECIES[1]

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The importance of the Mediterranean Basin as a long-term reservoir of biological diversity has been widely recognized, although much less effort has been devoted to understanding processes that allow species to persist in this area. *Ramonda myconi* (Gesneriaceae) is a Tertiary relict plant species restricted to the NE Iberian Peninsula. We used RAPD and chloroplast markers to assess the patterns of genetic structure in eight mountain regions covering almost the full species range, to identify the main historical processes that have shaped its current distribution and to infer the number and location of putative glacial refugia. While no cpDNA variation was detected, the species had relatively high levels of RAPD variation. Maximum levels of diversity were found within populations (71%), but there was also a significant differentiation between geographical regions (20%) and among populations within regions (9%). A spatial AMOVA identified three main groups of populations, corresponding to previously recognized centers of endemism and species richness. In addition, we found a marked geographical pattern of decreasing genetic diversity and increasing population differentiation from west to east. Our results support a complex phylogeographic scenario in the Iberian Peninsula of "refugia-within-refugia" and suggest that the higher diversity observed in western regions might be associated with prolonged and more stable climatic conditions in this area during the Quaternary.

Key words: chloroplast markers; fragmentation; Gesneriaceae; glacial refugia; Mediterranean climate; NE Iberian Peninsula; paleoendemic species; Quaternary climatic changes; *Ramonda myconi*; RAPD.

The Mediterranean Basin has been recognized as one of the biodiversity "hot spots" in the world (Myers et al., 2000), characterized by high levels of plant diversity and endemism. About 60% of the native species in the Mediterranean flora are endemic to the region (Greuter, 1991). The factors giving rise to the high species diversity in this area are manifold, including paleoclimatic history, ecogeographical heterogeneity, and the long-standing influence of man in shaping current landscape structure (Cowling et al., 1996; Blondel and Aronson, 1999; Thompson, 2005). The high proportion of plant endemics in the Mediterranean Basin can be explained, at least in part, by the ability of many species to survive the effects of Quaternary climatic changes (Blondel and Aronson, 1999). Good examples are provided by the so-called paleoendemisms, which represent the remaining evidence from the Tertiary paleoflora (Thompson, 2005).

The importance of the Mediterranean Basin as a reservoir of genetic diversity has been supported by the numerous phylogeographic studies conducted during the last decade. These studies have recognized the presence of three main glacial refugia in this area (Iberian Peninsula, Italy, and the Balkans) and have demonstrated the importance of these glacial refugia for the subsequent expansion and postglacial recolonization of the European continent (Comes and Kadereit, 1998; Taberlet et al., 1998; Hewitt, 1996, 1999, 2000), though there is increasing evidence of additional refugia for temperate species further north in central and eastern Europe (Kotlík et al., 2006; Magri et al., 2006, among others). This research area has benefited from the application of molecular markers and theoretical models of population genetics, beyond the limitations imposed by the quality of the fossil record for plant species other than wind-pollinated trees (Cruzan and Templeton, 2000).

At the continental scale, phylogeographic studies have mainly focused on the location of the major refugia, as well as on tracing the main migration routes followed by species during northward expansions (Hewitt, 1996, 1999; Taberlet et al., 1998). In contrast, much less is known about genetic diversity and population differentiation within southern refugia. This is somewhat surprising because these regions have enabled the long-term survival of species in spite of long periods of climate oscillations and they could have played a major role in the survival and evolution of many European taxa (Hampe and Petit, 2005). Thus, even though much work has been done to identify major refugia, relatively few studies have focused on the exact location of geographical areas that have prevented the extinction of species during glacial periods. Initially, southern refugia were thought to harbor more genetic diversity than recolonized areas, but recent studies have shown that this higher genetic diversity only holds at the regional scale and that most Mediterranean relict populations often have reduced within-population genetic diversity and high genetic distinctiveness, thereby increasing their conservation interest (Comps et al., 2001; Hampe et al., 2003; Petit et al., 2003). In the case of the Iberian Peninsula, for instance, a number of recent studies have depicted a high degree of genetic structure for many animal and plant species, thus favoring the notion of this region as a complex pattern of "refugia-within-refugia" (Gómez and Lunt, 2006, and references therein). Some examples include tree species (*Quercus*

[1] Manuscript received 4 October 2007; revision accepted 27 February 2008.

The authors gratefully acknowledge S.C. González-Martínez, J.A. Rosselló, Ll. Sáez, and V. Rull for constructively criticizing the manuscript. They also thank J. Terradas for financial support, M. Pitet for laboratory assistance, and X. Domene for field work. This research was supported by funds from Institut d'Estudis Catalans (project number LF22I003) and by a grant (2004FI01255) from DURSI (Departament d'Universitats, Recerca i Societat de la Informació, Generalitat de Catalunya) to M. Dubreuil.

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doi: 10.3732/ajb.2007320

L., Lumaret et al., 2002; Olalde et al., 2002; *Pinus pinaster* Ait. and *P. halepensis* Mill., Gómez et al., 2005), short-lived plants (*Senecio gallicus* Vill., Comes and Abbott, 2000), butterflies (*Erebia triaria* De Prunner, Vila et al., 2005), and amphibians (*Lissotriton boscai*, Lataste, 1879; Martínez-Solano et al., 2006).

In this context, the analysis of the genetic structure of populations of Tertiary paleoendemisms is particularly appropriate because they represent relict lineages that have survived in sheltered habitats during the strong Quaternary climatic oscillations. These organisms provide a good model system to ascertain how plant species have responded to previous climatic events. A good example of Tertiary relict species is found in the Gesneriaceae, a tropical family with a few extant species distributed in Europe: *Ramonda myconi* (L.) Rchb., *R. nathaliae* Pančić & Petrović, *R. serbica* Pančić, *Haberlea rodhopensis* Friv., and *Jankaea heldreichii* (Boiss.) Boiss. (Thompson, 2005). The long-lived *R. myconi* has a highly fragmented distribution, with populations restricted to isolated mountain areas in NE Iberian Peninsula. Like many other Mediterranean endemic plant species, populations are restricted to rocky habitats (Lavergne et al., 2004). Previous demographic studies have shown that the long term-dynamics of *R. myconi* populations is of the remnant type, that is, local populations within a region are able to persist because of the long life span of adult plants and episodic but successful recruitment events (Picó and Riba, 2002). Even though *R. myconi* belongs to a group of higher plants known as resurrection plants, a group of species that can withstand severe desiccation (Müller et al., 1997), plant survival, growth, and reproduction has been shown to be highly sensitive to drought conditions (Riba et al., 2002). These findings suggest that its long-term persistence might be threatened by the increased aridity predicted in the Mediterranean Basin.

In this study, we used both RAPD and chloroplast markers to investigate the geographic distribution of genetic diversity in the relict populations of *R. myconi*. The study was conducted covering nearly the whole species range to (1) identify the main evolutionary and historical processes that have shaped the present-day distribution of gene diversity and (2) to determine the number and location of putative glacial refugia for this Tertiary paleoendemism.

MATERIALS AND METHODS

The plant species—*Ramonda myconi* is usually found in the cooler and wetter, north-facing slopes of calcareous mountain ranges in northeastern Spain and the central Pyrenees (Fig. 1). The species is an iteroparous herb (Picó and Riba, 2002), grows rooted in rock crevices, and forms a basal rosette of leaves. Flowering takes place from May to August, depending on weather conditions and altitude. The flowers are hermaphroditic, visited mainly by generalist insects (bumblebees and hoverflies), and the results from crossing experiments suggest that plants are self-compatible but unable to set seed autonomously. Seeds are very small (1–2 mm) with no conspicuous appendages that might facilitate dispersal.

A demographic survey carried out on the same region-wide scale to the one considered in this study showed that plant sizes are highly variable, ranging from 1 to 2 leaves and 0.2 cm in rosette diameter (seedlings) to 20–30 leaves and 26 cm. Plants grow slowly, and adult established individuals are able to survive for many years (Picó and Riba, 2002). The results from a long-term demographic follow-up study from 1994 to 2006 on three populations in the Cerdanya mountains (eastern Pyrenees; M. Mayol et al., unpublished data) indicate that, under current environmental conditions, the survival of adult reproductive plants is indeed very high (96%), while that of seedlings is very low (26%). The life span of the plant is not known, but data on accumulated individual growth in size (number of leaves and rosette diameter) during the same 12-yr period suggests that, under current environmental conditions, the time from germination to attain the minimum size for reproduction is ca. 70 years. Similarly, the estimated age of a plant with 11 leaves and a rosette diameter of 12 cm (the median size of plants in all the regions) is 200–250 years. Previous demographic analyses (Riba et al., 2002) showed that, on a regional scale, both growth and fecundity are negatively affected by drought conditions, particularly during the summer period.

Plant sampling and DNA extraction—Because our main objective was to characterize the pattern of genetic structure across the species range, between-population sampling on a regional-wide scale was favored over within-population sampling. We selected eight mountainous regions (massifs) covering most of its natural range: Guara, Osca Nord, Montsec, Cerdanya, Gironès, Sant Llorenç, Montserrat, and Els Ports (Fig. 1). These mountain ranges provide strong spatial climatic heterogeneity due to both elevation gradients and contrasted exposures. Details on climatic heterogeneity, extent (area), and altitudinal range for each massif are given in Table 1. A minimum of 30 plants were sampled from between two to three populations within each region, except for Els Ports region where only 18 plants could be sampled in the single known population (Estrets), which was recently discovered (Table 2). For comparative purposes, a similar amount of sampling effort was devoted to obtain within-population estimates of genetic diversity, with 10–16 individuals per population, except for population Vadiello (Guara region) where we had access to only four plants (Table 2). Leaves from 252 individuals were dried in silica gel and stored at −20°C until DNA extraction. Population sizes were also roughly estimated as the number of flowering plants and categorized into three groups: less than 100, between 100–500, and more than 500 flowering plants (Table 2). Genomic DNA was extracted from 50 mg of dried leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quantity and quality of DNA was checked by running 5 μL of each sample in 1% agarose gels.

RAPD markers—We favored the use of RAPD markers over others (e.g., AFLP, nuclear (SSRs), or allozymes) for their technical simplicity and for providing genome-wide information from many loci. RAPD reactions were performed in 20 μL total volume, containing 1× reaction buffer, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 μM of the arbitrary decamer, and 1 U of *Taq* DNA polymerase (Ecogen, Barcelona, Spain). Amplifications were carried out in a Primus 96 Thermal Cycler (MWG Biotech, Ebersberg, Germany) programmed for an initial denaturation step at 94°C for 1 min, followed by 40 cycles of 1.5 min at 94°C, 1 min at 40°C, and 2 min at 72°C. The reaction was completed with a final elongation step at 72°C for 10 min. We initially tested 120 primers (kits E, F, G, H, I, J; Operon Biotechnologies, Alabama, USA) on one individual for each of the eight regions. Eleven primers that gave reproducible and polymorphic banding profiles were selected for the final study (Table 3). To test the reproducibility of the RAPD profiles, we ran random duplicate amplifications using different samples of each population with all the selected primers. Only bands consistently reproduced in both assays were considered. A negative control containing all components except genomic DNA was included in each set of reactions to check for possible contamination. In addition, the same individual (Nocito 1) was always included in each set of reactions to ensure *Taq* efficiency and also to verify the correspondence of bands between different gels. Amplification products were separated on 1.4% agarose gels in 1× TAE (Tris-acetate EDTA) buffer, then stained with ethidium bromide. Each gel included both the respective negative and positive controls, and band sizes were determined by comparison with AmpliSize Molecular Ruler (50–2000 bp ladder, Bio-Rad, Barcelona, Spain). The electrophoresis was run at 80 V for 75 min in 1× TAE buffer. RAPD bands were visualized under UV light and recorded with a digital camera.

Chloroplast markers—Variation in the chloroplast genome was explored using three different approaches. First, we amplified two noncoding cpDNA regions (*trnL* intron and *trnL-trnF* intergenic spacer) using the universal primer pairs described in Taberlet et al. (1991). The amplification products were screened for polymorphism using 16 individuals (two from each region). PCR reactions were performed in a total volume of 50 μL containing 10–20 ng of template DNA, 1× reaction buffer, 3 mM $MgCl_2$, 0.1 mM of each dNTP, 0.2 μM of each primer, and 1 U of *Taq* DNA polymerase (Ecogen). Amplifications were carried out in a Primus 96 Thermal Cycler (MWG Biotech) programmed as follows: one cycle of 2 min at 94°C, 30 cycles of 50 s denaturing at 94°C, 50 s annealing at 53°C and 1.5 min extension at 72°C; with a final extension of 3 min at 72°C. Ten microliters of the amplified products were digested in 20 μL reactions with 24 different restriction enzymes following

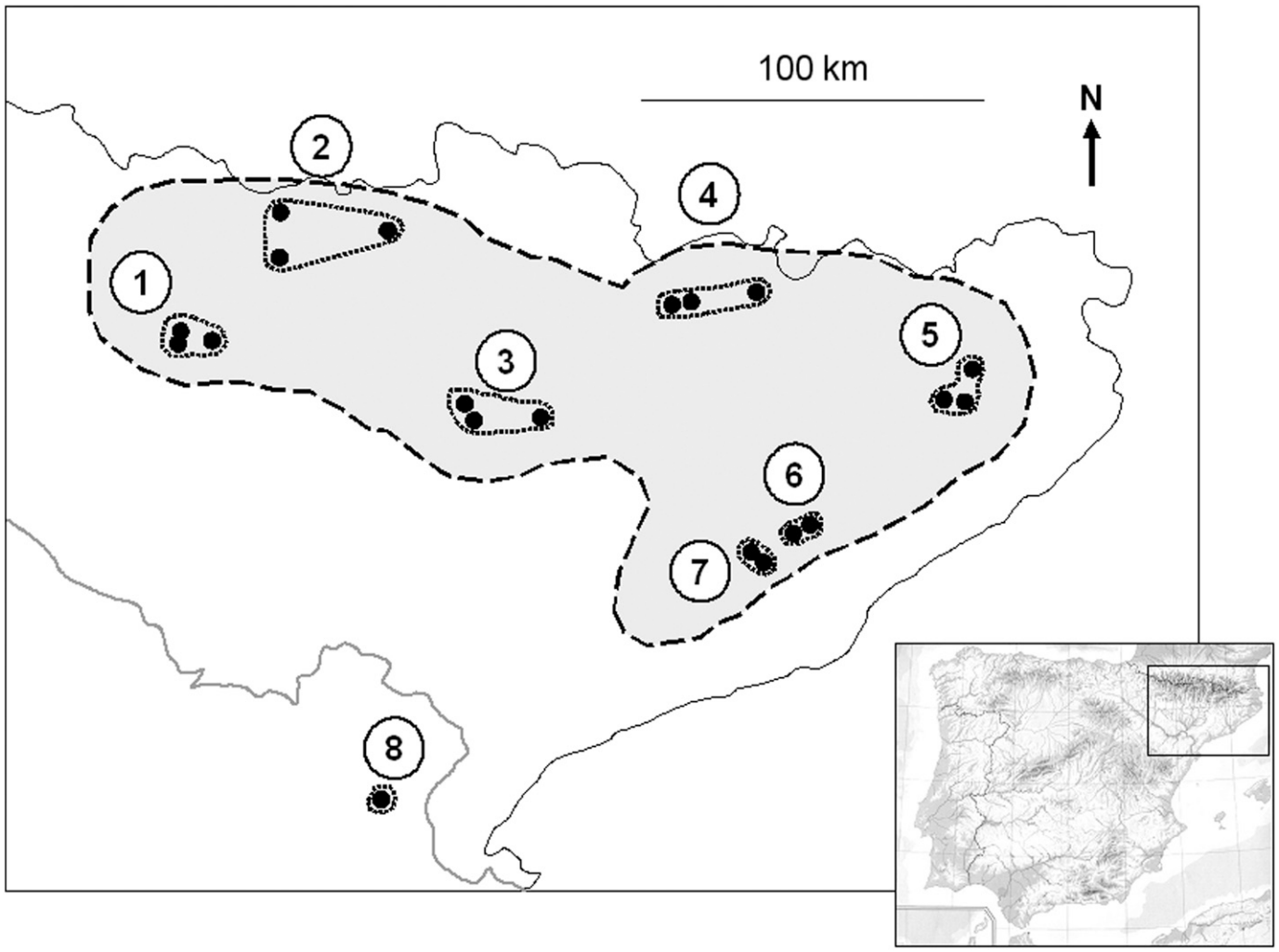

Fig. 1. Map showing the distribution range of *Ramonda myconi* (dashed line, gray shading), and the regions and the populations included in this study (dotted line). Sites are: 1 = Guara, 2 = Osca Nord, 3 = Montsec, 4 = Cerdanya, 5 = Gironès, 6 = Sant Llorenç, 7 = Montserrat, 8 = Els Ports. The gray line represents the river Ebro.

manufacturer's instructions: *Alu*I, *Dde*I, *Eco*RI, *Hae*III, *Hinf*I, *Hpa*II, *Mbo*I, *Taq*I, *Tru9*I (Promega, Madison, Wisconsin, USA) and *Apo*I, *Bfa*I, *Bsl*I, *Bst*NI, *CviKI*-1, *Dra*I, *Hpy188*I, *Hpy188*III, *HpyCH4*V, *Mbo*II, *Mly*I, *Psi*I, *ScrF*I, *Ssp*I, *Tsp509*I (New England Biolabs, Beverly, Massachusetts, USA). Restriction fragments were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed. Fragment sizes were estimated using the AmpliSize Molecular Ruler (50–2000-bp ladder, Bio-Rad).

TABLE 1. Main climatic and topographic features of the mountain regions (massifs) considered in our sampling of *Ramonda myconi* populations: extent (area), altitudinal range, and spatial variability (range) in rainfall (mm) and mean daily temperatures (°C) within each massif for June, the driest month during the summer. Climatic data obtained from Ninyerola et al. (2005).

Region	Area (km²)	Altitude (m a.s.l.)	Rainfall (mm)	Temperature (°C)
Guara	440	430–2070	75–90	17–20
Osca Nord	1265	750–3355	90–180	8–16
Montsec	200	400–1680	70–110	12–19
Cerdanya	385	1100–2600	90–140	7–15
Gironès	210	250–1100	75–120	15–20
Sant Llorenç	80	450–1100	60–75	15–19
Montserrat	50	250–1230	55–65	15–19
Els Ports	253	500–1450	45–60	15–20

Second, and given that PCR-RFLP usually detect low levels of polymorphisms, seven universal microsatellite (cpSSR) primers (ccmp2-ccmp7, ccmp10) described by Weising and Gardner (1999) and three microsatellites (NTCP13, NTCP37, NTCP39) described from *Nicotiana tabacum* (Bryan et al., 1999), were tested on the same samples used in the PCR-RFLP approach. DNA amplification were carried out in a Primus 96 Thermal Cycler (MWG Biotech), with the following PCR profile: one cycle of 5 min at 94°C, 25 cycles of 1 min denaturing at 94°C, 50 s annealing at 55°C and 1 min extension at 72°C; with a final extension of 10 min at 72°C. Reactions were performed in 10 μL total volume, containing 5–10 ng of template DNA, 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 0.15 U of *Taq* DNA polymerase (Ecogen). Amplified fragments were separated by capillary electrophoresis on an ABI 310 automated sequencer (Applied Biosystems, Foster City, California, USA), using GeneScan 350 ROX (Applied Biosystems) as the internal size standard.

Finally, we amplified and sequenced three additionally noncoding cpDNA regions: the 5′ *rpS12-rpL20* and *trnS-trnG* spacers (Hamilton, 1999) and the *trnT-trnL* spacer (Taberlet et al., 1991), using 1–3 individuals per population (a total of 20 individuals for 5′ *rpS12-rpL20*, 21 for *trnS-trnG* and 25 for *trnT-trnL*). Amplifications were performed in an MJ Research (Waltham, Massachusetts) PTC-100 Thermal Cycler using the same protocol described for the *trnL* intron and *trnL-trnF* spacer. Following amplification, PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), and

TABLE 2. Location and description of the sampled populations of *Ramonda myconi*. Population sizes were estimated as the number of flowering plants. *N* = number of sampled individuals. *D* = Mean distance among sampled populations (km). ? = Population size for Vadiello could not be estimated. Totals are in boldface.

Region	Locality	Location	Altitude (m)	Pop. size	*N*	*D*
Guara	Nocito	42.25°N 0.28°W	800	100–500	15	
	Mascún	42.29°N 0.07°W	700	100–500	16	
	Vadiello	42.24°N 0.28°W	700	?	4	
					35	12.6
Osca Nord	Eriste	42.61°N 0.47°E	1500	100–500	13	
	Pineta	42.67°N 0.08°E	1300	>500	11	
	Tella	42.57°N 0.17°E	1300	100–500	12	
					36	24.8
Montsec	Carbonera	42.06°N 0.72°E	1300	100–500	11	
	Meià	42.01°N 1.01°E	900	100–500	11	
	Colobor	42.03°N 0.78°E	1000	<100	11	
					33	16.7
Cerdanya	Urús	42.34°N 1.86°E	1350	100–500	10	
	Pi	42.30°N 1.75°E	1550	>500	14	
	Ingla	42.31°N 1.78°E	1350	>500	10	
					34	5.9
Gironès	Far	42.02°N 2.53°E	900	100–500	15	
	Rocacorba	42.06°N 2.68°E	750	<100	10	
	Tres Rocs	42.01°N 2.63°E	500	100–500	11	
					36	9.2
Sant Llorenç	Castellsapera	41.64°N 1.97°E	900	100–500	15	
	Santa Agnès	41.65°N 2.02°E	900	100–500	15	
					30	4.2
Montserrat	Agulles	41.61°N 1.79°E	950	100–500	15	
	Sant Jeroni	41.60°N 1.81°E	900	100–500	15	
					30	2.2
Els Ports	Estrets	40.89°N 0.29°E	550	<100	18	—
TOTAL					**252**	

sequenced with an ABI 377 automated sequencer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Samples were sequenced in both forward and reverse directions. The sequences obtained were compared to GenBank DNA sequence databases using BLAST (Altschul et al., 1990).

Data analysis—In the case of RAPD data, amplification products were scored as present or absent (1 and 0, respectively), and a binary matrix was constructed. Bands with the same gel mobility were assumed to be homologous, and following the suggestions of Grosberg et al. (1996), no attempts were made to code for band intensity. To avoid biased estimates of genetic diversity, we only included those bands whose observed frequency was less than $1 - (3/N)$, where N is the number of individuals in the analysis (Lynch and Milligan, 1994).

Genetic diversity within populations was estimated from the frequencies of the RAPD bands using Nei's unbiased gene diversity (*h*: Nei, 1987) and by Shannon's diversity index (Lewontin, 1972), which does not make assumptions regarding Hardy–Weinberg equilibrium. The percentage of polymorphic loci

TABLE 3. List of RAPD primers used to assess genetic polymorphism in *Ramonda myconi*, their sequences, the number of amplified bands, and the range of fragment sizes. M = monomorphic; P = polymorphic; T = total.

Primer	Nucleotide sequence (5'-3')	Number of bands			Fragment size range (bp)
		M	P	T	
OPE-10	CAC CAG GTG A	1	2	3	700–1500
OPE-11	GAG TCT CAG G	1	5	6	300–2000
OPE-17	CTA CTG CCG T	0	9	9	380–1550
OPF-14	TGC TGC AGG T	1	5	6	580–1500
OPF-16	GGA GTA CTG G	1	7	8	280–1350
OPG-12	CAG CTC ACG A	2	7	9	300–1950
OPG-13	CTC TCC GCC A	1	8	9	380–2200
OPI-08	TTT GCC CGG T	0	6	6	700–1800
OPI-10	ACA ACG CGA G	0	7	7	400–1250
OPJ-04	CCG AAC ACG G	1	6	7	300–1500
OPJ-05	CTC CAT GGG G	1	7	8	270–1500
TOTAL		**9**	**69**	**78**	

(P_p) was also calculated for each population. All genetic diversity parameters were computed with the program POPGENE version 1.32 (Yeh et al., 1997). Comparisons of population genetic diversity among geographical regions and among groups of populations with different population sizes were performed with one-way ANOVA (STATISTICA version 6.0; StatSoft, 2001). In the former case, we excluded Els Ports region because only one population was sampled and the population Vadiello (Guara region) because of its limited sample size.

The geographical structure of genetic variation was assessed by an analysis of molecular variance (AMOVA) following the approach by Excoffier et al. (1992) using the program ARLEQUIN version 2.000 (Schneider et al., 2000). A matrix of squared Euclidean distances among all individuals was computed from the binary data matrix and used as an input distance matrix for calculation of the variance components. The AMOVA analysis was performed at different hierarchical levels: (1) among and within populations, without regional grouping; and (2) among geographical groups (regions), among populations within regions and among individuals within populations. Geographical groups were defined according to the initial sampling scheme (eight mountain ranges). Significance levels of the variance components were obtained by nonparametric permutation using 10 000 replicates (Excoffier et al., 1992). In addition, SAMOVA (spatial analysis of molecular variance) was used to define groups of populations that were geographically homogeneous and maximally differentiated from each other (Dupanloup et al., 2002), using the program SAMOVA version 1.0 (website http://web.unife.it/progetti/genetica/Isabelle/samova.html). The most likely number of groups (K) was identified by repeatedly running the SAMOVA for 10 000 iterations for $K \in \{2, ..., 10\}$ from each of 500 random initial conditions and retaining the largest F_{CT} values (i.e., the largest proportion of total genetic variance due to differences between groups) as predictors of the best grouping of populations (Dupanloup et al., 2002). The relationships between the groups obtained were measured by computing a matrix of pairwise F_{CT} values using ARLEQUIN 2.000 (Schneider et al., 2000), and then using this matrix to construct a neighbor-joining tree using the program POPULATIONS version 1.2.28 (Langella, 2002).

Pairwise genetic population differentiation (F_{ST}) was computed using ARLEQUIN 2.000 (Schneider et al., 2000), and significance levels of the estimated values were obtained by permutation using 10 000 replicates. Isolation by distance between populations was tested according to Rousset (1997): the correlation between the matrix of genetic [$F_{ST} / (1 - F_{ST})$] and geographic distances (logarithmic scale) among pairs of populations was analyzed with a Mantel test (10 000 permutations) using the program MANTEL version 2.0 (Liedloff, 1999).

For chloroplast markers, the sequences obtained were edited using the program CHROMAS version 1.51 (Technelysium Pty., Queensland, Australia). Sequences were deposited in the GenBank database under the accessions EF589672–EF589675. Sequence data available from previously sequenced Gesneriaceae taxa were downloaded from GenBank and compared with the newly obtained sequences.

RESULTS

RAPD markers—A total of 69 reproducible polymorphic bands, ranging from 270 to 2200 bp, were obtained with 11 primers (Table 3). Only nine bands were monomorphic for the whole data set. No bands exclusive to populations or regions were found, but some of them had a marked geographic pattern: bands OPE-11(4) and OPF-16(5) were present only in Guara and Osca Nord regions; band OPG-13(9) was present in Guara, Osca Nord, and Montsec; band OPF-14(2) was exclusive to Guara, Osca Nord, and Els Ports; and band OPF-14(3) was only found in Guara, Osca Nord, Montsec, and Els Ports. All these bands were absent in the remaining regions. Conversely, only one band, OPI-10(1), was exclusive to the easternmost regions (Cerdanya, Gironès, Montserrat, and Sant Llorenç).

Values of genetic diversity are given in Table 4. All genetic diversity parameters (P_p, h, I) were highly correlated: P_p/h: $r = 0.910$, $P < 0.01$; P_p/I: $r = 0.933$, $P < 0.001$; I/h: $r = 0.997$, $P < 0.001$. Population Pi had the lowest within-population diversity ($P_p = 35.90$, $h = 0.1307$, I = 0.1830), whereas population Mascún ($P = 78.21$, $h = 0.2551$, I = 0.3694) was the most diverse.

TABLE 4. Genetic diversity values of *Ramonda myconi* populations according to the RAPD data. P_p = percentage of polymorphic loci. h = Nei's unbiased gene diversity (Nei, 1987) over all loci. I = Shannon's diversity index (Lewontin, 1972). The highest values of genetic diversity within regions are in boldface.

Region	Locality	Level of diversity	P_p	h	I
Guara	Nocito		73.08	0.2447	0.3515
	Mascún		**78.21**	**0.2551**	**0.3694**
	Vadiello		37.18[a]	0.2095	0.2275
		Regional	82.05	0.2591	0.3883
Osca Nord	Eriste		56.41	0.1946	0.2752
	Pineta		**67.95**	**0.2573**	**0.3525**
	Tella		55.13	0.1871	0.2641
		Regional	78.21	0.2234	0.3389
Montsec	Carbonera		53.85	**0.2191**	**0.2955**
	Meià		55.13	0.2014	0.2798
	Colobor		**56.41**	0.2098	0.2888
		Regional	71.79	0.2349	0.3483
Cerdanya	Urús		**43.59**	**0.1716**	**0.2319**
	Pi		35.90	0.1307	0.1830
	Ingla		42.31	0.1558	0.2134
		Regional	55.13	0.1624	0.2447
Gironès	Far		**48.72**	0.1590	0.2292
	Rocacorba		47.44	**0.1794**	**0.2445**
	Tres Rocs		42.31	0.1689	0.2290
		Regional	51.54	0.1924	0.2858
Sant Llorenç	Castellsapera		**48.72**	**0.1930**	**0.2683**
	Santa Agnès		46.15	0.1646	0.2340
		Regional	51.28	0.1900	0.2758
Montserrat	Agulles		**55.13**	**0.1918**	**0.2731**
	Sant Jeroni		44.87	0.1521	0.2184
		Regional	56.41	0.1758	0.2622
Els Ports	Estrets	*Regional*	44.87	0.1538	0.2213
		Species	88.46	0.2587	0.3986

[a]Low value of population Vadiello was probably due to the low number of sampled individuals.

Differences among populations in genetic diversity were not related to population size (ANOVA: $P = 0.69$, $P = 0.83$ and $P = 0.75$ for P_p, h and I, respectively). At the regional level, Guara was the region with the highest mean diversity values ($P_p = 82.05$, $h = 0.2591$, I = 0.3883) while Els Ports ($P_p = 44.87$, $h = 0.1538$, I = 0.2213) had the lowest. Gene diversity parameters differed significantly among regions (ANOVA: $P < 0.05$ for all diversity parameters) and revealed a marked geographical pattern, decreasing progressively along a west–east longitudinal gradient (Table 4, Fig. 2A). The correlation between diversity parameters and longitude was highly significant when Els Ports region was not taken into account (P_p: $r = -0.962$, $P < 0.001$; h: $r = -0.861$, $P < 0.05$; I: $r = -0.881$, $P < 0.01$).

The 78 RAPD markers defined 252 distinct phenotypes among the 252 individuals studied. As a result, partitioning of genetic variance revealed that most of the variation (72%) was present within populations (Table 5). However, a significant proportion of the variation was due to differences among populations. The overall F_{ST} value was 0.281 ($P < 0.001$), and all pairwise F_{ST} values were significant ($P < 0.05$; values ranging from 0.037 to 0.506), suggesting a limited gene flow among populations. Furthermore, the results obtained from AMOVA analysis also indicated the existence of high levels of isolation among disjoint mountain areas because the percentage of genetic variation explained by differences among the eight regions originally considered in our sampling was very high (20.19%; Table 5B).

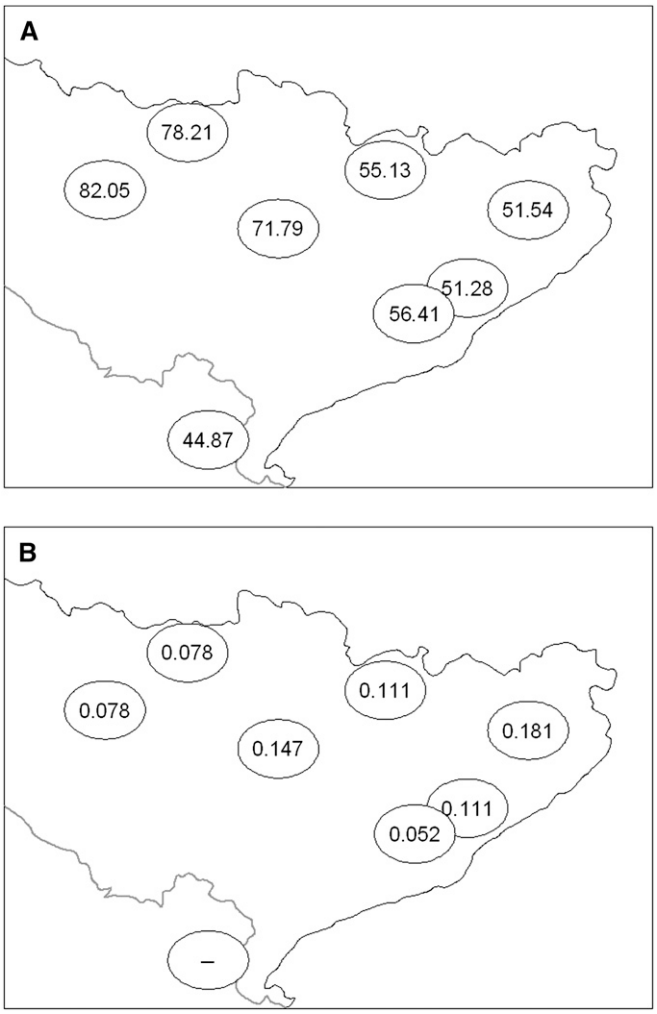

Fig. 2. Distribution of genetic diversity and population divergence at the regional level in *Ramonda myconi*. (A) Percentage of polymorphic loci. (B) Population pairwise genetic distances (F_{ST}). Values are means per region. Note that genetic diversity decreases and population differentiation increases progressively from west to east.

The SAMOVA algorithm also confirmed the existence of distinct groups of genetically defined sampling areas. F_{CT} values increased progressively with the number of groups considered, reaching a plateau at $K = 6$. For $K \geq 7$, some groups were made of only one population, indicating that the geographical structure was disappearing. Thus, we only retained for interpretation the grouping patterns between $K = 2$–6 (Fig. 3). The first level of divergence defined two groups, one containing the southernmost population of Estrets (Els Ports region) and another including the rest of populations (Fig. 3). In the analysis where $K = 3$, an additional partition subdivided northern sampling areas into distinct western (Guara, Osca Nord, Montsec) and eastern (Cerdanya, Gironès, Montserrat, Sant Llorenç) groups (Fig. 3). From this point, the increase of K produced two additional subdivisions of the eastern group, allowing the recognition of three small groups of populations maximally differentiated from each other (Cerdanya, Gironès, Montserrat-Sant Llorenç). The splitting of the western group into Guara-Osca Nord and Montsec was produced only when we considered $K = 6$.

TABLE 5. Analysis of molecular variance (AMOVA) based on RAPD data for *Ramonda myconi*. (A) Assuming no regional differentiation. (B) Regional grouping according to the eight mountain ranges initially considered in our sampling scheme (Guara, Osca Nord, Montsec, Cerdanya, Gironès, Sant Llorenç, Montserrat, Els Ports).

Source of variation	df	Sum of squares	Variance components	Percentage of variation
(A) Assuming no regional differentiation				
Among populations	19	823.71	2.867	28.11***
Within populations	232	1701.70	7.335	71.89
(B) Eight mountain ranges				
Among regions	7	601.29	2.094	20.19***
Among populations within regions	12	222.42	0.942	09.08***
Within population	232	1701.70	7.335	70.73***

Note: *** $P < 0.001$ (significant after 10 000 permutations)

This pattern of differentiation was confirmed by the unrooted neighbor-joining tree based on the pairwise F_{CT} distance matrix among these six groups of populations (Fig. 3), which revealed two main clusters: one cluster included the three groups located in the easternmost range (Cerdanya, Gironès, Montserrat-Sant Llorenç), and a second cluster included the southern (Els Ports) and western groups (Guara-Osca Nord, Montsec). The pairwise F_{CT} distances between neighboring groups in the eastern range were twice as high as those obtained for the groups in the western range (mean $F_{CT} = 0.219$ and 0.102, respectively), while there were no statistically significant differences for geographical distances within each area (mean distance among western groups: 74.5 km; among eastern groups: 73.6 km). Group 1, corresponding to the small and geographically marginal population of Els Ports, was quite distinct from the rest of the groups (pairwise F_{CT} values ranging from 0.222 to 0.464). However, this group was significantly more differentiated (ANOVA: $P < 0.05$) from the groups of the eastern range (mean $F_{CT} = 0.429$) than from those of the west (mean $F_{CT} = 0.253$).

These results are in accordance with the regional pattern obtained for population differentiation. As in the case of genetic diversity, mean population differentiation at the regional level revealed a clear pattern of increasing divergence toward the east (Fig. 2B). The only exception was the Montserrat region, that showed the lowest values for population differentiation ($F_{ST} = 0.052$), probably due to the geographic proximity of the two populations sampled in this region (2.2 km, Table 2).

Finally, the Mantel test showed that between-population differentiation increased significantly with the logarithm of geographical distance (Mantel test: $r = 0.3797$, $P < 0.001$), as illustrated in Fig. 4, suggesting a model of isolation by distance. The increase in population differentiation is particularly evident at geographical distances ≥75 km, which corresponds to the mean distance between mountainous regions, especially in the easternmost area of the species range (Fig. 4).

Chloroplast markers—No genetic variability was found using chloroplast markers. Both the *trnL-trnL-trnF* region and the microsatellite loci gave good amplification products, but failed to detect any intraspecific genetic variation in *R. myconi*. The 10 chloroplast microsatellites were monomorphic, and the restriction analysis of the *trnL* intron and *trnL-trnF* spacer lacked any differences in length of the digested DNA. This lack of variation was further confirmed by sequencing one sample from each region included in the study. In fact, our sequences were identical to that of *Ramonda myconi* chloroplast *trnL-trnL-trnF* deposited in the GenBank (AJ492301).

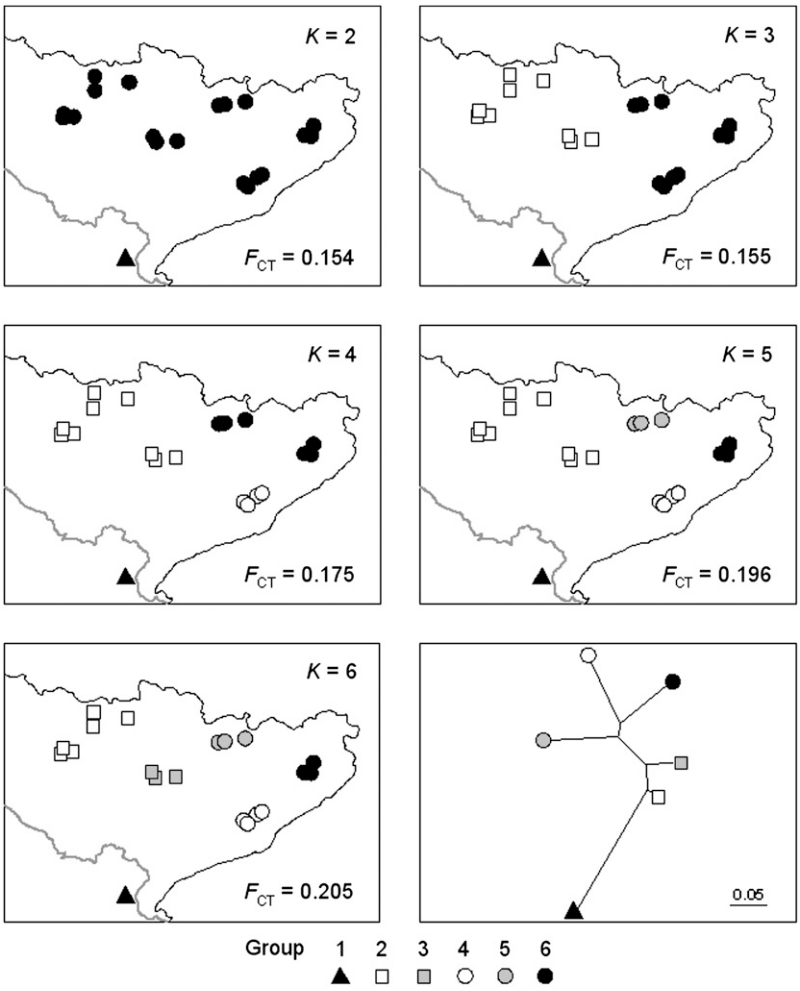

Fig. 3. Geographical distribution of groups defined by the spatial analysis of molecular variance (SAMOVA) of *Ramonda myconi* populations. The maximum value for F_{CT} was obtained for $K = 6$. The unrooted neighbor-Joining tree is based on the pairwise F_{CT} distance matrix among these six groups of populations. Group 1 = Els Ports; group 2 = Guara-Osca Nord; group 3 = Montsec; group 4 = Montserrat-Sant Llorenç; group 5 = Cerdanya; group 6 = Gironès.

Sequences obtained with the 5′*rpS12-rpL20* and *trnS-trnG* primer pairs were 827 and 796 bp long, respectively. The *trnT-trnL* spacer was easily amplified, but sequencing in the forward sense was difficult for many samples, so we could only use a 382-bp long sequence common to all analyzed individuals. The BLAST search revealed a close similarity to *Sinningia tuberosa trnT-trnL* intergenic spacer (AJ439287), to *S. speciosa rpl20-rps12* intergenic spacer (AY623369), and to *Vanhouttea* sp. *trnS-trnG* intergenic spacer (AJ438420), indicating an amplification of the correct part of the chloroplast genome. No polymorphisms were found for any of these three cpDNA regions, with all the analyzed individuals having the same sequences.

DISCUSSION

Genetic diversity for RAPD and chloroplast markers—Genetic diversity and structure of plant populations is strongly influenced by both historical events and current patterns of interpopulation genetic exchange (Schaal et al., 1998), as well as life history features (Austerlitz et al., 2000). Comparisons and inferences regarding the amount of within-population genetic variability based on our limited sample sizes (69 polymorphic loci and 10–18 individuals per population) must be made with caution. Indeed, variances in population genetic parameters are very high when less than 15–20 loci and 20–30 individuals per population are scored (e.g., Aagaard et al., 1998). Nevertheless, in some cases (Fischer and Matthies, 1998; Fischer et al., 2000), comparisons among populations based on the same kind of marker and on a very limited sample size (e.g., 4–10 plants per population and 20–40 loci) have proved useful in revealing the links between genetic variability, population size, and plant fitness. In our study, we did not find a significant relationship between within-population diversity and present-day population sizes. However, we did find significant differences among regions, as well as a significant west–east geographical trend. Such geographical pattern matches the one obtained for genetic differentiation, and it is probably best explained by demographic processes that might have operated in the distant past along the species range (discussed in the next section on genetic structure).

In contrast to the relatively high levels of genetic diversity detected with RAPDs, no variation was found using chloroplast markers. Although not all sampled individuals were included in

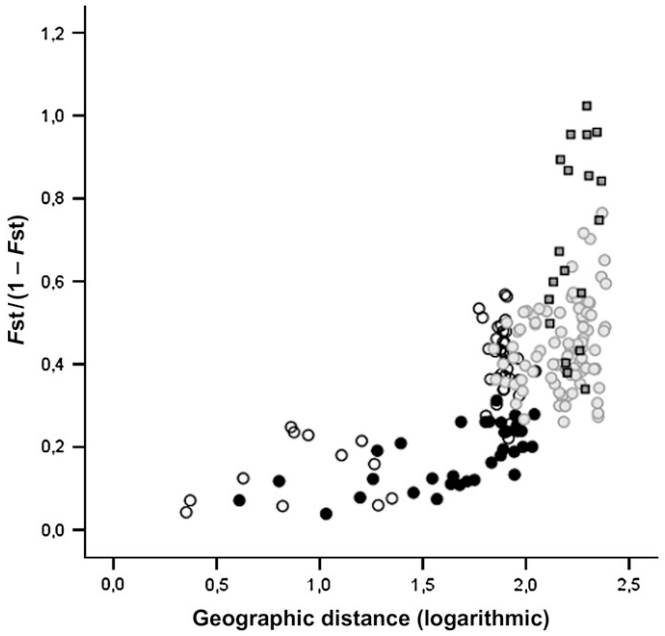

Fig. 4. Relationship between genetic [$F_{ST}/(1 - F_{ST})$] and geographic distances (km) among *Ramonda myconi* populations (Mantel test: $r = 0.3797$; $P < 0.001$). White circles, pairwise values for eastern populations (Cerdanya, Gironès, Montserrat, Sant Llorenç); black circles, pairwise values for western populations (Guara, Osca Nord, Montsec); gray circles, pairwise values among western and eastern populations; gray squares, pairwise values with the Estrets population.

our analysis, no variation was found among plants from very distant populations; hence it is unlikely that such lack of variability could be simply the result of limited sample size. Moreover, the lack of variation was common to all the cpDNA markers scored (1 intron, 4 intergenic spacers, 10 cpSSR), even though some noncoding cpDNA regions used in this study (cpSSRs, *trnS-trnG*, and *trnT-trnL* spacers) have consistently provided greater levels of variation as compared to other cpDNA regions (Provan et al., 2001; Shaw et al., 2005). The absence of variation in the chloroplast genome at the species level has been documented for other long-lived endemic or relict species, such as *Pinus torreyana* Parry ex Carrière (Provan et al., 1999), *Zelkova abelicea* (Lam.) Boisser and *Z. sicula* Di Pasquale (Fineschi et al., 2002), or *Heptacodium miconioides* Rehd. (Lu et al., 2006). This fact has usually been interpreted as the consequence of strong bottlenecks or genetic drift associated with small effective population sizes for maternally inherited markers (Birky et al., 1989). In fact, several other factors may have contributed to the lack of variation observed in *R. myconi*, including the low mutation rates associated with the chloroplast genome and the extremely slow population dynamics documented for the species (Picó and Riba, 2002), which altogether could have prevented the accumulation of mutations in the cpDNA. Finally, an alternative explanation for the discrepancy between diversity estimates made on nuclear and chloroplast markers could be that RAPD technique surveyed the whole genome, while the cpDNA fragments analyzed only covered a small proportion of the organelle DNA. Therefore, nuclear markers provide an alternative to reconstruct the population history in species where low levels of intraspecific variation are detected for chloroplast genome. Because RAPD markers are sometimes criticized for their low reproducibility, AFLPs have become the preferred markers in many studies because of higher reliability and precision. However, RAPDs have been applied satisfactorily both to assess genetic variability of endemic and/or relict species (e.g., Pye and Gadek, 2004; Renau-Morata et al., 2005; Andrianoelina et al., 2006; Romeiras et al., 2007) and to ascertain the Pleistocene population dynamics of some Pyrenean, arctic, and alpine plants (Gabrielsen et al., 1997; Bauert et al., 1998; Tollefsrud et al., 1998; Segarra-Moragues and Catalán, 2003). Moreover, Kjølner et al. (2004) addressed the congruence of the results obtained with RAPDs and AFLPs, concluding that RAPDs might be used as reliable method in small, low-tech laboratories.

Genetic structure at the regional scale: Inferences about refugia—High genetic differentiation was observed between geographical regions (20%) and among populations within regions (9%), indicating that gene flow is not strong enough to counteract genetic drift. The high correlation found between population genetic and geographic distances suggested an overall pattern of isolation-by-distance, where gene flow is much more likely to occur between neighboring populations (Wright, 1943; Kimura and Weiss, 1964). Because *R. myconi* is entomophilous and its seeds have no obvious morphological traits favoring dispersal in space, it is expected that the homogenizing effects of gene flow become less influential relative to drift as geographical distances between populations increase. Nevertheless, the relative roles of genetic drift and gene flow differed, depending on the geographical group considered. Significantly higher levels of population divergence were found in the easternmost regions as compared with the western part of the range, indicating that in the eastern part of the species range the effect of genetic drift has been stronger than that of gene flow. These contrasting patterns of genetic structure suggest that the regions under study could have experienced different evolutionary histories, as discussed further later.

The spatial genetic analysis (SAMOVA) allowed us to identify the most important genetic barriers between groups of populations in *R. myconi*. A primary discontinuity was detected separating the southernmost population of Estrets (Els Ports) and the remaining regions, while a secondary barrier divided the northern areas into a well-defined western (Guara, Osca Nord, Montsec) and eastern (Cerdanya, Gironès, Montserrat, Sant Llorenç) groups (Fig. 3). These groups were highly divergent, accounting for about 15% of the total genetic variation. This highly structured geographical pattern of genetic diversity suggests the existence of several allopatric refugia for *R. myconi* during the Pleistocene, supporting the multiple refugia scenario proposed for the Iberian Peninsula (Gómez and Lunt, 2006).

Several lines of evidence give support to the occurrence of important refugial areas in northern latitudes of the Iberian Peninsula. One of these refugia could be located near the central Pyrenees, as suggested both from palynological data (Jalut et al., 1992; Valero-Garcés et al., 2000; González-Sampériz et al., 2004) and patterns of phylogeographic concordance among different species (Gómez and Lunt, 2006). In addition, evidence for the presence of small eastern refugia located near the Mediterranean coast of Spain has also some support from pollen records and molecular data (Pérez-Obiol and Julià, 1994; Salvador et al., 2000; Lumaret et al., 2002). The strong differentiation found between the eastern regions located on each side of the Ebro Valley, Els Ports (Estrets population), and the rest of eastern populations, suggest that both regions might have acted as glacial refugia for *R. myconi* along the Mediterranean coast. In fact,

the population found in Els Ports was more closely related to western than eastern populations, which suggests that the Ebro Valley or the Iberian System have served as corridors for historical gene exchange through present-day extinct populations. A connection between the Pyrenees and Els Ports seems to have occurred on the basis of common biogeographical patterns for some plant species, such as *Brimeura amethystina* (L.) Chouard, *Pyrola chlorantha* Sw., or *Pyrola secunda* L. Moreover, pollen records showing the abundance of *Corylus* and evergreen *Quercus* during the Late Glacial indicate that suitable refugial zones for temperate trees occurred during the late Pleistocene along the Ebro Valley (Valero-Garcés et al., 2000; González-Sampériz et al., 2004). In agreement with this evidence, our results give support for this area to have allowed the persistence of *R. myconi* populations at least during the cold Quaternary stages.

Far beyond the regional groupings described, the results of SAMOVA also revealed that the western populations form a geographically more homogeneous group, characterized by higher genetic diversity and lower population differentiation, as compared to the other regions. As a result, a clear longitudinal gradient of decreasing genetic diversity and increasing population differentiation was observed from west to east (Fig. 2). Similar longitudinal trends as those detected in *R. myconi* have been previously reported for other plant species (*Silene* subgen. *Petrocoptis*, Mayol and Rosselló, 2001; white oaks from Iberian Peninsula, Olalde et al., 2002). For white oaks, Olalde et al. (2002) suggested that the higher genetic diversity in this area could be interpreted as the result of an admixture of lineages presumably arising from different glacial refugia extended along the Mediterranean and Atlantic coasts. Because of the expected reduced colonizing ability of *R. myconi* (no specific mechanisms for dispersal, low seedling establishment and growth), it is unlikely that the higher diversity found in the central Pyrenees could be the result of secondary contact between different migration fronts. Moreover, the highly fragmented present-day distribution of the species further suggests limited long-distance dispersal since the last glaciation.

Patterns of genetic diversity and structure are often interpreted as the result of recolonization processes following the last glaciation. However, Tertiary relict species survived in southern refugia during successive glacial and interglacial periods, and thus the observed genetic patterns in these species may be reflecting a combination of postglacial and more ancient events. The long-term ability of these species to withstand repeated episodes of climate oscillation would have required particularly stable ecological habitats for persistence during the fluctuations of the Quaternary. The existence of a stable ecological area allowing the persistence of temperate tree populations during the last 130 000 yr has been reported in western Greece (Tzedakis et al., 2002). This study highlights the importance of local intrinsic properties of refugia (e.g., high topographic variability) to buffer the impact of Quaternary climatic oscillations on vegetation communities. Therefore, the higher genetic diversity and lower differentiation observed in the westernmost regions (central Pyrenees and Guara) could simply reflect the existence of more suitable habitats during the ice ages. Indirect evidence supporting the existence of an important refugium during the Quaternary in the central Pyrenees are the diversity of habitats and the species richness present today in this area, the occurrence of some other Tertiary relicts [*Borderea pyrenaica* Bubani & Bordère ex Miégev., *B. chouardii* (Gaussen) Heslot], and the high level of plant endemics (63 of the 160 endemic plant species of the Pyrenean flora are exclusively from the central Pyrenees; Villar et al., 1997). In addition, this region was probably not so strongly affected by past climatic changes as other areas located near the Mediterranean coast. Recent studies indicate that climatic instability during the last glacial period differentially affected the Mediterranean and the Atlantic sides of the Iberian Peninsula, at least during the last 50 000 yr (Sánchez-Goñi et al., 2002; Moreno et al., 2005). Thermal and precipitation gradients occurred between the western and eastern part of Iberia during some Heinrich events (H4, H5), with more humid conditions in the Atlantic side and more arid conditions in the Mediterranean coast (Sánchez-Goñi et al., 2002). This west–east climatic gradient, similar to the one found at present in this area, divided the Iberian Peninsula into two climatic regions with contrasting patterns of precipitation (Rodó et al., 1997). Moreover, these arid episodes were not only present during glacial periods. In the western Mediterranean, the transition toward a Mediterranean climate in the present interglacial period has not been synchronous, and progressed following a south–north gradient from southern Spain to the Gulf of Lion (Jalut et al., 1997, 2000). All this evidence suggests that repeated critical periods in moisture availability affected *R. myconi* populations in a different way depending on their geographical situation, having more severe impact in Mediterranean coastal and southern populations. Although the species is able to withstand repeated periods of drought, traits related to growth and reproduction are particularly sensitive to temperature and water availability, especially during summer months (Riba et al., 2002). Hence, more pronounced oscillations between arid and humid periods in the easternmost regions might have historically led to decreased levels of genetic diversity as a consequence of more severe reductions in population sizes, limited gene flow, and enhanced genetic drift. In contrast, western populations may have remained comparatively large and less isolated, contributing to the maintenance of gene flow between neighboring populations, in accordance with the higher genetic diversity and lower population divergence found in this area.

Conclusions—Our results suggest that, in addition to life-history traits, the present-day genetic diversity and structure of *R. myconi* has been strongly influenced by several historical factors acting throughout the Pleistocene. The high regional genetic divergence observed suggests that, in spite of its small distribution range, three distinct areas might have acted as glacial refugia for this species during glacial cycles. These areas fit well with previously recognized centers of endemism and species richness and give support to a complex phylogeographic scenario in the Iberian Peninsula of "refugia-within-refugia" (Gómez and Lunt, 2006). In addition, the detected gradient of decreasing diversity and increasing population divergence from west to east is better explained by long-term range fragmentation processes than by founder effects following postglacial expansion. The higher diversity observed in the central Pyrenees may be associated with prolonged and more stable climatic conditions in this area when compared with the easternmost regions under the influence of the Mediterranean climate.

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