Molecular phylogeography of *Thymus herba-barona* (Lamiaceae): Insight into the evolutionary history of the flora of the western Mediterranean islands

Arántzazu Molins,[1] Gianluigi Bacchetta,[2] Marcela Rosato,[3] Josep A. Rosselló[3,4] & Maria Mayol[1]

1 *CREAF (Center for Ecological Research and Forestry Applications), Autonomous University of Barcelona, 08193 Bellaterra, Spain*
2 *Centro Conservazione Biodiversità, Dipartimento di Scienze Botaniche, Università degli Studi di Cagliari, S. Ignazio da Laconi 13, 09123 Cagliari, Italy*
3 *Jardín Botánico, ICBiBE, University of Valencia, c/Quart 80, 46008 Valencia, Spain*
4 *Marimurtra Botanical Garden, Carl Faust Fdn., P.O. Box 112, 17300 Blanes, Spain*
Author for correspondence: *Maria Mayol, Maria.Mayol@uab.es*

Abstract *Thymus herba-barona* is endemic to Majorca, Corsica, and Sardinia. In order to gain insight into its evolutionary history, we examined the genetic diversity and phylogeography of the species using sequences of the *trnT-trnL* intergenic spacer from 106 individuals belonging to 15 populations. We detected high within-species genetic diversity and strong among-population differentiation, but no evidence for phylogeographic structure. A haplotype network supported the occurrence of three main clades, the ancestral one being geographically restricted to the Gennargentu massif in Sardinia, while the two derived ones were relatively widespread. Coalescent-based analyses indicated deep divergence times and limited ongoing gene flow between major clades. The inferred evolutionary history of the species involved an early range expansion followed by successive fragmentation episodes, probably related to progressive climatic aridification since the Pliocene (3.2 Ma). Our data suggest that long-distance dispersal events played a minor role in the evolutionary history of the species. The exact origin of the species remains unclear. The highly structured pattern of genetic variability detected suggests that random genetic drift played a major role in structuring genetic variation in this endemic plant. The cytogenetic evidence does not support the proposed recognition of three taxonomic entities within *T. herba-barona* based on morphological discontinuities associated with different ploidy levels.

Keywords continental islands; fragmentation; genetic drift; plastid DNA; western Mediterranean basin

Supplementary Material Table S1 and Fig. S1 are available in the free Electronic Supplement to the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

The Mediterranean basin is characterized by high plant diversity and endemism (Greuter, 1991), and is recognized as a global biodiversity hotspot (Myers & al., 2000; Thompson, 2005). The high biodiversity is due, in part, to Mediterranean areas serving as refugia for many taxa during the Pleistocene glaciations, allowing the long-term persistence of populations and the formation of new species. The numerous islands present in the Mediterranean appear to have constituted major refugial areas (Médail & Diadema, 2009), and today harbour numerous narrowly endemic taxa (Médail & Quezel, 1997, 1999). In addition, the highly fragmented insular landscapes have promoted geographical and genetic isolation among plant populations, favouring allopatric speciation via selection and/or genetic drift (Thompson, 2005). In spite of the central role that islands play in our understanding of the evolution of biodiversity and despite a long tradition of studies on endemism in the Mediterranean flora (e.g., Favarger & Contandriopoulos, 1961; Contandriopoulos & Cardona, 1984), it is only recently that the Mediterranean islands have regained interest from evolutionists, leading to the study of plant evolution on the Aegean Islands (Affre & Thompson, 1997; Widén & al., 2002; Bittkau & Comes, 2005; Edh & al., 2007), the Balearic Islands (Sales & al., 2001; López de Heredia & al., 2005; Molins & al., 2009; Rosselló & al., 2009), and Corsica-Sardinia (Falchi & al., 2009).

The Balearic Islands, Corsica, and Sardinia, together with Sicily, are the largest islands in the western Mediterranean (together, they form the Tyrrhenian Islands). They have been identified as one of ten hotspots of plant diversity in the Mediterranean, with about 5500 vascular plants and a rate of endemism around 10% (Médail & Quezel, 1997; Médail, 2008). These islands have a complex palaeogeographical origin, linked to tectonic movements. According to geodynamic reconstructions of the western Mediterranean during the last 30 million years, the territories currently found in southern France, the Balearic Islands, Corsica, Sardinia, Kabylies (Algeria), Calabria, Sicily, northeastern Spain, and the Rif/Betic Range (North Africa/southeastern Spain) were all connected forming an Oligocenic land mass known as the Hercynian massif (Alvarez & al., 1974; Westphal & al., 1976; Rosenbaum & al., 2002; Speranza & al., 2002). Successive splitting of this massif during the Late Oligocene and the south-eastward rotation of land masses during the Miocene led to the current position of the different microplates.

The distribution of several endemic plants shared between the Balearic Islands, Corsica, and Sardinia has been attributed to the Oligocenic connections among these islands (Greuter 1995; Quézel, 1995; Thompson, 2005). Examples include *Arum pictum* L.f., *Arenaria balearica* L., *Cymbalaria aequitriloba* (Viv.) A. Chev., *Delphinium pictum* Willd., *Helicodiceros muscivorus* (L.f.) Engler, *Naufraga balearica* Constance & Cannon, *Soleirolia soleirolii* (Req.) Dandy, *Teucrium marum* L., and *Thymus herba-barona* Loisel. These species are considered as palaeoendemics. To date, however, only very few empirical studies provide explicit support for a relictual origin of the Hercynian endemic plants. Mansion & al. (2008) reported an Early Oligocene (~30 Ma) origin for *Helicodiceros muscivorus* and a Miocene (~16 Ma) origin for *Arum pictum*, the former apparently representing the first documented case of vicariance driven by the initial splitting of the Hercynian belt during the Oligocene.

Thymus herba-barona, the caraway thyme, is a perennial plant endemic to Majorca, Corsica and Sardinia. Together with *T. nitens* Lamotte, restricted to southern France, they are the only members of *Thymus* subsect. *Pseudopiperellae* Jalas (sect. *Serpyllum* (Mill.) Benth.), which is endemic to the western Mediterranean (Jalas, 1971). In Corsica and Sardinia, *T. herba-barona* is common in mountainous areas between 800 and 2000 m (Camarda, 1978). In Sardinia it is less widespread than in Corsica and mainly occurs in the Gennargentu massif and Marghine-Goceano region (Fig. 1A). In the Balearic Islands the only known population is from Majorca (Serra d'Alfàbia, ca. 900 m) and consists of approximately 150 individuals with an area of occupancy of only about 100 m² (Mayol & al., 1990, 1998). Until the discovery of the Balearic population, it was assumed that *T. herba-barona* was a palaeoendemic species that originated by hybridization between unknown ancestral species (Contandriopoulos, 1962). Its restricted occurrence on Majorca might be interpreted as resulting from a rare long-distance dispersal event from Corsica or Sardinia, or human-mediated introduction. This hypothesis does not fit, however, with the distribution of chromosome numbers in the species, including polyploid numbers on Corsica ($2n = 56$, tetraploid cytotype; Contandriopoulos, 1962) and Sardinia ($2n = 84$, hexaploid cytotype; Diana-Corrias, 1980), and diploidy on Majorca ($2n = 28$; Mayol & al., 1990; Rosselló & Castro, 2008). The diploidy on Majorca instead is suggestive of an origin or refugial status of the species on that island. The small, but constant morphological differences between the Balearic plants and those of Corsica and Sardinia also support the relictual nature of the Majorcan population or, alternatively, an ancient colonization of the island.

These morphological discontinuities have led to the recognition of different entities within the species. Mayol & al. (1998) proposed the recognition of the Balearic plants at the infraspecific level: *T. herba-barona* subsp. *bivalens* Mayol, L. Sáez & Rosselló). Camarda (2003) considered that the morphological discontinuities between the plants inhabiting the different islands, together with their different cytotypes, warrant the recognition of three separated entities at the species level: *T. herba-barona* from Corsica, *T. catharinae* Camarda from Sardinia, and *T. bivalens* (Mayol, L. Sáez & Rosselló) Camarda from Majorca.

In this study we used cpDNA sequences to examine the phylogeography of *T. herba-barona* s.l. throughout its distribution range, in order to gain more insight into its evolutionary history. We also have obtained new chromosome counts from Corsica and Sardinia to verify the previous ploidy levels reported for these islands. The main objectives of this work were: (1) to determine the relative role of the different evolutionary forces in shaping genetic variation; (2) to test whether the disjunct distribution of this Tyrrhenian endemism is due to a vicariant process congruent with the Oligocene geological splitting of the Hercynian massif, or other biological and/or anthropogenic processes must be invoked to explain its current distribution.

■ MATERIALS AND METHODS

Chromosome preparation. — Seeds were germinated on solid agar in Petri dishes at 20°C. Seedlings (2–4 days old) were pretreated with 2 mM 8-hydroxyquinoline for 2 h at 4°C, and then for 2 h at room temperature. The root tips were fixed in an ethanol–glacial acetic acid (3 : 1) mixture and stored at −20°C. For chromosome counts root tips were hydrolysed for 5–10 min in 1 M HCl at 60°C, washed and stained in Schiff reagent for 4–6 h. Stained meristems were squashed in a drop of 2% acetic-carmine, and permanent preparations were made by mounting in Canada balsam. Photomicrographs of well-spread metaphases were taken with an Olympus Camedia C-2000-Z digital camera. Chromosome counts were made from digital images using the processing image software ImageTool v.5.0.

Plant sampling and DNA extraction. — A total of 106 samples of *T. herba-barona* were collected from 15 populations covering the entire species range (Fig. 1A; Table 1). Seven to eight individuals were collected per locality. Voucher specimens from each location were deposited at BC and CAG. Leaves were dried in silica gel and stored at room temperature until being processed. Total genomic DNA was isolated and purified from 20 mg of dried leaf tissue using the DNeasy Plant Mini-kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity and quality of DNA was checked by running 5 µl of each sample in 1% agarose gels.

DNA amplification, sequencing, and alignment. — Amplification and sequencing of the *trnT-trnL* intergenic spacer was conducted using the universal primers "a" and "b" of Taberlet & al. (1991). PCR reactions were performed in 50 µl, containing 1× PCR buffer, 0.0001% Bovine Serum Albumin, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.6 µM of each primer, approximately 50–100 ng of genomic DNA, and 3 units of Taq polymerase (NETZYME DNA Polymerase, NEED S.L., Valencia, Spain). Thermal cycling started with a denaturation step at 94°C lasting 2 min, followed by 30 cycles each comprising 50 s denaturation at 94°C, 50 s annealing at 53°C, and 1 min 30 s elongation at 72°C, with a final elongation cycle of 3 m at 72°C. PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and sequenced with an ABI 3100 Genetic Analyser using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, U.S.A.). Samples

were sequenced in both directions and BLASTed against GenBank (Altschul & al., 1997). Alignment relied on ClustalX v.1.83 (Thompson & al., 1997). To confirm that *trnT-trnL* is evolving in a neutral manner, sequence variation was tested for significant deviation from neutrality using Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) tests, considering all individuals analysed as a single population. Both statistics were calculated using ARLEQUIN v.2.000 software (Schneider & al., 2000). Sequences were deposited in the GenBank database under the accession numbers JF999980–JF999996.

Genetic diversity and structure analysis. — The completeness of haplotype sampling based on the number of individuals analysed was estimated using the Stirling probability distribution method proposed by Dixon (2006). Parameters of genetic diversity and differentiation were estimated following the methods described by Pons & Petit (1995, 1996), using the programs HAPLODIV and HAPLONST (available at http://www.pierroton.inra.fr/genetics/labo/Software/), both at the species and the island level (Corsica, Sardinia). Within-population diversity, total diversity, and level of population differentiation, as well as their standard errors, were computed both by taking the distance between haplotypes into account (v_s, v_t, N_{st}) and by ignoring genetic distance between them (h_s, h_t, G_{st}). Values of N_{st} and G_{st} were compared using the *U*-statistics, to test for the existence of a phylogeographical structure (N_{st} significantly higher than G_{st}; Pons & Petit, 1996).

The geographical structure of genetic variation was assessed by the analysis of molecular variance (AMOVA, Excoffier & al., 1992) using ARLEQUIN. The total genetic variance was partitioned into covariance components at different hierarchical levels under two hypotheses: (1) all locations were treated as a single group to determine the amount of variation partitioned among and within populations; and (2) locations were grouped by origin to determine the amount of variation attributable to differences between islands, populations within each island, and individuals within populations. The significance levels of the variance components were obtained by nonparametric permutation using 10,000 replicates.

In addition, the approach proposed by Dupanloup & al. (2002) was used to define groups of populations that were geographically homogeneous and genetically differentiated from each other (spatial analysis of molecular variance; SAMOVA). The most likely number of groups (*K*) was identified by repeatedly running the program SAMOVA v.1.0 (Dupanloup & al., 2002) from $K = 2$ to $K = 10$, using 500 random initial conditions and performing 10,000 iterations. The largest F_{CT} values (i.e., the largest proportion of total genetic variance due to differences between groups) were chosen as predictors of the best grouping of populations (Dupanloup & al., 2002).

Haplotype network and nested clade analysis. — A haplotype network was constructed using statistical parsimony (Templeton & al., 1992) with the program TCS v.1.21 (Clement & al., 2000). Insertions and deletions were treated as a fifth character state, and coded as a single mutational event (Simmons & al., 2001). Since one of the polymorphisms detected consisted of a poly-A repeat, being more prone to homoplasy (Ingvarsson & al., 2003), an alternative haplotype network was also constructed excluding this site from the analysis. Two network ambiguities (loops) were resolved using predictions based on coalescent theory according to the rules outlined in Crandall & Templeton (1993) and Pfenninger & Posada (2002). The resulting haplotype network was nested into hierarchical clades using the automated implementation of Nested Clade Phylogeographic Analysis (NCPA, Templeton & al., 1995) provided by the program ANECA v.1.1 (Panchal, 2007). ANECA software implements both TCS v.1.18 and GEODIS v.2.2 (Posada & al., 2000), the inference key dated 11 November 2005, and automates the inference process, providing a framework for replicating analyses in an objective way.

Divergence time estimate. — After major clades of the network were identified, we estimated divergence times between the clades using the program MDIV (Nielsen & Wakeley 2001; available at http://people.binf.ku.dk/rasmus/webpage/mdiv.html). MDIV uses a Markov Chain Monte Carlo (MCMC) approach which allows the joint estimation of the posterior distribution of a variety of parameters: the divergence time since two populations diverged from a common ancestral population ($T = t_{div1} / N_e$), the time to most recent common ancestor (TMRCA = t_{div2} / N_e), the migration rate between populations since divergence ($M = N_e m$), and the relative size of each of the two current populations ($\theta = 2N_e\mu$), where N_e is the effective population size, m is the migration rate, and μ is the per-locus mutation rate. Initial runs were tested under a finite sites (HKY) model of evolution and default priors $M = 10$, $T = 20$ and $\theta = 10$, to explore the posterior distribution of scaled migration rate (M) and divergence time (T). Initial analysis indicated that migration among major clades was nearly zero, so we reanalysed the data with the migration prior set to $M = 0$ and the max $T = 20$. MDIV analyses were run for 2×10^6 generations following a burn-in period of 500,000 generations, and analyses were repeated three times to ensure convergence on the same posterior distributions for each parameter. The values of θ, scaled migration rate, and scaled time of divergence with the highest posterior probability were considered as the best estimates. These values were converted into years before present as $t_{div} = (T\theta) / 2\mu$, assuming a generation time of one year and a mutation rate of $1.1–2.9 \times 10^{-9}$ nucleotide substitutions per site per year (Wolfe & al., 1987; Clegg & al., 1994).

■ RESULTS

Chromosome numbers. — Chromosome numbers of plants from six populations of *T. herba-barona* are given in Table 1. Our results strongly suggest that ploidy levels are not exclusive to single islands. The diploid cytotype ($2n = 28$), considered restricted to Majorca was also found in a Corsican population (Haut Ascò), and the tetraploid cytotype ($2n = 56$), previously known only from Corsica, was found in populations from Corsica as well as Sardinia (Table 1). We could not verify the presence of the hexaploid cytotype ($2n = 84$) in *T. herba-barona*, even in the accessions from the Gennargentu massif (Bruncu Spina population), from where it was previously reported (Diana-Corrias, 1980).

Genetic diversity and population structure. — Sequences ranged from 673 to 691 base pairs (bp), with a total aligned length of 703 bp. Variation was neutral, judging from Tajima's (D = 1.781, P > 0.5) and Fu's tests (F_S = 2.313, P > 0.5). Thirteen polymorphic sites were detected, identifying a total of 17 haplotypes (Table S1 in the Electronic Supplement). This was the most likely number of haplotypes estimated using the Stirling probability, suggesting that we have sampled all existing haplotypes with 95% certainty. Only two haplotypes were shared between islands, haplotype I and haplotype L, which were present in both Corsica and Sardinia (Fig. 1A; Table 1). All the analysed individuals from Majorca shared haplotype O. In Sardinia, all populations located in the Gennargentu massif were polymorphic, harbouring between two and four haplotypes, while the remaining Sardinian populations were fixed for a single haplotype (Fig. 1A). Five out of the twelve haplotypes present in Sardinia were shared among two populations (haplotypes A, B, D, G, and J; Fig. 1A). All Corsican populations were polymorphic with a single exception (OLM), and four of the six haplotypes found in this island were shared by at least two populations (haplotypes H, K, L, and N; Fig. 1A).

These results were highly coincident with the results obtained excluding the poly-A repeat, although the number of haplotypes was considerably lower (10; Fig. S1 in the Electronic Supplement). Sardinia was again the island with the highest number of haplotypes (9), and only few (3) were shared among islands. All but one population located in the Gennargentu massif were polymorphic, harbouring between two and four haplotypes, while the remaining Sardinian populations belonged to a single haplotype. All Corsican populations were polymorphic with the single exception of OLM.

Parameters of genetic diversity and structure are given in Table 2. Total genetic diversity detected in *T. herba-barona* was high, with very similar values for ordered and unordered haplotypes (v_t = 0.977 ± 0.0575 and h_t = 0.979 ± 0.0082, respectively). Within-population variation, by contrast, was low, with average haplotypic diversity values of h_s = 0.334 ± 0.0858 and v_s = 0.376 ± 0.1058, based on unordered and ordered alleles, respectively. Genetic differentiation among all 15 populations was high, and was independent of the genetic distance between haplotypes (G_{st} = 0.659 ± 0.0899, N_{st} = 0.615 ± 0.0962; N_{st} = G_{st}, U = −0.81, P > 0.05). This was in accordance with the AMOVA results, revealing that about 67% of the chloroplast variation in *T. herba-barona* was explained by differences among locations (Table 3). Genetic differentiation among islands was low (7.71%) and non-significant (Table 3).

When islands were analysed separately, Sardinia revealed a stronger population differentiation than Corsica, as shown by the AMOVA (72.12% vs. 49.53%; Table 3) and HAPLONST analyses (G_{st} = 0.653 vs. 0.557, N_{st} = 0.659 vs. 0.464; Table 2). Neither Corsica (N_{st} = G_{st}, U = −0.39, P > 0.05) nor Sardinia (N_{st} = G_{st}, U = 0.14, P > 0.05) revealed significant phylogeographic structure. These results were in agreement with those obtained from the SAMOVA approach, since F_{CT} values were similar for all K analysed (range from 0.439 to 0.560), suggesting that there is not a single group of geographically homogeneous and maximally differentiated populations (data not shown).

Table 1. Location, description and codes of fifteen *Thymus herba-barona* populations included in this study. Geographical location of each population is shown in Fig. 1. Number of individuals carrying a given haplotype shown in brackets.

Island	Locality	Code	Latitude, Longitude	Altitude [m]	Sample size	Haplotypes	Chromosome number	References
Majorca	Alfàbia	ALF	39.73° N, 2.71° E	990	7	O (7)	$2n$ = 28	1, 2
Corsica	Olmi	OLM	42.43° N, 9.29° E	775	7	H (7)	–	–
	Haut Ascò	ASC	42.37° N, 8.91° E	1825	8	K (7), N (1)	$2n$ = 28	This work
	Lago di Capitello	CAP	42.21° N, 9.01° E	1850	7	K (1), N (6)	$2n$ = 56	This work
	Bocca d'Illarata	ILL	41.70° N, 9.21° E	905	7	H (2), L (3), M (2)	–	–
	Colle di Vizzavona	VIZ	42.11° N, 9.12° E	1150	7	H (2), I (1), L (4)	$2n$ = 56	This work
	Monte Reinoso						$2n$ = 56	3[a]
Sardinia	Monte Rasu	RAS	40.42° N, 9.00° E	1000	7	J (7)	–	–
	Badde Urbara	URB	40.16° N, 8.62° E	930	7	C (7)	–	–
	Montarbu di Seui	SEU	39.89° N, 9.38° E	1305	7	D (6), J (1)	–	–
	Monte Santa Vittoria	VIT	39.75° N, 9.31° E	1120	7	E (4), F (3)	–	–
	Monte Spada	SPA	40.07° N, 9.27° E	1300	7	B (3), G (4)	$2n$ = 56	This work
	Monte Novo S. Giovanni	GIO	40.12° N, 9.40° E	1210	7	A (2), B (2), G (2), L (1)	–	–
	Bruncu Spina	GEN	40.02° N, 9.30° E	1800	7	A (2), D (3), I (2)	$2n$ = 56; $2n$ = 84	This work; 4
	Monte Linas	MES	39.45° N, 8.62° E	975	7	P (7)	$2n$ = 56	This work
	Monte Limbara	LIM	40.85° N, 9.18° E	1200	7	Q (7)	–	–

References: 1, Mayol & al. (1990); 2, Rosselló & Castro (2008); 3, Contandriopoulos (1962); 4, Diana-Corrías (1980).

[a] Contandriopoulos (1962) also reports "un fort degreé de polyploïdie" from Vizzavona and Monte Foscu (Corsica), although the exact number of chromosomes is not specified.

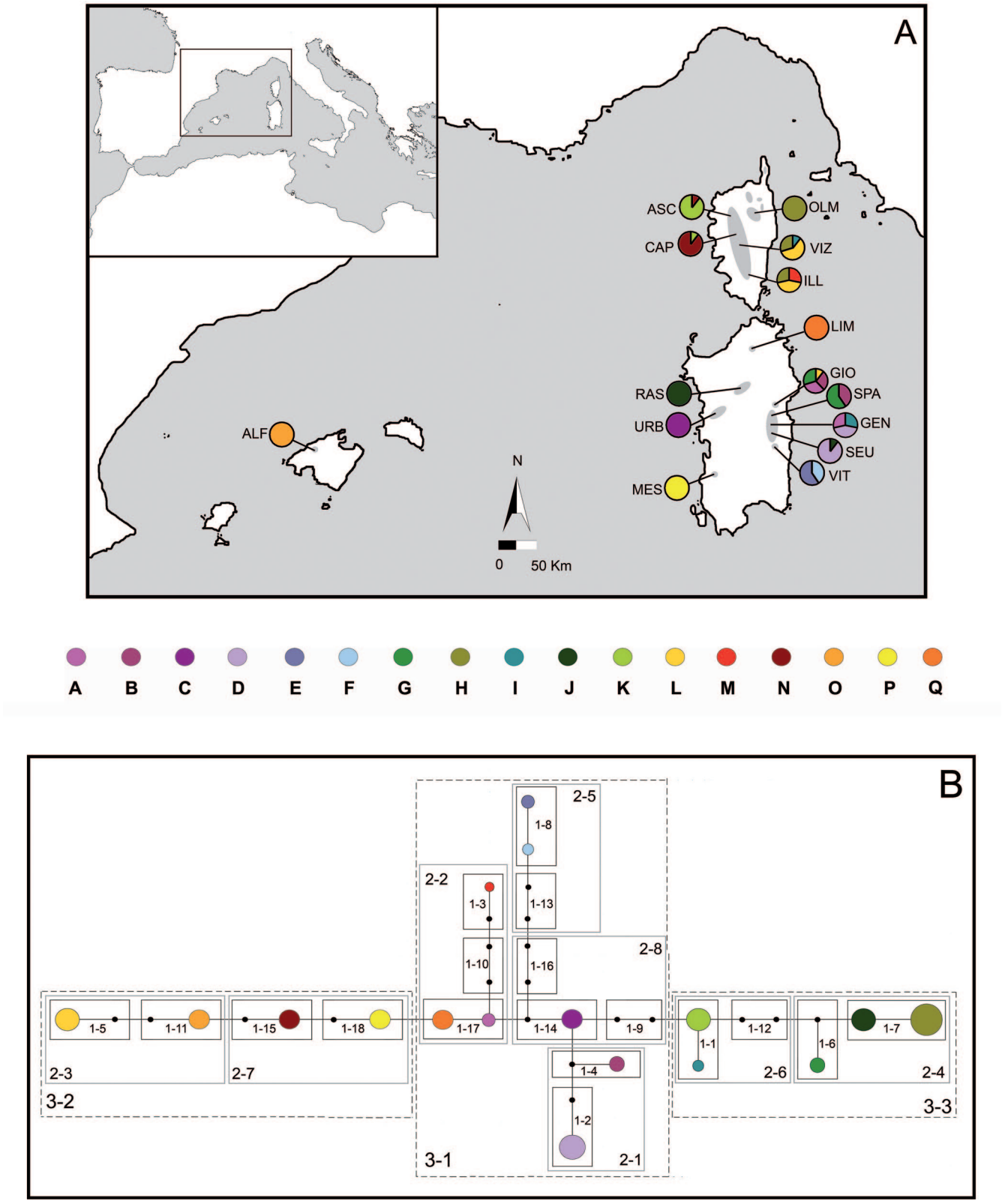

Fig. 1. A, Geographical distribution of the 17 chloroplast haplotypes found in this study for *Thymus herba-barona*. For population codes see Table 1. The grey area indicates the approximate distribution range of the species. **B,** Parsimony network showing the relationships among cpDNA haplotypes detected in *Thymus herba-barona*. The size of the circle is proportional to the frequency of each haplotype in the total sample. Each line in the network represents one mutational step. Small black circles represent missing haplotypes that were not observed in the data. Hierarchically nested clades are indicated by boxes and numbered clade designations.

Haplotype network and nested clade analysis. — TCS estimated a 95% connection limit of two mutational steps, leaving seven haplotypes unincorporated. By manually increasing the connection limit it was possible to connect all of them with a non-parsimonious connection of six steps (Fig. 1B). Multiple missing haplotypes (19) were inferred in the network. Two closed loops were detected, one linking haplotypes B, C, and two non-detected haplotypes, and the other linking two missing haplotypes. These network ambiguities could be resolved using Templeton's frequency and geographical criteria, and ultimately did not affect nesting design or conclusions inferred from the analysis. The hierarchical nested design identified 18 one-step, 8 two-step, and 3 three-step clades (Fig. 1B). The interior clade 3-1 comprised eight haplotypes (A, B, C, D, E, F, M, Q) that were distributed along the Gennargentu massif, northern Sardinia, and southern Corsica (Fig. 1). Clades 3-2 and 3-3 showed an external position in the network, and comprised four (L, N, O, P) and five (G, H, I, J, K) haplotypes, respectively (Fig. 1B). The former haplotype group was present in Majorca (O), Corsica (L, N) and Sardinia (L, P), while the latter was widely distributed in Corsica (H, I, K) and Sardinia (G, I, J). NCPA identified twelve clades for which the null hypothesis of no geographic structuring of haplotypes could be rejected (Table 4). Higher-level (older) clades indicated that the evolutionary history of *T. herba-barona* was characterized by an early range expansion event, followed by more recent fragmentation episodes, the main process detected for younger clades (Table 4). The alternative network, obtained when the polymorphic poly-A repeat was not taken into account, was highly congruent with these results (Fig. S1). Interior haplotypes were restricted to the Gennargentu massif, while external clades harboured haplotypes widely distributed in Corsica and Sardinia, or in Corsica, Majorca, and Sardinia (Fig. S1). Total cladogram also indicated an early range expansion event, while

Table 2. Estimates of genetic diversity and differentiation measures for ordered (v_s, v_t, N_{st}) and unordered (h_s, h_t, G_{st}) haplotypes in *Thymus herba-barona*, and their standard deviations. h_s and v_s, intrapopulation diversity, h_t and v_t, total diversity, G_{st} and N_{st}, pairwise differentiation between populations.

Parameter	Majorca	Corsica	Sardinia	Total value
No. of populations	1	5	9	15
No. individuals	7	36	63	106
No. of haplotypes	1	6	12	17
No. of private haplotypes	1	4	10	–
Average no. haplotypes	1	7.20	7.00	7.07
No. of polymorphic sites	–	9	13	13
h_s	–	0.393 ± 0.1410	0.339 ± 0.1189	0.334 ± 0.0858
h_t	–	0.887 ± 0.0460	0.976 ± 0.0179	0.979 ± 0.0082
G_{st}	–	0.557 ± 0.1731	0.653 ± 0.1264	0.659 ± 0.0899
v_s	–	0.467 ± 0.1708	0.333 ± 0.1347	0.376 ± 0.1058
v_t	–	0.871 ± 0.1053	0.976 ± 0.0721	0.977 ± 0.0575
N_{st}	–	0.464 ± 0.1685	0.659 ± 0.1371	0.615 ± 0.0962
$N_{st} - G_{st}$	–	−0.093	0.006	−0.044

Table 3. Analysis of molecular variance (AMOVA) based on *trnT-trnL* intergenic spacer sequence data for *Thymus herba-barona*.

	Source of variation	df	Sum of squares	Variance components	Percentage of variation
Assuming no regional differentiation	Among populations	14	233.64	2.20740	66.93*
	Within populations	91	99.27	1.09086	33.07
Majorca, Corsica, Sardinia	Among islands	2	46.00	0.26299	7.71 ns
	Among populations within islands	12	187.64	2.05887	60.33*
	Within populations	91	99.27	1.09086	31.96*
Corsica	Among populations	4	54.21	1.64997	49.53*
	Within populations	31	52.12	1.68145	50.47
Sardinia	Among populations	8	133.43	2.25794	72.12*
	Within populations	54	47.14	0.87302	27.88

* $P < 0.001$ (significant after 10,000 permutations), ns = not significant.

Table 4. Inferred historical processes affecting genetic structure of *Thymus herba-barona* populations based on nested clade analysis.

Clade	χ^2-statistic	Probability	Inference chain	Inferred event
1-1	11.00	<0.050	1–19–20–211–12 No	Contiguous range expansion
1-7	19.00	<0.001	1–19–20–2–11–12–13–14 No	Long-distance colonisation and/or past fragmentation
1-17	11.00	<0.010	1–19 No	Allopatric fragmentation
2-1	13.00	<0.010	1–19 No	Allopatric fragmentation
2-2	13.00	<0.050	1–19 No	Allopatric fragmentation
2-3	15.00	<0.001	1–19–20–2–11–12–13–14 No	Long-distance colonisation and/or past fragmentation
2-4	24.00	<0.001	1–2–11–12 No	Contiguous range expansion
2-7	14.00	<0.001	1–19 No	Allopatric fragmentation
3-1	109.62	<0.001	1–19–20–2–3–5-15 No	Long-distance colonisation and/or past fragmentation
3-2	29.00	<0.001	1–2–11–12 No	Contiguous range expansion
3-3	32.86	<0.001	1–2–3–4 No	Restricted gene flow with isolation by distance
Total cladogram	141.20	<0.001	1–2–11–12 No	Contiguous range expansion

the main processes detected for younger (lower-level) clades are allopatric fragmentation or restricted gene flow with isolation by distance.

Divergence time between the high-level clades of *Thymus herba-barona*. — Coalescent-based analyses indicated that our data were consistent with a model of large divergence times with limited gene flow between major clades, rather than a model of recurrent gene flow. Estimates of *T* for all pairwise comparisons were similar (clades 3-1/3-2 = 2.00; clades 3-1/3-3 = 2.40; clades 3-2/3-3 = 4.00), suggesting that all the events occurred approximately at the same time. Assuming mutation rates of $1.1–2.9 \times 10^{-9}$ substitutions per site per year (s/s/y), the divergence times between clades 3-1/3-2, clades 3-1/3-3, and clades 3-2/3-3 were placed during the Middle Pliocene–Lower Pleistocene, at 3.26–1.23, 3.66–1.39, and 3.90–1.48 Ma, respectively. For TMRCA, estimates were slightly greater, with values ranging from 4.96–1.88, 5.52–2.09 and 5.99–2.27 Ma among clade pairs 3-1/3-2, 3-1/3-3, and 3-2/3-3.

■ DISCUSSION

The distribution of genetic variation: The relative role of gene flow, selection, and drift. — The results obtained in this study for *Thymus herba-barona* indicate high levels of genetic diversity, both at the species and the within-population levels (h_t = 0.979; h_s = 0.334, respectively). We detected a total of 17 cpDNA haplotypes, which is similar to the number of haplotypes found in the widespread Mediterranean *Cistus creticus* L. on Corsica and Sardinia (16 haplotypes; Falchi & al., 2009). There is a general perception that endemic island plant species often have reduced genetic diversity compared to more common and widespread species (Frankham, 1997), but this trend is not consistent, and there is a scarcity of examples from the western Mediterranean basin. For example, high genetic variation has been detected in some narrowly distributed, endemic species from the Balearic Islands, such as *Crepis triasii* (Cambess.) Fries or *Hippocrepis balearica* Jacq. (21 and 18 haplotypes, respectively; our unpublished results), and in *Cyclamen creticum* Hildebr., endemic to the island of Crete (Affre & Thompson, 1997). Thus, there is a need for more studies on the genetic diversity present in endemic plants from this region.

In addition to the high within-population diversity, we found that approximately 67% of the chloroplast variation in *T. herba-barona* was found between populations, suggesting a limited extent of gene flow among the studied populations. Substantial levels of genetic structure have been reported for other Mediterranean island endemics, such as *Brassica insularis* Moris (Hurtrez-Boussès, 1996), *Cyclamen creticum* Hildebr. (Affre & Thompson, 1997), *Nigella arvensis* L. alliance (Bittkau & Comes, 2005), *Brassica cretica* Lam. (Edh & al., 2007), *Centaurea horrida* Badarò (Mameli & al., 2008), or *Senecio rodriguezii* Willk. ex J.J. Rodr. (Molins & al., 2009). Significant population divergence is expected to develop in species occurring in spatially isolated populations and with restricted dispersal capability, circumstances that are likely to reduce gene flow and enhance random genetic drift. Recently, drift has been invoked as a major evolutionary force driving plant diversification in the Aegean region, having greater impact on population structure than cytoplasmic gene flow (Widén & al., 2002; Bittkau & Comes, 2005; Edh & al., 2007). In the case of *T. herba-barona*, our results are consistent with this hypothesis, since most of the haplotypes found were exclusive to only one population (C, E, F, M, O, P, Q) or were restricted to a few geographically close localities (A, B, D, G, K, N). Moreover, smaller and more isolated populations (ALF, MES, URB, RAS, LIM, OLM; Fig. 1) were all randomly fixed for a single haplotype, a result that fits theoretical expectations suggesting that small populations are more prone to genetic drift as a consequence of random sampling effects (e.g., Ellstrand & Elam, 1993). In contrast, most of the Corsican populations and those from the Gennargentu massif (Sardinia), where the species is more widely distributed, harboured at least two different haplotypes (Fig. 1), and genetic divergence among Corsican populations was much lower than

among those from Sardinia (49.53 vs. 72.12%; Table 3). All these facts suggest that genetic drift might have acted as a major evolutionary force determining the patterns of genetic variability and structure observed, and that its effects have been higher in those areas where the species occurs in small and more isolated populations.

In gynodioecious species, the impairment of the pollen formation in functional females results from interactions between the nuclear and the mitochondrial genomes (CMS: cytoplasmic male sterility; reviewed in Ivanov & Dymshits, 2007). Selection targeting mitochondrial genes associated with CMS could influence cpDNA evolution as well, owing to gametic disequilibrium between cpDNA and mtDNA (Olson & McCauley, 2000; McCauley & Olson, 2003). Because in many gynodioecious species, such as *Thymus vulgaris* L., female monoecious plants produce more viable seeds than hermaphrodites (Thompson & Tarayre, 2000), this could result in an increased frequency of the mtDNA carrying a given CMS factor, but also in the frequency of the cpDNA haplotypes present in the same individuals. To our knowledge, there is no report on the reproductive system of *T. herba-barona*, but preliminary studies involving the single Balearic population (ALF), have detected coexisting hermaphrodite and female individuals, suggesting that it is also a gynodioecious species (J.M. Iriondo, Universidad Rey Juan Carlos, Spain, pers. comm.). Hence, we cannot rule out the possibility that such kind of hitchhiking-like effect could indeed have contributed to the structuring of genetic variation in some populations. The fact that all small and highly isolated populations are fixed for a single haplotype, however, suggests an important effect of genetic drift relative to other evolutionary processes, such as gene flow or selection. In any case, a detailed understanding of the role of cytoplasmic selection in this species requires specific studies dealing with this question.

Evolutionary history of *T. herba-barona*. — A striking result of this study is the lack of a phylogeographic break between populations from different islands, even though 15 of 17 haplotypes were restricted to single islands (one to Majorca, four to Corsica, ten to Sardinia; Table 2). The lack of phylogeographic structure was also evident within major islands (Corsica, Sardinia), meaning that similar haplotypes were not geographically close to each other. Instead, haplotypes belonging to clades 3-2 and 3-3, differing from each other by at least six mutations, were geographically widely distributed and shared by several populations (Fig. 1). In contrast, haplotypes from clade 3-1 were mainly restricted to the Gennargentu massif in Sardinia. This, together with the interior position of the clade 3-1 in the network, is consistent with a model of range expansion, where the older (interior) haplotypes are confined to the ancestral preexpansion area, and the younger (derived) haplotypes are geographically widespread (Templeton & al., 1995). Accordingly, the oldest event inferred from NCPA in the population history of *T. herba-barona* was a range expansion from the Gennargentu massif (Sardinia) to the remaining territories. After this range expansion, inferred population history from lower-level (younger) clades supports the occurrence of several fragmentation episodes (Table 4).

Setting an approximate time frame for the above-mentioned processes is complicated by the absence of any fossil record. In such cases, coalescent-based methods have proven a valuable tool for estimating demographic parameters and divergence times (Knowles & Maddison, 2002; Nielsen & Beaumont, 2009). Under the slower mutation rates assumed here (1.1×10^{-9} s/s/y), the time to common ancestry (TMRCA) among three-step clades ranged from 5.99 to 4.96 Ma. Reconciliation between the inferred ages and the palaeogeological history of the Mediterranean basin suggest that an ancestor of *T. herba-barona* dispersed from Sardinia to Corsica and Majorca during the desiccation of the Mediterranean Sea between 5.96 and 5.33 Ma (the Messinian salinity crisis; Krijgsman & al., 1999). After this date, the estimated divergence time between major clades (3.90–3.26 Ma) suggests that several fragmentation episodes took place within all islands, probably related to the onset of the Mediterranean climatic regime during the Pliocene (ca. 3.2 Ma; Suc, 1984) and the alternation of warm and cold periods during the Pleistocene glaciations (1.8 Ma to 15 ka). The progressive warming and drying of the climate has caused high levels of extinction in the pre-existing flora, as well as an important shift in the distribution of many species (Thompson, 2005). Mesophilous plants were replaced by thermophylous taxa or displaced to higher altitudes, leading to the fragmentation and isolation of formerly well-connected areas. Populations of *T. herba-barona* are found today at high altitude (above 800 m), suggesting that the recent climate may prevent these populations from occurring at lower altitudes, which may limit gene flow among populations. This is congruent with the lower among-population differentiation found in Corsica, where mountain areas occurring above the 1000 m are more frequent than in Majorca or Sardinia.

The Messinian salinity crisis has been advocated as one of the main events in shaping the biogeography of both animal and plant species in the western Mediterranean (e.g., Bocquet & al., 1978; Kiefer & Bocquet, 1979; Stöck & al., 2008). During this time, land bridges between different islands and the continent could have acted as corridors allowing the exchange of taxa. However, land connections among the Balearic Islands and the Corso-Sardinian archipelago during the Messinian salinity crisis have not been indicated (Alvarez & al., 1974; Rosenbaum & al., 2002). Hence, a long-distance dispersal event must be invoked to explain the occurrence of *T. herba-barona* in the Balearic Islands if we accept that the range expansion of the species has taken place during this period. While a long-distance dispersal event was incongruent with the cytogenetic evidence available from previous studies, the cytogenetic evidence reported in this study shows that the diploid cytotype occurs both on Corsica and Majorca (Table 1). Based on this new evidence, a long-distance colonization from diploid populations in Corsica to the Balearic Islands cannot be discarded, although it seems unlikely given the strong genetic divergence and reduced gene flow detected among populations from these islands. In addition, the presence of high ploidy levels (4*x*, 6*x*) in the Gennargentu massif, where the older haplotypes are confined, together with the fact that several ploidy levels were detected on the larger islands (Corsica, Sardinia), rather

suggests that the occurrence of polyploidy was a relatively recent event that occurred independently in each population, and thus may not be a reliable indicator to support a long-distance colonization of the Balearic Islands. Finally, the cytogenetic evidence does not support the taxonomic distinction of three entities within *T. herba-barona* proposed by Camarda (2003) based on morphological discontinuities associated with different ploidy levels.

The accuracy of our divergence-time estimates depends on the reliability of the substitution rate assumed. The mutation rates used have been inferred from cultivated plants (e.g., tobacco, wheat, rice; Wolfe & al., 1987; Clegg & al., 1994), whose sequence divergence could be faster than those of wild Mediterranean plants. Although the Mediterranean region has suffered significant geological and past climatic changes, the heterogeneous topography of the region has provided particular suitable habitats within a matrix of unsuitable landscapes, allowing the long-term persistence of populations and favouring evolutionary stasis of many palaeoendemisms (Hampe & Petit, 2005; Thompson, 2005). Several Hercynian palaeoendemics show little or no morphological differentiation despite a long history of isolation on small fragments of what were once larger microplates (e.g., *Arum pictum, Arenaria balearica, Helicodiceros muscivorus*, *Soleirolia soleirolii*, *Thymus herba-barona*). Slow sequence evolution is often associated with morphological stasis (Barraclough & Savolainen, 2001; Soltis & al., 2002). Thus, the long-term geographical persistence of Hercynian endemic plants could have resulted in slowed evolutionary rates, thereby preserving ancestral molecular variants. An illustrative example of slow rates of molecular change is that of *Quercus suber* L., whose populations preserve the genetic footprints of the Oligocene connections among the lands forming the Hercynian massif, suggesting that distinct haplotypes have persisted without detectable chloroplast modification for more than 15 million years (Magri & al., 2007). The highly structured pattern of the five distinct haplotypes found in *Cephalaria squamiflora* (Sieber) Greuter, a species complex restricted to few islands in the western and eastern Mediterranean region, has also been attributed to Oligocenic land connections although no more than four mutational steps were detected among the most distant haplotypes (Rosselló & al., 2009). Hence, it is likely that long periods of stasis favouring the preservation of ancestral molecular variants in different lineages have been a much more common situation in the Mediterranean region than previously thought. As to *T. herba-barona*, some evidence suggests an ancient origin of the phylogeographic pattern observed. First, the high cpDNA diversity detected may be explained by ancient presence of the species, allowing the accumulation of a high number of mutations. Second, the inference of multiple (19) missing intermediate haplotypes in the network (Fig. 1) supports a long evolutionary separation of populations leading to the extinction of ancestral haplotypes.

In summary, our results are not sufficiently conclusive to reject any of the two hypotheses, i.e., the current distribution of *T. herba-barona* being due to (1) a vicariant process congruent with the Oligocene geological splitting of the Hercynian massif (~30 Ma), or (2) a range expansion followed by long-distance colonization of Majorca during the Late Miocene (Messinian, ~6–5 Ma). However, deep coalescence times clearly suggest that the inferred evolutionary processes are not related to human activities. Additional studies are needed to better understand the complex evolutionary history of this endemic plant, increasing the number of sequenced genes in order to obtain better estimates of divergence times.

■ CONCLUSIONS

The results of this study show a highly structured pattern of genetic variation in *T. herba-barona*, suggesting an important effect of genetic drift relative to gene flow or selection in accordance with the results from recent studies on other Mediterranean insular systems. Its inferred population history supports an early range expansion event from the Gennargentu massif in Sardinia to Corsica and Majorca, followed by a more recent fragmentation of its distribution range. Our divergence-time estimates suggest that the current distributional range of the species, being restricted to mountain habitats, probably represents relict populations from once more widespread mesophytic ancestors during more favourable climatic periods. Progressive climatic warming since the Plio-Pleistocene (3.2 Ma) likely resulted in fragmentation and reduction of the range of the species. The presumed Hercynian origin of the species remains ambigous, however; more data from additional loci are needed in order to improve our understanding of the temporal and spatial origins of this endemic plant. Finally, the cytogenetic evidence does not support the taxonomic distinction of three entities within *T. herba-barona* proposed by Camarda (2003) based on morphological discontinuities associated with different ploidy levels.

■ ACKNOWLEDGEMENTS

This research was supported by funds from Spanish Ministry of Education and Science (Project CGL2004-00223/BOS) and Catalan Government (Consolidated Research Group 2009SGR608), and by a Ph.D. grant from Spanish Ministry of Education and Science to A. Molins (BES-2005-7898). We thank two anonymous reviewers for their helpful suggestions to improve the quality of the manuscript.

■ LITERATURE CITED

Affre, L. & Thompson, J.D. 1997. Population genetic structure and levels of inbreeding depression in the Mediterranean island endemic *Cyclamen creticum* (Primulaceae). *Biol. J. Linn. Soc.* 60: 527–49.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucl. Acids Res.* 25: 3389–3402.

Alvarez, W., Cocozza, T. & Wezel, F.C. 1974. Fragmentation of alpine orogenic belt by microplate dispersal. *Nature* 248: 309–314.

Barraclough, T.G. & Savolainen, V. 2001. Evolutionary rates and species diversity in flowering plants. *Evolution* 55: 677–83.

Bittkau, C. & Comes, H.P. 2005. Evolutionary processes in a

continental island system: Molecular phylogeography of the Aegean *Nigella arvensis* alliance (Ranunculaceae) inferred from chloroplast DNA. *Molec. Ecol.* 14: 4065–4083.

Bocquet, G., Widler, B. & Kiefer, H. 1978. The Messinian Model—A new outlook for the floristics and systematics of the Mediterranean area. *Candollea* 33: 269–287.

Camarda, I. 1978. Le piante endemiche della Sardegna: 21–23. *Boll. Soc. Sarda Sci. Nat.* 17: 227–241.

Camarda, I. 2003. *Thymus catharinae* (Lamiaceae), *Dianthus stellaris* (Caryophyllaceae) e *Rubus limbarae* (Rosaceae) species novae di Sardegna. *Parlatorea* 6: 83–93.

Clegg, M.T., Gautt, B.S., Learn, G.H., Jr. & Morton, B.R. 1994. Rates and patterns of chloroplast DNA evolution. *Proc. Natl. Acad. Sci. U.S.A.* 91: 6795–6801.

Clement, M., Posada, D. & Crandall, K.A. 2000. TCS: A computer program to estimate gene genealogies. *Molec. Ecol.* 9: 1657–1659.

Contandriopoulos, J. 1962. Recherches sur la flore endémique de la Corse et sur ses origines. *Ann. Fac. Sci. Marseille* 32: 1–354.

Contandriopoulos, J. & Cardona, M.A. 1984. Caractère original de la flore endémique des Baléares. *Bot. Helv.* 94: 101–132.

Crandall, K.A. & Templeton, A.R. 1993. Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics* 134: 959–969.

Diana-Corrias, S. 1980. Numerici cromosomici per la flora italiana. *Inform. Bot. Ital.* 12: 125–129.

Dixon, C.J. 2006. A means of estimating the completeness of haplotype sampling using the Stirling probability distribution. *Molec. Ecol. Notes* 6: 650–652.

Dupanloup, I., Schneider, S. & Excoffier, L. 2002. A simulated annealing approach to define the genetic structure of populations. *Molec. Ecol.* 11: 2571–2581.

Edh, K., Widén, B. & Ceptilis, A. 2007. Nuclear and chloroplast microsatellites reveal extreme population differentiation and limited gene flow in the Aegean endemic *Brassica cretica* (Brassicaceae). *Molec. Ecol.* 16: 4972–4983.

Ellstrand, N.C. & Elam, D.R. 1993. Population genetic consequences of small population size: Implications for plant conservation. *Annual Rev. Ecol. Syst.* 24: 217–42.

Excoffier, L., Smouse, P.E. & Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.

Falchi, A., Paolini, J., Desjobert, J.-M., Melis, A., Costa, J. & Varesi, L. 2009. Phylogeography of *Cistus creticus* L. on Corsica and Sardinia inferred by the *TRNL-F* and *RPL32-TRNL* sequences of cpDNA. *Molec. Phylog. Evol.* 52: 538–543.

Favarger, C. & Contandriopoulos, J. 1961. Essai sur l'endémisme. *Ber. Schweiz. Bot. Ges.* 77: 383–408.

Frankham, R. 1997. Do island populations have less genetic variation than mainland populations? *Heredity* 78: 311–327.

Fu, Y.X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147: 915–925.

Greuter, W. 1991. Botanical diversity, endemism, rarity and extinction in the Mediterranean area: An analysis based on the published volumes of Med-Checklist. *Bot. Chron. (Patras)* 10: 63–79.

Greuter, W. 1995. Origin and peculiarities of Mediterranean island floras. *Ecol. Medit.* 21: 1–10.

Hampe, A. & Petit, R.J. 2005. Conserving biodiversity under climate change: The rear edge matters. *Ecol. Letters* 8: 461–467.

Hurtrez-Boussès, S. 1996. Genetic differentiation among natural populations of the rare Corsican endemic *Brassica insularis* Moris: Implications for conservation guidelines. *Biol. Conservation* 76: 25–30.

Ingvarsson, P., Ribstein, S. & Taylor, D. 2003. Molecular evolution of insertions and deletions in the chloroplast. *Molec. Biol. Evol.* 20: 1737–1740.

Ivanov, M.K. & Dymshits, G.M. 2007. Cytoplasmic male sterility and restoration of pollen fertility in higher plants. *Russ. J. Genet.* 43: 354–368.

Jalas, J. 1971. Notes on *Thymus* L. (Labiatae) in Europe. I. Supraspecific classification and nomenclature. *Bot. J. Linn. Soc.* 64: 199–215.

Kiefer, H. & Bocquet, G. 1979. *Silene velutina* Pourret ex Loiseleur (Caryophyllaceae)—example of a Messinian destiny. *Candollea* 34: 459–472.

Knowles, L.L. & Maddison, W.P. 2002. Statistical phylogeography. *Molec. Ecol.* 11: 2623–2635.

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J. & Wilson, D.S. 1999. Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400: 652–655.

López de Heredia, U., Jiménez, P., Díaz-Fernández, P. & Gil, L. 2005. The Balearic Islands: A reservoir of cpDNA genetic variation for evergreen oaks. *J. Biogeogr.* 32: 939–949.

Magri, D., Fineschi, S., Bellarosa, R., Buonamici, A., Sebastiani, F., Schirone, B., Simeone, M.C. & Vendramin, G.G. 2007. The distribution of *Quercus suber* chloroplast haplotypes matches the palaeogeographical history of the western Mediterranean. *Molec. Ecol.* 16: 5259–5266.

Mameli, G., Filigheddu, R., Binelli, G. & Meloni, M. 2008. The genetic structure of the remnant populations of *Centaurea horrida* in Sardinia and associated islands. *Ann. Bot.* 101: 633–640.

Mansion, G., Rosenbaum, G., Schoenenberger, N., Bacchetta, G., Rosselló, J.A. & Conti, E. 2008. Phylogenetic analysis informed by geological history supports multiple, sequential invasions of the mediterranean basin by the angiosperm family Araceae. *Syst. Biol.* 57: 269–285.

Mayol, M., Rosselló, J.A., Mus, M. & Morales, R. 1990. *Thymus herba-barona* Loisel., novedad para España, en Mallorca. *Anales Jard. Bot. Madrid* 47: 516.

Mayol, M., Sáez, L. & Rosselló, J.A. 1998. *Thymus herba-barona* subsp. *bivalens*, a new endemic taxon from the Balearic Islands. *Flora Montiber.* 8: 61–64.

McCauley, D.E. & Olson, M.S. 2003. Associations among cytoplasmic molecular markers, gender, and components of fitness in *Silene vulgaris*, a gynodioecious plant. *Molec. Ecol.* 12: 777–787.

Médail, F. 2008. A natural history of the islands' unique flora. Pp. 26–33 in: Arnold, C. (ed.), *Mediterranean islands: A unique and comprehensive guide to the islands and islets of the Mediterranean*. London: Mediterranean Islands c/o Survival Books.

Médail, F. & Diadema, K. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *J. Biogeogr.* 36: 1333–1345.

Médail, F. & Quézel, P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. *Ann. Missouri Bot. Gard.* 84: 112–127.

Médail, F. & Quézel, P. 1999. Biodiversity hotspots in the Mediterranean Basin: Setting global conservation priorities. *Conservation Biol.* 13: 1510–1513.

Molins, A., Mayol, M. & Rosselló, J.A. 2009. Phylogeographical structure in the coastal species *Senecio rodriguezii* (Asteraceae), a narrowly distributed endemic Mediterranean plant. *J. Biogeogr.* 36: 1372–1383.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B. & Kent, J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

Nielsen, R. & Beaumont, M.A. 2009. Statistical inferences in phylogeography. *Molec. Ecol.* 18: 1034–1047.

Nielsen, R. & Wakeley, J. 2001. Distinguishing migration from isolation: A Markov Chain Monte Carlo approach. *Genetics* 158: 885–896.

Olson, M.S. & McCauley, D.E. 2000. Linkage disequilibrium and phylogenetic congruence between chloroplast and mitochondrial haplotypes in *Silene vulgaris*. *Proc. Roy. Soc. Biol. Sci. Ser. B* 267: 1801–1808.

Panchal, M. 2007. The automation of nested clade phylogeographic analysis. *Bioinformatics* 23: 509–510.

Pfenninger, M. & Posada, D. 2002. Phylogeographic history of the land snail *Candidula unifasciata* (Helicellinae, Stylommatophora): Fragmentation, corridor migration and secondary contact. *Evolution* 56: 1776–1788.

Pons, O. & Petit, R.J. 1995. Estimation, variance and optimal sampling of gene diversity. I. Haploid locus. *Theor. Appl. Genet*. 90: 462–470.

Pons, O. & Petit, R.J. 1996. Measuring and testing genetic differentiation with ordered and unordered alleles. *Genetics* 144: 1237–1245.

Posada, D., Crandall, K.A. & Templeton, A.R. 2000. GeoDis: A program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molec. Ecol.* 9 : 487–488.

Quézel, P. 1995. La flore du bassin méditerranéen: Origine, mise en place, endémisme. *Ecol. Medit.* 21: 19–39.

Rosenbaum, G., Lister, G.S. & Duboz, C. 2002. Reconstruction of the tectonic evolution of the western Mediterranean since the Oligocene. *J. Virtual Explor.* 8: 107–130.

Rosselló, J.A. & Castro, M. 2008. Karyological evolution of the angiosperm endemic flora of the Balearic Islands. *Taxon* 57: 259–273.

Rosselló, J.A., Cosín, R., Bacchetta, G., Brullo, S. & Mayol, M. 2009. Nuclear and chloroplast DNA variation in *Cephalaria squamiflora* (Dipsacaceae), a disjunct Mediterranean species. *Taxon* 58: 1242–1253.

Sales, E., Nebauer, S.G., Mus, M. & Segura, J. 2001. Population genetic study in the Balearic endemic plant species *Digitalis minor* (Scrophulariaceae) using RAPD markers. *Amer. J. Bot.* 88: 1750–1759.

Schneider, S., Roessli, D. & Excoffier, L. 2000. Arlequin, version 2.000: A software for population genetic data analysis. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.

Simmons, M.P., Ochoterena, H. & Carr, T.G. 2001. Incorporation, relative homoplasy, and effect of gap characters in sequence-based phylogenetic analyses. *Syst. Biol*. 50: 454–462.

Soltis, P.S., Soltis, D.E., Savolainen, V., Crane, P.R. & Barraclough, T.G. 2002. Rate heterogeneity among lineages of tracheophytes: Integration of molecular and fossil data and evidence for molecular living fossils. *Proc. Natl. Acad. Sci. U.S.A.* 99: 4430–35.

Speranza, F., Villa, I.M., Sagnotti, L., Florindo, F., Cosentino, C., Cipollari, P. & Mattei, M. 2002. Age of the Corsica-Sardinia rotation and Liguro-Provençal basin spreading: New paleomagnetic and Ar/Ar evidence. *Tectonophysics* 347: 231–251.

Stöck, M., Sicilia, A., Belfiore, N.M., Buckley, D., Lo Brutto, S., Lo Valvo, M. & Arculeo, M. 2008. Post-Messinian evolutionary relationships across the Sicilian channel: Mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. *B.M.C. Evol. Biol.* 8: 56. DOI: 10.1186/1471-2148-8-56.

Suc, J.-P. 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature* 307: 429–438.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Pl. Molec. Biol*. 17: 1105–1109.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123: 585–595.

Templeton, A.R., Crandall, K.A. & Sing, C.F. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* 132: 619–633.

Templeton, A.R., Routman, E. & Phillips, C.A. 1995. Separating population structure from population history: A cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics* 140: 767–782.

Thompson, J.D. 2005. *Plant evolution in the Mediterranean*. Oxford: Oxford University Press.

Thompson, J.D. & Tarayre, M. 2000. Exploring the genetic basis and proximate causes of female fertility advantage in gynodioecious *Thymus vulgaris*. *Evolution* 54: 1510–1520.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. 1997. The ClustalX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 25: 4876–4882.

Westphal, M., Orsini, J. & Vellutini, P. 1976. Corsica-Sardinia microcontinent, its initial position — paleomagnetic data and geological fitting. *Tectonophysics* 30: 141–157.

Widén, B., Andersson, S., Rao, G.Y. & Widén, M. 2002. Population divergence of genetic (co)variance matrices in a subdivided plant species, *Brassica cretica*. *J. Evol. Biol.* 15: 961–970.

Wolfe, K.H., Li, W.H. & Sharp, P.M. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNA. *Proc. Natl. Acad. Sci. U.S.A.* 84: 9054–9058.

Appendix. Collection and voucher information for populations of *Thymus herba-barona* included in this study. Collectors: *Ad, C. Adamo; An, R. Angius; Ar, F. Argagnon; Ba, G. Bacchetta; Br, S. Brullo; Ca, T. Carai; Co, A. Congiu; Cs, M. Casti; D, A. Demurtas; F, G. Fenu; G, J.L. Gradaille; J, N. Jimenez; L, G. Iiriti; Ma, M. Mayol; Mu, M. Mus; Mt, E. Mattana; N, F. Navarro; Pi, E. Pirodda; Po, L. Podda; Pt, C. Pontecorvo; R, J.A. Rosselló.*

Country, Island , Province, Locality, Code, Date, Collectors, Accession number

Spain, Majorca, Balearic Islands, Alfàbia, ALF, 08.VIII.1989, *G, Ma, Mu, R,* BC808332. **France,** Corsica, Bastia, Olmi, OLM, 28.V.2004, *Ar, Ba, Cs, J, N,* CAG GB315/04. **France,** Corsica, Bastia, Haut Ascò, ASC, 22.VII.2004, *Ad, Ba, Ca, L, Pt,* CAG GB446/04. **France,** Corsica, Bastia, Lago di Capitello, CAP, 23.VII.2004, *Ad, Ba, Ca, L, Pt,* CAG GB455/04. **France,** Corsica, Ajaccio, Bocca d´Illarata, ILL, 27.V.2004, *Ba, Cs, J, N,* CAG GB278/04. **France,** Corsica, Bastia, Colle di Vizzavona, VIZ, 27.V.2004, *Ba, Cs, J, N,* CAG GB294/04. **Italy,** Sardinia, Sassari, Monte Rasu, RAS, 30.V.2004, *Ba, Cs, J, N,* CAG GB350/04. **Italy,** Sardinia, Oristano, Badde Urbara, URB, 30.V.2004, *Ba, Cs, J, N,* CAG GB356/04. **Italy,** Sardinia, Ogliastra, Montarbu di Seui, SEU, 18.VII.2004, *An, Ba, Br, Mt,* CAG GB431/04. **Italy,** Sardinia, Nuoro, Monte Santa Vittoria, VIT, 20.VII.2004, *D, F, Mt, Pi, Po,* CAG GB441/04. **Italy,** Sardinia, Nuoro, Monte Spada, SPA, 25.V.2004, *Ba, Cs, J, N,* CAG GB233/04. **Italy,** Sardinia, Nuoro, Monte Novo S. Giovanni, GIO, 25.V.2004, *Ba, Cs, J, N,* CAG GB244/04. **Italy,** Sardinia, Nuoro, Bruncu Spina, GEN, 22.I.2007, *Ba, F, Pt, R,* CAG GB004/07. **Italy,** Sardinia, M.Campidano, Monte Linas, MES, 26.VI.2008, *Ba,* CAG GB057/08. **Italy,** Sardinia, Olbia-Tempio, Monte Limbara, LIM, 19.I.2008, *Ba, Co,* CAG GB001/08.