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Short Communication

Phylogeography of *Cistus creticus* L. on Corsica and Sardinia inferred by the *TRNL-F* and *RPL32-TRNL* sequences of cpDNA

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1. Introduction

Cistus is a genus of evergreen shrubs native to the Mediterranean region, including the Canary Islands (where four endemic species occur) and Madeira. Plants of the genus are self-incompatible, which leads to crossing between species. Many *Cistus* species are important constituents of the flora of the Corsican bush (termed maquis). They produce large quantities of seed, the germination of which is influenced by fire. The adaptation of this genus to the Mediterranean environment is due to ecological factors including fire events and insect pollination (Farley and McNeilly, 2000). The genus *Cistus* comprises about 30 species native to the Mediterranean area, among which *C. monspelliensis*, *C. ladaniferus*, *C. salviifolius*, *C. laurifolius* and *C. albidus* are the most common (Gaussen et al., 1982).

C. creticus L. (syn. *Cistus incanus* subsp. *creticus* or syn. *Cistus villosus*) is abundant in the oriental Mediterranean basin; it is a shrub (less than 1 m high) with simple opposed leaves and pink flowers (blossoming time, May–June) (Jeanmonod and Gamisans, 2007).

The genetic structure of *C. creticus* remains poorly understood. Guzman and Vargas (2005) proposed a phylogenetic hypothesis for 20 species of the family Cistaceae in Spain, based on plastid and nuclear DNA sequence data. They concluded that *Halimium* and *Cistus* form a natural group, with purple-flowered and white-flowered forms defining two major lineages. Isoenzyme loci analysis has shown high genetic divergence in *C. osbaeckiaefolius*, *C. chinamadensis* and *C. symphytifolius* from the Canary Islands (Batista et al., 2001). Intraspecific variability has been demonstrated in Mediterranean populations of *C. salviifolius* using isoenzyme markers (Farley and McNeilly, 2000), and in *C. ladaniferus* populations using RAPD markers (Quintela-Sabaris et al., 2005). In these studies no correlation was found among isoenzymes, RAPD diversity and environmental factors (Farley and McNeilly, 2000; Quintela-Sabaris et al., 2005).

The evolution of plant diversity in the Mediterranean basin has been influenced by geological history and climatic oscillations (Medail and Quezel, 1999). Mediterranean islands have evolved a large number of endemic plant species and infraspecific taxa (Lopez de Heredia et al., 2005). The islands of Corsica and Sardinia provide a model system to address the impact of long-term isolation on genetic diversity and gene flow. Compared with populations on the continent, the island populations have less genetic variability because habitat limitations have resulted in fewer populations and individuals, promoting effects of bottlenecks or genetic drift. Few studies using molecular genetics methods have focused on the within-region or within-population phylogeographic structure.

The genetic structure of a population is the result of migration and isolation. Phylogeography concerns the correlation between genetic data and geographical distribution. The concept of phylogeography (Avise et al., 1987) has had a large impact on animal systems research, but its application to botanical systems has been relatively slow.

The maternal cytoplasmic DNA (cpDNA) lineages in natural populations often display distinct geographic distributions (Avise, 2000), and noncoding regions of cpDNA have been used successfully in phylogeography studies (Petit et al., 1997; Caron et al., 2000; Dutech et al., 2000; Raspé et al., 2000). Because of its maternal inheritance in angiosperms, the cpDNA transmitted by seeds

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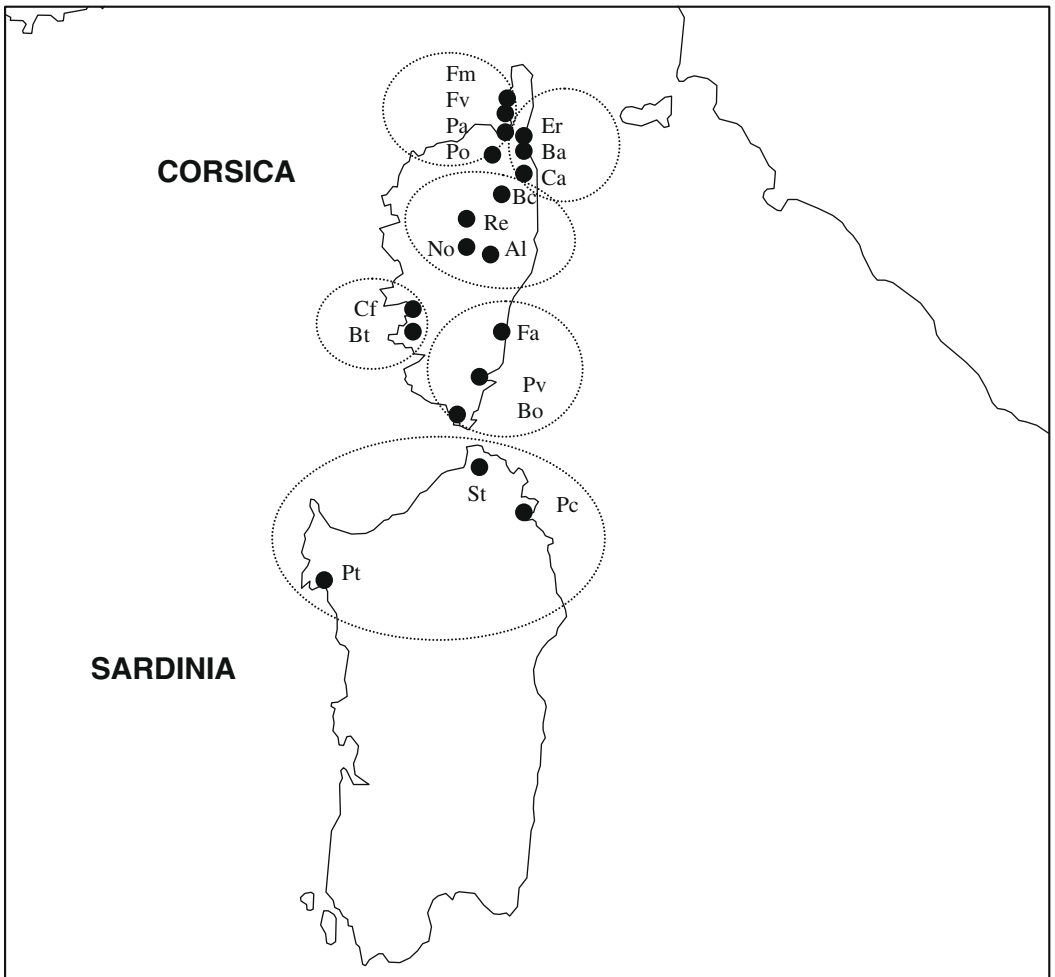
has less gene flow than nuclear DNA transmitted by pollen dispersal.

The coding regions of cpDNA of higher plants are highly conserved. This has led to the design of universal primers that can amplify intergenic regions in most plants. The noncoding sequences have frequently been used to survey plant intraspecific phylogeny, population genetic structure and phylogeography (Sang, 2002). In addition, as a consequence of its low rate of evolution, and low homoplastic and neutral properties (Schaal et al., 1998), cpDNA can be used to study the genetic effects of migration and colonization over long periods (Wolfe et al., 1987; Ennos et al., 1999); several studies have involved the use of cpDNA to investigate vegetation changes resulting from climatic variation during the Pleistocene period (Sewell et al., 1996; King and Ferris, 1998; Tremblay and Schoen, 1999; Caron et al., 2000; Dutech et al., 2000).

All phylogeographic studies involving limited geographic areas have suggested a degree of concordance in terms of distribution patterns, whereas continent-wide surveys suggest more nonconcordant patterns (Taberlet, 1998; Petit and Vendramin, 2007). We restricted our study to the area delimited by the former CorsicaSardinia microplate. Corsica and Sardinia offer particular advantages for phylogeographical and population genetic studies. These islands are characterized by a high level of endemism, a large number of endemic species, and a complex paleogeographical history.

This study is the first to combine molecular phylogenetic and biogeographic approaches to elucidate the evolution of plant diver-



		Location		Haplotype			Location		Haplotype
NE	Ba	Bastia	9°.27' 42°.41'	II	SW	Bt	Bastelicaccia	8°.49' 41°.55'	XVI
	Ca	Casamozza	9°.26' 42°.31'	I		Cf	Capo di Feno	8°.36' 41°.57'	XV
	Er	Erbalunga	9°.28' 42°.46'	II	SE	Pv	Porto Vecchio	9°.17' 41°.34'	IX
CE	Bc	Barchetta	9°.22' 42°.30'	I		Bo	Bonifacio	9°.09' 41°.23'	XI
	Re	Restonica	9°.08' 42°.17'	IV		Fa	Favona	9°.23' 41°.46'	X
	Al	Altiani	9°.17' 42°.14'	III	NS	Pc	Porto Cervo	9°.39' 40°.52'	XIII
	No	Noceta	9°.12' 42°.11'	V		St	Santa Teresa	9°.14' 41°.14'	XII
NW	Fv	Farinole-village	9°.20' 42°.44'	VI		Pt	Porto Conte	9° 00' 40° 56'	XIV
	Fm	Farinole-mer	9°.20' 42°.43'	VI					
	Pa	Patrimonio	9°.21' 42°.42'	VIII					
	Po	Poggio d'Oletta	9°.20' 42°.37'	VII					

Fig. 1. Sample location and haplotype definition of *C. creticus* (NE, northeast Corsica; CE, central Corsica; NW, northwest Corsica; SW, southwest Corsica; SE, southeast Corsica; NS, north Sardinia).

Table 1
The 16 haplotypes of the *TRNL-F* and *RPL32-TRNL* intergenic regions of *C. creticus* cpDNA.

Haplotypes	N	5 0	1 4	2 4	3 6	3 3	9 3	1 5	1 7	2 8	3 0	3 5	4 7	4 3	4 1	5 1	6 1	6 7	6 8	6 9	7 6	7 0	7 5	9 9	9 1	9 8	1 0	1 0	1 1	1 2	1 2	1 2	1 2	1 3	1 3	1 3	1 4	1 5	1 5	1 6	1 7	1 7	1 7
								R	P	L		-	T	R	N	L																											
I	2	T	G	G	T	G	A	G	T	T	C	C	C	T	A	G	A	C	A	G	A	C	C	G	T	T	G	A	1	1	1	2	2	3	3	3	4	4	5	5	6	7	A
II	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
III	1	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
IV	1	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
V	1	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
VI	2	.	A	.	.	.	A	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.
VII	1	.	.	.	.	.	A	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.
VIII	1	.	.	.	.	.	A	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.	.
IX	1	.	.	.	.	C	.	.	A	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	G
X	1	.	.	.	.	C	.	.	A	.	.	.	.	.	G	.	.	.	.	G	.	.	.	.	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	A
XI	1	.	.	.	.	C	.	.	A	.	.	T	.	.	G	A	.	.	.	G	.	.	.	A	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	A
XII	1	.	.	.	.	C	.	.	A	.	.	T	.	.	G	A	.	.	.	G	T	.	.	A	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	A
XIII	1	.	.	.	.	C	.	.	A	.	.	T	.	.	G	.	.	T	.	G	T	.	.	.	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	A
XIV	1	.	.	.	.	C	.	.	A	.	.	T	.	.	G	.	.	.	.	G	T	.	.	.	C	.	.	.	.	.	C	T	.	.	.	.	.	T	.	.	.	.	A
XV	1	.	.	A	.	.	.	A	C	.	.	T	T	.	.	.	T	.	.	G	.	.	.	.	C	.	G	.	.	.	A	C	.	.	.	.	.	.	.	.	C	.	A
XVI	1	.	.	A	.	.	.	A	C	.	.	T	T	.	C	G	.	.	A	G	.	.	.	.	C	.	G	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	A

sity on Corsica and Sardinia. We chose Corsica and Sardinia because (i) they are two of the biggest islands in the western Mediterranean, (ii) they have a high level of endemicity (340 species), and (iii) they have a common geological history. The main goal of the study was to investigate whether the past geological connections and climate history of Corsica and Sardinia could explain the current distribution of their plants.

In this study, the *TRNL-F* and the *RPL32-TRNL* noncoding sequences of cpDNA were used to examine the phylogeographical pattern of 19 populations of *C. creticus* distributed across Corsica and Sardinia.

2. Materials and methods

We sampled 19 populations of *C. creticus* growing wild in Corsica and north Sardinia (Fig. 1). For each population the leaves of three individual shrubs, chosen at random, were collected during spring 2008. Genetic analysis was performed on the leaves of all 57 individuals.

Total DNA was extracted from approximately 0.1 g of fresh leaf material using the DNeasy plant mini kit (Qiagen S.A., Courtaboeuf, France), according to the manufacturer's instructions.

Amplification of DNA was performed in a 25 μl volume PCR mixture containing 2 mM $MgCl_2$, 1 μM of each primer and 12 μl of Hot Start DNA Polymerase Master Mix (Qiagen S.A., Courtaboeuf, France). Amplifications were carried out in a Gene Amp PCR System 9700 (Applied Biosystems; Perkin Elmer, France) with an initial denaturation/activation at 94 °C for 15 min, followed by 30 cycles comprising 30 s at 94 °C, 1 min at 56 °C and 2 min at 72 °C. A final extension for 10 min at 72 °C was included. The *TRNL-F* region was amplified with the primers 5′-ATTTGAACTGGTGACACGAG-3′ and 5′-CGAAATCGGTAGACGCTACG-3′ (Taberlet et al., 1991), and the *RPL32-TRNL* region was amplified with the primers 5′-CTGCTTCCTAAGAGCAGCGT-3′ and 5′-CAGTTCCAAAAAAACGTACTTC-3′ (Shaw et al., 2007).

The amplified products were run in a 1.2% agarose gel, stained with ethidium bromide, and visualized and photographed under UV light (Kodak Gel Logic, Sciencetech, Les Ulis, France). The purified PCR products were sequenced in both directions in an ABI 377 automated sequencer, following standard methods and using the primers used for amplification. Sequences were aligned using the CLUSTAL X program (Thompson et al., 1997). Haplotype and nucleotide diversity were determined using the DNASP 4.0 program (Rozas et al., 2003). Neutrality was assessed using the Tajima D test, and the Fu and Li test. A maximum parsimony analysis was conducted by heuristic search using the MEGA 4 program (Kumar et al., 2008). The robustness of nodes was inferred from a bootstrap analysis of 1000 replicates. Relationships among haplotypes were inferred using TCS software (Clement et al., 2000), which uses a statistical parsimony method to estimate ancestral or intermediate haplotypes.

A nonparametric analysis of molecular variance (AMOVA) was performed using squared Euclidean distances (Excoffier et al., 2005). Variance was apportioned to the following components: among individuals within a population, among populations within a region, and among regions. Genetic analysis and gene flow (*Nm*) was performed using ARLEQUIN version 3.0 software (Excoffier et al., 2005). Because cpDNA is haploid and uniparentally inherited, *Nm* was estimated using the expression $F_{st} = 1/(1 + 2Nm)$, where *N* is the female effective population size and *m* is the female migration rate.

3. Results

The nucleotide sequences of the two cpDNA noncoding regions were determined for 57 leaf samples of *C. creticus* collected from 19

sites on Corsica and Sardinia. The total length of the noncoding regions was 879 bp for *TRNL-F* and 900 bp for *RPL32-TRN L*. The sequences obtained were deposited in the GenBank database with Accession Numbers EU684549EU684567 for *TRNL-F* and EU684568EU684586 for *RPL32-TRNL*. Individuals from the same location (Fig. 1) had identical sequences for each of the two cpDNA regions (Table 1).

The total number of polymorphic sites was 19 (2.13%) for the *TRNL-F* spacer and 20 (2.22%) for the *RPL32-TRNL* spacer. The calculated values of haplotype diversity were 0.965 for *TRNL-F* and 0.930 for *RPL32-TRNL*. The values for nucleotide diversity were 0.00631 and 0.00603 for *TRNL-F* and *RPL32-TRNL*, respectively. Based on the similarity of the genetic diversity parameters for the two cpDNA regions, we concatenated the two spacer sequences for later analysis. The haplotype diversity was 0.982 and the nucleotide diversity was 0.00617 for the concatenated sequences. We designated 16 haplotypes using Roman numerals (Table 1).

In the maximum parsimony analysis (Fig. 2A) the distribution of the cpDNA haplotypes showed strong geographical structuring, with bootstrap values exceeding 50%. The haplotype distribution indicates that the populations form two distinct groups, one in north Corsica and the other in south Corsica and north Sardinia.

The topology of genealogy generated using TCS software with a 95% cutoff value to construct the significant connection (Fig. 2B) was consistent with the maximum parsimony study (Fig. 2A). Haplotype I from central Corsica was identified as an ancestral haplotype, and was similar to other central and northeast Corsican haplotypes (II, III–V). The haplotypes of northwest Corsica (VI–VIII) were separated by five mutations (three for *RPL32-TRNL* and two for *TRNL-F*). The southern haplotypes were differentiated by 8 mutations and formed two subgroups; southwest Corsica (haplotypes XV and XVI), and southeast Corsica and north Sardinia (haplotypes IX–XIV). These two subgroups were differentiated by seven mutations.

Table 2
AMOVA analysis of the two groups: north (populations: northeast, northwest and central Corsica) and south (populations: southeast and southwest Corsica and north Sardinia). Individuals are defined in Fig. 1.

Source of variation	d.f.	Sum of squares	Percentage of variation
Among groups	1	43.883	52.15***
Among populations			
Within groups	4	33.720	36.90***
Within populations	14	10.750	10.94***

*** $P < 0.001$.

Sequence variation demonstrated nonsignificant deviation for expectations of neutrality by both the Tajima test ($D = -0.06769$; $P > 0.10$) and the Fu and Li ($Fs = 0.41982$; $P > 0.10$) test.

The AMOVA, based on north (northeast, central and northwest) and south (southwest and southeast, including north Sardinia) partitions, showed that a large proportion of the total variance (52.15%, $P < 0.001$) was explained by differences between north and south Corsica (including north Sardinia) (Table 2). The variation among populations within groups was 36.90% ($P < 0.001$), and within populations was 10.94% ($P < 0.001$). This result suggests that these two groups have strong genetic differentiation. This was evident in the estimates of *Nm* and F_{st}. *Nm* was 0.04911 between northeast and southwest Corsica, and the highest gene flow was detected between northeast and central Corsica (*Nm* = 0.2500). F_{st} varied 0.66667 between northeast and central Corsica, and 0.91507 between northeast and southwest Corsica. The means value of gene flow between north and south Corsica (including north Sardinia) was 0.06305, and for F_{st} was 0.88896.

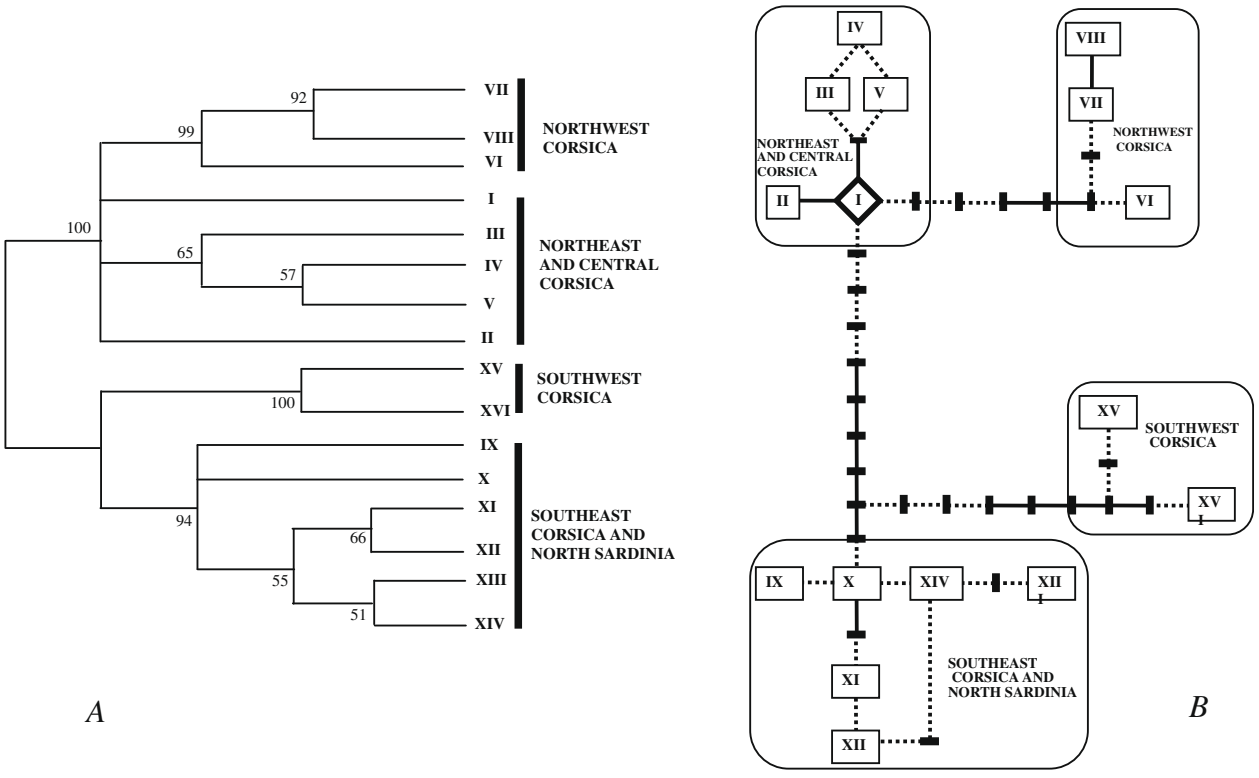


Fig. 2. (A) Consensus tree of 16 haplotypes of *C. creticus*, inferred from the 857 most parsimonious trees (the numbers at nodes are bootstrap values, 1000 replicates). The consistency index is 0.9394, the retention index is 0.99798 and the composite index is 0.9204 for 31 parsimony informative sites. (B) Minimum spanning network of the haplotypes (solid lines represent *TRNL-F* mutations, dotted lines represent *RPL32-TRNL* mutations; haplotype I in the diamond represents the ancestral haplotype).

4. Discussion

In this study we demonstrated genetic differentiation and phylogeographical patterns for *C. creticus* in Corsica and Sardinia, based on variability in the *TRNL-F* and *RPL32-TRNL* spacers of cpDNA.

The *Cistus* genus has a dominant role in the woodland understory and the evergreen scrub of the Mediterranean region (Médail and Quézel, 1997). The major centre of species diversity is in the western Mediterranean, particularly on both sides of the Strait of Gibraltar. Using cpDNA and ITS sequences, Guzman et al. (2005) showed that the differentiation of extant *Cistus* occurred in the western Mediterranean. Despite the present distribution and diversity of *Cistus* species, paleobotanical data strongly suggest that the centre of origin of *Cistus* was not the Mediterranean region. The oldest reported pollen record for the Cistaceae (*Cistaceae-rumpollenites*), from at least 23 million years ago (mya) in the Lower Miocene, comes from the Czech Republic (Guzman et al., 2005). However, this report should be treated cautiously as there are difficulties in identifying pollen at the genus level. The use of molecular data to estimate the time of divergence of *C. creticus* in the Tyrrhenian Islands could result in misinterpretations, because no absolute substitution rate (molecular clock) has been estimated for the Cistaceae. In addition, the heterogeneous mismatch distribution (results not shown) does not establish the time of divergence (Rogers and Harpending, 1992).

The phylogenic reconstruction (Fig. 2A) separated the populations into two distinct groups; one in north Corsica (including the central area) and the other in south Corsica (including north Sardinia). The ancestral origin of the two groups appears to be in central Corsica, with a low differentiation among northern populations (Fig. 2B). The AMOVA also revealed differentiation in the genetic structure between the two regions (Table 2).

This differentiation was also correlated with geological data. Corsica can be divided into two geologically distinct parts by a line drawn from the northwest, near Poggio d'Oletta, to the southeast coast, near Favona; this line corresponds to a 2800 m mountain barrier that shows traces of former glaciers (Jolivet et al., 1990). West of this line, the island is composed chiefly of a granite geological feature that is also evident at a lower altitude in northern Sardinia. To the east of this line, the predominant rocks are schists of unknown age (Jolivet et al., 1990).

Between these parts, a central depression 10 km wide contains the ancestral haplotype I (Fig. 2B). Because cpDNA is transmitted by seeds, the results suggest that low seed flow was highly dependent on geographical coercion, as indicated by the low *Nm* values between the north and south, and the high number of mutational events between these areas (Fig. 2B).

According to paleomagnetic, stratigraphic and geomorphological data from the Tyrrhenian area, Corsica and Sardinia separated as a single landmass from the continent in the Miocene period, about 29 mya. After the initial disjunction, a rotation took place that brought the two islands to their approximate present positions. The pattern and timing of the separation of the two islands is more complex. Although episodic contacts between southern Corsica and northern Sardinia may have persisted until very recently, the separation of these islands may have begun as long as 15 mya, and was certainly complete by 9 mya (Radicati di Brozolo and Giglia, 1973).

Notable biogeographic aspects are the haplotype similarities between southern Corsica and northern Sardinia (Fig. 2A and B), and the differentiation of extant *C. creticus*, which appears to have occurred before separation of the two islands.

The most distinctive phylogeographical pattern observed for *C. creticus* was the pronounced vicariance pattern along the south Corsica (south Corsica and north Sardinia)–north Corsica line. The small differentiation between Sardinia and southern Corsica suggests that the sea transgression had no effect on the fragmentation of *C. creticus*, as shown by the low number of mutational events between these populations (Fig. 2B).

However, based on the low mutation rate reported for the chloroplast genome (Wolfe et al., 1987), the high cpDNA diversity found in *C. creticus* could be explained by the ancient presence of this species, which allowed the accumulation of a significant number of mutations.

The influence of the nature of the soils also needs to be considered, as these correlated with the distribution of haplotypes; granite in southern Corsica and northern Sardinia, and schist in central and northern Corsica.

Analysis of additional loci is probably necessary to enable a more reliable interpretation. In particular, the inclusion of nuclear DNA polymorphisms could provide valuable information on the importance of gene flow between the areas studied.

Study of the genetic variation in *C. creticus* from Corsica and Sardinia will allow better understanding of the biological and historical processes that affect its differentiation and distribution, and could underpin the development of conservation strategies.

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