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Low genetic diversity and significant structuring in the endangered *Mentha cervina* populations and its implications for conservation

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ABSTRACT

Eighteen populations of the endangered aromatic and medicinal plant *Mentha cervina* (Lamiaceae) were sampled across its natural range, in the western half of the Iberian Peninsula, and inter-simple sequence repeats (ISSRs) markers were used to assess genetic diversity and population structure. *M. cervina* populations exhibited a relatively low genetic diversity (percentage of polymorphic loci PPB = 14.2–58.3%, Nei's genetic diversity H_e = 0.135–0.205, Shannon's information index I = 0.08 – 0.33). However, the genetic diversity at species level was relatively high (PPB = 98.3%; H_e = 0.325; I = 0.23). The results of the analysis of molecular variance indicated very structured populations, with 50% of the variance within populations, 44% among populations and 6% between regions defined by hydrographic basins, in line with the gene differentiation coefficient (G_{ST} = 0.532). A Mantel test did not find significant correlation between genetic and geographic distance matrices (r = 0.064), indicating that isolation by distance is not shaping the present genetic structure. The levels and patterns of genetic diversity in *M. cervina* populations were assumed to result largely from a combination of evolutionary history and its unique biological traits, such as breeding system, low capacity of dispersion, small effective size and habitat fragmentation. The high genetic differentiation among populations indicates the necessity of conserving the maximum possible number of populations. The results also provide information to select sites for *ex situ* conservation. Optimal harvesting strategies, cultivation and tissue culture should also be developed as soon as possible to guarantee sustainable use of the species under study.

1. Introduction

Plants belonging to the genus *Mentha* (Lamiaceae) have evolved in nature through natural hybridization and selection, showing substantial variation in terms of their natural habitats, growth characteristics, and aromas (Tutin et al., 1972; Franco, 1984). In this genus, which includes many herbs that are used for medicines, cosmetics, and spices, particular attention has been given to some cultivated species, namely peppermint (*Mentha piperita* L.) and spearmints (*Mentha spicata* L. and *Mentha cardiaca* Baker) because of their essential oil (EO) compositions (Kokkini, 1991). The EOs from *Mentha* species have been used

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since ancient times for the treatment of many digestive tract diseases and in culinary (İşcan et al., 2002), and they are known to have antimicrobial properties (Flamini et al., 1999; Naigre et al., 1996). As such, mints are valuable crops with a substantial importance in the botanical economy and to the pharmaceutical industry.

Mentha cervina L. (commonly known as hart's pennyroyal) is an aromatic plant that is traditionally used in Portugal to flavour food dishes and for its medicinal properties, preventing different gastric disorders and inflammations of the respiratory tract (Monteiro et al., 2007; Póvoa et al., 2006; Rodrigues et al., 2008, 2012). It is a spreading herbaceous perennial with slender, lance-shaped, mid-green leaves and whorls of white or lilac flowers from midsummer into autumn. It has a western steno-Mediterranean distribution, and is found in France, Portugal, Spain, Morocco, Algeria and is presumably extinct in Italy (Rhazi and Grillas, 2010). It occurs mainly in river banks and other damp and wet places, which require a longer flooded period that is characteristic of the priority habitat Mediterranean temporary ponds (3170) (Silva et al., 2009). According to our field survey, areas that had previously been reported in herbaria as species habitats have now been severely deteriorated or fragmented largely due to anthropogenic activities (e.g., deforestation, over-exploitation) and in one case the population had completely disappeared due to a hydroelectric dam construction (Póvoa et al., 2006). With the growth of commercial demands in recent years, the excessive harvesting from the wild (main source of plant material), overgrazing and the unfavorable conservation status of these habitats have shrunk the natural resources of *M. cervina* to a narrow distribution (Póvoa et al., 2006). Nowadays, it is considered to be decreasing in number and classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi and Grillas, 2010). A previous study (Rodrigues et al., 2008) found no chemical polymorphism in the EOs obtained in populations from different provenances. This surprising uniformity, in a species of a rather polymorphic genus, suggested a lack of variation and a need to assess genetic diversity. Up to date, no study has targeted the genetic diversity and structure, although this information is essential for the formulation of effective conservation strategies in threatened species (Holsinger and Gottlieb, 1991; Escudero et al., 2003; Shah et al., 2008).

The use of molecular markers to evaluate neutral genetic variation has become an important tool to study population genetics. Among various molecular tools, ISSRs (Inter-simple sequence repeats) have gained increasing interest because they have greater reliability and reproducibility of banding patterns when compared to RAPD (random amplified polymorphic DNA) primers (Culley and Wolfe, 2001), and at the same time, the cost of the analyses is relatively lower than that of some other markers such as AFLPs and microsatellites (Fang and Roose, 1997). Therefore, the recent use of ISSRs has been extensive in population genetics studies with wide applications in genetic diversity studies of species with conservation concerns (Esselman et al., 1999; Ge et al., 2005; McGlaughlin et al., 2002; Smith and Bateman, 2002; Xia et al., 2007), including Lamiaceae species (Liu et al., 2006; Mendes et al., 2009). ISSRs are especially useful in detecting diversity in closely related, or even clonal, individuals (Chen et al., 2006; Esselman et al., 1999; Han et al., 2007; Zietkiewicz et al., 1994).

Because of the medicinal and aromatic potential of this species (Gonçalves et al., 2007; Rodrigues et al., 2012) and current threats for its conservation, we in the present study use ISSRs to assess levels of variation, identify the degree of genetic differentiation among populations and provide guidelines for the conservation and sustainable use of this species in the range sampled.

2. Materials and methods

2.1. Plant material

In 2009 and 2010, several field trips were conducted across the geographic range of *M. cervina*. A total of 192 individuals, which correspond to 18 populations with different geographic origins were included in the analysis (Table 1). It is important to mention that despite our efforts to collect samples from a higher number of populations no more than 18 were found. Geographic distances between populations vary from 10 (between Mc37 and Mc38) to 487 km (between Mc33 and Mc46). Due to the limited availability of individuals in the populations, each population was evaluated by analyzing 10–15% of the individuals. These individuals were collected, throughout the entire range of each location, taking in to account distances between plants and accompaniment of the plant rhizomes to avoid duplication of individuals. From each sampled individual, fresh leaves were collected and dried in silica gel for subsequent DNA extraction. Vouchers for each population were deposited in the LISI Herbarium (Table 1).

2.2. DNA extraction and ISSR-PCR amplification

Total DNA was extracted from silica gel-dried leaves following the protocol of the plant mini kit (Quiagen), using 100–200 mg of leaf material grounded to fine powder in liquid nitrogen. The quantity of DNA extracted was evaluated by electrophoresis in 0.8% agarose gels in TBE buffer (50 mM Trisma, 50 mM boric acid, 2.5 mM EDTA, pH 8.3), and each DNA sample was diluted to 10 ng/μl for PCR amplification.

All the tested primers were synthesized by Stab Vida (Lisbon, Portugal). Twenty primers were initially screened, and 10 of them, which yielded bright and discernible bands, were used for the analysis of all 192 samples.

PCR reactions were standardized and run on an I-cycler, Bio-Rad thermalcycler. For every 20 μL reaction, 10 ng of DNA, 1× reaction buffer, 0.5 μM of primer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 2% of DMSO and 0.5 units Taq DNA polymerase were included. The amplification conditions were performed with the following program: initial denaturation at 94 °C for

Table 1
Location of *Mentha cervina* populations and number of individuals sampled in the present study.

Populations	Sample sizes	Specific sample collection sites				
		Localization	Altitude (m)	Latitude	Longitude	Hydrographic basin
Mc10	9	Ouguela, Campo Maior	207	39°4′54.96″N	7°0′4.33″W	Guadiana
Mc29	9	Ponte da Ajuda, Elvas	166	38°46′32.02″N	7°10′29.61″W	Guadiana
Mc32	12	Vilar seco, Miranda do Douro	725	41°31′25.48″N	6°24′5.56″W	Douro
Mc33	13	Póvoa, Miranda do Douro	750	41°34′22.71″N	6°19′17.53″W	Douro
Mc34	5	Bagaúste, Peso da Régua	50	41°9′0.41″N	7°45′2.24″W	Douro
Mc35	14	Escarigo, Figueira de Castelo Rodrigo	560	40°50′34.73″N	6°49′33.62″W	Douro
Mc36	9	Segura, Idanha-a-Nova	235	39°49′11.06″N	6°58′52.99″W	Tejo
Mc37	16	Salvaterra do Extremo, Idanha-a-Nova	253	39°53′37.50″N	6°54′18.38″W	Tejo
Mc38	12	Monfortinho, Idanha-a-Nova	255	39°59′9.96″N	6°52′50.23″W	Tejo
Mc39	9	Oledo, Idanha-a-Nova	335	39°58′10.64″N	7°18′27.85″W	Tejo
Mc40	9	Montalvão, Nisa	116	39°39′50.86″N	7°32′19.27″W	Tejo
Mc41	9	Valência de Alcântara	313	39°28′1.17″N	7°12′24.16″W	Tejo
Mc42	8	Torrão, Alcácer do Sal	50	38°17′0.32″N	8°13′57.81″W	Sado
Mc43	15	Entradas, Castro Verde	154	37°44′36.51″N	7°58′44.60″W	Guadiana
Mc44	9	La Codosera	298	39°16′48.08″N	6°52′20.89″W	Guadiana
Mc45	9	Alburquerque	234	39°11′0.69″N	7°1′59.03″W	Guadiana
Mc46	14	Gomes Aires, Almodôvar	200	37°30′58.11″N	8°11′5.17″W	Guadiana
Mc47	11	Castro Marim	50	37°11′21.63″N	7°27′27.81″W	Guadiana

4 min, followed by 40 cycles of 30 s at 94 °C to denature, 45 s at 48 °C to anneal the primers and 2 min at 72 °C to extend the primers. The last cycle was followed by 45 s at 94 °C, 45 s at 44 °C and a final extension at 72 °C for 5 min. A negative control with no DNA added was included in each PCR reaction. The amplified products were separated by electrophoresis in horizontal 1.5% agarose gels in 1× TBE buffer, at 100 V constant for 2 h. The gels were stained with ethidium bromide (0.5 μg/ml), and visualized in ultraviolet light by using GEL DOC 2000 (Bio-Rad Gel Documentation System). The size of the amplified products was determined by comparison with 100 bp ladder. To verify the repeatability of the results, each PCR amplification and gel running was repeated twice, and only the amplified ISSR fragments present in both runs where considered.

2.3. Data analysis

Since ISSR markers are dominantly inherited, each band was assumed to represent the phenotype at a single diallelic locus (Williams et al., 1990). Consistently reproducible amplified ISSR fragments, between 300 and 1800 bp, were scored as present (1) our absent (0), each of which was treated as an independent character regardless of its intensity. Fragments of the same molecular weight were considered as the same locus.

The binary matrix (1/0), constructed with GELCOMPARII (Applied Maths), was used in parameter estimation with multiple algorithms and methodologies.

POPGENE 1.32 (Yeh et al., 1997) was used to calculate various genetic diversity parameters: percentage of polymorphic bands (PPB), expected heterozygosity (H_e), genetic diversity among populations (D_{ST}), Nei's (1973) coefficient of gene differentiation (G_{ST}) and Shannon's index of diversity (I).

Genetic diversity was also estimated as heterozygosity using the Bayesian approach of Holsinger et al. (2002), with the analysis program HICKORY version 1.1 (Holsinger and Lewis, 2003). Several runs were carried out with default sampling parameters (burn-in = 5,000, sample = 25,000 and thin 5) to ensure consistency of results and the full model was selected.

Grouping of the individuals using the Principal Coordinates Analysis (PCoA) was done with GENALEX 6 program (Peakall and Smouse, 2006). The additional measurement of partitioning genetic variation was obtained with the hierarchical analysis of molecular variance (AMOVA) analysis, using GENALEX 6 program, with 9999 permutations.

To illustrate the relationship among populations, the UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrograms were generated using the software package AFLPSURV (Vekemans et al., 2002) and PHYLIP (Felsenstein, 1989) based on Nei's genetic distance. This method was implemented with bootstrapping (1000 replicates), to assess the statistical support of each branch, and then majority-rule consensus trees were generated for each type of distance, using the modules NJ and CONSENSE in the PHYLIP package. The trees were viewed and drawn using TREEVIEW program (Page, 1996).

To further understand the relationships among populations, a Bayesian analysis with the software STRUCTURE (Pritchard et al., 2000) was used to reveal the number of genetic pools, assign individuals to populations and identify migrants and admixed individuals. Several runs were carried out with default sampling parameters (burn-in = 50.000, number of MCMC runs after burn-in = 500.000, using the admixture model and allele frequencies correlated) and K calculated by Evanno et al. (2005).

Geographical distances were calculated by Google Earth program. To test the correlation between Nei's genetic distance (D) between populations and geographic distances (in km) among populations, a Mantel (Mantel, 1967) test was performed using GENALEX 6. Clonality was also tested using the multilocus genotypes analysis of the GENALEX 6 program.

Table 2
Primers used in ISSR analysis of *Mentha cervina* and number of reproducible bands.

Primer code	Sequence[a]	Reproducible bands
ISSR1	(CA)8RG	21
ISSR3	(GA)8YT	14
ISSR4	(GA)8YC	16
ISSR5	(GA)8YG	18
ISSR6	(AG)8YT	15
ISSR7	(AG)8YC	18
ISSR8	(AC)8YA	16
ISSR10	(GT)8YC	19
ISSR15	(GACAC)3	20
ISSR898	(CA)6-RY	18
	Mean	17.5

[a] Y = C or T; R = A or G.

3. Results

3.1. Genetic diversity

Among the 20 ISSR primers tested for their ability to detect polymorphic bands (putative loci) in a subset of *M. cervina* samples, 10 primers generated interpretable polymorphic amplifications. In these 10 selected primers, the number of bands per primer (loci) varied from 14 (ISSR3) to 21 (ISSR1) with an average of 17.5 bands per primer (Table 2). Genetic diversity estimates from ISSR are summarized in Table 3. ISSR amplification of the 192 individuals, gave a total of 175 bands that could be scored, corresponding to an average of 79.9 fragments per individual. Of these bands, 172 were polymorphic. All the primers produced polymorphic bands when all 18 populations were considered. Private bands, absent in all populations except one, were not observed. The proportion of polymorphic bands at the population level varied from 14.2% (Mc34) to 64.6% (Mc43), with a mean of 44.4%. This figure was 98.3% at the species level. Nei's gene diversity (H_e) and Shannon's information index (I) showed a similar trend to PPB. As indicated by these three indices, the least genetically diverse populations are Mc29 ($H_e = 0.0802$, $I = 0.117$, PPB = 20.6%) and Mc34 ($H_e = 0.0512$, $I = 0.076$, PPB = 14.2). The most diverse populations are Mc33 ($H_e = 0.222$, $I = 0.332$, PPB = 64%) and Mc43 ($H_e = 0.219$, $I = 0.328$, PPB = 64.6) (Table 3).

3.2. Genetic differentiation

According to Nei's analysis of gene diversity, the percentages of genetic variation among *M. cervina* populations were 53.2% (G_{st}) and 46% (Theta-B value, an estimate of G_{st} obtained by HICHORY analysis) which indicated elevated

Table 3
Measures of genetic diversity in the *Mentha cervina* species and for each population. PPB, percentage of polymorphic loci (at the 5% level); *I*, Shanon's Information index; H_e, Nei's gene diversity.

Population	PPB		*I*	H_e
	Number	Percentage		
Mc10	101	57.7	0.29	0.193
Mc29	36	20.6	0.12	0.080
Mc32	102	58.3	0.31	0.208
Mc33	112	64.0	0.33	0.222
Mc34	25	14.2	0.08	0.051
Mc35	93	53.1	0.28	0.187
Mc36	57	32.6	0.18	0.124
Mc37	95	54.3	0.24	0.156
Mc38	91	52.0	0.24	0.159
Mc39	66	37.7	0.19	0.128
Mc40	47	26.9	0.14	0.097
Mc41	54	30.9	0.17	0.116
Mc42	63	36.0	0.20	0.131
Mc43	113	64.6	0.33	0.219
Mc44	64	36.6	0.18	0.117
Mc45	85	48.6	0.25	0.169
Mc46	105	60.0	0.29	0.193
Mc47	90	51.4	0.26	0.170
Mean	78	44.4	0.23	0.151
Total	172	98.3	0.23	0.325

Table 4
Analysis of molecular variance (AMOVA) of intersimple sequence repeat (ISSR) data using GENEALEX, to determine the genetic structure for different hierarchical levels of the eighteen *Mentha cervina* populations. d.f., degree of freedom; SSD, sums of squares; MSD, mean square deviations; variance component estimates; percentage variation is the distribution of variation at a given level of hierarchy (among groups/among populations/within populations) and *P* value is the significance of variance after 9999 permutations.

Source of variation	d.f.	SSD	MSD	Variance component	Percentage	Θ_{ST}	*P* value
Mentha cervina							
Among populations	17	3104.54	182.62	15.671	49%	0.493	<0.001
Within populations	174	2807.174	16.133	16.133	51%		
Four groups: Douro, Tejo, Sado, Guadiana							
Among groups	3	752.34	250.78	1.96	6%	0.061	<0.001
Among populations	14	2352.20	168.01	14.26	44%	0.469	<0.001
Within populations	174	2807.17	16.13	16.13	50%	0.501	<0.001

interpopulation genetic differentiation. The AMOVA, that considered only one hierarchical level (Table 4), showed that most of the variation was found within populations (51%), providing additional evidence for high genetic structuring of populations. Considering two levels, when populations were grouped by hydrographic basin, the proportion of total variance residing within populations was 50%, among populations 44% and among basins 6% ($P = 0.001$), which indicates that there is only a small proportion of the variation associated with grouping by river basin, even though it is statistically significant (Table 4). The nearly identical Φ_{ST} from the AMOVA analysis (0.469) and the G_{ST} from the POPGENE and from HICHORY analysis provide additional support of the statistics used in this study and robustness of the results.

3.3. Genetic relationships

The STRUCTURE analyses pointed out that the eighteen populations of *M. cervina* in the present study share 7 genetic pools, with migrants and admixed individuals. Mc37 and Mc38 are considered to share the same genetic pool in the two analyses, which is not surprising since they are geographically very close (10 km) and share the same river basin. The populations from the north region constitute another gene pool (Mc32, Mc33, Mc34 and Mc35) and 3 of them grouped together in the UPGMA tree (Fig. 2) (although with weak bootstrap support). The populations from the center-south are from two gene pools, that were also clustered in the UPGMA tree (although only with moderate bootstrap support) and the other 3 gene pools are found in the midland with weak and arbitrary clustering (Figs. 2 and 3).

The PCoA (Fig. 4) provided additional evidence for the highly structured populations, and in each population the individuals formed cohesive clusters. Overall the populations were grouped in 4 main groups, and the relationships were more or less in agreement to that implied by the Bayesian analysis (STRUCTURE) and the cluster analysis. The first two components, accounted for 48.87% (axis 1 = 27.15%; axis 2 = 21.71%) of the total variability. The clearest separation indicated in this analysis was of the southern populations (Mc42, Mc43, Mc44, Mc45, Mc46 and Mc47). The relationship of groups in the center and northern range presented broad overlap of populations (Mc10, Mc29, Mc32, Mc33, Mc34, Mc35, Mc39, and Mc41). The spread also indicated likely admixture of gene pools in some populations, namely Mc33, Mc35, and Mc43. This is in agreement with the mixed gene pools inferred for up to 25–30% of these populations in the STRUCTURE analysis (Fig. 1). This admixture and the complex pattern of relationships which does not indicated strong geographical groups are corroborated by the Mantel test which did not find significant correlation between genetic and geographic distance matrices ($r = 0.064$, $P < 0.298$, 9999 permutations), indicating that the isolation by distance shaping the genetic structure pattern present in *M. cervina* population. Also, the analysis of clonality found no matching multilocus genotypes.

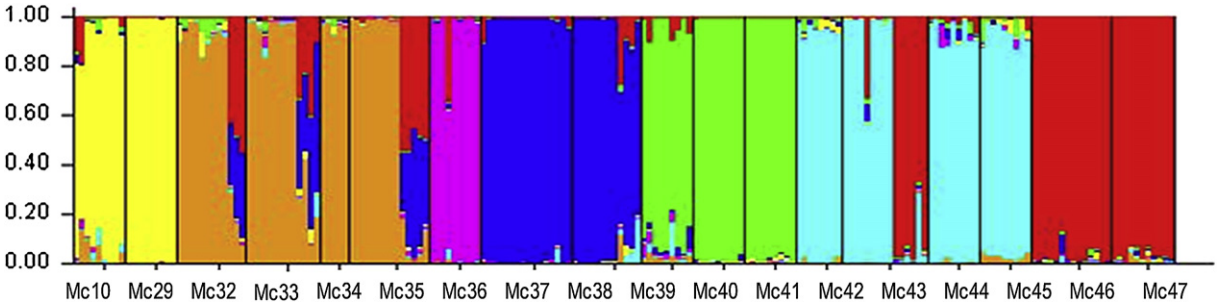

Fig. 1. Bayesian admixture proportions of *M. cervina* ($K = 7$).

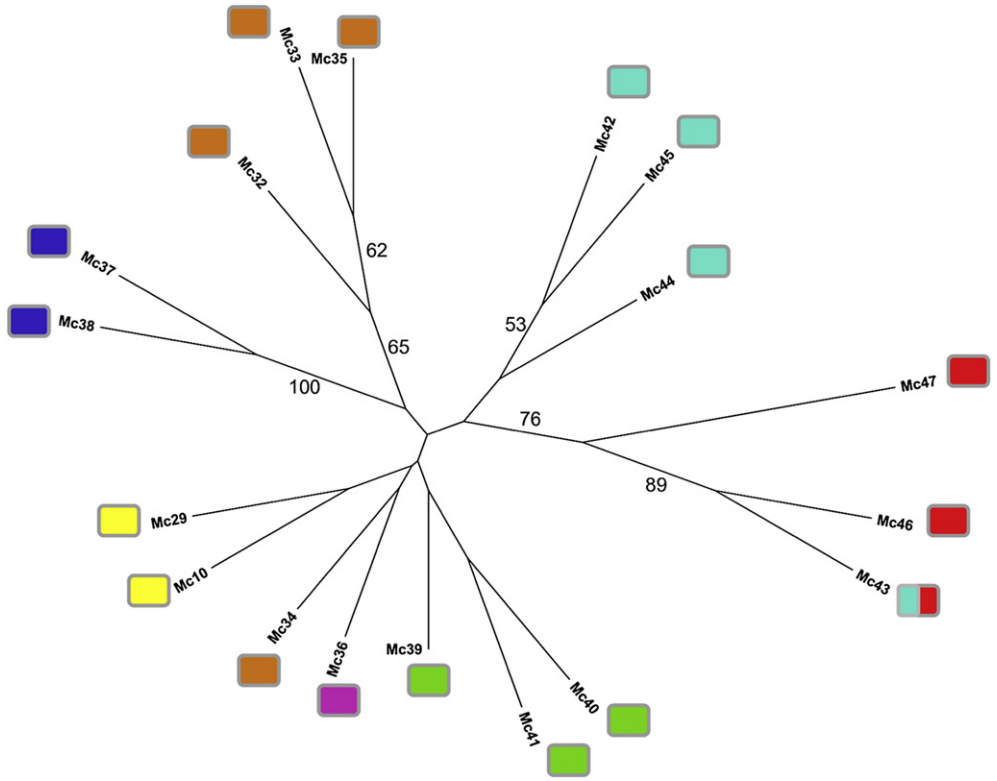

Fig. 2. UPGMA Dendrogram based on Nei's genetic distance matrix for *Mentha cervina* populations. One thousand replicates of bootstrapping analysis were used to assess the statistical support of each branch. Numbers in branches correspond to the bootstrap analyses (50% or more). Square colour blocks correspond to the structuring of populations according to the STRUCTURE analysis (Fig. 1). See Table 1 for population abbreviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Genetic diversity and differentiation

Geographic range is usually regarded as an approximate measure of the total number of individuals of a species, so we can expect species with a wider distribution to tend to have higher genetic diversity than rare and endangered species (Karron, 1987; Sheeja et al., 2009; Xiao et al., 2004). However, many studies have shown that endangered or endemic species can also maintain high genetic diversity (Ellis et al., 2006; González-Astorga and Castillo-Campos, 2004; Luan et al., 2006; Zhang et al., 2010). *M. cervina* has a large extent of occurrence (from Italy to North Africa), but a restricted area of occupancy (approximately 600 km^2) (Rhazi and Grillas, 2010). In Portugal, the same pattern is observed, and populations can be found from north to south, but in each location the area of occupancy is also very restricted, and most of the populations are found growing near our in river banks, with very limited number of individuals (10–1000).

The overall genetic diversity of *M. cervina,* based on ISSR analysis, is relatively high ($H_e = 0.323$; $I = 0.226$; PPB = 98.3%) at the species level, however, is relatively low at the population level. The PPB values ranged from 14.19 to 64.57%, averaging 44.4%, and the parameters H_e (ranging from 0.0802 to 0.222, with an average of 0.151) and I (ranging from 0.076 to 0.332, with an average of 0.2264) display a similar trend. It is clear from data in Table 5, that the two indices of genetic diversity (PPB and H_e) have the highest value for *M. cervina* at the species level in comparison with all other species. However, at the population level, the values are lower than the genetic diversity reported for other populations of Lamiaceae species and are consistent with other populations of endangered species (Table 5 and references there in). The high overall genetic diversity displayed by the species itself can be explained mostly by differences among populations due to the high genetic structure.

The present diversity pattern could not be explained by the isolation by- distance model, as revealed by Mantel test ($r = 0.064$). Although evolutionary divergence could be associated with rivers, because they are believed to be major geographical barriers that might largely hinder gene flow via seed and pollen dispersal among populations (Pfeifer and Jetschke, 2006), they also can act as dispersal routes for species that grow nearby or in the water. The AMOVA analysis revealed a weak partitioning of variation associated with the share of the river basin of populations, suggesting that each population analyzed is genetically defined and structured as a distinct genetic pool. Therefore, although highly structured, the

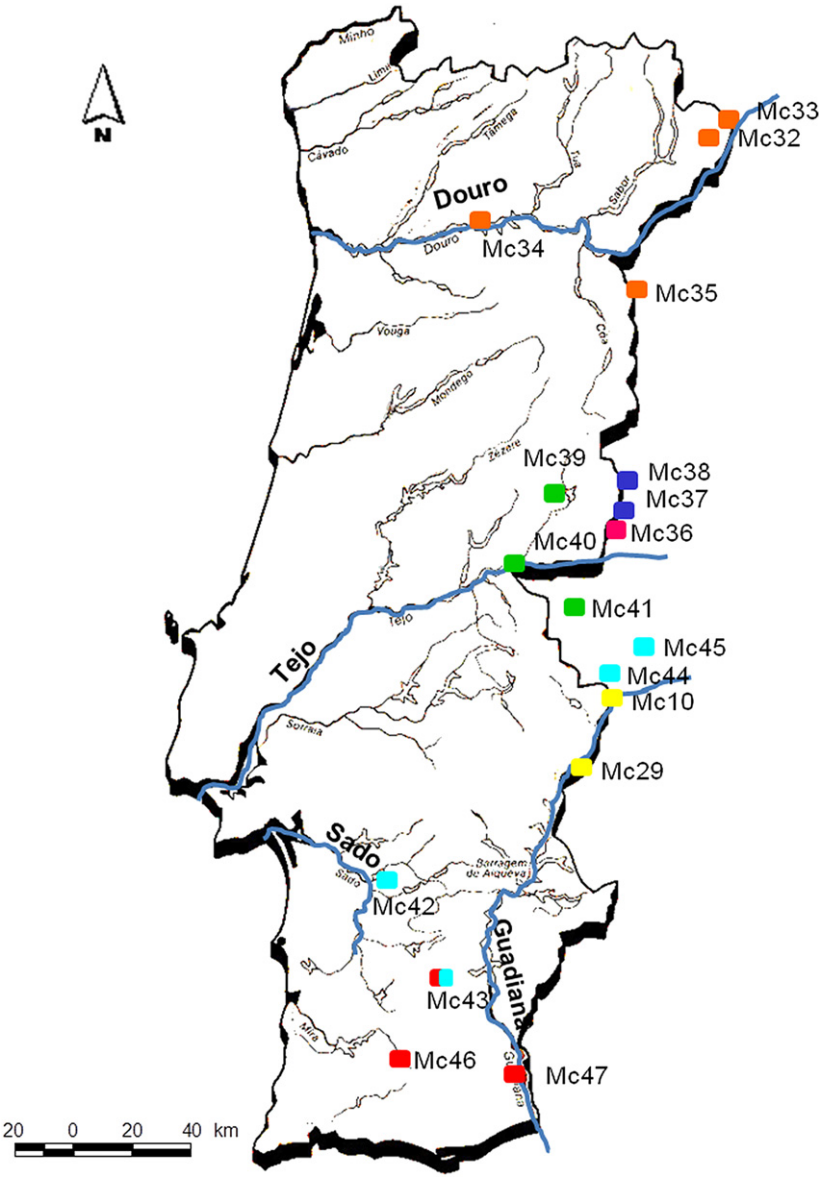

Fig. 3. Map of Portugal with the location of *Mentha cervina* populations analysed. Square colour blocks correspond to the structuring of populations according to the STRUCTURE analysis. See Table 1 for population abbreviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

populations of *M. cervina* are structured without a strong geographical pattern and in a more or less stochastic fashion, indicating a predominance of stochastic processes shaping the genetic variation.

M. cervina was usually observed in severely fragmented habitats and with small population sizes (from 10 to 1000 individuals), which make this species extremely vulnerable to fluctuations in climate and habitat disturbance (Travis et al., 1996). Indeed, populations with small population size and severe human disturbance (MC29, MC34, MC36 and Mc40), showed the lowest genetic diversity, while population MC33 and MC43, with relatively large population size and limited human disturbance, showed higher genetic diversity. It seems that populations in fragmented habitats and small effective size, are more subjected to stochastic events, genetic drift and inbreeding (Hartl and Clark, 1997), leading to a low genetic diversity and the high genetic structure pattern observed.

Another effect of habitat fragmentation and other human disturbance is being reported on plant breeding systems, with an increase in self-fertilization (Aguilar et al., 2008; Eckert et al., 2009). In plants, breeding system can significantly affect genetic diversity and it's partitioning within and among populations (Hamrick and Godt, 1996; Nybom, 2004). Selfing species are expected to have reduced effective population sizes (Ingvarsson, 2002), to have lower genetic diversity within populations and a partition of diversity of about 50%, whereas outcrossing species partition on average 20% among populations (Evans et al., 2000;

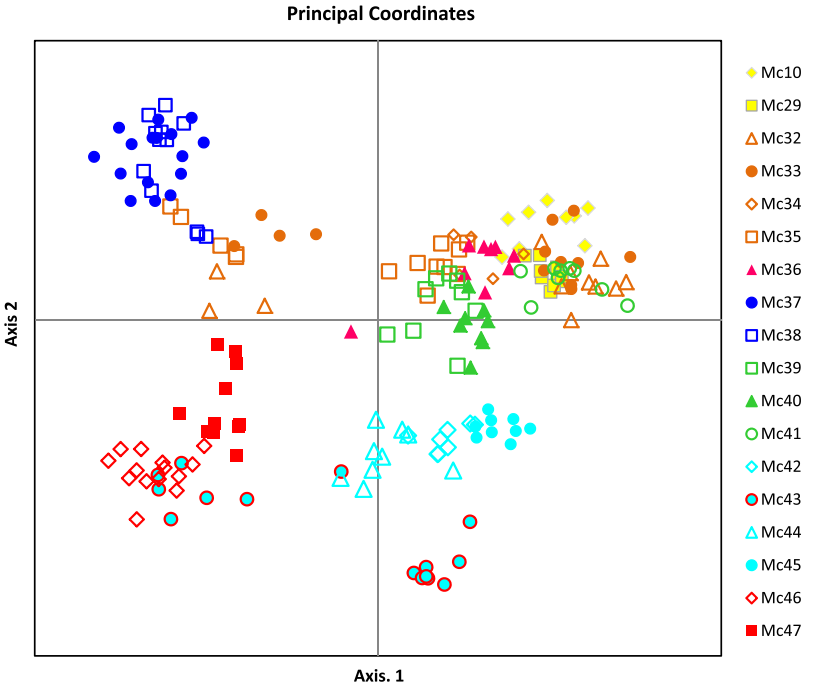

Fig. 4. Representation of the scores on the first two axes of the principal coordinate analysis (PCoA) from the matrix of genetic distances of 192 individuals from 18 populations of *M. cervina*, based on 175 ISSR loci. Percentage of variance accumulated on the first two axes = 48.87% (axis 1 = 27.15%, axis 2 = 21.71%). For population names see Table 1.

Hamrick and Godt, 1989; Nybom and Bartish, 2000; Tarayre and Thompson, 1996). Although there are no reports on the breeding system of *M. cervina*, it is likely an outcrossing species based on the breeding features of its closely related species (Judd et al., 1999), and so, most of the genetic diversity of *M. cervina* was expected to be partitioned within populations (Hamrick and Godt, 1996). Instead, the intra- and inter-population genetic partitioning were on average 51% and 49%, respectively.

M. cervina exhibits vigorous vegetative growth by rhizomes and an almost absent seed production (personal observations). Furthermore, seed dispersal is not likely to be very efficient in *M. cervina* because seeds are very small and light and lack any apparent dispersal structures.

Based on the ISSR data and on our field survey of habitats, the low levels of variability within populations and the high genetic structuring among populations probably resulted from 1) genetic drift and inbreeding dictated by fragmented habitat

Table 5
Genetic diversity measurements in *Mentha cervina*, in other Lamiaceaes and in endangered species. PPB percentage of polymorphic loci, H_e Nei's gene diversity: Numbers between () correspond to population values, and the other number to the mean value of the populations; G_{st} coefficient of gene differentiation.

	PPB		H_e		G_{st}	Methodology	Reference
	Species	Population	Species	Population			
Lamiaceae							
Mentha cervina	98.29	44.4 (14.2–64.6)	0.325	0.151 (0.08–0.222)	0.532	ISSR	Present study
Lamiophlomis rotata	96.7	51.8 (37.9–69.2)	0.291	0.291	0.430	ISSR	Liu et al., 2006
Lamiophlomis rotata	93.13	47.5 (23.6–64.4)	0.287	0.287	0.422	RAPD	Liu et al., 2006
Hemigenia exilis	97.00	72.0 (45.5–91.7)	0.27	0.39 (0.355–0.431)		RAPD	Mattner et al., 2002
Phlomis purpurea			0.085	0.085		Allozymes	Aparicio et al., 2000
Phlomis composita			0.079	0.079		Allozymes	Aparicio et al., 2000
Mentha pulegium		72.0 (60–90)	0.229	0.30 (0.40–0.21)		Allozymes	Ben and Boussaid, 2004
Endangered species							
Cycas guizhouensis	38.9	14.2 (8.9–20.5)	0.1082	0.0597 (0.036–0.088)		ISSR	Xiao et al., 2004
Camellia nitidissina	75.24	42.4	0.2302	0.098	0.575	ISSR	Bin et al., 2005
Camellia nitidissina	63.22	18.8 (11.5–24,1)	0.1561	0.083 (0.051–-0.105)	0.406	ISSR	Xiao et al., 2008
Sinocalycanthus chinensis	73.08	23.65	0.1987	0.084	0.578	ISSR	Jin and Li 2007
Emmenopterys henryi	56.05	22.56	0.191	0.071 (0.053–0.105)		ISSR	Li and Jin 2008
Gynostemma pentaphyllum	96.4	8.9 (1–25.3)	0.2624	0.026 (0.004–0.084)	0.889	ISSR	Wang et al., 2008
Saruma henryi	73.7	22.8 (10.3–36.6)	0.260	0.086 (0.045–0.124)	0.690	ISSR	Zhou et al., 2010
Vellozia gigantea	88.8	56.6 (47.2–68.5)	0.256	0.183 (0.140–0.225)	0.280	ISSR	Lousada et al., 2011
Astragalus nitidiflorus	51.3	31.8 (28.2–37.2)	0.171	0.129 (0.109–0.146)	0.242	ISSR	Vicente et al., 2011

and small population sizes and 2) a low seed setting/germination/dispersion. In this context, the genetic diversity within population is mostly dependent on the first colonizing plants, and works in a very stochastic manner.

Although ISSRs markers are considered to be neutral and thus to provide no direct assessment of fitness, the diminished genetic diversity found within these populations, despite the high genetic diversity at the species level, might explain the lack of phytochemical diversity at the essential oil composition reported in Rodrigues et al. (2008). Nevertheless, to better understand the patterns of genetic diversity and structure further studies on the quantitative genetic differentiation, species breeding system and the effects of habitat fragmentation and other human disturbance on plant diversity will be needed.

4.2. Implications for conservation

The main goal of current conservation plans is mainly focus in maintaining species diversity, in detriment of the intraspecific genetic diversity (Margules and Pressey, 2000). Nevertheless, the intraspecific genetic diversity is the primary source of diversity and has suffered extinction rates three to eight times higher than species extinction rates (Hughes et al., 1997). In *M. cervina*, the high genetic diversity at the species level is coupled with significant structuring and very low diversity at the population level. For this reason for satisfactory conservation of genetic diversity it should be considered the intraspecific genetic diversity together with the habitat preservation in an integrative approach to species conservation.

In situ conservation is usually the preferred strategy for most wild plant species because allows populations to continue to be exposed to evolutionary processes in its natural habitat enabling the perpetuation and integration of co-adapted gene complexes, especially in producing new resistances to stresses (pests, diseases, climate changes) (Aga et al., 2005; Vinceti et al., 2004). Because *M. cervina* populations currently face the problem of conservation link to the disappearance of the species habitat (general problem of wetland conservation) and the harvest pressure on wild populations, preserving and expanding the habitat at each site to allow natural expansion of populations would be a good strategy for its conservation before populations become too small to persist naturally. According to the field survey, the construction of a hydroelectric dam has flooded the habitat of population Mc29, and so this population is already lost. Given that Mc10 is genetically more close to Mc29, the survival of this population should be assigned priority for the conservation plan.

In conservation biology, genetic diversity is recognized as an important criterion to consider when prioritizing populations for protection, but conservation and management measures in a long-term perspective should ensure that the vast majority of the diversity is preserved for upcoming adaptations (i.e. the highest neutral genetic diversity that may be the future target of natural selection) (McKay and Latta, 2002). And so, preserve the populations that together maximize the species genetic diversity should be applied in opposition to the traditional approach of targeting populations that are the most diverse individually. Considering the different genetic pools found it would be worthy to prioritize populations in a matter as to represent all the genetic pools, and within these the most diverse populations. Populations Mc10, Mc33, Mc36, Mc37, Mc39 and Mc45 can be good representatives.

In order to increase the genetic diversity of *M. cervina* populations, the transfer of individuals between populations should also be considered. Nevertheless, one should take into consideration that when populations are genetically highly structured, outbreeding depression might be a potential genetic threat for already weakened populations (Sagvik et al., 2005). And so, it may be wiser to protect a network of populations that exchange genetic material and are able to reinforce each other. Given the genetic clusters found, we can suggest that only individuals within these clusters should be exchanged.

Moreover, considering that the samples collected in this study provide a snapshot of the species distribution area as a whole in Portugal, it would be also wise to preserve populations in different regions in order to limit population declines caused by large-scale environmental catastrophes and also to harbour possible local adaptative variation. Commercial harvesting of *M. cervina* for essential oil extraction is one of the major forms of its disturbance because its composition determines that the most favorable harvesting season is before seed dispersion. To meet the commercial demand for this herb and reduce harvesting pressure in *M. cervina* wild populations, cultivation by seed and tissue culture, should be carried out as soon as possible as an alternative source of raw materials for trade.

Not only in Portugal, but also throughout its range, the populations of *M. cervina* are suffering severe and rapid declines and are therefore classified as Near Threatened in the IUCN Red List of Threatened Species (Rhazi and Grillas, 2010). The populations once known in Italy (Abruzzi) are presumed extinct. In France, it is known in six departments and is considered as vulnerable (one level upward of the Near Threatened, according to the IUCN nomenclature) and in North Africa it is considered rare (Rhazi and Grillas, 2010). Taking in to account the levels of diversity of Portuguese *M. cervina* populations, its threatened habitat status and the high harvesting pressures, it is also suggested to consider *M. cervina* as Endangered Species in Portugal in one of the forthcoming volumes of the IUCN Red List of Threatened Species.

Enlarge sampling to represent the full distribution range of *M. cervina* and complete the genetic landscape picture of this species is needed for the effective conservation management of this medicinal and aromatic species.

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References

Aga, E., Bekele, E., Bryngelsson, T., 2005. Inter-simple sequence repeat (ISSR) variation in forest coffee trees (*Coffea arabica* L.) populations from Ethiopia. Genetica 124, 213–221.

Aguilar, R., Quesada, M., Ashworth, L., Herrerias-Diego, Y., Lobo, J., 2008. Genetic consequences of habitat fragmentation in plant populations: susceptible signals in plant traits and methodological approaches. Mol. Ecol. 17, 5177–5188.

Aparicio, A., Albaladejo, R.G., Porras, M., Ceballos, G., 2000. Isozyme evidence for natural hybridization in *Phlomis* (Lamiaceae): hybrid origin of the rare *P.* x *margaritae*. Ann. Bot. 85, 7–12.

Ben Fadhel, N., Boussaid, M., 2004. Genetic diversity in wild Tunisian populations of *Mentha pulegium* L. (Lamiaceae). Genet. Resourc. Crop Ev. 51, 309–321.

Bin, X.Y., Tang, S.Q., Zhou, J.Y., et al., 2005. ISSR analysis on genetic diversity of *Camellia nitidissima* Chi (Theaceae) in China. J. Wuhan. Bot. Res. 23, 20–26.

Chen, W.R., Gituru, Y.H., Wang, Q.F., 2006. The extent of clonality and genetic diversity in the rare *Caldesia grandis* (Alismataceae): comparative results for RAPD and ISSR markers. Aquat. Bot. 84, 301–307.

Culley, T.M., Wolfe, A.D., 2001. Population genetic structure of the cleistogamous plant species *Viola pubescens* Aiton (Violaceae), as indicated by allozyme and ISSR molecular markers. Heredity 86, 545–556.

Eckert, C.G., Kalisz, S., Geber, M.A., Sargent, R., Elle, E., Cheptou, P.-O., Goodwillie, C., Johnston, M.O., Kelly, J.K., Moeller, D.A., Porcher, E., Ree, R.H., Vallejo-Marín, M., Winn, A.A., 2009. Plant mating systems in a changing world. Trends Ecol. Evol. 25, 35–43.

Ellis, J.R., Pashley, C.H., Burke, J.M., McCauley, D.E., 2006. High genetic diversity in a rare and endangered sunflower as compared to a common congener. Mol. Ecol. 15, 2345–2355.

Escudero, A., Iriondo, J.M., Torres, M.E., 2003. Spatial analysis of genetic diversity as a tool for plant conservation. Biol. Conserv. 113, 351–365.

Esselman, E.J., Jianqiang, L., Crawford, D.J., Wimdus, J.L., Wolfe, A.D., 1999. Clonal diversity in the rare Calamagrostis porteri ssp. Insperata (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Mol. Ecol. 8, 443–451.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620.

Evans, M.E.K., Dolan, R.W., Menges, E.S., Gordon, D.R., 2000. Genetic diversity and reproductive biology in *Warea carteri* (Brassicaceae), a narrowly endemic Florida scrub annual. Am. J. Bot. 87, 372–381.

Fang, D.Q., Roose, M.L., 1997. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor. Appl. Genet. 95, 408–417.

Felsenstein, J., 1989. PHYLIP phylogeny inference package (version 3.2). Cladistics 5, 164–166.

Flamini, G., Cioni, P.L., Puleio, R., Morelli, I., Panizzi, L., 1999. Antimicrobial activity of the essential oil of *Calamintha nepeta* and its constituent pulegone against bacteria and fungi. Phytother. Res. 13, 349–351.

Franco, J.A., 1984. Nova Flora de Portugal (Continente e Açores), vol. II. Lisboa.

Ge, X.J., Zhou, X.L., Zhong, C.L., Hsu, T.W., Schaal, B.A., Chiang, T.Y., 2005. Low genetic diversity and significant population structuring in the relict *Amentotaxus argotaenia* complex (Taxaceae) based on ISSR fingerprinting. J. Plant Res. 118, 415–422.

González-Astorga, J., Castillo-Campos, G., 2004. Genetic variability of the narrow endemic tree Antirhea aromatic Castillo-Campos and Lorence, (Rubiaceae, Guettardeae) in a tropical forest of Mexico. Ann. Bot. 93, 521–528.

Gonçalves, M., Vicente, A., Cavaleiro, C., Salgueiro, L., 2007. Composition and antifungal activity of the essential oil of *Mentha cervina* from Portugal. Nat. Prod. Res. 21, 867–871.

Hamrick, J.L., Godt, M.J.W., 1989. Allozyme diversity in plant species. In: Brown, A.H.D., Clegg, M.T., Kahler, A.L., Weir, B.S. (Eds.), Plant Population Genetics, Breeding, and Genetic Resources. Sinauer Associates, Sunderland, Massachusetts, USA, pp. 43–63.

Hamrick, J.L., Godt, M.J.W., 1996. Effect of life history traits on genetic diversity in plant species. Philos Trans. R. Soc. London. B. Biol. Sci. 351, 1291–1298.

Han, Y.C., Teng, C.Z., Zhong, S., Zhou, M.Q., Hu, Z.L., Song, Y.C., 2007. Genetic variation and clonal diversity in populations of *Nelumbo nucifera* (Nelumbonaceae) in central China detected by ISSR markers. Aquat. Bot. 86, 69–75.

Hartl, D.L., Clark, A.G., 1997. Principles of Population Genetics, third ed. Sinauer, Sunderland.

Holsinger, K.E., Gottlieb, L.D., 1991. Conservation of rare and endangered plants: principles and prospects. In: Falk, D.A., Holsinger, K.E. (Eds.), Genetics and Conservation of Rare Plants. Oxford Univ. Press, New York, pp. 195–208.

Holsinger, K.E., Lewis, P.O., 2003. Hickory (Version 1.1). Department of Ecology & Evolutionary Biology. The University of Connecticut, Connecticut.

Holsinger, K.E., Lewis, P.O., Dey, D.K., 2002. A Bayesian approach to inferring population structure from dominant markers. Mol. Ecol. 11, 1157–1164.

Hughes, J.B., Daily, G.C., Ehrlich, P.R., 1997. Population diversity: its extent and extinction. Science 278, 689–692.

Ingvarsson, P.K., 2002. A metapopulation perspective on genetic diversity and differentiation in partially self-fertilizing plants. Evolution 56, 2368–2373.

İşcan, G., Krmer, N., Kürkcüolu, M., Hüsnü, K., Başer, C., Demrc, F., 2002. Antimicrobial screening of *Mentha piperita* essential oils. J. Agric. Food Chem. 50, 3943–3946.

Jin, Z.X., Li, J.M., 2007. ISSR analysis on genetic diversity of endangered relic shrub *Sinocalycanthus chinensis*. Chin. J. Appl. Ecol. 18, 247–253.

Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F., 1999. Plant Systematics: a Phylogenetic Approach. Sinauer, Sunderland.

Karron, J.D., 1987. A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. Evol. Ecol. 1, 47–58.

Kokkini, S., 1991. Chemical races within the genus *Mentha* L. In: Liskens, H.F., Jackson, J.F. (Eds.), Essential Oils and Waxes. Springer-Verlag, Berlin, pp. 63–78.

Li, J.M., Jin, Z.X., 2008. Genetic structure of endangered Emmenopterys henryi Oliv. based on ISSR polymorphism and implications for its conservation. Genetica 133, 227–234.

Liu, J., Wang, L., Geng, Y., Wang, Q., Luo, L., Zhong, Y., 2006. Genetic diversity and population structure of *Lamioplomis rotate* (Lamiaceae), an endemic species of Qinghai–Tibet plateau. Genetica 128, 385–394.

Lousada, J.M., Borb, E.L., Ribeiro, K.T., Ribeiro, L.C., Lovato, M.B., 2011. Genetic structure and variability of the endemic and vulnerable *Vellozia gigantea* (Velloziaceae) associated with the landscape in the Espinhaço Range, in southeastern Brazil: implications for conservation. Genetica 139, 431–440.

Luan, S., Chiang, T.Y., Gong, X., 2006. High genetic diversity vs. low genetic differentiation in *Nouelia insignis* (Asteraceae), a narrowly distributed and endemic species in China, revealed by ISSR fingerprinting. Ann. Bot. (Lond) 98, 583–589.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer. Res. 27, 209–220.

Margules, C.R., Pressey, R.L., 2000. Systematic conservation planning. Nature 405, 243–253.

Mattner, J., Zawko, G., Rossetto, M., Krauss, S.L., Dixon, K.W., Sivasithamparam, K., 2002. Conservation genetics and implications for restoration of *Hemigenia exilis* (Lamiaceae), a serpentine endemic from Western Australia. Conserv. Biol. 107, 37–45.

McGlaughlin, M., Karoly, K., Kaye, T., 2002. Genetic variation and its relationship to population size in reintroduced populations of pink sand verbena, *Abronia umbellata* subsp. *breviflora* (Nyctaginaceae). Conserv. Genet. 3, 411–420.

McKay, J.K., Latta, R.G., 2002. Adaptive population divergence: markers, QTL and traits. Trends Ecol. Evol. 17, 285–291.

Mendes, M., Trindade, H., Figueiredo, A.C., Pedro, L.G., Barroso, J.G., 2009. Chaerophyllum azoricum Trel. grown in the Azores archipelago, Portugal: evaluation of the genetic diversity using molecular markers and comparison with volatile oils profiles. Flavour Fragr. J. 24, 259–265.

Monteiro, A., Póvoa, O., Marinho, S., Rodrigues, L., Monteiro, P., 2007. *Mentha pulegium* e *Mentha cervina*, Os Poejos na boa Cozinha Portuguesa. ISA Press, Lisboa.

Naigre, R., Kalck, P., Roques, C., Roux, I., Michel, G., 1996. Comparison of antimicrobial properties of monoterpenes and their carbonylated products. Planta Med. 62, 275–277.

Nei, M., 1973. Analysis of genetic diversity in subdivided populations (population structure/genetic variability/hetrozygosity/gene differentiation). Proc. Natl. Acad. Sci. U S A 70, 3321–3323.

Nybom, H., 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol. Ecol. 13, 1143–1155.
Nybom, H., Bartish, I.V., 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspect Plant Ecol. Evol. Syst. 3, 93–114.
Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 112, 357–358.
Peakall, R.O.D., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295.
Pfeifer, M., Jetschke, G., 2006. Influence of geographical isolation on genetic diversity of *Himantoglossum hircinum* (Orchidaceae). Folia Geobot. 41, 3–20.
Póvoa, O., Farinha, N., Marinho, S., Nunes, P., Godinho, D., Mata, F., Rodrigues, L., Monteiro, A., 2006. Pennyroyal (*Mentha pulegium* L.) and Hart's Pennyroyal (*Mentha cervina* L.) biodiversity in Alentejo. Acta Hort. 723, 91–97.
Pritchard, J., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.
Rhazi, L., Grillas, P. 2010. Mentha cervina. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.2. www.iucnredlist.org. Downloaded on 31 January 2012.
Rodrigues, L., Monteiro, P., Póvoa, O., Teixeira, G., Moldão, M., Figueiredo, A.C., Monteiro, A., 2008. Morphology of secretory structures and essential oil composition in *Mentha cervina* L. from Portugal. Flavour Fragr. J. 23, 340–347.
Rodrigues, L., Duarte, A., Figueiredo, A.C., Brito, L., Teixeira, G., Moldão, M., Monteiro, A., 2012. Chemical composition and antibacterial activity of the essential oils from the medicinal plant *Mentha cervina* L. grown in Portugal. Med. Chem. Res. 21, 3485–3490.
Sagvik, J., Uller, T., Olsson, M., 2005. Outbreeding depression in the common frog, Rana temporaria. Conserv. Genet. 6, 205–211.
Shah, A., Li, D.Z., Möller, M., Gao, L.M., Hollingsworth, M.L., Gibby, M., 2008. Delimitation of *Taxus fuana* Nan Li & R.R. Mill (Taxaceae) based on morphological and molecular data. Taxon 57, 211–222.
Sheeja, G., Jyotsna, S., Vern, L.Y., 2009. Genetic diversity of the endangered and narrow endemic *Piperia yadonii* (Orchidaceae) assessed with ISSR polymorphisms. Am. J. Bot. 96, 2022–2030.
Silva, V., Póvoa, O., Espírito-Santo, M.D., Vasconcelos, T., Monteiro, A., 2009. *Mentha cervina* communities in Portugal. Lazaroa 30, 73–79.
Smith, J.F., Bateman, T.A., 2002. Genetic differentiation of rare and common varieties of *Eriogonum shockleyi* (Polygonaceae) in Idaho using ISSR variability. West N. Am. Naturalist 62, 316–326.
Tarayre, M., Thompson, J.D., 1996. Population genetic structure of the gynodioecious *Thymus vulgaris* (Labiateae) in Southern France. J. Evol. Biol. 10, 157–174.
Travis, S.E., Maschinski, J., Keim, P., 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. cremnophylax, a critically endangered plant, using AFLP markers. Mol. Ecol. 5, 735–745.
Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A., 1972. Flora Europaea. In: Diapensiaceae to Myoporaceae, vol. 3. Cambridge University Press, Cambridge.
Vekemans, X., Beauwens, T., Lemaire, M., Roldan-Ruiz, I., 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Mol. Ecol. 11, 139–151.
Vicente, M.J., Segura, F., Aguado, M., Migliaro, D., Franco, J.A., Martínez-Sánchez, J.J., 2011. Genetic diversity of *Astragalus nitidiflorus*, a critically endangered endemic of SE Spain, and implications for its conservation. Biochem. Syst. Ecol. 39, 175–182.
Vinceti, B., Van Breuge, P., Amaral, W., 2004. The practical implications of research outputs from forest genetic studies. In: Vinceti, B., Amaral, W., Meilleur, B. (Eds.), Challenges in Managing Forest Genetic Resources for Livelihoods. International Plant Genetic Resources Institute, pp. 245–267.
Wang, C., Zhang, H., Qian, Z.Q., Zhao, G.F., 2008. Genetic differentiation in endangered *Gynostemma pentaphyllum* (Thunb.) Makino based on ISSR polymorphism and its implications for conservation. Biochem. Syst. Ecol. 36, 699–705.
Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531–6535.
Xia, T., Chen, S., Chen, S., Zhang, D., Zhang, D., Gao, Q., Ge, X., 2007. ISSR analysis of genetic diversity of the Qinghai–Tibet Plateau endemic *Rhodiola chrysanthemifolia* (Crassulaceae). Biochem. Syst. Ecol. 35, 209–214.
Xiao, L.Q., Ge, X.J., Gong, X., Hao, G., Zheng, S.X., 2004. ISSR variation in the endemic and endangered plant *Cycas guizhouensis* (Cycadaceae). Ann. Bot. (Lond) 94, 133–138.
Xiao, W., Hong-Lin, C., Yun-Sheng, J., Wan-Hui, Y., Xue-Jun, G., Feng, L., 2008. Population genetic structure of *Camellia nitidissima* (Theaceae) and conservation implications. Bot. Stud. 49, 147–153.
Yeh, F.C., Yang, R., Boyle, T., 1997. POPGENE Version 1.32. Ag/For Molecular Biology and Biotechnology Centre. University of Alberta and Center for International Forestry Research.
Zhang, D.Q., Gao, L.M., Yang, Y.P., 2010. Genetic diversity and structure of a traditional Chinese medicinal plant species, *Fritillaria cirrhosa* (Liliaceae) in southwest China and implications for its conservation. Biochem. Syst. Ecol. 38, 236–242.
Zhou, T.H., Qian, Z.Q., Li, S., Guo, Z.G., Huang, Z.H., Liu, Z.L., Zhao, G.F., 2010. Genetic diversity of the endangered Chinese endemic herb *Saruma henryi* Oliv. (Aristolochiaceae) and its implications for conservation. Popul. Ecol. 52, 223–231.
Zietkiewicz, E., Rafalski, A., Labuda, D., 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20, 176–183.