Biochemical Systematics and Ecology 51 (2013) 50–59

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Genetic diversity in *Mentha cervina* based on morphological traits, essential oils profile and ISSRs markers

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ARTICLE INFO

Article history:
Received 25 June 2013
Accepted 11 August 2013
Available online

Keywords:
Mentha cervina
Morphological trait
Essential oil
Genetic diversity
ISSRs
Conservation genetics

ABSTRACT

Morphological, phytochemical and genetic differences were studied to evaluate the level and distribution of diversity in twelve populations of the Portuguese endangered medicinal plant *Mentha cervina* L. Morphological variation was correlated with ecological conditions at the site of origin. Pulegone was the major essential oils compound in all of the populations collected at full flowering (68–83%), in different growing conditions (51–82%), and for all the developmental stages studied (47–82%). Although clusters were defined, the analysis revealed a high chemical correlation among all populations ($S_{corr} \geq 0.95$%). Inter-simple sequence repeats markers were used to assess the population structure and genetic variation. Populations exhibited a relatively low genetic diversity (PPB = 14.3–64.6%, H_e = 0.051–0.222, I = 0.076–0.332), with high structuring between them (G_{ST} = 0.51). However, the genetic diversity at species level was relatively high (PPB = 97.7%; H_e = 0.320). The levels and patterns of genetic diversity were assumed to result largely from a combination of evolutionary history and its unique biological traits, such as breeding system, clonal growth, low capacity of dispersion and habitat fragmentation. The relatively low genetic diversity in the populations analyzed indicates that the maintenance of their evolutionary potential is at risk if population sizes are maintained and if there is no protection of the habitats.

1. Introduction

Genetic variation is fundamentally involved in the ability of a species to adapt to biotic and abiotic changes and in its evolution. Recognition of the levels and distribution of genetic variation within and among populations of a species is the base for development and selection of plant genotypes in breeding programs and increases the understanding of the historical

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http://dx.doi.org/10.1016/j.bse.2013.08.014

processes underlying the genetic diversity providing information for the management and preservation of endangered and geographically restricted species (Escudero et al., 2003; Shah et al., 2008).

Plants belonging to the genus *Mentha* L. (Lamiaceae) have evolved in nature through natural hybridization and selection, showing substantial variation in terms of their natural habitats, growth characteristics, and aromas (Franco, 1984; Tutin et al., 1972). They have a substantial importance in the botanical economy and to the pharmaceutical industry, mainly because of the essential oils produced and their antimicrobial properties, used since ancient times for the treatment of many digestive tract diseases and in culinary (İşcan et al., 2002).

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). It has a western steno-Mediterranean distribution, found in France, Portugal, Spain, Morocco, Algeria and it is presumed extinct in Italy (Rhazi and Grillas, 2010). In Portugal 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). The growth of commercial demands in recent years, the excessive harvesting from the wild, overgrazing and the unfavourable conservation status of their habitats, has shrunk the natural resource 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).

Morphological, molecular and biochemical markers are complementary in determining the genetic similarity of inter- and intra-species and the relationship between the populations (Chahal and Gosal, 2002; Kohler and Friedt, 1999). Given the well-known genus chemical variability, the essential oil composition from cultivated *M. cervina* populations cultivated (Alentejo Region, Portugal) was recently examined (Rodrigues et al., 2008). This study showed no essential oil chemical polymorphism despite the cultivated population's different provenance. A low genetic diversity associated with high differentiation among populations was also observed when *M. cervina* genetic diversity was assessed by inter-simple sequence repeats (ISSRs) (Rodrigues et al., 2013c).

Given the medicinal and aromatic potential of this species and its current threatened situation, the present study aims at assessing *M. cervina* genetic diversity level in Portugal based on the combination of molecular, phytochemical and morphological traits and also to provide guidelines for the conservation and sustainable use of this medicinal species.

2. Materials and methods

2.1. Plant material

A total of 12 populations of *M. cervina* with different geographic origins were included in the analysis. Geographic distances between populations vary from 9 km (between Mc32 and Mc33) to 450 km (between Mc33 and Mc43). Vouchers for each population were deposited in the LISI Herbarium (Table 1).

2.2. Morphological study

In this study, the 12 populations of *M. cervina* were kept in the same culture conditions, in the essay field at the Elvas Agrarian School (Alentejo), Portugal (Table 1). For each population, 24 plants were employed, in three lanes 1 m apart. The soil was soft and well drained. Dripping wings for irrigation and fertilization were placed among the lanes throughout their length. The cultural operations, until harvesting, consisted of manual elimination of weeds. For each population, 15 plants

Table 1
Data on collection site and sample type of *Mentha cervina* populations studied.

Populations	Sample type	Specific sample collection sites					Voucher
		Location	Altitude (m)	Latitude	Longitude	Hydrographic basin	
Mc10	M/G/W/C/DS	Ouguela, Campo Maior	207	39°4′54.96″N	7°0′4.33″W	Guadiana	532/2005
Mc32	M/G/W	Vilar Seco, Miranda do Douro	725	41°31′25.48″N	6°24′5.56″W	Douro	759/2008
Mc33	M/G/W	Póvoa, Miranda do Douro	750	41°34′22.71″N	6°19′17.53″W	Douro	760/2008
Mc34	M/G/W	Bagaúste, Peso da Régua	50	41°9′0.41″N	7°45′2.24″W	Douro	761/2008
Mc35	M/G/W	Escarigo, Figueira de Castelo Rodrigo	560	40°50′34.73″N	6°49′33.62″W	Douro	762/2008
Mc36	M/G/W/C/DS	Segura, Idanha-a-Nova	235	39°49′11.06″N	6°58′52.99″W	Tejo	763/2008
Mc39	M/G/W/C/DS	Oledo, Idanha-a-Nova	335	39°58′10.64″N	7°18′27.85″W	–	764/2008
Mc41	M/G/W/C/DS	Valência de Alcântara	313	39°28′1.17″N	7°12′24.16″W	Tejo	766/2008
Mc42	M/G/W	Torrão, Alcácer do Sal	50	38°17′0.32″N	8°13′57.81″W	Sado	767/2008
Mc43	M/G/W	Entradas, Castro Verde	154	37°44′36.51″N	7°58′44.60″W	Guadiana	768/2008
Mc44	M/G/W/C/DS	La Codosera	298	39°16′48.08″N	6°52′20.89″W	Guadiana	769/2008
Mc45	M/G/W/C/DS	Alburquerque	234	39°11′0.69″N	7°1′59.03″W	Guadiana	770/2008

Morphological study (M); genetic study (G); and phytochemical study from wild grown plants (W), from cultivated *vs* wild growing conditions (C), and at developmental stages (DS).

(5 plants per lane) were observed. Because no morphological descriptor list was developed yet for *Mentha*, morphological variables observed were adapted from the morphological descriptor list developed for *Coriander* by Diederichsen (1996). In a first stage (2 years observation), 35 morphological variables were scored. Analyses of correlation coefficients between all pairs of morphological variables, cluster analyses and principal components analysis allowed elimination of 24 that had or low discrimination value. In total, 11 morphological variables were scored in this study (Table 2).

Data analysis. Morphological variables discriminant analysis was used to assess the degree of separation of the populations by multivariate measurements and to test the impact of individual variables on the discrimination (Sokal and Rohlf, 1995). The cluster analysis was performed using the unweighted pair-group method with arithmetic average (UPGMA) and the Euclidean distance as the similarity coefficient, in the STATISTICA software (StatSoft). In the discriminant analysis, 15 measurements were used for each population, and in the Cluster analyses the score for each character was the mean value of the 15 measurements.

2.3. Phytochemical study

Three populations, from the 20 populations analyzed in Rodrigues et al. (2008) in addition to other 9 populations were included in the present study. To characterize the essential oil composition and identify possible chemotypes, the 12 populations were collected, during the flowering phase, from natural habitats. In order to understand the evolution in essential oil composition and yield along the plant life cycle, and compare cultivated with wild growing conditions, a time-course study was undertaken. In this study, 6 of these populations (15 plants per population), where collected from the wild, transported in containers and transplanted to the essay field at Instituto Superior de Agronomia, Lisbon, Portugal. Plants were planted 50 cm apart, in 2 m^2 plots, and drip irrigated periodically (each 7–10 days). Samples from the 6 populations, in the wild and in the cultivated essay field, were harvested at the vegetative, pre-flowering and full-flowering phases. The essential oils were isolated by hydrodistillation and analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) as reported by Rodrigues et al. (2008). The percentage composition of the oils was computed by the normalization method from the GC peak areas, which were calculated as mean values of two injections of each oil sample, without using response factors. The identity of the components was assigned by comparison of their retention indices, relative to a C_9–C_{17} hydrocarbon standard mixture, and with GC–MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards.

Data analysis. The essential oils composition was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) as reported by Rodrigues et al. (2008).

2.4. Genetic study

In this study, DNA extraction and amplification of the 121 individuals (corresponding to the 12 populations) with 10 ISSRs markers, and data analysis was performed as reported by Rodrigues et al. (2013b).

To test the correlation between Nei's genetic distance (D), morphological distance matrix, essential oil composition distance matrix and geographic distances (in km) between populations, Mantel tests (Mantel, 1967) were performed using GenAlex 6 program (Peakall and Smouse, 2006). All matrices were transformed to zero mean and unit variance before performing Mantel tests.

3. Results and discussion

3.1. Morphological study

The results of assessment between the 11 morphological variables, using statistical analyses, showed that the plant height, stem length and the number of flowers in the first inflorescence variables had maximum coefficient of variance, respectively.

Table 2
Morphological variables examined.

Morphological variables	Abbreviation and units
Plant height	Alt (cm)
First basal leaf length	comp1fba (cm)
Stem length	comC_tt (cm)
First inflorescence leaf length	c_foflor (cm)
Stem diameter at the plant base	diam_ba (cm)
Stem diameter at the first inflorescence	diam_inf (cm)
First basal leaf width	lrg_foba (cm)
First inflorescence leaf width	lrg_foi (cm)
Number of nodes until first inflorescence	nos_cauP
Number of flowers in the first inflorescence	n_flor
Number of flowered verticillaster at full flowering	nv_flor

The stem diameter at the first inflorescence, the first basal leaf width and length had minimum diversity variance coefficient (Table 3).

Discriminant analysis revealed that the three first functions represented 91% of the total variation in the data set (Table 3, Fig. 1). The first discriminant function accounted for 70% of the total variance, and separated cluster II from the others (Fig. 1). The standardized coefficients of function 1 were highest for plant height and stem length parameters. Function 2 represented another 16% of the total variance and roughly separated cluster I and cluster III from cluster IV (Fig. 1). This function was related to the number of flowers in the basal inflorescence and stem length. Function 3 accounted for 6% of the total variation and was a number of flowered verticillaster at full flowering and number of nodes until 1st inflorescence function.

Cluster analysis was used to investigate further the inter-relationships of these populations (Fig. 2). The clusters formed were similar to the relationships observed in the discriminant analysis. The results allowed the discrimination of the populations from the north (cluster II) in both, the cluster and the discriminant analysis.

The multivariate taxonomic distance matrix for all traits showed no significant association with geographic distance ($P = 0.25$, $r = 0.198$).

In this study, multivariate analyses revealed a structure in which the populations from the north region (Mc32, Mc33, Mc34 and Mc35) were differentiated from those in the more central-south localities. Nevertheless, the observed trend of morphological variation seems not to be associated with the inter-population distance. Several studies have indicated that morphological variation is apparently the result of an adaptive response to the environment. For example, variation of some traits is associated with a latitudinal and altitudinal range (Kleinschmit, 1993). In the present study, the morphological variation pattern, linked with the short plant height and stem length, suggests adaptation to the contrasting climatic conditions.

3.2. Phytochemical study

The essential oil yield, in the 12 wild populations of *M. cervina*, collected at full flowering ranged from 0.4% to 1.6% (w/d.w.), averaging 1.0% (w/d.w) (Table 4). These values are in accordance with the reported oil yield study by Vázquez Vicente (1981) and with some reported oil yields at full flowering for wild *Mentha* (*Mentha pulegium* 1.2%, Hassanpouraghdam et al., 2011; *Mentha arvensis* 1.7%, *Mentha piperita* 1.2%, *Mentha spicata* 1.2%, and *Mentha longifolia* 1.0%, Hussain et al., 2010). In the cultivated populations the essential oil yield at full flowering ranged between 0.3 and 1.1% (w/w.d.), less than half the yield found (2.4–4.0% w/d.w.) for cultivated populations in the Alentejo region in the study by Rodrigues et al. (2008). According to Voirin et al. (1990), the oil yield is favoured with higher temperatures, water deficit and higher summer sunshine, which is the case in the Alentejo Region, but not so much in the Lisbon Region, which may explain the difference in the yields found.

Thirty three components were identified, ranging from 92 to 100% of the total oil composition. The identified oil components are listed in Table 4 in order of their elution on the DB-1 column, arranged according to the four types of essential oils obtained by agglomerative cluster analysis (Fig. 3), with the lowest and the highest percentages found for each component in each volatile oil type.

Mostly quantitative rather than qualitative variation was observed in all the essential oils analyzed. Oxygen-containing monoterpenes (80–97%) were dominant in all oils, Table 4. Pulegone was the major compound in all of the populations (68–83%) at full flowering, followed by isomenthone (0.1–22%), limonene (3–9%), and menthone (1–2%). Cluster analysis (Fig. 3), confirmed a high chemical correlation among all populations ($S_{corr} \geq 0.95\%$). Even though, two clusters were defined in the bases of isomenthone relative amounts. In cluster I, which included 11 out of the 12 samples, isomenthone ranged from 0.1 to 15%, whereas the one sample cluster II showed a higher percentage (22%). No correlation was detected between the clusters and the geographical collection site. Sub-cluster a has a relative amount between 0.4 and 7%, sub-cluster b between

Table 3
Summary table obtained by stepwise discriminant analysis and standardized coefficients for the first three discriminant functions based on quantitative values of morphometric plant characters of 12 *Mentha cervina* populations.

	Stepwise discriminant analysis summary				Standardized function coefficients		
	N	*F* statistics	R^2	Wilks' lambda	Function 1	Function 2	Function 3
Plant traits							
Alt	1	65.5	0.14	$6\ 10^{-3}$	−0.798		
comC_tt	2	39.9	0.71	$5\ 10^{-3}$	−1.094	0.552	
n_flor	3	31.2	0.20	$3\ 10^{-3}$		−0.543	
c_foflor	4	26.0	0.27	$3\ 10^{-3}$			
nv_flor	5	22.0	0.60	$3\ 10^{-3}$			0.93
nos_cauP	6	19.1	0.23	$3\ 10^{-3}$			0.569
diam_ba	7	16.9	0.11	$3\ 10^{-3}$			
lrg_foi	8	15.4	0.15	$3\ 10^{-3}$			
diam_inf	9	14.1	0.30	$3\ 10^{-3}$			0.535
comp1fba	10	12.8	0.14	$3\ 10^{-3}$	−0.209		
lrg_foba	11	11.9	0.16	$3\ 10^{-3}$			
Cumulative variation					70.2	85.7	91.2

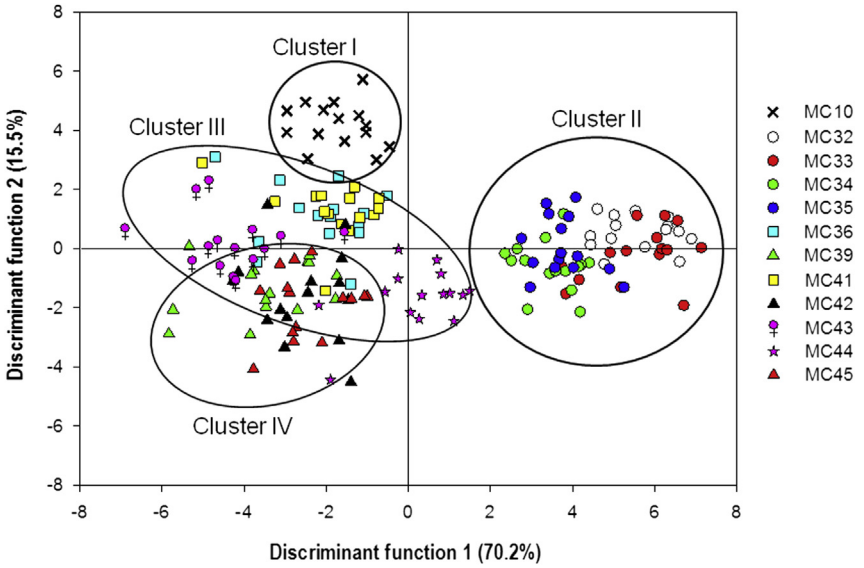

Fig. 1. Discriminant analysis based on the morphological trait in the 12 *Mentha cervina* populations. For samples grouped on each of the clusters, see Fig. 2.

9 and 15% and sub-cluster c (Mc34) has the lower relative amount (0.1%). No significant correlation was detected between the clusters and the geographical collection site ($P = 0.070$, $r = 0.301$).

All the essential oils studied belonged to the pulegone chemotype, in accordance with the results of Rodrigues et al. (2008) on cultivated Portuguese populations collected during the flowering phase. *M. cervina* essential oils studied until now showed high uniformity, which is not usual in *Mentha* species and hybrids that are known to have different chemotypes (Kokkini and Vokou, 1989).

In plant developmental terms, the essential oil yield had a different behaviour according to the growing conditions. The yield of the essential oils isolated from wild growing populations increased rapidly from the vegetative stage (mean value 0.4% w/d.w.) until full flowering, June and July (mean value 1.1% w/d.w.). In the cultivated ones, the essential oil yield increased from the vegetative until the pre-flowering phase (1.2% w/d.w) and then it started to decrease towards the flowering stage (0.7% w/d.w.).

The analysis of the main essential oil constituents revealed that pulegone remained the major constituent, along the life cycle of the plant, for both growing conditions, although the behaviour of the main components was slightly different (Fig. 4).

In the essential oil isolated from cultivated populations, pulegone relative amount increased from the vegetative until the pre-flowering phase and then it started to decrease towards the full-flowering phase. The pulegone relative amount from wild growing populations increased until the vegetative phase and then stabilized towards the end of the plant life cycle. These changes were followed by changes in the main components relative amounts, whenever the pulegone decreased, the isomenthone and menthone tended to increase (Fig. 4), in particular the isomenthone. Physiological variations (i.e. organ and

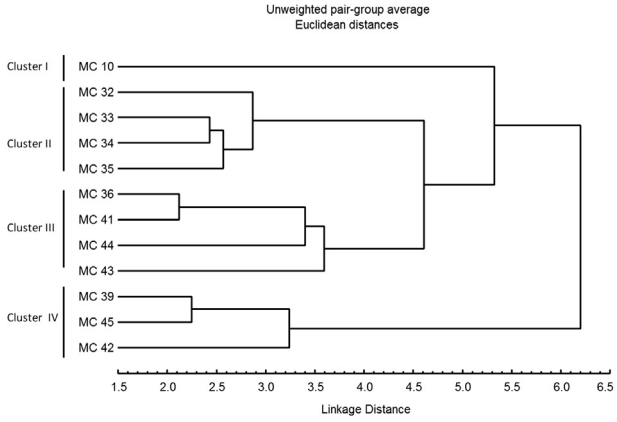

Fig. 2. Hierarchical cluster analysis dendrogram of Euclidean similarity distance showing the relationship among 12 *M. cervina* populations based on average values of morphological variables.

Table 4
Minimum and maximum percentage range of components identified in the essential oil, isolated from the aerial parts of 12 *Mentha cervina* wild populations collected at full-flowering phase. For samples grouped on each of the clusters, see Fig. 3.

Components	RI	Cluster I					Cluster II
		a		b		c	
		Min	Max	Min	Max	Mc34	Mc36
3-Methyl cyclohexanone	914	t	t	t	t	t	t
α-Thujene	924	t	t	t	t	t	t
α-Pinene	930	0.2	0.6	0.3	0.5	0.1	0.3
Camphene	938	t	t	t	t	t	t
Sabinene	958	t	0.4	0.1	0.2	0.2	0.1
3-Octanone	961	t	t	t	t	t	t
β-Pinene	963	0.2	1.1	0.3	0.4	0.3	0.3
2,5-Dimethyl-1-hexene[a]	970	t	t	t	t	t	t
3-Octanol	974	0.7	1.6	1.2	1.5	1.2	1.0
β-Myrcene	975	0.4	1.1	0.8	1.0	0.8	0.7
p-Cymene	1003	t	t	t	t	t	t
1,8-Cineole	1005	t	t	t	t	t	t
Limonene	1009	2.6	6.7	2.8	4.8	8.6	3.1
cis-β-Ocimene	1017	t	t	t	t	t	t
trans-β-Ocimene	1027	t	t	t	t	t	t
γ-Terpinene	1035	t	t	t	t	t	t
n-Octanol	1045	t	t	t	t	t	t
cis-Linalol oxide	1045	t	t	t	t	t	t
trans-Limonene oxide	1112	t	t	t	t	t	t
Menthone	1120	0.6	1.7	1.0	1.6	1.2	1.8
Isomenthone	1126	0.4	6.7	9.4	15.4	0.1	21.8
Menthofuran	1134	t	0.2	t	t	t	t
cis-Isopulegone	1134	0.5	1.6	0.3	1.1	1.0	0.9
Terpinen-4-ol	1148	t	t	t	t	t	t
Verbenone	1164	t	t	t	t	t	t
Myrtenol	1168	t	t	t	t	t	t
Pulegone	1210	78.4	83.4	71.9	75.7	73.7	68.1
Piperitone	1211	t	t	t	t	t	t
Carvotanacetone[a]	1222	t	t	t	t	t	t
Piperitenone	1289	0.3	3.9	0.3	0.9	9.2	0.4
β-Caryophyllene	1414	t	0.1	t	0.1	0.2	t
β-Caryophyllene oxide	1561	t	0.3	t	0.4	0.2	t
Humulene epoxide	1579	0.2	1.3	0.5	1.7	0.5	0.2
% Identification		97.4	99.5	98.0	99.3	92.1	98.9
Grouped components							
Monoterpene hydrocarbons		3.5	9.9	4.4	6.9	10.1	4.6
Oxygen-containing monoterpenes		80.3	97.4	82.9	94.6	85.2	93.0
Sesquiterpene hydrocarbons		t	0.1	t	0.1	0.2	t
Oxygen-containing sesquiterpenes		0.2	1.6	0.5	2.1	0.7	0.2
Others[b]		0.7	1.6	1.2	1.5	1.2	1.0
Oil yield (v/w)		0.9	1.5	0.7	1.6	0.4	1.1

RI: retention index relative to C_9–C_{16} *n*-alkanes on the DB-1 column; t: traces (<0.05%).
[a] Identification based on mass spectra only.
[b] Components that do not fit on the classification of terpene or phenylpropanoid and which are mainly non-aromatic alcohols, ketones and alkenes.

leaf position), environmental conditions (i.e. harvest date and planting time), geographic variations and genetic factors and evolution are known to affect the biosynthesis of the essential oils (Figueiredo et al., 2008). Thus, these type of variations, that where already seen in *M. pulegium* (Rodrigues et al., 2013a) may be due to the influence of the developmental stage and environmental conditions on the regulation of the essential oil biosynthesis.

3.3. Molecular study

ISSR amplification of the 121 individuals, gave a total of 175 bands that could be scored, corresponding to an average of 82.4 fragments per individual. Of these bands, 171 were polymorphic (97.7%). Genetic diversity estimates from ISSR are summarized in Table 5.

Based on ISSR analysis, the genetic diversity of *M. cervina* at the species level is relatively high ($H_e = 0.323$; $I = 0.226$; PPB = 98%), however, in contrast, relatively low genetic diversity occurred at the population level (Table 5). The proportion of polymorphic bands at the population level varied from 14% to 65%, with a mean of 45%. The least genetically diverse population is Mc34 with 5% gene diversity and the most diverse populations is Mc43 with 22% gene diversity (Table 5). These

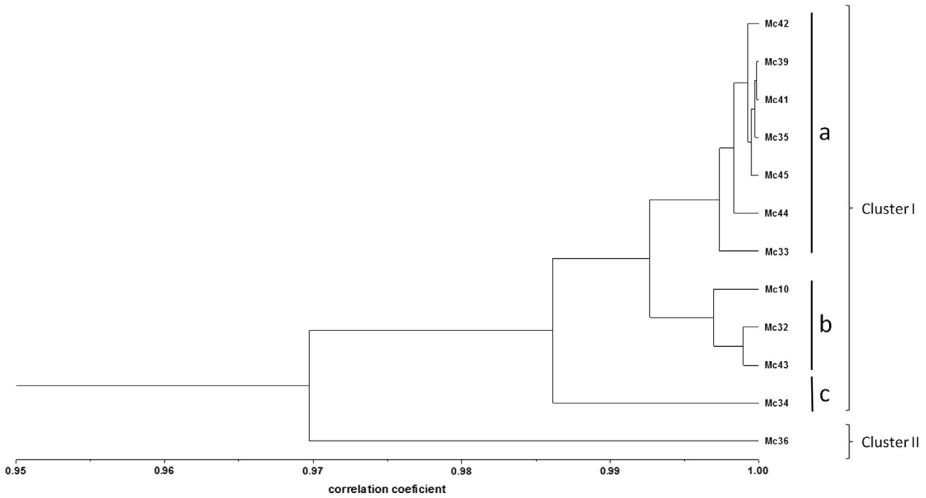

Fig. 3. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Mentha cervina* samples examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). For abbreviations, see Table 1.

values are lower than the genetic diversity reported for other Lamiaceae species (Aparicio et al., 2000; Ben Fadhel and Boussaid, 2004; Liu et al., 2006; Mattner et al., 2002) and are consistent with other endangered species (Bin et al., 2005; Jin and Li, 2007; Li and Jin, 2008; Vicente et al., 2011; Wang et al., 2008; Xiao et al., 2004; Zhou et al., 2010).

According to Nei's analysis of gene diversity, the percentages of genetic variation among *M. cervina* populations were 51% (G_{st}) which indicated a high inter-population genetic differentiation. The AMOVA showed that most of the variation was found within populations (52%) provided additional evidence for the genetic structuring of populations. The nearly Φ_{ST} from the AMOVA analysis (0.478) and the G_{st} from the POPGENE software analysis (Yeh et al., 1997) provide additional support for the robustness of ISSR markers used in this study.

The high level of genetic differentiation ($G_{st} = 0.532$) detected among *M. cervina* populations suggest that each population analyzed is genetically defined and structured as a distinct genetic pool. Also, the values of heterozygosity found for the fragments analyzed (0.051–0.222) are lower than the average outcrossing-animal species ($H_e = 0.260$), and for some of the populations also lower than self-pollinating plants ($H_e = 0.091$), using RAPD markers (Nybom and Bartish, 2000), which indicates inbreeding within *M. cervina* populations. The reason behind this low heterozygosity may be partly attributed to the clonal growth of this species and the low seed setting and dispersion (Rodrigues et al., 2013b). Clonal growth can significantly decrease the effective population size, and hence contribute to the loss of genetic diversity and the genetic differentiation via increased levels of genetic drift and inbreeding (Erickson and Hamrick, 2003). Also, *M. cervina* was usually observed in severely fragmented habitats and with small population sizes (from 10 to 1000 individuals), which make this species

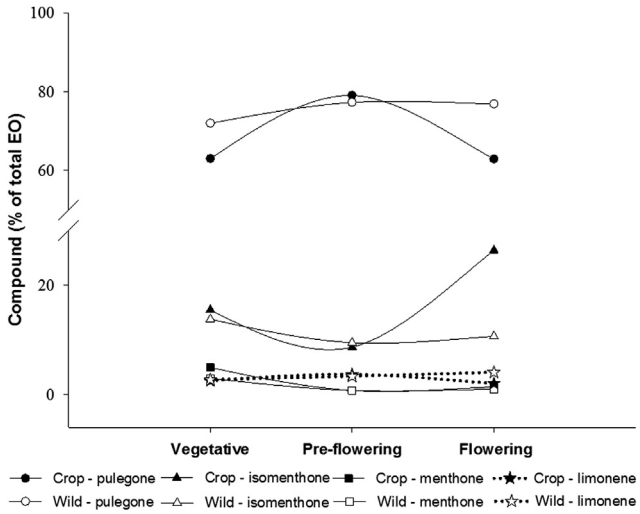

Fig. 4. Time-course study of the main components of the *Mentha cervina* essential oils isolated in wild (open symbols) and cultivated (closed symbols) growing conditions at different developmental stages. The values are the mean values from 6 populations.

Table 5
Measures of genetic diversity in each population and the entire data in *Mentha cervina*. PPB, percentage of polymorphic loci; *I*, Shannon's information index; H_e, Nei's gene diversity.

Population	PPB		*I*	H_e
	Number	Percentage		
Mc10	101	57.7	0.290	0.193
Mc32	102	58.3	0.310	0.208
Mc33	112	64.0	0.332	0.222
Mc34	25	14.3	0.076	0.051
Mc35	93	53.1	0.278	0.187
Mc36	57	32.6	0.181	0.124
Mc39	66	37.7	0.191	0.128
Mc41	54	30.9	0.170	0.116
Mc42	63	36.0	0.195	0.131
Mc43	113	64.6	0.328	0.219
Mc44	64	36.6	0.178	0.117
Mc45	85	48.6	0.253	0.169
Mean	78	44.5	0.232	0.155
Total	171	97.7		0.320

extremely vulnerable to stochastic events, genetic drift and inbreeding (Hartl and Clark, 1997), leading to a low genetic diversity and the high genetic structure pattern observed.

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 and a cluster analysis (UPGMA) was also used to generate an unrooted tree based on Nei's genetic distances (Fig. 5). The STRUCTURE analyses revealed that the twelve populations of *M. cervina* in the present study share 5 genetic pools, with migrants and admixed individuals. The populations from the north region constitute one gene pool (Mc32, Mc33, Mc34 and Mc35) and grouped together in the UPGMA tree (despite the week

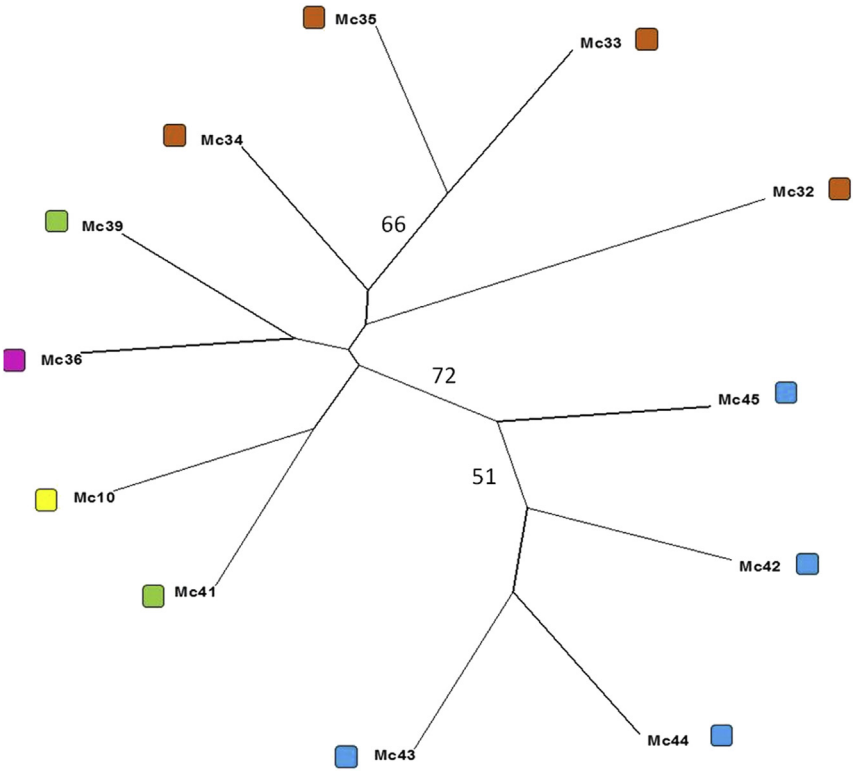

Fig. 5. UPGMA dendrogram based on Nei's genetic distance matrix for *Mentha cervina* populations. One thousand replicates of bootstrapping analysis were used to assess the statistical support of each branch. Numbers in branches correspond to the bootstrap analyses (50% or more). Square colour blocks represent the different gene pools 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.)

bootstrap support). The two populations from the south share the gene pool with two populations from the centre-south and were also clustered in the UPGMA tree (with moderate bootstrap support) and the other 3 gene pools are encountered in the midland with week and arbitrary clustering (Fig. 5).

On the other hand, Mantel test did not find significant correlation between genetic and geographic distance matrices ($r = 0.031$, $P < 0.5$, 999 permutations), indicating that the isolation by distance is not shaping the present *M. cervina* population genetic structure.

3.4. Correlation between morphological, phytochemical and molecular traits

The obtained results showed that four populations (Mc32, Mc33, Mc34 and Mc35), out of 12, grouped together, in both the morphological and molecular studies, whereas the essential oils correlation evaluation grouped the populations in an entirely different way (Figs. 2, 3 and 5). Several reasons can determine this different grouping:

ISSRs are considered to be neutral and thus to provide no direct assessment of fitness. The forces that cause differentiation for these markers would be the result of mutation, genetic drift, low gene flow and no selection. Conversely, morphological traits and phytochemical profiles are generally believed to be subject to natural selection, and their expression is partially under the influence of environmental factors (Bruschi et al., 2003). Adding to this, the ISSRs are random markers that show differences in the whole genome, and are not necessarily related to a specific morphological trait or secondary compound. However, previous studies on *Salvia fruticosa* (Skoula et al., 1999), *Ocimum gratissimum* (Vieira et al., 2001) *Tanacetum vulgare* (Keskitalo et al., 2001), *Primula ovalifolia* (Nan et al., 2003) and *Vitex rotundifolia* (Hu et al., 2007) reported that the patterns of relatedness observed in chemical profiles seemed to correspond well with the genetic profiles generated by RAPDs and ISSRs. On the other hand, there are also studies, where no correlation could be found among collection site, chemical and molecular analysis (Trindade et al., 2008 in *Thymus caespititius*). The same pattern of correlations can be observed for the morphological traits. Liu et al. (2007) and Hamza et al. (2004) demonstrated a clear correlation between the morphological traits and the detection of the genetic variability as revealed by RAPD analysis. Conversely, Schut et al. (1997) and Eshraghi et al. (2006) reported a few correlations between the molecular and the morphological traits. Together these studies, suggests that may be there is a genetic basis for the chemical profiles and morphological traits that can be observed with the ISSRs markers, although they are not clear.

At last, although essential oils may evolve more rapidly than morphological traits, the rather unusual uniformity found in the essential oils composition in populations with different geographic provenances, at different developmental stages and in different growing conditions, may explain why the morphological traits were more correlated with the genetic variation, than the phytochemical ones.

From a conservation perspective, the low genetic and phytochemical diversity observed, within the populations tested is symptomatic and a signal that ecological management of *M. cervina* habitats is necessary to prevent the consequent decline in population size that could increase the risk of extinction due to demographic and environmental stochasticity.

Acknowledgements

The financial support of Fundação para a Ciência e Tecnologia (FCT/CBAA and PhD Grant SFRH/BD/38143/2007 of Leandra Sofia Rodrigues) is gratefully acknowledged.

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