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Diversity distribution analysis in *Minthostachys verticillata* Epling (Griseb) (Lamiaceae) (peperina) populations by EST-SSR markers

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Abstract

A variability genetic analysis was carried out among *Minthostachys verticillata* populations collected in the central and northwest region of Argentina. 93 plants from nine locations were analyzed by PCR, developing EST-SSR primers from the database of *Mentha spp*. AMOVA analysis revealed that variability was higher among populations than within them (93% vs. 7%).The highest percentage of polymorphic loci corresponded to the locations from Padre Monti (75%) and Cortaderas (70.83%). Three groups were identified by cluster analysis following a geographic gradient. The genetic variability found in this study is greater than the phytochemical variability represented in previous studies.

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Introduction

Minthostachys verticillata (peperina) is a native species, which is distributed between central and northwest of Argentina (Cabrera, 1976; Schmidt-Lebuhn, 2007, 2008). It has a large phytochemical variability, primarily among the populations of these two regions (Lizzi and Retamar, 1975; Retamar *et al.*, 1996; Zigadlo *et al.*, 1996; Ojeda *et al.*, 2001; Bandoni *et al.*, 2002; Ojeda, 2004; Elechosa *et al.*, 2005; Arteaga *et al.*, 2013; Arteaga and Gil, 2013). Its area of distribution ranges from humid forests (yungas) in the Northwest, to semi-arid areas in the center of the country. Peperina is widely used in traditional medicine and local industries in the manufacture of soft drinks, several teas and aromatic herbs throughout its natural area of dispersion (Bonzani and Ariza-Espinar, 1993; Martínez and Planchuelo, 2003). As a result of this and other factors such as changes in land use, natural populations are declining to the point of being considered endangered species (Bustos and Bonino, 2005; Barboza *et al.*, 2009). While there are many studies about the phytochemical variability in peperina, there are few in terms of its genetic variability. The chemical profile and its variability are conditioned by the degree of genetic heterogeneity of individuals within populations and plastic responses due to changes in the environment (Harborne, 1991; Gershenson, 1994; Langenheim, 1994; Croteau *et al.*, 2005; Rios-Estepa *et al.*, 2010).

The study of genetic variability by using molecular markers can provide an important measure of the genetic differentiation of populations that occupy different geographical areas and complement studies of chemical profiles (Skoula *et al.*, 1999; Trindade *et al.*, 2008, 2009; Chen *et al.*, 2009; Honermeier, 2010).

Microsatellites or Simple Sequence Repeat (SSR) markers are regions of short sequences (2 to 10 base pairs) of DNA repeated throughout the entire genome, being able to be associated or not to genes. Due to its high variability, these markers are suitable for obtaining polymorphisms (Tanksley, 1993). The microsatellites markers have been generated in large numbers in most of the cultured species while its development is very demanding in time, infrastructure and economic resources (Varshney *et al.*, 2005), being its main limitations.

However in recent years it was noted a large increase in the availability of DNA sequence data in a wide variety of taxa, including an abundance of expressed sequences (ESTs) markers available in public databases (Pashley *et al.*, 2006). Thus the use of these databases is a fast and economical alternative for the development of SSR through the use of computer tools (Gupta *et al.*, 2003; Bhat *et al.*, 2005). The transferability of polymorphic EST-SSR markers has been demonstrated in numerous cases, including aromatic and medicinal species (Varshney *et al.*, 2005; Ellis and Burke, 2007). Tripathi *et al.* (2008) obtained ESTs-SSR from secondary metabolites of medicinal and aromatic plants, such as alkaloids and terpenoids, demonstrating the potential of bioinformatic tools in the development of markers for genetic analysis in these species. In oregano were used SSR markers from ESTs database to identify and characterize species of *Origanum vulgare* and *Origanum majoricum* (Novak *et al.*, 2008). However, there are few precedents of transferability of these markers between species at family level.

In the present study, we employed EST-SSR markers to investigate the genetic diversity of nine natural *M. verticillata* populations distributed in three provinces of Argentina.

Materials and methods

Plant material

Ninety-three plants were collected in nine locations from the Central and Northern regions of Argentina covering the provinces of San Luis, Cordoba and Tucuman (Table 1 and Fig. 1). Geographic distances between populations cover a range of nearly 700 km from Cortaderas in the south (San Luis) to Padre Monti to the North (Tucumán).

Table 1. Geographical location of *M. verticillata* populations and number of individuals/population (N).

Province	Populations	N	Geoposicion		Altitude (masl)
			Latitude	Longitude	
San Luis	Cortaderas (COR)	22	32 29,169 S	64 58,472 O	1047
	Pasos Malos (PMA)	11	32 19,164 S	64 58,824 O	1094
Cordoba	Hornillos (HOR) 7	31	54,227 S	64 58,706 O	1084
	Chacras1 (CHA1)	8	32 13,417 S	65 25,769 O	1000
	Chacras2 (CHA2)	8	32 13,549 S	65 00,525 O	941
	Embalse (EMB)	2	32 11,154 S	64 23,368 O	526
	Unquillo (UNQ)	8	31 11,534 S	64 21,767 O	800
	Ongamira (ONG)	10	30 46,013 S	64 27,656 O	1277
Tucuman	Padre Monti (PMO)	17	26 29,377 S	64 59,521 O	982

N = number of individuals analyzed; masl= meters above the sea level.

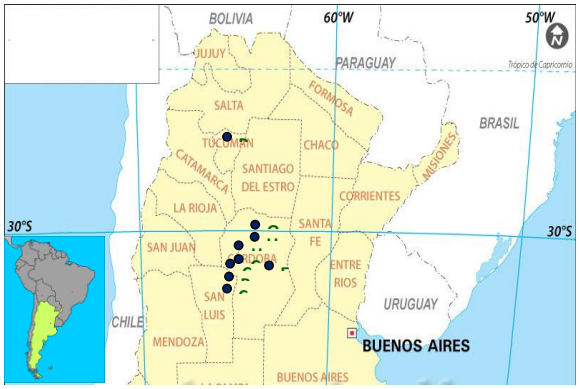


Fig. 1. Map of geographic distribution of the nine populations of *M. verticillata* within three provinces of Argentina.

DNA extraction and PCR amplification

Young leaf samples were collected directly from the field and were kept on silica gel until processing in the laboratory. DNA extraction was performed using a modified CTAB method described by Murray and Thompson (1980) for *Mentha spp*. (Shiran *et al.*, 2005). The quantity of DNA extracted was evaluated by electrophoresis in 0.8% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.5), and each DNA sample was diluted to 10 ng/µl for PCR amplification.

The amplification by PCR was performed in a final volume of 18 µL that included: 20 ng of DNA, 1X reaction Buffer (Inbio-Highway), 1.5 mM of $MgCl_2$, 0.3 µM of each primer, 200 µM of each dNTPs, and 0.5 units of Taq DNA polymerase (Inbio-Highway). PCR was carried out using PTC-100 Thermal Cycler machine (MJ Research, Inc. Waltham, USA) with the following profile: initial denaturation at 94°C for 4 minutes, followed by 40 cycles of 40 s at 94°C (denature), 45 s at 55 or 60°C (depending of primer) (annealing) and 50 sec at 72°C (elongation). The last cycle was followed by a final extension at 72°C for 8 min.

PCR products were visualized at 6% (w/v) denaturing polyacrylamide gels stained with silver nitrate and revealed with sodium carbonate. Band size was determined by comparison with DNA 10 bp DNA Ladder (Invitrogen).

Search and identification of ESTs containing SSR

The EST search was conducted in the TrichOME V3 database (http://www.planttrichome.org/trichomedb/estbyspecies.jsp). The BatchPrimer3 v1.0 software (freely available at http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) (You *et al.*, 2008) was used to identified microsatellites in the EST sequences and design the flanking primers. The major parameters for designing the primers were: primer length from 18 to 24 nucleotides, with 22 as the optimum, PCR product size from 120 to 300 bp, optimum annealing temperature 60°C, and GC contents with 50% as optimum. The others parameters were left by default. The EST-SSR primer pairs were synthesized in Bio-Synthesis, Inc. (Lewisville, Texas, USA).

Data analysis

The tested primers produced different patterns of bands that were classified as polymorphic, monomorphic (band of the same molecular weight in all individuals) and non-specific, when the banding pattern was not clear in all individuals, in addition to non-reproducible.

The multilocus data was transformed into a binary matrix of presence (1), absence (0) of each allele for each individual, where each band was seen as a locus. Genetic diversity parameters analyzed include percentage of polymorphism (%*P*), number of different alleles (*Na*), number of alleles (*Ne*), index of Shannon (*I*), and expected heterozygosity (*He*). All these parameters were analyzed with the statistical software GenAlEX 6.5 (Peakall and Smouse, 2012) on the basis of the data matrix built, assuming a population in Hardy-Weinberg equilibrium. This software was also used to carry out an Analysis of Molecular Variance (AMOVA) among and within populations, in order to evaluate the structure of the observed genetic variation. The significance of PhiPT among populations was determined with probability of non-differentiation (FST= 0) estimated about 9,999 permutations. GenAlEx 6.5 was also used to evaluate genetic relationships between populations through of Principal Coordinate Analysis (PCoA), in addition to the correlation analysis between Nei's genetic distance and geographic distance (in Km) between populations through Mantel test (Mantel, 1967) with 999 permutations. Clustering analysis was conducted using the UPGMA method (Unweighted Pair Group Method with Arithmetic mean) using the POPGENE 1.32 software (Yeh *et al.*, 1997). The phenogram was obtained from Nei's (1972) genetic distance matrix generated through 1000 permutations.

Results

Genetic diversity and genetic differentiation among populations

From the twenty primers that were used, five showed no amplification product, seven were polymorphic and the rest were monomorphic or showed no reproducibility. In total, twenty-four alleles were generated.

The highest percentage of polymorphic loci corresponded to PMO (75%) and COR (70.83%) populations (Table 2). These two populations showed also private bands that were not found in the other populations (data not shown). The lowest values of *I* and *He* were in EMB with 0.05 and 0.035 respectively, while PMO had the highest values for these two parameters (*I*= 0.29 and *He*= 0.18). In the range of these values we can distinguish three groups of populations in terms of genetic diversity: the highest group composed by COR, PMA, PMO and ONG with $I > 0.2$, an intermediate group composed by HOR, CHA1 and UNQ with *I*= 0.1 to 0.2, and an lowest group composed by CHA2 and EMB with $I < 0.1$. However, for *He* we have distinguished two groups, where COR, CHA1, PMA, PMO and ONG had values higher than 0.1, and HOR, CHA2, EMB and UNQ with values lower than 0.1 (Table 2).

The AMOVA analysis indicated that variability was higher within populations than among them (93% vs. 7%) (Fig. 3). The PhiPT value (0,074, *P*= 0,010) showed a low differentiation among populations (Table 4), indicating that the greater genetic diversity occurs within populations.

Genetic relationships and population structure

The Nei's genetic distance matrix analysis showed that the greater genetic distance corresponds to PMO-EMB (0.1394) (Table 3), which is consistent with the greater geographical distance between them. The shortest genetic distance corresponds to the populations of COR-PMA (0.0065), geographically closest. The Mantel test showed a positive and significant correlation ($r = 0.65$, $P< 0.05$) between genetic and geographic distances to all populations (Fig. 2).

The Principal Coordinate Analysis (PCoA) showed that the first axis explained 58.88% of total variation, while the second axis explained 32.54% (Fig. 4). We

observed the existence of three groups of populations following a gradient of geographical distribution in the North-South direction, with PMO and ONG to the North, UNQ, HOR and EMB on the center and CHA2, PMA and COR to the South.

This is consistent with as shown on the dendrogram (Fig. 5), where populations of COR, PMA and CHA2 closest geographically, are grouped together with the minor genetic dissimilarity among them. By contrast, the population of PMO in Tucuman is the most distant geographical and genetically from the rest.

Table 2. Parameters mean values for each population.

Population	***% P***	***Na***	***Ne***	***I***	***He***
COR	70,83	1,458	1,174	0,201	0,117
HOR	25,00	0,625	1,113	0,118	0,075
CHA1	45,83	1,000	1,147	0,174	0,105
PMA	58,33	1,208	1,188	0,220	0,132
PMO	75,00	1,500	1,289	0,290	0,182
ONG	50,00	1,042	1,210	0,212	0,134
CHA2	20,83	0,500	1,091	0,088	0,056
EMB	8,33	0,333	1,059	0,050	0,035
UNQ	37,50	0,833	1,128	0,143	0,087
TOTAL	43,52	0,944	1,155	0,166	0,102

% P = Percentage of Polymorphic Loci; Na = Number of Different Alleles; Ne = Number of Effective Alleles; I = Shannon's Information Index; He = Expected Heterozygosity.

Table 3. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the *M. verticillata* populations.

Pop.	**COR**	**HOR**	**CHA1**	**PMA**	**PMO**	**ONG**	**CHA2**	**EMB**	**UNQ**
COR	****	0.9786	0.9829	0.9936	0.9346	0.9633	0.9887	0.9383	0.9893
HOR	0.0217	****	0.9684	0.9644	0.9069	0.9559	0.9686	0.9596	0.9899
CHA1	0.0172	0.0321	****	0.9863	0.9239	0.9694	0.9852	0.9340	0.9910
PMA	0.0065	0.0363	0.0138	****	0.9339	0.9661	0.9910	0.9274	0.9836
PMO	0.0677	0.0977	0.0792	0.0684	****	0.9644	0.9223	0.8699	0.9278
ONG	0.0373	0.0451	0.0311	0.0345	0.0363	****	0.9621	0.9263	0.9738
CHA2	0.0114	0.0319	0.0149	0.0091	0.0809	0.0386	****	0.9263	0.9876
EMB	0.0637	0.0412	0.0683	0.0754	0.1394	0.0765	0.0765	****	0.9527
UNQ	0.0107	0.0101	0.0090	0.0166	0.0749	0.0265	0.0125	0.0484	****

Pop.= Population; ****= invalid data.

Table 4. Analysis of molecular variance (AMOVA) showing the partitioning of genetic variation within and between nine populations of *M. verticillata*.

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Population	8	54,051	6,756	0,300	7%
Within Population	84	316,390	3,767	3,767	93%
Total	92	370,441		4,067	100%

df= degrees of freedom, SS= Sum of Squares, MS= Mean Squares, Est. Var.= estimate of variance, %= percentage of total variation.

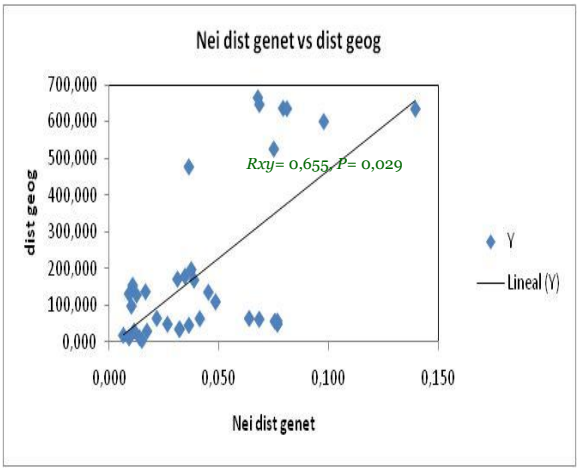


Fig. 2. Relationship between genetic and geographic distance (Km) of *M. verticillata* populations.

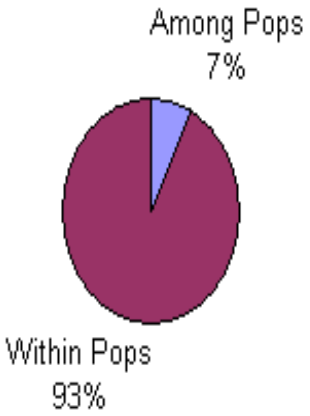


Fig. 3. Percentages of Molecular Variance.

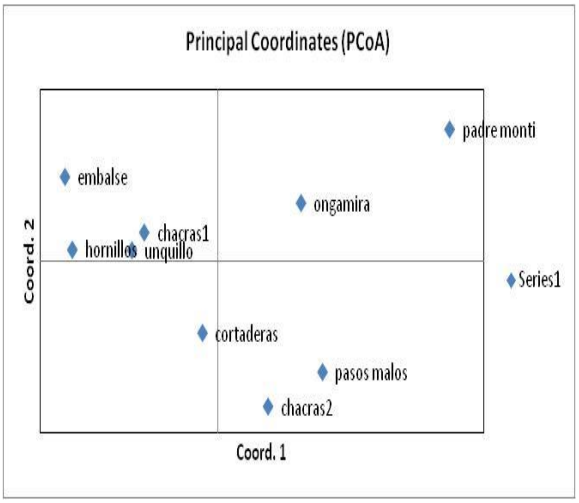
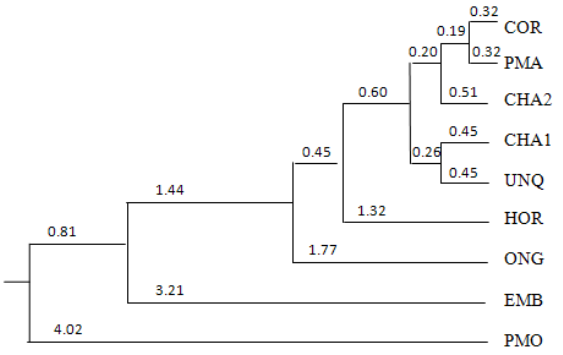


Fig. 4. Principal Coordinate Analysis (PCoA) of nine populations of *M. verticillata* based on the two principal axes.



Numbers above branches represent bootstrap support for 1000 replicates.

Fig. 5. UPGMA phenogram based on Nei's (1972) genetic distances among populations of *M. verticillata*.

Discussion

Genetic diversity and differentiation

The microsatellite markers developed in peperina from *Mentha spp*., generated polymorphic bands in spite of being species of different genus, which allowed us differentiate the nine populations throughout their range. While the number of alleles was low, due to these are transferred SSR, was markedly higher than that obtained by Novak *et al.* (2008) in oregano, although in that case the species were the same genus.

The distribution of variability displayed in the AMOVA, is characteristic of species that are crossed to short distance. The species of this genus in general have high percentages of progenies and pollinate through flies and bees (Schmidt-Lebuhn *et al.*, 2007). The highest percentages of polymorphisms and exclusive bands observed in PMO and COR could have a relationship with the degree of conservation of these sites of collection, due to a minor anthropogenic pressure.

The populations exhibited a low genetic diversity, according that was expected for the type of marker used (EST-SSR) because they are in conserved sequences. The values of diversity genetic found in our work are consistent with those found by

Rodrigues *et al.* (2013) in *Mentha cervina* (*He* = 0.051–0.222, *I* = 0.076–0.332). The highest value of genetic diversity (*I*= 0.29, *He*= 0.18, *%P*= 75 %) shown by PMO can be explained by its location in the region of the Yungas, area where is located the largest biodiversity in Argentina (Brown, 1995).

On the other hand, the analysis of the lowest values of *I*, *He* and *%P* for EMB, must be taken into account the low number of individuals sampled due to be subject to a higher pressure extractive, favored by the lower altitude (526 masl) and easy access to the villagers. This evidenced in the least amount of individuals available for the collection.

Genetic variation and its phytochemical implication

The genetic similarity analysis revealed a latitudinal gradient in which the closest populations geographically have greater similarity (Fig. 5). This was also observed in other native species (Inza *et al.*, 2012). PMA, CHA1 and ONG populations belong to the central region which covers the provinces of San Luis and Cordoba, where are the typical peperinas. These populations remain high values of *I* and percentages of polymorphism in the order of 50 %, indicating that there would be genetic diversity despite the large decrease in the population.

Most of the studies conducted in peperina have been phytochemicals and it is one of the most studied species of the genus in that sense (Schmidt-Lebuhn, 2008). The largest phytochemical variability occurs among populations, being the Northwest region the most diverse and the Central region more homogeneous. It was found that populations from Cordoba and San Luis have pulegone and menthone as main compounds from its essential oil, decreasing this last compound its concentration to the North of Argentina. Other compounds are cited such as thymol, carvacrol, limonene, linalool and carvone (Zigadlo *et al.*, 1996; Elechosa *et al.*, 2005; Ojeda *et al.*, 2001; Ojeda, 2004; Retamar, 1996), indicating the existence of a high chemical variability in this species, which would increase inversely to latitude.

Conclusions

The genetic diversity of *M. verticillata* was large within populations and small among populations. The Mantel test, demonstrate a positive and significantly correlation between genetic and geographic distance. In spite of occupy a wide area of distribution with a large anthropic disturbance, this species still maintain reserves of variability. The use of EST-SSRs in native species can be a useful tool for the analysis of natural populations, such as complement of chemical analysis and the development of markers associated with compounds in native species.

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