

# Genetic diversity and spatial genetic structure within a population of an aromatic shrub, *Lippia organoides* (Verbenaceae), in the Chicamocha Canyon, northeastern Colombia

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## Summary

The geographical scale of genetic structure in a continuous population is highly dependent on its breeding system and dispersion capabilities, and this knowledge is important for the study of population dynamics as well as for conservation purposes. In the present study, spatial autocorrelation statistics and intersimple sequence repeat (ISSR) markers were used to describe the genetic structure of a natural population of a prominent aromatic plant, *Lippia organoides*, native to the Chicamocha Canyon in northeastern Colombia. For this purpose, individuals were sampled from two localities within the Chicamocha Canyon, where the species is abundant and continuously distributed. Cluster (principal coordinate analysis (PCO) and unweighted pair group method using arithmetic averages (UPGMA)), analysis of molecular variance (AMOVA) and Bayesian analyses revealed a low level of genetic differentiation among the two localities, suggesting that they belong to a single population. Genetic diversity levels in this population, described as the percentage of polymorphic loci ( $P=86.21\%$ ) and quantified using Shannon's diversity index ( $I=0.453$ ) and the average panmictic heterozygosity ( $H_B=0.484$ ), were shown to be comparable to or higher than that in other plant species with allogamous breeding systems and to other related Verbenaceae species. Fine-scale autocorrelation analyses showed a pattern consistent with the classical model of isolation by distance with moderate but significant levels of local spatial structure. Our results suggest that sampling individuals at distances greater than  $\sim 1.2$  km may result in the collection of different genotypes, which could help preserve the levels of genetic diversity in a propagation programme. The causes of this spatial pattern are currently unknown and could be influenced by many contemporary factors such as restricted seed dispersal and/or short-distance pollen movement, among others.

## 1. Introduction

In natural populations, the spatial genetic structure, i.e. the non-random spatial distribution of genotypes, can result from a number of evolutionary and ecological processes, including the mating system (Kalisz *et al.*, 2001) and the capacity of the species for gene dispersal (i.e. seed and pollen dispersal mechanisms) (Epperson, 2000; Kalisz *et al.*, 2001; Hardy *et al.*, 2004). The patchy spatial distribution of genotypes

can occur at different scales, e.g. at the level of populations, subpopulations, or even among neighbouring individuals (Escudero *et al.*, 2003). At a fine spatial scale, the most prevalent cause for spatial structure is probably limited gene dispersal, which results in the formation of local pedigree structure (Epperson, 2000; Vekemans & Hardy, 2004). In sexually reproducing plants, the dispersal function is mediated by the male gametophyte (pollen) and the young sporophyte (seed), and other propagules may also be involved in vegetatively reproducing plants (Ward, 2006). Frequently, pollen and seeds show moderate to strong spatial restriction in their dispersal (Vekemans

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& Hardy, 2004), therefore generating processes of fine-scale population genetic structure (Gehring & Delph, 1999; Hardy *et al.*, 2004; Tero *et al.*, 2005). These processes are mainly a result of isolation by distance, namely, the significantly positive correlation between genetic relatedness among individuals and their spatial proximity (Wright, 1946; Hardy *et al.*, 2005).

Currently, spatial autocorrelation analyses are used to detect and interpret spatial patterns of genetic variation within plant populations (He *et al.*, 2000; Zhang *et al.*, 2007). Spatial autocorrelation methods make no assumptions about the scale of the structure, which is especially convenient when there is no previous knowledge of the spatial distribution of genotypes in the species under study, and also because processes that generate spatial structure may operate at different scales and there is thus no single 'correct' scale at which to describe populations (Levin, 1992).

The species under study, *Lippia origanoides*, Kunth, Verbenaceae, is a shrub that can reach up to 3.5 m in height, and whose leaves, flowers and stems present a characteristic Artemisia or marjoram odour (Lopez, 1977). This is a promising species with essential oils of high quality that are used mostly in cosmetics and perfumery, and that have significant antioxidant activity and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Candida tropicalis* (Santos *et al.*, 2004; Oliveira *et al.*, 2007; Stashenko *et al.*, 2008). The leaves of *L. origanoides* are used as a substitute for common marjoram (Maisch, 1885; Oliveira *et al.*, 2007).

According to herbarium records (Missouri Botanical Garden Herbarium, St. Louis, MO, USA), *L. origanoides* occurs mainly in the Neotropical region, specifically in northern South America (Colombia, Venezuela and Guyana), with one record occurring in central Chile. It is generally found in dry environments, where the soils are shallow, stony and poor in organic matter (Albesiano *et al.*, 2003). In Colombia, northern South America, herbarium records of *L. origanoides* suggest that the species occurs in the Andean region of Santander in northeastern Colombia (specifically in the Chicamocha Canyon) (Albesiano *et al.*, 2003), the Atlantic Coast in northern Colombia (Herbarium Federico Medem Bogotá, Instituto Alexander von Humboldt (FMB)) and in the inter-Andean valleys in southern Colombia (Universidad del Cauca (CAUP) Herbario, Popayán, Cauca, Colombia). According to Albesiano *et al.* (2003), in Santander, *L. origanoides* is associated with the vegetation type known as thorny scrub, which is distributed in the semi-arid area of the central basin of the Chicamocha River in the localities of Pescadero and Cepitá, especially in small hillsides with smooth or steep slopes, soils with poor content of organic matter where lixiviation processes are frequent and

with relatively high erosion rate. In these localities, the excessive shepherding of goats has played an important role in the vegetation distribution and has favoured the proliferation of *L. origanoides* and other species that are not directly grazed by these herbivores due to toxic tannins and defensive structures like spines and thorns (Albesiano *et al.*, 2003). Although there is no evidence that *L. origanoides* is an endangered species in Colombia (Betancur *et al.*, 2006), it has been suggested that human disturbance, including deforestation, wildfires and transitory cultivation, has played an important role in reducing the distribution of this species in the Chicamocha Canyon (Malagon *et al.*, 1995; Albesiano *et al.*, 2003; Serrato, 2007). Also, in the Atlantic Coast in northern Colombia, recent urbanization activities in areas around the city of Santa Marta in the department of Magdalena have reduced and fragmented the distribution extent of *L. origanoides* (A. Suárez, unpublished observations).

Despite being a promising aromatic plant, very little is known about basic biological aspects of *L. origanoides*, e.g. its breeding system and dispersal capabilities, which have a direct influence on genetic diversity levels and the spatial distribution of genotypes inside natural populations (Wright, 1946). It is expected that the odoriferous compounds present in these plants attract pollinators, thus favouring high levels of gene flow within populations (Levin *et al.*, 2001; Albre *et al.*, 2003).

In this sense, highly polymorphic genetic markers are a useful tool to infer genetic structure and dispersal patterns (Hardy *et al.*, 2005). Intersimple sequence repeats (ISSRs) are arbitrary multilocus markers produced by PCR amplification using anchored-microsatellite primers (Zietkiewicz *et al.*, 1994). This method has the advantage of generating relatively stable PCR products that show high levels of polymorphisms without requiring prior genomic information (Bornet & Branchard, 2004). As a result, ISSRs have been widely applied in studies of genetic diversity and population structure (Camacho & Liston, 2001; Culley & Wolfe, 2001; Sica *et al.*, 2005). Additionally, this technique constitutes an alternative for taxa for which little or no research has been done at the molecular level (Liu & Wendel, 2001). Nevertheless, ISSRs are dominant markers and their principal limitation for population genetic studies is the biased estimation of allele frequencies, which is usually done by assuming Hardy–Weinberg equilibrium (HWE) inside populations (Zhivotovsky, 1999; Culley & Wolfe, 2001). The estimation bias of this method is particularly serious in presumably outcrossing species where the frequency of the null allele may not be confidently estimated from the proportion of individuals that lack the marker since a relatively high proportion of the null allele will be present in

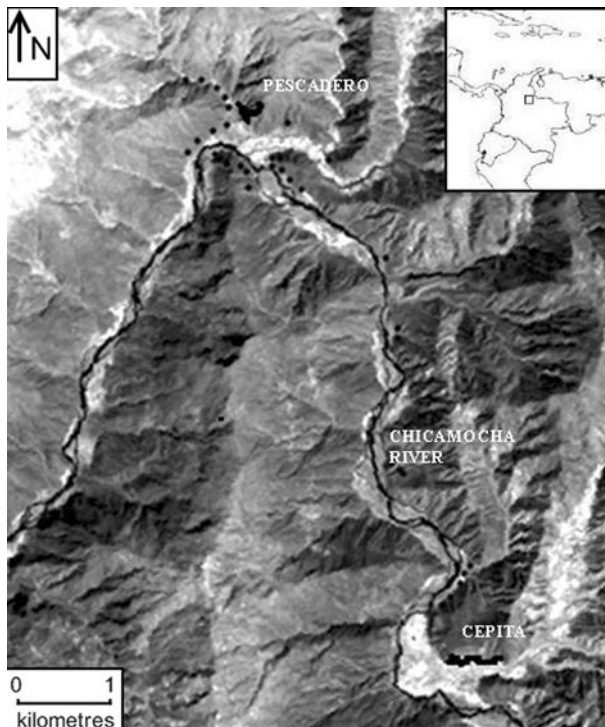


Fig. 1. Map of the distribution of the *Lippia origanoides* individuals sampled from the Chicamocha Canyon. The sampling strategy reflected the current distribution and abundance of the species within each locality (Pescadero and Cepitá). Samples from 62 and 39 individuals were collected in Pescadero and Cepitá, respectively, at elevations ranging from 550 to 830 m.a.s.l. Samples were also taken from ten individuals along a transect connecting these two localities on the east side of the Chicamocha River, where *L. origanoides* is relatively less abundant (see the text for details). The dark dots indicate collection points.

the non-identifiable heterozygotes (Lynch & Milligan, 1994). Thus, to counteract this limitation, different types of alternative methods have been proposed, such as a Bayesian approach, which takes full advantage of data provided by dominant markers without assuming HWE (Zhivotovsky, 1999; Holsinger *et al.*, 2002).

In this context, the aim of the present study was to measure genetic diversity levels of *L. origanoides* from the central basin of the Chicamocha River, in north-eastern Colombia, and to document fine population structure at different spatial scales using spatial autocorrelation analyses.

## 2. Materials and methods

### (i) Sample collection

Samples from 111 individuals of *L. origanoides* were collected in the central basin of the Chicamocha River in the localities of Pescadero (6°49'N, 73°00'W) and Cepitá (6°45'N, 72°58'W) (Fig. 1). Sampling was

performed in these two localities because they provide relatively homogeneous areas dominated by the species thus minimizing the possible effects of selection on genetic structure due to microhabitat heterogeneity. The sampling strategy reflected the current distribution and abundance of the species within each locality. In Pescadero, *L. origanoides* is abundant and continuously distributed on both sides of the Chicamocha River, whereas in Cepitá, *L. origanoides* is found on the east side of the Chicamocha River, and at this locality, sampling was performed along two small transects separated by farms and cultivated land. Samples from 62 and 39 individuals were collected in Pescadero and Cepitá, respectively, at elevations ranging from 550 to 830 m.a.s.l. For the purpose of examining larger distance classes, samples were also taken from ten individuals along a transect connecting these two localities on the east side of the Chicamocha River where *L. origanoides* is relatively less abundant (Fig. 1). For each sample collected, latitudinal, longitudinal and elevation data were recorded using a GPS (geographical positional system) from Garmin (USA).

Samples from each individual, consisting of 20–30 young leaves, were collected and preserved in zip-lock plastic bags containing silica gel and stored at  $-20^{\circ}\text{C}$  until DNA extraction. Herbarium samples were sent to the Colombian National Herbarium (COL) for taxonomic determination under the accession number 522 847.

### (ii) DNA extraction and PCR amplification

Genomic DNA was isolated from young leaves. Leaf samples were finely ground in liquid nitrogen ( $-80^{\circ}\text{C}$ ) and DNA extraction was made using a modification of the CTAB method reported by Pirttilä *et al.* (2001). The resulting DNA solution was stored at  $-20^{\circ}\text{C}$  in TE buffer for later use.

A set of 50 ISSR primers (obtained from the University of British Columbia, Vancouver, ON, Canada) was screened for amplification success and polymorphisms in a representative sample of *L. origanoides*. PCR reactions were carried out in a total volume of  $15\ \mu\text{l}$  containing 1 unit of *Taq* polymerase (Promega),  $1\times$  PCR buffer (Promega),  $0.2\ \text{mM}$  dNTPs,  $0.5\ \mu\text{M}$  of the ISSR primer and 10–20 ng of extracted DNA. Amplifications were performed on a PTC-100TM thermocycler (MJ Research) following Wolfe (2005) cycling conditions. PCR products were loaded onto 2% agarose gels in  $0.5\times$  TBE buffer, run at constant voltage (50 V) for 3 h and detected by staining with ethidium bromide. Each ISSR band was considered an independent character or locus, and polymorphic bands were scored visually as either absent (0) or present (1). Qualitative differences in band intensity were not considered (Assefa *et al.*, 2003).

Two independent scorings were made on each gel, and only those bands consistently scored were considered for analysis. Also, replicate samples were analysed for each locus and only reproducible bands were considered for analysis.

### (iii) Data analysis

Genetic diversity was described as the percentage of polymorphic loci ( $P$ ), and quantified as Shannon's diversity index ( $I$ ) (Shannon & Weaver, 1949) using POPGENE v.1.31 (Yeh *et al.*, 1995), which assumes that populations are in HWE. Also, a Bayesian approach, which does not assume HWE (Zhitovskiy, 1999; Holsinger *et al.*, 2002), was used to estimate the average panmictic heterozygosity ( $H_B$ ) using the HICKORY software (Holsinger & Lewis, 2003). This software uses Markov chain Monte Carlo simulations to produce posterior distributions based on the data (Holsinger *et al.*, 2002).

The genetic structure of *L. origanoides* from the two localities of the central basin of the Chicamocha River was first explored by means of a principal coordinate analysis (PCO) using a squared Euclidean distance matrix from which principal coordinates were extracted. Also, a matrix of pairwise similarities among individuals was obtained using the Nei & Li (1979) coefficient and this matrix was employed to build a dendrogram by using the unweighted pair group method using arithmetic averages (UPGMA) algorithm. The PCO and UPGMA analyses were carried out using MVSP 3.1 (Kovach, 1998). Population structure was further examined by means of analysis of molecular variance (AMOVA) with the program GENALEX version 6.1 (Peakall & Smouse, 2006), and by applying the Bayesian hierarchical model implemented in HICKORY (Holsinger & Lewis, 2003).

The correlation between a genetic Euclidean distance matrix and a geographical distance matrix was assessed by means of Mantel tests using GENALEX version 6.1 (Peakall & Smouse, 2006). Since Mantel tests are not so powerful to detect fine-scale structure because they are only sensitive to linear relationships of spatial autocorrelation (Heywood, 1991), additional spatial autocorrelation analyses were done under the Moran's  $I$  and the normalized Mantel  $r_m$  approaches. The purpose of the Moran's  $I$  coefficient is to quantify the correlation of the values of a single variable (in this case the first extracted axis of a PCO) between pairs of individuals located at a given physical distance (Fortin *et al.*, 2002). On the other hand, the normalized Mantel  $r_m$ , which is based on a Mantel statistic, estimates the linear relationship between two sets of variables taken at the same sampling locations (Escudero *et al.*, 2003). Normalized Mantel  $r_m$  coefficients were calculated using the pairwise Euclidean

genetic distances extracted from GENALEX version 6.1 (Peakall & Smouse, 2006).

It has been suggested that the sampling design can affect the estimation of the spatial genetic structure (Vekemans & Hardy, 2004). Vekemans & Hardy (2004) proposed that sampling nearby individuals at different locations, and then organizing them into subgroups could be an alternative for characterizing fine genetic structure when there is not a homogeneous sampling. For this reason, the autocorrelation analyses were performed not only for the entire region (all 111 individuals) but also separately for each of the two localities (Pescadero and Cepitá). Every possible pair of individuals was considered as a join and assigned to 1 of 10 distance classes (according to the Euclidean distance separating the pair) in Pescadero and Cepitá, and 20 distance classes in the entire region. Distance classes were based on either equally sized or arbitrarily chosen intervals, each one containing at least 30 pairs of data. For the entire region and Pescadero, several intervals were tested ranging from 20 to 200 m, at regular increments of 10 m, and maximum distance classes of 4.8 and 1.4 km, respectively. For Cepitá, tested intervals ranged from 40 to 80 m, at regular increments of 10 m, with a maximum distance class of 590 m. These distance classes accounted for the smallest and largest spatial resolution possible for our dataset. The analyses were done following the classical rule of only considering pairs of points separated by less than half the maximum distance observed among all pairs (Wagner *et al.*, 2005). Moran's  $I$  and normalized Mantel  $r_m$  coefficients were calculated using the PASSAGE software (Rosenberg, 2001). For these two approaches, the significance of individual coefficients was determined from their moments (Sokal & Oden, 1978), and significance for the entire correlograms was calculated using a Bonferroni criterion (Oden, 1984).

## 3. Results

### (i) Genetic diversity

A total of 58 scorable bands, ranging from 300 bp to more than 1000 bp, were generated using five ISSR primers containing CT or TC repeat units in 111 individuals. The five primers used are the following: no. 813 (CTC TCT CTC TCT CTC TT), no. 814 (CTC TCT CTC TCT CTC TA), no. 824 (TCT CTC TCT CTC TCT CG), no. 843 (CTC TCT CTC TCT CTC TRA) and no. 845 (CTC TCT CTC TCT CTC TRG). Of these bands, 50 were polymorphic ( $P = 86.21\%$ ). Genetic diversity indexes, calculated either assuming HWE ( $I$ ) or with the Bayesian approach ( $H_B$ ), did not differ significantly ( $0.453 \pm 0.009$  and  $0.484 \pm 0.227$ , respectively). Additionally, there was

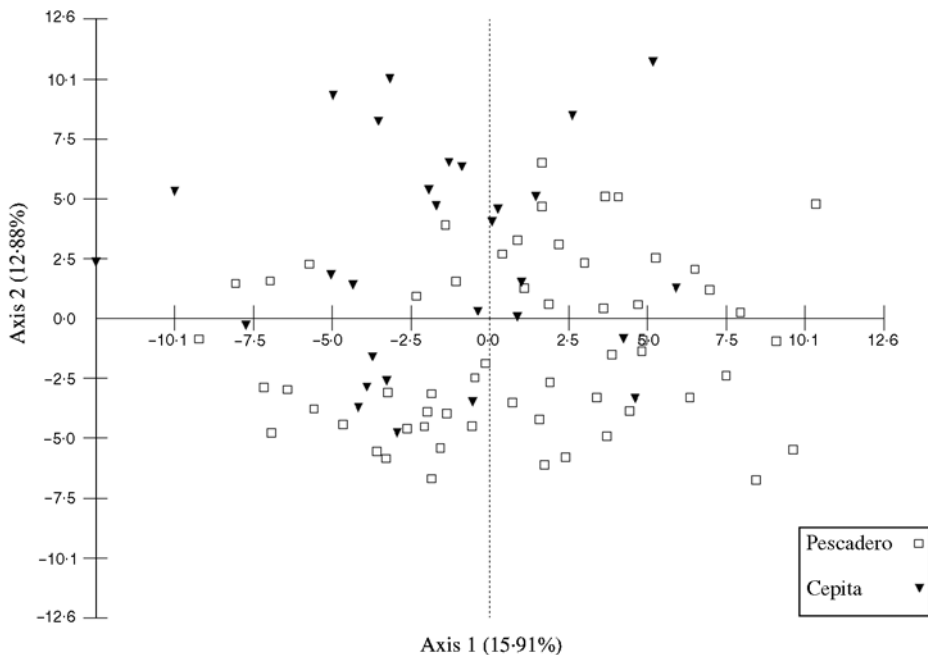


Fig. 2. The first two coordinates from the PCO, using squared Euclidean genetic distance, explained 28.8% of the total variability. The high overlap of individuals shows a pattern of very low differentiation between the two localities.

not a significant difference between the genetic diversity values between the localities of Pescadero and Cepitá ( $I=0.435 \pm 0.006$  and  $H_B=0.495 \pm 0.229$  for Pescadero;  $I=0.441 \pm 0.005$  and  $H_B=0.493 \pm 0.231$  for Cepitá).

### (ii) Population structure

Results of the PCO analysis are shown in Fig. 2. The high overlap of individuals shows a pattern of very low differentiation between the two localities. In addition, the dendrogram constructed with UPGMA using the similarity matrix based on the Nei and Li coefficient did not suggest the presence of discrete geographical groups among individuals (data not shown). The average genetic similarity as measured by the Nei and Li coefficient (Nei & Li, 1979) was 0.84 and ranged from 0.987 to 0.672. Pairs of genetically distant individuals (Nei and Li coefficient close to 0.672) were found within the two localities (inside Pescadero and inside Cepitá) and also between the two localities. The AMOVA results indicate that most of the molecular variation in *L. origanoides* from the Chicamocha Canyon is found within localities (97%), with much less amount of variation among localities (3%) ( $\Phi_{PT}=0.034$ ,  $P<0.001$ ). Similarly, the Bayesian analysis shows a low level of genetic differentiation among localities ( $\theta_{II}=0.032 \pm 0.021$ ). All these results suggest the existence of a single population of *L. origanoides* in the central basin of the Chicamocha River.

### (iii) Spatial genetic structure

The presence of spatial genetic structure in the population of *L. origanoides* from the Chicamocha Canyon was evaluated by means of a Mantel test and fine-scale autocorrelation analyses. The Mantel test was carried out for the entire region (all 111 individuals) and showed a weak but significant correlation among genetic and geographical distance ( $r=0.074$ ,  $P<0.05$ ), suggesting that genetic variation within this population may be spatially structured. To further explore this, three separate fine-scale autocorrelation analyses (for the entire region, for Pescadero and for Cepitá) were conducted using both Moran's  $I$  and normalized Mantel  $r_m$ . For the entire region and for Pescadero, autocorrelograms were conducted at distance class intervals ranging from 20 to 200 m. In both cases, significant autocorrelations were observed in all distance class intervals analysed and for intervals smaller than 150 m only positive spatial autocorrelations were observed (data not shown). Negative correlations were only observed when larger intervals were considered (i.e. 150–200 m). The correlograms for 150 m intervals are shown in Figs 3 and 4. For the entire region, the Moran's  $I$  autocorrelogram (Fig. 3a) shows significant positive values at the first interval (0–150 m) and at the intervals 750–900 m, 900–1050 m and 1050–1200 m, indicating high genetic similarity shared by individuals within these distances. Figure 3a also shows significant negative values at the intervals 1200–1350 m and 3500–4000 m, indicating that individuals

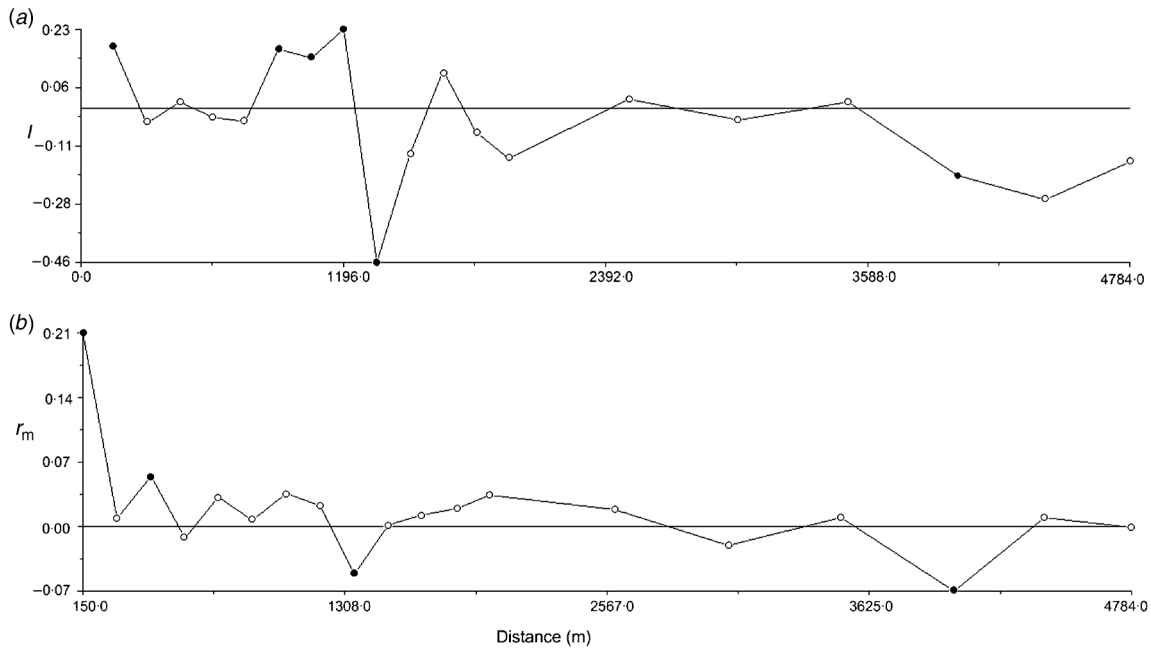


Fig. 3. Spatial genetic structure in the entire region. Distance classes were defined at 150 m intervals. Black dots represent significant ( $P < 0.05$ ) Moran's  $I$  and normalized Mantel  $r_m$  values. (a) Moran's  $I$  correlogram shows significant positive values at the intervals 0–150 m, 750–900 m, 900–1050 m and 1050–1200 m. At the intervals 1200–1350 m and 3500–4000 m, values were significantly negative. (b) Mantel correlogram shows significant positive values at the intervals 0–150 m and 300–450 m, and significant negative values at the intervals 1200–1350 m and 3500–4000 m.

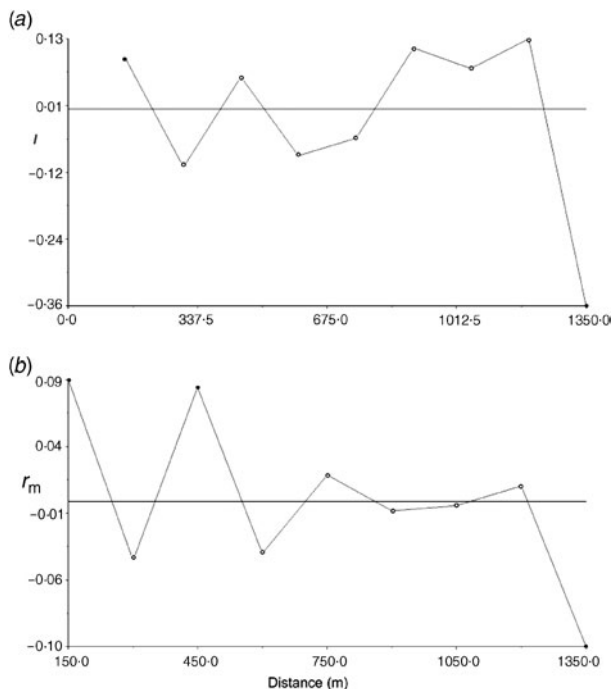


Fig. 4. Spatial genetic structure in Pescadero. Distance classes were defined at 150 m intervals. Black dots represent significant ( $P < 0.05$ ) Moran's  $I$  and normalized Mantel  $r_m$  values. (a) Moran's  $I$  correlogram shows significant positive autocorrelation at the first interval (0–150 m) and significant negative autocorrelation at the last interval of 1200–1350 m. (b) Mantel correlogram shows similar results with an additional significant positive value at the interval 300–450 m.

separated for more than  $\sim 1.2$  km are likely to be genetically more differentiated than expected from a spatial random distribution of genotypes. Similar results were obtained for the correlogram based on the normalized Mantel  $r_m$  coefficient (Fig. 3b) with significant positive values for the intervals 0–150 m and 300–450 m, and significant negative values for the intervals 1200–1350 m and 3500–4000 m. For Pescadero, the Moran's  $I$  autocorrelogram showed significant positive autocorrelation at the first interval (0–150 m) and significant negative autocorrelation at the last interval of 1200–1350 m (Fig. 4a). This result was similar for the normalized Mantel  $r_m$  autocorrelogram with an additional significant positive value at the interval 300–450 m (Fig. 4b). In Cepitá, autocorrelograms for distance class intervals ranging from 40 to 80 m were obtained and a similar correlation pattern to that observed for the entire region and for Pescadero was found, but at a smaller scale (Fig. 5). The Moran's  $I$  autocorrelogram exhibited significant positive correlations at the first two distance intervals ranging from 0 to 120 m, whereas at the distance interval of 420–490 m, significant negative correlations were observed (Fig. 5a). Similar results were obtained for the normalized Mantel  $r_m$  autocorrelogram with an additional significant negative value for the last interval of 490–590 m (Fig. 5b).

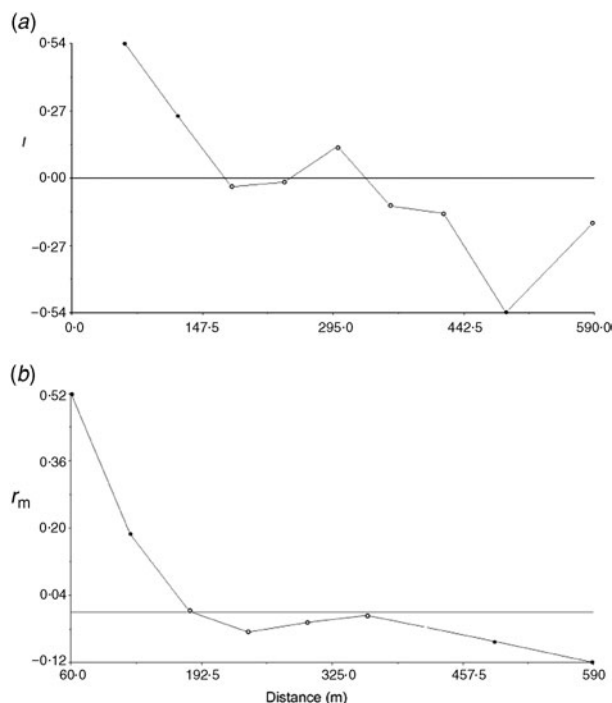


Fig. 5. Spatial genetic structure in Cepitá. Distance classes were defined at 60 m intervals. Black dots represent significant ( $P < 0.05$ ) Moran's  $I$  and normalized Mantel  $r_m$  values. (a) Moran's  $I$  correlogram shows significant positive correlations at intervals ranging from 0 to 120 m and significant negative correlations at the interval 420–490 m. (b) Mantel correlogram showed similar results with an additional significant negative value for the last interval of 490–590 m.

#### 4. Discussion

##### (i) Genetic diversity

*L. origanoides* from the Chicamocha Canyon exhibited relatively high levels of genetic variation indicated by a high  $P$  (86.21%) and relatively high  $I$  and  $H_B$  (0.453 and 0.484, respectively). This level of genetic variation is higher than those reported previously for widely distributed species (Loveless & Hamrick, 1984; Gitzendanner & Soltis, 2000; Nybom, 2004). However, for a more meaningful comparison of genetic diversity, data from close relatives (e.g. congeners) should be analysed (Gitzendanner & Soltis, 2000). As far as we know, there have been no reports of genetic diversity in *Lippia* species, except for an unpublished study of *Lippia alba* and *Aloysia triphylla* (a species related to the genus *Lippia*) using ISSR markers (F. Martínez, G. Castillo & M. I. Chacón, 2008, personal communication). In that study, *L. alba* showed relatively high diversity values as measured by  $P$  (85.23%),  $I$  (0.418) and  $H_B$  (0.436), and *A. triphylla* also showed a similar pattern for the same indexes:  $P$  (95.45%),  $I$  (0.436) and  $H_B$  (0.461). Genetic diversity levels of these two widely distributed Verbenaceae species are similar or slightly higher than those

obtained for *L. origanoides*. Other reports from widely distributed Verbenaceae species, which are not so closely related to *L. origanoides*, show comparatively similar or lower levels of genetic diversity. For example, reports from *Vitex negundo* L. in China have shown  $P$  ranging from 81 to 85% in some populations (Su *et al.*, 2003; Zhang *et al.*, 2007). *Vitex rotundifolia* L., an aromatic and medicinal plant from China, has shown moderate  $P$  (22.6%) and  $I$  (0.275) (Hu *et al.*, 2008).

In contrast with these results, a parallel analysis of DNA polymorphisms in four non-coding chloroplast DNA regions showed no genetic diversity among a set of *L. origanoides* individuals from the Chicamocha Canyon (Suárez *et al.*, 2007). ISSR polymorphisms used in the present study are presumably mostly from nuclear DNA. In this way, these apparently contrasting results may be explained by a higher mutation rate of microsatellite regions from the nuclear genome compared with the chloroplast genome (Morgante & Olivieri, 1993), and by the different modes of transmission of the complete chloroplast DNA (cpDNA) (usually maternally) and ISSRs (usually biparentally) (Birky, 1995). The levels of genetic variation within populations may not only be influenced by the mating system of the species but also by its effective population size (Wright, 1946; Culley & Wolfe, 2001; Muir *et al.*, 2004; Mable & Adam, 2007), which is expected to be smaller for uniparentally than for biparentally inherited markers. However, it is important to note that the sampling scheme analysed in Suárez *et al.* (2007) was rather small; thus more samples are needed to assess intraspecific variation in the chloroplast genome.

There are several reports where outcrossing may yield high levels of genetic variation in a population (Culley & Wolfe, 2001; Muir *et al.*, 2004; Mable & Adam, 2007). Given the relatively high levels of genetic diversity found in *L. origanoides* and the lack of a significant difference between the genetic diversity indexes calculated either assuming HWE or with the Bayesian approach, an important influence of outcrossing on the reproduction of this species could be inferred. However, this is a subject that deserves further investigation.

##### (ii) Population genetic structure

In this study, different approaches were used to estimate the level of genetic differentiation between individuals within and among localities in the central basin of the Chicamocha Canyon (Pescadero and Cepitá). Results from the PCO, UPGMA, AMOVA and the Bayesian approach (based on partitioned heterozygosity) showed a very low genetic differentiation among localities, which may suggest that in this region there is a single population where neither

the river nor human disturbance is acting as a barrier to gene flow.

However, the spatial autocorrelation analyses using the Moran's  $I$  and normalized Mantel  $r_m$  coefficients indicate that the distribution of genetic diversity within this population is not random among and within localities. The shape of the correlograms revealed significant fine-scale genetic structure within the Chicamocha Canyon. The Moran's  $I$  correlogram corresponding to the entire region (Fig. 3) showed what seems to be a sinusoidal variation in the first nine distance classes. This pattern may indicate a patchy distribution of the variation (Radeloff *et al.*, 2000). However, at greater distances, only significant negative values or no significant values were displayed.

In general, there was a strong positive spatial association between individuals distributed within 0–1200 m from each other (0–120 m for Cepitá), whereas individuals distributed at a distance greater than ~1200 m from each other (490 m for Cepitá) showed significant negative autocorrelation (Figs 3–5). These spatial patterns of genetic relatedness are consistent with the classical model of isolation by distance, where there is a decline of genetic similarity with increasing geographical distance (Wright, 1946). Among the different evolutionary and ecological processes that may lead to the establishment of this spatial genetic structuring, the most widely studied is limited gene dispersal (Epperson, 2000; Vekemans & Hardy, 2004). A variety of empirical data indicate that in several cases seeds show a localized dispersal rate resulting in patches of plants, whereas pollen may show a long-distance dispersal occurring at a higher frequency, which is enough to counteract the effects of genetic drift in small populations (Wright, 1946; Ouborg *et al.*, 1999; Kalisz *et al.*, 2001). ISSR polymorphisms are mostly located in the nuclear genome, which in most angiosperms shows a biparental inheritance, thus reflecting the combined effect of the relative movement of seeds and pollen. The moderate but significant levels of local spatial structure in *L. origanoides*, revealed by the spatial autocorrelation analyses, could be due in part to relatively restricted seed dispersal capabilities as it has been reported for different plant populations (e.g. Kalisz *et al.*, 2001; Hardy *et al.*, 2004; Hardesty *et al.*, 2005). Also, it could be the result of short-distance pollen movement, as it has been reported for some shrubs and herbs (Marr *et al.*, 2000; Barthelmess *et al.*, 2006; Byrne *et al.*, 2007). Additional studies, including those that make use of different types of genetic markers (e.g. markers from cpDNA), will permit to evaluate the relative movement of seeds and pollen (McCauley, 1994; Trapnell & Hamrick, 2004; Petit *et al.*, 2005) and their influence on fine-scale population structure in this species.

The results from the present study are comparable with those obtained for tree species where distance classes of hundreds of metres were analysed (Doligez & Joly, 1997; Degen *et al.*, 2001) and where fine genetic structure at distance classes of hundreds of metres was found (Latouche-Hallé *et al.*, 2003; Hardesty *et al.*, 2005). Numerous analyses in shrubs have found spatial genetic structure at finer scales, from 1 to 10 m (Loiselle *et al.*, 1995; England *et al.*, 2003; Tang *et al.*, 2007). Future analyses may include a sampling strategy where shorter distances are considered.

Autocorrelation analyses are greatly affected by the sampling strategies used (Vekemans & Hardy, 2004). However, in the three autocorrelation analyses performed (Figs 3–5), a relatively large number of pairs of data were assigned to each distance class analysed; therefore, it is unlikely that the significant genetic structures detected in our analyses within proximate distances are artefacts of the sampling scheme.

### (iii) *Implications for conservation and breeding*

The wide distribution and the relatively high genetic diversity estimated in the present study lead us to conclude that *L. origanoides* is not yet an endangered species. However, appropriate conservation and breeding strategies should be implemented if a massive oil production is planned. Previous studies on promising species from Africa and China have reported how species with wide distributions have become vulnerable because of the overharvesting of natural stands (Muchugi *et al.*, 2006; Tang *et al.*, 2007). Currently, human disturbance is taking place in regions where *L. origanoides* exhibits a natural wide distribution (Malagon *et al.*, 1995; Albesiano *et al.*, 2003; Serrato, 2007). Habitat fragmentation caused by deforestation and transitory cultivation could lead to significant concerns regarding the conservation of this species. The estimation of the average size of genetic patches can be helpful in designing collection strategies for proper propagation and breeding purposes. Usually, the first  $x$ -intercept in the correlogram has been used as an estimate of the diameter of the patch (Peakall *et al.*, 2003). However, this interpretation might be problematic since this distance is not a characteristic of the populations studied and depends strongly on the sampling scheme (Vekemans & Hardy, 2004). For these reasons, the present study does not allow us to predict the patch size appropriate for an accurate collection, but it does allow us to suggest that sampling individuals at distances greater than ~1.2 km along the transects where the samplings were conducted could result in the collection of different genotypes, which could help preserve the levels of genetic diversity in a propagation programme. Since different chemotypes have been



identified within *L. origanoides* in the Chicamocha Canyon (Stashenko *et al.*, 2008), additional research is needed to establish the relationship between essential oil composition and genetic variation, which will undoubtedly contribute to future breeding initiatives.

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