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Population Structure and Genetic Diversity of the Medicinal and Aromatic Plant Cunila spicata Benth. (Lamiaceae) Based on Inter-Simple Sequence Repeat (ISSR) Markers

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ABSTRACT

Cunila spicata is an endangered aromatic and medicinal plant of South Brazil. In the present paper, the ISSR technique was employed to study the intra- and inter-population genetic diversity of this species. Nine primers generated a total of 109 amplification products, most of which were polymorphics. Low genetic diversity at population level (H_E= 0.053) and species level (H_T=0.196), and high differentiation among populations (G_{ST}= 0.727) were detected in *C. spicata*. The genetic diversity, low estimated genetic flow and absence of correlation between genetic distances, geographic distances and chemical composition, indicates that genetic drift and inbreeding may be the main factors involved in the genetic structure of *C. spicata* populations. Based on these findings, strategies are proposed for the genetic conservation and management of this species.

Indexing terms/Keywords

Cunila spicata, ISSR, genetic diversity, conservation

Academic Discipline And Sub-Disciplines

Biotechnology

SUBJECT CLASSIFICATION

Biology

TYPE (METHOD/APPROACH)

Experiment

INTRODUCTION

Cunila spicata Benth. is one of the 12 South American species of the genus *Cunila* (Lamiaceae). The South American species of this genus are botanically separate in three sections (Coelho de Souza 1997), of which two have been recently confirmed by molecular analysis (Agostini et al. 2008). *Cunila spicata* belongs to the *Spicatae* section which is formed by shrubs with terminal or sub-terminal spikes.

Popularly known as “poejo” or “poejo-do-banhado”, *C. spicata* is an herbaceous perennial and aromatic plant with small leaves covered by multiple glandular trichomes. Their small white flowers with characteristic purple spots are distributed in terminal 5-9 cm long spikes. This species grows on marginal areas of marshes and swamps in Argentina, Paraguay, Uruguay and South Brazil (Coelho de Souza 1997). In the last decades, due to intense anthropic interference (agriculture and cattle associated with global warming and drier summers), *C. spicata* populations are becoming rarer and smaller.

Infusions and ethanolic extracts of aerial parts of *C. spicata* are used in popular medicine for the treatment of respiratory disorders due to their pectoral, anticatarrhal, antitussive, anti-inflammatory, decongestant, depurative and expectorant properties (Pio Correa 1974, Toursarkissian 1980). Ethanolic, chloroformic and ethyl acetate extracts of *C. spicata* have *in vitro* inhibitory activity against Herpes Simplex Hominis Virus (HSV), Polyovirus (PV) and Vesicular Stomatite Bovine Virus (Simões et al. 1994). Moreover, Apel *et al.* (2009) showed that the essential oil of *C. spicata* is efficient to control cattle tick (*Riphicephalus microplus*).

The analysis of *C. spicata* extracts allowed identifying β-betulenal, isorosiridol diacetate, β-sitosterol, phytol, cadinol, linalool, dihydrocarveol, geraniol and neryl acetate, as well as several glycosidic terpenoids (Manns and Hartmann 1992, Manns 1995). Studies on the essential oil composition of different populations of *C. spicata* collected in south Brazil allowed us to identify four chemotypes of this species with high concentrations linalool/1,8-cineole, 1,8-cineole, carvone/carveol, and 1,8-cineole/limonene, respectively (Echeverrigaray et al. 2009). These chemotypes differed from an Argentinian accession characterized by three main constituents: limonene, geranyl acetate and linalool (Van Barem 2001).

In the last decades, molecular markers arose as powerful tools in areas of genetic diversity, conservation, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species. Among molecular markers, inter simple sequence repeats (ISSR) markers (Zietkiewicz 1994) have been successfully used to identify and

determine relationships at the species, population and cultivar levels in many plant species (Reddy et al. 2002, Xiao et al. 2006, Wang et al. 2012), including *Cunila* (Agostini et al. 2008, Fracaro et al. 2005). This method is widely applicable to aromatic and medicinal plant species because it is rapid, reliable, inexpensive, requires little amounts of template DNA and, unlike other markers, do not require prior knowledge of DNA sequences (Trindade 2010).

Considering the potential of *C. spicata* as an aromatic and medicinal plant, and the limited knowledge regarding the genetic diversity of this endangered species, the present work aimed to evaluate the genetic structure and diversity of *C. spicata* populations, and its relation with the chemical composition and geographic distribution. These results will have important implications for the effectiveness and efficiency of conservation programs.

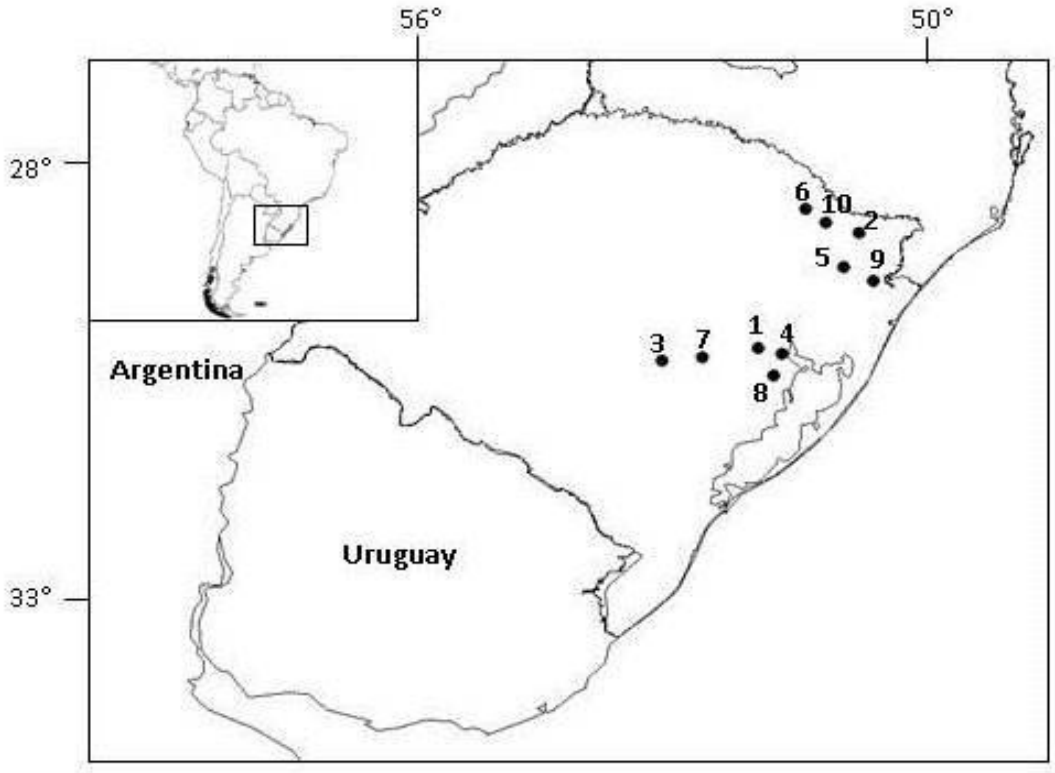

Figure 1. Geographical origin of the 10 populations of *Cunila spicata* analyzed in this study: 1. Arroio dos Ratos, 2. Bom Jesus, 3. Cachoeira do Sul, 4. Guaíba, 5. Lageado Grande, 6. Muitos Capões, 7. Pântano Grande, 8. Sertão Santana, 9. Tainhas, 10. Vacaria.

MATERIALS AND METHODS

Plant material

Cunila spicata flowering plant samples were collected at 10 locations in Rio Grande do Sul State, Brazil, between September and November 2013, classified and deposited in the Herbarium of the University of Caxias do Sul. Each population was represented by 10 plants. Representatives of *C. galioides*, *C. menthoides* and *C. origanoides*, other species of the genus *Cunila*, were also included. *C. spicata* populations were collected in two phytogeographical regions (Figure 1): (1) the grasslands of high altitude (850-1400m) of South Brazil characterized as a steppe associated with the Araucaria forest, represented by the accessions Bom Jesus, Vacaria, Tainhas, Lageado Grande and Muitos Capões, and

(2) the grasslands (up to 360m) of the Southeast range with a characteristic savanna vegetation, represented by the accessions Arroio dos Ratos, Guaiba, Cachoeira do Sul, Pântano Grande and Sertão Santana (Joli et al. 1999).

DNA extraction and PCR amplification

Leaf materials were placed in porcelain mortars chilled with liquid nitrogen and were ground with a pestle to a fine powder. Total DNA were extracted by the CTAB method described by Doyle and Doyle (1987). DNA samples were quantified in spectrophotometer at 260nm. The $A_{260/280}$ readings of DNAs ranged from 1.8 to 1.9. PCR reactions were performed in a total reaction volume of 25µl. The ISSR mix included 50mM KCl, 10mM Tris-HCl (pH 8.3), 3mM $MgCl_2$, 2% formamida , 0.75mM de cada dNTP (dATP, dCTP, dTTP, e dGTP), 0.4mM of primer, 30 ng of genomic DNA, and 1.5 units of Taq DNA polimerase (Invitrogen).

The ISSR amplification conditions were an initial step of 5 min at 94ºC followed by 40 cycles of 1 min at 94ºC for

denaturation, 45 sec at 48 to 52ºC for annealing, and 2 min at 72ºC for extension, and a final extension at 72ºC for 5 min.

Amplification products were resolved by electrophoresis in horizontal 1.5% agarose gels in 1 X TBE buffer (50mM Tris, 50mM Boric Acid, 2.5mM EDTA, pH 8.3) under a constant voltage of 90V. Gels were stained with ethidium bromide (1µg/ml), and digitalized under UV light. The size of the amplicons was determined by comparison with a Lambda DNA digested with *EcoRI* and *HindIII* restriction enzymes.

Data analysis

Amplicons were visually scored considering (1) as the presence of a band, and (0) its absence. Only bands that appeared consistently between two independent runs were rated. The binary (1/0) data matrix was used to calculate Jaccard´s similarity coefficients, and associations were revealed by cluster analysis using the UPGMA algorithm using SPSS software version 10.1 (SPSS Inc., Chicago, Illinois).

POPGENE v. 1.31 (Yeh et al. 1999) was used to estimate several parameters: the percentage of polymorphic loci (P), the total gene diversity (H_E), the Shannon diversity (SI), the coefficient of gene differentiation (G_{ST}), and the gene flow ratio (Nm). The proportion of diversity between populations was calculated as (SI_{SP}-SI_{POP})/SI_{SP}, where SI_{POP} is the mean intra- population diversity, and SI_{SP} is the Shannon diversity of *C. spicata*. AMOVA analyses and Mantel test were performed using GenAlEx (Peakall and Smouse 2001).

RESULTS

Initially 20 primers of ISSR were evaluated against nine plants of *Cunila spicata*, three from Guaíba, Muitos Capões and Cachoeira do Sul populations, to select a set of primers that allowed to amplified polymorphic segments of medium to hard intensity, between 200 and 2000 bp, and with high reproducibility in three independent amplification experiments. Of these primers, four produced no distinct bands, three resulted in faint bands on a smeary background, and four produced a low number of bands (2 or 3 bands) all of them monomorphic. The results obtained allowed to select nine ISSR primers (Table

1) that produced robust and reproducible patterns, used to evaluate intra- and inter-population variability in *C. spicata*.

Pattern obtained with primer $(GA)_8T$ are exemplified in Figure 2.

Table 1. RAPD and ISSR primers used, annealing temperatures, number of amplicons and number of polymorphic bands within *C. spicata*.

Primer	Sequence	Annealing temperature (ºC)	Total number of bands	Number of bands within *C. spicata*	Polymorphic bands within C. spicata
ISSR 1	$(AC)_8T$	48	14	9	6
ISSR 2	$(AG)_8YT$	50	17	12	11
ISSR 3	$(GA)_8T$	50	17	13	11
ISSR 4	$(CA)_8T$	50	10	6	3
ISSR 5	$(CT)_8G$	48	17	13	12
ISSR 6	$(GTGC)_4$	54	27	22	21
ISSR 7	$(CT)_8A$	48	12	7	3
ISSR 8	$(GA)_8YC$	50	23	16	15
ISSR 9	$(CTC)_4RC$	50	15	11	10
			152	109	92

Using the nine selected primers, 152 and 109 ISSR bands were scored in *Cunila* and *C. spicata,* respectively, ranging in size between 450 and 2027 bp. The number of amplification products per primer varied from 6 [$(CA)_8T$] and 22 [$(GTGC)_4$] in *C. spicata*. Including the outgroup species (*C. galioides*, *C. menthoides,* and *C. origanoides*) in the analysis 99.3% of the bands were polymorphic, but this percentage fell to 84.4% within *C. spicata* plants.

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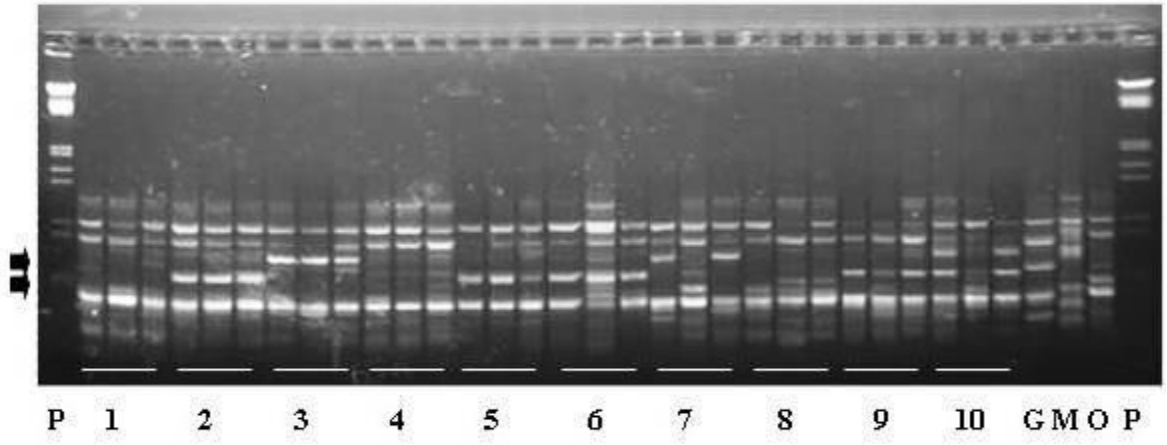

Figure 2. primer $(GA)_8T$ P. Lambda *Eco*RI/*Hind*III, 1. Arroio dos Ratos, 2. Bom Jesus, 3. Cachoeira do Sul, 4. Guaíba, 5. Lageado Grande, 6. Muitos Capões, 7. Pântano Grande, 8. Sertão Santana, 9. Tainhas, 10. Vacaria, G- *C. galioides*, M- *C. menthoides,* O- *C. origanoides*. Black arrows indicate population specific bands.

The percentage of polymorphic loci (P) at the population level was 13.94% on average, ranging from 7.34% (Cachoeira do Sul) to 20.18% (Arroio dos Ratos), but was 85.32% at the *C. spicata* species level (Table 2). Of the 109 bands scored in *C. spicata*, 57% amplicons were found in less than 20%, and 21% in more than 90% of the sampled individuals.

Assuming Hardy-Weinberg equilibrium, Nei´s genetic diversity was estimated to be 0.053, on average, at the *C. spicata* population level and 0.196 at the species level, while Shannon indices were 0.079 and 0.316, respectively (Table 2). Among the 10 populations, the level of variability ranged between H_E= 0.079 and SI= 0.117 for Arroio dos Ratos to H_E= 0.027 and SI= 0.041 for Cachoeira do Sul (Table 2). Ten out of 109 bands were present in >80% of the individuals of a given population, 12 bands were specifics of two or three populations, and one band was present in all the individuals of five populations (Figure 2).

Table 2. Genetic variability within and among populations of *C. spicata*, as revealed by ISSR analysis. P, percentage of polymorphic loci; ne, effective number of alleles; H, Nei´s gene diversity; SI- Shannon´s diversity index.

Population	P	Ne	H_E	SI
Arroio dos Ratos	20.18	1.139	0.079	0.117
Bom Jesus	16.51	1.090	0.056	0.086
Cachoeira do Sul	7.34	1.046	0.027	0.041
Guaiba	12.84	1.089	0.051	0.074
Lageado Grande	14.68	1.101	0.058	0.085
Muitos Capões	11.01	1.057	0.036	0.056
Pantano Grande	11.93	1.085	0.048	0.070
Sertâo Santana	17.43	1.137	0.075	0.107
Tainhas	9.17	1.068	0.038	0.055
Vacaria	18.35	1.112	0.067	0.101
Intra-population mean	13.94		0.053	0.079
Inter-population values	85.32	1.304	0.196	0.316

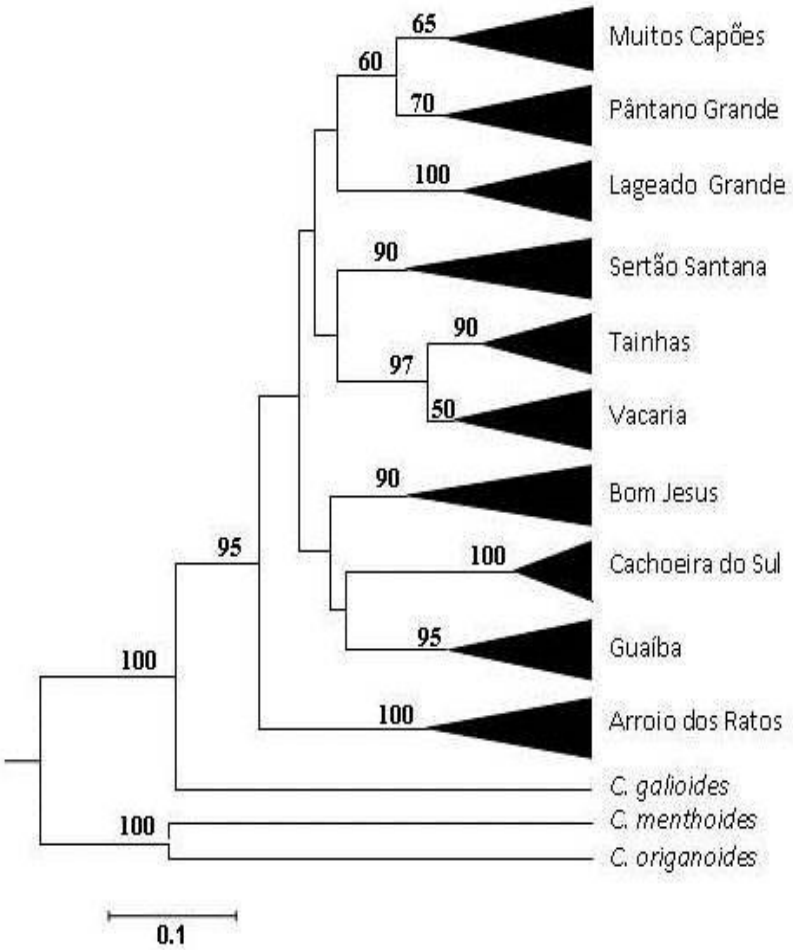

Figure 3. UPGMA dendrogram of ten *C. spicata* populations and three outgroup species of the genus *Cunila* based on ISSR patterns (Jaccard´s similarity coefficients). The numbers indicte bootstrap values.

Cluster analysis of the similarity values (Jaccard´s coefficient) allowed separating all the populations, as well as *C. spicata* from other species of the genus (Figure 3). Genetic structuring of *C. spicata* populations, graphically observed in Figure 3, was confirmed by the high coefficient of genetic differentiation (G_{ST}) and predicted proportion of diversity between populations, 0.727 and 75.0%, respectively.

There was no significant correlation between genetic and geographic distances, and between the genetic variation and the geographic regions (the grasslands of high and low altitude of South Brazil). However, a discriminant analysis allowed separating the populations by their region of origin, showing that a group of 15 out of 92 polymorphic bands are region specifics.

In a previous work we reported the essential oil composition of these populations (Echeverrigaray et al. 2009). Although a no clear relation was observed between genetic differentiation and the essential oil profiles, the low intra-population and high inter-population diversity can explain the low chemical variation observed within populations and the relatively high number of chemotypes identified in this species. Moreover, the Tainhas and Vacaria populations characterized by their high concentration of 1,8-cineole/α-terpineol/limonene were close related.

DISCUSSION

DNA analysis using polymerase chain reaction (PCR)-based molecular markers have proved to be efficient tools to assess genetic diversity, contributing to determine genetic relationships, DNA fingerprinting, population genetic studies, and conservation of aromatic and medicinal plants (Xiao et al. 2006, Trindade 2010). Among PCR based markers, ISSR, which involves amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction, represents a robust, reproducible, universal, rapid and low cost option for the

study of genetic variability in both crop and wild plants (Reddy et al. 2002, Xiao et al. 2006). In this study, a great number of bands (109 bands), most of which polymorphic (84.4%) were obtained with the selected ISSR primers (Table 1), and comparing the electrophoretic profiles, 28 out of 30 accessions of *C. spicata* could be distinguished.

The percentage of polymorphic bands of *C. spicata* at the species level was higher than that previously reported in *C. galioides* (Fracaro et al. 2005) and other Lamiaceae species (Skoula et al. 1999, Echeverrigaray et al. 2001), indicating a higher overall genetic variability in *C. spicata*.

An analysis of ISSR allelic frequencies on *C. spicata* showed a high number of rare alleles (57%) and common alleles (21%), presented in less than 20% and more than 90% of the individuals, respectively. This U-shape distribution of the allelic frequencies observed in *C. spicata* is common in natural populations of outcrossing plant species with a large genetic background (Chakraborty et al. 1980), particularly considering the dominant nature of ISSR markers.

In general, genetic parameters (Table 2) showed that there is a high level of genetic diversity among populations and low level of genetic variation within natural populations of *C. spicata* from South Brazil, with a high coefficient of genetic differentiation (0.727) and predicted proportion of diversity between populations (75%).

The genetic differentiation of plant populations reflects the interaction of several factors like habitat fragmentation, genetic drift, mating systems, gene flow and selection (Schaal et al. 1998). The high genetic differentiation among populations, associated with a low value of estimated genetic flow between populations (0.187), indicates that the strong differentiation in *C. spicata* may be mainly due to genetic drift. Moreover, habitat fragmentation by intense agriculture and cattle ranching in its area of distribution may also contribute to the genetic isolation and small size (20-50 plants) of *C. spicata* populations.

Cluster analysis confirmed the genetic structure of *C. spicata,* separating all individuals by their original population, but no significant grouping was evidenced among *C. spicata* populations (Figure 3), indicating that the genetic variation is distributed all along the populations. This observation is reinforced by the absence of correlation between genetic and geographic distances, and between genetic variation and phytogeographic regions. These data differs from those obtained in *C. galioides*, another *Cunila* species of botanical section *Spicatae*, in which strong correlations were detected between the genetic distances (RAPD markers), chemical composition and geographical distribution (Fracaro et al. 2005), and ISSR analysis of *Vitex rotundifolia* populations, a medicinal plant from China (Hu et al. 2008). However, absence of correlation between genetic and geographical distances are not rare in natural plant species (Xiao et al. 2006).

The analysis of genetic variability, population analysis, evaluation of gene flow, among other parameters, integrating information obtained by molecular analyses and ecological studies allow the development of conservation strategies (Hu et al. 2008, Heywood et al. 2003, Escudero et al. 2003). The low intra- and high inter-population variability of *C. spicata* indicates that the conservation of one or few populations is insufficient to conserve all the variation of the species. The priority in this case must be to protect a high number of the existing populations *in situ*, and to collect and maintain a group of populations previously selected based on their genetic diversity and allelic constitution. In this sense, the ten populations analyzed in the present study have been successfully maintained separately at the experimental field of the Institute of Biotechnology for the last two years, and represent the first permanent germplasm collection of *C. spicata.* The genetic integrity of these populations has been evaluated using ISSR markers.

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Author' biography with Photo

Dr. Sergio Echeverrigaray is pos graduated im Genetics and completed his PhD in Agronomy from University of São Paulo . His current interests are on topics of Molecular Biology, Microbial Genetics, Applied Microbiology, Population Genetics, Biotic and Abiotic Stress Response.