Intra and inter populational genetic variability in *Maytenus ilicifolia* Mart. ex Reiss. 1861, through RAPD markers

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(With 1 figure)

Abstract

Maytenus ilicifolia is a medicinal plant largely used in the South Brazilian folk medicine. The aim of this study was to quantify the intra and inter populational genetic variability in three populations of *M. ilicifolia*, focusing on the genetic conservation of this species, which has been threatened by anthropic action. RAPD (Random Amplified Polymorphic DNA) markers were used to analyze 30 plants of each of the three populations collected in the Alto Uruguai Gaúcho region. Fourteen selected primers generated a total of 158 bands, 71.5% of which were polymorphic. The comparison of Jaccard's distances showed that the intra populational variation was higher than the inter populational variability, and cluster analysis allowed the separation of the three populations. Just 7.6% of the bands were specific of at least two populations. Data indicate that the analyzed *M. ilicifolia* populations represent a single genetic pool, and therefore any of the population thoroughly can represent the overall genetic variability of the species in the sampled region.

Keywords: conservation, espinheira santa, native populations, molecular markers.

Variabilidade genética intra e interpopulacional em *Maytenus ilicifolia* Mart. ex Reiss, utilizando marcadores RAPD

Resumo

Maytenus ilicifolia é uma planta medicinal bastante utilizada na medicina popular da região sul do Brasil. O objetivo deste estudo foi quantificar a variabilidade genética intra e interpopulacional em três populações de *M. ilicifolia* visando a conservação genética desta espécie, que se encontra ameaçada pela ação antrópica. Marcadores RAPD (*Random Amplified Polymorphic DNA*) foram utilizados para analisar 30 plantas de cada uma das três populações coletadas na região do Alto Uruguai Gaúcho. Foram selecionados 14 *primers*, que geraram 158 bandas, das quais 71,5% foram polimórficas. A comparação das distâncias de Jaccard mostraram que a variabilidade intra populacional foi maior que a interpopulacional, e a análise de agrupamentos permitiu a separação das três populações. Somente 7.6% das bandas foram específicas de pelo menos duas populações. Os resultados indicam que as populações de *M. ilicifolia* analisadas representam um único conjunto gênico, de tal forma que qualquer uma das populações pode representar a variabilidade genética geral da espécie na região.

Palavras-chave: conservação, espinheira santa, populações naturais, marcadores moleculares.

1. Introduction

The use of medicinal plants in Brazil is intense, especially by populations of reduced income; exotic plants introduced by immigrants, as well as native plants, are used for this purpose. Brazilian flora has a high biodiversity, and it has a great potential to study and to discover new phytotherapics. However, the little knowledge and the indiscriminated use of natural resources have lead to the inclusion of several plants in the list of threatened species. The anthropic action as well as fragmentation of ecosystems might provoke a reduction on the gene flow between populations and the increase of inbreeding, leading to the reduction of the genetic variability and enhancing the vulnerability (Kageyama et al., 1998).

The genus *Maytenus* belongs to the *Colastraceae* family, and several species of this genus are recognized and used as medicinal plants in several countries, standing out *M. chubutensis* (Speg) Lourteig and O'Donell and Sleumer, 1955 in Chile, *M. horrida* Reiss, 1861 in Paraguay, *M. macrocarpa* (Ruiz and Pav.) Briq., 1919 and *M. krukovii* A. C. Sm, 1939 in Ecuador and Peru, *M. diversifolia* (Maxim.) Ding Hou, 1962 and *M. emarginata* (Willd.) Ding Hou, 1962 in Taiwan, *M. rothiana* (Laws.) Lobeau-Callen, 1975 in India, *M. mossambicensis* (Klotzsch) Blakelock, 1957 in Mozambique, *M. senegalensis* (Lam.) Exell, 1892 in Tanzania, and *M. canariensis* (Loes) Kunkel and Sunding, 1971 in Canaries Islands, among others.

In Southeast and South Brazil, the most important species of the genus used in popular medicine are *M. ilicifolia* Mart. ex Reiss. 1861 and *M. aquifolia* Mart. 1841. Due to their morphologic similarities, these two species are frequently erroneous identified. *M. ilicifolia* is a sub shrub, branched out since its base, measuring about five meters height, the glabrous shrubs are tetra or multicarenated, and the leaves are coriaceous, glabrous with full margin or one to several thorns (Carvalho-Okano, 1992). Throughout observations, this author suggests that due to the reduction of reproductive organs, there might be a tendency of transition from bisexuality to unisexuality of flowers in this species.

Studies performed with *M. ilicifolia* leaves extracts pointed its anti ulcerogenic (Carlini and Braz, 1998; Souza-Formigoni and Oliveira, 1991; Niero et al., 2001), analgesic (Oliveira et al., 1991), antitumoral (Fox, 1991), and abortive (Montanari and Bevilacqua, 2002) properties.

Maytenus ilicifolia occurs mainly in the south region of Brazil and is found preferably in the understory or on the margins of rivers. In Rio Grande do Sul State, *M. ilicifolia* is present in the different phytogeographic regions (Mossi et al., 2002). The low frequency of occurrence (Siqueira, 1994.), the intense use as phytotherapic, and the antropic action in the region of its natural occurrence have lead to the inclusion of this species in the official list of endangered plants of the Brazilian flora.

In this sense, genetic studies are fundamental for the management and conservation of this species. The use of molecular markers is a powerful tool in the genetic study of populations, and the RAPD (Random Amplified Polymorphic DNA) technique is suitable for the analysis of genetic diversity in natural populations of dioicious species (Ferreira and Grattapaglia, 1996). As far as we know, the genetic studies of *M. ilicifolia* populations are restricted to a study of the genetic variability of two natural populations from Paraná State using RAPD markers (Bittencourt, 2000), and a study that includes a population of *M. ilicifolia* from Santa Catarina State using isoenzymatic markers (Perecin and Kageyama, 2002).

This work aims to analyze intra and inter populational genetic variability, with the use of molecular markers (RAPD), in three native populations of *Maytenus ilicifolia* located within the same ecological region in the north of Rio Grande do Sul State.

2. Materials and Methods

In the present work three native populations of *Maytenus ilicifolia* from Erechim, Barão de Cotegipe, and São José (North of Rio Grande do Sul State, Brazil) were analyzed. The precise location, altitude, and distance between populations are presented in Table 1. This region is characterized by a subtropical climate, red latossol with a high content of aluminium and iron (typical haplorthox soil), and a semi-caducifolius seasonal forest. The areas where the populations were collected had low anthropic perturbation.

For the purpose of this work, the group of plants geographically isolated was considered as a population. Thirty adult plants randomly collected and representing the whole sampled area represented each population. The minimum distance between two plants was 10 m. The collected leaves were immediately placed in liquid nitrogen and, afterwards, stored in a freezer at –80 °C until DNA extraction (as shown in Table 1).

For the isolation of total DNA of each plant, a method described by Doyle and Doyle (1988) modified for *M. ilicifolia* (Bittencourt, 2000) was used. The basic process consists of macerating about 150 mg of leaves in liquid nitrogen; adding 750 µL of extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0, 0.2% 2 Mercaptoetanol, 0.01% Proteinase K, 1% PVP); keeping in immersion for 45 minutes at 65 °C, removing residual protein with two volumes of 24:1 chloroform: isoamyl alcohol until total cleaning of DNA; precipitation with 2/3 volume of isopropanol, fol-

Table 1. Geographic coordinates, altitude, and linear distances between the three populations of *Maytenus ilicifolia* included in the study.

Region	Coordinates	Altitude (m)	Distance (km)	
			Erechim	Barão de Cotegipe
Erechim	28° 38' 300'' S and 52° 17' 500'' W	770	-	-
Barão de Cotegipe	27° 34' 352'' S and 52° 20' 892'' W	740	19.4	-
São José	27° 32' 454'' S and 52° 20' 892'' W	720	22.5	3.4

lowed by two washes with 1,000 µL of 70% ethanol; and finally resuspending the pellet in 150 µL of TE (Trisma: EDTA 10:1). All centrifugations were performed at 13,000 rpm. DNA was quantified by UV spectrophotometry at 260 nm, and its integrity was evaluated by gel electrophoresis.

For RAPD amplification the reaction described by Williams et al. (1990) was used, with some modifications. The reaction mixture contained: 50 mM KCl; 3 mM $MgCl_2$; 50 mM Tris HCl (pH 9.0); 0.25 mM Triton-X-100; 1.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies); 200 mM of each dNTP; 0.2 mM of primer, and about 40 ng of *M. ilicifolia* DNA.

OPA, OPB, OPD, OPF, OPH, OPW and OPY kits from Operon Technologies, with 20 primers each, were used, aiming to identify those which presented the best results for *Maytenus ilicifolia* of Erechim, Barão de Cotegipe, and São José. Primers were selected based on the number of amplified bands, their intensity, their repetitiveness, and the level of polymorphism. The amplification was carried out in a MJ Research INC thermocycler. The process of amplification was based on the following sequence: 3 min. at 92 °C, 40 cycles of 1 minute at 92 °C, 1 minute at 35 °C and 2 minutes at 72 °C, including a final elongation step of 3 minutes at 72 °C, and 4 °C until the retreating of the samples.

Electrophoretic separations were performed in horizontal 1.4% agarose gels in TBE 1X buffer (0.089 M Trisma, 0.089 M Boric Acid, and 0.008 M EDTA) with a constant voltage of 90 V. A 100 bp DNA Ladder from Gibco (BRL) was used as a molecular weight marker. The visualization of fragments was done with ethidium bromide and the observation was performed using UV light. The gels were photographed using the digital CEL PRO System (Media Cybernetics).

To determine the genetic variability, the obtained data through the determination of presence or absence of bands was used to construct a matrix, which was analyzed through the computer program NTSYS-pc, version 1.7 (Rohlf, 1992). Genetic distances were calculated using Jaccard´s similarity coefficients, and the dendrograms were built by UPGMA algorithm (Unweighted Pair Group Method using Arithmetic Averages), described by Sokal and Michener (1958). Confidence limits for groupings were calculated by randomizing 100 samples using Winboot program (Yap and Nelson, 1996). The allele frequency was determined by the percentage of fragments present in the 30 analyzed plants of each population, as comparing to each allele in the three populations.

3. Results and Discussion

In the study of intra and inter populational variability in the three populations, 14 primers from Operon Technologies (as shown in Table 2) were used. These primers were selected from 140 decamer primers from kits OPA, OPB, OPD, OPF, OPH, OPW, and OPY. The selection of the primers was based on the quantity, intensity, and repetition of the amplified fragments, and results obtained by Bittencourt (2000). Considering that the amplification of fragments can be affected by factors such as concentration of reaction components and different conditions of amplification cycles (Kresovich et al., 1992; Weeden et al., 1992), only those amplified fragments that presented high intensity and reproductivity

Table 2. Primer sequences, number of RAPD fragments, and polymorphic products obtained in the analysis of three populations of *Maytenus ilicifolia*.

Primer	Sequence (5' to 3')	Number of amplified fragments			Number of polymorphic fragments		
		Erechim	Barão de Cotegipe	São José	Erechim	Barão de Cotegipe	São José
OPA-01	CAGGCCCTTC	12	11	12	7	4	7
OPA-02	TGCCGAGCTG	13	13	13	9	9	8
OPA-08	GTGACGTAGG	11	11	12	8	10	10
OPA-09	GGGTAACGCC	11	11	11	8	9	10
OPB-03	CATCCCCCTG	11	11	10	8	8	10
OPB-07	GGTGACGCAG	9	9	9	6	6	8
OPD-20	ACCCGGTCAC	12	12	11	6	5	7
OPF-01	ACGGATCCTG	14	14	13	7	8	4
OPF-10	GGAAGCTTGG	12	12	11	4	4	3
OPH-08	GAAACACCCC	10	10	8	2	5	4
OPW-04	CAGAAGCGGA	14	14	15	6	4	8
OPW-08	GACTGCCTCT	11	11	11	7	6	7
OPY-11	AGACGATGGG	5	5	6	2	1	3
OPY-13	GGGTCTCGGT	10	10	8	3	4	4
Total		155	154	150	83	83	93
Polymorphism					53.5%	53.9%	62.0%

over three repetitions were considered on the subsequent analysis.

Taking into consideration the 90 studied individuals, a total of 158 fragments were identified, and 113 of these fragments (71.52%) were polymorphic. In the 30 representatives of each population, considered for the purposes of this analysis, 155 fragments for the Erechim population, 154 for Barão de Cotegipe, and 150 for São José, matched the above criteria and were considered for the multivariate analysis (as shown in Table 2). These amplified fragments presented 50 to 2,200 base pairs (bp).

The average number of fragments per primer was relatively higher than that previously found by Bittencourt (2000). This difference can be attributed to distinct laboratorial conditions, to the primers used, but mostly to the criteria established for the selection of fragments. The percentage of polymorphic bands was 53.5, 53.9%, and 62% for the populations of Erechim, Barão de Cotegipe, and São José, respectively (as shown in Table 2). These values can be considered low if compared to the results obtained by Bittencourt (2000) in *M. ilicifolia* (84.6%), and other arboreal species such as *Acacia raddiana* with 90.69% (Shrestha et al., 2002), and *Olea europaea* with 91.5% (Mekuria et al., 2002) polymorphic bands.

The average Jaccard´s coefficients within population were: 0.78 (0.71 to 0.98), 0.79 (0.69 to 0.98), and 0.76 (0.67 to 0.87) for Barão de Cotegipe, Erechim, and São José populations, respectively (as shown in Table 3). High intra populational variability was also evidenced by Bittencourt (2000) studying two populations of *M. ilicifolia* from Paraná State. However, the amplitude of variation observed by Bittencourt (2000) was higher than that obtained in Rio Grande do Sul populations. As can be observed in Table 3, the similarity coefficients between plants of different populations varied from 0.62 to 0.87 with an average of 0.72.

The variance data for within populations and among populations (see Table 3) show that intra populational variances (0.102 to 0.140) are superior to the variances between populations (0.076 to 0.099). These results indicate that the allelic frequencies in the three populations might be similar, and the number of population-specific alleles (bands) could be relatively low.

The coefficient of variation calculated among populations showed a higher proximity between Erechim and São José (3.94) compared to Erechim and Barão de Cotegipe (4.13), and Barão de Cotegipe and São José (4.44). This result can not be explained by geographical distribution, since Erechim population is 19.4 km away from Barão de Cotegipe and 22.5 km away from São José, while Barão de Cotegipe is 3.4 km away from São José.

The analysis of grouping through UPGMA algorithms (see Figure 1) allowed to separate the 90 analyzed plants in three groups correspondent to each of the evaluated populations (bootstrap > 95%), except a plant of Barão de Cotegipe's population that was grouped with those of Erechim population. Moreover, as expected considering the high intra populational variability, the significance values obtained through the bootstrap analysis did not support the separation (> 40%).

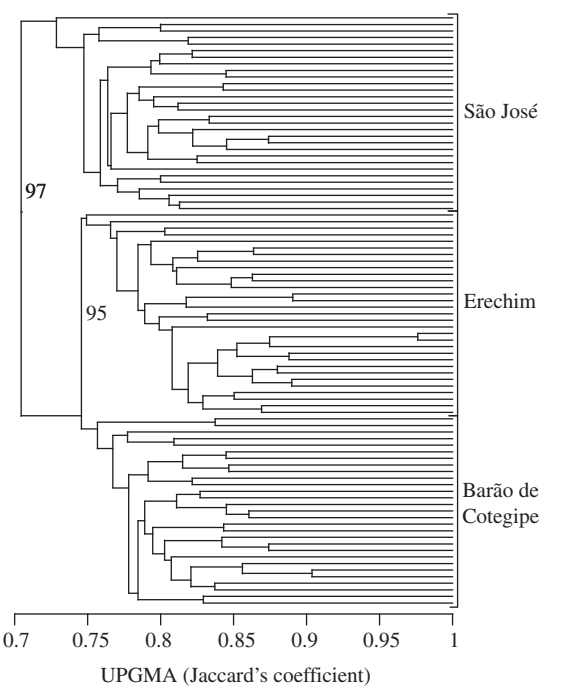

Figure 1. Cluster analysis of 90 plants from three populations of *Maytenus ilicifolia* based on RAPD data using the UPGA algorithm.

Table 3. Analysis of the intra e inter populational similarity of three populations of *Maytenus ilicifolia* from Alto Uruguai, RS, based on RAPD results.

Population	Means	Minimum	Maximum	Variance	Standard derivation	Correlation (%)
Barão de Cotegipe	0.78	0.71	0.90	0.107	0.0327	4.19
Erechim	0.79	0.69	0.98	0.140	0.0374	4.73
São José	0.76	0.67	0.87	0.102	0.0319	4.19
Barão de Cotegipe – Erechim	0.75	0.67	0.87	0.096	0.0310	4.13
Barão de Cotegipe – São José	0.71	0.62	0.81	0.099	0.0315	4.44
Erechim – São José	0.70	0.62	0.80	0.076	0.0276	3.94

Considering the allelic frequency (bands) in the three populations: 28.48% of the bands had the same frequency in the three populations, 39.6% had low frequency differences (< 50%) in at least two populations, and 31.01% had important frequency differences (< 50%) between populations. Frequency differences between populations greater than 50% confirm the results obtained by Perecin and Kageyama (2002) using isoenzymatic markers. Of the total of 158 bands, just 12 (7.6%) were specific of one or other population, and can be considered as rare alleles.

As pointed out by Perecin and Kageyama (2002), the allele differences between populations may be associated to the reproductive system of the species, a relatively low gene flow between populations, and a large original genetic pool.

The obtained results lead to some considerations concerning the conservation of the genetic base in *Maytenus ilicifolia* at the Alto Uruguai region: 1) the high intra populational variability and the absence of well defined groups between populations indicate that the conservation of a high number of individual of a single population will represent a large fraction of the overall genetic variability of the species within the region; and 2) any measure to be taken in the sense of preserving the germoplasms ex situ should be accompanied by a program of in situ conservation to prevent the loss of low frequency or rare alleles.

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