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Genetic variation in natural populations of maté (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) using RAPD markers

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This study characterized the genetic diversity of four populations of *Ilex paraguariensis*, a plant species native to South America, using random amplified polymorphic DNA (RAPD) markers. A total of 341 different RAPD bands were generated by the 15 primers analysed. High genetic variability was detected within each population, with an average diversity of 0.163. The within-population variation was large, probably as a result of the life history characteristics of *I. paraguariensis*. The average distance between individuals from each population was 0.392 and that between populations was 0.433, indicating a low between-population divergence. Most bands were common to all populations and the population-specific bands occurred at low frequencies. Partitioning of the genetic diversity indicated that 85% of the variation occurred within populations and only 15% between populations. Although most of the variability is within populations, clusterings of plants in each population were observed in the dendrogram.

Keywords: genetic variation, *Ilex paraguariensis*, natural populations, RAPD.

Introduction

Ilex paraguariensis A. St.-Hil. (Aquifoliaceae), commonly called maté, is native to South America, and is common in the southern States of Brazil, in Paraguay, Argentina and parts of Uruguay. The Brazilian distribution of the species (Fig. 1) includes the States of Rio Grande do Sul, Santa Catarina, Paraná, Mato Grosso do Sul, Minas Gerais, São Paulo and Rio de Janeiro (Grondona, 1954).

Maté is a 12–30 m tree, which can live for up to 100 years. It is an obligately outcrossing, dioecious, insect-pollinated, diploid ($2n = 40$) species (Niklas, 1987). It shows great variability in leaf characters, flowers from September to December and fruits in March (Winge *et al*., 1995).

Besides its use in 'chimarrão' and 'tererê', traditional beverages in Brazil, Uruguay and Argentina, many other uses in canned drinks, soluble teas, cosmetics, colourings, medicines and as a caffeine source are now known. However, despite its socio-economic importance, the biology and genetics of maté are poorly understood.

Scientific approaches to diversity conservation, the exploration of plant genetic resources and the design of plant improvement programmes require a detailed knowledge of the amount and distribution of genetic diversity within species. Molecular genetic markers can provide a relatively unbiased method of quantifying genetic diversity in plants. In 1990, Williams *et al*. and Welsh & McClelland described a procedure for the identification of polymorphism based on PCR (polymerase chain reaction), which is independent of DNA sequence knowledge. Randomly amplified polymorphic DNA (RAPD) markers are based on the amplification of unknown DNA sequences using single, short, random oligonucleotide primers. The RAPD method overcomes many of the technical limitations of RFLP (restriction fragment length polymorphism) and has been used in many genetic analyses, including population genetic studies in a number of genera (Chalmers *et al*., 1992; Ashburner *et al*., 1997; Gallois *et al*., 1998). This study investigated the levels of RAPD diversity within and among populations of *I. paraguariensis*, across its geographical range.

Materials and methods

Sample collection

Young leaves were collected from plants selected at random from four populations in four states of Brazil:

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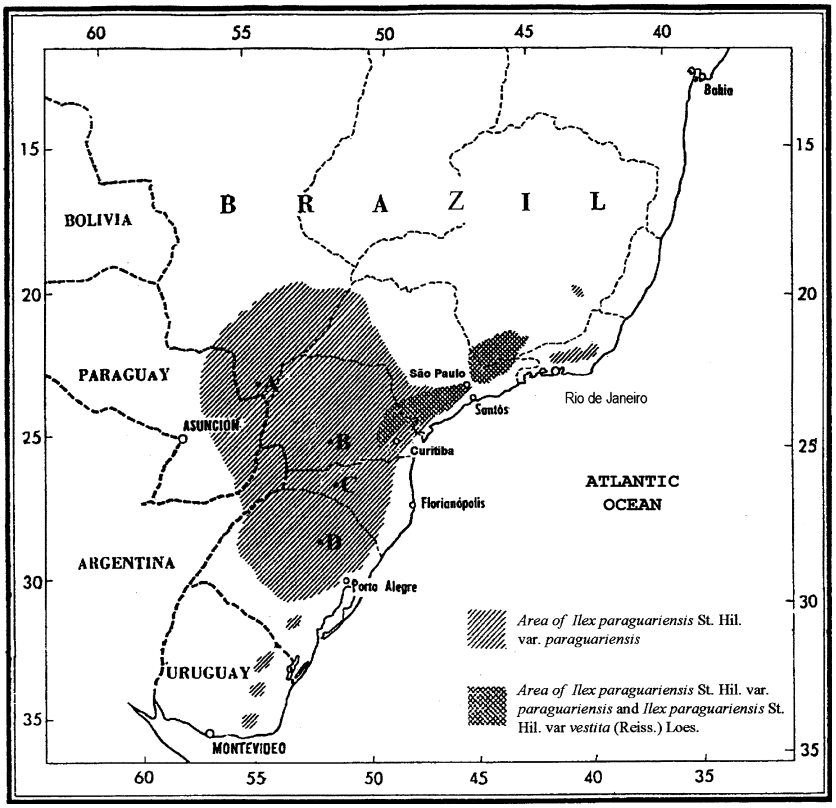

Fig. 1 Distribution of maté (*Ilex paraguariensis*) in South America (from Grondona, 1954), showing the location of the populations analysed in this study. A: population from Municipal district of Iguatemi and Tacuru, State of Mato Grosso do Sul (MS); B: population from Municipal district of Guarapuava and Pinhão, State of Paraná (PR); C: population from Municipal district of Catanduvas, State of Santa Catarina (SC); and D: population from Municipal district of Ilópolis, State of Rio Grande do Sul (RS).

Mato Grosso do Sul (MS), Paraná (PR), Santa Catarina (SC), and Rio Grande do Sul (RS). These populations are approximately 250 km from each other and follow a north-west–south-east transect (Fig. 1). Forty-three plants from MS, 35 from PR, 31 from SC, and 39 from RS were used in the analysis.

DNA extraction

DNA was extracted from 120 to 150 mg of leaf material using a modified Doyle & Doyle (1987) protocol. Leaf material was ground to a fine powder in liquid nitrogen and then placed in a microcentrifuge tube with 1 mL of extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA · Na_2 · $2H_2O$, 1.4 M NaCl, 1% PVP 40000, and 0.01% proteinase K) plus 20 μL of 2-β-mercaptoethanol. Following incubation at 65°C for 30 min, 700 μL of chloroform:isoamyl alcohol (24:1) was added, mixed, centrifuged at 13 800 ***g*** for 15 min, the supernatant transferred to a new tube and then repeated. Nucleic acids were precipitated with isopropanol (2/3 volume of supernatant), the mixture centrifuged at 13 800 ***g*** for 15 min, the supernatant discarded and the remaining pellet washed in 76% ethanol containing 10 mM ammonium acetate for 20 min. The pellet was dissolved in 100 μL of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA · Na_2 · $2H_2O$) and nucleic acid reprecipitated with 1/2 volume of ammonium acetate and 2.5 volumes of ethanol. After centrifuging at 13 800 ***g*** for 15 min the pellet was redissolved in TE buffer with 10 μg/mL RNase and the solution was kept at 30°C for 30–60 min. The DNA was kept at 4°C for 24 h, dispensed in aliquots, and stored at −20°C. DNA concentration of each sample was estimated by visual assessment compared to λ-phage DNA (Amersham Pharmacia Biotech, Uppsala, Sweden) of different known concentrations.

DNA amplification

Approximately 35 ng of genomic DNA were amplified in a volume of 25 μL containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.025 mg of BSA, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.2 μM 10-mer primer (Operon Technologies, Inc., Alameda, CA, USA), and 1 unit of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) by means of a thermal cycler (Perkin-Elmer Corporation, mod. 480, Norwalk, CT, USA). The cycling programme began with an initial 3 min at 94°C followed by 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and a final 5 min at 72°C. Each reaction vial was overlaid with 10 μL of mineral oil. A negative control was added in each run to test for contamination. The set

Table 1 Codes and sequences of primers analysed, total number of bands analysed and fragment size

Primer	Sequence (5′ → 3′)	Total no. of bands	Fragment size range (bp)
OPA-01	CAGGCCCTTC	21	450–2800
OPA-02	TGCCGAGCTG	25	350–2500
OPF-01	ACGCATCCTG	25	420–2800
OPF-03	CCTGATCACC	22	400–2500
OPF-05	CCGAATTCCC	22	400–2800
OPF-14	TGCTGCAGGT	27	500–2700
OPH-03	AGACGTCCAC	20	350–1750
OPH-04	GGAAGTCGCC	22	720–2400
OPH-05	AGTCGTCCCC	28	400–2500
OPH-08	GAAACACCCC	21	350–2200
OPH-12	ACGCGCATGT	22	280–1800
OPH-13	GACGCCACAC	27	420–2100
OPH-15	AATGGCGCAG	24	400–1900
OPH-18	GAATCGGCCA	19	500–1900
OPH-19	CTGACCAGCC	16	350–1700
Total		341	280–2800
Mean/primer		22.7	—

of 15 primers analysed is shown in Table 1. Amplification products were separated by electrophoresis in 1.4% agarose gel containing 1 μg/mL ethidium bromide and TBE buffer (0.178 M Tris borate, 2 mM EDTA · Na_2 · $2H_2O$, pH 8.2). Ten microlitres of amplified DNA was mixed with 3 μL of BFF (1.2 mg/mL bromophenol; 125 mg/mL Ficoll) and 10 μL of this mixture was applied in each well of the gel. DNA molecular weight markers (100-bp ladder; Amersham Pharmacia Biotech, Uppsala, Sweden) were added to each gel. The gels were run at a current of 50 mA until the bromophenol had migrated 9 cm from the well. The bands were then visualized under UV light and photographed. Reproducibility of the RAPD analytical procedure was investigated with repeated analysis of several samples. Only those bands which showed consistent amplification were considered in this study.

Data analysis

Each RAPD band was assigned a number (1, 2, 3,…, n) and treated as a unit character coded as 1 (present) or 0 (absent). Genetic diversity was estimated by the Shannon information index (Lewontin, 1972):

$$H = \sum_{i=1}^{k} p_i \log_e p_i$$

where k is the number of RAPD bands, and p_i is the frequency of the ith band in a given population. H is the population RAPD diversity for each primer. These data were averaged to obtain estimates of within-population RAPD diversity (H_O). The average diversity for all populations (H_{POP}) was calculated at two levels: for each primer as the average of H and over all primers as the average of H_O. RAPD diversity for the species (H_{SP}) was calculated using pooled band frequencies of all individuals. The proportion of within-population diversity relative to total diversity is given by H_{POP}/H_{SP} and that between populations by $(H_{SP} - H_{POP})/H_{SP}$. Genetic distance ($D$) was estimated using the complementary value of Nei & Li's (1979) similarity coefficient:

$$D = 1 \quad SC = 1 \quad [2N_{AB}/(N_A + N_B)],$$

where SC is the similarity coefficient, N_A is the number of bands in individual A, N_B is the number of bands in individual B, and N_{AB} is the number of bands present in both A and B. The Nei & Li's (1979) distance was calculated using TREECON for Windows (Van de Peer & De Wachter, 1994). In order to compare the results with those obtained by other authors, Jaccard's (1908) similarity coefficient was also calculated:

$$S_J = a/(a + u),$$

where a is the number of bands in which the two OTUs (operational taxonomic units) agree and u the number of bands present in one OTU but absent in the other one. Jaccard's (1908) similarity coefficient was calculated using NTSYS-pc software (Rohlf, 1987). These two coefficients omit the sharing of negative bands, which is the most appropriate analysis for RAPD data where the nonamplification of a DNA fragment, and therefore the absence of a band, can result from different mutations. Nei & Li's (1979) distance was used to construct a neighbour-joining dendrogram using TREECON for Windows (Van de Peer & De Wachter, 1994).

Results

The RAPD profile

Three hundred and forty-one different RAPD bands were generated by the 15 primers analysed. The total number of bands scored per primer ranged from 16 (OPH-19) to 28 (OPH-5), with an average of 22.7 bands per primer. The size of the amplified fragments ranged from 280 to 2800 base pairs (bp). Table 1 summarizes these data.

Within-population variability

Many different RAPD patterns were detected within each population of *I. paraguariensis*. Figure 2(a) shows

examples of RAPD profiles illustrating this variability. Among the 341 scored bands, only four bands (1.17%) were monomorphic for population MS, four (1.17%) for PR, six (1.76%) for SC and five (1.47%) for RS.

The average population diversity using the Shannon information index was 0.163 and ranged from 0.153 for population MS to 0.176 for population PR (Table 2). The average distance between individuals from each population was 0.392 (Table 3). The four populations of *I. paraguariensis* showed only slight differences in the levels of genetic distance, ranging from 0.377 (RS) to 0.408 (SC). Figure 3 shows a dendrogram for the individuals from the four populations analysed. Grouping of plants from each population was observed on the dendrogram, particularly for populations MS and RS, which showed most individuals together (74% and 77%, respectively). Trees from populations SC and PR formed smaller groups. This result may indicate a greater within-population similarity for MS and RS,

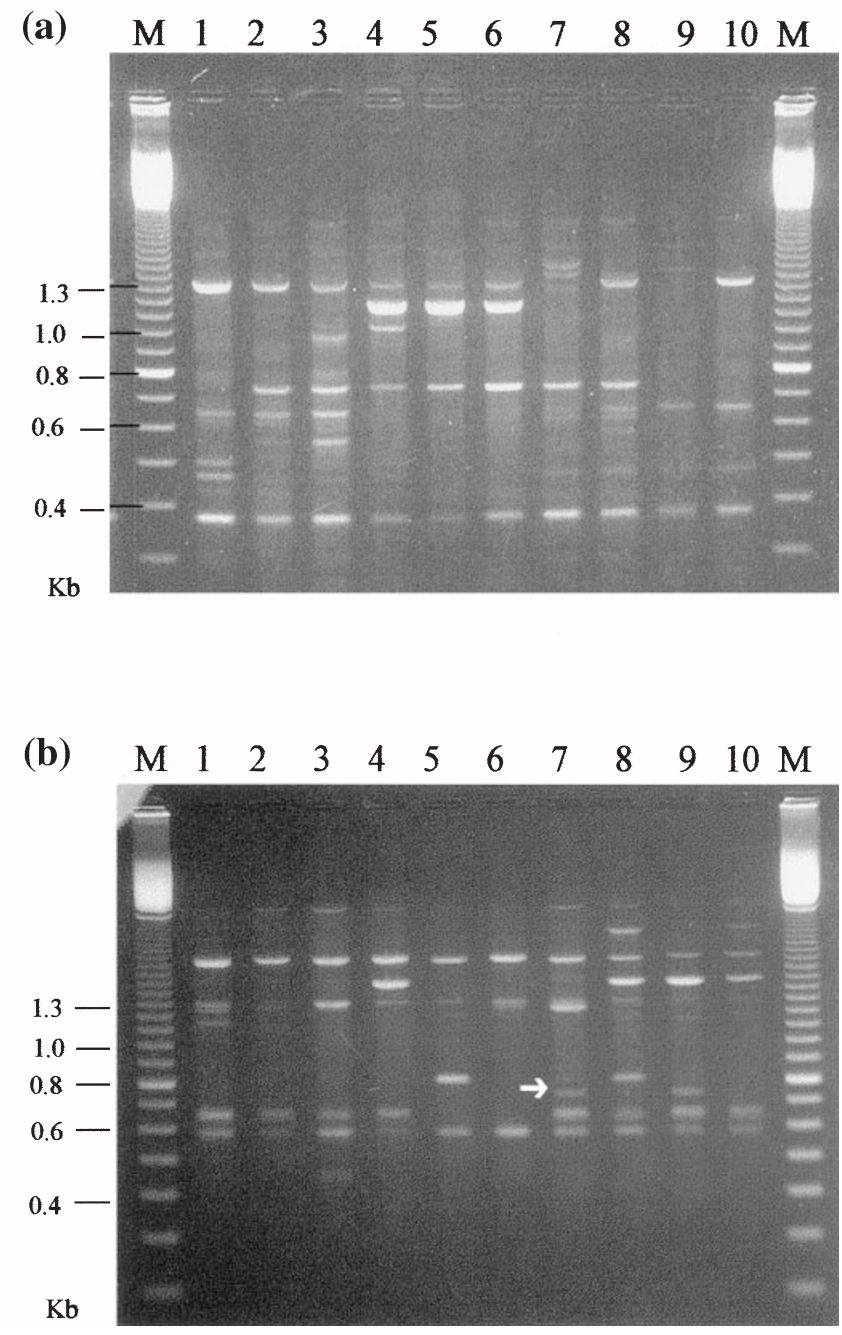

Fig. 2 RAPD polymorphism in natural populations of *Ilex paraguariensis* generated by primer (a) OPA-02 and (b) OPA-01. (a) Ten individuals from population PR (1–10); (b) two individuals from population SC (1–2) and eight individuals from population PR (3–10). One band specific from population PR is indicated by an arrow. M indicates the DNA molecular size marker (100-bp ladder).

Table 2 Genetic diversity of four maté populations and partitioning of the genetic diversity within and between populations (Shannon's index) for the 15 primers analysed

	H							
Primer	MS	PR	SC	RS	H_{POP}	H_{SP}	H_{POP}/H_{SP}	$(H_{SP} - H_{POP})/H_{SP}$
OPA-01	0.115	0.193	0.127	0.155	0.148	0.173	86	14
OPA-02	0.229	0.267	0.236	0.200	0.233	0.264	88	12
OPF-01	0.157	0.200	0.188	0.233	0.195	0.221	88	12
OPF-03	0.160	0.212	0.192	0.137	0.175	0.199	88	12
OPF-05	0.182	0.184	0.133	0.205	0.176	0.189	93	7
OPF-14	0.183	0.126	0.189	0.158	0.164	0.184	89	11
OPH-03	0.117	0.187	0.162	0.109	0.144	0.175	82	18
OPH-04	0.154	0.134	0.143	0.138	0.139	0.167	83	17
OPH-05	0.151	0.187	0.177	0.146	0.169	0.183	92	8
OPH-08	0.167	0.166	0.186	0.171	0.173	0.197	87	13
OPH-12	0.138	0.132	0.105	0.077	0.113	0.144	78	22
OPH-13	0.147	0.166	0.159	0.177	0.162	0.187	86	14
OPH-15	0.232	0.188	0.182	0.155	0.190	0.211	90	10
OPH-18	0.176	0.150	0.121	0.132	0.145	0.165	88	12
OPH-19	0.220	0.123	0.132	0.176	0.163	0.226	72	28
	H_O							
Total	0.153	0.176	0.164	0.169	0.163	0.192	85	15

H, within-population genetic diversity per primer; H_O, mean within-population genetic diversity; H_{POP}, mean within-population genetic diversity for all populations; H_{SP}, genetic diversity of species; H_{POP}/H_{SP}, % of diversity within populations; $(H_{SP} - H_{POP})/H_{SP}$, % of diversity between populations.

Table 3 Average distance (Nei & Li, 1979) coefficients within (diagonal line in bold) and between populations of *Ilex paraguariensis*

	MS	PR	SC	RS
MS	**0.394**			
PR	0.443	**0.393**		
SC	0.459	0.423	**0.408**	
RS	0.433	0.412	0.424	**0.377**
	D_{within}: 0.392		$D_{between}$: 0.433	

although this had not been observed using Nei and Li's coefficient.

Between-population diversity

Of the 341 bands analysed, 23 (6.7%) were exclusive to MS, 17 (5%) to PR, 11 (3.2%) to SC and 10 (2.9%) to RS. Nevertheless, these unique bands occurred at low frequencies in each population (<10% for most of them). One hundred and seventy-six bands (51.6%) were detected in all populations, of which 5.9% occurred at frequencies greater than 80%, and 8.5% with frequencies between 50 and 79%. Figure 2(b) illustrates the between-population diversity.

Pair-wise comparisons revealed an average (Nei & Li, 1979) distance among populations of 0.433 (Table 3). Interpopulation distances were very similar and ranged from 0.412 (PR and RS) to 0.459 (MS and SC).

The dendrogram (Fig. 3) indicates that PR and SC are the most similar populations, whereas population MS is the most distinct. A tendency of clustering following a north-west–south-east gradient can be observed on the dendrogram, i.e. the populations show a degree of genetic differentiation correlated with the geographical distance.

Variation partitioning

Shannon's index of phenotypic diversity was used to partition RAPD diversity into within- and between-population components (Table 2). The average diversity of RAPD markers for *I. paraguariensis* (H_{SP}) was 0.192, and an assessment of the proportion of diversity present within populations (H_{POP}/H_{SP}) indicates that most of the diversity (85%) was detected within populations.

Discussion

Within-population variability

Several studies have demonstrated considerable differences among species in the level of genetic variation

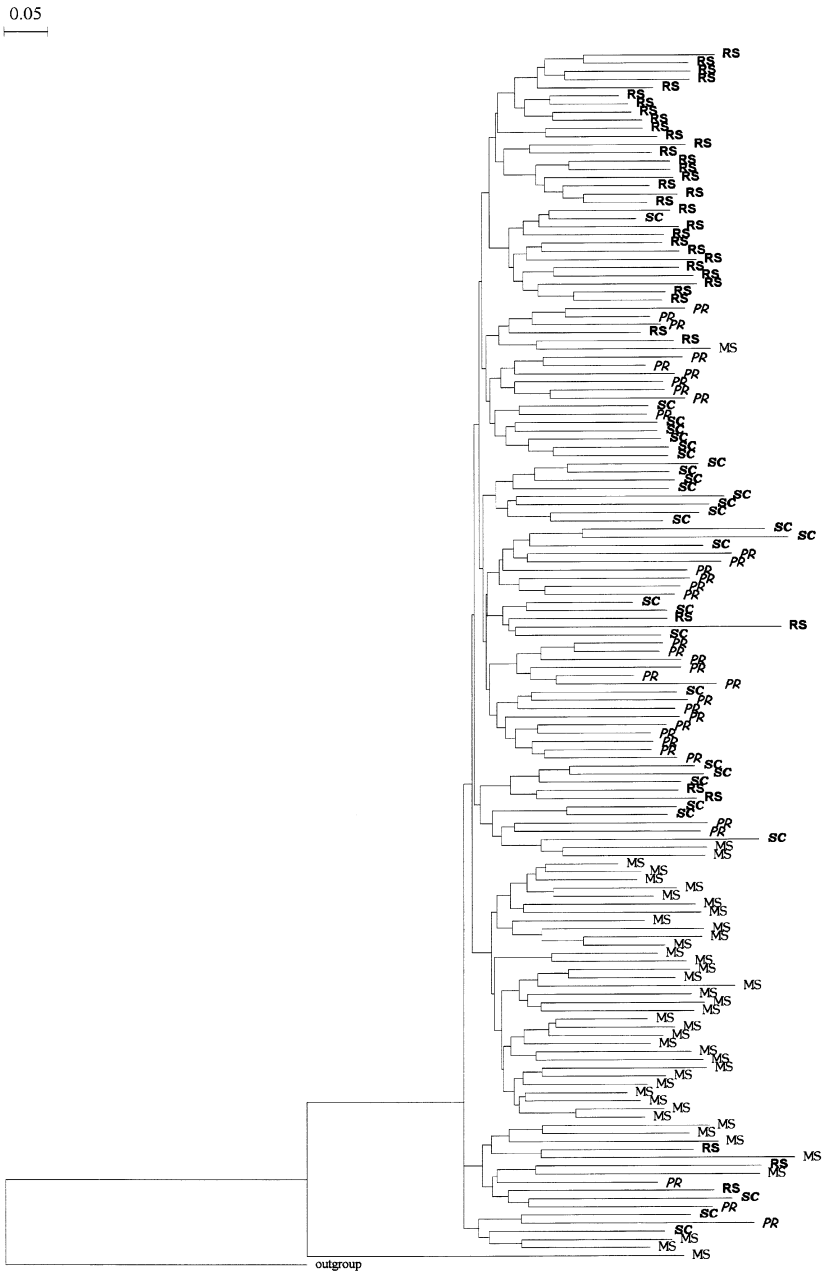

Fig. 3 Dendrogram of 148 trees from four populations of *Ilex paraguariensis* based on a pair-wise distance matrix formed using Nei & Li's (1979) distance of RAPD markers and clustered using neighbour-joining.

within natural populations of plants (Hamrick, 1979). Table 4 shows data obtained by other authors using RAPDs. The number of bands per primer obtained for *I. paraguariensis* was higher than found in other species. *Ilex paraguariensis* showed a high distance between individuals from each population (0.392), higher than the values observed for other species except *Banksia cuneata*.

Intrapopulation variability in *I. paraguariensis* has been estimated using isoenzymes (Winge *et al*., 1995) and seed storage proteins (Gregianini, 1999). The isoenzyme analysis was performed with F_1 seedlings from three southern Brazilian populations for two systems (esterases and acid phosphatases – two highly polymorphic systems), with a total of five loci analysed. The mean number of alleles per locus (A) and the expected heterozygosity (H) were high ($A = 3.94$ and $H = 50\%$). The study with seed storage proteins used seeds collected from the same plants as in the present investigation and employed SDS–PAGE vertical electrophoresis. All populations analysed showed a high intrapopulation variability [expressed by a low mean Jaccard's (1908) similarity coefficient of $S_J = 0.374$], similar to that obtained for RAPD markers ($S_J = 0.345$). Nevertheless,

Table 4 Measures of genetic diversity in natural populations of some species of plants

Species	Life history characteristics (*)	No. of pops.	No. of primers	Mean no. of bands/primer	Nei & Li's (1979) distance: within between	Partitioning of diversity (Shannon's index) % within pops. % between pops.	References
Alkanna orientalis	Long-lived perennial, mainly outcrossing	4	7	6.43	— —	62 38	Wolff *et al.* (1997)
Amentotaxus formosana	Endemic, dioecious	2	20	14.5	0.028 0.034	—	Wang *et al.* (1996)
Banksia cuneata	Rare and endangered species, outcrossing	10	5	26.02	0.700 —	—	Maguire & Sedgley (1997)
Camellia sinensis	—	5	21	6.62	— —	70 30	Wachira *et al.* (1995)
Cocos nucifera	Perennial, mainly outcrossing	12	14	8.8	— —	60 40	Ashburner *et al.* (1997)
Fagus sylvatica	—	4	59	11.64	0.226 —	—	Gallois *et al.* (1998)
Gliricidia sepium	Leguminous trees, obligately outcrossing	8	11	—	0.170 0.337	40 60	Chalmers *et al.* (1992)
Tylosema esculentum	Leguminous, long-lived perennial, mainly outcrossing	3	11	7.8	— —	84.6 15.4	Monaghan & Halloran (1996)
Panicum virgatum	Perennial, outcrossing	14	5	18.2	0.186 0.348	—	Gunter *et al.* (1996)
Vicia pisiformis	Outcrossing, small populations without gene flow between them	9	6	13.6	— —	19 81	Gustafsson & Gustafsson (1994)
Ilex paraguariensis	Obligately outcrossing, long-lived perennial, large populations	4	15	22.73	0.392 0.433	85 15	This paper

Pops, populations.
*Data available in the original paper.

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cluster analysis with seed storage proteins indicated a higher intrapopulation variability than that with RAPD data, because the latter resulted in a better clustering of the trees from each population.

Thus, within-population polymorphism of *I. paraguariensis* has been analysed using three different markers, RAPD, seed storage proteins, and isoenzymes, which have different evolutionary dynamics and therefore can show distinct genetic variability. Seed storage proteins are expected to be more variable than isoenzymes and RAPD, and RAPD more variable than isoenzymes. But different degrees of variation have been found on grouping enzymes by functional characteristics. Enzymes active in energy metabolism and characterized by only one physiological substrate show less polymorphism than those involved in peripheral metabolism and with multiple physiological substrates (Gillespie & Langley, 1974). All the approaches detected high intrapopulation variability in *I. paraguariensis*. However, all molecules analysed are highly polymorphic, even the enzyme systems studied, which may have caused an overestimation of the true polymorphism of this species.

The high within-population variability observed in natural populations of maté, detected by RAPD and other methods, might be related to life history characteristics of the species which, according to Hamrick *et al.* (1979) and Hamrick & Godt (1989), can affect the genetic structure of plant populations and have effects on genetic variation. *Ilex paraguariensis* is an obligately outcrossing, perennial, long-lived species and a positive association between these characters and genetic variation has already been found (Gottlieb, 1973; Rick & Fobes, 1975; Hamrick, 1979). Moreover, the maté populations analysed are large, which reduces the effects of drift and may allow the preservation of high variation (Hamrick, 1979).

For the breeders, the high genetic variability present in the populations of *I. paraguariensis* is an advantage because it is necessary for improvement programmes. For conservation of genetic diversity, i.e. establishment of germplasm banks, the results indicate that a fairly large sample is necessary to represent the variability in this species.

Between-population divergence

The divergence observed among the four populations of *I. paraguariensis* was similar to that found for *Gliricidia sepium* and *Panicum virgatum*, but not for *Amentotaxus formosana* (Table 4). Nevertheless, the mean values obtained for intra- and interpopulation divergences for maté are very similar, which indicates a low divergence among the populations. This conclusion is supported by the fact that most bands are common to all populations and the population-specific bands occurred at low frequencies, which has also been observed in other species (Monaghan & Halloran, 1996; Wang *et al.*, 1996).

The data obtained with RAPD markers for *I. paraguariensis* are in agreement with the results from seed storage proteins (Gregianini, 1999), which also showed low between-population divergence. The mean Jaccard's (1908) similarity coefficient for storage proteins was 0.308 and for RAPD was 0.320.

Population relationship

The results indicate that PR and SC are the most similar populations, and that MS is the most distinct one (Fig. 3). Nevertheless, many clusters included trees from different populations, indicating that some plants can be more similar to those from other populations than to those of their own population.

Partial data on caffeine and theobromine in the leaves of plants from the same populations of this paper showed a tendency to a north-west–south-east gradient. Caffeine concentration is higher in plants from MS and lower in those from RS. For theobromine, the situation is the inverse (M. L. Athayde, C. G. Coelho & E. P. Schenkel, pers. comm.). The analysis made by Butzke *et al.* (1992) also shows a north-west–south-east gradient in speed of seed germination for three populations of maté. PR showed the fastest germination, followed by SC and then RS. A tendency to a north-west–south-east gradient was also found in the present study using RAPD markers.

Partitioning of variation

The proportion of the total diversity found within populations was 85%, leaving only 15% of the diversity between populations. This finding is in agreement with the observation that outcrossing plants retain considerable variability and that most variation is exhibited within populations (Hamrick *et al.*, 1979). Nevertheless, the proportion of variability occurring within populations of maté is higher than for other outcrossing plant species (Table 4), comparable only to that of *Tylosema esculentum*. This low divergence among the populations of *I. paraguariensis* could be explained by the occurrence of gene flow among populations, but this seems not to be the current situation, because the populations analysed are nowadays about 250 km distant from each other.

Ilex paraguariensis is an insect-pollinated plant probably without pollinator-specificity (Ferreira *et al.*, 1983). There is little information about its seed dispersal. Farmers report that birds eat maté fruits and then transport the seeds to surrounding areas, but this dispersal would not be enough to maintain such high

interpopulation similarity levels. Another explanation could be a recent fragmentation of these populations. The geographical isolation of these populations may have begun in the early 20th century with the colonization by settlers, who deforested the native areas, and became more intense three decades ago when there was a reduction of natural forests to increase plantation areas intended for large-scale crops. It is unlikely that forests with *I. paraguariensis* were continuous, but the populations were probably geographically closer, allowing gene flow between them. So the plants analysed in this study could have been living when the populations were not yet isolated, or could be plants of only one generation after isolation, thus giving insufficient time to allow differentiation among the populations.

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