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Genetic differentiation of *Euterpe edulis* Mart. populations estimated by AFLP analysis

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Abstract

Heart-of-palm (*Euterpe edulis* Mart.) is a wild palm with a wide distribution throughout the Atlantic Rainforest. Populations of *E. edulis* represent important renewable natural resources but are currently under threat from predatory exploitation. Furthermore, because the species is indigenous to the Atlantic Rainforest, which is located in the most economically developed and populated region of Brazil, social and economic pressures have devastated heart-of-palm forests. In order to estimate the partitioning of genetic variation of endangered *E. edulis* populations, 429 AFLP markers were used to analyse 150 plants representing 11 populations of the species distribution range. Analysis of the genetic structure of populations carried out using analysis of molecular variance (AMOVA) revealed moderate genetic variation within populations (57.4%). Genetic differentiation between populations (F_{ST} = 0.426) was positively correlated with geographical distance. These results could be explained by the historical fragmentation of the Atlantic coastal region, together with the life cycle and mating system. The data obtained in this work should have important implications for conservation and future breeding programmes of *E. edulis*.

Keywords: AFLP, Atlantic Rainforest, conservation biology, *Euterpe edulis*, genetic variation

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Introduction

The geographical range of the Atlantic Rainforest has been reduced during the last 500 years from 12% (1.1 million km²) to 1% of the Brazilian territory. Because of the proximity of urban centres this complex ecosystem, the fifth most threatened in the world, has been the subject of environmental degradation by social and economic pressures (Turner & Corlett 1996). In addition, there is evidence that deforestation and fragmentation of the Atlantic Rainforest could have been occurring as a result of the native population crop habits for at least 1500 years before the arrival of Europeans to South America, with consequences to the spatial distribution of plant populations (Dean 1996). The Atlantic Rainforest is a centre of biodiversity and represents one of the tropical regions with the largest number of species per unit area. Most natural populations of these species are comprised of a low number of individuals (Kageyama & Gandara 1993) and it is believed that for every two endangered Brazilian trees, one is found exclusively in the Atlantic Rainforest ecosystem (Consórcio Mata Atlântica 1992). Tropical palms represent highly diverse, renewable natural resources of ecological, medicinal and agricultural importance (Balick 1986), and the Atlantic Rainforest is home to nine genera and 39 species of palms, of which one genus and 32 species are endemic (Glasman 1972). Palm studies in tropical forests have revealed significant variation in density and species composition related to topography and edaphic conditions (Chazdon 1996).

Euterpe edulis Mart. (heart-of-palm), which belongs to the Palmae family, has a wide distribution throughout the Atlantic Rainforest. This palm species grows under the shadow of the dossel species to 20 m or more in height. The plant is dotted with unisex flowers that are borne in threes, comprising a central female and two lateral male inflorescences (Henderson *et al*. 1995). Flowers are insect pollinated, and the opening of male flowers before female flowers in the same panicles seems to benefit outcrossing. In contrast, the long flowering period, with one or three flowerings per reproductive cycle, opens the possibility of inbreeding between flowers from different raquila in the

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same plant (Reis 1996). Therefore, the mating system of the species has not been elucidated completely. Because of the high level of fruit production and the high density of *E. edulis* individuals in populations, its fruits are consumed extensively by birds and mammals (Resende 1996). The shoot apical meristem of the solitary stem is soft and tasty and represents an important product in the Brazilian food industry. Wild-gathered heart-of-palm represents the principal source of heart-of-palm for the industry, because agricultural production is insignificant in Brazil (Leão & Cardoso 1974; Henderson *et al*. 1995; Resende 1996). Both predatory collection and environmental degradation of the Atlantic Rainforest have been related to the decline in distribution and abundance of heart-of-palm populations and as a result, initial steps towards the domestication of this species are now being undertaken, aimed at the rapid growth of plants with multiple stems and improved product quality (Carvalho 1994).

Molecular marker techniques have provided valuable information about the genetic structure of natural plant populations (for reviews see Hamrick 1990; Powell *et al*. 1995, 1996a,b; Ouborg *et al*. 1999). Of the new, polymerase chain reaction (PCR)-based methods, the amplified fragment length polymorphism (AFLP; Vos *et al*. 1995) technique is considered a highly informative fingerprinting tool (Powell *et al*. 1996b) and has been used successfully to analyse the genetic structure of populations of tropical tree species (Muluvi *et al*. 1999; Russell *et al*. 1999). *E. edulis* is one of the most studied palms in Brazil but, despite its economical and ecological value, little is known about the genetic variability and structure of the remaining populations. A recent study using AFLP analysis in germplasm collections of coconut highlighted the convenience of using genetic data instead of depending on only morphological traits and provenance for breeding programs (Perera *et al*. 1998). This is especially true for plants with a long generation time such as *E. edulis*. Molecular characterization of the variability may help to manage and preserve genetic resources for long-term survival of species and for further applications such as domestication and use in breeding programmes.

In this study we used AFLP to characterize the genetic variability and its partitioning within and between 11 *E. edulis* populations along the Brazilian Atlantic coast. The main goal of this work was to establish priority areas for conservation of endangered *E. edulis* populations in the Atlantic Rainforest, based on identifying the distribution of the observed genetic variation.

Materials and methods

Plant material

Leaves from 150 individuals were sampled from 11 geographically distant areas of the Atlantic Rainforest, from 14–26°S and 50–38°W. The locations of the populations are shown in Fig. 1. The number of samples per population varied from 11 to 22 for AFLP analysis (Table 1). Young leaves were sampled from trees at least 8 m high and 50 m apart to reduce the chances of consanguinity. Plant material was dried in silica gel for at least 48 h before storing at –70 °C.

DNA isolation

DNA was isolated from dehydrated leaf material as described by Cardoso *et al*. (1998). Briefly, 0.4 g of leaf tissue was ground into a fine powder in liquid nitrogen using a pestle and mortar and mixed with 5 mL of prewarmed extraction buffer (0.1 M Tris–HCl, pH 8.0; 1.25 M NaCl; 0.02 M EDTA; 2% MATAB and 0.1% β-mercaptoethanol, added just before use). Samples were vortexed and incubated at 65 °C for 1 h. After cooling at room temperature, they were extracted twice with 2.5 mL of chloroform/isoamyl alcohol 24 : 1, mixed carefully and centrifuged at 1500 *g* for 10 min. To the supernatant 0.8 vol. of isopropanol was added and mixed. DNA was collected with a Pasteur pipette, resuspended in 500 mL of TRIS-EDTA (TE) and incubated at room temperature for 1 h to dissolve. Fifty millilitres of 5 M NaCl and 1 mL of absolute ethanol were then added. The collected DNA was rinsed in washing solution (76% ethanol and 0.2 M sodium acetate) 1 mL, for 20 min. Finally, the DNA was transferred to 500 µL of TE. The DNA content of samples was measured using a fluorimeter.

AFLP analysis

The AFLP protocol was carried out as described by Vos *et al*. (1995) using the AFLP Analysis System I kit (Life Technologies, Inc.), with a few modifications to the manufacturer's instructions. Genomic DNA (0.36 mg) was digested by two restriction enzymes (*Eco*RI/*Mse*I) at 37 °C for 2 h in a 12.5-µL volume. To the digested DNA was added 12 µL of the ligation adapters *Eco*RI and *Mse*I solution and 0.5 µL of T4 DNA ligase (0.5 U) and the reaction was incubated at 20 °C for 2 h.

PCR reactions were performed after diluting the ligated DNA 5–10-fold with sterile distilled water. Fragments were pre-amplified by 28 PCR cycles (94 °C for 30 s, 56 °C for 60 s, 72 °C for 60 s), using two primers having a single selective nucleotide. PCR products from the pre-amplification reaction were diluted 10–50-fold and used as templates for the selective amplification using *Eco*RI primer +3 (AFLP starter primer kit; Life Technologies, Inc.) radioactively end-labelled with 1 µCi γ-[^{33}P] ATP (Amersham, UK) and unlabelled *Mse*I +3 primer. AFLP reactions were performed for one cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, followed by reduction of the annealing temperature at each cycle by 0.7 °C for 12 cycles; the annealing temperature was maintained at 56 °C for the remaining 23 cycles. Five pairs of primers were used

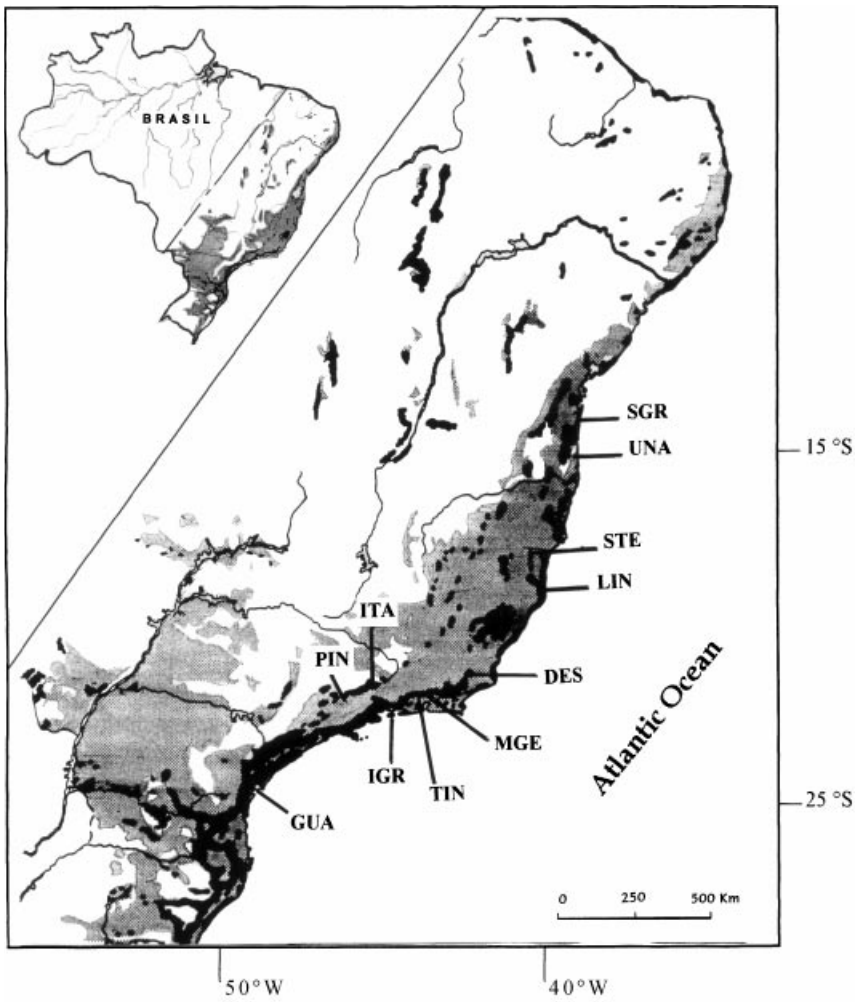

Fig. 1 Original (■) and remaining (■) Atlantic Rainforest. Sampled *Euterpe edulis* populations are labelled as follows: GUA, Guaraqueçaba; PIN, Pindamonhangaba; ITA, Itatiaia; IGR, Ilha Grande; TIN, Tinguá; MGE, Magé; DES, Desengano; STE, Santa Teresa; LIN, Linhares; UNA, Una; SGR, Serra Grande.

Table 1 Average diversity values detected with five primers pairs for *Euterpe edulis* populations (n = number of polymorphic loci; H = genetic diversity; N = sample size)

		Primer pair/Total number of loci											
		ACA/CTC (66)		ACC/CAA (85)		AAG/CTG (68)		ACT/CAT (120)		AAC/CTT (90)		Average over loci	
Populations	N	n	H	n	H	n	H	n	H	n	H	n	H
Guaraqueçaba	16	29	0.168	40	0.164	29	0.165	40	0.110	46	0.193	184	0.160
Pindamonhangaba	17	30	0.168	33	0.135	29	0.147	42	0.113	39	0.161	173	0.144
Itatiaia	12	32	0.184	27	0.122	24	0.127	38	0.104	32	0.138	153	0.135
Ilha Grande	11	17	0.101	21	0.099	13	0.065	24	0.076	16	0.074	91	0.083
Tingua	14	23	0.114	25	0.105	20	0.105	39	0.099	29	0.126	136	0.110
Magé	14	22	0.136	17	0.063	31	0.147	49	0.118	44	0.178	163	0.128
Desengano	22	32	0.183	23	0.096	26	0.134	56	0.120	41	0.173	178	0.141
Santa Teresa	11	27	0.150	28	0.123	19	0.109	36	0.101	30	0.132	140	0.123
Linhares	12	21	0.132	15	0.062	19	0.109	21	0.056	19	0.080	95	0.089
Una	11	44	0.186	17	0.073	13	0.068	30	0.075	33	0.140	137	0.108
Serra Grande	11	22	0.122	16	0.064	21	0.114	26	0.074	19	0.078	104	0.090
Average over populations		63	0.149	80	0.101	65	0.117	102	0.095	85	0.134	395	0.119

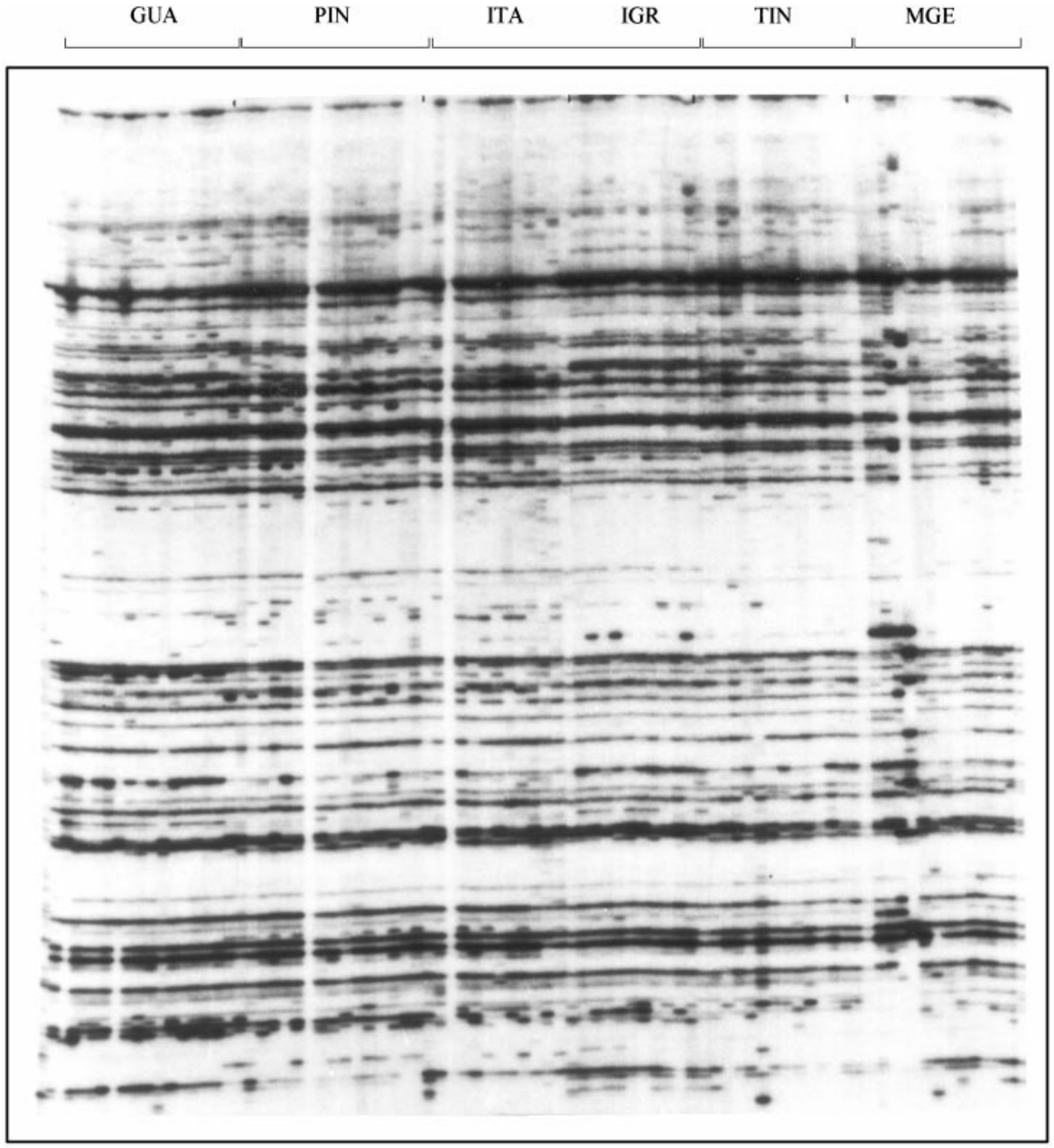

Fig. 2 Subset of amplification products generated using E-ACT and M-CAT primers from six populations.

to amplify the DNA of 150 samples: E-ACA/M-CTC (E 5′-GACTGCGTACCAATTCACA-3′/M 5′-GATGAGTCCTGAGTAACTC-3′); E-ACC/M-CAA (E 5′-GACTGCGTACCAATTCACC-3′/M 5′-GATGAGTCCTGAGTAACAA-3′); E-AAG/M-CTG (E 5′-GACTGCGTACCAATTCAAG-3′/M 5′-GATGAGTCCTGAGTAACTG-3′); E-ACT/M-CAT (E 5′-GACTGCGTACCAATTCACT-3′/M 5′-GATGAGTCCTGAGTAACAT-3′); E-AAC/M-CTT (E 5′-GACTGCGTACCAATTCAAC-3′/M 5′-GATGAGTCCTGAGTAACTT-3′). The reaction products were analysed on 5% denaturing polyacrylamide gels and exposed to XK-1 Kodak film.

Data analysis

AFLP products were scored as the presence (1) and absence (0) of bands. Within-population diversity values were calculated using Nei's unbiased diversity statistic (Nei 1987), averaging over individual AFLP products. A UPGMA dendrogram showing the relationships between populations based on Nei's genetic distance (Nei 1978) was constructed using POPGENE version 1.31 (Yeh *et al*. 1997). A genetic distances matrix was also used to perform a hierarchical analysis of molecular variance (AMOVA, Excoffier

Table 2 Genetic distance between 11 natural populations of *Euterpe edulis* based on AFLP data

Pop	GUA	PIN	ITA	IGR	TIN	MGE	DES	STE	LIN	UNA	SGR
GUA	—										
PIN	0.059	—									
ITA	0.078	0.031	—								
IGR	0.067	0.090	0.103	—							
TIN	0.099	0.073	0.067	0.114	—						
MGE	0.111	0.084	0.090	0.125	0.048	—					
DES	0.100	0.064	0.053	0.114	0.033	0.060	—				
STE	0.110	0.066	0.070	0.149	0.089	0.088	0.072	—			
LIN	0.157	0.106	0.095	0.191	0.115	0.140	0.100	0.066	—		
UNA	0.160	0.123	0.114	0.193	0.111	0.130	0.110	0.095	0.109	—	
SGR	0.157	0.122	0.116	0.187	0.105	0.129	0.104	0.100	0.113	0.052	—

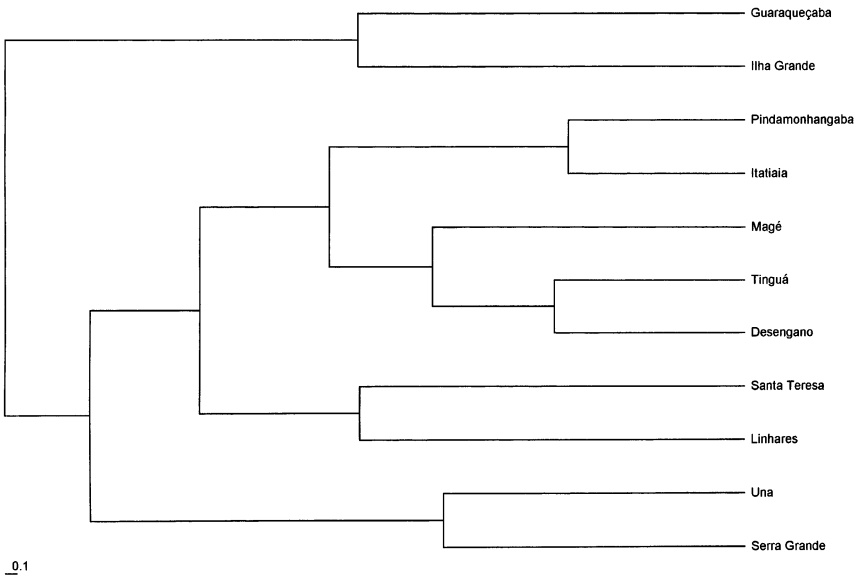

Fig. 3 UPGMA dendrogram based on pairwise genetic distance showing relationships between *Euterpe edulis* populations. Population codes are as in Fig. 1.

et al. 1992), essentially as described by Huff *et al*. (1993) using ARLEQUIN software (V. 1.1; Schneider *et al*. 1997).

Results

AFLP analysis of 150 individuals using five pairs of primers provided a total of 429 markers, 395 (92.07%) of which were polymorphic. An example of the amplification products obtained is shown in Fig. 2. The primers differed in their ability to detect polymorphism within populations. The average over all populations of polymorphic bands amplified per primer pair combination varied from 63 (E-ACA/M-CTC) to 102 (E-ACT/M-CAT) and from 13 (E-AAG/M-CTG in Ilha Grande and Una) to 56 (E-ACT/M-CAT in Desengano) for individual populations. Estimates of Nei's genetic diversity (Nei 1987) for all loci in individual populations showed most diversity in Guaraqueçaba (H = 0.160) and least diversity in Ilha Grande (H = 0.083; Table 1).

The genetic distance matrix (Nei 1978) was used to establish the level of genetic divergence between the populations (Table 2). Estimates of genetic distance using AFLP data ranged from 0.031 for the most closely related populations (PIN and ITA), to 0.157 in the most divergent populations (GUA and SRG). There was a positive correlation between geographical and genetic distance. In general, the Ilha Grande population was the most divergent, probably due to geographical isolation of this island. However, unexpectedly low values of genetic distance were found between Pindamonhagaba and Santa Teresa (0.066), and Itatiaia and Santa Teresa (0.070). These results indicate that genetic distance is not solely dependent on geographical distance, although the dendrogram shows that in most cases, clustering based on genetic distances reflects geographical relationships (Fig. 3). For example, the cluster formed by the Pindamonhangaba and Itatiaia populations is located on the mountain ridge complex of Mantiqueira, whereas Tinguá, Magé and Desengano are located on the mountain ridge complex of the Serra do Mar. Populations of Guaraqueçaba and Ilha Grande form a cluster more distant from the other populations, which may reflect their relative geographical isolation. The overall genetic differentiation between populations was highlighted by

Source of variation	d.f.	Sum of squares	Variation Variance	%	*P*
Among populations	10	2619.052	17.48015	42.60	<0.001
Within populations	140	3297.007	23.55005	57.40	<0.001
Total	150	5916.059	41.03020		

Table 3 Analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) based on 429 AFLP amplification products

AMOVA, which showed that 42.6% of total variation was attributed to between-population variation (F_{ST} = 0.426) (Table 3).

Discussion

The proportion of polymorphic loci amplified in *Euterpe edulis* was 92%, which is similar to figures reported for another tropical tree *Caesalpinia echinata* Lam. (Cardoso *et al*. 1998) and to those reported in *Populus tremuloides* (Yeh *et al*. 1995) using random amplified polymorphic DNA (RAPD). In contrast, a lower percentage of polymorphic loci was observed in *Gliricidia sepium* (Chalmers *et al*. 1992) and *Theobroma cacao* (Russell *et al*. 1993) using RAPDs, and in *Moringa oleifera* using AFLPs (Muluvi *et al*. 1999). This suggests high levels of genetic variation in heart-of-palm. Despite this, the proportions of polymorphic loci for some of the populations using single primer pairs were quite low (between 23% in Ilha Grande and 46% in Guaraqueçaba). Nevertheless, at the species level the results are consistent with data from other tree species, in which high genetic variation has been related to life history and ecological characteristics such as a wide geographical range, primarily outcrossing and animal-seed dispersal mechanisms (Hamrick & Loveless 1989).

Reis (1996) reported minimal genetic divergence using allozyme markers in populations of *E. edulis* from different elevational levels in the Southern region of Brazil. However, this study was conducted in geographically adjacent populations. In contrast, the distance between heart-of-palm populations sampled in this study varied from 40 to 1700 km. Therefore, the values of genetic distance observed here were notably higher than those reported previously. In addition, when compared with AFLP data from another widely distributed tropical tree, *Calycophylum spruceanum* (Russell *et al*. 1999), heart-of-palm populations generally showed higher values for genetic divergence. The highest genetic distance values were found between the island population of Ilha Grande and the other populations, which probably reflects its geographical isolation (Fig. 1). The unexpectedly low value between Guaraqueçaba and Ilha Grande (0.067), which are 512 km apart, could be partly explained by the fact that they are located on a coastal zone in the lowest part of Serra do Mar, a mountain ridge complex with a large number of migratory birds (Zimmermann 1991). Accordingly, the topography of the Atlantic Rainforest, which favours the linking of several populations by mountain ridge complexes, may contribute to allele exchange between them.

The partitioning of genetic variation observed in natural populations of *E. edulis*, obtained by AFLP markers was 57.4% within populations and 42.6% among populations. The value for the within-population component was lower than most of those demonstrated previously for woody, widely distributed, predominantly outcrossed, long-lived perennial species using different molecular markers (e.g. Hamrick & Loveless 1989; Nesbitt *et al*. 1995; Yeh *et al*. 1995; White & Powell 1997; Russell *et al*. 1999). In contrast, previous studies using RAPD markers have shown higher levels of population differentiation in tropical trees (Chalmers *et al*. 1992 and Dawson *et al*. 1995 in *Gliricidia sepium*; Gillies *et al*. 1997; Cardoso *et al*. 1998). In addition, a recent study with *Moringa oleifera*, based on AFLP data, found similar values of partitioning of genetic variation (Muluvi *et al*. 1999). The F_{ST} value of 0.426 revealed by AFLP data indicates relatively high levels of genetic differentiation between the populations studied. Considering that *E. edulis* is predominantly outcrossed, with protandrous dicogamy, male flowers opening before female flowers limiting the potential for self-pollination, higher levels of variability within populations would have been expected. However, this species has a long flowering period and produces one or three flowerings per reproductive cycle, increasing the possibility of inbreeding by male and female flowers from different inflorescences on the same plant (Reis 1996). Inbreeding may partly explain the lower within-population variability (Hamrick & Loveless 1989). To test this hypothesis, additional studies of reproductive biology should be carried out to confirm self-compatibility, which seems to be more widespread in tropical plants than was first thought (Bawa & Ashton 1991).

It has previously been suggested that *E. edulis* plants would not reach more than 50 years old (Silva 1991). Moreover, Dean (1996) reported that deforestation and fragmentation of the Atlantic Rainforest, due to the farming habits of the Indians, may have occurred for at least 1500 years before the arrival of Europeans to Brazil. The observed genetic divergence suggests that *E. edulis* populations might have gone through multiple generations partly isolated as a consequence of forest fragmentation and the plant's life cycle. As a consequence, the genetic differentiation observed between populations of *E. edulis*

may be the result of the life cycle, unsynchronized flowering between populations and mating system, associated with the historical fragmentation process of the Atlantic Rainforest, which together have determined the patterns of gene flow in the species. Several authors have highlighted ecological factors that could lead to genetic structuring within natural plant populations, which are often difficult to resolve without using molecular markers (e.g. Hamrick & Loveless 1989; Dawson *et al*. 1995; Godt & Hamrick 1999; Muluvi *et al*. 1999; Palacios *et al*. 1999; Russell *et al*. 1999; White *et al*. 1999). Gene flow and the mating system have been referred to as the most important determinants of the genetic structure of plant populations (Clegg 1980; Loveless & Hamrick 1984; Hamrick 1990), but for many tropical plants there is little information on the reproductive biology and survival mechanisms of the species. Additional studies are required before conclusions can be drawn on the relative roles of seed dispersal, pollination mechanism, patterns of juvenile survival, outcrossing, geographical distance, heterozygote advantage and/or the long period of fructifying and flowering, in shaping the patterns of observed spatial genetic structure.

E. edulis has maintained a widespread distribution throughout the Atlantic Rainforest, and therefore, conservation at the species level is not yet critical. However, at the population level, the species is under continuous threat of degradation; this situation could compromise the survival of populations and in the long-term the species itself would be at risk. Furthermore, genetically depauperate populations can be disadvantageous for domestication and breeding programmes in this species, which are being carried out by many researchers. In most cases, sampled populations are fragmented remnant forests surrounded by urban or cultivated land under continuous pressure. In addition, predatory collection of plants for commercialization of the meristem is quite intense, even in protected areas. Our results suggest that the priorities for conservation of *E. edulis* should take into account the geographical variation between-population observed. Hence, at the species level, action should be taken to protect the populations Guaraqueçaba, Itatiaia, Desengano and Una because they present the highest levels of within-population genetic diversity. Conversely, the most divergent populations harbour genetic variation not found in any of the other populations and this should be taken into account when managing the species at the population level. In future, we plan to carry out additional studies to explain the contribution of ecological and biological factors, as well as the impact of habitat fragmentation of Atlantic Rainforest, on the observed genetic structure. In this respect, microsatellite markers may provide the most useful tool for better understanding the *E. edulis* mating system and gene flow.

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