

## A Preliminary Study of Genetic Variation in Populations of *Monstera adansonii* var. *klotzschiana* (Araceae) from North-East Brazil, Estimated with AFLP Molecular Markers

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- **Background and Aims** This study sought genetic evidence of long-term isolation in populations of *Monstera adansonii* var. *klotzschiana* (Araceae), a herbaceous, probably outbreeding, humid forest hemi-epiphyte, in the brejo forests of Ceará (north-east Brazil), and clarification of their relationships with populations in Amazonia and the Atlantic forest of Brazil.
- **Methods** Within-population genetic diversity and between-population dissimilarity were estimated using AFLP molecular markers in 75 individuals from eight populations located in Ceará, the Brazilian Atlantic Forest and Amazonia.
- **Key Results** The populations showed a clinal pattern of weak genetic differentiation over a large geographical region ( $F_{ST} = 0.1896$ ). A strong correlation between genetic and geographical distance (Mantel test:  $r = 0.6903$ ,  $P = 0.002$ ) suggests a historical pattern of isolation by distance. Genetic structure analysis revealed at least two distinct gene pools in the data. The two isolated Ceará populations are significantly different from each other (pairwise  $\Phi_{PT} = 0.137$ ,  $P = 0.003$ ) and as diverse (Nei's gene diversity, average  $H_e = 0.1832$ , 0.1706) as those in the Atlantic and Amazon forest regions. The population in southern Brazil is less diverse (Nei's gene diversity, average  $H_e = 0.127$ ) than the rest. The Ceará populations are related to those of the Atlantic forest rather than those from Amazonia (AMOVA, among-groups variation = 11.95%,  $P = 0.037$ ).
- **Conclusions** The gene pools detected within an overall pattern of clinal variation suggest distinct episodes of gene flow, possibly correlated with past humid forest expansions. The Ceará populations show no evidence of erosion of genetic diversity, although this was expected because of their isolation. Their genetic differentiation and relatively high diversity reinforce the importance of conserving the endangered brejo forests.

**Key words:** AFLP, Amazonia, Araceae, Atlantic forest, brejo forest, French Guiana, *Monstera adansonii* var. *klotzschiana*, north-east Brazil, population genetic variation.

### INTRODUCTION

The brejo forests have long interested palaeobiogeographers (e.g. Ab'Sáber, 1982; Andrade-Lima, 1982) because they are considered to represent relicts of former continuous humid forest in the dry north-east of Brazil. Studies of angiosperm species composition have so far shown only low levels of endemism (Sales *et al.*, 1998), especially when compared with the similarly fragmented, montane 'campo rupestre' vegetation of central Bahia (Stannard, 1995). In this study a population-level approach was taken and with a focus on the root-climbing aroid *Monstera adansonii* Schott var. *klotzschiana* (Schott) Madison, a widespread species ecologically restricted to humid forests (Mayo, 1984). Using AFLP markers, we looked for evidence of genetic distinction and divergence between the brejo populations of this taxon in Ceará (Fig. 1A: KCEpa, KCEub), where they are most isolated ecologically, and between them and conspecific

populations in the Atlantic (Fig. 1A: KBA, KES, KPER, KRJSP) and Amazon (Fig. 1A: KFG, KPA) forest regions.

#### *The brejo forests: biogeography and history*

The predominant ecosystem in north-east Brazil is the caatinga thorn forest, characterized by its dry climate and high temperatures (Sampaio *et al.*, 2002). Within it lies a mosaic of humid forested montane areas above 500 m elevation with a mean annual rainfall over 1200 mm, known as 'brejos' (Sales *et al.*, 1998; Pôrto *et al.*, 2004). The brejo forests are classified as humid evergreen montane forest within the Brazilian Atlantic forest system (Rodal *et al.*, 1998; Sales *et al.*, 1998). In Ceará State there are seven brejo forest fragments designated by Figueiredo (1997) as tropical semi-evergreen moist forest, of which we studied two. Biological studies of the brejos of north-east Brazil have mostly focused on species composition and have shown many disjunctions with the Amazon or Atlantic forests and some cases of endemism

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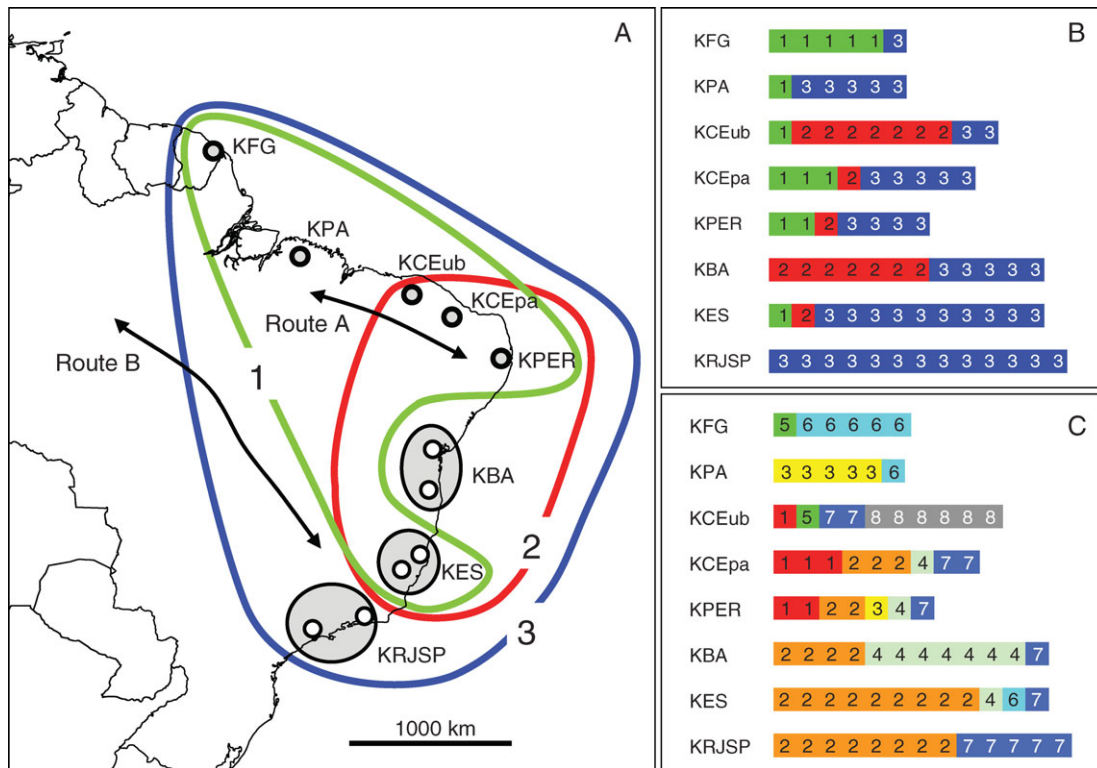


FIG. 1. *Monstera adansonii* var. *klotzschiana*. (A) Location of sampled populations in French Guiana and Brazil; see Table 1 for population codes (populations KBA, KES and KRJSP represent amalgamations of individuals from two subpopulations as shown here diagrammatically and in Table 1). Route A, Route B: hypothesized humid forest biota migration routes between Amazonia and the Atlantic forest (see text). Coloured lines: populations with members in three gene pool clusters revealed by hierarchical, optimization and Bayesian clustering methods; Cluster 1 (green), Cluster 2 (red), Cluster 3 (blue). (B, C) Modification of the diagram outputs from STRUCTURE ver. 2.1, showing the proportion of individuals from each population assigned to clusters resulting from Bayesian cluster analysis. Each numbered element represents one individual; the different colour codes and integers represent the clusters. (B) Three-cluster model; (C) eight-cluster model (populations arranged in north–south order).

(Andrade-Lima, 1966; Plowman, 1986; Sales *et al.*, 1998; Borges-Nojosa and Caramaschi, 2003; Pôrto *et al.*, 2004).

The brejo forests are thought to be natural fragments representing relicts of previous expansions of the Atlantic and Amazon forests during more humid periods of the Pleistocene and Holocene (e.g. Ab’Sáber 1982; Andrade-Lima, 1982; Bigarella and Andrade-Lima, 1982;

Borges-Nojosa and Caramaschi, 2003). In a Quaternary pollen study, Oliveira *et al.* (1999) discussed two hypothesized corridors of humid forest that could have linked the Amazon and Atlantic forests during cooler and more humid periods of the Holocene. Route A (Fig. 1A) followed the north-eastern coast from Maranhão to the region around Salvador in Bahia and Route B traversed the interior of

TABLE 1. *Monstera adansonii* var. *klotzschiana*, geographical details of sampled populations from Brazil and French Guiana

Country and region	Brazilian state	Population code	Latitude and longitude	Geographical details	Number of samples
Brazil: north-east	Bahia	KBA	13-933S, 39-271W (mean value for two localities)	Recôncavo 12-839S, 39-272W Cacao Zone (mean value for two localities) 15-0275S, 39-270W	4 8
Brazil: north-east	Ceará	KCEpa	3-956S, 38-629W	Serra de Aratanha, Pacatuba	9
Brazil: north-east	Ceará	KCEub	3-833S, 40-940W	Serra de Ibiapaba, Ubajara	10
Brazil: south-east	Espírito Santo	KES	19-559S, 40-302W (mean value for two localities)	Linhares 19-151S, 40-071W Santa Tereza 19-966S, 40-533W	7 5
French Guiana		KFG	4-624 N, 52-533W (mean value for four localities)	Petit Saut, Kaw, Matoury, Arataí	6
Brazil: Amazonia	Pará	KPA	1-509S, 48-300W	Acará, Marituba	6
Brazil: north-east	Pernambuco	KPER	8-005S, 34-946W	Recife, Dois Irmãos	7
Brazil: south-east	Rio de Janeiro, São Paulo	KRJSP	23-411S, 44-684W (mean value for two localities)	Jardim Botânico and Vista Chinesa 22-967S, 43-228W Bertioga 23-854S, 46-139W	4 9

southern Maranhão, Piauí, Bahia and central Brazil and corresponds to the gallery forest links proposed by Oliveira-Filho and Ratter (1995). Borges-Nojosa and Caramaschi (2003), based on lizard and worm lizard (amphisbaenid) biogeography, suggested that the Ceará brejos are relicts from at least two expansions of humid forest from Amazonia and the Atlantic forest during the Pleistocene (40 000–23 000 years BP) and the Holocene (6000–5000 years BP). According to Oliveira *et al.* (1999), from 11 000 to 5000 years BP the palaeoclimates hypothesized for the south-east, south and central regions of Brazil (Ledru, 1993; Behling, 1998) were similar to those for the north-east, but from about 4000 years BP north-east Brazil became much drier, while rainfall in the other regions gradually increased.

#### *Monstera adansonii*: ecology and reproductive biology

*Monstera adansonii* var. *klotzschiana* is a root-climbing hemi-epiphyte in the family Araceae (Mayo *et al.*, 1997) that occurs in humid forest environments. It is a common taxon in the eastern half of Amazonia, including the Guianas, and in the Brazilian Atlantic forest as defined by Fundação SOS Mata Atlântica (2007). It has not been observed in caatinga or other kinds of seasonal forest and is rare in undisturbed primary forest. Typically it flowers on large tree boles in secondary moist tropical forest with a well-developed canopy. Within this habitat, it flowers most conspicuously where light levels are higher, at the edge of clearings and along forest edges. It exploits natural clearings caused by tree-fall and is most abundant in habitats affected by human disturbance, but is intolerant of low humidity and unshaded positions. Our field observations in Ceará confirmed that this plant occurs only in habitats near waterfalls and streams that are permanent throughout the year, and where there is no exposure to dry winds or full sun.

The growth cycle of this taxon has been studied by Andrade and Mayo (1998) in the Atlantic forest of Pernambuco (NE Brazil). When conditions are favourable, the plant becomes robust and produces the mature perforated leaf blades typical of many *Monstera* species. Flagelliform branches of very different morphology descend to the ground and seek out new tree boles to colonize, usually forming small-leaved, terrestrial populations and hence extensive clones.

The reproductive biology of *Monstera adansonii* var. *klotzschiana* has not yet been studied in detail. The chromosome number is reported as  $2n = 60$  by Ramalho (1994). The inflorescence is a dense spike (spadix) of small, bisexual flowers without a perianth (perigone) and is protogynous as is normal in Araceae (Mayo *et al.*, 1997). The stigmas are wet at anthesis and by the time the anthers ripen and shed their pollen, the stigmas have shrivelled and are probably no longer receptive. Our assumption, based on these observations, is that outbreeding is predominant in this taxon. This is supported by Chouteau *et al.* (2006), who studied pollen/ovule ratios in Araceae from French Guiana and reported results for *Monstera adansonii* that are consistent with xenogamy.

According to Gibernau (2003), pollination in *Monstera* has been reported by only three authors (Madison, 1977; Ramirez and Gomez, 1978; Ramirez, 1980; he also mentions Grayum, unpublished), the pollinators being either trigonid bees or scarabaeid beetles. Recently, Chouteau *et al.* (M. Gibernau, Toulouse, pers. comm.) have demonstrated that the morphological and phenological syndrome of the inflorescence in *Monstera* corresponds to that of Araceae genera that are known to be bee-pollinated. Grayum (1990: p. 663, and pers. comm.), however, is doubtful that trigonid bees play any part in pollination of *M. oreophila* Madison in Panama, which appears to be pollinated before the spathe opens. However, field observations by the first author (IMA) show that the stigmas of flowering spadices of *M. adansonii* var. *klotzschiana* from Maranguape in Ceará are still wet and presumably receptive when the spathe is open, with at least the anterior part of the spadix exposed.

Madison reports the ripe infructescences and fruits of *M. adansonii* var. *klotzschiana* as medium-yellow to orange, but our observations and those of Ramalho (1994) in north-east Brazil are that they are white. The infructescences are held erect as dense spikes of fruits, which ripen by shedding the styler portion of the pistils to reveal black seeds embedded in pulp. Andrade and Mayo (1998) reported the abundant production of fertile seeds of *M. adansonii* var. *klotzschiana*, which germinated freely on the forest floor. Data on dispersal vectors is sketchy. Scott Mori and Tatyana Lobova (New York Botanical Garden, pers. comm.) have commented that the white fruits of *M. adansonii* could well be dispersed by bats, but there is no hard evidence as yet. Madison (1977) reported that the seeds of *M. adansonii* and *M. deliciosa* lose their viability rapidly if allowed to dry, but when moist they can remain viable for several months.

## MATERIALS AND METHODS

### Sampling

Seventy-five individuals were sampled from eleven humid forest localities in French Guiana, Pará, Ceará and the Brazilian Atlantic forest (Table 1, Fig. 1A). A further 24 individuals were sampled but gave only partial results and hence were later omitted. Smaller samples from different but neighbouring localities were amalgamated to form single population units (KBA, KCEub, KES, KFG, KRJSP). Because of the likelihood of extensive clonal growth in this species, care was taken to sample from plants that were widely distant in the local populations visited, and to avoid sampling from plants on the same host tree; this reduced the scope for larger samples in many populations.

### Specimen identification

*Monstera adansonii* var. *klotzschiana* is characterized by its floral and vegetative morphology and preference for shaded habitats. The stigma is elliptic and raised on a shallow but distinct styler prominence at the apex of

the gynoecium; the mature leaf blade is typically leathery, more than twice as long as wide and ovate-elliptic to oblong-elliptic with a somewhat asymmetric, acute-to-rounded leaf base. In all populations, fertile vouchers were prepared, but when fertile individuals were not seen at the time of sampling, identification of plants sampled for DNA was made in the field from living, non-flowering material using vegetative and habitat features.

Voucher specimens of all populations sampled (see Supplementary Information available online) are deposited at the following herbaria: Herbário, Universidade Federal do Ceará, Fortaleza (EAC); Herbário, Universidade Estadual de Feira de Santana (HUEFS); Herbarium, Royal Botanic Gardens, Kew (K). Species names in Araceae follow CATE Araceae (2007).

### Molecular techniques

Genomic DNA was extracted from leaf fragments dried in silica gel following the 2× CTAB protocol of Doyle and Doyle (1987) with modifications (10 mL extraction with 40 µL β-mercaptoethanol, followed by precipitation in isopropanol). All samples were then cleaned using QIAquick columns (QIAGEN Inc., Warrington, Cheshire, UK). AFLP reactions (Vos *et al.*, 1995) were conducted according to the AFLP™ Plant Mapping Protocol of PE Applied Biosystems Inc. (Applied Biosystems, 1996), using EcoRI and MseI. Eight primer combinations (with three and four selective bases on the EcoRI- and MseI-based primers, respectively) were tested on a small number of samples. The two combinations that gave the best results in terms of clarity of traces, numbers of bands and numbers of polymorphic bands, were EcoRI-ACT/MseI-CTAG and EcoRI-AGG/MseI-CTAG, and these were then used with all samples. Fragments were separated using an ABI 3100 sequencer and analysed using Genescan and Genotyper 2.0 software (PE Applied Biosystems Inc.). Amplified fragments between 50 and 500 base pairs were scored by visual inspection for presence (1) or absence (0) of peaks in the output traces. Only distinct peaks were scored as present and the manual scoring procedure was repeated three times on separate occasions to reduce to a minimum inconsistencies in scoring.

### Genetic data analysis

The resulting binary matrices of AFLP bands were used to carry out various genetic similarity analyses. Jaccard's similarity coefficient (Sneath and Sokal, 1973) was computed for all pairs of individuals using the SIMQUAL module in NTSYSpc ver. 2.20d (Rohlf, 2005), and then transformed into a dissimilarity measure as (1 – Jaccard's similarity).

Agglomerative hierarchical cluster analysis was carried out on this dissimilarity matrix using the SAHN module in NTSYSpc 2.20d with the Flexible (FLEXI) and UPGMA (unweighted pair-group method, arithmetic average) clustering methods. The Flexible clustering method was originally introduced by Lance and Williams (1967) and is defined by the parameter values of a

general recurrence formula, which determines the shortest distance between a group  $k$  and a group previously formed by the fusion of two other groups ( $i$  and  $j$ ) (Everitt *et al.*, 2001). We used a Beta parameter value of –0.25, the default setting in NTSYSpc.

A 1000 replicate bootstrap analysis of the dissimilarity matrix was carried out in NTSYSpc ver. 2.2d using the RESAMPLE and SAHN modules successively to produce 1000 bootstrapped FLEXI trees. The CONSENS module was then used to compute a majority-rule consensus tree. The original non-bootstrapped FLEXI tree with the >50 % bootstrap values added can be seen in Fig. 2.

We analysed the data using Ward's method in the PAST package (PAST ver. 1.42, Hammer *et al.*, 2001), based on a Euclidean distance matrix computed from the original binary matrix. Ward's method is an agglomerative algorithm that minimizes the increase in total error sum of squares when a new group is added to the existing one (Everitt *et al.*, 2001).

Optimization clustering was carried out on the binary matrix using the K-means algorithm implemented in PAST (Hammer *et al.*, 2001), in which, starting from a random assignment of individuals to clusters, the individuals are iteratively moved to the cluster with the closest (in Euclidean distance) centroid. K-means clustering requires an *a priori* choice of cluster number ( $k$ ), and we repeated the analysis separately for  $k$  values of 2, 3, 4 and 5.

Two model-based (Bayesian) approaches were used to investigate structure at the level of the individuals. The software package STRUCTURE version 2.1 (Pritchard *et al.*, 2000) was applied with a 'no admixture' model, 50 000 Burn-in period length and 50 000 MCMC replicates after Burn-in. This approach requires that the number of clusters ( $k$ ) is predefined, and the analysis then assigns the individuals to the clusters probabilistically. We performed five runs for each value of  $k$  (2 to 12). The output (see Supplementary Information available online) uses colour coding to show the assignments of individuals in each population to the clusters. STRUCTURE is widely used for inferring gene-pool structure in genetic data.

The other model-based cluster analysis carried out used the software package MCLUST Version 3 for R (Fraley and Raftery, 2006; R Development Core Team, 2006). The initial data frame consisted of a matrix of scores on the first 33 principal coordinate axes (encompassing 99.42 % of the variance), derived from a principal coordinate analysis (PCoA) carried out in NTSYSpc ver. 2.2d (modules DCENTER, EIGEN, MXPLOT) on the dissimilarity (1 – Jaccard's similarity) matrix. The function *mclustBIC* was used, specifying a prior distribution (*prior = priorControl()*) to provide a complete set of values for BIC (Bayesian Information Criterion), which is the parameter used to assess which of the ten models tested fits the data best.

ARLEQUIN ver. 2.000 (Schneider *et al.*, 2000) was used to compute the number of AFLP phenotypes observed in each population, using the setting 'Infer haplotype definitions from distance matrix'. Diversity measures for each population were calculated using (1) GenAEx 6 (Peakall and Smouse, 2006), which computes average expected

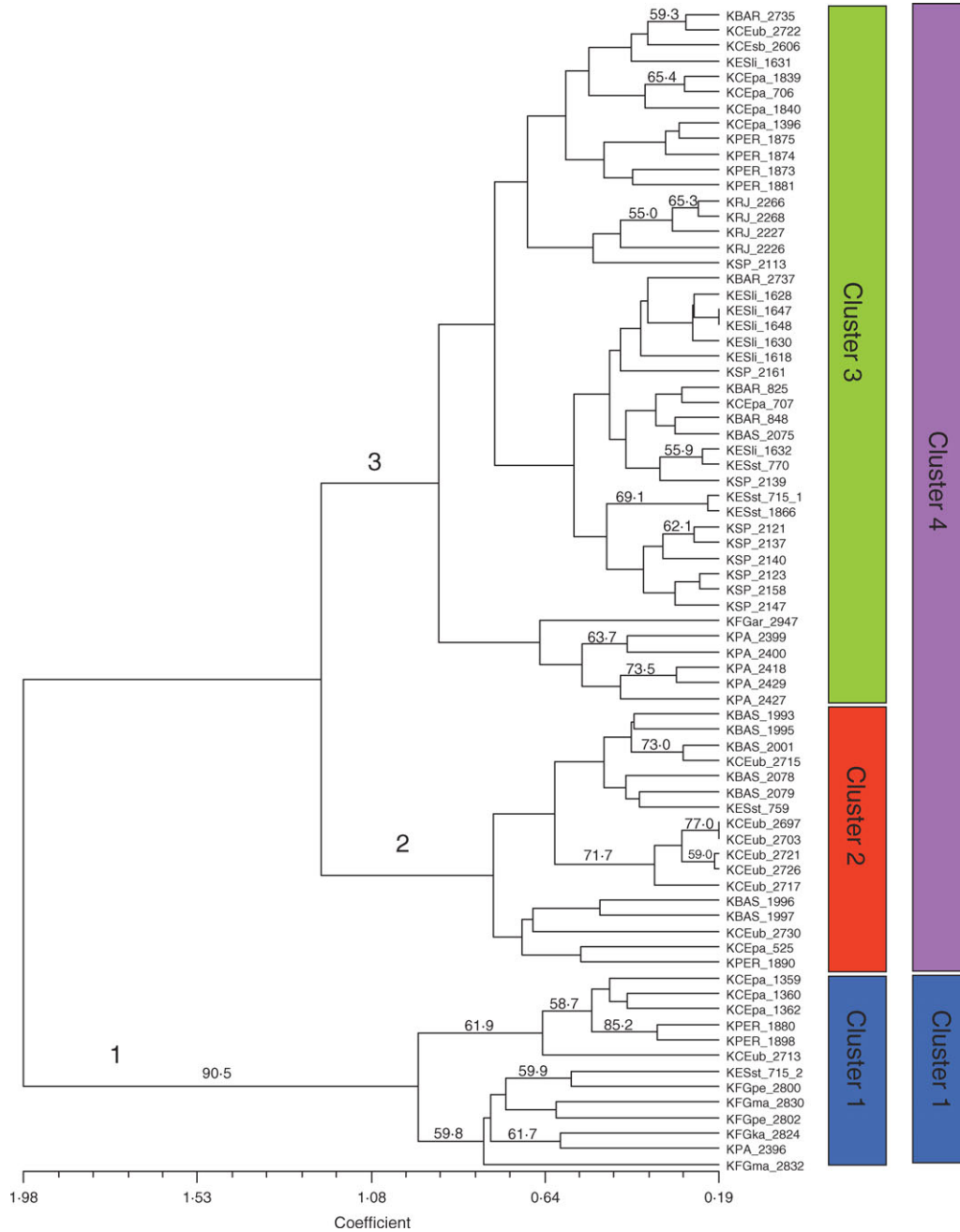


FIG. 2. *Monstera adansonii* var. *klotzschiana*, cluster analysis of 75 individuals from eight populations. Dendrogram shows hierarchical clustering using Flexible method (computed with NTSYSpc ver. 2.2), with bootstrap values >50 % from a 1000-replicate analysis (computed with NTSYSpc ver. 2.2) indicated on the branches. Coloured bars show the two-cluster and three-cluster results from other types of cluster analysis. Two-cluster model (clusters 1 and 4) was found by Bayesian analysis (STRUCTURE ver. 2.1, MCLUST ver. 3), Ward's method (PAST ver. 1.42) and K-means clustering (PAST ver. 1.42). The three-cluster model (clusters 1, 2, 3) was found by all methods except MCLUST ver. 3. Cluster 1 is robust in all analyses.

heterozygosity ( $H_e$ , equivalent to Nei's gene diversity) for each population from binary data assuming Hardy–Weinberg equilibrium, and (2) POPGENE (Population Genetic Analysis) version 1.31 (Yeh *et al.*, 1999), which computed percentage of polymorphic loci ( $p$ ) and Nei's (1973) gene diversity ( $H$ ).

Population genetic structure was studied using AFLP-SURV version 1.0 (Vekemans, 2002; Vekemans

*et al.*, 2002). The programme estimates allele frequencies at each marker locus in each population, assuming that markers are dominant and that there are two alleles per locus (presence of the band being dominant and absence recessive). We used the default option, i.e. Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), and assumed Hardy–Weinberg equilibrium for the data. Wright's fixation

index  $F_{ST}$  (Hartl and Clark, 1997) was computed using the method of Lynch and Milligan (1994) and tested by a permutation procedure of 1000 replicates, which randomly permuted individuals among the populations and then recalculated  $F_{ST}$  for each permutation; the observed value of  $F_{ST}$  was then compared to the distribution of randomized  $F_{ST}$  values.

A distance matrix of  $F_{ST}$  between every pair of populations was calculated in AFLP-SURV as a measure of interpopulation genetic differentiation, from which 1000 bootstrapped replicate matrices were then computed. The software package PHYLIP version 3.66 (Felsenstein, 2006) was used to carry out the bootstrap analysis: the module NEIGHBOR was used to compute UPGMA dendrograms for all bootstrapped matrices and an extended majority-rule consensus tree was produced using the module CONSENSE (see Fig. 3). Interpopulation genetic distances were also computed and tested using the software package GenAlEx 6 (Peakall and Smouse, 2006), which computes  $\Phi_{PT}$ , a metric analogous to  $F_{ST}$  and especially suitable for binary data. The significance of these interpopulation  $\Phi_{PT}$  distances was evaluated by a permutation test of 1000 permutations.

Analysis of Molecular Variance (AMOVA) was carried out using ARLEQUIN version 2.000 (Schneider et al., 2000).

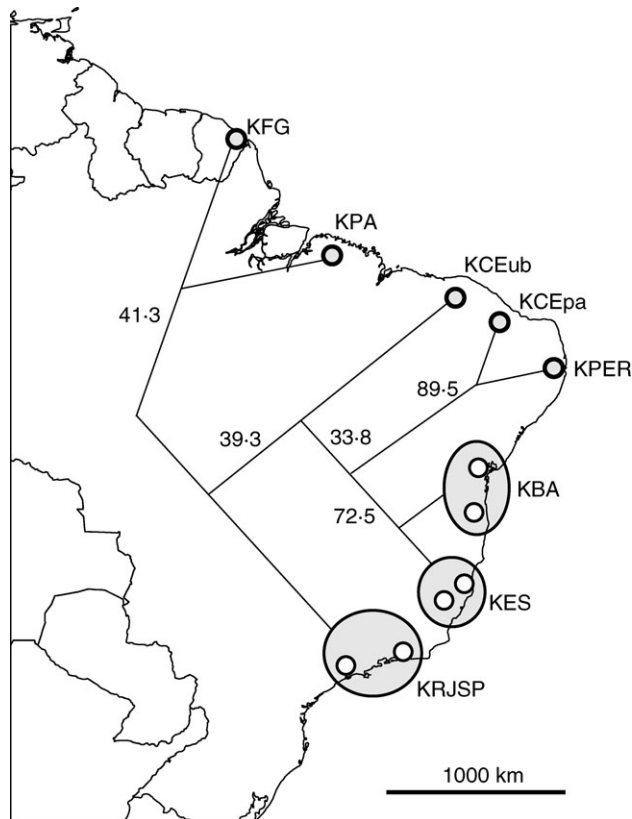


FIG. 3. *Monstera adansonii* var. *klotzschiana*, UPGMA dendrogram superimposed on map of eight populations; bootstrap values computed with PHYLIP ver. 3.66. Based on a distance matrix of  $F_{ST}$  values between every pair of populations, from which 1000 bootstrapped replicates were computed with AFLP-SURV ver. 1.0.

Genetic structure and  $F$  statistics are computed from a matrix of Euclidean squared distances between every pair of individuals. Four models of the eight populations were tested to investigate regional relationships. The first treated all populations as a single group, i.e. a two-level analysis, to obtain a value for  $F_{ST}$  as an overall measure of population divergence. The other three models divided the populations into two higher groups, which were subjected to three-level hierarchical analyses; the groupings differed as follows: (1) Ceará and Atlantic forest populations together; (2) Ceará and Amazonian populations together; and (3) Ceará, Pernambuco and Amazonian populations together (Table 2).

Isolation-by-distance was investigated by computing the correlation between geographical and genetic ( $F_{ST}$ ) population pair-wise distance matrices and applying the Mantel test, using NTSYSpc version 2.2d (Rohlf, 2005). The significance of the Mantel  $z$ -statistic value was tested non-parametrically by creating a null distribution of  $z$

TABLE 2. Analysis of Molecular Variance (AMOVA) in *Monstera adansonii* var. *klotzschiana* from Brazil and French Guiana, for 75 individuals from eight populations using 223 AFLP markers. Computed with ARLEQUIN ver. 2.0. The P values represent the probability of obtaining an equal or more extreme value by chance, estimated from 10 100 permutations

Model	Partitioning	Variance (%)	$F$ -statistic	P
Two levels: (KBA, KCEpa, KCEub, KES, KFG, KPA, KPER, KRJSP)	Among populations	18.96	$F_{ST} = 0.190$	< 0.001
	Within populations	81.04		
Three levels: Ceará populations grouped with Atlantic Forest	Among groups	11.95	$F_{CT} = 0.119$	< 0.05
Two groups: (KFG, KPA), (KBA, KCEpa, KCEub, KES, KPER, KRJSP)	Among populations within groups	13.69	$F_{SC} = 0.155$	< 0.001
	Within populations	74.36		
Three levels: Ceará populations grouped with Amazonia	Among groups	2.88	$F_{CT} = 0.029$	< 0.001
Two groups: (KFG, KPA, KCEpa, KCEub), (KBA, KES, KPER, KRJSP)	Among populations within groups	17.11	$F_{SC} = 0.176$	< 0.001
	Within populations	80.01		
Three levels: Ceará and Pernambuco populations grouped with Amazonia	Among groups	4.44	$F_{CT} = 0.044$	< 0.001
Two groups: (KFG, KPA, KCEpa, KCEub, KPER), (KBA, KES, KRJSP)	Among populations within groups	16.04	$F_{SC} = 0.168$	< 0.001
	Within populations	79.52		
	Among populations		$F_{ST} = 0.205$	< 0.001

using 10 000 random permutations and comparing the observed  $z$  value.

Population structure was also investigated by the Bayesian software HICKORY version 1.0.4 (Holsinger *et al.*, 2002; Holsinger and Lewis, 2003), in order to assess the importance of inbreeding in the data and the assumption of Hardy–Weinberg equilibrium. HICKORY makes it possible to evaluate departures from Hardy–Weinberg equilibrium in dominant as well as co-dominant markers. The AFLP data were fitted to four models, ‘full model’, which allows for inbreeding, ‘ $f=0$ ’ model, which implies lack of inbreeding, ‘ $\theta=0$ ’ model, which implies a zero-valued  $F_{ST}$  analogue (i.e. no differentiation between populations), and ‘ $f$  free’ model, which decouples the estimates of  $f$  and  $\theta$ . Computations were carried out using the default values, as recommended in the manual (Holsinger and Lewis, 2003): burn-in = 5000, number of samples = 25 000 and thinning factor = 5. To estimate the best fit of the four models, the criteria  $DIC$  (deviance information criterion),  $Dbar$ ,  $Dhat$  and  $pD$  were used, with interpretations following the recommendations given in the manual.

## RESULTS

The matrix of 75 individuals from which satisfactory results were obtained in both primer combinations was made up of 223 fragments; 88.79 % of the scored marker bands were polymorphic. All AFLP phenotypes recorded were unique to an individual and we tentatively concluded that no resampling of clones had occurred during the field collections (see Discussion).

### *Hierarchical and model-based cluster analysis*

Agglomerative hierarchical cluster analysis using flexible method (FLEXI in NTSYSpc) and Ward’s method (in PAST) resulted in dendrograms in which either two or three major clusters were differentiated with the same composition of individuals (Clusters 1–4, Fig. 2); cluster 1 had 90.5 % bootstrap support (1000 replicates). Optimization methods (K-means analysis in PAST) and the Bayesian analysis package STRUCTURE also found these clusters for settings of  $k=2$  and  $k=3$  ( $k$  = number of clusters; Figs 1B and 2). Principal coordinate analysis (PCoA, see Supplementary Information available online) showed a clear differentiation of cluster 1 and a lesser distinction of clusters 2 and 3; the first two principal coordinate axes accounted for a total of 34.2 % of the total variance in the data.

In the genetic structure analysis (STRUCTURE), the highest estimate of the likelihood of the data, given the number of clusters chosen, was obtained when  $k=8$ , i.e. with eight clusters. The diagram showing assignment of individuals to the clusters revealed a clinal structure to the data (Fig. 1C, see Supplementary Information available online). However, for models with more than three clusters ( $k > 3$ ) cluster composition became variable between runs. Reflecting this, the variance values of the five runs fluctuated considerably once the  $k$  value exceeded  $k=4$  and

increased progressively; the largest  $k$  value for which cluster composition was constant was found to be  $k=3$  (Table 3). Figure 1A shows the occurrence of the clusters (gene pools) in the sampled populations for the three-cluster model, and the assignment of individuals to both three- and eight-cluster models (Fig. 1B, C). Gene pool 3 is the most widespread pattern, represented in all populations and the only one found in the southernmost population (KRJSP). Gene pool 2 is the most restricted, occurring only in north-east Brazil and Espírito Santo. Gene pool 1 is absent from the southern Brazilian and Bahian populations.

Model-based clustering using MCLUST found the best fit with a two-cluster solution (Fig. 2, Clusters 1 and 4), when scores from all 33 principal coordinates were used (comprising 99.42 % of total variance). The highest values for BIC all favoured the VEI model (Volume = variable, Shape = equal, Orientation = coordinate axes) and were, in descending order, 6734.082 for a two-cluster solution, 6663.384 for a three-cluster solution and 6583.755 for a four-cluster solution. The two clusters of the optimal model correspond in composition to Cluster 1 and Cluster 4 in the hierarchical cluster analyses (Fig. 2). Analyses of scores from the first ten principal coordinates (comprising 67.81 % of total variance), found an optimal six-cluster solution (see Supplementary Information available online), in which the groups corresponding to clusters 2 and 3 in the other analyses were further subdivided. In a similar way to the eight-group STRUCTURE solution, this seems to reflect the presence of further substructure in the data, which is not sufficiently well-defined to emerge consistently in all analyses.

The two-cluster structure (Fig. 2, Clusters 1 and 4) is clear in all the different types of cluster analysis and consistent in assignment of individuals to the clusters. All analytical approaches except MCLUST also agreed in the composition of the three-cluster structure. It thus seems reasonable to infer that there are at least two distinct

TABLE 3. Genetic structure analysis of eight populations of *Monstera adansonii* var. *klotzschiana*. Summary of results of the three-cluster model (identical values obtained over five runs) computed with STRUCTURE version 2.1. Values are percentage representation of each gene pool in each population sampled. Populations arranged in geographical order from north to south

Population	Sample size	Cluster (inferred gene pool)		
		1	2	3
French Guiana (KFG)	6	83.30	0.00	16.70
Pará (KPA)	6	16.70	0.00	83.30
Ubajara, Ceará (KCEub)	10	10.00	70.00	20.00
Pacatuba, Ceará (KCEpa)	9	33.30	11.10	55.60
Pernambuco (KPER)	7	28.60	14.30	57.10
Bahia (KBA)	12	0.00	58.30	41.70
Espírito Santo (KES)	12	8.30	8.30	83.30
Rio-São Paulo (KRJSP)	13	0.00	0.00	100.00

historical gene pools represented within the overall range sampled and probably three (Fig. 1).

#### Population genetic diversity

The POPGENE and GenAlEx 6 packages agreed precisely in values for average expected heterozygosity or Nei's gene diversity (Table 4). The results showed relatively higher diversity for the two Ceará populations and lowest diversity for the southernmost population (KRJSP). Comparison with the analyses of individuals (Fig. 1A, B) suggests that this may be due to the presence in the KRJSP population of only one gene pool.

Hierarchical cluster analysis (UPGMA), using interpopulation distance matrices of population pair-wise  $F_{ST}$  (see Supplementary Information available online), grouped the Ceará populations with those in the Atlantic forest, the two Amazonian populations forming a separate subgroup (Fig. 3). The generally low bootstrap support, except for the two pairs KCEpa/KPER (89.5 %) and KBA/KES (72.5 %), shows that over the large region sampled (3232 km maximum extent), geographical structure is relatively weak.

The permutation test of inter-population distance using GenAlEx 6 ( $\Phi_{PT}$ , 1000 permutations), found all but two pairs (KCEpa/KPER, KPA/KPER) significantly different ( $P < 0.02$ ). The genetic distance separating the two populations from Ceará (pairwise  $F_{ST} = 0.137$ ,  $P = 0.003$ ) was statistically significant, although they are geographically closer than any other pair (257 km). KPA is inconsistent in pairing with KFG in the UPGMA population cluster analysis, but preferentially with KPER in the  $\Phi_{PT}$  pairwise distance analyses. This also suggests generally weak genetic structure at the population level.

The two-level AMOVA in ARLEQUIN found an  $F_{ST}$  value of 0.1896 ( $P < 0.001$ ), with 18.96 % of variation among populations and 81.04 % within populations

TABLE 4. Genetic diversity measures in eight sampled populations of *Monstera adansonii* var. *klotzschiana* from Brazil and French Guiana, computed with POPGENE ver. 1.31 and GenAlEx 6. See Table 1 for key to population codes

Population code	Sample size	Percentage polymorphic loci ( $p$ )	Average expected heterozygosity ( $H_e$ ) equivalent to Nei (1973) gene diversity	
			Mean	s.d.
KBA	12	51-12	0.1575	0.013
KCEpa	9	60-54	0.1832	0.012
KCEub	10	57-85	0.1706	0.013
KES	12	59-64	0.1641	0.013
KFG	6	61-43	0.1672	0.011
KPA	6	45-74	0.1503	0.013
KPER	7	51-57	0.1589	0.012
KRJSP	13	42-60	0.1272	0.012

(Table 2). Three-level hierarchical AMOVA analyses of the two-group models (Table 2) supported the results of cluster analysis in favouring the association of the Ceará populations with those of the Atlantic Forest rather than with the Amazonian ones. Percentage variation among groups was 11.95 % when the Ceará populations are grouped with those of the Atlantic forest, in comparison to values of 2.88 % and 4.44 % for the other two models, which grouped the Ceará populations with those in Amazonia.

A Mantel test showed strong correlation between the geographical distance matrix and the matrix of pair-wise  $F_{ST}$  derived from AFLP-SURV [Fig. 4,  $P$  (random  $z \geq$  observed  $z$ ) = 0.002,  $r = 0.6903$ ]. This result allows us to infer that over the sampled range, *M. adansonii* var. *klotzschiana* shows a historical pattern of isolation-by-distance (Wright, 1943, 1946), i.e. that during the period of time represented by these patterns, gene flow and genetic drift were in equilibrium.

The Bayesian software HICKORY results showed that inbreeding was unlikely to be a major driving force in determining the gene pool structure in this taxon (Table 5). Although the  $DIC$  parameter was lower in the *Full* model than in the  $f = 0$  model (difference in  $DIC$  value = 17.74), the difference was more than accounted for by the number of parameters in the two models (difference in  $pD = 33.85$ ), and the other two parameters ( $Dbar$

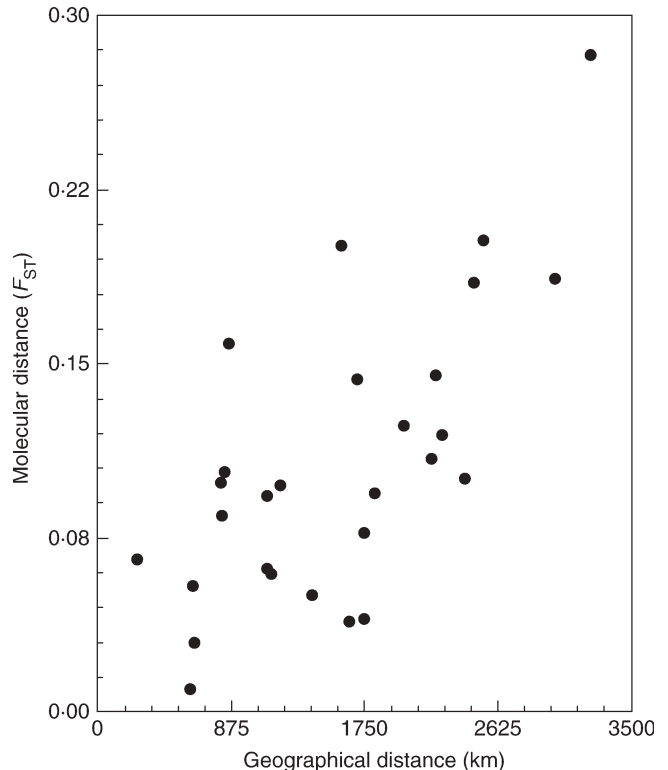


FIG. 4. *Monstera adansonii* var. *klotzschiana*, bivariate plot showing significant correlation between matrices of pair-wise geographical (km) and genetic distance ( $F_{ST}$ ) in eight populations, comprising 75 individuals [matrix correlation (= normalized Mantel statistic  $z$ ):  $r = 0.6903$ ,  $P$  (random  $z \geq$  observed  $z$ ) = 0.002]. Computed with NTSYSpc ver. 2.2.

TABLE 5. Genetic structure analysis using HICKORY ver. 1.0.4 (Holsinger and Lewis, 2003). Default values for computations were used as follows: Burnin 5000; sample 25000; thin 5. See text for further explanation

Model	Parameter					
	<i>Dbar</i>	<i>Dhat</i>	<i>pD</i>	<i>DIC</i>	<i>f</i>	<i>Theta B</i>
<i>Full</i>	3607-6247	2916-1145	691-5102	4299-135	0-9849	0-1753
<i>f = 0</i>	3591-5243	2866-169	725-3554	4316-8797		0-1262
<i>Theta B = 0</i>	5589-0359	5399-6654	189-3704	5778-4063	0-9828	
<i>f free</i>	3630-996	2906-9112	724-0847	4355-0807	0-4981	0-1609

and *Dhat*) were lower in the  $f = 0$  model ( $Dbar = 3591.52$ ,  $Dhat = 2866.17$ ) than in *Full* model ( $Dbar = 3607.62$ ,  $Dhat = 2916.11$ ). Because the model with the lowest *Dbar* value is that which best fits the data, this pattern of results allows the  $f = 0$  model (inbreeding equals zero) to be considered at least as likely as the *Full* model, according to the HICKORY manual (Holsinger and Lewis, 2003).

## DISCUSSION

This study is the first known to us that compares genetic patterns in plants at population level in the brejo forests of north-east Brazil and as such is preliminary, given the absence of strictly comparable work in the region and the small sample sizes we had available. Instead of showing strong local differentiation in the brejo forest populations and marked differences between the Amazonian and Atlantic forest, the results present a picture of a widespread taxon with a clinal pattern of genetic variability and rather low differentiation over a subcontinental geographical region. The overall  $F_{ST}$  value of 0.1896 in *M. adansonii* var. *klotzschiana* is comparable with other plants having outcrossing or mixed mating systems, reviewed by Morjan and Rieseberg (2004 : Table 3 and Supplementary Information), although rather lower than in cases sampled across comparably large geographical regions. The low  $F_{ST}$  value represents high within-population variation and low differentiation between populations. Assogbadjo *et al.* (2006) reported a comparable result for *Adansonia digitata* (Malvaceae,  $\Phi_{PT} = 0.176$ , 17.63 % variation among populations, 82.37 % within populations), considered an outbreeder on other evidence. Cardoso *et al.* (2005), in a study of isolated populations of *Caesalpinia echinata* (Leguminosae) from eastern Brazil, found a strong correlation between genetic and geographical distance, but over half (61 %) of total genetic variation partitioned between populations. They proposed that this could have resulted from ancient fragmentation of seasonally dry vegetation within a predominant matrix of moist Atlantic forest, a comparable but inverse scenario to that of *Monstera adansonii*.

The strong pattern of isolation-by-distance and the lack of evidence for inbreeding suggest that this is a historical pattern brought about by extensive gene flow which, as the distance between localities increases, has had a decreasing effect in cancelling out genetic differentiation caused by genetic drift. In a study of frog molecular phylogeography, Carnaval (2002) concluded that geologically recent, but not present-day, gene flow had taken place between

brejo forest and coastal populations in the Atlantic forest of the north-eastern Brazilian states of Pernambuco and Alagoas.

Genetic structure analysis of the individuals shows evidence that two distinct gene pools are represented in the data, suggesting that different historical phases of widespread gene flow have taken place in the region sampled. Many of the analyses also indicated the presence of a third gene pool, and the eight-cluster result from the Bayesian STRUCTURE analysis even hinted at gradual and successive replacement of locally occurring, but weakly differentiated, genetic patterns from French Guiana in the north-west to São Paulo in the south-east (Fig. 1C). *Monstera adansonii* var. *klotzschiana* is restricted to humid forest habitats and although information on its ecology and population biology is sparse, it nevertheless seems unlikely that gene flow by pollen or seed dispersal can occur between populations separated by semi-arid environments. We therefore tentatively conclude that the hypothesized gene flow episodes could have been correlated with cycles of humid forest expansion across eastern South America.

The two brejo forest populations sampled in Ceará were found to be well differentiated from each other and as diverse or more so than those elsewhere. Our initial expectation had been that the Ceará populations would show lower-than-average diversity, due to effects of genetic drift and restricted gene flow in these small, ecologically and geographically isolated, natural forest ‘refugia’. Instead, it was the southernmost population from the Rio de Janeiro–São Paulo region that showed lower-than-average genetic diversity. Further studies are needed to explore these patterns, but there is at least an indication in our results that successive population expansions involving either Amazonia or the Atlantic forest, or both, have led to an accumulation of diversity in the forest fragments in Ceará that has counteracted genetic erosion.

The among-population relationships show that the Ceará populations are related to those of the Atlantic forest in north-east Brazil rather than to Amazonian ones. Although bootstrap values are mostly rather low in the UPGMA tree (Fig. 3), this interpretation is supported by the hierarchical AMOVA analyses (Table 2). Our results are in agreement with the current classification of the Ceará brejo forests as part of the Atlantic forest of Brazil (Fundação SOS Mata Atlântica, 2007). A similar conclusion was reached for the herpetofauna of the Baturité massif in Ceará by Borges-Nojosa (1991), who reported a

biogeographic pattern of disjunctions dominated by Atlantic forest taxa.

From the conservation standpoint the results show that in north-east Brazil isolated populations in small and threatened areas of forest with low plant species endemism have unique intraspecific genetic diversity patterns and population diversity at least as high as that in conspecific populations from known biodiversity hotspots, such as the Atlantic forest and the Belém region of Amazonia. Future genetic investigations of other long-lived and widely distributed brejo forest species such as the trees *Schefflera morototoni* (Araliaceae), *Tapirira guianensis* and *Thyrsodium schomburgkianum* (both Anacardiaceae) would be interesting to compare with our results.

Our interpretations and conclusions are necessarily tentative at this stage, but the study is a useful basis from which to develop more comprehensive and detailed work in the future. Larger samples are needed to confirm the gene pool cluster patterns and population diversity levels reported here. Analysis of duplicates from the same individuals is needed to improve error estimates in AFLP band scoring and to inform the evaluation of clonality in the populations. More sampling localities are required, especially in the southern Atlantic forest, where genetic diversity was found to be least, and in Amazonia, where *Monstera* species diversity is much greater. Future work should focus on co-dominant markers such as nuclear microsatellites. Given the preliminary nature of this project, we chose to use AFLP as an efficient technique for obtaining a broad picture of molecular patterns in a previously unstudied taxon. However, AFLP markers are dominant and provide no information on heterozygote frequencies and hence no direct estimates of inbreeding. Although we found no evidence of inbreeding from HICKORY software analysis, the use of co-dominant markers would provide confirmation of this and better understanding of the processes that have conditioned the observed genetic structure. The current patchy understanding of the mating system also prevented us from inferring the contribution of inbreeding to the genetic structure observed, pointing to the need for new research on reproductive ecology in *M. adansonii*.

In conclusion, the AFLP markers in populations of *M. adansonii* var. *klotzschiana* show a pattern of relatively high within-population diversity and relatively low, although still significant, among-population divergence. For the populations in Ceará, these results were unexpected given their geographical and ecological isolation, since their within-population diversity was as high as or higher than that of populations in the Atlantic forest and Amazonia. The widespread pattern of isolation-by-distance and the distinct gene pool patterns found over the range sampled suggest that episodes of extensive gene flow have occurred involving populations in Amazonia, Ceará and the Atlantic forest of Brazil. We hypothesize that this is a legacy from an epoch when gene flow within continuous humid forest habitat was possible, rather than evidence of present-day long-distance gene dispersal, and suggest that our results are consistent with theories proposed by various previous authors of Holocene migration of forest

biota between Amazonia and the Atlantic forest. The genetic differentiation and relatively high diversity of the separate brejo populations in Ceará emphasize their biological uniqueness and scientific importance, despite their threatened status and highly disturbed condition. Measures to reinforce conservation of these forests are urgently needed.

#### SUPPLEMENTARY INFORMATION

Supplementary information is available online at <http://aob.oxfordjournals.org/> and gives the following: (1) Voucher herbarium specimens documenting the populations sampled; (2) Gene pool cluster diagrams from STRUCTURE ver. 2.1, showing clinal structure in three separate runs of the eight-cluster model; (3) Principal Coordinates Analysis (PCoA) ordination showing six-cluster model computed with MCLUST ver. 3; (4) PCoA ordination showing the three-cluster model; and (5) Tables of pairwise inter-population  $F_{ST}$ ,  $\Phi_{PT}$  and geographical distance.

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