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Population differentiation and species cohesion in two closely related plants adapted to neotropical high-altitude 'inselbergs', *Alcantarea imperialis* and *Alcantarea geniculata* (Bromeliaceae)

T. BARBARÁ,* G. MARTINELLI,‡ M. F. FAY,* S. J. MAYO† and C. LEXER*
**Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, UK, †Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK, ‡Instituto de Pesquisas Jardim Botânico do Rio de Janeiro (JBRJ), Rua Pacheco Leão 915, 22460-030 Rio de Janeiro, Brazil*

Abstract

Isolated granitic rock outcrops or 'inselbergs' may provide a window into the molecular ecology and genetics of continental radiations under simplified conditions, in analogy to the use of oceanic islands in studies of species radiations. Patterns of variability and gene flow in inselberg species have never been thoroughly evaluated in comparison to related taxa with more continuous distribution ranges, or to other species in the same kingdom in general. We use nuclear microsatellites to study population differentiation and gene flow in two diploid, perennial plants adapted to high-altitude neotropical inselbergs, *Alcantarea imperialis* and *Alcantarea geniculata* (Bromeliaceae). Population differentiation is pronounced in both taxa, especially in *A. imperialis*. Gene flow in this species is considerably lower than expected from the literature on plants in general and Bromeliaceae in particular, and too low to prevent differentiation due to drift ($N_em < 1$), unless selection coefficients/effect sizes of favourable alleles are great enough to maintain species cohesion. Low gene flow in *A. imperialis* indicates that the ability of pollinating bats to promote gene exchange between inselbergs is smaller than previously assumed. Population subdivision in one inselberg population of *A. imperialis* appears to be associated with the presence of two colour morphs that differ in the coloration of rosettes and bracts. Our results indicate a high potential for inselbergs as venues for studies of the molecular ecology and genetics of continental radiations, such as the one that gave rise to the extraordinary diversity of adaptive strategies and phenotypes seen in Bromeliaceae.

Keywords: adaptive radiation, bromeliad, gene flow, inselberg, microsatellites, species cohesion

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Introduction

Experimental observations on islands have a long tradition in studies of biogeography and evolution (Darwin 1859; MacArthur & Wilson 1967). Similar to oceanic islands, isolated granitic rock outcrops or inselbergs (from German Insel = island and Berg = mountain) have been suggested as model systems for ecological and evolutionary studies (Prance 1996; Porembski & Barthlott 2000). The insular nature of these ancient monoliths harboring specialized animal and plant life has raised the expectation that inselbergs may indeed represent 'terrestrial habitat islands', resembling oceanic islands as models for studying ecological and evolutionary processes (Porembski & Barthlott 2000). If this view is correct, then inselbergs may contribute to much-needed research towards integrating genetics into the ecological theory of adaptive radiation, as encouraged by Schluter (2000). In particular, inselberg radiations may then provide a window on the molecular ecology and genetics of continental adaptive radiations, complementary to the much celebrated examples of radiations on islands (Schluter 2000; Emerson 2002).

Correspondence: Thelma Barbará, Fax: +44(0)20 8332 5310; E-mail: thelmabarbarakew@yahoo.co.uk and t.barbara@kew.org

An important aspect is that inselbergs are thought to provide a simpler setting for evolutionary studies than other systems of terrestrial habitat islands, such as continental mountain ranges (Schonswetter *et al*. 2005; Hughes & Eastwood 2006) or the 'sky islands' of western North America (Knowles 2001; DeChaine & Martin 2005; Smith & Farrell 2005). Indeed, from an experimental evolutionary biologist's point of view, inselbergs may be more similar to terrestrial lakes, such as those utilized for studies of ecotype differentiation, speciation, and adaptive radiation in lake whitefish (Campbell & Bernatchez 2004), cichlids (Barluenga *et al*. 2006), or sticklebacks (Schluter 2000). To our knowledge, however, patterns of genetic variability and gene flow in inselberg species have never been evaluated in comparison to related taxa with more continuous distribution ranges, or to patterns of gene flow in other organisms in general. Here, we aim at filling this gap. A recent review of the role of gene flow and selection in the maintenance of species cohesion (Morjan & Rieseberg 2004) allows us to put our data in the context of results in many other species.

Our study is focused on inselberg-dwelling plants in the Bromeliaceae (bromeliads), a family that represents a particularly striking and phylogenetically well-characterized continental adaptive radiation (Brown & Gilmartin 1989; Givnish *et al*. 1997; Smith & Till 1999; Benzing 2000; Barfuss *et al*. 2005). Bromeliaceae is one of the predominant plant families found on South American inselbergs (Barthlott *et al*. 1993; Safford & Martinelli 2000). Population genetic studies exist for several bromeliads in different subfamilies (Soltis *et al*. 1987; Murawski & Hamrick 1990; Izquierdo & Pinero 2000; Sarthou *et al*. 2001; Gonzalez-Astorga *et al*. 2004; Sgorbati *et al*. 2004; Cavallari *et al*. 2006), which allows us to compare genetic attributes of related species with varying breeding systems. Here, we focus on *Alcantarea imperialis* (Carriere) Harms and *Alcantarea geniculata* (Wawra) J.R. Grant, two inselberg-adapted bromeliads in the Atlantic Rainforest of Brazil, one of the world's most important and vulnerable biodiversity 'hotspots' (Myers *et al*. 2000).

Alcantarea imperialis and *A. geniculata* are perennial, rupicolous plants (they grow on rocks with very limited substrate). They are characterized by hermaphrodite flowers and mixed mating or outcrossing breeding systems with animal pollination and wind-based seed dispersal (Martinelli 1994; Safford & Martinelli 2000). Their vegetative structures form 'tanks' that are able to hold many litres of water (up to 40 L in some cases), thus providing an important resource base for associated biota in this harsh high-altitude environment (Benzing 2000). *Alcantarea imperialis* and *A. geniculata* are part of a larger radiation that includes numerous other taxa of the closely related genera *Alcantarea* and *Vriesea*, many of which occur on inselbergs (Safford & Martinelli 2000). However, these two diploid perennial species are especially suitable for our purpose. Pollinator observations and hand-pollination experiments in *A. imperialis* indicate that it favours outcrossing over selfing (Martinelli 1994), and thus this species represents a conservative model case for assessing the effect of inselberg adaptation on population connectivity. Less information exists about the pollination syndrome of the closely related *A. geniculata* (Porsch 1935; Martinelli 1994), but we anticipated that inspection of Hardy–Weinberg proportions would provide us with clues about its breeding system at the outset of the study. The two species are clearly recognizable based on consistent vegetative features (Martinelli 1994) and they co-occur on some inselbergs, which makes a joint analysis sensible.

Here, we address the following questions related to the molecular ecology and population genetics of *Alcantarea* spp. on neotropical high-altitude inselbergs:

1 How do levels of population differentiation in these inselberg species compare to other bromeliads, or to other plants in general?
2 Are the observed levels of differentiation compatible with the view of inselbergs as 'terrestrial islands', and if yes, what are the relative roles of gene flow and selection in maintaining species cohesion in the face of fragmentation?
3 To what extent are molecular marker-based breeding system parameters congruent with knowledge of the pollination syndromes of these two closely related congeners?
4 Is there evidence for population subdivision on inselbergs?

We use our data to assess the usefulness of inselbergs as model cases for studying the molecular ecology and genetics of a continental plant radiation, and we comment on implications for conservation in this world biodiversity 'hotspot'.

Materials and methods

Population sampling

A total of eight populations of *Alcantarea imperialis* and *Alcantarea geniculata* were sampled on high-altitude granitic inselbergs located in the Atlantic Rainforest of southeastern Brazil (states of Rio de Janeiro and Minas Gerais) (Fig. 1). Sampling on high-altitude outcrops in the tropics is a costly and demanding procedure (few localities are accessible without a helicopter and rappelling), and thus our sampling design was chosen to extract a maximum of information without sampling all populations of each species. Rather, populations were sampled in such a way as to represent the species range of each of these endemics and to provide a broad range of geographical distances between populations, including both neighbouring and also more distant population pairs for each species. The

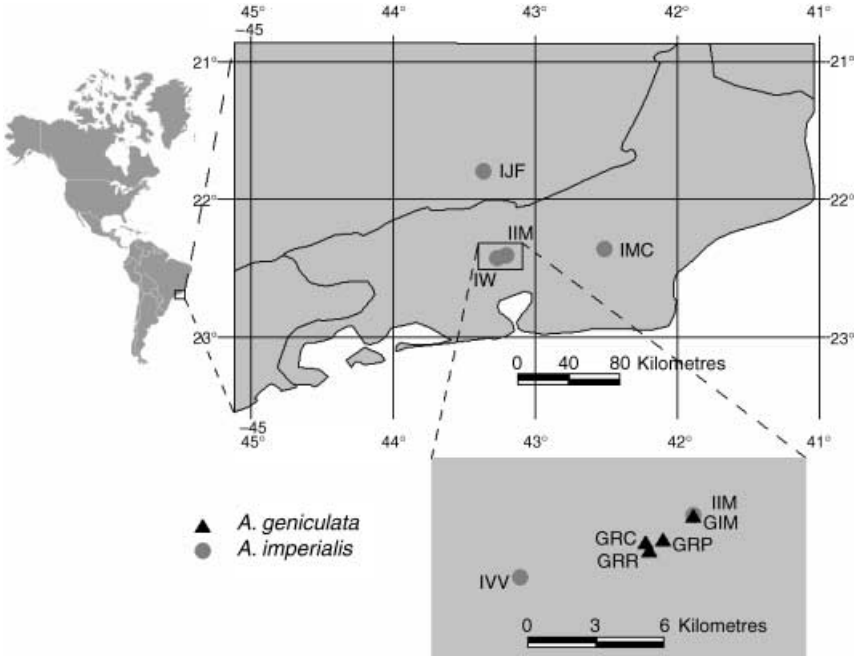

Fig. 1 Distribution map of *Alcantarea* inselberg populations sampled in the Atlantic Rainforest of Brazil. For population abbreviations and details see Materials and methods.

names, abbreviations, and geographical coordinates of the sampled populations are as follows: *A. imperialis*: Imperialis 'Irmã Menor' or IIM (22°24.401'S, 43°12.143'W), Imperialis 'Macaé-de-Cima' or IMC (22°22.176'S, 42°29.774'W), Imperialis 'Juíz-de-Fora' or IJF (21°47.922'S, 43°22.243'W), and Imperialis 'Vale das Videiras' or IVV (22°25.870'S, 43°16.228'W); *A. geniculata*: Geniculata 'Irmã Menor' or GIM (22°24.401'S, 43°12.143'W), Geniculata 'Ricardo Clearing' GRC (22°25.044'S, 43°13.262'W), Geniculata 'Ricardo Rock' GRR (22°25.232'S, 43°13.183'W), and Geniculata 'Reserva Privada' GRP (22°24.960'S, 43°12.844'W).

The altitudes of the sampled populations ranged between 872 m and 1310 m above sea level. Geographical distances between populations ranged from 7 to 110 km with an average of 68 km for the Atlantic Rainforest endemic *A. imperialis*, and from 0.4 to 2.4 km with an average of 1.4 km for the narrow endemic *A. geniculata*. The species co-occur on one of the sampled inselbergs, namely the rock outcrop 'Irmã Menor' (populations IIM and GIM). Population IMC of *A. imperialis* consisted of plants of two different colour morphs: one with green and one with red rosettes and bracts. The two colour morphs were found interdigitated at roughly equal frequency with no obvious spatial pattern. Inspection of juvenile plants in the population suggested that colour morphs segregated like a heritable character with simple Mendelian mode of inheritance, but controlled crosses have not yet been analysed. Sample sizes for all populations are given in Table 2. For each plant, leaf material for DNA extraction was collected in silica gel.

Molecular markers and genotyping assays

Six of the eight microsatellite markers used in this study were assayed for the first time in *Alcantarea* spp. by cross-species amplification from the related bromeliad genera *Tillandsia* and *Guzmania* (markers e6, p2p19, e19, e6b, and CT5; Boneh *et al*. 2003) and from *Pitcairnia* (Pit8; Sarthou *et al*. 2003). Two additional markers, Ai4.10 and Ai4.3, were isolated de novo from *A. imperialis* and are reported in more detail elsewhere, in combination with new markers from another bromeliad species (Palma-Silva *et al*. 2007). Repeat types and molecular size ranges for all eight markers in *A. imperialis* and *A. geniculata* are given in Table 1.

For molecular genotyping, total genomic DNA was extracted from silica gel-dried leaves using a modified approach based on Doyle & Doyle (1987), and DNA was quantified using an Eppendorf BioPhotometer. The eight nuclear microsatellites were polymerase chain reaction (PCR)-amplified following methods described previously by Burke *et al*. (2002), making use of a standard touchdown cycling programme with an annealing temperature (T_a) of 48 °C and either FAM- or JOE-labelled forward primers, or a three-primer protocol including unlabelled M13-tagged forward and unlabelled/untagged reverse primers for each marker, and a third 'universal' M13-primer labelled with one of the fluorescent dyes, FAM or JOE (Applied Biosystems). Microsatellite genotypes were resolved on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems), making use of the different fluorescent dyes for duplexing. Molecular sizes in base pairs were determined using the

Table 1 Characterization of microsatellite markers in 'inselberg' populations of *Alcantarea imperialis* and *Alcantarea geniculata*, including marker source, repeat type, molecular size range in each *Alcantarea* spp. in base pairs (bp), number of alleles (A), expected (H_E) and observed (H_O) heterozygosity, within-population inbreeding coefficient (F_{IS}), and total-population inbreeding coefficient (F_{IT}) in each species

		A. imperialis						*A. geniculata*					
Locus	Repeat type	Size range (bp)	A	H_E	H_O	F_{IS}	F_{IT}	Size range (bp)	A	H_E	H_O	F_{IS}	F_{IT}
Ai4.10†	di	184–188	3	0.446	0.066	–0.007	+0.879	184–191	4	0.554	0.408	+0.175	+0.297
E19‡	di	115–123	4	0.509	0.402	+0.000	+0.264	105–139	6	0.104	0.083	+0.196	+0.202
E6‡	tri	107–128	3	0.510	0.405	+0.002	+0.309	107–128	3	0.275	0.301	–0.215	–0.058
Ai4.3†	di	191–195	3	0.496	0.279	+0.002	+0.389	191	1	—	—	—	—
CT5‡	di	157–195	14	0.723	0.448	–0.002	+0.346	159–187	8	0.707	0.570	+0.060	+0.238
E6b‡	tri	128–146	7	0.781	0.475	+0.001	+0.420	128–153	5	0.135	0.128	+0.043	+0.054
P2p19‡	tri	185–214	9	0.812	0.491	+0.001*	+0.324	178–208	5	0.590	0.526	+0.062	+0.128
Pit8§	di	280–318	9	0.646	0.328	–0.002	+0.575	280–314	7	0.635	0.481	+0.210***	+0.255

†Microsatellite markers isolated de novo from *Alcantarea imperialis* in the Jodrell Laboratory at RBG Kew (Palma-Silva *et al*. 2007). ‡Markers isolated by Boneh *et al*. (2003). §Marker isolated by Sarthou *et al*. (2003). Significant F_{IS} values for particular loci are indicated by asterisks (*$P < 0.05$, ***$P < 0.005$).

Table 2 Characterization of 'inselberg' populations of *Alcantarea imperialis* and *Alcantarea geniculata* with eight nuclear microsatellite markers, including the number of chromosomes sampled (N), variance in allele size (Var), allelic richness, as well as expected (H_E) and observed (H_O) heterozygosities. Departures from Hardy–Weinberg equilibrium are indicated by asterisks (*$P < 0.05$, ***$P < 0.005$)

Species	Population	N	Var	Allelic richness	H_E	H_O
A. imperialis	IIM	40	16.4	2.20	0.327	0.304
	IMC	112	13.3	2.29	0.395	0.365
	IJF	52	28.6	2.68	0.452	0.400*
	IVV	44	21.9	2.75	0.421	0.362
	Overall:	248	27.96	6.25	0.615	0.362
A. geniculata	GIM	52	26.5	2.66	0.455	0.408
	GRC	62	22.9	2.45	0.383	0.333***
	GRR	18	17.4	2.13	0.341	0.355
	GRP	36	21.5	2.21	0.343	0.330
	Overall:	168	23.84	5.25	0.429	0.357

GENESCAN-500 ROX size standard (Applied Biosystems), and result files from the sequencers were analysed using GENESCAN and GENOTYPER software (Applied Biosystems).

Data analysis

Genetic diversity of the sampled loci and populations. In order to characterize the microsatellite loci in the two study species, the number of alleles (A), expected heterozygosity (H_E), observed heterozygosity (H_O), and the within- and total-population inbreeding coefficients F_{IS} and F_{IT} were calculated for each locus using the computer programs MSA (Dieringer & Schlotterer 2003) and FSTAT (Goudet 1995). In addition, departures from Hardy–Weinberg equilibrium (HWE) for each locus within populations of each species were tested using FSTAT, in order to explore the possibility that particular loci may deviate from HWE within populations because of null alleles (= allele nonamplification). Subsequently, each population was characterized using the variance in allele size (Var), H_E, and H_O calculated by MSA and allelic richness in FSTAT (Goudet *et al*. 1995). All genetic diversity parameters were corrected for sample size in MSA and FSTAT. Departures from HWE for each population were identified using exact tests in GENEPOP (Raymond & Rousset 1995).

Tests of basic assumptions underlying indirect estimation of gene flow

To determine if the sampled *Alcantarea* inselberg populations are likely to meet the equilibrium conditions required for the indirect estimation of gene flow via F-statistics, the possibility of founder effects due to recent colonization (genetic 'bottlenecks') was tested using the 'sign test' and 'Wilcoxon sign-rank' test in the BOTTLENECK program (Piry *et al*. 1999). Both tests are able to detect recent reductions in effective population size due to genetic bottlenecks. The analyses were carried out both for the 'infinite allele model' (IAM) and for the 'two-phased mutation model' (TPM) recommended for microsatellites in the user manual. The detection of recent bottlenecks would indicate that *Alcantarea* inselberg populations have not yet reached an equilibrium between gene flow and genetic drift, which may render the indirect estimation of gene flow via F_{ST} difficult (Whitlock 1992). Also, correlations between genetic and geographical distance (isolation by distance; Slatkin 1993) were tested using nonparametric

Mantel tests in FSTAT with 10 000 randomizations for each species. Isolation by distance may lead to serious departures from an island migration model which may complicate the indirect estimation of gene flow (Whitlock 1992).

Indirect analysis of gene flow via F*-statistics and population phylogeny*

Analysis of molecular variance (AMOVA) in ARLEQUIN (Excoffier *et al.* 2005) was used to obtain *F*-statistics for microsatellites at different hierarchical levels. We tested the hierarchies 'among species', 'among populations within species', and 'within populations' for the entire data set. Subsequently, separate AMOVA models were analysed to test the distribution of genetic variance among and within populations of each species. In addition, F_{ST} between pairs of populations and between the two colour morphs present in population IMC was estimated using MSA (Dieringer & Schlotterer 2003). The significance of each *F*-statistic was tested through 10 000 permutations at the appropriate hierarchical level in ARLEQUIN or MSA. To depict relationships between populations and species in a graphical way, a neighbour-joining (NJ) tree was constructed based on the chord distance of Cavalli-Sforza & Edwards (1967). One thousand bootstrap replicates of the distance matrix were obtained in MSA, and NJ trees were generated and analysed in PHYLIP 3.6 (Felsenstein 2004).

Effective population sizes and migration rates

Theta ($4N_e\mu$, with N_e = effective population size and μ = mutation rate) for populations of *A. imperialis* and *A. geniculata* and the effective number of migrants (N_em) between pairs of populations were estimated following a coalescent theory and maximum-likelihood-based approach using MIGRATE 2.0.6 (Beerli & Felsenstein 1999). Pairwise analysis was used because of unfavourable trade-offs between analysis run-time and gains in precision when multiple populations are analysed simultaneously. Local gene flow between pairs of populations can be estimated with confidence even if not all populations were sampled, as long as migration rates from unsampled populations are expected to be low (Beerli 2004). Pairwise analysis allowed the estimation of intraspecific gene flow (N_em) into each inselberg population in each species. In addition, interspecific N_em for populations of *A. imperialis* and *A. geniculata* co-occurring in sympatry on inselberg Irmã Menor (populations IIM and GIM) was estimated in both directions. In each case, genetic divergence between pairs of populations (F_{ST}; Weir & Cockerham 1984) was used to obtain initial start values for the estimation of theta and N_em. The computations were carried out under both the IAM and the stepwise mutation model (SMM), and effective population sizes were estimated from theta values by assuming a microsatellite mutation rate of 10^{-3} per gamete per generation (Zhang & Hewitt 2003).

Bayesian genetic structure analysis

Bayesian analysis in STRUCTURE version 2 (Pritchard *et al.* 2000) was used to obtain additional insights into patterns of gene flow and population subdivision within *Alcantarea* inselberg populations. Our aim was to determine the most likely number of populations (*K*) for each species, and to estimate admixture proportions (*Q*) for individuals of each population. Preliminary runs revealed that the variances of likelihood estimates relative to the actual likelihoods for models with different *K* were not satisfactory in *A. geniculata*, despite excessive run lengths. This probably was the case because allelic diversities were low in this narrow endemic, and one locus (Ai4.3) was even fixed for a single allele in this species (Table 1). Therefore, Bayesian genetic structure analysis was carried out for the more variable species *A. imperialis* only. 'Burn-in' lengths of 50 000 and run lengths of 1 000 000 were identified as being appropriate based on the diagnostic tools available in STRUCTURE. The analyses were carried out under the admixture model for independent allele frequencies, and all possible models for *A. imperialis* from $K = 1$ to $K = 8$ were evaluated based on the natural logarithm of their probability and on their variances. Individual and average admixture proportions (*Q*) for each sampled population in each genetic cluster found by STRUCTURE were recorded for the model with the highest probability.

Results

Genetic diversity and equilibria in Alcantarea *inselberg populations*

All eight microsatellite loci were polymorphic, with up to 14 alleles per locus and gene diversities (H_E) of up to 0.812 in *Alcantarea imperialis*, and up to eight alleles per locus and H_E up to 0.707 in *Alcantarea geniculata* (Table 1). One locus, Ai4.3, was fixed for a single allele in the narrow endemic *A. geniculata* but was nevertheless polymorphic in *A. imperialis* (Table 1). Low or nonsignificant within-population inbreeding coefficients (F_{IS}) for most loci indicate near-random mating within populations, whereas high total-population inbreeding coefficients (F_{IT}) reflect species-level homozygote excess due to genetic subdivision ('Wahlund' principle; Hartl & Clark 1997) (Table 1). One locus (P2p19) displayed an overall departure from Hardy–Weinberg equilibrium (HWE) within populations in *A. imperialis* in the form of a heterozygote deficit and one did so in *A. geniculata* (Pit8), resulting in significantly positive within-population inbreeding coefficients (F_{IS}) in these two cases (Table 1). As no locus displayed consistent departures from HWE across all

populations, it is likely that the observed departures from HWE reflect occasional departures from random mating rather than the presence of null alleles.

Genetic diversity evaluated at the population level was always higher in *A. imperialis* than in *A. geniculata*, regardless of whether it was estimated via the variance in allele size (Var), allelic richness, expected (H_E), or observed heterozygosities (H_O) (Table 2), which probably reflects the different geographical distribution ranges of the two species (endemic vs. narrow endemic) or differences in pollination syndromes (discussed below). Population IJF in *A. imperialis* and population GRC in *A. geniculata* displayed significant heterozygote deficits, whereas all other populations were in HWE (Table 2). Neither the sign test nor the Wilcoxon sign-rank test for recent population bottlenecks was significant for any of the populations, regardless of the mutation model used (not shown). This indicates that populations of the two species are not recently colonized and are therefore likely to have reached equilibrium, which is a prerequisite for the indirect estimation of gene flow. Mantel tests in each species indicated no significant correlation between geographical and genetic distance (not shown), thus suggesting the absence of isolation by distance in inselberg populations of *Alcantarea*.

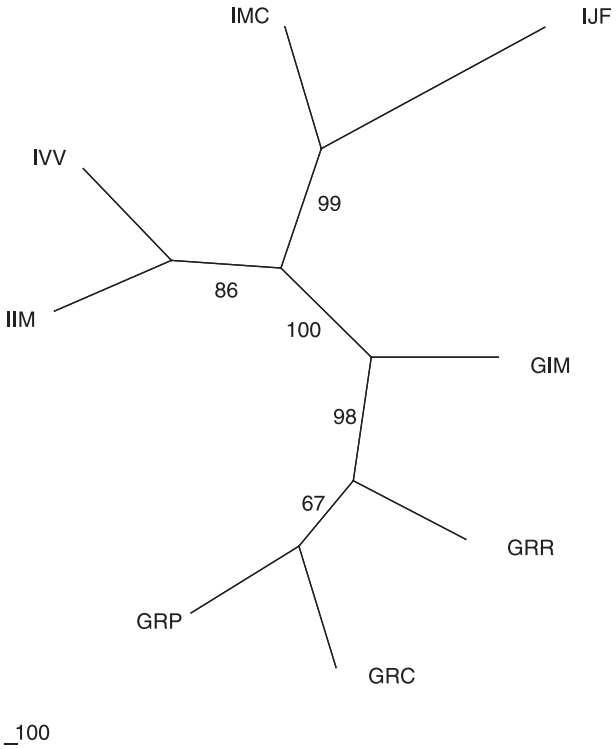

Fig. 2 Unrooted neighbour-joining tree of populations based on Cavalli-Sforza & Edward's (1967) chord distance, including bootstrap support values in percent. A scale for genetic distance is provided at the bottom of the graph. For population abbreviations see Materials and methods.

Genetic structure and gene flow between Alcantarea *inselberg populations*

An NJ tree based on microsatellite genetic distances (Fig. 2) separated inselberg populations of *A. imperialis* and *A. geniculata* with high bootstrap support. As indicated by the branch lengths in Fig. 2, genetic distances between populations of *A. imperialis* were sometimes nearly as large as genetic distances between the two species, reflecting a high degree of genetic isolation among *A. imperialis* inselberg populations. In agreement with these results, AMOVA attributed a significant proportion of the genetic variance (28%; $P < 0.05$) to the 'among species' level, and a similarly high and significant proportion to 'among populations within species' (25%; $P < 0.001$) (Table 3). Separate AMOVA models for each species revealed that a higher proportion of the genetic variance resided 'among populations' in *A. imperialis* (44%; $P < 0.001$) than in *A. geniculata* (11%; $P < 0.001$) (Table 3). Individual F_{ST} estimates between pairs of populations ranged from 0.166 to 0.535 for *A. imperialis* and from 0.082 to 0.142 for *A. geniculata* (all P values < 0.005

Table 3 Results of analysis of molecular variance (AMOVA) for three different hierarchical models, a three-level model including both *Alcantarea* species, and separate two-level models for each species. The significance of each F_{ST} analogue was tested through 10 000 permutations at the appropriate hierarchical level

Model	Partitioning	Variation (percentage)	*F*-statistic	*P*
Three levels — both species	Among species	28	$F_{CT} = 0.280$	< 0.05
	Among populations within species	25	$F_{SC} = 0.351$	< 0.001
	Within populations	47	$F_{ST} = 0.533$	< 0.001
Two levels — *A. imperialis*	Among populations	44	$F_{ST} = 0.434$	< 0.001
	Within populations	56	$F_{IS} = 0.099$	
			$F_{IT} = 0.490$	
Two levels — *A. geniculata*	Among populations	11	$F_{ST} = 0.111$	< 0.001
	Within populations	89	$F_{IS} = 0.094$	
			$F_{IT} = 0.195$	

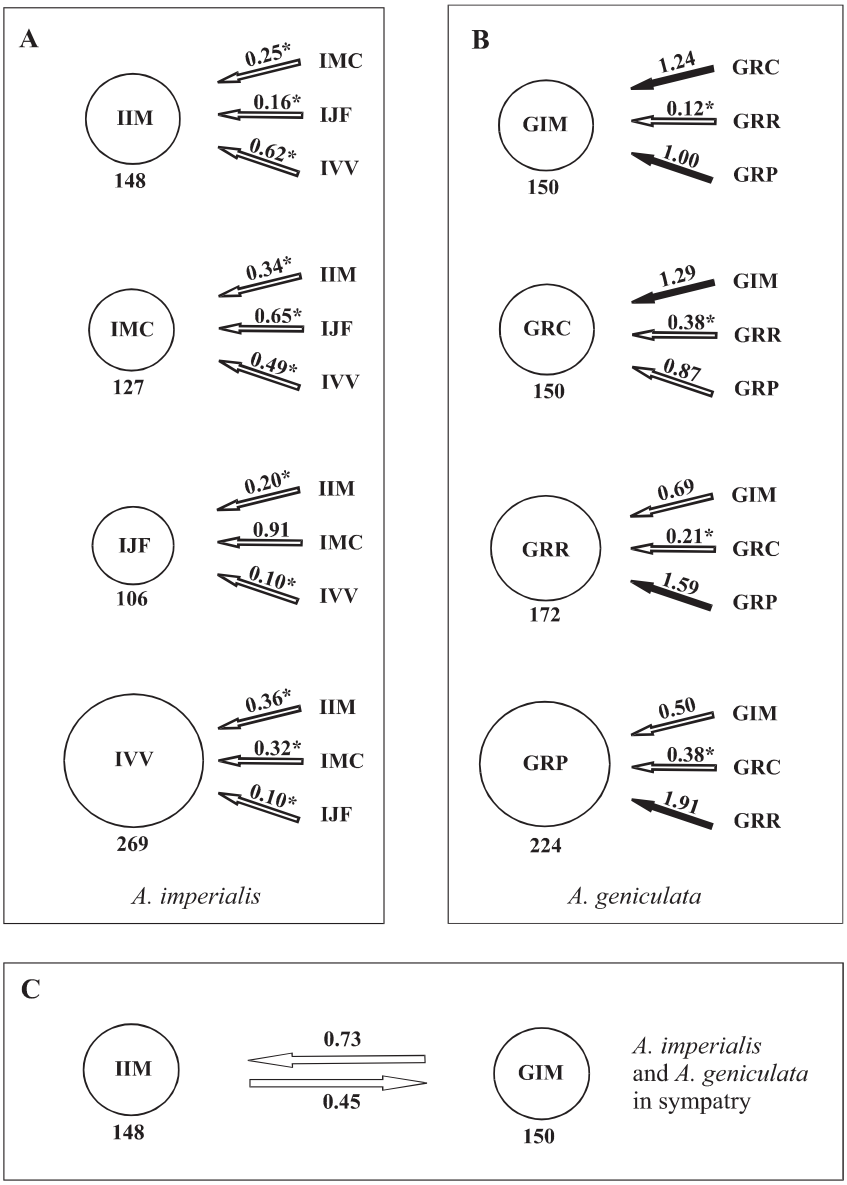

Fig. 3 Effective population sizes (N_e) and effective number of migrants (N_em) for inselberg populations of *Alcantarea imperialis* and *Alcantarea geniculata*, estimated following Beerli & Felsenstein (1999) under the SMM with MIGRATE 2.0.6. (A) Effective population size for each sampled population of *A. imperialis* and migration into that population from other inselbergs. (B) Effective population size for each population of *A. geniculata* and migration into that population from other inselbergs. (C) Effective population size and migration for populations of *A. imperialis* and *A. geniculata* co-occurring on one 'inselberg', Irmã Menor. Population comparisons with $N_em < 1$ are indicated by empty arrows, and comparisons for which intraspecific N_em (A and B) was smaller than interspecific N_em in sympatry (C) are indicated by asterisks. For population abbreviations see Materials and methods.

in both species; not shown). Interspecific pairwise F_{ST} averaged 0.496 (maximum = 0.554) and was lowest between the sympatric populations IIM and GIM (F_{ST} = 0.412) and between population GIM of *A. geniculata* and the neighbouring population IVV of *A. imperialis* (F_{ST} = 0.368; distance = 7 km). A high and significant proportion of the genetic variance was observed within populations of each species (56% and 89% in separate AMOVA models for *A. imperialis* and *A. geniculata*, respectively) (Table 3), as expected for perennial plants with mixed or outcrossing breeding systems.

Maximum-likelihood-based estimates of migration rates (N_em; 'gene flow') were low in *A. geniculata* and extremely low in *A. imperialis* (Fig. 3), consistent with the microsatellite genetic distances (Fig. 2) and *F*-statistics from AMOVA (Table 3; above). In fact, all N_em estimates for *A. imperialis* were < 1 migrant per generation, which has traditionally been regarded as the minimum required for maintaining species cohesion (Fig. 3). In *A. geniculata*, seven out of 12 N_em estimates were < 1. Migration rates between the only pair of populations of *A. imperialis* and *A. geniculata* sampled in sympatry on the same inselberg were N_em = 0.45 and 0.73 migrants per generation, depending on the direction of the analysis, suggestive of very low levels of interspecific gene flow in sympatry (Fig. 3).

Bayesian genetic structure analysis in A. imperialis

Bayesian genetic structure analysis confirmed the pronounced genetic structure in *A. imperialis* and yielded

Table 4 Average admixture proportion for each sampled population of *Alcantarea imperialis* (rows) among each of five 'genetic clusters' (columns) inferred by Bayesian analysis in STRUCTURE (Pritchard *et al.* 2000). For population abbreviations see Materials and methods

	Cluster				
Population	I	II	III	IV	V
IIM	0.010	0.011	0.019	0.019	0.942
IMC	0.015	0.691	0.280	0.006	0.007
IJF	0.961	0.014	0.015	0.006	0.005
IVV	0.051	0.008	0.014	0.438	0.489

additional insights into patterns of gene flow and population subdivision in this species. A model of $K = 5$ populations was best able to capture the variation in the data, based on both an abrupt slowing down of the change in probability of the data (ln prob) for models with a number of populations $K > 5$ and a noticeable increase in the variance with $K > 5$; the ln prob for the $K = 5$ population model was –1329.4 with a variance of ln likelihood of 109.7, whereas for the $K = 6$ model the parameter values were –1319.4 and 136.2 for ln prob and variance, respectively. Based on the small difference in ln prob and the increase in variance, the simpler $K = 5$ model was chosen to represent the data.

The average admixture proportion for each population of *A. imperialis* among the five different 'genetic clusters' found by the Bayesian analysis is given in Table 4, and the admixture proportions (Q) of individual plants in each of the four clusters are depicted graphically in Fig. 4. The graph reveals that some plants from population IVV are admixed with alleles from the same 'genetic cluster' that makes up population IIM. These two inselberg populations are separated by only 7.5 km. Also, population IMC, the population containing plants of two different colour morphs, showed signs of admixture between two 'genetic clusters' not found elsewhere in the data set (Fig. 4).

Discussion

Population differentiation and species cohesion in fragmented Alcantarea *inselberg populations*

The forces responsible for the maintenance of species cohesion have received great interest in recent years (Ehrlich & Raven 1969; Morjan & Rieseberg 2004), and this allows us to put our present results on *Alcantarea* inselberg species in the context of gene flow estimates from many other taxa. The genetic parameters estimated here indicate that gene flow between inselberg populations of *Alcantarea imperialis* and *Alcantarea geniculata* is much weaker than normally expected for diploid perennial outcrossing plants. Estimates of the effective number of migrants (N_em) indicate that gene flow is < 1 for all pairwise population comparisons of *A. imperialis* and for seven out of 12 comparisons in *A. geniculata* (Fig. 3). Thus gene flow in *A. imperialis* would appear to be too low to prevent differentiation because of genetic drift (Wright 1931), unless selection coefficients/effect sizes of favourable alleles are great enough to maintain species cohesion (Morjan & Rieseberg 2004). This pattern is less pronounced in the narrow endemic *A. geniculata*, where each population is interconnected to at least one other population with $N_em > 1$ (Fig. 3). In accordance with low levels of gene flow (N_em), genetic distances and branch lengths in our neighbour-joining tree of populations are almost as large within as between species (Fig. 2). Likewise, the proportion of genetic variance of AMOVA residing between populations of each species approaches that of the variance residing between species (25% vs. 28%, respectively; Table 3). Strong population subdivision is also visible from high total inbreeding coefficients F_{IT} (Wahlund effect; Hartl & Clark 1997) despite low estimates of within-population inbreeding F_{IS} (Table 1; Table 3) and only modest departures from HWE for most populations (Table 2).

If we compare genetic differentiation between populations of *A. imperialis* ($F_{ST} = 0.434$ in single species AMOVA; Table 3) with estimates of F_{ST} from the literature database of Morjan & Rieseberg (2004), a surprising result emerges: F_{ST} in *A. imperialis* falls far outside the interquartile range

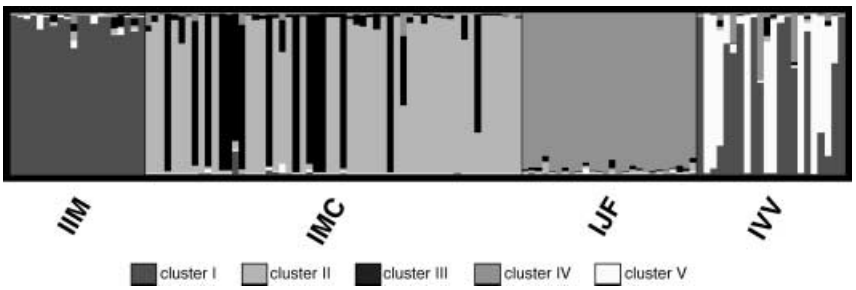

Fig. 4 Bayesian admixture proportions (Q) of individual plants of *A. imperialis* for a $K = 5$ population model. The $K = 5$ 'genetic clusters' identified by STRUCTURE are indicated in different shades of grey. For population abbreviations see Materials and methods.

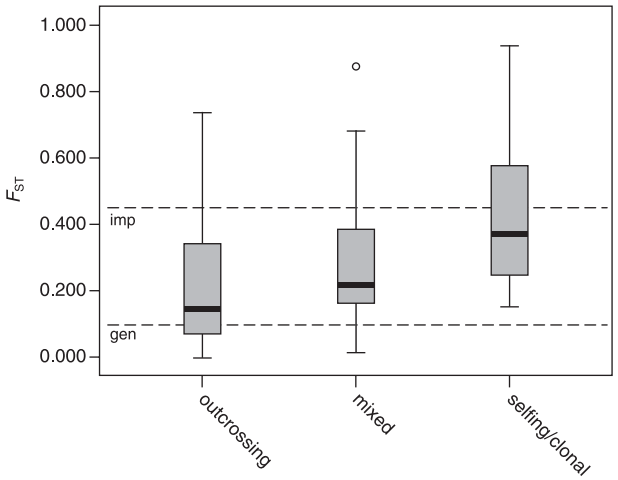

Fig. 5 Comparison of F_{ST} in *Alcantarea imperialis* and *Alcantarea geniculata* with estimates from the plant literature. F_{ST} in *A. imperialis* (imp) and *A. geniculata* (gen) is compared to 135 nuclear marker-based estimates of population divergence (F_{ST} or G_{ST}) in plants with outcrossing, mixed, or selfing breeding systems from the literature database of Morjan & Rieseberg (2004). The boxplots allow comparison of F_{ST} in *Alcantarea* spp. with the interquartile ranges (boxes) for plants with different breeding systems.

(= typical expectation) for F_{ST} in outcrossing plants or plants with mixed mating systems (Fig. 5). Rather, it falls within the interquartile range of plants with selfing mating systems (Fig. 5). We note that controlled pollination experiments and observations of ovule penetration in *A. imperialis* suggest that this species, although self-compatible, favours outcrossing (Martinelli 1994). Ovule penetration was clearly higher after cross-pollination (range: 52–75%) compared to self-pollination (range: 0–29%) at 96 h, the earliest time point at which penetration was observed in this species (Martinelli 1994). Although mixed pollination experiments would be desirable, the concordance of most of our studied populations with HWE (Table 2) confirms the predominantly outcrossing nature of this species. Also, our molecular marker data did not reveal a single case of clonal origin for plants at the adult stage (= all sampled plants had unique genotypes). Such unusually high population divergence in a preferentially outcrossing species indicates strongly restricted gene flow due to fragmented distribution on inselbergs. Genetic divergence for *A. geniculata*, on the contrary, was more similar to what would be expected for a predominantly outcrossing species (F_{ST} = 0.111; Fig. 5), most likely due to greater population connectivity associated with its much narrower endemic range.

How does F_{ST} in *A. imperialis* compare with other bromeliads then? Population divergence in bromeliads with comparable (mixed or outcrossing) mating systems and more continuous distributions are typically much lower than in *A. imperialis*, for example F_{ST} = 0.043 (*Tillandsia ionantha*; Soltis *et al*. 1987), 0.196 (*Aechmea tuitensis*; Izquierdo & Pinero 2000), or 0.080–0.160 (*Encholirium* spp.; Cavallari *et al*. 2006), with no obvious difference between the size of geographical distribution ranges or the type of molecular marker used. Again, bromeliads with F_{ST} values as high as those in *A. imperialis* are normally taxa with increased levels of selfing/cloning: population divergence was estimated as F_{ST} = 0.390 for *Tillandsia achyrostachys* (clonal reproduction is documented for this species; Gonzalez-Astorga 2004), and as F_{ST} = 0.356 for *Aechmea magdalenae* (tendency to clone documented as well; Murawski & Hamrick 1990). In *Tillandsia recurvata*, a species expected to self-pollinate based on its floral morphology, F_{ST} was as high as 0.906 (Soltis *et al*. 1987), and in *Puya raimondii*, a notorious inbreeder, the related differentiation parameter G_{ST} was as high as 0.961 (Sgorbati *et al*. 2004). This translates into F_{ST} = 0.966 following Cockerham & Weir (1987). Hence, just as in our earlier comparison to the general plant database, *A. imperialis* exhibits much greater population differentiation than expected based on its well-documented outcrossing mating system. Again, this points to restricted gene flow due to fragmented distribution on inselbergs as the most likely cause of high F_{ST}. The only other genetic survey of inselberg bromeliads that we are aware of, on the mixed outcrosser *Pitcairnia geyskesii* on rock outcrops of French Guiana, estimated F_{ST} = 0.322 (Sarthou *et al*. 2001), which is lower but similar to our estimate for *A. imperialis*. Taken together, their study and ours indicate high degrees of population isolation in inselberg species for different subfamilies of Bromeliaceae.

Correspondence between molecular marker data and pollination syndromes of Alcantarea *spp.*

Previous work on *Alcantarea* spp. (Martinelli 1994; Safford & Martinelli 2000) allows us to place our molecular marker data in the context of the pollination syndromes of these species. Extensive fieldwork on *A. imperialis* (Martinelli 1994) indicates that flowers are visited by a wide range of animals including hummingbirds (*Clytolaema rubricauda*, *Leucochloris albicollis*, *Heliothryx aurita*, and *Melanothrochilus fuscus*), hawkmoths (Sphingidae), and bats (*Anoura caudifera* and *Artibeus lituratus*). However, hummingbirds visit flowers only during the beginning of anthesis and after senescence (the flowers are open during the night), and only bats appear to be able to promote pollination (Martinelli 1994). With this in mind, the extremely low levels of gene flow observed in *A. imperialis* are surprising — bats are thought to be efficient pollinators and should thus be able to promote gene exchange between plant populations situated on inselbergs (Sazima *et al*. 1989; Sazima *et al*. 1999). Our data indicate that the ability of bats to promote gene flow between inselberg populations may be smaller than previously assumed.

Suggested pollinators of *A. geniculata* are bees and sphingid moths (Knuth 1904; Porsch 1935), but these suggestions may be based on superficial observation of visitors to flowers or simply on speculative comments based on floral morphology of cultivated specimens (Martinelli 1994). Nevertheless, owing to its narrowly endemic range, the geographical distances between outcrops on which these populations occur tend to be very small (often < 1 km). These distances may easily be covered by any of these potential pollinators, and dispersal of the small seeds by wind should easily be facilitated as well. Future studies of *Alcantarea* spp. should include molecular-marker-based estimation of breeding system parameters from progeny arrays (Ritland 2002), to allow a more accurate assessment of breeding systems under natural conditions.

Population subdivision on inselbergs

Our Bayesian analysis following Pritchard *et al*. (2000) provides some first insights into fine-scale genetic structure in *A. imperialis* inselberg populations. First, fine-scale patterns of gene flow between the geographically neighbouring populations IIM and IVV become apparent in Fig. 4, with population IVV exhibiting a proportion of plants belonging to the same 'genetic cluster' as all plants of its geographical neighbour IIM (cluster 1; Fig. 4; Table 4). Second, population subdivision becomes apparent for IMC, the population with two different colour morphs present (plants with red and plants with green rosettes and bracts; see Materials and methods). The Bayesian analysis identified two genetic clusters exclusive to this population (clusters 2 and 3; Fig. 4; Table 4). Genetic divergence between the two sympatric morphs was low ($F_{ST} = 0.099$) but indeed significant at the 0.001 level when compared to 10 000 random permutations of alleles between morphs. It is thus possible that genetic subdivision in population IMC is indeed associated with colour morphs, although the origin of morphs (secondary contact vs. origin in sympatry), their genetic basis (monogenic or oligogenic), and the actual mechanism maintaining isolation (ecological or pollinator-mediated), must remain speculative at present. It is certain that colour morphs arise easily and repeatedly in Bromeliaceae, as evidenced by the multitude of spontaneous colour variants found in breeders' collections and among horticultural varieties.

Inselbergs as venues for molecular ecology studies

Patterns of diversity and gene flow in *A. imperialis* indicate that fragmented distribution on inselbergs can lead to unusually high levels of population differentiation, thus indicating restricted gene flow and decreased population connectivity even in species with predominantly outcrossing mating systems. This supports the view that these granitic rock outcrops, widely distributed throughout tropical and temperate regions of the world (Porembski & Barthlott 2000), may indeed be comparable to oceanic islands in their effects on patterns of genetic variability and gene flow. Consequently, inselbergs are indeed promising venues for ecological and evolutionary studies as suggested by ecologists (Porembski & Barthlott 2000), similar to the use of oceanic islands in biogeography and evolutionary biology (MacArthur & Wilson 1967).

When compared to other types of 'terrestrial archipelagos', inselbergs may be most similar in nature to the 'sky island' populations of the North American cordillera system. Phylogeographical studies based on mitochondrial DNA in animals and plastid DNA polymorphisms in plants indicate that 'sky island' populations in the western USA underwent extensive genetic divergence in response to fragmentation associated with recent palaeoclimatic cycles (Knowles 2001; DeChaine & Martin 2005; Smith & Farrell 2005). Although tropical inselberg populations may have been affected by range expansions and contractions of similar magnitude, they differ from North American 'sky islands' in many ways. Perhaps most importantly, gradients in humidity, temperature, and irradiation between tropical inselbergs and their surrounding rainforest will be much steeper for a herbaceous plant. Also, changes in soil substrates will be more severe for tropical inselbergs, with tremendous differences in nutrient availability between outcrops and their surrounding forest (Porembski & Barthlott 2000). It is this clear ecological differentiation between outcrops and their surrounding forest — an inhospitable matrix that prevents gene flow — that has encouraged comparisons with oceanic islands.

The inselbergs of the South American Atlantic Rainforest offer a huge variety of abiotic environmental conditions (Safford & Martinelli 2000), ranging from high-altitude rock outcrops (studied here) to coastal lowland inselbergs subject to completely different temperature regimes and high salinity from ocean spray — the preferred habitats of closely related species such as *Alcantarea glaziouana* or *Alcantarea regina* (Martinelli 1994). Such island-like distributions of populations across different environments are typically regarded as conducive to ecological speciation and adaptive radiation (MacArthur & Wilson 1967; Schluter 2000). We also note that low levels of interspecific gene flow (N_em) appear to be possible on inselbergs on which *A. imperialis* and *A. geniculata* co-occur (Fig. 3), in agreement with the view that hybridization may form an integral part of species radiations (Seehausen 2004).

Implications for conservation

The Brazilian Atlantic Rainforest is one of the world's most critically endangered biodiversity hotspots (Myers *et al*. 2000), the primary conservation problem from a genetic

standpoint being rapid landscape and habitat fragmentation due to logging and urban development. Although neither *A. imperialis* nor *A. geniculata* occur in the rainforest matrix in which their habitats are embedded, their pollinators may nevertheless depend directly on the forest for shelter, breeding, and alternative sources of food. Preservation of surrounding forest may therefore be more crucial to the survival of these inselberg species than their naturally fragmented distribution patterns suggest.

High levels of population differentiation in *A. imperialis* (43% of genetic variance residing between populations; Table 3) indicate the need to consider a sufficient number of inselbergs when devising *in situ* or *ex situ* conservation plans in this ecologically and horticulturally important species. For *A. geniculata*, the need to preserve many locations is less clear from the partitioning of the genetic variance (11% of variance between populations; Table 3), but its extremely narrow range and lower diversity (Table 2) call for close monitoring and preventive action. Calamities such as natural or man-induced fires in some of its locations would easily make this species vulnerable to stochastic factors typical for small populations (Frankham *et al*. 2002). On a positive note, one population of *A. geniculata* studied here, population GRP, is already under protection. This population is located in a Private Nature Reserve (RPPN 'Pedra do Amarylis'). Genetic diversity in this population is within the normal range of this species (Table 2), and its effective population size (N_e) is the largest observed for *A. geniculata* to date (Fig. 3). Thus, community-based *in situ* conservation of this narrow endemic is already proving effective.

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This work forms part of Thelma Barbará's PhD thesis work on the molecular population genetics of *Alcantarea* spp. adapted to inselbergs in the Atlantic Rainforest of Brazil. Gustavo Martinelli has long-standing interests in the ecology, systematics, and reproductive biology of Bromeliaceae in southeastern Brazil. Simon Mayo's and Mike Fay's work is focused primarily on the evolution and systematics of tropical and temperate monocot families. Christian Lexer's main interest is on the genetics of speciation in selected plant groups.
