Genetic fingerprinting of two endemics from the Seychelles - *Medusagyne oppositifolia* (Medusagynaceae) and *Rothmannia annae* (Rubiaceae)

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Abstract: Levels of genetic variation in two endemic woody species, *Medusagyne oppositifolia* from Mahé and *Rothmannia annae* from Aride, were studied using amplified fragment length polymorphism (AFLP) fingerprinting and shown to be relatively low (6-7%). In *M. oppositifolia* six wild plants (four from the main population on Mt. Bernica and two more isolated plants from Copolia and Mt. Sébért) were sampled, and these were compared to cultivated material of *M. oppositifolia* from Kew and Nancy. The sample from Mt. Sébért was genetically the most distinct. Cultivated plants from both gardens had similar genotypes to plants from Mt. Bernica, although one plant from Nancy appeared to be slightly more distinct than the others in the PCOA analysis. In *R. annae*, 20 samples collected on Aride were studied, and many individuals were genetically indistinguishable as they had no unique markers. Three individuals (two from the core area and one isolated plant in the west) appeared to be relative genetic outliers. The results are discussed in terms of the conservation of the species.

Keywords: Aride, Mahé, AFLP, genetic fingerprinting, *Medusagyne oppositifolia*, *Rothmannia annae*

Introduction

There is now a general consensus of opinion that management of rare and endangered taxa can be improved if it is informed by genetic data at various levels (Mace *et al.* 1996). In a world of limited resources isolated lineages should be accorded higher priority for conservation. Thus an endangered monotypic family such as Medusagynaceae (see below) has greater significance in terms of biodiversity than a rare subspecies of an otherwise common species, and this significance should be reflected in the establishment of conservation priorities (Vane-Wright *et al.* 1991). DNA sequencing allows us to investigate these issues by placing narrow endemics within a larger context (Fay *et al.* 1997; Chase & Fay 1997).

Within species, the loss of genetic variation is of major concern as it can preclude the ability of a species to respond to natural selection and consequently limit its evolutionary potential. In the long term, genetic variation can be a critical factor for persistence in a changing environment (Frankel & Soulé 1981; Lande & Barrowclough 1987). Small populations are often subject to the loss of alleles through genetic drift. Reduced population size can also result in inbreeding depression and a decline in population fitness caused by increased homozygosity and the unmasking of recessive deleterious alleles. As a result, the success of any genetic conservation programme is dependent on an understanding of the level and distribution of genetic variation present in the genepool.

At the population level, DNA sequences are not generally variable enough to be informative and to acquire knowledge of the distribution of genetic variation within and be-

tween populations, one of a range of techniques, collectively referred to as genetic fingerprinting, is often used to produce this important information. Genetic fingerprinting involves the production of a set of DNA fragments which reflects the genetic constitution of an individual. These fragments can be visualised as bands in gel electrophoresis using radioactivity, silver staining or fluorescent dyes to label the DNA. For a fuller discussion of the different molecular techniques for studying population genetics, see Qamaruz-Zaman *et al.* (1998a).

One of the first methods to be developed was restriction fragment length polymorphisms (RFLPs). However, this technique is not appropriate for use with rare species or small populations since it requires relatively large amounts of DNA and, hence, also a large quantity of leaves or other plant material.

In the nineteen nineties, genetic fingerprinting techniques incorporating the technology of the polymerase chain reaction (PCR) were developed, thus allowing fingerprinting studies to be carried out with much smaller quantities of DNA and consequently reducing the initial amount of plant material required, an added benefit when dealing with rare and endangered plants. Several fingerprinting techniques using PCR have been developed. One of the first of these was random amplified polymorphic DNAs (RAPDs), and this has been widely used with cultivated and wild species. However, the technique suffers from lack of reproducibility.

In 1995, AFLP"! (amplified fragment length polymorphism) was developed by Keygene Inc. (Vos *et al.* 1995) and is the most sensitive fingerprinting technique currently available suitable for use with rare and endangered taxa. The technique has several advantages over other currently used fingerprinting methods:

1. It is fast (the technique has been automated).
2. It requires relatively small quantities of DNA, thus making it suitable for work with rare species.
3. It provides 10-100 times more markers and is thus more sensitive than some other fingerprinting techniques (e.g. RAPDs).
4. It is highly reproducible.

Developed for use with crop plants, this technique has been used in the study of a number of rare and endangered taxa from the temperate regions, including *Astragalus cremnophylax* (Travis *et al.* 1996), *Orchis simia* (Qamaruz-Zaman *et al.* 1998b), *Populus euphratica* (Fay *et al.* 1999a) and *Phylica* spp. from St. Helena and Tristan da Cunha (Richardson 1999). However, there are relatively few examples of its use with tropical taxa e.g. *Alstroemeria* spp. from Peru and Brazil (Han *et al.* 1999) and *Phylica nitida* from Mauritius and Reunion (Richardson 1999). Here we illustrate the use of AFLPs in the investigation of genetic diversity in two endemic trees from the Seychelles, *Medusagyne oppositifolia* Baker and *Rothmannia annae* (E.P.Wright) Keay. This paper is based on two unpublished conservation genetic reports (Fay & Beltran 1999; Fay *et al.* 1999b).

Medusagyne oppositifolia is the sole species in the family Medusagynaceae. An endemic to the Seychelles, it is only found on the island of Mahé, where it is known as the jellyfish tree or bois méduse. The botanical and common names refer to the gynoecium which resembles a gorgon's head or the umbrella (shaped larval stage of a jellyfish due to the pres-

ence of up to 25 stalked capitate stigmas). The unusual mode of dehiscence of the valves of the septicidal capsule from the base results in a structure at maturity that resembles the ribs of an umbrella. This species was assumed extinct from 1903 until its rediscovery in 1970 (Robertson *et al.* 1989). The family is monotypic, and its affinities have been obscure.

Originally described by Baker (1877) as a member of Ternstroemiaceae, *M. oppositifolia* was judged to be the sole member of a separate family by Engler & Gilg (1924). A fuller account of Medusagynaceae was published by Engler & Melchior (1925), and the family has been recognised in all subsequent systems of classification (Dickison 1990a). Affinities have been suggested to, among others, Caryocaraceae, Clusiaceae, Eucryphiaceae, Ochnaceae, Paracryphiaceae and Quiinaceae (Robertson *et al.* 1989, Dickison 1990a, b). Molecular studies carried out at Kew (Fay & Chase 1996; Fay *et al.* 1997) have shown that Medusagynaceae is most closely related to Ochnaceae and Quiinaceae. The molecular results indicate that *M. oppositifolia* is a relict palaeoendemic, and it should be given high priority conservation (Vane-Wright *et al.* 1991).

The present habitats occupied by this species indicate that it originally flourished in exposed areas prone to drought. There has been no recent natural regeneration from seed, all plants apparently being a similar age (Lucas & Synge 1978; Gerlach 1997; Wise 1998). Propagation of the species is difficult, and although it has been achieved from seed at Nancy and in tissue cultures derived from seed at Kew, it has thus far not proved possible to generate large numbers of plant *ex situ* (Wilkinson & Staniforth, pers. comm.). Given these problems, *M. oppositifolia* is in severe danger of extinction and has an IUCN category of Critically Endangered (CR; Gerlach, 1997).

As part of the Threatened Plants Appeal organised by the Friends of Kew, we extended our studies of this enigmatic species. It is only known in cultivation from three collections, one at Kew, donated by Whitehead in 1981, and two at Nancy, donated by Friedman in 1983 and 1984. However, it was unclear whether these collections are distinct from the remaining trees on Mahé, and consequently how significant they might be in the conservation of *M. oppositifolia*. To address the questions relating to the origin and distinctness of the cultivated plants and the level of genetic variation existing in this species we carried out genetic fingerprinting studies on cultivated material from Kew and Nancy and samples from the wild populations.

Rothmannia annae (Rubiaceae) is the only species of the genus found in the Seychelles, all others being from southern Africa. Now found only on Aride Island, the most northern of the granitic islands in the Seychelles archipelago, it is thought to have occurred previously on Mahé, Praslin, Silhouette and Félicité. It is known as Wright's gardenia or bois citron. There are currently approximately 1200 surviving trees (Gerlach 1997; Wise 1998).

Aride Island is 72ha in area, 1.6x0.4 km, and the majority of the *R. annae* trees grow in shallow soils on granite on the upper slope of a wooded hill that runs east-west along the length of the island. The dominant tree species in the woodland is *Pisonia grandis* Vieill. ex Guillaumin (Nyctaginaceae), and *Ficus nautarum* Baker, *F. reflexa* Thunb. (Moraceae) and *Euphorbia pyrifolia* Lam. (Euphorbiaceae) are also frequent (Cadbury, pers. comm.). Although locally frequent *R. annae* is categorised as vulnerable with IUCN ratings of VU (A1d and D) due to the decline in numbers in recent years and the small distribution area (Gerlach 1997).

The Nature Preservation Trust of Seychelles and the Royal Society for Nature Conservation (which owns and manages Aride) approached Kew to ask if we would be able to undertake a genetic fingerprinting project on *R. annae* to allow the production of an informed management plan for this species.

Materials and Methods

Plant materials:

Frauke Fleischer-Dogley co-ordinated the collection of plant material from wild plants of *M. oppositifolia* on Mahé. Cultivated material from the Conservatoire et Jardins Botaniques de Nancy, France (provided by the Curator, Romaric Pierrel) and from RBG Kew was also included. The samples in this study are listed in Table 1. The collection of leaf material from wild plants of *R. annae* on Aride was carried out by Mike Betts, James Cadbury and Elaine Harper. The samples in this study are listed in Table 2. Map numbers refer to localities shown on Fig. 1. Each tree is tagged and these numbers and the DNA bank accession numbers are also given in Table 2. All samples were of leaf material dried in silica gel following the technique of Chase & Hills (1991), except that of *M. oppositifolia* from Kew, which was fresh material from tissue culture. DNAs were extracted using a modified 2(CTAB (cetyltrimethyl-ammonium bromide)) procedure (Doyle & Doyle 1987) followed by purification on caesium chloride/ethidium bromide gradients (1.55 g/ml density) using standard techniques.

Genetic Fingerprinting:

AFLPs were produced following the AFLP Plant Mapping Protocol of PE Applied Biosystems Inc. (1996). Two primer combinations were used with *M. oppositifolia* and three with *R. annae*. The fragments were separated on acrylamide gels using an Applied Biosystems Automated Sequencer. Only amplified fragments with sizes ranging from 50-500 base pairs were included in the analysis as bands outside this size range cannot be accurately sized. Gel analysis was carried out with Genescan 3.1 and Genotyper 2.0 (PE Applied Biosystems Inc.). The bands were scored as either present (1) or absent (0) for all individuals, resulting in a binary matrix which was analysed using the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) method of genetic distance analysis and by principle coordinates analysis (PCOA).

Table 1. Samples of *M. oppositifolia* used in this study

Origin	Field Notes/Accession	No. plants	Origin	Field Notes/Accession	No. plants
Mahé (wild)	Mt. Bernica	4	Nancy (cultivated)	833163	1
	Mt. Sébert	1		903519A	1
	Copolia	1		903519A	1
Kew (cultivated)	MWC 670	1			

Table 2. Samples of *R. annae* used in this study

Map no.	1	2	3	4	5	6	7	8	9	10
Tree tag no.	1001	1111	1112	1113	1114	1115	1117	1118	1119	1120
Kew DNA Bank no.	6652	6653	6654	6655	6656	6657	6658	6659	6660	6661
Map no.	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Tree tag no.	1121	1123	1124	1125	1126	1127	1128	0678	0677	1129
Kew DNA Bank no.	6662	6663	6664	6665	6828	6992	6830	6651	6650	6831

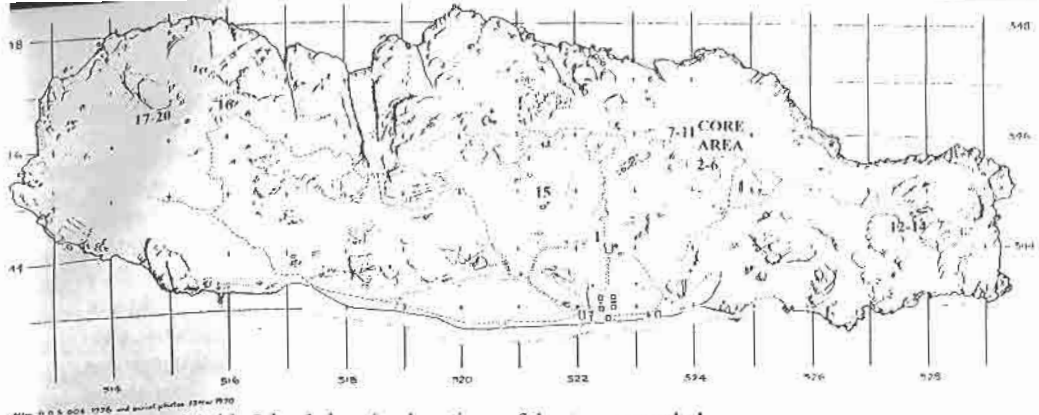

Fig. 1. Map of Aride Island showing locations of the trees sampled

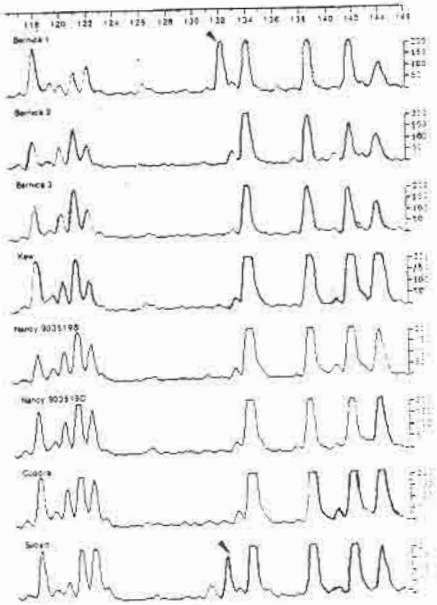

Fig. 2. Specimen traces for samples of *Medusagyne oppositifolia*, showing a general high degree of similarity. An example of a polymorphic band, shared by individuals Bernica 1 and Sébert, is indicated by arrows in the top and bottom traces. Numbers at the top of the figure indicate the sizes of the fragments in base pairs and numbers at the side indicate the strength of the peaks in arbitrary units of florescence.

Results & Discussion

Medusagyne oppositifolia:

In total, 119 bands were scored, 16 of which were polymorphic. The level of sampling of wild plants in this study was low as a result of the difficulty in reaching some of the localities where the remaining trees grow, but the level of variability in the individuals tested was also low, as might be expected given the recent history of this species. The maximum genetic distance within the samples of *M. oppositifolia* was approximately 6.8%, and there was little clear geographical structure in the results for *M. oppositifolia*, either in UPGMA or PCOA. Of the plants sampled, that from Mt. Sébért was genetically the most distinct. Cultivated plants from both gardens had similar genotypes to plants from Bernica, although one plant from Nancy appeared to be slightly more distinct than the others in PCOA. Examples of the fingerprint traces are shown in Fig. 2. The cluster diagram derived from the UPGMA analysis is shown in Fig. 3 and the plot of the first two axes from PCOA (accounting for 31.4 and 19.1% of the variation, respectively) is shown in Fig. 4.

Plants at Nancy and Kew should be conserved as a valuable *ex situ* resource in the conservation of this species, and they should be maintained in the collections at both gardens. However, these cultivated plants do not represent lineages no longer found on Mahé, and there thus appears to be no benefit to be gained from repatriation of material in terms of widening the genetic base of this species in the wild.

If it is possible to obtain further samples of leaves from other wild plants, these should be included in an expanded analysis. In the meantime, any seeds produced by the cultivated plants should be stored in a seed bank under controlled conditions. Consideration should also be given to storing seeds from wild plants to ensure that the genetic variability found in *M. oppositifolia* is maintained.

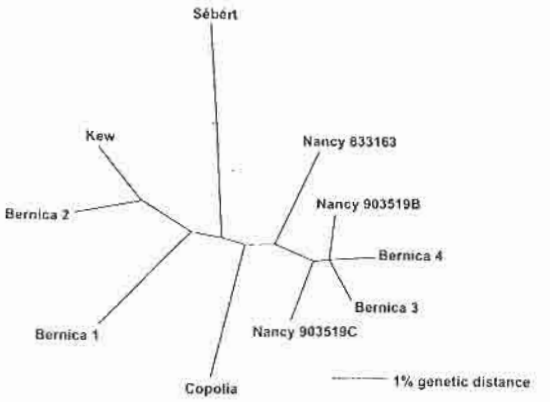

Fig. 3. UPGMA cluster diagram of *Medusagyne oppositifolia* individuals based on AFLPs

Rothmannia annae:

In all, 122 bands were scored, of which 14 were polymorphic. In the UPGMA analysis, the maximum genetic distance within the samples of *R. annae* was approximately 6%, with two individuals (tag nos. 1118 and 1127) falling outside the main cluster. One of these, 1118, is an individual from the core area, whereas the other is an isolated individual from the west of the island (see Fig. 1). In the main cluster, genetic variability was low, with the maximum genetic distance being <2%. Within this cluster there were four smaller clusters, comprising of seven, four, two and two individuals, within which the individuals were not distinguishable from each other as they showed identical AFLP profiles. The remaining three individuals also fell within the main cluster but differed from the other individuals by at least one band. Examples of the fingerprint traces are shown in Fig. 5. The cluster diagram derived from the UPGMA analysis is shown in Fig. 6 and the plot of the first two axes (accounting for 30.6 and 9.1% of the variation, respectively) in the PCOA is shown in Fig. 7. There was no clear geographical structure in the variation with similar genotypes occurring in different populations.

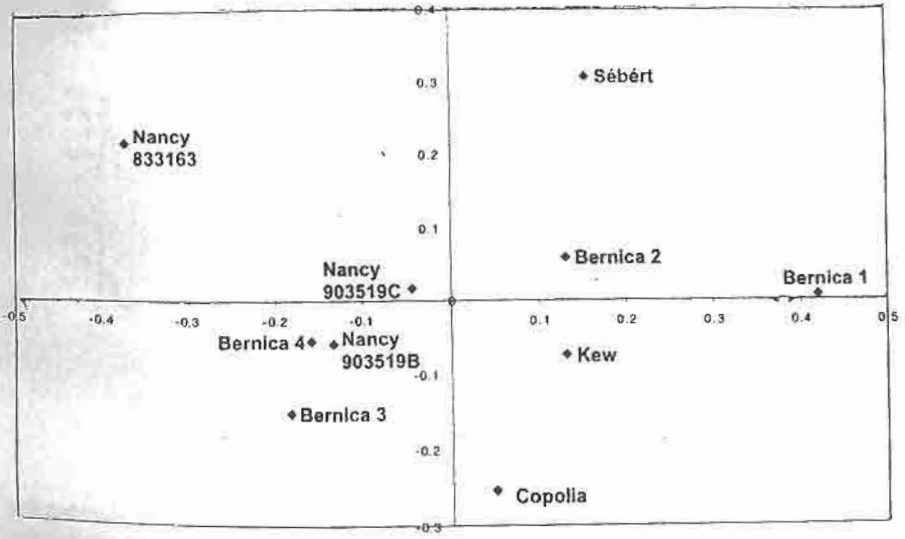

Fig. 4. Plot of the first and second axes from a PCO analysis of *Medusagyne oppositifolia*

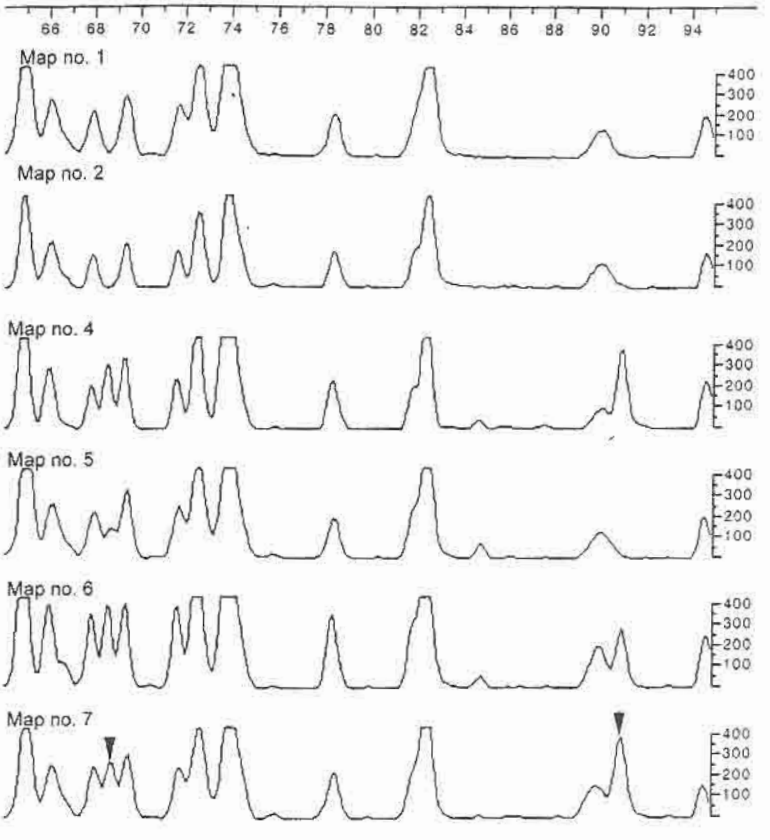

Fig. 5. Specimen traces for samples of *Rothmannia annae* from Aride Island, showing the general high degree of similarity. Polymorphic bands are indicated by arrows in the bottom trace. Numbers at the top of the figure indicate the sizes of the fragments in base pairs and numbers at the side indicate the strength of the peaks in arbitrary units of fluorescence.

Proving that individuals are genetically identical is considerably more difficult than proving them to be distinct, and the individuals that are not distinguishable from each other in this analysis are not necessarily genetically identical. However, the AFLP traces for these individuals bear the same degree of similarity to each other as those for material of known clonal origin (e.g. with *Cosmos atrosanguineus* (Hook.) Stapf (Asteraceae); Fay unpublished data) and Wilkinson 2000) and AFLPs have been used as supporting evidence for the clonal origin of plants in populations of *Populus euphratica* Olivier (Salicaceae; Fay *et al.* 1999) and *Salix* spp. (Salicaceae; Beismann *et al.* 1997).

Given the low level of genetic variation, for the continued survival of *R. annae* it is clear that every effort should be made to maintain the current level of genetic diversity, and that relative genetic outliers such as 1118 and 1127 are likely to be important in this process. More detailed sampling would be required to ascertain whether these are representative of other genetic clusters not otherwise represented in this sample or whether they are truly distinct genotypes. This study provides the background for a project with increased sampling.

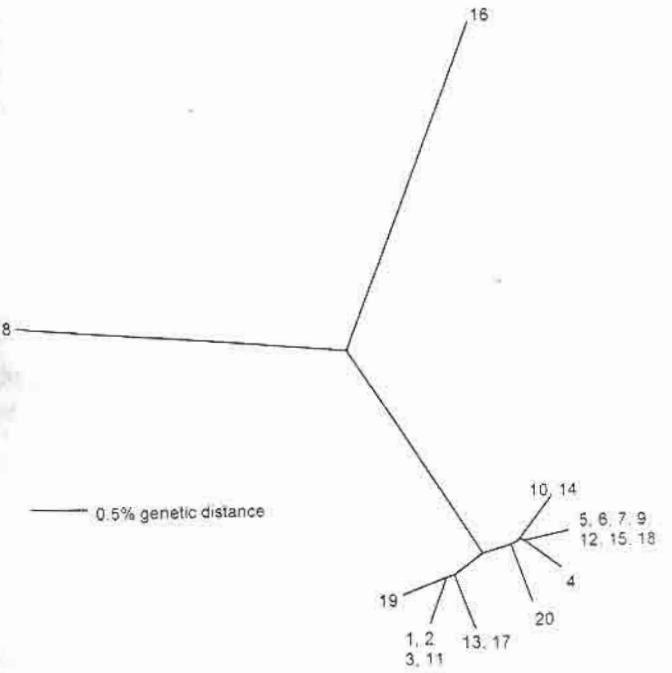

Fig. 6. UPGMA cluster diagram of *Rothmannia annae* individuals from Aride Island based on AFLPs. Numbers refer to the locations shown on Fig. 1.

Given the lack of geographical structure, it is not possible to designate populations of *R. annae* on Aride that are more important genetically than others. The small size of the island probably means that pollination is still occurring between the remaining populations and that there is therefore geneflow between them. The genetic data presented here support that hypothesis. Rather than conserving individual genotypes, as the distinct genotypes are distributed across the different populations on the island, it appears to be more important to control factors that lead to the population decline in this species.

In conclusion, genetic fingerprinting using AFLPs has been shown here to be an appropriate tool for the assessment of levels of genetic variation in the remaining populations of two endemic species from the Seychelles. The data collected have been used to inform the management of these species for their conservation and will, we hope, contribute to the continued survival and wellbeing of these species.

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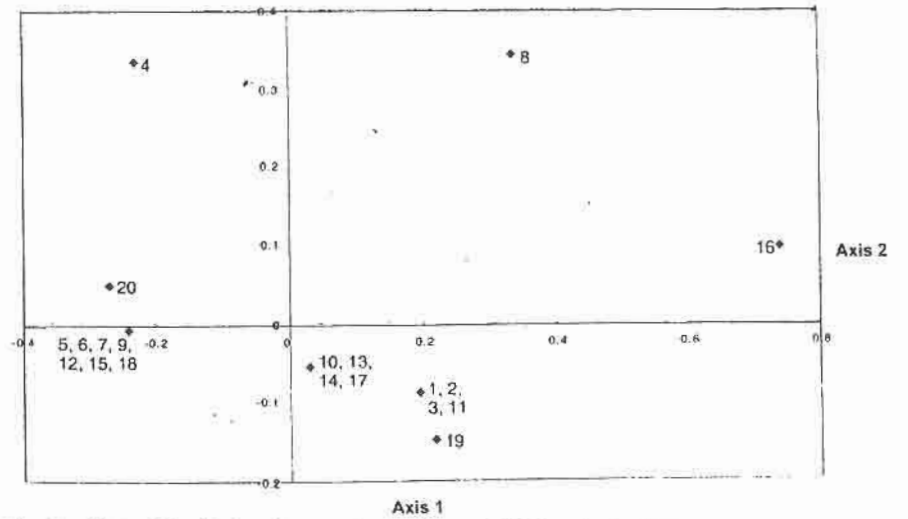

Fig. 7. Plot of the first and second axes from a PCO analysis of *Rothmannia annae* individuals from Aride Island. Numbers refer to the locations shown on Fig. 1.

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