ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 4 (4): 267-274, 2005
ISSN 1682-296X

Detection of Genetic Diversity in *Trochetia boutoniana* using Random Amplified Polymorphic DNA (RAPD) Markers

D. Puchooa and K. Venkatasamy
Department of Agricultural and Food Science,
Faculty of Agriculture, University of Mauritius, Réduit, Mauritius

Abstract: The reliability of Random Amplified Polymorphic DNA (RAPD) techniques to detect genetic diversity between *Trochetia boutoniana* species from six different locations in Mauritius was investigated. DNA fragments generated by 10-base primers were compared and variation could be detected. OPC-18 for example generated banding patterns unique to *Trochetia boutoniana* from three of the six locations. Investigation indicates that RAPD markers can be used to characterize *Trochetia boutoniana* species and that the technique is sensitive enough to reveal differences within this species.

Key words: Genetic diversity, primers, RAPDs, *Trochetia boutoniana*

INTRODUCTION

The genus *Trochetia*, member of the Sterculacea family, is an important endemic group to the Mascarene Islands and consists of six recognised species, namely, *Trochetia granulata, Trochetia uniflora, Trochetia triflora, Trochetia boutoniana, Trochetia blackburniana* and *Trochetia parviflora*[1]. Out of these, the last five are from Mauritius. *T. boutoniana* is a shrub 2-3 m high with a very short trunk which branches profusely at the base. It bears spectacular earring-shaped carmine red colour flowers locally known as Boucle d`oreille. These flowers have greatly captured the interest of botanists and it was declared the National flower of Mauritius in 1992. The most important population is located on the cliffs of Le Morne Brabant in the south-west of Mauritius but other discrete individuals scattered over the island have also been reported. Unfortunately, the plant is on the red list of critically endangered species[2]. The invasion of goyave de chine species has stifled the Trochetia species by competing with them for space and sunlight. Also, removal of flowers by visitors and attack by monkeys are the key reasons for the dwindling of the *Trochetia`s* population. Recently, the Ministry of Agriculture, Food Technology and Natural Resources have been working in the regeneration of *Trochetia boutoniana* by conventional seed propagation. However, proper identification of the species seems to be a problem. Identification, especially genetic variation, is indispensable to effective management and use of genetic resources.

Traditionally, diversity has been assessed by measuring variation in phenotypic traits such as flower colour, growth habit or quantitative agronomic traits like yield potential and stress tolerance. This approach, however, has certain limitations: Genetic information provided by morphological characters is often limited and expression of quantitative traits is subjected to strong environmental influence. Biochemical methods based on seed protein and enzyme electrophoresis have been useful in analysis of genetic diversity as they reveal differences between seed storage proteins or enzymes encoded by different alleles at one (allozymes) or more gene loci (isozymes). Use of biochemical methods eliminates the environmental influence; however, their usefulness is limited due to their inability to detect low levels of variation. DNA-based techniques introduced over the past two decades have potential to identify polymorphisms represented by differences in DNA sequences. These methods are being used as complementary strategies to traditional approaches for assessment of genetic diversity. The major advantage being that they analyse the variation at the DNA level itself, excluding all environmental influences. The analysis can be performed at any growth stage using any plant part and it requires only small amounts of material. Following the advances in molecular biology in the last decade, a variety of different methods have been developed for analysis of genetic diversity.

PCR-based techniques such as Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and Simple Sequence Repeats

Corresponding Author: Dr. D. Puchooa, Department of Agricultural and Food Science, Faculty of Agriculture, University of Mauritius, Réduit, Mauritius

(SSRs, microsatellites) have proved especially important in diversity studies. Newer and more powerful molecular techniques that detect variation at specific gene loci, which can be automated for high throughput of samples, are becoming available[3], permitting precise and versatile analyses of genetic variation. Molecular markers have been applied to study genetic diversity from natural populations and formulate efficient sampling strategies to capture maximum variation for genetic resources conservation. Molecular techniques have proved useful in a number of ways to improve the conservation and management of plant genetic resources. In particular, genetic diversity data provides information on gaps in terms of coverage in gene pools as well as redundancies, i.e., material with similar characteristics that wastes resources through increased cost of management. For example, RAPD analysis in *Brassica oleracea* revealed that 14 phenotypically uniform accessions could be reduced to 4 groups with minimal loss of genetic variation[4]. Molecular markers have been used to identify groups from which core collection accessions can be selected or to monitor the effectiveness of one or the other strategy in capturing genetic diversity found in the whole collection. Variation within species has also been studied to explore geographic or ecological patterns of distribution of diversity in many different crops and their wild relatives that include wild bean (*Phaseolus vulgaris*)[5], banana[6], mango[7], bambara groundnut[8], vetch[9], *Cicer* sp.[10], sorghum[11], sweet potato[12], tea[13] and chicory[14]. Marker-assisted analysis revealed limited genetic diversity within and between sites, when Fajardo *et al.*[15] studied genetic diversity in sweet potato accessions collected from four provinces of Papua New Guinea, a secondary centre of diversity. Ude *et al.*[16] used AFLP and RADP markers to detect polymorphism and assess genetic relationships in a set of 25 plantains from Western and Central Africa. A small group of genetically distinct cultivars from Cameroon was separated from the bulk of other plantains, suggesting that Cameroon may harbour accessions with useful or rare genes for widening the genetic base of breeding populations. RAPDs have also been used to assay genetic variation and establish genetic relationships among the wild species of *Arachis*[17]. As there is no published information regarding genetic diversity studies in *Trochetia boutoniana*, an investigation was carried out to determine the sensitivity of RAPD in the detection of genetic diversity within *Trochetia boutoniana* species in Mauritius.

MATERIALS AND METHODS

Plant samples: Plant materials were obtained from six different locations around the island (Table 1).

Table 1: *Trochetia boutoniana* species evaluated by Random Amplified Polymorphic DNA analysis

Location	Sample identity
Le Morne Brabant	T_1
Réduit-University of Mauritius	T_2
Réduit-Mauritius Institute of Education	T_3
Pamplemousses Botanical Garden (2 shrubs)	T_4 and T_5
Curepipe-Ministry of Agriculture, Food Technology and Natural Resources (2 shrubs)	T_6 and T_7
Mahebourg (2 shrubs)	T_8 and T_9

DNA extraction: Young, tender and unbruised leaves of *Trochetia boutoniana* were used as plant materials. For all DNA extraction procedures fresh materials were used to avoid any DNA degradation which might occur during freezing. The leaves were chosen for DNA extraction due to their continued availability whole year round.

Solutions: An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris-HCl (pH 8.8), 20 mM EDTA (pH 8.8), 1.6 M NaCl, 2.5% PVP (Mr 10,000) and 0.5% β-mercaptoethanol was prepared. Chloroform: Isoamylalcohol (24:1), 70 and 95% ethanol, 3 M NaCl and a TE buffer consisting of 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8.4) were also needed.

DNA isolation and purification: 0.5 g of fresh leaves was ground in liquid nitrogen using a motar and a pestle. Fifteen milliliter of preheated (60°C) extraction buffer were then added and the tubes mixed by inversion. After mixing for some minutes the tubes were incubated in a water-bath at 60°C for 35 min. The tubes were then allowed to cool to room temperature before adding an equal volume of chloroform:octanol (24:1). After mixing of the tubes by inversion to form an emulsion, the tubes were centrifuged at 8000 g for 15 min at room temperature. After centrifugation the top aqueous solution was transferred to new tubes using a micropipette. The chloroform:octanol extraction step was repeated to remove cloudiness in the aqueous phase. The tubes were gently inverted about 12 times. To accentuate precipitation of DNA, the mixtures were placed in freezer (-20°C) for 10 min. The DNA was pelletted by centrifugation at 8000 g for 10 min at 4°C. Then the supernatant were poured off and the resulting pellets were washed twice with 70% ethanol and allowed to air dry for 1 h. The pellets were then dissolved in 300 μL TE buffer. To remove any RNA from the preparation, 7 μL Rnase A was added and the tubes were incubated at 37°C for 1 h. An equal volume of chloroform-isoamylalcohol was added and the mixture centrifuged at 8000 g for 5 min and the upper aqueous layer was gently removed with the help of a micropipette. To this supernatant 1/10 volume of 3 M NaCl was added followed by 2 volumes of cold absolute ethanol. The mixture was placed in freezer at

Table 2: List of primers used and their sequence

Oligo-primer name	Primer sequence
OPI-03	CAG AAG CCC A
OPI-06	AAG GCG GCA G
OPA-10	GTG ATC GCA G
OPJ-13	CCA CAC TAC C
OPJ-16	CTG CTT AGG G
OPR-03	ACA CAG AGG G
OPR-09	TGA GCA CGA G
OPR-18	CCT CCT CAT C
OPJ-04	CCG AAC ACG G
DK-01	CCC TCT GCG G
DK-02	CGA CCG CCA A
OPW-08	GAC TGC ATC T
OPW-01	CTC AGT GTC C
OPW-02	ACC CCG CCA A
OPW-04	CAG AAG CGG A
OPW-09	GTG ACC GAG T
OPB-16	TTT GCC CGG A
OPB-17	AGG GAA CGA G
OPC-05	GAT GAC CGC C
OPC-14	GTG ACA GGC T

-20°C for 1 h followed by centrifugation at 12000 g for 10 min. The resulting pellets were washed with 70% ethanol, air dried and resuspended 100 µL of 0.1xTE.

Measurement of amount and purity of DNA: The DNA yield per gram of leaf tissue was measured by using a UV-VIS Spectronic Genesys 5 (Milton Roy) spectrophotometer at 260 nm. DNA purity was determined by calculating the absorbance ratio $A_{260/280}$. Pure DNA has a ratio of 1.8±0.1[18] Polysaccharide contamination was assessed by calculating the absorbance ratio $A_{260/230}$. DNA samples from the leaf tissues were electrophoresed on a 1% agarose gel.

RAPD procedure: The primers used for RAPD analysis and their sequence is shown in Table 2. A modification of the procedure of Williams *et al.*[19] was used. RAPD reactions were performed in a total volume of 30 µL containing: 1.0 U *Taq* polymerase, 2.5 mM $MgCl_2$ and 1X PCR buffer (supplied with *Taq* polymerase), 15 pM primer, 0.25 mM of DNA polymerization mix (containing dATP, dCTP, dGTP and dTTP) and 20 ng template DNA. For control reactions, template DNA was excluded from the reaction mixture. Polymerase Chain Reaction (PCR) cycling conditions were as follows: 1 cycle of 90s at 95°C, followed by 40 cycles of 30s at 92°C, 1 min at 35°C, 3 min at 72°C and a final time delay cycle of 5 min at 72°C. PCR reactions were performed in a BIO-RAD MyCycler Thermal Cycler. Amplified products were separated by electrophoresis in a 2.0% agarose gel. A Tris-borate EDTA (TBE) buffer system was used.

RESULTS

Quality and amount of DNA: From the absorbance results at 260 and 280 nm, purity of the DNA from nine locations

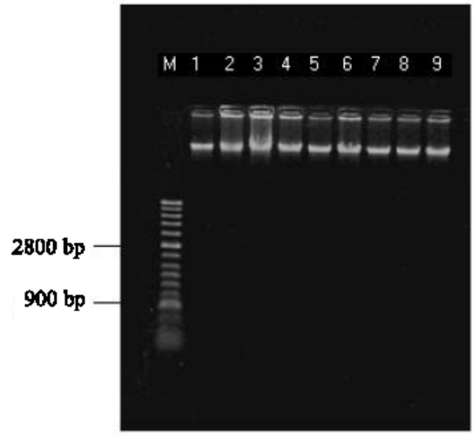

Fig. 1: 1% agarose gel showing results of DNA extracted from *Trochetia boutoniana* from six different locations. Lane M: Molecular Marker, Lane 1: DNA from T_1, Lane 2: DNA from T_2, Lane 3: DNA from T_3, Lane 4: DNA from T_4, Lane 5: DNA from T_5, Lane 6: DNA from T_6, Lane 7: DNA from T_7, Lane 8: DNA from T_8, Lane 9: DNA from T_9

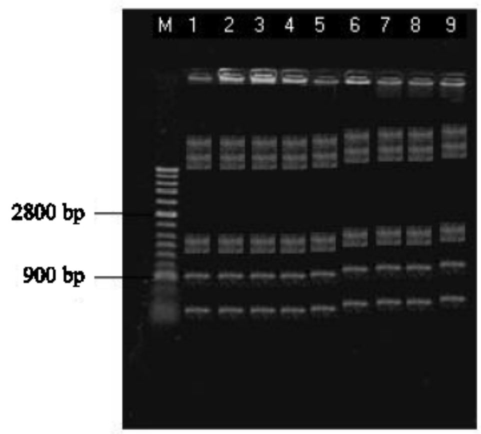

Fig. 2: 2% agarose gel showing results of RAPD using primer OPA 10. Lane M: molecular Marker, Lane 1: DNA from T_1, Lane 2: DNA from T_2, Lane 3: DNA from T_3, Lane 4: DNA from T_4, Lane 5: DNA from T_5, Lane 6: DNA from T_6, Lane 7: DNA from T_7, Lane 8: DNA from T_8, Lane 9: DNA from T_9

were calculated and $A_{260/280}$ ratios ranging from 1.640 to 1.740 were obtained. The average yield was 283.5 µg DNA per gram of leaf material. Upon electrophoresis on 1% agarose gel, thick and distinct bands were obtained (Fig. 1).

Screening of explant with 10-base primers: Initial screening of explants from *Trochetia boutoniana* species obtained from different regions was performed with twenty 10-base primers. Analysis was based on the visual

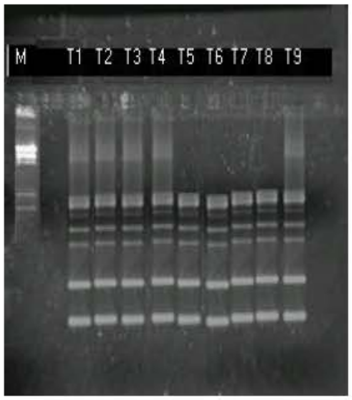

Fig. 3: 2% agarose gel showing results of RAPD using primer OPA 18. Lane M: Molecular Marker, Lane T_1: DNA from T_1, Lane T_2: DNA from T_2, Lane T_3: DNA from T_3, Lane T_4: DNA from T_4, Lane T_5: DNA from T_5, Lane T_6: DNA from T_6,Lane T_7: DNA from T_7, Lane T_8: DNA from T_8, Lane T_9: DNA from T_9

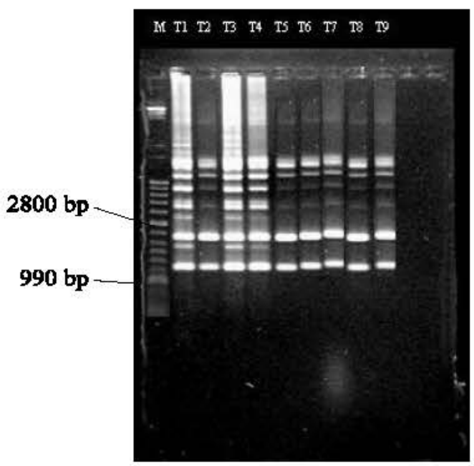

Fig. 4: 2% agarose gel showing results of RAPD using primer OPC 14. Lane M: Molecular Marker, Lane T_1: DNA from T_1, Lane T_2: DNA from T_2, Lane T_3: DNA from T_3, Lane T_4: DNA from T_4, Lane T_5: DNA from T_5, Lane T_6: DNA from T_6,Lane T_7: DNA from T_7, Lane T_8: DNA from T_8, Lane T_9: DNA from T_9

resolution of DNA bands on agarose gels. Criteria for selection were based on each primer's ability to produce clearly discernable bands for evaluation and comparison. Based on these criteria, the following 5 primers were selected: OPA10, OPA18, DK02, OPC14 and OPC18. The DNA fingerprints produced by the five selected primers are shown in Fig. 2-6. Each primer investigated produced a distinct pattern of amplified bands. The difference in

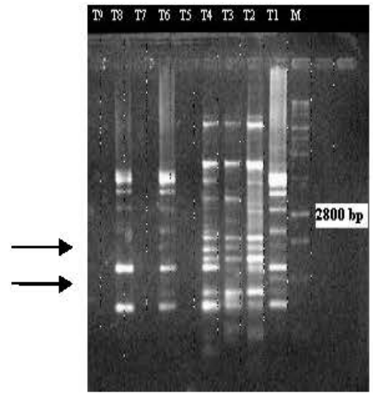

Fig. 5: 2% agarose gel showing results of RAPD using primer OPC 18. Lane M: Molecular Marker, Lane T_1: DNA from T_1, Lane T_2: DNA from T_2, Lane T_3: DNA from T_3, Lane T_4: DNA from T_4, Lane T_6: DNA from T_6, Lane T_7: DNA from T_7, Lane T_8: DNA from T_8, Lane T_9: DNA from T_9

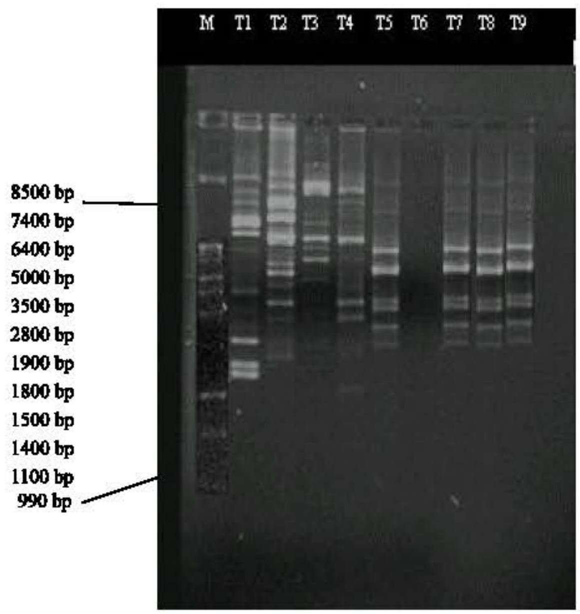

Fig. 6: 2% agarose gel showing results of RAPD using primer DK 02. Lane M: Molecular Marker, Lane T_1: DNA from T_1, Lane T_2: DNA from T_2, LaneT_3: DNA from T_3, Lane T_4: DNA from T_4, Lane T_5: DNA from T_5, Lane T_7: DNA from T_7, Lane T_8: DNA from T_8, Lane T_9: DNA from T_9

fingerprints generated is also evident when primers OPC 18 and DK 02 were used (Fig. 5 and 6, respectively).

The stability of amplified polymorphisms produced by primers was assessed for ten DNA extractions of the same location and ten separate PCR reactions and the bands generated on 1% agarose gel. Consistencies in

Table 3: Representative banding profile of *Trochetia boutoniana* samples generated using OPC 18

Band size (bp)	Sample identity T_1	T_2	T_3	T_4	T_6	T_8
990	++	++	++	++	++	++
1000	0	0	+	0	0	0
1100	v	++	++	++	v	0
1400	+	v	0	0	0	0
1500	++	++	+	++	++	++
1600	v	++	++	++	v	0
1800	v	++	++	++	v	v
1900	v	++	++	++	v	v
2000	++	+	0	v	v	v
2900	++	++	v	+	+	+
3000	0	++	0	0	0	0
3200	+	0	+	+	0	0
3500	++	v	v	v	++	++
3600	++	++	0	++	++	++
4000	v	++	++	++	0	0
7500	v	++	++	++	0	0

V= Faint bands amplified for some PCR reactions; + = Presence of intense bands amplified for all reactions but which vary in intensity; ++ = Very strong bands present in all reactions; 0 = Absence of a band. As samples T_5, T_7 and T_9 were from the same regions as T_4, T_6 and T_8, respectively, they were not included in the reaction

DNA banding profiles were obtained although the intensity of some bands generated varied for some PCR reactions. Primer OPA 10 produced 7 clear and scorable bands. Identical amplification products were obtained for *Trochetia boutoniana* from the different locations. The amplified products ranged in size between 1000 and 11000 bp. Primers OPA 18 and OPC 14 also produced identical amplification products for *T. boutoniana* species obtained from the different locations with 8 (OPA 18) and 14 (OPC 14) scorable bands. The RAPD fingerprints of these primers (OPA 10, OPA 18 and OPC 14), therefore, did not reveal any variation in the *Trochetia boutoniana* samples obtained from the different locations. Among the polymorphic primers, DK 02 generated a maximum of six polymorphic bands (Fig. 6) whereas, OPC 18 (Fig. 5) produced a minimum of one polymorphic band. Primer DK 02 produced differences in banding patterns in DNA of *T. boutoniana* obtained from Le Morne Brabant (T_1), Réduit (T_2 and T_3) and Pamplemousses Botanical Garden (T_4 and T_5). Also, using this primer, different banding patterns were obtained for the *Trochetia boutoniana* species obtained from the University of Mauritius (T_1) and the Mauritius Institute of Education (T_2) although both were from Réduit. More surprisingly, the fingerprints of the two shrubs obtained from the Pamplemousses Botanical Gardens (T_4 and T_5) also produced different banding patterns using primer DK 02. However, primer DK 02 produced identical banding patterns for T_5, T_7, T_8 and T_9. Primer OPC 18 produced similar bands for T_1, T_6, and T_8, at least two bands were common to all (Fig. 5). Although, differences in banding patterns were observed between T_2, T_3 and T_4 using primer OPC 18, nevertheless, there were at least 8 monomorphic bands.

A number of bands, namely 990 bp, 1500 bp, were common to all samples tested. For others, such as the bands at 2900 and 3500 bp, there were clear differences in the intensity of the bands from very faint to very intense. The band at 1000 bp was present only in sample T_3. Bands at 1100 bp and 1600 bp were absent in T_8 while being present at very high intensity in T_2, T_3 and T_4 while very faintly in T_1 and T_6. For the bands at 1800 and 1900 bp, high intensity bands were observed for samples T_2, T_3 and T_4 while, very faint bands were obtained for T_1, T_6 and T_8. The bands at 2000 and 3600 bp were absent in T_3 only while band at 3000 bp was present in T_2 only and at high intensity. The bands at 4000 and 7500 bp were absent in both T_6 and T_8, present very faintly in T_1 while present at very high intensity in T_2, T_3 and T_4 (Table 3).

DISCUSSION

Trochetia boutoniana, whose flower has been declared as the national flower of Mauritius, is listed as being critically endangered[2]. The mother population of the species is situated on the rocky cliffs of Le Morne Brabant, situated in the southwest coast of the island. The Forestry Department as well as the National Parks Conservation Unit of the Ministry of Agriculture, Food Technology and Natural Resources are actively involved in the conservation and propagation of this species. Observations made by the Forestry Department suggest that the handful of *T. boutoniana* plants scattered over the island bear certain morphological differences such as leaf shape. No doubt evolutionary forces and various ecological stresses must have played a key role in shaping these plants' phenotypes. The present study provides the first information on genetic variation among *Trochetia boutoniana* species using RAPD analysis. The polymorphism was due to the occurrence of variation in only nine plants obtained from six different locations around the island and which are not morphologically indistinguishable. The results also show that the endemic Mauritian *T. boutoniana* species exhibit marked genetic variability. This is highly suggestive of an existing gene flow within the *T. boutoniana* population. Such gene flow can simply be equated to the exchange of genetic traits between *T. boutoniana* populations through the movement of individuals from one place to another. Also with a sustained condition of low population size and genetic isolation, it seems that the *T. boutoniana* species have diverged from each other thus exhibiting variations among the populations scattered over the island.

The DNA profiles generated in this study of *Trochetia boutoniana* from six different locations around the island, demonstrate the sensitivity of RAPD techniques for differentiating within cultivars. Repeated PCR reactions of the same DNA samples and separate DNA isolates, generated reproducible amplified products with each primer investigated. The reliability of amplified DNA profiles generated by RAPD techniques have been questioned in the past[20,21]. Factors such as the quality and quantity of sample DNA, reaction parameters and the type of thermal cycler used have influenced the profile of the DNA bands generated[20]. It was observed that while there may be variation in results obtained between laboratories, consistent results can be maintained within a particular laboratory as long as the reaction parameters remain the same for all reactions.

RAPD markers have been successfully used by a number of researchers to differentiate among cultivars and to establish phylogenetic relationship. For example, genetic variation within and between natural populations of *Digitalis obscura* was quantified using RAPDs and the results were used for optimizing sampling strategies for conservation of genetic resources of the species[22]. Recommendations to focus on the sampling of marginal pawpaw (*Asimina triloba*) populations in future collection missions were derived from the genetic structure across natural distribution areas, established by RAPD analysis[23]. Recent examples of use of molecular markers to identify redundancies in collections include perennial kales[24], wheat[25], grapevine[26], sorghum[27], cassava[28], flax[29] and barley[30]. Using RAPD markers, Skroch *et al.*[31] found no significant differences in genetic diversity between core collection and a chosen set of accessions, randomly chosen from the whole collection of common bean germplasm, indicating that the method used to develop the core collection had not been very efficient in capturing diversity. Grenier *et al.*[32] evaluated different sampling methods to constitute a core collection of sorghum landraces for the extent of genetic diversity captured based on microsatellites analysis. Molecular markers have been employed for fingerprinting, verification of accession identity and genetic contamination. RAPDs were used to identify dwarf off-types arising from micropropagation of banana cultivars[33] and incorrectly labeled accessions in a barley germplasm[34]. Del Rio *et al.*[35], Wu *et al.*[36] and Börner *et al.*[37] using RAPDs studied the changes in genetic diversity following regeneration of potato, rapeseed and wheat accessions, respectively.

It is concluded that the high degree of polymorphism obtained with RAPD analysis is very useful in fingerprinting and determining the genetic diversity within *Trochetia boutoniana*. Amplification with a minimum of 2 primers could distinguish the *Trochetia boutoniana* from different locations around Mauritius used in this study and can provide a method to avoid mislabeling. Knowledge of the degree of genetic relationship will be of utmost importance for the establishment of a core collection as part of the germplasm collection management.

ACKNOWLEDGEMENTS

The authors wish to thank the Forestry Department and the Conservation Unit of the Ministry of Agriculture, Food Technology and Natural Resources and the SSR Botanical Garden Trust for providing plant materials for this study. The support of the Faculty of Agriculture of the University of Mauritius for this study is also acknowledged.

REFERENCES

1. Strahm, A.W., 1993. The conservation and restoration of the flora of Mauritius and Rodrigues. Ph.D Thesis, University of Reading, pp: 15-45.
2. IUCN., 1994. IUCN Red list categories. Gland, Switzerland: IUCN World Conservation Union, pp: 46-55.
3. Sicard, D., S.S. Woo, R. Arroyo-Garcia, O. Ochoa, D. Nguyen, A. Korol, E. Nevo and R. Michelmore, 1999. Molecular diversity at the major cluster of disease resistance genes in cultivated and wild *Lactuca* sp. Theor. Applied. Genet., 99: 405-418.
4. Phippen, W.B., S. Kresovich, F.G. Candelas and J.R. McFerson, 1997. Molecular characterization can quantify and partition variation among genebank holdings: A case study with phenotypically similar accessions of *Brassica oleracea* var. capitata L. (cabbage) 'Golden Acre'. Theor. Applied Genet., 94: 227-234.
5. Tohme, J., D.O. Gonzalez, S. Beebe and M.C. Duque, 1996. AFLP analysis of gene pools of a wild bean core collection. Crop Sci., 36: 1375-1384.
6. Pillay, M., E. Ogundiwin, D.C. Nwakanma, G. Ude and A. Tenkouano, 2001. Analysis of genetic diversity and relationships in East African banana germplasm. Theor. Applied Genet., 102: 965-970.
7. Kashkush, K., F. Jinggui, E. Tomer, J. Hillel and U. Lavi, 2001. Cultivar identification and genetic map of mango (*Mangifera indica*). Euphytica, 122: 129-136.
8. Massawe, F.J, M. Dickinson, J.A. Roberts and S.N. Azam Ali, 2002. Genetic diversity in bambara groundnut (*Vigna subterranean* (L.) Verdc) landraces revealed by AFLP markers. Genome, 45: 1175-1180.

9. Potokina, E., F. Blattner, T. Alexandrova and N. Bachmann, 2002. AFLP diversity in common vetch (*Vicia sativa* L.) on the world scale. Theor. Applied Genet., 105: 58-67.
10. Iruela, M., J. Rubio, J. Cubero, I. Gill and T. Millan, 2002. Phylogenetic analysis in the genus *Cicer* and cultivated chickpea using RAPD and ISSR markers. Theor. Applied Genet., 104: 643-651.
11. Nkongolo, K.K. and L. Nsapato, 2003. Genetic diversity in *Sorghum bicolor* (L.) Moench accessions from different ecogeographical regions in Malawi assessed with RAPDs. Genet. Resour. Crop Evol., 50: 149-156.
12. Gichuki, S.T., M. Berenyi, Zhang DaPeng, M. Hermann, J. Schmidt, J. Glossl and K. Berg, 2003. Genetic diversity in sweetpotato (*Ipomoea babatas* (L.) Lam) in relationship to geographic sources as assessed with RAPD markers. Genet. Resour. Crop Evol., 50: 429-437.
13. Balasaravanan, T., Pius, P.K., R.R. Kumar, N. Muraleedharan and A.K. Shasany, 2003. Genetic diversity among south Indian tea germplasm *(Camellia sinensis, C. assamica and C. assamica* sp. *lasiocalyx)* using AFLP markers. Plant Sci., 165: 365-372.
14. Van Cutsem, P., P. du Jardin, C. Boutte, T. Beauwens, S. Jacqmin and X. Vekemans, 2003. Distinction between cultivated and wild chicory gene pools using AFLP markers. Theor. Applied Genet., 107: 713-718.
15. Fajardo, D.S., D.R. La Bonte and R.L. Jarret, 2002. Identifying and selecting for genetic diversity in Papua New Guinea sweetpotato *Ipomoea batatas* (L.) Lam. Germplasm as botanical seed. Genet. Resour. Crop Evol., 49: 463-470.
16. Ude, G., M. Pillay, D. Nwakanma and A. Tenkouano, 2003. Analysis of genetic diversity and sectional relationships in *Musa* using AFLP markers. Theor. Applied Genet., 104: 1239-1245.
17. Santos, V.S.E.D., M.A. Gimenes, J.M.F. Valls and C.R. Lopes, 2003. Genetic variation within and among species of five sections of the genus *Arachis* L. (Leguminosae) using RAPDs. Genet. Resour. Crop Evol., 50: 841-848.
18. Clark, M.S., 1997. In: Plant Molecular Biology-A Laboratory Manual, Springer-Verlag Berlin Heidelberg, New York, pp: 305-328.
19. Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.
20. Munthali, M., B.V. Ford-Lloyd and H.J. Newbury, 1992. The random amplification of polymorphic DNA for fingerprinting plants. PCR Methods and Application, 1: 274-276.
21. Levi, A., Rowland, L.J. and J.S. Hartung, 1993. Production of reliable randomly amplified polymorphic DNA (RAPD) markers from DNA of woody plants. Hortic. Sci., 28: 1188-1190.
22. Nebauer, S.G., L. del Castillo-Agudo and J. Segura, 1999. RAPD variation within and among natural populations of outcrossing willow-leaved foxglove (*Digitalis obscura* L.). Theor. Applied Genet., 98: 985-994.
23. Huang, H.W., D.R. Layne and T.L. Kubisiak, 2000. RAPD inheritance and diversity in pawpaw (*Asimina triloba*). J. Am. Soc. Hortic. Sci., 125: 454- 459.
24. Zeven, A.C., K.J. Dehmer, T. Gladis, K. Hammer and H. Lux, 1998. Are the duplicates of perennial kale (*Brassica oleracea* L. var. ramose DC) true duplicates as determined by RAPD analysis? Genet. Resour. Crop Evol., 45: 105-111.
25. Cao, W.G., P. Hucl, G. Scoles and R.N. Chibbar, 1998. Genetic diversity within spelta and macha wheats based on RAPD analysis. Euphytica, 104: 181-189.
26. Cervera, M.T., J.A. Cabezas, J.C. Sancha, F. Martinez de Toda and J.M. Martinez-Zapater, 1998. Application of AFLPs to the characterization of grapevine *Vitis vinifera* L. genetic resources. A case study with accessions from Rioja (Spain). Theor. Applied Genet., 97: 51-59.
27. Dean, R.E., J.A. Dahlberg, M.S. Hopkins S.E. Mitchell and S. Kresovich, 1999. Genetic redundancy and diversity among 'orange' accessions in the US National Sorghum Collection as assessed with Simple Sequence Repeat (SSR) markers. Crop Sci., 39: 1215-1221.
28. Chavarriaga-Aguirre, P., M.M. Maya, J. Tohme, M.C. Duque, C. Iglesias, M.W. Bonierbale, S. Kresovich, G. and G. Kochert, 1999. Using microsatellites, isozymes and AFLPs to evaluate genetic diversity and redundancy in the cassava core collection and to assess the usefulness of DNA-based markers to maintain germplasm collections. Mol. Breed., 5: 263-273.
29. van Treuren, R., L.J.M. van Soest and T.H.J.L. Hintum, 2001. Markerassisted rationalisation of genetic resources collections: A case study in flax using AFLPs. Theor. Applied Genet., 103: 144-152.
30. Lund, B., R. Ortiz, Skovgaard and R. Waugh, 2003. Analysis of potential duplicates in barley gene bank collections using re-sampling of microsatellite data. Theor. Applied Genet., 106: 1129-1138.

31. Skroch, P.W., J. Nienhuis, S. Beebe, J. Tohme and F. Pedraza, 1998. Comparison of Mexican common bean (*Phaseolus vulgaris* L.) core and reserve germplasm collections. Crop Sci., 38: 488-496.
32. Grenier, C., M. Deu, S. Kresovich, P.J. Bramel-Cox and P. Hamon, 2000. Assessment of genetic diversity in three subsets constituted from the ICRISAT sorghum collection using random vs non-random sampling procedures B. Using molecular markers. Theor. Applied Genetics, 101: 197-202.
33. Damasco, O.P., G.C. Graham, R.J. Henry, S.W. Adkins, M.K. Smith and I.D. Godwin, 1996. Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* sp. AAA) bananas. Plant Cell Rep., 16: 118-123.
34. Poulsen, D.M.E., H.L. Ko, J.G. vanderMeer, P.M. vandePutte and R.J. Henry, 1996. Fast resolution of identification problems in seed production and plant breeding using molecular markers. Aus. J. Exp. Agric., 36: 571-576.
35. Del Rio, A.H., J.B. Bamberg and Z. Huaman, 1997. Assessing changes in genetic diversity of potato genebanks. 1. Effects of seed increase. Theor. Applied Genet., 95: 191-198.
36. Wu, X.M., N.F. Wu, X.Z. Qian, R.G. Li, F.H. Huang and L. Zhu, 1998. Phenotypic and genotypic changes in rapeseed after 18 years of storage and regeneration. Seed Sci. Res., 8: 55-64.
37. Börner, A., S. Chebotar and V. Korzun, 2000. Molecular characterization of the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term maintenance. Theor. Applied Genet., 100: 494-497.