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Molecular Characterization of *Musa* spp. by Simple Sequence Repeats (SSR)

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ABSTRACT. *Biological diversity in banana is rich in Sri Lanka, and may carry genes resistant to major pests and diseases. It is a source of germplasm for improvement of banana. Although morphological characterization of banana germplasm is being done at the Plant Genetic Resources Center in Sri Lanka, molecular characterization allows identification of duplicates, determination of phylogenetic relationships and estimation of the extent of genetic diversity. To identify cultivars and phylogenetic relationships, DNA was extracted from fourteen AA, AAA, AAB, ABB cultivars and one wild species (Musa acuminata Colla.). Nine simple sequence repeat primer pairs were used and polymerase chain reaction products were subjected to urea PAGE followed by silver staining. A total of 44 alleles were detected in 11 polymorphic loci. Even though diploid and triploid 'A' genomes exhibited high polymorphism, the presence of 'A' genome can be identified in interspecific hybrid cultivars. It provides evidence that 'A' genome in the genotypes may have come from different sources or it may have evolved from long term mutations. Multivariate analysis of molecular data allowed clustering of germplsm into 7 major groups. Cultivars in 4 clusters were in agreement with morphological classification, whereas the other clusters consisted of several genome groups. Wild diploid M. acuminata, ratambala and diploid cultivar navari showed distinct molecular differences in contrast to all other cultivars. Theses results support the view that diploid unel and navari are distinct genotypes. They can be utilized in future banana improvement programs.*

INTRODUCTION

Bananas and plantains (*Musa* spp.) are of vital importance to millions of people in developing countries. The total world production is about 70 million tons per year. Sri Lanka produces 460,500 tons annually in an extent of 54,800 ha and has a high local demand and export potential. Therefore, improvement of *Musa* remains a high priority in Sri Lanka. Twenty nine cultivars (Chandraratne and Nanayakkara, 1951) and two wild species (*Musa acuminata* and *M. balbisiana* Colla.) are distributed from sea level to 2000 m amsl in Sri Lanka representing almost all agro-ecological regions. Limited studies on the local banana germplasm shows apparent resistance to major biotic and abiotic stresses (Chandraratne and Nanayakkara, 1951). This diversity is a great asset for improvement of *Musa*, if conserved and properly characterized. Currently, germplasm collections are available at the Plant Genetic Resources Center (PGRC) and in the other research centers (200 accessions). It is doubtful whether these collections represent total diversity and

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further collection missions may be necessary to fill the gaps. Morphological characterization of large collections of banana is laborious and takes a long time as one has to wait until bunches are developed. Some morphological traits are environmentally dependant. Furthermore some physiological characters are also not reflected in the morphology of plants. Application of molecular markers such as Randomly Amplified Polymorphic DNA (RAPD) or Simple Sequence Repeats (SSR) are useful alternative means of characterization (Kaemmer *et al.*, 1992; Bhat *et al.*, 1995). The data generated by these techniques could be used to remove duplicates in the collections, determine phylogenetic relationships among cultivars and wild species, and to study the diversity within the cultivars and wild species. Therefore, the objectives of this study were to determine phylogenetic relationships between cultivars and wild species and also identify genetic markers for *Musa* spp.

MATERIALS AND METHODS

Fourteen banana cultivars and a diploid wild species, namely unel (*M. acuminata*) were selected for the investigation (Table 1). This germplasm is maintained in the fields of the PGRC, Gannoruwa. For DNA extraction, samples were collected from unopened, whitish leaf blades of young leaves using the modified CTAB method (Saghai-Maroof *et al.*, 1984; Doyle and Doyle, 1987; Rogers and Bendich, 1988).

Table 1. List of *Musa* spp. used in the study.

Number	Musa spp.	Suggested genomic group
1	Unel (wild, seedy)	AA
2	Navari (seedless)	AA
3	Binkesel	AAA
4	Ratambala	AAA
5	Galanamalu	AAA
6	Sapuanamalu	AAA
7	Anamalu	AAA
8	Ambon	AAA
9	Ratahondarawala	AAB
10	Embul	AAB
11	Puwalu	AAB
12	Muwanethikesel	AAB
13	Angawiaru	AAB
14	Bahu	ABB
15	Mondan	ABB

Source: Simmonds, 1996.

DNA extraction and purification

Three grams of leaf samples were ground in liquid nitrogen until a powder was formed. The powder was transferred quickly into 15 ml of pre warmed (60°C) 4% CTAB buffer with 0.1% β-mercaptoethanol. After incubating for 30 min at 60°C, 15 ml of 24 : 1 of Chloroform : Isoamyl alcohol was added and gently shaken for 10 min. The solution was centrifuged at 5000×g (10 min) and supernatant was filtered into 50 ml glass tubes. Then, 0.6 volume of ice cold isopropanol was added and gently mixed by inverting. Precipitated DNA was hooked out and placed in an eppendorf tube and washed twice with washing solution (1 M ammonium acetate and 70% ethanol) and air-dried. Pellet was dissolved in 100-500 µl of TE buffer at 4°C. Presence of DNA was confirmed in an agarose mini gel and stained with ethidium bromide. The extract was further purified from polysaccharides (by bringing the DNA solution to 1 M NaCl concentration) and RNA (by adding RNAse A to 100 µg/ml).

Quantification of DNA was done using 260 nm and 280 nm wave lengths in UV spectrophotometer by diluting stock 200 times. Finally, a sample of the stock was diluted to 50 ng/µl to be used in PCR.

PCR Amplification

PCR amplification was done using modified procedure of Kaemmer *et al.* (1997). PCR was performed using 94°C for 2 min for the initial denaturing and then 35 cycles of [30 sec 94°C denaturing, 30 sec 72°C annealing temperature 55°C, 30 sec 72°C extension] and a final extension at 72°C for 5 min. Reaction volume was 25 µl. Final concentration in the reaction solution was 0.5 µM for each forward and reverse primer (Table 2), 200 µM for dNTPs each, 2.5 mM for $MgCl_2$ and 0.025 U/µl of Taq DNA polymerase (Abgene®) and 5 ng/µl of DNA template. Twenty percent of loading buffer (95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue and 10 mM NaOH) was added to PCR products. Samples and the 1 kb DNA ladder were then denatured for 5 min in the boiling water.

Electrophoresis and silver staining of denaturing polyacrylamide gels

Six percent polyacrylamide 40 cm gels containing 7 M urea in 0.5 × TBE were prepared. The gel was pre run (at 50 W) for 45-60 min to pre-warm to 45°C. Five micro liters of products were run at 1250 V for 3-3 ½ h. After electrophoresis, the gels were soaked in 10% acetic acid for 20 min, washed 2 times for 3 min each in deionized water and stained for 20 min in 0.1% $AgNO_3$ containing 0.06% formaldehyde. After a brief rinse (10 sec) in de-ionized water signals were developed by soaking the gels in 200 ml of 3% Na_2Co_3 with 400 µl of 10% solution of $Na(S_2O_3)_2$ and 3 ml 38% formaldehyde. Development was terminated by soaking gels in 10% acetic acid for 2-3 min. Finally, gels were rinsed with de-ionized water and air dried for 1 h. Gels were scanned using photo editor computer package.

Scoring of bands and data analysis

Presence of clear bands representing alleles of each loci were scored as '1' and absence as '0'. The data generated were used for cluster analysis and principle component analysis using SPLUS 2000.

Table 2. SSR primers used in the analysis.

Primer	Sequence 5 ------- 3'	Product size (bp)
AGMI 59 (F)	AATCGAAATCGAGTCAACAAGG	309
AGMI 60 (R)	TTTTGTGGATGGTTGGTTCC	-
AGMI 95 (F)	ACTTATTCCCCCGCACTCAA	220
AGMI 96 (R)	ACTCTCGCCCATCTTCATCC	-
AGMI 101 (F)	TGCAGTTGACAAACCCCACACA	189
AGMI 102(R)	TTGGGAAGGAAAATAAGAAGATAGA	-
AGMI 103 (F)	ACAGAATCGCTAACCCTAATCCTC	181
AGMI 104 (R)	CCCTTTGCGTGCCCCTAA	-
MaSSR 7a (F)	AAGAAGGCACGAGGGTAG	212
MaSSR 7b (R)	CGAACCAAGTGAAATAGCG	-
MaSSR 12a (F)	TGTCGAAGCATCCTACATC	262
MaSSR 12b (R)	CTTGGAAACATGAGAAACATAC	-
MaSSR 19a (F)	AGAGTTCTTTTATACTAGGGG	144
MaSSR 19a (R)	AACCCGGATATTTCATTGTA	-
MaSSR 20a (F)	GAAATGGAGTTGGAGAAACA	222
MaSSR 20b (R)	CACATATCCTTGTCGGAAGT	-
MaSSR 24a (F)	GACCCCSTTAAGCTGAACA	172
MaSSR 24b (R)	CCGACGGTCAACATACAATACA	-

F - Forward; R – Reverse
Source: Kaemmer *et al.*, 1997.

RESULTS AND DISCUSSION

Nine primers amplified a total of 44 alleles in 11 polymorphic loci. Number of alleles observed ranged from 2-7 per locus depending on the primer and genotype. Primer pairs (referred to as primers) AGMI 59/60, 95/96, 101/102, 103/104, MaSSR 7a/7b, 24a/24b and MaSSR 12a/12b exhibited higher number of alleles (Plate 1, 2 and 3). Primers MaSSR 19a/19b and MaSSR 20a/20b were specific to a few genotypes (Plate 4). Both AGMI primers 95/96 and 103/104 amplified two loci each while the rest showed a single locus. Kaemmer *et al.*, (1997) reported that the product of AGMI 95/96

is about 220 bp and AGMI 103/104 is 181 bp in length. However, in this investigation both primers have amplified an additional locus of size about 100 bp. The MaSSR 24 produced a total of seven alleles with all 15 genotypes. AAA cultivar ratambala, AAB cultivars (ambul and puwalu) and bahu (ABB) showed triple bands. Thus, identification of AAB and ABB inter-specific hybrids from AAA (except ratambala) was possible with the primer MaSSR 24 (Plate 2). The *Musa* genotypes used in this study (Table 1) demonstrate a high level of SSR polymorphism even within AAA genotypes. It shows evidence that 'A' genome in these genotypes may have come from different sources or it may have evolved from long term mutation. Some specific bands were observed in navari (AA) and bahu (ABB). Navari produced specific bands for MaSSR7 (lane 2, Plate 2) and MaSSR20 (lane 2, Plate 3). Since this cultivar is compared here with the other *M. acuminta* genotypes including a wild species (AA and AAA) they can be considered as SSR makers for identification of navari. Bahu also amplified a specific band with AGMI 95/96 (Plate 1).

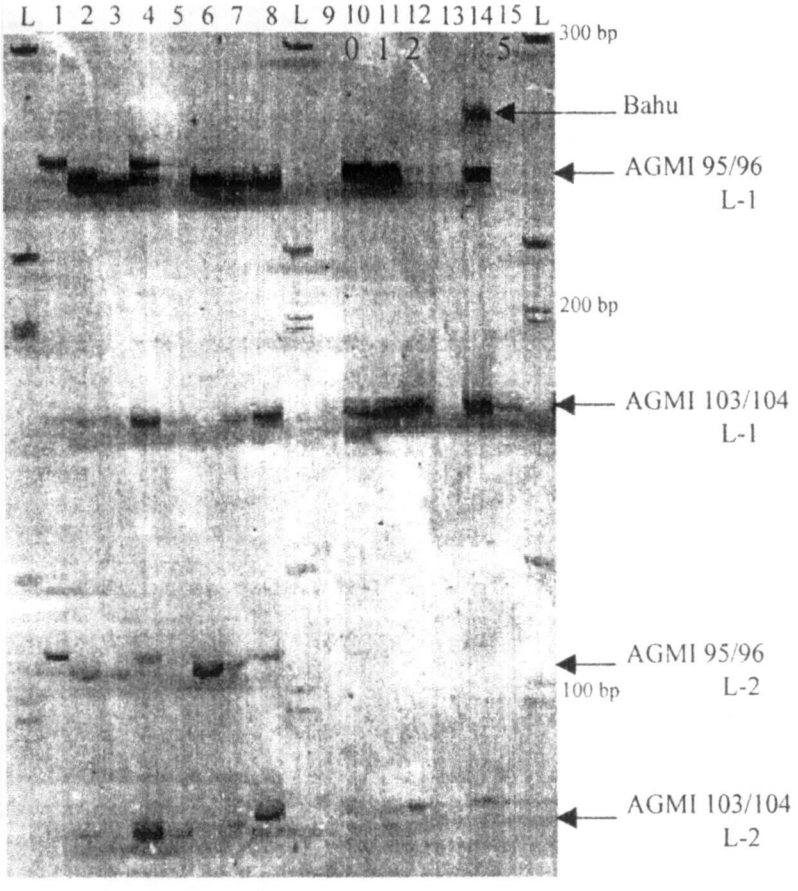

Plate 1. Silver stained urea PAGE with PCR products of SR primers AGMI 95/96 and 103/104.

[Note: Genotypes of lane 1- 15 are shown in Table 1]

In the dendogram (Fig. 1) groupings were done based on their similarity. Grouping with this SSR data and the genomic grouping based on morphological data showed considerable differences. Clustering classified the cultivars and wild species *M. acuminata* into 7 logical groups (Fig. 1). Unel (1), ratahondarawalu (9), puwalu (11) and navari (2) were considerably distant to all other genotypes (Fig. 2).

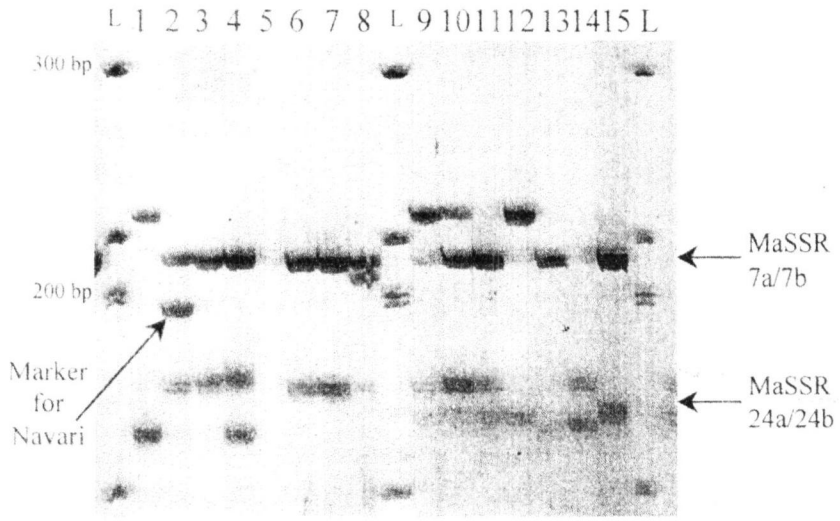

Plate 2. Silver stained urea PAGE with PCR products of SSR primers MaSSR 7a/7b and 24a/24b.

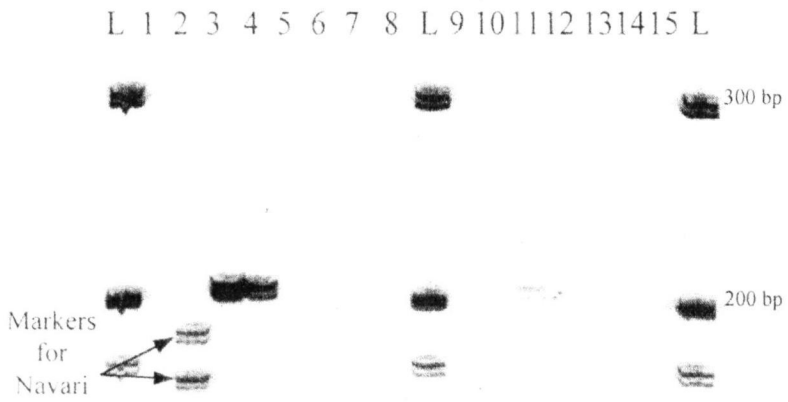

Plate 3. Silver stained urea PAGE with PCR products of SSR primer 20a/20b.
[Genotypes of lane 1-15 are shown in Table 1]

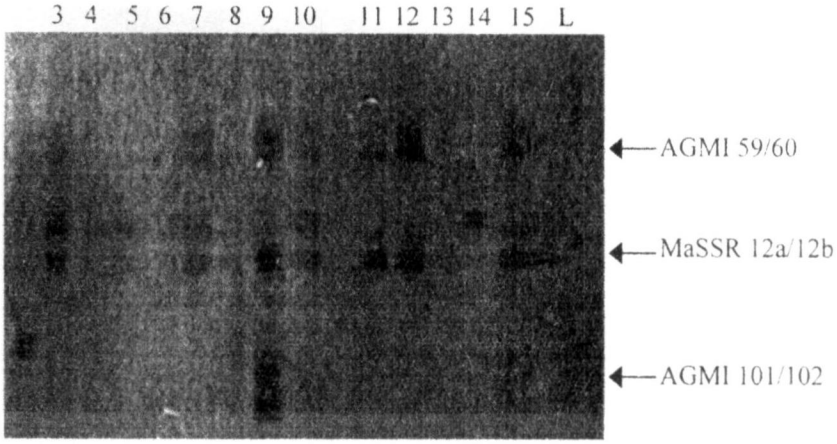

Plate 4: Silver stained urea PAGE with PCR products of primer AGMI 59/60, MaSSR 12a/12b and AGMI 101/102

[Note: Genotypes of lane 3-15 are shown in Table 1].

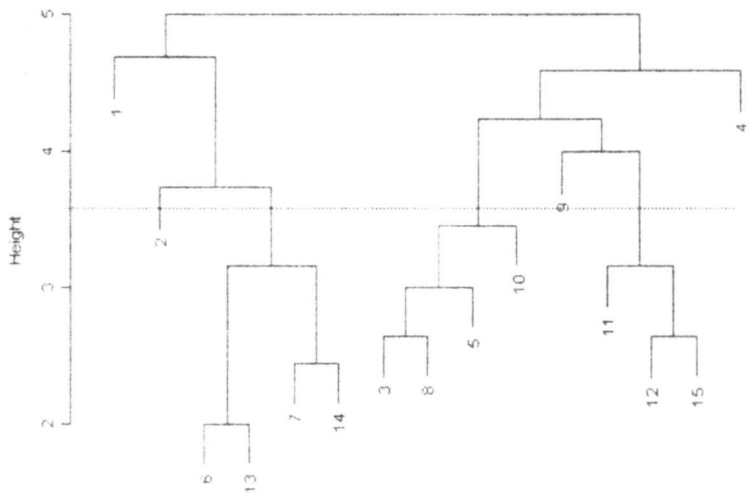

Fig. 1. Hierarchical cluster tree indicates grouping and genetic distances among *Musa* cultivars and wild species.

[Note: Group = (G)1-1 Uncl, (G)2-2 Navari, (G)3-6 Sapuanamalu, 13 Angaviaru, 7 Anamalu, 14 Bahu, (G)4-3 Binkesel, 8 Ambon, 5 Galanamalu, 10 Embul, (G)5-9 Ratahondarawala, (G)6-11 Puwalu, 12 Muwanethikesel, 15 Mondan, (G)7 Ratambala].

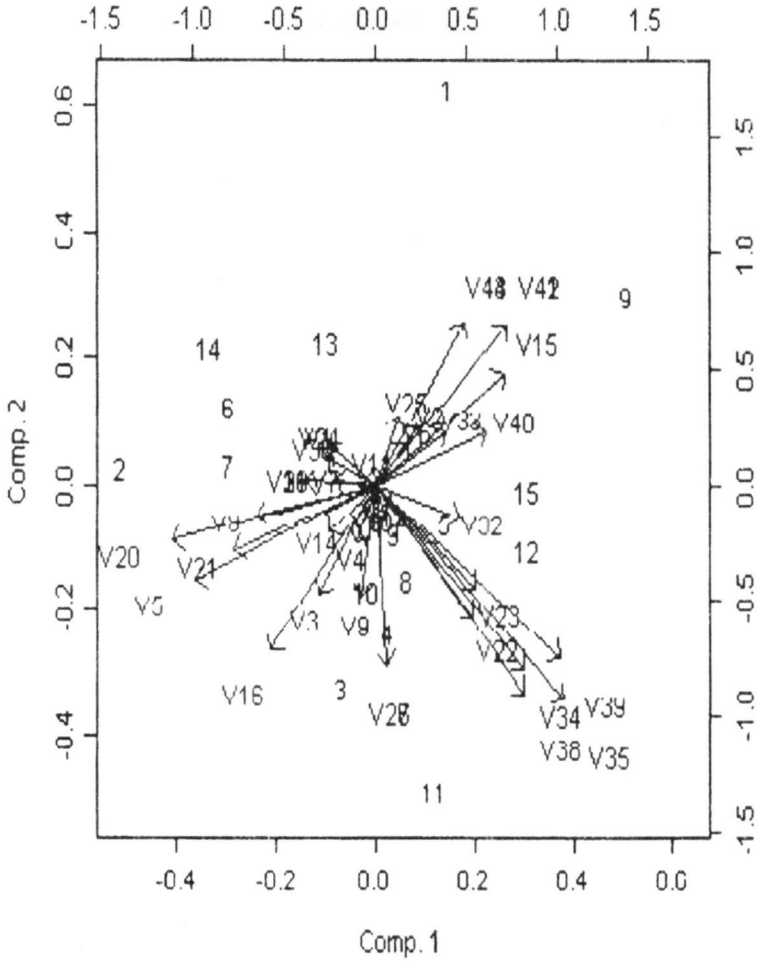

Fig. 2. Principle component analysis indicating genetic distances among cultivars and wild species.

[Note: Values given in Arabic numbers represent cultivars whereas numbers with letter 'V' represent alleles].

Extremely distant unel is a wild seedy diploid, which can be used for genomic mapping and banana breeding. Although navari is a seedless diploid, crossing with the pollen of *M. balbisiana* (BB) was successful initially. However, post zygotic incompatibility was observed (Samarasinghe, 1998) and therefore, raising plants through embryo rescue may be possible after crossing. Thus, navari is a valuable rare cultivar, which will be important in banana breeding. This also supports the view that the navari is a probably endemic cultivar to Sri Lanka (Chandrarathne and Nanayakkara, 1951). Traditional cultivar, ratahondarawala also separated individually, and is

important as a useful germplasm. Cultivar embon (AAA) and bin kesel (AAA) indicated much genetic similarity, which is in agreement with morphological characterization. Even though they belong to different genomic groups, these results show a close association of sapuanamalu, angaviaru, anamalu and bahu.

CONCLUSIONS

SSR primers MaSSR 7a/7b, 12a/12b, 20a/20b, 24a/24b and AGMI 59/60, 95/96, 101/102 and 103/104 can be used to classify cultivars possessing 'A' genome. To analyse 'B' genome, primers developed from 'B' genomic libraries will be more useful. MaSSR 24ab can be used to differentiate inter-specific cultivars (AAB or ABB) from AA or AAA genotypes. MaSSR 7a/7b and MaSSR 20a/20b markers can be used to identify navari in crossing programs. Navari is a probable endemic cultivar valuable for breeding programs. Unel is also an important germplasm, which will be useful in future banana improvement programs.

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