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# DNA TYPING OF DESSERT BANANA CULTIVAR '*KOLIKUTTU*' ('SILK') ACCESSIONS BY MICROSATELLITE MARKERS

HWL Pushpakumari[1*], WLG Samarasinghe[2], and SGJN Senanayake[1]
[1]Dept of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya
[2]Plant Genetic Resources Centre, Gannoruwa, Peradeniya

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## ABSTRACT

**Fourteen *Kolikuttu* accessions showing morphological variation and *Fusarium* wilt tolerance were collected. Extracted DNA was subjected to PCR using six SSR primers. PCR products were separated by PAGE and bands were visualized, scored and statistically analyzed using POPGENE 1.32 version to obtain the dendrogram by genetic distance matrix. Out of 19 SSR alleles amplified, 12 were polymorphic resulting in 63.16% loci polymorphism. Based on the dendrogram, three different '*Kolikuttu'* accessions known as "*Athdath Puwalu*", the accession collected from Telijjawila, and accession collected from Angunakolapelassa were clustered separately. These three accessions could be considered as different genotypes.**

***Key words**:* DNA Typing, Dessert Banana, Cultivar Kolikuttu

## INTRODUCTION

Banana belongs to the Genus *Musa* that is classified again into four recognized sections, namely *Eumusa*, *Rhodochlamys, Australimusa,* and *Callimusa*. Eumusa includes all species of true bananas (Chandraratna 1951). There is a wide range in cultivated or true bananas. In Sri Lanka, twenty nine banana cultivars and two wild species have been reported (Chandraratna & Nanayakkara 1951). Five out of twenty nine cultivars are cooking types and the rest are dessert types. Dessert type, '*Kolikuttu*' (Silk, Apple) may be originated in India and has AAB genome. Of the numerous fruit crops grown in Sri Lanka, banana is cultivated in about 48,000 ha contributing 46% of the total fruit production (AgStat 2004; Rajapakse *et al.* 2005). '*Kolikuttu* 'is one of the highly prized fruit in Sri Lanka but is highly susceptible to *Fusarium* wilt disease (Panama wilt) (Rajapakse *et al.* 2005).

Study on the genotypic variation among individuals of a crop is the primary requirement for any crop improvement programme. To perform breeding programme it is essential to evaluate genetic variation if it is unknown. Therefore, it is necessary to evaluate genetic diversity of *Kolikuttu* germplasm in Sri Lanka to exploit genetic variation for desired characters such as *Fusarium* wilt tolerance. Simple Sequence Repeat (SSR) markers are routinely used for diversity analysis and molecular breeding in many crops because of their high level of polymorphism, co-dominant nature, efficiency and cost effectiveness. Microsatellite markers have been utilized for numerous applications in *Musa* (Crouch *et al*. 1998b). The experiment was carried out with the objectives to estimate the genetic diversity of selected *Kolikuttu* accessions and to genotype selected *Kolikuttu* accessions.

The experiment was carried out at the Plant Genetic Resources Centre, Peradeniya, Sri Lanka from March to August 2008. Fourteen '*Kolikuttu*' accessions were collected based on morphological variations and *Fusarium* wilt tolerance from different locations while an '*Ambul'* accession was used as the control (Table 1). Immature unopened whitish colour banana cigar leaves were taken for DNA extraction.

DNA was extracted according to the CTAB (Centylmethyl Ammonium bromide) procedure (Weising *et al*. 1995), which was modified for *Musa* by Samarasinghe *et al*. (2001).

Three grams of each leaf samples were ground in liquid nitrogen until a fine powder was formed. The powder was transferred quickly into 15ml of pre-warmed ($60^0$C) 4% CTAB extraction buffer with 0.1% ß–mercaptoethanol. After incubating for 30 min at $60^0$C, 15ml of 24:1 Chloroform: Isoamyl alcohol was added and gently shaken for 20 min. The solution was centrifuged at 5000 rpm (10 min) and supernatant was transferred to another autoclaved "Falcon" centrifuge tube. Then 0.6 volume of ice cold isopropanol was added and mixed gently by inverting. Precipitated DNA was spooled out and placed in an eppendorf tube and washed with washing solution (1M ammonium acetate and 70% ethanol). Mixture was centrifuged at 5000rpm for 10min. The washing solution was drained and DNA pellet was air dried. Pellet was dissolved in 100-500µl of TE buffer at $4^0$C. Presence of DNA was confirmed on an agarose gel and stained with ethidium bromide. The extract was further purified from RNA by adding RNAse to 50µg/ml.

*Corresponding author:*layanthi.agru2@gmail.com*
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**Table 1. *Kolikuttu* accessions used in the experiment and their collection sites** [HORDI- Horticultural Research and Development Institute, PGRC- Plant Genetic Resources Centre]

| Identity no. | Location of sample collected | Original collection site (District) | Vernacular name | Reason for collection |
|---|---|---|---|---|
| 1 | HORDI | Divulapitiya (Gampaha) | Kolikuttu | To evaluate for *Fusarium* wilt disease tolerance |
| 2 | HORDI | Halpitiya (Kegalle) | Kolikuttu | do |
| 3 | HORDI | Sevanagala (Monaragala) | Kolikuttu | do |
| 4 | HORDI | Embilipitiya (Ratnapura) | Kolikuttu | do |
| 5 | HORDI | Embilipitiya (Ratnapura) | Kolikuttu | do |
| 6 | PGRC | Embilipitiya (Ratnapura) | Idal Puwalu | Had lax bunches |
| 7 | PGRC | Embilipitiya (Ratnapura) | Kutti Puwalu | Had compact bunches |
| 8 | PGRC | Siyambalanduwa (Monaragala) | Kotiyagala Type | Had compact bunches |
| 9 | PGRC | Puttlam (Puttlam) | Athdath Puwalu | Had long fruits |
| 10 | PGRC | Angunukolapalassa (Hambanthota) | Kolikuttu | Not affected by *Fusarium* wilt |
| 11 | Telijjawila | Matara | "Agra" | Newly released variety and has not affected by *Fusarium* wilt over ten generations and no hard lumps formation in fruits |
| 12 | Telijjawila | Matara | Kolikuttu | Used for consumption |
| 13 | Jaffna | Jaffna | Kolikuttu | Used for domestic consumption |
| 14 | Angunukolapalassa | Hambanthota | Kolikuttu | *Fusarium* wilt Susceptible plant |
| 15 | HORDI | Gannoruwa (Kandy) | Ambul | Control |

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

PCR amplification was carried out with six simple sequence repeats (SSR) primers (Table2). Final concentration in the reaction solution was 0.5pM for each forward and reverse primer, 0.2mM for dNTPs each, 2.5mM for $MgCl_2$ and 0.025U/µl of Taq DNA polymerase (Promega Corporation, USA) and 50µg/µl of DNA template in a 15ul reaction volume.

PCR was performed using $94^0$C for 2 min for the initial denaturing and then 35cycles of (30 sec $94^0$C denaturing, 30sec at annealing temperature $55^0$C, 30 seconds $72^0$C extension) and a final extension at $72^0$C for 5min for primers MaSSR 18, MaSSR 20 and MaSSR 24. As the annealing temperatures of the primers Mb1- 134, Mb1-113 and Mb1-69 ranged from $59^0$C to $62^0$C, two categories of touchdown temperature cycles were used, consisting of initial denaturation of 3min at $95^0$C for both profiles, followed by five cycles reduced by $1^0$C per cycle (either 60-$55^0$C or 55-$45^0$C), culminating with 30 cycles of denaturation for 20s at $94^0$C, annealing for 20s at either 56 or $48^0$C respectively, extension for 30s at $72^0$C, and a final extension at $72^0$C for 10 min (Buhariwalla *et al*., 2005).

Eight percent polyacrylamide 40cm gels containing 7M urea in 0.5× TBE were prepared. The gel was pre run at (at 50 W) for 45-60 min to prewarm to $45^0$C. Six micro liters of products were run at 1300V for about one and half hours and at 1500v for about three hours for efficient separation of bands. After electrophoresis, the gels were soaked in 10% acetic acid for 20min, washed 3 times for 3min each in deionized water and stained for 20min in 0.1% $AgNO_3$ containing 0.15% formaldehyde. After a brief rinse (5sec) in de-ionized water signals were developed by soaking the gels in 500ml of 7.5% solution of NaOH and 3.75ml formaldehyde. Development was terminated by soaking gels in

**Table 2. Six SSR primers used in the analysis**

| Primer pair | Sequence of 5' to 3'primer pairs | Annealing Temperature | Expected Product size (bp) |
|---|---|---|---|
| MaSSR 18a (F) | CGTCACAGAAGAAAGCACTTG | $55^0$C | 200 |
| MaSSR 18b (R) | CCTTCTCCATCGTCATCAATC | | |
| MaSSR 20a (F) | GAAATGGAGTTGGAGAAACA | $55^0$C | 222 |
| MaSSR 20b (R) | CACATATCCTTGTCGGAAGT | | |
| MaSSR 24a (F) | GACCCCCTTAAGCTGAACA | $55^0$C | 172 |
| MaSSR 24b (R) | CCGACGGTCAACATACAATACA | | |
| Mb1-69a (F) | CTGCCTCTCCTTCTCCTTGGAA | 48 ºC | 386 |
| Mb1-69b (R) | TCGGTGATGGCTCTGACTCA | | |
| Mb1-113a (F) | AGGTGCCACACAGTTCAGACA | 56 ºC | 399 |
| Mb1-113b (R) | CAACCCAAACCTGTTCGACCAA | | |
| Mb1-134a (F) | ATGCCCAAGAAGGGAAGGGAA | 56 ºC | 398 |
| Mb1-134b (R) | TAATGCCGGAGGATCAGTGTGA | | |

F- Forward; R- Reverse
Source: Samarasinghe *et al*., 2002 and Buhariwalla *et al*., 2005

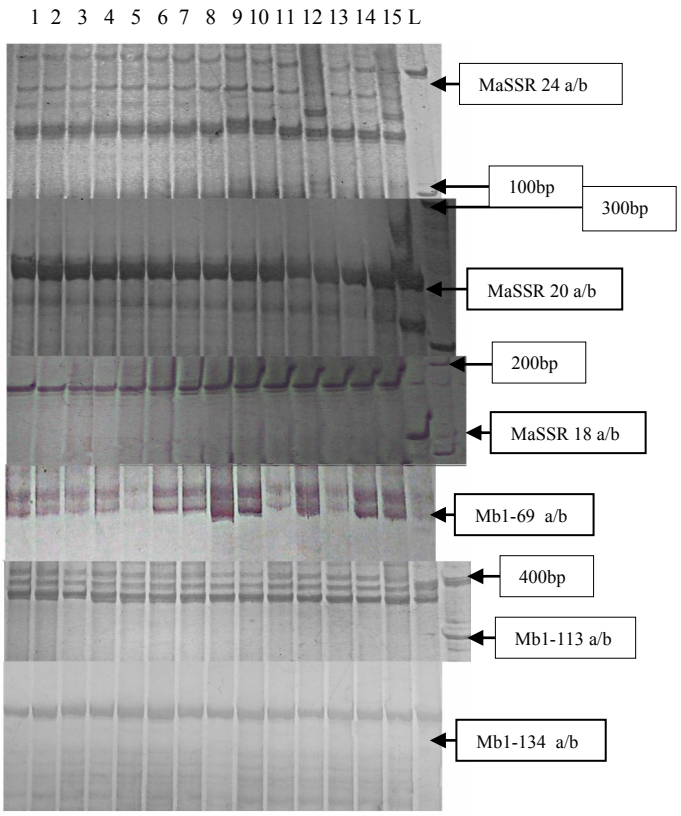


**Plate 1**: Polyacrylamide gel (8%) picture of the SSR products of 14 "*Kolikuttu*" accessions and "Ambul" accession amplified by six primers– [Sample descriptions of lane 1-15 are given in Table 1]

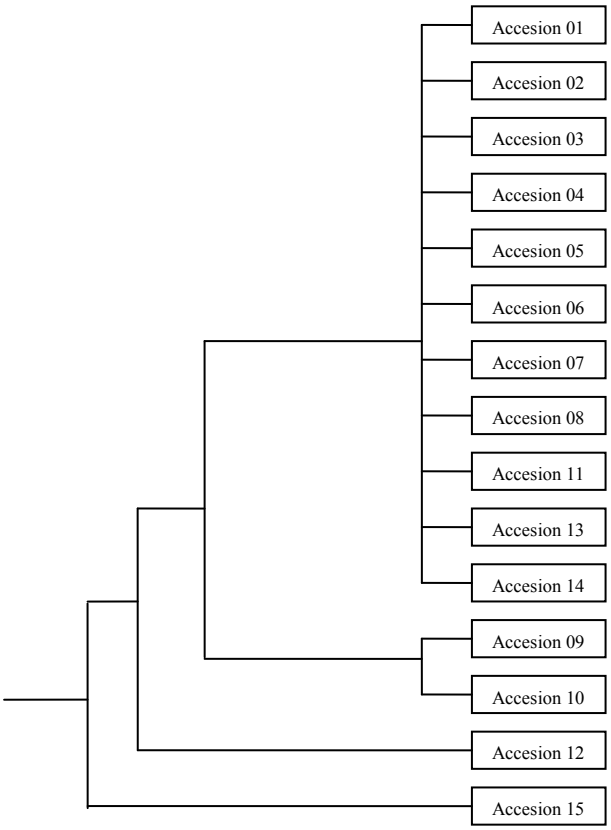


**Figure 1. Hierarchical cluster tree (Dendrogram) indicates grouping among accessions**
[Note: Accessions 1-5 HoRDI collection, 6-Idal puwalu, 7- Kutti puwalu, 8-Kotiyagala type, 9- Athdath puwalu, 10- PGRC[Plant Genetic Resources Centre] (Angunakolapalessa), 11- Agra, 12- Tellijjawila, 13- Jaffna, 14- *Fusarium* wilt susceptible, 15- Control(Ambul)

10% acetic acid for 2-3min. Finally, gels were rinsed with de-ionized water.

Each polyarylamide gel was scored visually for each primer and data were analyzed using POPGENE version 1.32. Presence of a band scored as "1" and absence of a band scored as "0" whereas non amplified bands were considered as missing data and denoted as "." for the software.

Three different *Kolikuttu* genotypes were identified among examined accessions. The accession known as "*Athdath Puwalu*" collected from *Puttlam* area (Genetic distances with the accession 11 is 0.1112, with the accession 12 is 0.3054 and with the control is 0.9985) is clustered separately while the accession collected from *Telijjawila* also is shown as a different genotype (Genetic distances with the accession 13 is 0.1719 and with the control is 0.4595) (Table 3) whereas accessions known as "*Kutti Puwalu*" , "*Idal Puwalu*" and "*Kotiyagala type*" have included in the same group (Fig. 1).

Accession collected from *Angunakolapelassa* which had not affected from *Fusarium* wilt is unique genotype clustered separately from other *Fusarium* wilt tolerant accessions. The accession known as "Agra" collected from *Telijjawila* is a newly released variety [by Dr. (Mrs) Sujatha Werasinghe of Agriculture Research station, Telijjawila, Matara] with neither hard lump formation in fruits nor affected by *Fusarium* wilt over ten generations. But it has not shown a genetic variation at analyzed SSR loci with reference to either morphological variation or *Fusarium* wilt tolerance (Fig. 1). Accessions 1 to 5 were collected from Horticultural Research and Development Institute (HORDI) to evaluate *Fusarium* wilt disease tolerance. However, they have clustered together with *Fusarium* wilt susceptible accession (accession No. 14) and therefore, a clear relationship between these two types could not obtain and further investigations are needed.

Among the analyzed SSR primers, MaSSR 24 a/b was the best primer for genotyping "Kolikuttu" accessions. Despite a considerable morphological variation observed, analyzed accessions showed a lower genetic variation with respect to the analyzed SSR loci. This concludes that the most of the morphological variations in 'Kolikuttu' were due to the environmental effect than the genetic effect.

However, three different genotypes were identified among examined *Kolikuttu* accessions which

**Table 3: Nei's pairwise Genetic Distances (1972)**

| pop ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 2 | 0.0000 | **** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 3 | 0.0000 | 0.0000 | **** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 4 | 0.0000 | 0.0000 | 0.0000 | **** | 1.0000 | 1.0000 | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 5 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | **** | 1.0000 | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 6 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | **** | 1.0000 | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 7 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | **** | 1.0000 | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 8 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | **** | 0.8947 | 0.8947 | 1.0000 | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 9 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | **** | 1.0000 | 0.8947 | 0.7368 | 0.8947 | 0.8947 | 0.3684 |
| 10 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.1112 | 0.0000 | **** | 0.8947 | 0.7368 | 0.8947 | 0.8947 | 0.3684 |
| 11 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.1112 | 0.1112 | **** | 0.8421 | 1.0000 | 1.0000 | 0.4737 |
| 12 | 0.1719 | 0.1719 | 0.1719 | 0.1719 | 0.1719 | 0.1719 | 0.1719 | 0.1719 | 0.3054 | 0.3054 | 0.1719 | **** | 0.8421 | 0.8421 | 0.6316 |
| 13 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.1112 | 0.1112 | 0.0000 | 0.1719 | **** | 1.0000 | 0.4737 |
| 14 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.1112 | 0.1112 | 0.0000 | 0.1719 | 0.0000 | **** | 0.4737 |
| 15 | 0.7472 | 0.7472 | 0.7472 | 0.7472 | 0.7472 | 0.7472 | 0.7472 | 0.7472 | 0.9985 | 0.9985 | 0.7472 | 0.4595 | 0.7472 | 0.7472 | **** |

[Nei's genetic identity (above diagonal) and genetic distance (below diagonal)]

will be useful in variety improvement. The accession located at Angunakolapallessa can be considered as an important genotype for further evaluation for *Fusarium* wilt tolerance. Analyzing these samples with more primers may help for further genotyping.

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