Molecular variability of plantain ecotypes from the genus *Musa* (Musaceae)

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Abstract: Twelve plantain ecotypes were characterized for molecular variability using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Out of the 60 RAPD and 40 ISSR primers tested, 16 RAPD and 14 ISSR primers were found polymorphic, and were used for molecular profiling of the plantain ecotypes. The average number of bands per primer, average number of polymorphic bands per primer, mean polymorphic information content (PIC) value, resolving power (Rp), and marker indices (MI) for RAPD primers were 8.63, 3.25, 0.87, 15.49, and 2.86, whereas the respective values for the ISSR assay were 7.93, 4.14, 0.85, 12.49, and 3.55. The genetic similarity coefficient was calculated using the Jaccard coefficient. The unweighted pair group method with arithmetic averages (UPGMA)-based clustering pattern remained more or less similar for RAPD, ISSR, and combined RAPD and ISSR data. Clustering was strongly supported by high bootstrap values. The genotypes Njockkon and Changalikodan (similarity coefficient > 0.94) and Manjeri Nendran (a) and Manjeri Nendran (b) (similarity coefficient > 0.89) were more closely related than the other ecotypes. The principal component analysis (PCA) showed results similar to those of the dendrogram. The results revealed that ISSR would be a better tool than RAPD for evaluation of genetic diversity in plantain ecotypes. Further, huge variability exists among the plantain ecotypes, which can be utilized for germplasm collection, in situ conservation, and development of high yielding nutritionally superior cultivars in *Musa* L. breeding programs.

Key words: Banana, genetic diversity, ISSR, Nendran plantain, RAPD

1. Introduction

Banana (*Musa* L. species (Musaceae)) is considered as an important food-fruit crop and is cultivated commercially in more than 120 countries. India is a major banana producing nation with an area of 0.79 million hectares and productivity of 28.4 metric tonnes per hectare (NHB, 2011). A wide range of diploids, triploids (2n = 3x = 33), and tetraploid cultivars/landraces of banana are known that have evolved from interspecific hybrids of the 2 wild diploid species, *Musa acuminate* Colla (contributing 'A' genome) and *Musa balbisiana* Colla (contributing 'B' genome). Despite botanical homogeneity, plantains manifest wide and unique phenotypic variability regarding plant size, bunch type, bunch and fruit orientation, fruit apex shape, pseudostem, and fruit color. Nendran (*Musa*

AAB group) is one of the leading banana cultivars belonging to the plantain subgroup and is one of the most prized plantain varieties of India. However, biodiversity in the Nendran cultivar is complex and is represented by clones distinguishable by variation in plant stature, bunch, fruit morphology, and degree of development of the male phase (Menon et al., 2002). Therefore, the present study was carried out with the objective to characterize variability in plantain ecotypes of banana using molecular markers. The development and application of molecular markers provide powerful tools to reveal polymorphism, are robust to detect genetic variability (Simmons et al., 2007), and are not influenced by environment or developmental stages of the plant, thus making them an ideal tool for genetic relationship studies. However, the potential usefulness of

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molecular techniques in identifying genetic relationships varies from plant to plant because of the uniqueness of each genome.

Random amplified polymorphic DNA (RAPD) has proved an efficient tool in detecting genetic variations and genetic relationships among germplasm of many plants, including banana (Crouch et al., 2000). It is relatively quick and inexpensive, and requires a small amount of DNA and no prior sequence information of the target genome (Solouki et al., 2008). Inter simple sequence repeats (ISSR), another polymerase chain reaction (PCR)-based technique, is a reliable marker system for many organisms, especially plants (Modgil et al., 2005), because of its simplicity, speed, high stability, no prior requirement of sequence information, cost effectiveness, and versatility of markers that amplify DNA repeat sequences using single primers. It involves amplification of the DNA segment present at an amplifiable distance between 2 identical microsatellite repeat regions oriented in opposite directions (Zietkiewicz et al., 1994). Therefore, ISSR has been widely used for varietal fingerprinting or genetic diversity analysis (Bornet et al., 2002). The dominant marker RAPD and the co-dominant marker ISSR sample multiple loci from across different genomes and help to resolve phylogenetic relationships. Although application of RAPDs in variability analysis of plantain (*Musa* AAB) has been carried out by various researchers, the use of ISSR for variability analysis of *Musa* spp. (AAB) has not received wide attention so far. The present study, therefore, used both RAPD and ISSR markers to compare their effectiveness and to assess the levels of genetic diversity in plantain (*Musa* AAB) ecotypes of banana.

2. Materials and methods

2.1. Plant material
Twelve plantain ecotypes comprising 9 Nendran, an exotic French plantain (Njockkon), a False Horn plantain (Big Ebanga), and a Horn plantain (Zanzibar) ecotypes were collected from the field gene bank of the Banana Research Station, Kannara (Kerala, India).

2.2. DNA extraction, purification, and quantification
Young and tender leaf tissue (1 g) was weighed and ground in liquid nitrogen using a mortar and pestle along with 50 μL of β-mercaptoethanol (BME) and a pinch of polyvinylpyrrolidone (PVP). The sample was further ground using excess of liquid nitrogen and 4 mL of extraction buffer (2X) and the powder was transferred to a sterile 50-mL centrifuge tube containing 3 mL of pre-warmed extraction buffer. The homogenate was incubated for 30 min at 65 °C with intermittent mixing. Equal volumes of chloroform and isoamyl alcohol (24:1) were added, centrifuged at 10,000 rpm for 15 min at 4 °C, and supernatant was collected. Next, 0.6 volume of

chilled isopropanol was added to the collected supernatant followed by incubation at –20 °C for 30 min. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C. The pellet was then harvested and washed with 5 mM ammonium acetate in 70% ethanol and later with 100% ethanol. The DNA samples were air dried for 30 min at room temperature and dissolved in 100 μL of TE buffer. The DNA samples were treated with RNase-A (Bangalore GeNei, India) at 37 °C for 1 h to remove RNA contamination. The quantity and quality of genomic DNA were estimated using a Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) and the samples were diluted to a concentration of 50 ng/μL.

2.3. Selection of primers
Initially, 60 RAPD and 40 ISSR primers were screened for polymorphism against the plantain ecotypes. Of these, 16 RAPD and 14 ISSR primers (Table) produced distinct banding patterns with good quality of amplification and reproducibility; however, no band was detected in any negative control.

2.4. RAPD amplification
PCR reactions were carried out in an Eppendorf Master Cycler (Eppendorf, USA). Each reaction tube contained 100 ng of template DNA, 2.5 mM MgCl$_2$, 100 μM of dNTPs, 1X *Taq* buffer, 20 pM of 10-mer RAPD primer, and 1 unit of *Taq* DNA polymerase made up to a final volume of 20 μL. DNA amplification was performed using the following thermal profile: 94 °C for 5 min (1 cycle); 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min (35 cycles); final extension at 72 °C for 10 min (1 cycle) and cooling of samples at 4 °C.

2.5. ISSR amplification
In the case of ISSR primers, optimal annealing temperature varied according to the base composition of the primers. The PCR reactions were carried out in a column gradient PCR program (Eppendorf, USA). PCR was performed using the following thermal profile: 94 °C for 5 min (1 cycle); 94 °C for 45 s, 46–56 °C for 1 min, 72 °C for 90 s (35 cycles); final extension at 72 °C for 7 min (1 cycle) and cooling of samples at 4 °C.

2.6. Agarose gel electrophoresis
The amplified products were run on 1.4% (RAPD) and 1.6% (ISSR) agarose gel using 1X TAE buffer stained with ethidium bromide along with 1 kb marker (λDNA/EcoRI, Fermentas, USA). The profile was visualized under a UV transilluminator and documented using the gel documentation system BIO-RAD (Biorad, USA). The documented RAPD and ISSR profiles were carefully examined for polymorphism. The number of bands produced by each primer was counted and tabulated.

Table. RAPD and ISSR primers used to detect total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (%P), polymorphic information content (PIC), and resolving power (Rp).

RAPD primers

No.	Primer name	Primer sequence (5' to 3')	TNB	NPB	P%	PIC	Rp
1	OPS-6	TGC TCT GCC C	6	2	33.33	0.81	10.17
2	OPS-7	GGT GAC GCA G	6	1	16.67	0.83	11.33
3	OPS-12	CCT TGA CGC A	7	3	42.86	0.81	9.33
4	OPS-19	ACC CCC GAA G	7	4	57.14	0.86	13.17
5	OPS-21	CAG GCC CTT C	9	4	44.44	0.89	16.67
6	OPS-31	CAA TCG CCG T	14	8	57.14	0.91	18.00
7	OPS-35	TTC CGA ACC C	10	2	20.00	0.90	18.83
8	OPS-36	AGC CAG CGA A	7	2	28.57	0.84	11.83
9	OPS-37	GAC CGC TTG T	14	5	35.71	0.92	24.33
10	OPS-40	GTT GCG ATC C	5	2	40.00	0.79	17.33
11	OPS-80	ACT TCG CCA C	9	2	22.22	0.89	17.50
12	OPS-127	CCG ATA TCC C	8	2	25.00	0.87	15.33
13	OPS-141	CCC AAG GTC C	8	1	12.50	0.87	15.00
14	OPS-182	GGC ACT GAG G	12	7	58.33	0.91	21.33
15	OPS-461	GTG GGC TGA C	9	5	55.56	0.88	14.67
16	OPB-15	GGA GGG TGT T	7	2	28.57	0.85	13.00
	Total		138	52	37.68	13.84	247.83

ISSR primers

No.	Primer name	Primer sequence (5' to 3')	TNB	NPB	P%	PIC	Rp
1	UBC-814	CTCTCTCTCTCTCTCTA	7	4	57.14	0.85	12.17
2	UBC-820	GTGTGTGTGTGTGTGTC	5	3	60.00	0.79	7.83
3	UBC-834	AGAGAGAGAGAGAGAGYT	10	8	80.00	0.83	9.83
4	UBC-835	AGAGAGAGAGAGAGAGYC	5	1	20.00	0.77	8.33
5	UBC-836	AGAGAGAGAGAGAGAGYA	7	4	57.14	0.84	11.50
6	UBC-843	CTCTCTCTCTCTCTCTRA	9	4	44.44	0.89	16.67
7	UBC-844	CTCTCTCTCTCTCTCTRC	7	3	42.86	0.83	10.67
8	UBC-845	CTCTCTCTCTCTCTCTRG	8	3	37.50	0.87	15.00
9	UBC-848	CACACACACACACACARG	9	4	44.44	0.86	13.67
10	UBC-857	ACACACACACACACACYG	11	9	81.82	0.89	15.67
11	UBC-868	GAAGAAGAAGAAGAAGAA	8	3	37.50	0.85	12.50
12	UBC-890	VHVGTGTGTGTGTGTGT	7	2	28.57	0.84	12.00
13	ISSR-3	CTCTCTCTCTCTCTCTTG	8	4	50.00	0.85	12.17
14	ISSR-6	GTGTGTGTGTGTCC	10	6	60.00	0.90	16.83
	Total		111	58	52.25	11.87	174.83

2.7. Data collection and analysis

Amplification profiles of 12 plantain ecotypes were compared with each other and bands of DNA fragments scored manually for the presence or absence of a particular band for RAPD, ISSR, and the combined data set. The data were analyzed using a numerical taxonomy and multivariate analysis system (NTSYSpc 2.02i) software package (Rohlf, 2000). The dendrogram was based on the proximity matrix obtained from the Jaccard coefficient and sequential agglomerative hierarchical nonoverlapping (SAHN) and clustering was done using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973). Principal component analysis (PCA) was also undertaken to estimate relationships

among ecotypes. PCA was performed using the Jaccard coefficient, EIGEN, and PROJ modules of NTSYSpc and bootstrapping was done using WINBOOT software (Yap and Nelson, 1996) with 5000 replications.

The resolving power (Rp) of a primer was calculated as the sum of band informativeness of all the bands produced by the primer (Prevost and Wilkinson, 1999). Rp of a primer = Σ IB, where IB (band informativeness) takes the value of: 1 – [2 × (0.5 – p)], p being the proportion of the 12 genotypes (plantain ecotypes analyzed) containing the bands. The polymorphic information content (PIC) value was calculated using the formula $1 - \Sigma pi^2$, where pi is the frequency of the ith allele (Smith et al., 1997). The number of alleles here refers to the number of scored bands and the frequency of an allele was obtained by dividing the number of ecotypes where it was found by the total number of ecotypes. Marker index (MI) for a primer was calculated as the product of PIC and the number of polymorphic bands (Manimekalai and Nagarajan, 2006).

3. Results

3.1. RAPD analysis
Out of the 60 RAPD primers screened, 16 primers that showed polymorphism among the 12 plantain ecotypes were selected for further study. The number of bands produced by the polymorphic primers varied from 5 to 14 and the molecular weight of bands from 2.876 to 0.564 kb. The total number of polymorphic bands and the percentage of polymorphism were 52 and 37.68, respectively. The marker indices (MI) of the primers varied from 0.83 (OPS-7) to 7.28 (OPS-31) with an average of 2.86. Total number of bands (TNB), number of polymorphic bands (NPB), percentage polymorphism (%P), polymorphic information content (PIC), and resolving power (Rp) obtained by each primer are summarized in the Table. Rp of the random primers ranged between 9.33 (OPS-12) and 24.33 (OPS-37) with an average of 15.49. However, the PIC values derived from allelic diversity and frequency among the genotypes were not uniform for all the RAPD loci tested. The PIC value varied between 0.79 (OPS-40) and 0.92 (OPS-37) with a mean of 0.87 (Table).

The genetic similarity matrix constructed using Jaccard coefficients showed the highest similarity coefficient value for Changalikodan with Njockkon (0.9420). The lowest similarity coefficient value (0.7753) was obtained between Kottayam and Attu Nendran. The dendrogram grouped plantain ecotypes into 3 major clusters on the basis of the reference line drawn at a similarity coefficient of 0.88 (Figure 1a). The first cluster included Kottayam, Manjeri Nendran (a), Myndoli, Thiruvadon, and Zanzibar. The second cluster is marked by the 6 ecotypes of Kaliethen, Nedu Nendran, Big Ebanga, Manjeri Nendran (b), Njockkon, and Changalikodan. The third singleton cluster included Attu Nendran.

Clustering based on PCA resulted in 4 clusters more or less similar to those of dendrogram clustering (Figure 1b). The first, second, and third principal components explain 86.67%, 3.79%, and 2.36% of overall variation, respectively. Cluster I consisted of 8 ecotypes, i.e. Nedu Nendran, Zanzibar, Manjeri Nendran (b), Big Ebanga, Njockkon, Changalikodan, Myndoli, and Kaliethen, and cluster II consisted of 2 ecotypes, i.e. Thiruvadon and Manjeri Nendran (a). Clusters III and IV included singleton clusters Kottayam and Attu Nendran, respectively.

On comparison of the above 2 methods, it was observed that the ecotype Kottayam, which was a part of cluster I formed a similar distinct cluster (cluster III) in the PCA. Similarly, Manjeri Nendran (a) and Thiruvadon, members of cluster I, grouped together in the cluster II of PCA. All 6 ecotypes (Kaliethen, Nedu Nendran, Big Ebanga, Manjeri Nendran (b), Njockkon, and Changalikodan) of cluster II of the RAPD-based dendrogram were combined in cluster I of the PCA. However, Attu Nendran formed a distinct cluster in both the RAPD-based dendrogram and the PCA.

3.2. ISSR analysis
A sum total of 14 primers showed polymorphism among the 12 plantain ecotypes. The number of bands produced by the polymorphic primers varied from 5 to 11 and the molecular weight of bands from 1.584 to 0.564 kb. The total number of polymorphic bands and the percentage of polymorphism was 58 and 52.25, respectively. The MI was lowest with the primer UBC-835 (0.77) and highest with the primer UBC-857 (8.01) with an average of 3.55. The TNB, NPB, %P, PIC, and Rp obtained by ISSR primers are summarized in the Table. The Rp of ISSR primers ranged between 7.83 (UBC-820) and 16.83 (ISSR-6) with an average of 12.49. The PIC values derived from allelic diversity and frequency among the genotypes were also not uniform for all the ISSR loci tested. The PIC values for the 14 primers varied between 0.77 (UBC-835) and 0.90 (ISSR-6) with an average of 0.85 (Table).

The similarity matrix constructed using Jaccard coefficients ranged from 0.6486 to 0.9459. The dendrogram grouped plantain ecotypes on the basis of ISSR markers into 3 clusters (Figure 2a). The first cluster included most of the ecotypes, whereas Thiruvadon and Big Ebanga belonged to the second cluster and Nedu Nendran formed a distinct singleton cluster. As per the ISSR-based dendrogram, the ecotypes Njockkon and Changalikodan, and Manjeri Nendran (a) and Manjeri Nendran (b) were quite similar and showed a similarity coefficient of more than 0.94.

The PCA showed a result more or less similar to that of dendrogram clustering (Figure 2b). The first, second, and third principal components explained 82.78%, 3.93%, and 3.24% of overall variation, respectively. PCA of the plantain ecotypes resulted in 4 clusters. Cluster I contained most of the ecotypes, whereas cluster II consisted of 2 ecotypes,
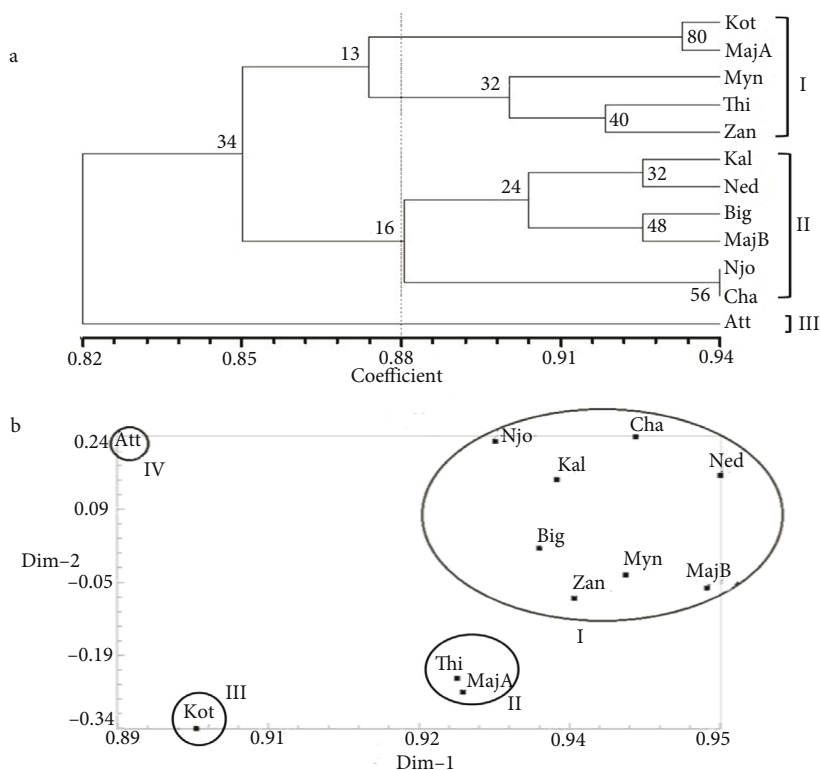
Figure 1. Unweighted pair group method with arithmetic averages cluster analysis (a) and principal component analysis ordination (b) of the plantain ecotypes using RAPD.

i.e. Thiruvadon and Big Ebanga, and Nedu Nendran was present as singleton cluster III. On comparison of the above 2 methods, overall similar results were obtained in both the ISSR-based dendrogram and the PCA.

3.3. Combined analysis of RAPD and ISSR data
The dendrogram obtained from the combined analysis of RAPD and ISSR data set grouped plantain ecotypes into 5 clusters (Figure 3a). Cluster I based on the RAPD and ISSR data consisted of 8 plantain ecotypes, whereas the rest of the ecotypes, Thiruvadon, Attu Nendran, Nedu Nendran, and Big Ebanga, were present as singleton clusters II, III, IV, and V, respectively. The highest similarity coefficient of 0.94 was observed between Njockkon and Changalikodan. The PCA showed a result more or less similar to that of clustering except for Kottayam, which was present in the largest cluster (cluster I) on the basis of dendrogram based on the combined analysis (Figure 3b). Therefore, it can be said that the PCA exhibited the lays, orientations, and positions of the plantain ecotypes tested and explained their relationships more explicitly than the cluster dendrogram did.

4. Discussion
Polymorphism in a given population is often due to the existence of genetic variants represented by the number of

alleles at a locus and their frequency of distribution in a population (Lawrence, 2000). Heterozygosity corresponds to a probability that 2 alleles taken at random from a population can be distinguished using the marker in question. Thus a convenient quantitative estimate of marker utility and the polymorphism detected can be given in terms of Nei's genetic diversity (h), Shannon's information index (I), and total genotypic diversity among the population (Ht) (Zhao et al., 2006).

The RAPD data observed for number of alleles (na), effective number of alleles (ne), Nei's genetic diversity, and Shannon's information index were analyzed for all the 12 plantain ecotypes using RAPD primers and their respective values were found to be 1.3768, 1.2165, 0.1269, and 0.1916. The value for total genotype diversity among the population was calculated as 0.1269. The Shannon's information index indicates low informativeness in RAPD markers. Since all the plantain ecotypes were from a common genetic base (plantain AAB), Nei's genetic diversity and total heterozygosity were very low. Genetic divergence was found to be quite low within the plantain ecotypes gene pool, which is consistent with the proposed evolution of this germplasm through somatic mutation of a relatively small number of introductions (Crouch et al., 2000). However, this is in contrast to the results
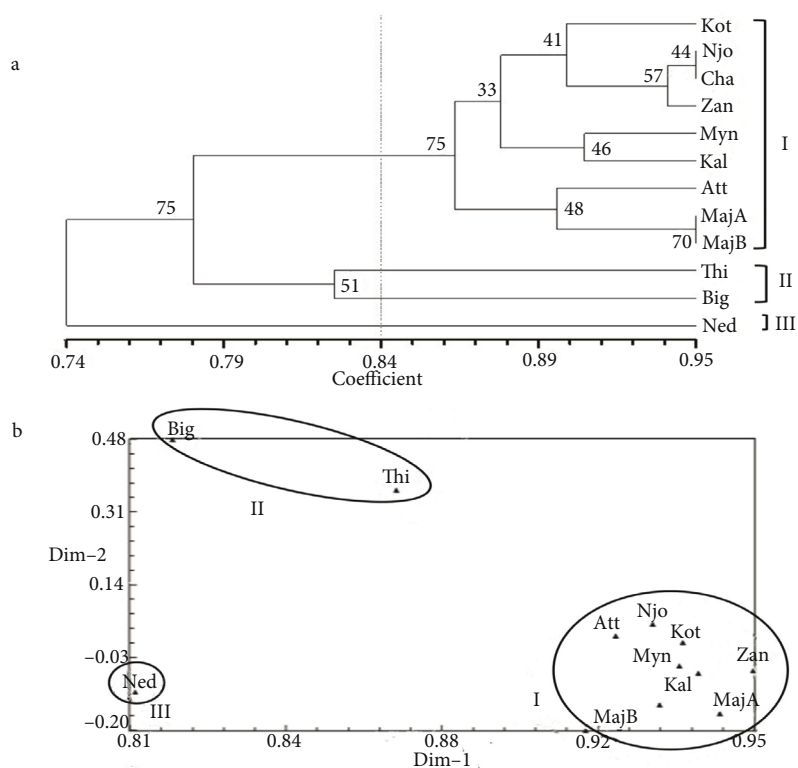
Figure 2. Unweighted pair group method with arithmetic averages cluster analysis (a) and principal component analysis ordination (b) of the plantain ecotypes using ISSR.

obtained by Mukherjee et al. (2013) in analysis of some economically important species, varieties, and cultivars of the genus *Allium* using RAPD and ISSR markers, where they got higher values.

The ISSR data observed for number of alleles, effective number of alleles, Nei's genetic diversity, and Shannon's information index for all the 12 plantain ecotypes were also analyzed using ISSR primers and their respective values were 1.5225, 1.2534, 0.1585, and 0.2464. The value for total genotypic diversity among the population was 0.1585. Shannon's information index and Nei's genetic diversity indicate high informativeness in ISSR compared to RAPD. ISSR studies by Alam et al. (2008) revealed higher mean value for Shannon's information index and Nei's genetic diversity among populations of *Podophyllum hexandrum* Royle. The reason for this could be the longer nucleotide sequences and occurrence of these microsatellites more frequently throughout the genome.

Both RAPD and ISSR analyses grouped plantain ecotypes in different clusters but only few of them maintained the same associations in RAPD as compared to the ISSR dendrogram. The highest similarity index (0.94) in RAPD and ISSR was seen between Njockkon and Changalikodan, which indicates the closeness between these genotypes. The high similarity between

these ecotypes may be due to the fact that Njockkon is an exotic French plantain introduced into India and Changalikodan is an ecotype of Nendran, which is also a French plantain. High similarity was also observed between Manjeri Nendran (a) and Manjeri Nendran (b) as per the ISSR (similarity index 0.94) and combined RAPD and ISSR (similarity index 0.92) marker systems. They had similar morphological characters with slight differences in the duration of the crop cycle, plant height, and yield (Menon and Aravindakshan, 1998). There was a close relationship between some of the plantain ecotypes used in this study, as these ecotypes may have been derived from the same pedigree or the amplification of the same nucleotide sequence present between the simple sequence repeats. Similar results have been found by Poerba and Ahmad (2010) for 36 triploid banana accessions with an estimated 46% genetic similarity based on RAPD and ISSR marker data. ISSR markers have also been used for genetic diversity analysis in *Musa* spp. (Racharak and Eiadthong, 2007; Venkatachalam et al., 2008). These data indicated that RAPD and ISSR markers were effective for diversity studies in plantain ecotypes.

The formation of subclusters within the cluster varied more between the PCA and RAPD-based dendrogram as compared to ISSR and the combined RAPD and ISSR
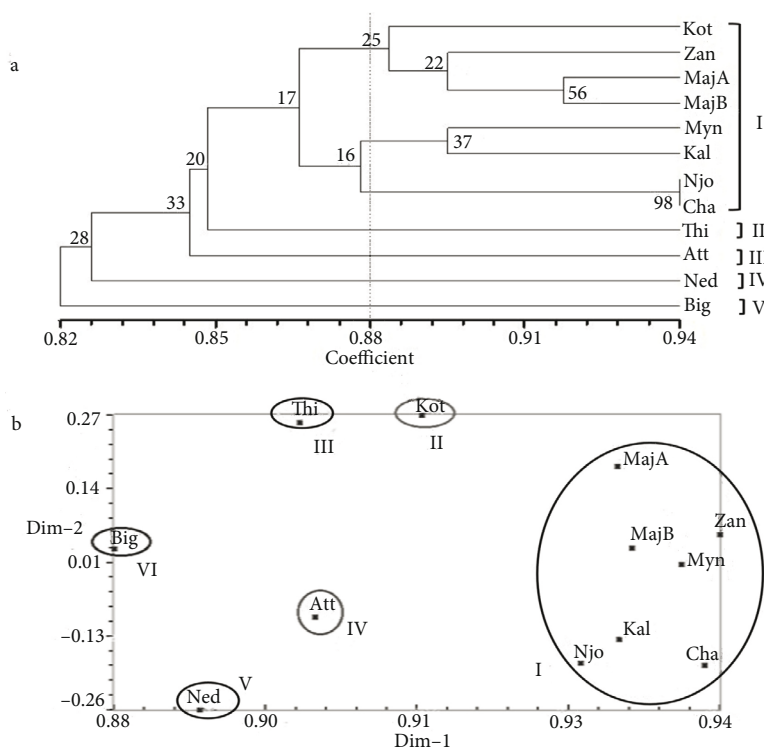
Figure 3. Unweighted pair group method with arithmetic averages cluster analysis (a) and principal component analysis ordination (b) of the plantain ecotypes using RAPD + ISSR data sets.

marker system. RAPD showed the plantain ecotypes of Kottayam with Attu Nendran (0.7753) as the most divergent. Kottayam and Attu Nendran are marked by morphological deviations among them regarding pseudostem shape and color, blotches at the petiole, bunch appearance, male bud shape and bract lifting, fruit position, and transverse section of fruit (Menon and Aravindakshan, 1998). However, ISSR and combined RAPD and ISSR dendrogram respectively showed the ecotypes Nedu Nendran with Big Ebanga (0.6486) and Thiruvadon with Nedu Nendran (0.7831) as most divergent. Thiruvadon and Nedu Nendran are ecotypes of Nendran and is also a French plantain, whereas Big Ebanga is a False Horn plantain and is an introduction to India. They possess morphological differences in the pseudostem height, blotches at the petiole and margin, bunch position and appearance, rachis type, male bud type and shape, and fruit shape. Nevertheless, Thiruvadon and Nedu Nendran have normal male buds but Big Ebanga has a degenerating male bud (Menon and Aravindakshan, 1998). This revealed the existence of a sufficient amount of genetic variability among the ecotypes of the same variety, which could be exploited further in breeding programs. Crouch et al. (2000) also observed large variations among plantain ecotypes (*Musa* spp., AAB group) using agronomic and molecular markers. These studies showed

that RAPD and ISSR marker systems can be effectively used in the determination of genetic relationships among plantain ecotypes. However, in our study ISSR shows better results than RAPD due to its higher reproducibility, Nei's genetic diversity, and Shannon's information index. Ray et al. (2006) detected ISSR having more reproducibility than RAPD in banana cultivars. Therefore, it is concluded that ISSR would be a better tool than RAPD for phylogenetic studies in banana and plantains. However, knowledge of the degree of the genetic relationship between these ecotypes will be important for germplasm collection, in situ conservation, and *Musa* breeding programs. The results of the present study will be useful in plantain DNA fingerprinting and in determining genetic diversity among plantain ecotypes.

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