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Evaluation of Genetic Diversity in Some Banana Hybrids using ISSR Markers

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A B S T R A C T

Bananas (*Musa* spp.) are mostly diploid or triploid with various combinations of genomes inherited from their ancestors. Inter simple sequence repeat (ISSR) markers were used for evaluation of genetic diversity of some synthetic Banana Hybrids. A total of 10 primers were evaluated for banana hybrids based on ISSR polymorphism. Diversity analysis carried out by using ISSR markers in some Banana hybrids revealed the confirmation of the hybridization along with the phylogenetic relationship of the hybrids with their parents. Out of the 10 primers studied, six primers exhibited scorable markers. A total of 54 markers were produced. The markers ranged from 5 to 8 in different primers. Of the 54 markers produced, 30 were polymorphic which account to 53.83 per cent polymorphism. Among the six primers studied ISSR 812 (UBC- 812) produced maximum number of polymorphic bands among the banana genotypes and might be useful for genetic diversity or DNA finger printing studies.

Introduction

Bananas and plantains belong to the family *Musaceae* and are cultivated throughout the humid tropics and sub-tropics. This crop is perennial with a faster relative growth rate compared to other fruit crops, while producing fruit all year round. Because of their nutritional value, bananas and plantains are considered the fourth most important crop worldwide after rice, wheat and corn. DNA marker technologies have been widely used in banana genetics and diversity analysis, e.g., in taxonomy, cultivar true-to-type assessment and genetic linkage map development. The various DNA fingerprinting techniques have been used to study the genetic diversity and taxonomy of bananas. These include isozyme analysis Bhat *et al.*, (1992), restriction fragment length polymorphism (RFLP) Jarret *et al.*, (1992), Kaemmer *et at.*, (1992) and Bhat *et al.*, (1994), inter simple sequence repeat (ISSR) markers Godwin *et al.*, (1997), sequence tagged microsatellite (STMS)

Grapin *et al.,* (1998) and Kaemmer *et at*.,(1997) and amplified fragment length polymorphism (AFLP) Loh *et al.,* (2000) and Wong *et al.,* (2001) Random Amplified Polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based technique which use random primers to generate DNA fragments which can be used as genetic markers, Williams *et al.,*(1990), Welsh and Mc Clelland, (1990), Howell, *et al.,* (1994) and Pillay *et al.,* (2000) is another such technique which allows one to easily back taxon names with a molecular identification system in the form of a barcode inherent to the plant. Using this technique, an unlimited number of polymorphic bands can be produced with relative ease form minute amounts of genomic DNA, Welsh and McClelland, (1990) allowing simultaneous screening of a large number of accessions. RAPD based fingerprinting has been successfully applied to the characterization of diverse *Musa* germplasms, Bhat and Jarret, (1995), Onguso *et al.,* (2004), analysis of *Musa* breeding populations Crouch *et al.,* (1999) and detection of somaclonal variants, Grajal Martin *et al.,* (1998). Banana breeding programme in India was started as early as 1949 at aduthurai. Several South Indian bananas were found to be more closely related to *M. bulbisiana* than *M. acuminate* as revealed by the metroglyph, Raman *et al.,* (1968). Bhakthavatsalu and Sathiamoorthy (1979) also elaborated upon the genomic status and breeding potential of many South Indian banana. As many synonyms exist for each clone and are differently name at various regions, much confusion prevails on the nomenclature of banana clones. In this situation, molecular characterization helps to identify clones unambiguously. In this context, banana breeding was initiated at Tamil Nadu Agricultural University, Coimbatore and many interdiploid crosses were attempted (Table 4). This had resulted in many diploids, triploids and tetraploids hybrids which were evaluated for their resistance against nematodes in both field and pot conditions. The hybrids were developed with male parent as resistant sources. These hybrids have to be confirmed for their hybridity taste and see the banding patterns of each and every hybrids, for which RAPD is an efficient way. ISSR analysis has been used to classify genotypes of *Mus*a representing the AA, AAA, AAB and BBB genome, Howell *et al.,* (1994). Molecular characterization of different clones of banana has been attempted by many authors in India. Jagannath *et al.,* (2003) characterized many AA and AB diploids using RAPD markers. Rekha *et al.,* 2004 made an attempt to study the variability between the AB cultivars. The evolved hybrids were subjected to diversity analysis to study the phylogenetic relation of the hybrids with their pedigree through ISSR markers. The main objective of the study is to evolve new synthetic hybrids by crossing the commercial triploids with improved resistant potential diploids.

Materials and Methods

The present study was taken up at the College orchard, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore during the period from 2005 to 2007, to evolve new banana hybrids for yield and quality combined with resistance to nematodes. The commercial cultivars and existing female fertile resistant hybrids were crossed with potential male donors (Pisang Lilin, Anaikomban, Rose and H 201) to evolve new hybrids as described in Table 3. Total of 7550 crosses were made with 63 different cross combinations, out of which 122 seedling have been generated and synthetic hybrids used for the experiment. The new hybrids evolved were planted in the main field for evaluation. The seedlings at twenty leaf stage were transplanted in the main field without trimming the roots since the corm was

too small. The main field selected was highly infested with nematodes (more than two nematodes per gram of soil), so as to facilitate screening the hybrids for nematode resistance. Seven parents were used in the crosses are shown in the table 4, out of which male parents Pisang Lilin used as resistant cheek and Rasthali used for susceptible cheek in the present experiment.

DNA extraction, quantification and quality cheek

Fresh leaf samples (from unfurling leaves) of all the 18 genotypes (hybrids and parents) collected from the field were used for isolation of genomic DNA (Table 4). Isolation was done following the method of Gawel and Jarret (1991), with a slight modification. Mercaptoethanol (1%) and Polyvinyl pyrrolidone (PVP) 0.2 % were added to the extraction buffer to remove the phenolics. Leaf tissue (0.5 g) was ground with liquid nitrogen and to this powder 750 µl of preheated CTAB buffer (65ºC) was added. It was then incubated at 65ºC in a water bath for 1 hour. After bringing the tubes to room temperature, equal volume (750 µl) of chloroform: isoamyl alcohol (24: 1) was added and the contents were mixed well for 10 minutes to form an emulsion.

It was then centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred to a fresh tube and re-extracted with chloroform: isoamyl alcohol. The aqueous phase was transferred to a new tube and 2/3 rd volume of ice cold isopropanol was added and incubated in a freezer overnight. The contents were then centrifuged at 10,000 rpm for 10 minutes at 4ºC. The pellet was washed with 70 % ethanol and air dried. The crude DNA was purified by RNase (10mg/ml) treatment followed by phenol: chloroform extraction. The DNA was ethanol precipitated and finally dissolved in TE buffer, pH-8.0, (10 mM Tris, 1mM EDTA). The DNA was quantified by a UV-Spectrometer at 260 nm, adjusted to the concentration of 100ng/µl, and stored at -20^{0}C in 25µl volumes until use. To check the quality and quantity of the extracted genome DNA, gel electrophoresis was carried out on 0.8 per cent agarose gel. DNA concentration for PCR amplification was estimated by comparing the band intensity of a sample with the band intensities of known dilutions that gave good amplifications. The dilutions were carried out by dissolving the genomic DNA in appropriate quantity of TE buffer (pH 8.0) as postulated by Meenakshisundaram (2002).

ISSR analysis

DNA from the selected genotypes were used for ISSR analysis following the method recommended by Bhat and Jarret (1995) with require modification. A total of ten ISSR primers (as described by University of British Columbia, Canada) synthesized at Sigma – Aldrich (USA), Bangalore, were used for amplifying the genomic DNA. Amplification was carried out in 15 µl reactions containing 20- 30 ng of genomic DNA, 1.5 µl of 1.5 mM of assay buffer, 0.75 µl of 10.0 mM of d NTPs, 1.2 µl of 0.5 µM of primer, 0.18 µl 0.25 mM of $MgCl_2$ and 0.18 µl of 3U/µl Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore) and 8.19 µl of sterile water. Amplifications were performed in PTC Thermal Cycler (MJ Research Inc.,) programmed for an initial denaturation at 94 ^{o} C for 5 min., 40 cycles of 1 min denaturation at 94^o C, 1 min annealing at 41^o C and 1 min. extension at 72^o C and a final extension of 5 minutes at 72^o C and at 4^o C for 30 minutes. PCR amplified products (4 µl) were resolved in a 4 % denaturing poly acrylamide gel by electrophoresis (PAGE) at 300 volts, 400 mA and 100 watts for 3.5 hours and viewed by silver staining, Panaud *et al.*, (1996). Sizes of the identified bands were determined relative to a 1 kb ladder (MBI Fermentas, Germany).

Electrophorosis

The ISSR-PCR products were separated in 1.5 per cent *(w/v)* agarose gel in 1 x TAE buffer (0.4M Tris, 0.2M acetic acid, 10mM EDTA, pH8.4) containing 0.5 µ*g/ml* ethidium bromide (Sambrook *et al.,* 1989). Along with the PCR amplified products, Lambda DNA (*Eco*RI and *Hind*III double digest) as standard marker were subjected to electrophoresis in 1.5 per cent agarose gel in 1x TBE buffer at 8V/centimetre for 4-5 hours. The electronic image of the ethidium bromide stained gel was visualized and documented in gel documentation system (Model Alpha imager 1200, Alpha Innotech Crop., USA). The 100bp DNA ladder was used as a size marker.

Loading DNA and running the gel

A piece of parafilm was placed on the solid surface and 1µl of genomic DNA (25 ng /µl) was pipetted on it. The contents were loaded into the wells carefully with the help of micropipette. Lambda DNA (*Eco* RI and *Hin*d III double digest) used as the standard marker. The gel was run for 1-2 hours until the tracking dye reaches half of the gel and bands were visualized and documented in gel documentation system (Model Alpha imager^{TM} 1200, Alpha Innotech Corp., California, USA).

Scoring and cluster analysis

In ISSR only two states (‘1’, present and ‘0’, absent) can be distinguished at each band position. Each band position corresponds to a locus with two alleles, presence and absence of the band, respectively. Polymorphism was scored as presence or absence from distinct reproducible ISSR bands obtained from all 10 primers across all the 18 genotypes. The average polymorphism information content (PIC) and marker index (MI) were calculated by applying the formulas given by Powell *et al.,* (1996) and Smith *et al.,* (1997). The binary data matrices were entered into the NTSYS pC package (Exeter softwere, New york). The data were analysed using qualitative routine to generate Jaccard’s Similarity construct a dendrogram using UPGMA (Unweighted pair Group Method with Arithmetic Average) and the SHAN (Sequential Heirarchial and Nested Clustering) routine in the NTSYS programme. Data analysis was done using NTSYSPC version 2.02 by Rohlf, (1994).

Results and Discussion

Result of DNA amplification showed that the 18 accession of banana hybrids and parents produced a wide array of strong and weak bands. However, only distinct, reproducible well-resolved fragments were scored as present or absent band for each of ISSR primers with 18 accessions. Figure 1 and 2 illustrated the typical level of polymorphisms observed among the 18 accession for primer UBC 812. Out of 10 primers used, six produced scorable markers. A total of 54 markers were produced. The markers ranged from 5 to 8 in different primers. The average number of markers / primer was 9 and 54 markers produced, 30 were polymorphic which account to 53.83 per cent polymorphism (Table 1, 2 and 3). Cluster analysis was performed on Jaccard's similarity coefficient matrices calculated for ISSR markers to generate a dendrogram for 18 hybrids and parents. The similarity co-efficients ranged from 0.31 to 1.00. The dendrogram separated the 18 hybrids and parents into two major clusters (Table 5). The first cluster had only hybrid H 504. All the other hybrids and parents were grouped into one major cluster (Table 4, Fig. 3). Number of DNA amplification bands depended on how primer attached to its homolog at DNA template, Tingey *et al.,* (1994). DNA fingerprinting has been carried out as a result

of substantial achievements in marker isolation and protocol optimisation. Nevertheless, commercial imperatives demand that DNA fingerprinting accomplishes more than just technological advances. International consensus is being reached on general guidelines for employing DNA fingerprints as legal evidence. Advances in the technology to economise the procedure and international consensus in operational framework are critical to the future of DNA fingerprinting. Molecular markers are of more recent development, and possess many advantages which make them superior to morphological markers. Molecular markers can be used to directly examine the DNA in order to find polymorphisms in the sequence. This offers the possibility of finding large numbers of polymorphisms which are not subject to environmental influence. Each molecular marker technique varies from each other in the number of loci examined, dominance or co-dominance and ease of application.

DNA based marker techniques can supply additional information which are not available from the examination of morphological characteristics alone by Jarret and Gawel (1995). The Inter Simple Sequence Repeats (ISSR) are DNA-based marker techniques that has been successfully used to determine genetic diversity and relationships *Musa* germplasms Kaemmer *et al.,* (1992), Howell *et al.,* (1994), Bhat and Jarret (1995), Crouch *et al., (*2000), Jain *et al.,* (2007), Racharak and Eidathong (2007), Ruangsuttapha *et al.,* (2007), Agoreyo *et al.*,(2008), Brown *et al.*,(2009) and for genome identification, Howell *et al.,* (1994), Pillay *et al.,* (2000), analysis of *Musa* breeding populations, Crouch *et al.,* (1999), detection of somaclonal variants, Grajal-Martin *et al.,* (1998) and genetic stability, Harirah and Khalid (2006), Ray *et al., (*2006), Lakshmanan *et al.,* (2007) and Venkatachalam *et al.*,(2007). Using these techniques an unlimited number of polymorphic bands can be produced with relative ease from minute amounts of genomic DNA, Welsh and McClelland(1990), Godwin *et al.,* (1997), Reddy *et al.,* (2002) allowing simultaneous screening of a large number of accessions. ISSR and RAPD were used in determinate genetic stability of three economically important banana (*Musa* spp.) cultivars and showed that ISSR detected more polymorphism than RAPD, Ray *et al.,* (2006). Similarly, ISSR were used for detection of genetic uniformity of micropropagated plantlets, Rout *et al.,* (2009) and for screening *in vitro* mutagenesis and variance Khatri *et al.,* (2011). The study also reported the use of ISSR to assess the genetic diversity and classification of 27 wild banana accessions and showed that the collected germplasm was derived from diverse origins, Qin *et al.,* (2014).

Table.1 Level of polymorphism detected by ISSR analysis banana hybrids and parents

1	**Genotypes screened**	**18**
2	Type of marker	**ISSR**
3	Number of primers used	**6**
4	Total number of markers	**54**
5	Range of markers across primers	**5-18**
6	Average number of markers/primer	**9**
7	Number of monomorphic markers	**24**
8	Number of polymorphic markers	**30**
9	**Percentage polymorphism**	**53.83**

Table.2 ISSR products generated by 6 primers for selected phase banana hybrids

ISSR primer	Total markers	Polymorphic markers	Polymorphism %
ISSR 835	7	4	57.14
ISSR 834	11	4	36.36
ISSR 823	5	1	20.00
ISSR 812	6	6	100.00
ISSR 811	18	12	66.66
ISSR 808	7	3	42.85

Table.3 List of primers used for ISSR analysis

S.No	Primer Details	Sequence information (5' – 3')	No.of bases
1.	UBC - 808	**AGA GAG AGA GAG AGA GC**	17
2.	UBC- 809	**AGA GAG AGA GAG AGA GG**	17
3.	UBC- 811	**GAG AGA GAG AGA GAG AC**	17
4.	UBC- 812	**GAG AGA GAG AGA GAG AA**	17
5.	UBC- 823	**TCT CTC TCT CTC TCT CC**	17
6.	UBC- 834	**AGA GAG AGA GAG AGA GYT***	18
7.	UBC- 835	**AGA GAG AGA GAG AGA GYC***	18
8.	UBC- 840	**GAG AGA GAG AGA GAG AYT***	18
9.	UBC- 841	**GAG AGA GAG AGA GAG AYC***	18
10.	UBC- 844	**CTC TCT CTC TCT CTC TRC***	18

* Single letter abbreviations for mixed base positions
R = (A, G), Y = (C, T)

Table.4 Details of banana hybrids and parents used in the present study

S. No	Hybrids	Parentage	Genome
1	H-504	H-03-09 x PL	AAABB
2	H-508	ANK x PL	AA
3	H-511	H-02-34 x Ykm#5	AABB
4	H-515	Mano. x ANK	AAA
5	H-516	ANK x PL	AA
6	H-531	Poovan x PL	AAB
7	H-532	H-201 x Mano.	AAB
8	H-534	H-03- 13 x Rose	AAB
9	H-537	(H-201 x PK) x Rose	AABB
10	H-540	(H-201 x PK) x Rose	AAABB
11	H 542	H-02-34 x ANK	AABB
12	H 571	H-04-05 x Ykm#5	AABB
13	H 572	H-03-35 (OP)	AAB
14	H-573	H-03-12 x Rose	AAABB
15	H 576	H-201 (OP)	AB
16	H 589	H-03-19 (OP)	AABB
Parents			
1	PL	Highly Resistance source (Check)	AA
2	ANK	Resistance source	AA
3	PK	Resistance source	ABB
4	Ykm#5	Resistance source	AAA
5	Mano.	Resistance source	AAA
6	Rose	Resistance source	AA
7	Rasthali	Highly Susceptible source(Check)	AAB

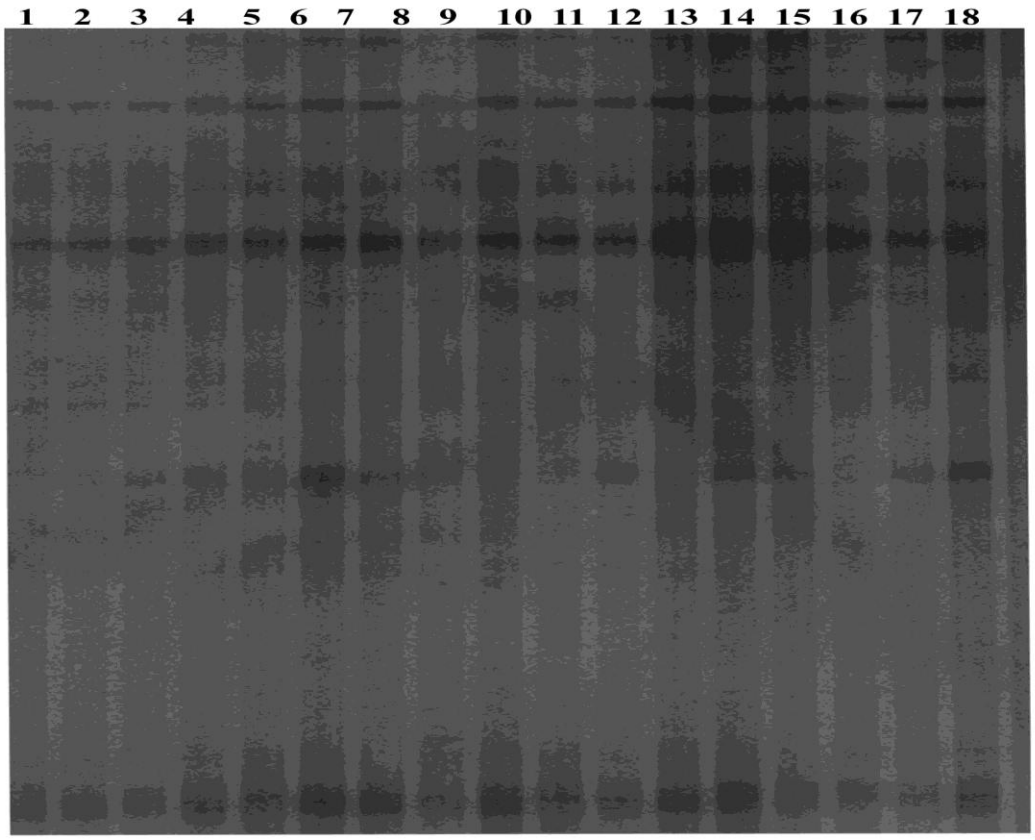
ANK – Anaikomban; PL – Pisang Lilin; PK-Peykunnan; Mano- Manoranjitham OP-Open pollinated

Table.5 Dendrogram of 18 genotypes of *Musa* species on 6 ISSR primers constructed using UPGMA based on Jaccard's Coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1.000																	
2	0.769	1.000																
3	0.678	0.884	1.000															
4	0.800	0.958	0.846	1.000														
5	0.551	0.678	0.777	0.642	1.000													
6	0.807	0.884	0.785	0.920	0.655	1.000												
7	0.692	0.769	0.807	0.800	0.666	0.807	1.000											
8	0.800	0.807	0.714	0.840	0.586	0.846	0.730	1.000										
9	1.000	0.769	0.678	0.800	0.551	0.807	0.692	0.800	1.000									
10	0.807	0.884	0.851	0.846	0.714	0.851	0.740	0.846	0.807	1.000								
11	0.769	0.920	0.814	0.880	0.620	0.884	0.703	0.807	0.769	0.884	1.000							
12	0.869	0.875	0.769	0.913	0.571	0.840	0.720	0.760	0.869	0.769	0.800	1.000						
13	0.666	0.793	0.892	0.758	0.821	0.827	0.785	0.700	0.666	0.827	0.793	0.689	1.000					
14	0.612	0.733	0.828	0.700	0.758	0.766	0.721	0.700	0.612	0.766	0.733	0.633	0.931	1.000				
15	0.538	0.555	0.535	0.576	0.464	0.592	0.538	0.640	0.538	0.653	0.555	0.500	0.533	0.533	1.000			
16	0.307	0.285	0.370	0.296	0.458	0.275	0.307	0.296	0.307	0.321	0.285	0.320	0.379	0.379	0.363	1.000		
17	0.750	0.760	0.666	0.791	0.535	0.800	0.615	0.653	0.750	0.666	0.760	0.863	0.655	0.600	0.520	0.280	1.000	
18	0.291	0.375	0.360	0.391	0.333	0.360	0.409	0.391	0.291	0.360	0.375	0.304	0.321	0.321	0.227	0.105	0.260	1.000

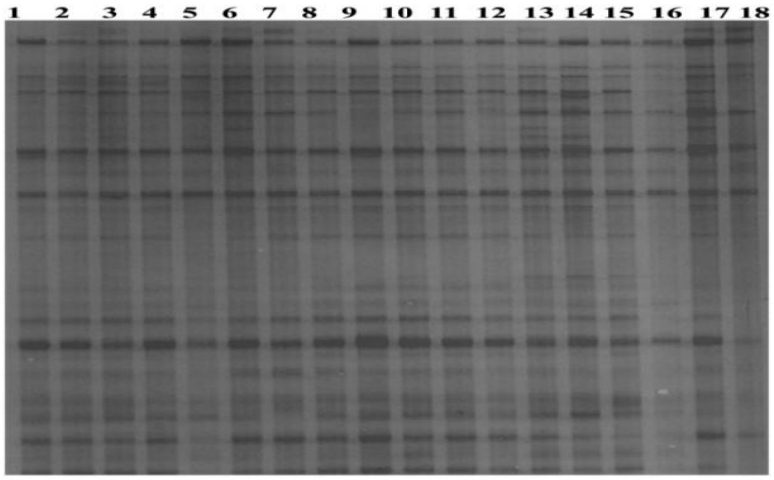
1. Pisang Lilin 2. Anaikomban 3. Ykm#5 4. Poovan 5. Rasthali 6. H 504 7. H 508 8. H 511 9. H 515 10. H 529 11. H 531 12. H 534 13. H 537 14. H 540 15. H 542 16. H 556 17.H 571 18. H 589

Fig.1 ISSR analysis of phase I banana hybrids using primer UBC 812



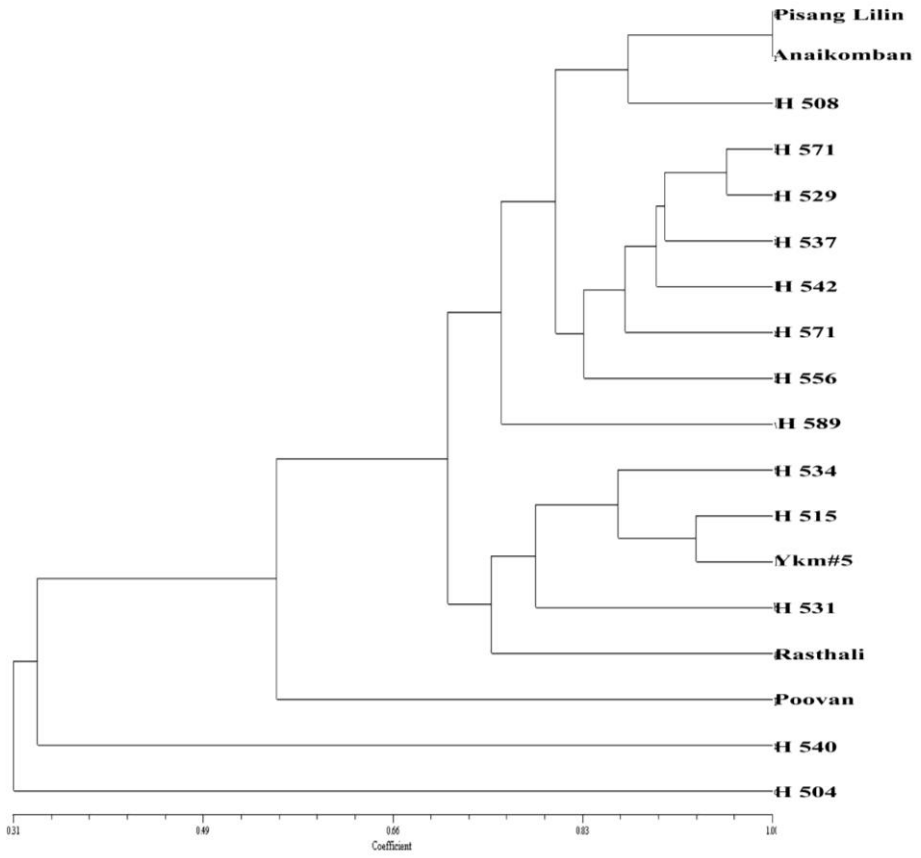
1. Pisang Lilin 2. Anaikomban 3. Ykm#5 4. Poovan 5. Rasthali 6. H 504 7. H 508 8. H 511 9. H 515 10. H 529 11. H 531 12. H 534 13. H 537 14. H 540 15. H 542 16. H 556 17.H 571 18. H 589

Fig.2 ISSR analysis of phase I banana hybrids using primer UBC 812



1. Pisang Lilin 2. Anaikomban 3. Ykm#5 4. Poovan
5. Rasthali 6. H 504 7. H 508 8. H 511 9. H 515
10. H 529 11. H 531 12. H 534 13. H 537 14. H 540
15. H 542 16. H 556 17.H 571 18. H 589

Fig.3 Dendrogram of 18 genotypes of Musa hybrids and referent cultivars on 6 ISSR primers constructed using UPGMA based on Jaccard's coefficient



ISSR were employed for molecular assessment of genetic identity and genetic stability in banana cultivars, Lu *et al.,* (2011). Recently, ISSR were used to analyse the pattern of genetic variation and differentiation in 32 individuals along with two reference samples of wild *Musa*, which corresponded to three populations across the biodiversity-rich hot-spot of the southern Western Ghats of India (Padmesh *et al.,* 2012).

The ISSR profiles indicated that each primer could generate polymorphisms among the accessions. The polymorphism may be due to mutation at priming sites and/or insertion/deletion event within the SSR region; and the extent of polymorphism also varies with the nature and the sequence repeat of the primer used by Reddy *et al.,* (2002). In order to supplement with the results of ISSR analysis was also carried out. To confirm in the present study, ISSR markers were used. Six primers were used to screen 18 hybrids and parents which produced 54 markers with a polymorphism percentage of 53.83. Cluster analysis grouped the accessions into two major clusters (Tables 5 and Fig. 3). A maximum of 18 markers per primer was observed in ISSR, which showed the informative nature of these markers. The average number of markers per primer in ISSR was nine and ISSR markers gave a polymorphism of 53.83 per cent by using just six primers (Table 1 and 2). This shows that even a few ISSR primers are sufficient to provide information needed in diversity analysis, thereby saving resources. ISSRs segregate mostly as dominant markers following simple Mendelian inheritance, Gupta *et al.,* (1994). Out of six primers studied ISSR primer UBC 812 produced maximum number of polymorphic (100.00%) bands among the banana genotypes and might be useful for genetic diversity studies. ISSR remain attractive options despite availability of sophisticated techniques because they are easy, quick, simple and economical. Neither sequence information nor any prior genetic studies are required for these analyses.

Based on the results obtained in the present study, it can be concluded that ISSR markers which are independent of environmental conditions and show higher levels of polymorphism can serve as an efficient tool in molecular diversity studies. Assessment of genetic diversity is important for parent selection in crop improvement, management of germplasm and IPR issues, and for ascertaining evolutionary relationships. Thus, in the above study Diversity analysis carried out by using ISSR markers in some banana hybrids revealed the confirmation of the hybridization along with the phylogenetic relationship of the hybrids with their pedigree.

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