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CONVERSION OF MONOMORPHIC BAND INTO POLYMORPHIC PATTERN USING NUCLEOTIDE SEQUENCING DATA IN *MUSA* VARIETIES

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Summary: Molecular markers are popularly used to characterize plant genetic diversity. Using sequencing data an attempt was made to convert monomorphic band to polymorphic pattern. For this, di, tri, tetra and penta nucleotide repeat ISSR primers were used for four *Musa* varieties. Sixteen out of thirty primers gave 23.08% polymorphism in four *Musa* varieties. One of the monomorphic bands (primer I-16) was selected for nucleotide sequencing. The obtained sequences were subjected to multiple sequence alignment (MSA) which revealed significant differences in nucleotide sequences. According to the analysis of ISSR band patterns and MSA of nucleotide sequence, UPGMA relationship was derived, both have divided the four varieties into two clusters. The varieties Khasdi, Red, Desi were clustered in the same group whereas var. Soneri was separated from them. Further, we detected cutting sites of various restriction enzymes to convert monomorphic band into polymorphic and a unique marker of known bp fragment for each variety. These results promote the initiative to integrate monomorphic bands in plant breeding applications, and DNA fingerprinting.
The sequence data from this study have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) under accession Nos. (Red, JN247634, Khasdi JN247635, Desi JN232197and Soneri JN24736.

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Keywords: *Musa*; polymorphism; molecular analysis; nucleotide sequencing

Abbreviations: ISSR – inter simple sequence repeat; SSR – simple sequence repeat, MSA – multiple sequence alignments, UPGMA – unweighted pair-group method for arithmetic averages analysis.

INTRODUCTION

Banana (*Musa* spp.) is the fourth most important fruit crop in the world. The estimated current world production of banana is 97.5 million tons per year, covering 10 million ha land. India is the largest producer of banana in the world contributing 19.71% to the total global production of banana (Sahijram et al.,

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2003). It is a rich source of carbohydrates and vitamins, particularly vitamins B; it is also a good source of potassium, phosphorus, calcium and magnesium (Wall, 2006). It helps reducing risk of heart diseases when used regularly and is recommended for patients suffering from high blood pressure, arthritis, ulcer, gastroenteritis, and kidney disorders (Bazzano et al., 2002). The multipurpose use has attracted global attention which prompted us to conduct preliminary studies on genetic diversity in *Musa* spp.
Molecular marker techniques play a crucial role in identification of plant at varietal levels. Numbers of the marker techniques are listed in the literature for discrimination of plants from one another. Microsatellite markers are one of those with 1-6 base pair repeats and show uniqueness with plant species studied (Joshi et al., 2000) and it is not affected by environmental conditions. Although SSR is a new generation powerful marker for eukaryotic system, it is hindered by the requirement of sequence information of flanking region from which primer is designed for PCR amplification (Powell, 1996).

A modification of SSR-based marker systems, i.e. ISSR analysis (Wolfe and Liston, 1998), circumvents this requirement for flanking sequence information and thus, has found wide application in plant systems. Many authors have used this technique for wheat (Zar and Ahmadi, 2011), pea cultivars (Reis and Diogo, 2012) and *Leucadendron* (Pharmawati et al., 2005) and have distinguished plants using monomorphic (i.e. similar molecular weight band amplified in each samples tested) or polymorphic (dissimilar length/molecular weight fragments) bands pattern developed on gel electrophoresis. The ISSR analyses are based on monomorphic and polymorphic band patterns and frequently in such analyses the most distance/similarity index is often used to construct a phylogenetic tree. The controversy remains over whether or not a particular monomorphic band in an amplicon (PCR product/or amplification of DNA) has similar nucleotide sequence and such assignments are not appropriately taken into account. In this paper, we present an innovative strategy to exploit monomorphism. Rather than identifying a polymorphic band with marker technology problems, we have constructed a sequence data set comprised exclusively of monomorphic bands.

To evaluate the potential of this approach, we performed multiple sequence alignments (MSA) within the sequence of monomorphic bands. We used this approach to test (i) to infer the nucleotide sequence variation, (ii) the interrelationships of *Musa* varieties and (iii) the availability of restriction sites. Thus, the resulting possibilities of polymorphism in monomorphic bands are discussed. We discussed also the 30 ISSR primers to construct the phylogenetic relationship and to select monomorphic bands for this study.

MATERIALS AND METHODS

Plant material and PCR amplification

Musa varieties (i) Desi, (ii) Red, (iii) Khasdi and (iv) Soneri were collected from Visanvel, Junagadh (Gujarat) and maintained in the Botanical Garden, Saurashtra University, Rajkot. Fresh, young, and expanded leaves were collected for DNA extraction. The genomic DNA

extraction was carried out with some modifications following the protocol reported by Mandaliya et al. (2010a) for high yield and quality DNA extraction. The DNA concentration was determined at 260 nm and purity was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Matasyoh et al., 2008) using the μQuant microplate reader, Bio-Tek instruments incorporation, USA. The extracted DNA was subjected to ISSR analysis using selected primer enlisted in Table 1. Each reaction mixture consisted of total 12.5 μL. PCR amplification was carried out in 3 stages according to Mandaliya et al. (2010b) in a Veriti (96 Well Fast Thermal Cycler), Applied Biosystems, USA. Electrophoresis loading samples were prepared according to Sheidai et al. (2007). Electrophoresis was carried out on 2% agarose gel at constant voltage of 50V in 1x TAE buffer. High Range Ruler and Supermix DNA Ladder, both from Bangalore Genei, India, were used as molecular size standards.

Statistical Analysis

Jaccard's similarity coefficient values for each pair wise comparison between *Musa* varieties were calculated and a silarity coefficient matrix was constructed with the help of Free tree software (Rana et al. 2005). This matrix was subjected to unweighted pair-group method for arithmetic averages analysis (UPGMA) to generate dendrograms (Hussein et al. 2007) using CLC Main Workbench5 for *Musa* varieties. This software can be obtained from http://www.clcbio.com.

Nucleotide sequencing

The primer products of I-16 were synthesized once again and run on 2 % low melting agarose gel at constant voltage of 50V in 1x TAE buffer. The selected bands were eluted and purified using Quiagen make QIAquick gel extraction kit for nucleotide sequencing. The purified products were cycle sequenced using Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Chemistry on ABi Genetic Analyzer 3130 (Fernandez-Carvajal et al., 2009). Sequences from the ISSR monomorphic bands were compared with NCBI database using BLAST program (Ghany and Zaki, 2003). Then, multiple sequence alignment (MSA) was performed on CLC Main Workbench5 to construct phylogenetic relationship and to confirm availability of restriction sites.

RESULTS

The 30 ISSR primers including di, tri, tetra and penta nucleotide repeats (Table 1) were tested in this study. These had amplified DNA fragments (amplicons) ranging from 100 to 5000 bp length from the genomic DNA of the studied *Musa* varieties. Amongst the primers studied, 16 primers generated sharp, clear and reproducible bands in all four *Musa* varieties. Figure 1 shows the reproducible amplification product of *Musa* varieties (i) Desi, (ii) Red, (iii) Khasdi and (iv) Soneri produced by the I-16 primer. The total number of bands generated from 16 primers was 182 including 42 polymorphic and 140 monomorphic (Table 2); thus, the percentage of polymorphic and monomorphic bands observed was 23.08% and 76.92%, respectively. The average 2.06 polymorphic amplicon per primer was observed. The maximum number of the amplified product was 20 (I-16) and the minimum was 2 (I-3). A dendrogram was

Table 1. ISSR primers.

No.	Seq.	Name	No.	Seq.	Name
I-1	(AT)8C	UBC803	I-16	(GGAGA)3	UBC880
I-2	(AG)8C	UBC808	I-17	(GGGGT)3G	UBC881
I-3	(CT)8A	UBC814	I-18	HBH(AG)7	UBC884
I-4	(CA)8A	UBC817	I-19	BHB(GA)7	UBC885
I-5	(TC)8A	UBC822	I-20	VDV(CT)7	UBC886
I-6	(AC)8C	UBC826	I-21	BDB (CA)7	UBC888
I-7	(AG)8TT	UBC834	I-22	DBD(AC)7	UBC889
I-8	(GA)8TT	UBC840	I-23	HVH (TG)7	UBC891
I-9	(AGC)6	UBC862	I-24	(AGC)5GC	ISSR1
I-10	(ATG)6	UBC864	I-25	(CA)7AC	ISSR2
I-11	(GGC)6	UBC867	I-26	(GT)7AC	ISSR3
I-12	(GAA)6	UBC868	I-27	GCA(GA)7	ISSR4
I-13	(GATA)4	UBC872	I-28	(GA)9C	ISSR5
I-14	(GACA)6	UBC873	I-29	(GA)9A	ISSR6
I-15	(GATA)2(GACA)2	UBC876	I-30	(CG)8C	ISSR7

(Where, M=AC; R=AG; W=AT; S=GC; Y=CT; K=GT; V=AGC; H=ACT; D=AGT; B=GCT; N=AGCT)

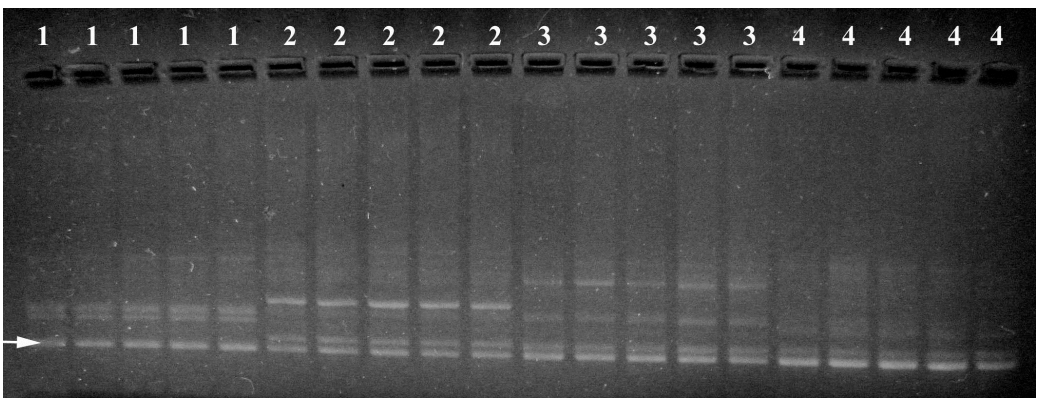

Figure 1. The amplification product of *Musa* varieties: (1) Desi, (2) Red, (3) Khasdi and (4) Soneri produced by I-16. The arrow indicates monomorphic bands selected for nucleotide sequencing.

Table 2. Consolidated ISSR amplicon profile, % monomorphism and % polymorphism prepared from detailed listing of each band amplified by ISSR primers studied.

Primer	Total Amplicon	Polymophic Amplification	Monomorphic Amplification	Total Band	Poly-morphic	Mono-morphic	% poly-morphic	% mono-morphic
I-2	4	1	3	9	1	8	11.11	88.89
I-3	2	2	0	2	2	0	100.00	0.00
I-4	3	0	3	9	0	9	0.00	100.00
I-5	6	1	5	17	1	16	5.88	94.12
I-7	3	2	1	7	3	4	42.86	57.14
I-8	7	6	1	14	10	4	71.43	28.57
I-10	10	4	6	21	4	17	19.05	80.95
I-12	1	0	1	4	0	4	0.00	100.00
I-13	1	0	1	2	0	2	0.00	100.00
I-15	8	4	4	13	4	9	30.77	69.23
I-16	10	5	5	20	5	15	25.00	75.00
I-18	3	0	3	12	0	12	0.00	100.00
I-21	4	1	3	12	1	11	8.33	91.67
I-22	5	1	4	15	1	14	6.67	93.33
I-24	5	2	3	9	2	7	22.22	77.78
I-26	6	4	2	16	8	8	50.00	50.00
Total	78	33	45	182	42	140		
Average	4.88	2.06	2.81				23.08	76.92

Table 3. Jaccard distance/similarity matrix among Four *Musa* varieties.

	Desi	Red	Khasdi	Soneri
Desi		0.55	0.47368	0.375
Red	0.55		0.67241	0.42254
Khasdi	0.47368	0.67241		0.46032
Soneri	0.375	0.42254	0.46032	

constructed based on the Jaccard distance/ similarity matrix (Table 3) using UPGMA method (Fig. 2a) and it indicated that the four varieties could be divided into two clusters. The varieties Khasdi, Red, Desi were clustered in the same group whereas var. Soneri was separated from them.

One of the monomorphic bands

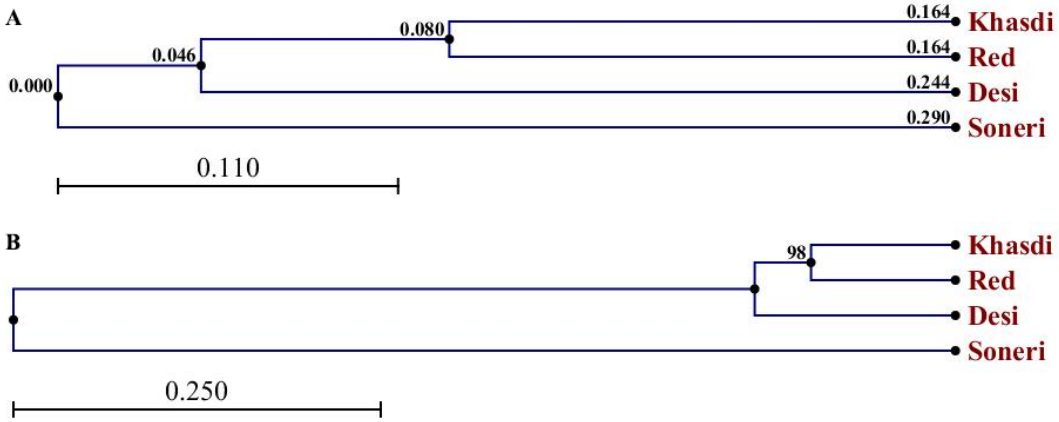

Figure 2. A) UPGMA relationship amongst four *Musa* varieties based on ISSR analysis from the details in Table 3. B) UPGMA relationship amongst four *Musa* varieties based on monomorphic bands performed on CLC Main Workbench5 **(**Red, JN247634, Khasdi JN247635, Desi JN232197and Soneri JN24736).

presented as a sharp and clear band in all *Musa* varieties indicated with an arrow in Fig. 1 was selected for nucleotide sequencing. The obtained sequences were 557-565 bp long. The sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and their accession numbers are Red, JN247634, Khasdi JN247635, Desi JN232197and Soneri JN247636.

Further, these sequences were analyzed by MSA (Fig. 3). The sequences were near to similar in length. The UPGMA relatedness derived from MSA is presented in Fig. 2b. Further analysis with CLC also revealed that each sequence had a unique cutting site for RENs and hence can be converted to polymorphic bands (Fig. 4). Cutting site for Eco RI was present only in var. Soneri, while Hind III was present in var. Khasdi. Bam HI gave 3 fragments of monomorphic band in var. Desi and two in var. Red. Thus, these monomorphic bands can be converted into polymorphic using appropriate RENs to obtain known bp fragments.

DISCUSSION

The identification of desired varieties of *Musa* is of prime importance for crop improvement programs. However, the existences of numerous synonyms, the long vegetative phase, and ambiguities in scoring morphologic traits complicate the process of identification even of known varieties. These limitations are surmountable by the DNA-fingerprinting approach. Using ISSR markers, it is now possible to make direct inferences about genetic variability and interrelationships among organisms at the DNA level without the confounding effects of environment. A number of studies reported in the literature suggest ISSR analysis of plants (Pharmawati et al., 2005). The present study was focused on ISSR analysis based on monomorphic and polymorphic band patterns in *Musa*.

Lakshmanan et al. (2007) compared and first reported the use of genetic markers to establish the genetic fidelity of RAPD and ISSR patterns of 11 plants

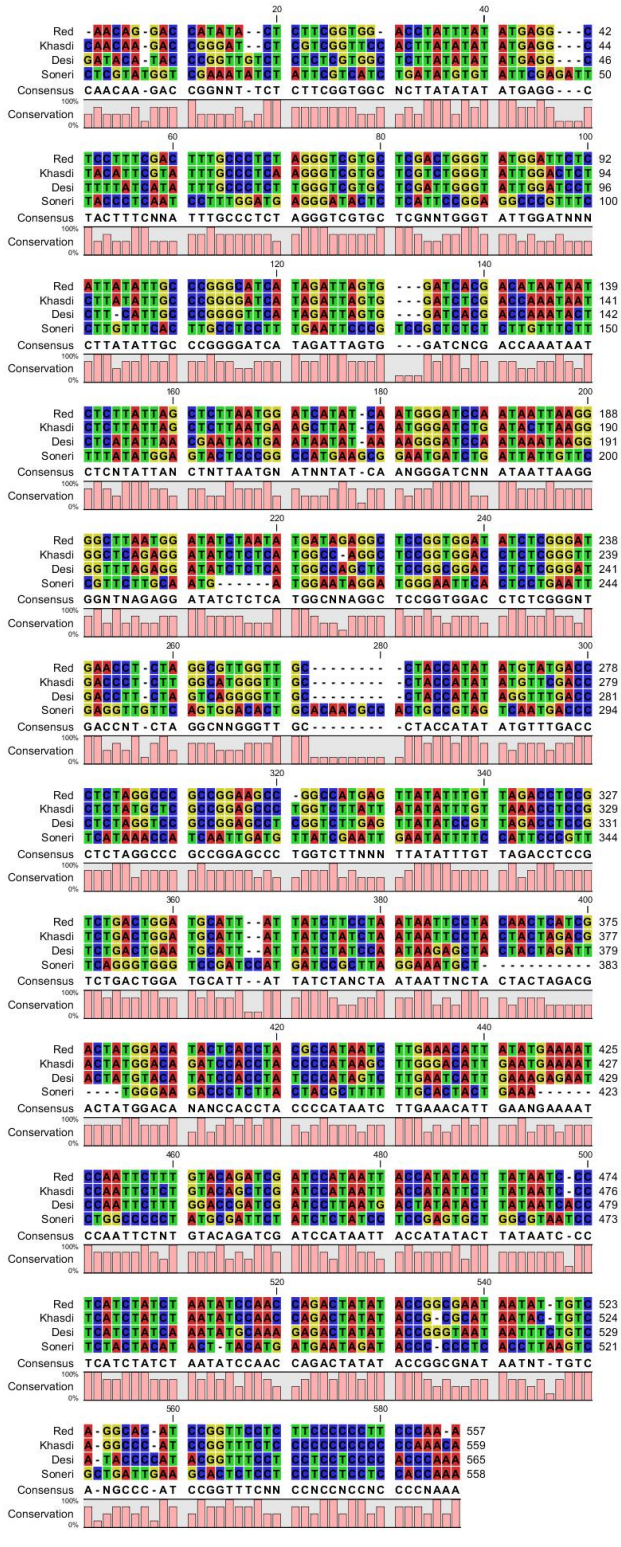

Figure 3. Multiple sequence alignment (MSA) of monomorphic band pattern of four *Musa* varieties performed on CLC Main Workbench5 **(**Red, JN247634, Khasdi JN247635, Desi JN232197and Soneri JN247636).

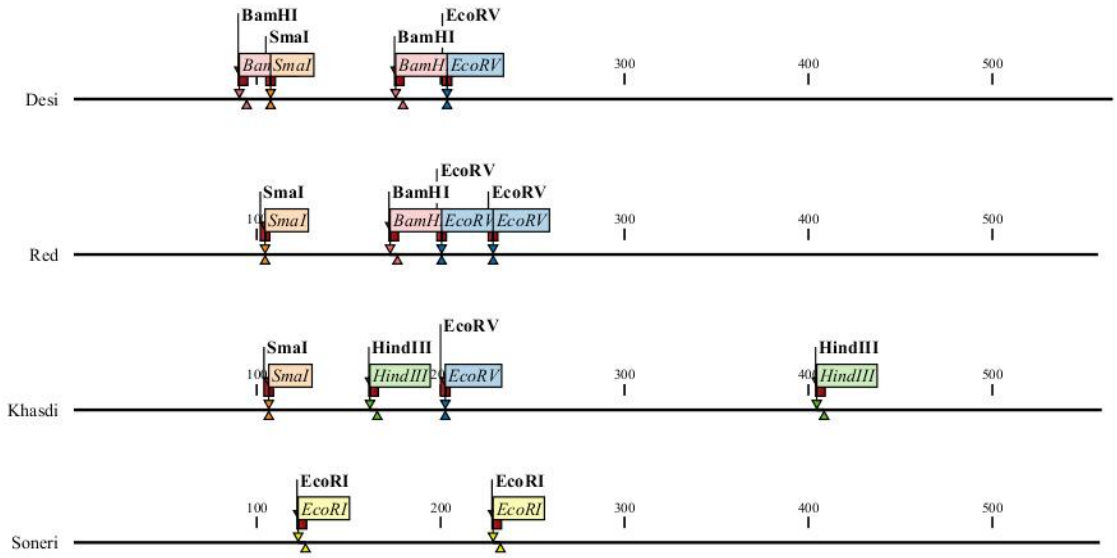

Figure 4. Assessment of restriction sites on monomorphic bands of four *Musa* varieties performed on CLC Main Workbench5 **(**Red, JN247634, Khasdi JN247635, Desi JN232197and Soneri JN247636).

chosen from 4000 *Musa* plantlets with a control plant. A total of 377 scorable bands were generated out of 30 RAPD primers and 47 scorable bands were generated from 5 ISSR primers. They observed only monomorphic patterns across all the plantlets analyzed. Venkatachalam et al. (2007) also observed a total of 16,875 bands from micropropagated plants and 14,175 bands from regenerated plants, which is highest compared to all other studies reported so far (Carvalho et al., 2004; Martins et al., 2004; Bennici et al., 2004; Ray et al., 2006). The primers they selected were based on earlier report(s) where such primers were found to be of great use in differentiating the varieties and developing phylogenetic relationships in banana (Pillay et al., 2001; Ude et al., 2003; Onguso et al., 2004). The band profile in their studies showed monomorphic pattern. In numerous studies the primers did not produce a recordable profile in repeated experiments. The failure of several primers to give informative banding patterns may be due to technical problems as those primers required special amplification conditions, such as alternative chemical stabilizers of different annealing temperatures (Pharmawati et al., 2005). The type of gel electrophoresis and the staining method used can also influence the number of scorable bands and the level of polymorphism obtained (Wiesner and Wiesnerova, 2003). In studies where polyacralamide gel was used with silver staining instead of agarose gel with ethidium bromide staining higher resolutions were obtained (Charters et al., 1996; Mathew et al., 1999). Godwin et al. (1997) reported that polyacralamide gels visualized by autoradiography of radiolabelled samples yielded good results.

Bands with same mobility were considered as identical irrespective of their

band intensity (Rout et al., 2009). Thus, PCR based amplification of gel helps to presume that somewhat similar bp (length) monomorphic band pattern with the identical primer among the varieties may express similar genomic region. However, the reliability and efficiency of molecular markers in detecting large scale genome arrangements have been frequently questioned and it is difficult to conclude about the variations in the amplified genomic region with limitations in the technique. So, controversy remains over whether or not a particular monomorphic band observed by various authors (Lakshmanan et al., 2007; Venkatachalam et al., 2007) as discussed above in each amplicon has similar nucleotide sequence.

To address this problem, one of the monomorphic bands (Fig. 1) that showed a sharp and clear band pattern in all *Musa* varieties was eluted from the gel and sequenced. The obtained sequences were 557-565 bp long, and were further analyzed for MSA in CLC. Although the sequences were near to similar in length, no 100% similarity in the nucleotide sequence was observed (Fig. 3). The UPGMA relatedness derived from the sequences in CLC is presented in Fig. 2b. The pattern of UPGMA relatedness was similar to that obtained from scoring monomorphic and polymorphic bands using total 16 ISSR primers (Fig. 2a). However, it is interesting to note that a similar trend was also obtained using sequences of a single monomorphic band using only one (I-16) primer. Both dendrograms indicate that the four varieties can be divided into two clusters. The varieties Khasdi, Red, Desi were clustered in the same group whereas Soneri was separated from them. The amplification products could result from changes in either the sequences of the primer binding sites or from changes that could have altered the sizes of the DNA fragments (of template). Variations could also result from prevention of the successful amplification of a target DNA fragment (e.g., insertions, deletions, and inversions).

Further analysis with CLC also revealed that each sequence had a unique cutting site for RENs and hence can be converted to polymorphic bands (Fig. 4). The cutting site for Eco RI was recorded only in var. Soneri, while Hind III was present in var. Khasdi, and selection of Bam HI gave polymorphic bands in both varieties Desi and Red. Thus, these monomorphic bands can be converted into polymorphic using appropriate RENs to obtain known bp fragments. We demonstrate the usefulness of this approach for the identification of the *Musa* varieties.

In conclusion, we report on results obtained using 30 ISSR primers for four *Musa sps.*, where 16 primers showed recordable monomorphic and polymorphic band patterns. One of the monomorphic bands generated using I-16 primer was sequenced to study the detailed nucleotide pattern. Significant variations were observed in DNA sequence in all four *Musa* varieties studied which can be converted to polymorphic using RENs cutting sites present on them. Furthermore, sequences generated for varieties Desi, Red, and Khasdi have shown similarity to other Musa BAC clones in the database 333(http://www.ncbi.nlm.nih.gov/Genbank/) but it is noteworthy that cultivar Soneri (JN247634) sequence was unique and showed distinct phylogenic trend. This technique could be used in

genetic mapping studies of plant species as well as to understand their phylogenic relationship. The polymorphism observed in the monomorphic bands based on sequence difference can be explored in precise identification of varieties using RENs cutting sites.

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Supplementary Table for ISSR amplification profile.

		Desi	Red	Khasdi	Soneri
I-2	1	0	0	0	1
	2	0	1	0	1
	3	1	1	1	1
	4	0	1	1	0
I-3	1	1	0	0	0
	2	1	0	0	0
I-4	1	1	1	1	1
	2	1	1	1	1
	3	1	1	1	1
I-5	1	1	1	1	1
	2	1	1	1	1
	3	0	0	1	0
	4	0	1	1	0
	5	1	0	0	1
	6	1	1	1	1
I-7	1	1	1	1	1
	2	0	0	1	1
	3	0	1	0	0
I-8	1	0	0	0	1
	2	1	1	1	1
	3	1	1	0	0
	4	1	0	0	0
	5	1	1	0	0
	6	1	1	0	0
	7	1	1	0	0
I-10	1	0	1	1	1
	2	0	1	1	1
	3	0	1	1	1
	4	0	1	0	0
	5	0	0	0	1
	6	0	1	1	1
	7	0	1	1	0
	8	0	0	0	1
	9	0	1	1	1
	10	0	1	0	0
I-12	1	1	1	1	1
I-13	1	0	1	1	0
I-15	1	0	0	0	1
	2	0	1	1	1
	3	0	0	0	1
	4	0	1	1	0
	5	0	0	0	1
	6	0	0	0	1
	7	1	0	0	1
	8	0	1	0	1
I-16	1	0	0	0	1
	2	0	0	1	1
	3	0	0	0	1
	4	0	1	0	0
	5	0	0	0	1
	6	0	1	0	1
	7	0	1	0	0
	8	1	1	1	1
	9	1	1	1	0
	10	1	1	1	1
I-18	1	1	1	1	1
	2	1	1	1	1
	3	1	1	1	1
I-21	1	1	1	1	0
	2	0	0	1	0
	3	1	1	1	1
	4	1	1	1	1
I-22	1	1	1	1	0
	2	1	1	1	0
	3	0	0	1	0
	4	1	1	1	1
	5	1	1	1	1
I-24	1	1	0	0	0
	2	0	0	0	1
	3	1	1	0	0
	4	1	0	0	1
	5	1	1	1	0
I-26	1	1	1	1	1
	2	1	1	0	0
	3	0	0	0	1
	4	1	1	1	1
	5	1	1	1	0
	6	0	1	1	0