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RESEARCH ARTICLE

Genetic Diversity Analysis of Bitter Gourd (*Momordica charantia* L.) Germplasm using Molecular Markers

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Abstract

Bitter gourd [*Momordica charantia* L.] is an important cucurbitaceous crop of medicinal and nutraceutical value. The present study was conducted to compare the efficacy of random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) molecular markers for genetic diversity analysis of bitter gourd. Twenty-one genotypes of bitter gourd, collected from different parts of India, were used for diversity analysis using two different types of markers. RAPD and ISSR markers yielded an average of 7.16 and 5.73 amplicon per primer, respectively. In the present study, RAPD and ISSR showed 89 and 63% polymorphic bands, respectively. Combined data analysis of RAPD and ISSR markers provided 73.66% polymorphism with 5.33 polymorphic amplicons per primer. The better discriminatory power of ISSR markers over RAPD markers may be due to comparatively higher values of average polymorphic information content (0.175) as well as the diverse nature of the genotypes. The information generated in this study would be helpful in designing breeding strategies for bitter gourd improvement.

Keywords: Bitter gourd, Cluster, Genetic diversity, Inter simple sequence repeats, Random amplified polymorphic DNA.

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Introduction

Bitter gourd (*Momordica charantia* L.) is a flowering vine of important cucurbitaceous vegetables grown in the tropics and subtropics (Behera, 2004). It is considered an important vegetable with exceptional nutritional properties like high vitamin C and iron content (Singh *et al.*, 2013). It is a slender, climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf axils (Anilaumar *et al.*, 2015; Kumar and Khurana, 2016). *M. charantia* L. (Karela) is a flowering climber having actinomorphic and unisexual flowers (Mishra *et al.*, 2015). The fruit has a distinct warty look of exterior and an oblong shape (Gupta *et al.*, 2011). Bitter gourd is a monoecious crop with small yellow flowers with a high male-to-female ratio that varies from 9:1 to 48:1 (Thomas, 2008). This type of flowering behavior is undesirable because it results in poorer fruit set and yield, a common problem in bitter gourd cultivation (Adarsh *et al.*, 2019). To have a higher yield the ratio of staminate and pistillate flowers to be synchronized (Islam *et al.*, 2014). Androecy and gynoecycan usually be altered in bitter gourd by environmental factors such as temperature, photoperiod and nutrition (Dabholkar, 2006; Thomas, 2008). The bitter gourd plant parts possess a large number of medicinal properties and have been used as traditional medicine for diabetes as well as source of other health-related ailment treatment substances such as charantin (Yeh *et al.*, 2013; Grover and Yadav, 2004 and Dandawate *et al.* 2016).

Genetic diversity is the basic raw material for trait improvement in any crop species. Among the various forms of genetic resources, landraces play an important role in breeding for developing high-

yielding variety for both qualitative and quantitative traits where the crop's fruit yield depends on the variety's genetic potential (Dey *et al.*, 2007). Genetic diversity assessment based on the phenotypic characters is generally inaccurate, because environmental factors and plant developmental stages affect the morphological characters of the plant (Govindaraj *et al.*, 2015). Contrarily, the DNA markers-based diversity assessment is considered more accurate and unbiased since it is independent of stage of plant and tissue and also more reliable (Fan *et al.*, 2010; Saleh, 2011). Among the various DNA markers available, the random amplified polymorphic DNA (RAPD) has been widely used because it does not require prior sequence information of the genome and could be applied in any crop species. Further, this technique involves polymerase chain reaction (PCR) so very low quantity of DNA is needed for generation of markers. The RAPD technique provides for a quick way to survey polymorphism at a very large number of loci and has been used for genetic diversity analysis in a range of crop species, including bitter gourd (Dey *et al.*, 2007; Behera *et al.*, 2008; Collard *et al.*, 2005). Similarly, inter simple sequence repeats (ISSR) marker is also quite useful in detecting genetic polymorphism among genotypes as it can generate large number of markers that targeted multiple microsatellite loci distributed across the genome and has been used for genetic diversity studies in bitter gourd (Verma *et al.*, 2017; Pandey *et al.*, 2019). In the past few years, advancement in sequencing has enabled development of simple sequence repeat (SSR) markers in bitter gourd (Saxena *et al.*, 2015; Alhariri *et al.*, 2021; Cui *et al.*,2022). The SSR markers are locus-specific and thus amplification profile generated using these markers are highly reliable. The main limitation of this approach is that a large number of primers targeting various SSR motifs need to be synthesized and analyzed to find the polymorphic marker which is not affordable to laboratories with limited funding. In contrast to SSR marker-based analysis, RAPD and ISSR marker analyses are less expensive since the primers used in these approaches are generic, which implies that once the primers for these techniques are synthesized, they can be used to research genetic diversity in other species as well. Further, RAPD and ISSR are shown to reveal polymporhism more efficiently than SSR markers. The present study used RAPD and ISSR techniques because they are inexpensive and primers were already available in our laboratory.

In the present study, 21 diverse genotypes of bitter gourd from different locations of India were collected and screened for genetic diversity using RAPD and ISSR markers to identify useful germplasm for crop improvement in India for characterizing numerous undocumented indigenous bitter gourds.

Materials and Methods

Twenty-one bitter gourd genotypes used in this study (Table 1) were collected from different sources and grown and maintained at the vegetable research farm Bihar Agricultural University, Sabour, Bhagalpur, Bihar.

Table 1: Bitter gourd genotypes and their sources

Accession name	*Fruit color*	*Source*
Bitter Kathi	Dark green	VNR seeds
Line-114	Small light green	Sasaram collection
Line-214	Medium green	Madhubani collection
Line-314	Dark green	Sasaram collection
BRBTL	Light green	Local collection
Line-514	White	Madubani collection
Line-814	Light green	Muzaffarpur collection
Meghdoot	Small dark green	Nalanda collection
Preethi	Whitish green	KAU
Gangajali Small	Small light green	Nalanda collection
Jhalari	Medium green	Rasi seeds
Indira	Dark green	Seeds of India
Pusa Ausadhi	Medium green	IARI
Pirpaiti Local	Medium green	Pirpaiti collection
Konkan Tara	Medium green	BSKV Dapoli
Pusa Rasdar	Light green	IARI
BRBTW	Milky white	Local collection
Swarnayamini	Medium green	ICARNER, Patna
BRBTG	Light green	Sasaram collection
Leena	Green	VNR seeds
Pallee	Dark green	East West seeds

DNA Isolation

The genomic DNA from young, healthy leaves was extracted using the cetyl trimethyl ammonium bromide (CTAB) method described previously (Anu *et al.*, 2015).

Marker Analysis

RAPD analysis was carried out with 10 decamer random primers procured from Operon Technologies, California, USA. RAPD analysis was performed in 25 µL volume containing 1X Taq DNA polymerase buffer, 200 µM dNTPs mixture, 0.5 µM primer, 25 ng of template DNA and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a thermal cycler (Eppendorf™ Nexux, Germany). The PCR conditions included initial denaturation DNA at 94°C for 4 minutes; followed by a 45-cycle amplification (94°C, 1-minute; 38°C, 1-minute; 72°C, 2 minutes) and a final extension step at 72°C for 7 minutes.

For ISSR analysis also, primers were obtained from Operon Technologies, California, USA. ISSR amplification was performed in 25 µL volume containing 1X Taq DNA polymerase buffer, 200 µM dNTPs mixture, 0.5 µM primer, 25 ng of template DNA and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a thermal cycler performed in 25 µL volume containing 1X Taq DNA polymerase. The reagents were mixed thoroughly and then placed on a thermal cycler for cyclic amplification and the conditions for amplification. DNA denaturation was done at 94.8°C for 5 minutes, followed

by a 40 cycle amplification (94°C for 1-minute; 36°C for minutes; 72°C for 2 minutes) followed by last step of final extension at 72°C for 5 minutes. Amplification products were then subjected to electrophoresis in 1.2% agarose gel using 1X TBE and detected by ethidium bromide staining, viewed under ultraviolet light and photographed with a gel-documentation system.

Data Analysis

NTSYS-pc Version 2.02 (Numerical Taxonomic System) software (Rohlf, 2000) was used to calculate Jaccard's similarity coefficients between genotypes. The similarity matrix was subjected to the cluster analysis of the unweighted paired group method using arithmetic averages (UPGMA) and dendrograms were generated. The polymorphic information content (PIC) was estimated by using the formula PIC = 2F (1-F), where F is the frequency of band present (Lynch and Walsh, 1998).

Results

Identification and Evaluation of RAPD & ISSR Markers for Genetic Diversity Analysis

A total of 15 ISSR and 25 RAPD markers were tested for amplification in bitter gourd genotypes. Eleven ISSR and 18 RAPD markers yielded reproducible and scorable amplified products in all the genotypes of bitter gourd studied. A list of ISSR and RAPD markers used in this study is shown in Tables 2 and 3.

RAPD Analysis

Out of 129 reproducible amplicons generated using 18 RAPD primers in 21 bitter gourd genotypes, 89% were polymorphic (Table 1). The size of the amplified products varies from approximately 100 to 1000 bp. The number of amplicons per primer ranged from four (OPA-4, OPB-8) to eleven (OPA-10, OPA-19), averaging 7.167 amplicons. The number of polymorphic amplicons per primer ranges from two (OPA-4) to nine (OPA-19 and OPC-14) with an average of 4.94. The percentage of polymorphism ranged from 42.86% (OPB-6 and OPC-10) to 90.00% (OPC-14), with an average of 68.001% (Table 1). The average PIC value was 0.160 and ranged from 0.043 (OPC-8) to 0.277 (OPA-12) (Table 1). The Jaccard's similarity coefficients of 21 bitter gourd genotypes based on 18 RAPD markers were computed. The similarity coefficients ranged from 0.52 to 0.93 with an average correlation coefficient of 0.72 (Figure 1).

ISSR Analysis

The size of the amplified products varies from ~100 bp to 1200 bp (Table 2). The number of amplicons per primer ranged from four (ISSR-51) to twelve (ISSR-8) with

Table 2: Banding pattern and statistics of RAPD primers of 21 genotype

S. No.	*Primer (Operon code)*	*Sequences*	*Total no. of amplified fragments*	*Monomorphic bands*	*Polymorphic bands*	*Range of amplicon size (bp)*	*Polymorphism (%)*	*PIC*
1	OPA-4	AATCGGGCTG	4	2	2	350–1500	50.00	0.067
2	OPA-5	AGGGGTCTTG	7	2	5	350–1500	71.43	0.212
3	OPA-8	GTGACGTAGG	5	1	4	300–1000	80.00	0.084
4	OPA-9	GGGTAACGCC	6	3	3	250–1000	50.00	0.243
5	OPA-10	GTGATCGCAG	11	5	6	300–2000	54.54	0.101
6	OPA-12	TCGGCGATAG	7	3	4	250–1000	57.14	0.277
7	OPA-15	TTCCGAACCC	6	2	4	300–2000	66.67	0.132
8	OPA-17	GACCGCTTGT	8	1	7	300–1800	87.50	0.162
9	OPA-18	AGGTGACCGT	7	2	5	300–1500	71.43	0.154
10	OPA-19	CAAACGTCGG	11	2	9	250–1000	81.82	0.231
11	OPB-1	GTTTCGCTCC	9	2	7	300–1500	77.78	0.222
12	OPB-6	TGCTCTGCCC	7	4	3	250–1200	42.86	0.144
13	OPB-8	GTCCACACGG	4	1	3	250–1300	75.00	0.131
14	OPB-12	CCTTGACGCA	6	1	5	300–1400	83.33	0.125
15	OPC-8	TGGACCGGTG	8	2	6	300–1200	75.00	0.043
16	OPC-10	TGTCTGGGTG	7	4	3	250–1200	42.86	0.206
17	OPC-14	TGCGTGCTTG	10	1	9	300–1200	90.00	0.202
18	OPC-15	GACGGATCAG	6	2	4	300–1000	66.67	0.146
	Total		129	40	89			
	Average		7.167	2.222	4.944		68.001	0.160

Table 3: Banding pattern and statistics of ISSR primers of 21 genotype

S. No.	*Primer (Operon code)*	*Sequences*	*Total no. of amplified fragments*	*Monomorphic bands*	*Polymorphic bands*	*Range of amplicon size (bp)*	*Polymorphism (%)*	*PIC*
1	ISSR4	ACACACACACACACACAG	6	1	5	250–800	83.33	0.138
2	ISSR8	ATGATGATGATGATGATG	12	2	10	300–1200	83.33	0.208
3	ISSR9	GAGGAGGAGGAGGAGGAG	5	1	4	300–2000	80.00	0.240
4	ISSR22	ACACACACACACACACAA	11	2	9	250–1000	81.82	0.134
5	ISSR23	TGTGTGTGTGTGTGTGAT	8	1	7	350–1300	87.50	0.159
6	ISSR24	TGTGTGTGTGTGTGTGAG	8	3	5	300–1500	62.50	0.221
7	ISSR35	AGAGAGAGAGAGAGAGGTA	6	1	5	250–1000	83.33	0.159
8	ISSR42	TGTGTGTGTGTGTGTGGC	6	1	5	250–1200	83.33	0.168
9	ISSR43	ACACACACACACACACCG	7	1	6	280–1100	85.71	0.254
10	ISSR44	ACACACACACACACACGA	6	2	4	260–1200	66.67	0.236
11	ISSR51	TGTGTGTGTGTGTGTGAT	4	1	3	250–1500	75.00	0.125
Total			79	16	63			
Average			7.18	1.45	5.73		79.32	0.190

an average of 7.18 amplicons per primer (Table 2). The average number of polymorphic amplicons per primer was 5.73, ranging from three (ISSR-51) to ten (ISSR-8). The percentage of polymorphism ranged from 62.50 (ISSR24) to 85.71% (ISSR-43), with an average of 79.32%. The maximum number of polymorphic amplicons was obtained with the primers ISSR8 (10), ISSR22 (9) with an average of 5.73 per primer. The average PIC value was 0.19 and ranged from 0.125 (ISSR-51) to 0.254 (ISSR-43). In all the primers, ISSR-43, ISSR-44, ISSR-9, and ISSR-8 had higher PIC values. The Jaccard's similarity coefficients of 21 bitter gourd lines based on 11 ISSR markers were computed (Figure 2). The similarity coefficients ranged from 0.52 to 0.91, with an average correlation coefficient of 0.71.

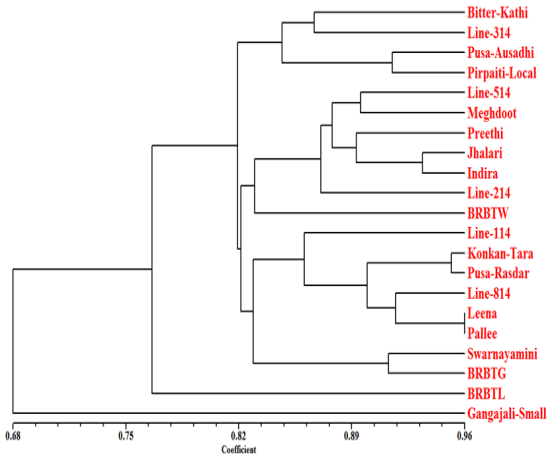

Figure 1: Dendrogram based on RAPD markers using Jaccard coefficient

Polymorphism Analysis using RAPD and ISSR Markers

Out of 208 reproducible amplicons generated by RAPD and ISSR primers in combination, 152 were found to be polymorphic. The average amplicons per primer were 7.173 of which 5.335 were polymorphic. The average percentage of polymorphism was 73.661. The average value of PIC, was 0.175, respectively. The Jaccard's similarity coefficients for combinations of 21 bitter gourd lines based on combined data analysis was computed (Figure 3). The coefficients ranged from 0.50 to 0.93.

Cluster Analysis Based on RAPD Analysis

The phylogenetic tree of the relationship among the 21 bitter gourd lines using 18 RAPD primers was shown in Figure 1. All genotypes were grouped in five clusters, namely, I (similarity coefficient = 0.52), II (similarity coefficient = 0.62), III (similarity coefficient = 0.72), IV (similarity coefficient = 0.82), and V (similarity coefficient = 0.93). The maximum number of genotypes was divided into two clusters, I and II. Cluster I was divided into two sub-cluster I and II. The sub-cluster was divided into three categories i.e. A1, A2, and A3. The category A-2 contains a large number of genotypes, followed by A-3 and A-1. The category A-2 was divided into two sub-categories A-1-1 and A-2-1. The category A-2-1 contains line-514, Meghdoot, Preethi, Jhalari, Indira and line-214, whereas A-2-2 contains BRBTW. Category A-3-1 contained line -814, Konkan Tara, Pusa rasdar, Leena and Pallee whereas category A-3-2 contained swarnayamini and BRBTG. The category A-1-1 contains bitter Kathi, and Line-314, whereas A-1-2 contains Pusa Aushadhi and Pirpaiti local. The sub-cluster II contained one genotype *i.e.* Gangajalee Small. The association amongst different genotypes was presented as a dendrogram. The resemblance coefficient

between the two genotypes is the value at which their branches join. The dendrogram also showed the relative magnitude of resemblance among different clusters.

Cluster Analysis Based on ISSR Analysis

The dendrogram generated from the Jaccard's similarity values using NTSYS software based on 11 ISSR primers presented in Figure 2. The genotypes were grouped into clusters I and II with a similarity coefficient (0.71). Cluster I was divided into two sub-cluster I, II. The sub-cluster I of cluster I was divided into Cat-A and Cat-B. The Cat –A contained bitter kathi and line-514. The Cat-B contained in Preethi, Gangajalee small, Pusa Aushadhi, Jhalari, Indra and Pirpaiti local. Sub-cluster-II was divided into Cat-A and Cat-B. Cat-A contained BRBTL and line -314, whereas Cat-B contained meghdoot and line-214. Cluster II was divided into sub-cluster-I -I and sub-cluster-II. Subcluster I was divided into Cat-A and Cat-B in which Cat-A contained line-814, Konkan Tara, Pusa Rasdar and BRBTW whereas Cat-B contained Leena and Palee. The sub-cluster –II had only one category, i.e., Cat-A. It contained Swarnayamini and BRBTG. The dendrogram also showed the relative magnitude of resemblance among different clusters.

Cluster Analysis Based on RAPD and ISSR Analysis

The dendrogram generated by combined RAPD and ISSR marker analysis (Figure 3) categories the genotypes into five clusters, I (similarity coefficient = 0.53), II (similarity coefficient = 0.63), III (similarity coefficient = 0.72), IV (similarity coefficient = 0.82) and V (similarity coefficient = 0.92). All the lines were divided into two clusters. Cluster I contained one line *i.e.* gangajalee small and cluster II were divided into two sub-cluster. Sub-cluster I contains only one line, *i.e.,* line-414 (BRBTL) while sub-cluster II was divided into two categories. Categories A contained were divided into sub-categories. Categories IA contained bitter kathi and Line-314, whereas categories IB contained Line-514, Meghdoot, line -214, Preethi, Pusa Aushadhi, Pirpaiti local, Jhalari and Indira. The categories IIA contained line-814, Konkan Tara, Pusa Rasdar, Leena and Pallee, whereas line II B contained Karela Safed (BRBTW), Swarnayamini and BRBTG.

Discussion

Genetic diversity is a pre-requisite for any crop improvement program to assess the variation among the plant's genotypes, genes or whole genomes. Genetic characterization of different bitter gourd genotypes provides an opportunity to broaden the genetic base of bitter gourd (Behera *et al.*, 2008). In the present study, the average percentage of polymorphism obtained by RAPD markers was 68.00 which was higher than 41.34% (Dalamu *et al.*, 2012), 36.5% (Dey *et al.*, 2006) and 48.3% (Rathod *et al.*, 2008).

However, percentage polymorphism (79.32%) revealed by ISSR markers was higher than those obtained by Dey *et al.* (2006) in bitter gourd (36.50%) Verma *et al.* (2007) in ash gourd (>80%), Behera *et al.* (2008) in bitter gourd (74.7%) and Dalamu *et al.* (2012) in bitter gourd (70.0%). The average number of polymorphic amplicons per RAPD primer was 4.94 and 5.35 in ISSR primers. However, Dalamu *et al.* (2012) obtained a higher number of average polymorphic amplicons per primer, 3.72 amplicons per ISSR primer whereas Behera *et al.* (2008) obtained 6.3 amplicons per primer.

The relative efficiency of marker types for genetic analysis varies among crop species. The present study concluded that ISSR markers were more effective than RAPD analysis. The better discriminatory power of ISSR markers over RAPD markers may be due to comparatively higher values of average PIC; 0.190 (ISSR) versus 0.160 (RAPD).

In our study, ISSR marker-based analysis of genetic diversity of bitter gourd genotypes revealed greater diversity when compared with RAPD and ISSR analyses in previous studies with different sets of bitter gourd germplasm [RAPD (36.5% polymorphism); Dey *et al.* (2006) and ISSR (74.5% polymorphism); Singh *et al.*, 2015]. In the present study, the average number of polymorphic bands was 4.94 per RAPD primer and 5.35 per ISSR primer. The values for polymorphism per primer in the similar range have been reported by Dey *et al.*, (2006). [RAPD (2.62) and ISSR (6.30)]. Similar results for RAPD and ISSR polymorphism was observed in relation to other crops like watermelon (Paris *et al.*, 2003), and watermelon *Cucumis lanatus* (Levi *et al.*, 2004). When compared to other arbitrary primers like

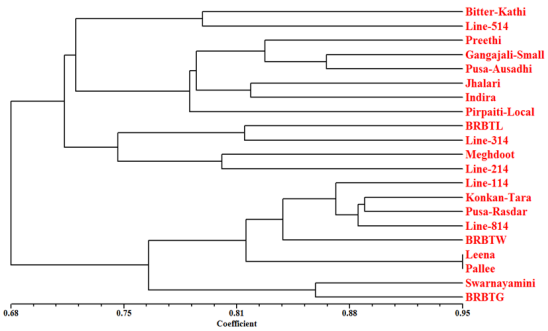

Figure 2: Dendrograms based on ISSR primers

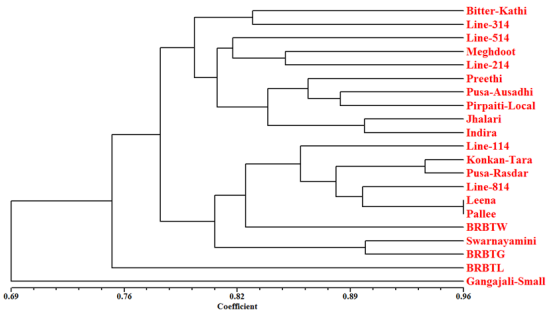

Figure 3: Dendrogram generated by combined 18 RAPD and 11 ISSR primers

RAPDs, ISSRs offer enormous potential for resolving intra- and inter-genomic relationships (Zietkiewicz *et al.*, 1994) and genus-specific bands, thus in an earlier study, this marker system was utilized for defining the uniqueness and genetic-relatedness among various cucurbitaceous members (Payel *et al.*, 2015). The increased level of diversity observed in our study was likely due in part to the number and comparatively high discriminatory power of ISSR markers employed and the diverse nature of the germplasm examined.

The Jaccard's similarity coefficient values for RAPD, ISSR and combined RAPD and ISSR data showed a wide range, suggesting that the bitter gourd germplasm collection represents a genetically diverse population. The similar range of Jaccard's similarity coefficient values for RAPD (0.72–0.97) and ISSR (0.50–0.95) markers were reported on previous study as well (Dalamu, 2011). Similarly, in another study, Behera *et al*. (2008) reported Jaccard's similarity coefficient values ranging between 0.57 to 0.93 for RAPD and between. 48 to 0.91 for ISSR were observed for the bitter gourd accessions that they had studied.

The genotypes lying closer to each other in dendrograms were more similar to those lying apart. The dendrogram showed the relative magnitude of resemblance among different clusters. In the UPGMA dendrogram drawn combined RAPD and ISSR data, genotypes from different sources were also clustered together, which may be attributed to sharing germplasm lines among the public sector institutions. The two genotypes (Leena and Pallee) from the private seed companies could not be distinguished even in the dendrogram drawn using combined RAPD and ISSR. Further, the dendrogram and genetic similarity matrix produced from marker's data was compared and revealed similar but not identical phylogenetic relationships. The results obtained from these two marker systems were in agreement. Based on DNA profiling using ISSR and RAPD PCR analysis, similar magnitudes of correlation were also found by Dalamu *et al.* (2011) and Behera *et al.* (2008) in bitter gourd and Paris *et al*. (2002) in *Cucurbita pepo*.

When RAPD, ISSR, and RAPD + ISSR derived dendrograms were compared, the discrimination among genotypes within the clusters was more effective than using them separately. The most of the accessions studied in the present experiment have differed with their geographic origin. Eastern India has been proposed as one of the centers for the domestication of bitter gourd (Behera *et al.*, 2008; Yang and Walters, 1992).

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