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Genetic diversity in Indian snapmelon (*Cucumis melo* var. *momordica*) accessions revealed by ISSR markers

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Abstract

Snapmelon (*Cucumis melo* var. *momordica*) is native to India and many of its accessions have been used as source for disease and insect pest resistance, worldwide. Inter simple sequence repeat (ISSR) markers were used to evaluate intra-specific genetic diversity among twenty-two snapmelon accessions, variable for fruit cracking, peeling patterns, fruit shape, and flesh colour. Of the 32 ISSR markers tested, three produced monomorphic products, nine markers failed to amplify, and rest of the 20 markers produced 127 amplification products, of which 74 (58.38%) were polymorphic. Although the accessions varied greatly in terms of fruit traits, the pair-wise Jaccord's similarity coefficient ranged from 0.59 to 0.88, revealing a narrow diversity in the studied samples owing to dominant nature of the ISSR markers. The dendrogram prepared through unweighted pair group method with arithmetic mean (UPGMA) distinguished two main clusters, cluster I consisting of 8 accessions, while cluster II contained 14 accessions. UPGMA clustering was also supported by principal components analysis (PCA). The first three PCs contributed 21.1, 18.9, and 8.7% of the variation, respectively. The first three PCs contributed for 48.7% variation in the studied accessions. This study could provide useful information for Indian snapmelon germplasm management activities, leading to development of a core collection for use in breeding and conservation programs.

Keywords: *Cucumis melo* var. *momordica*, ISSR, cluster analysis, dendrogram.
Abbreviations: ISSR: Inter simple sequence repeat, PCA: Principal component analysis, TSS: Total soluble solids, PIC: Polymorphic information content.

Introduction

Snapmelon (*Cucumis melo* L. var. *momordica* (Roxb.) belongs to family *Cucurbitaceae*, is a native of India (Duthie, 1905), and is used as vegetable in a variety of ways. The availability of rich diversity in Indian snapmelon accessions in terms of leaf lobe and fruit cracking and peeling patterns indicates that India is a centre of diversity (Dhillon et al., 2007). Ripe fruits of snapmelon have a specific characteristic of splitting (cracking), therefore in India, it is locally known as 'phut' (meaning 'to split'). Immature fruits of snapmelon are generally used as salad, while ripe fruits are consumed as dessert. In recent times, the juice of snapmelon is gaining popularity as a refreshing drink due to its cooling effects (Pareek et al., 1999). Snapmelon is rich in nutritional attributes; 100 g edible fruit of snapmelon contains 15.6 g carbohydrates, 18.6 mg vitamin C, and provides 74.0 kcal energy (Goyal and Sharma, 2009). However, the sweetness of snapmelon is lower compared to that of muskmelon (Dhillon et al., 2007). Snapmelon was reported to be intensively grown in the 19th century in northern part of India (Duthie, 1905). In India, records of melon dates back to 2000 BC, when various wild types of melons, *viz*., *momordica*, *acidulous*, and *flexuosus* came into cultivation; these were non-sweet types, mainly consumed as vegetable, and clearly separated from other botanical varieties (Pitrat et al., 2000). In India, snapmelon is generally grown during rainy season as an intercrop with maize and sorghum by the resource poor farmers for their own consumption.

India being a centre of diversity is endowed with great variability in terms of morphological characters, especially, fruit size and shape, fruit cracking and peeling patterns, flesh colour, skin texture, and primary and secondary colour of fruit skin (Pandey et al., 2009; 2011). Indian snapmelon accessions have been reported to be a good source for disease and insect pest resistance, and many of them are used as reference accessions worldwide (Pitrat et al., 2000; Cohen et al., 2003). Snapmelon is not covered in conservation priority in India and does not face an immediate threat of genetic erosion (Pandey et al., 2009). Despite its economic importance, and some of the extensive studies for identifying resistance sources, limited attention has been paid to genetic characterization and diversity studies of Indian snapmelon accessions. Previously, genetic diversity among snapmelon accessions, including varieties and populations, were based on pedigree data and morphological traits (Pandey et al., 2009; 2011). However, genetic diversity studies in melons are now employing various DNA markers, which are

environmentally neutral and independent of plant development phase. Different marker systems such as, restriction fragment length polymorphisms (RFLP; Neuhausen, 1992), randomly amplified polymorphic DNA (RAPD; Sensoy et al., 2007), amplified fragment length polymorphism (AFLP; Garcia-Mas et al., 2000), inter simple sequence repeat (ISSR; Fabriki et al., 2008), and simple sequence repeat (SSR; Escribano et al., 2012) markers have been used to analyse genetic diversity in melons (*C. melo*). Recently, 43 wild melon accessions including few snapmelon accessions were characterized using morphological and SSR marker data (Dhillon et al., 2005; Roy et al., 2012). However, there is no report on molecular markers-based genetic diversity analysis of Indian snapmelon (*C. melo* var. *momordica*) accessions. The knowledge about nature and magnitude of genetic diversity present in snapmelon would aid to a rational choice of the characters for which selections could be made. The objective of present study was to analyse genetic diversity in 22 snapmelon accessions, including six exotic collections from USA, using ISSR markers.

Results and Discussion

Fruit trait variability among accessions

A detailed description of the fruit-related characters of the 22 monoecious snapmelon accessions used in the study is presented in table 1 and some of the representative types are photographed in Fig. 1. Based on fruit cracking and peeling patterns, the 16 Indian accessions were grouped into four different types, *viz*., blossom end cracking (04), random skin peeling (04), random cracking (01), and longitudinal cracking (05); in the remaining two accessions no cracking was observed. However, among the six accessions from USA, two showed blossom end type of cracking, while the remaining four did not show cracking at maturity. Out of the 16 Indian accessions, 10 had elongated fruits. Majority of the accessions from U.P. (India) had either yellow or creamish secondary fruit colour. Overall, about half of the accessions had cream colour flesh, while rest had white/orange flesh colour. No association was observed between flesh and fruit skin colour.

Screening of primers for marker polymorphism

Of the 32 ISSR primers used in this study, 20 produced reproducible and polymorphic amplification products, three of the markers produced monomorphic pattern, while nine of the primers failed to amplify any fragment (Table 2). The 20 primers yielded a total of 127 amplification products, of which 74 (58.38%) were polymorphic. The size of the amplified products ranged from 300 to 2100 bp, while the number of polymorphic bands per primer ranged from 2 to 6, the average being 3.7 (Table 2). On the basis of polymorphic information content (PIC) value, primers UBC-848 [$(CA)_8$GG; 0.79], UBC-855 [$(AC)_8$TT; 0.75], and UBC-836 [$(AG)_8$TA; 0.71] were the most informative, while the primer UBC-843 showed least PIC value (0.19; Table 3). The average PIC value of the 20 ISSR primers was 0.48 (Table 2). The amplification product profiles generated from the primer UBC-834 is presented in Fig. 2. Polymorphic information content values of ISSR markers ranged from 0.19 to 0.79. It is worthwhile to mention that in previous study only RAPD primers were used in genetic diversity analysis in snapmelon accessions (Dhillon et al. 2007). ISSR markers are expected to be more informative mainly due to the fact that they amplify DNA segments present at an amplifiable distance in between two identical microsatellite regions oriented in opposite directions. In addition, such markers are highly reproducible due to the use of longer primers (16-25 nucleotides) leading to higher stringency compared to that of random decamer primers used in RAPD (Reddy et al., 2002). Analysis of genetic diversity using DNA markers in combination with morphological traits is useful for curators and breeders as it helps to define accessions by geographical regions and provides a reference data for future improvements (Dhillon et al., 2007).

UPGMA and PCA based cluster analysis

The genetic coefficients measured through ISSR marker data revealed varying degrees of genetic relatedness among the snapmelon genotypes. Jaccard's similarity coefficient ranged from 0.59 to 0.88 among 22 snapmelon accessions (data not shown). Snapmelon accessions DR/KPS-21 and EC-424817 revealed the maximum similarity of 0.88 followed by DR/KPS-48 and VRSM-73 (0.84), and DR/KPS-45 and EC-424820 (0.82). Accessions VRSM-58 and EC-428160 showed the least genetic similarity of 0.59 followed by VRSM-41 and VRSM-25 (0.60), and VRSM-41 and VRSM-10 (0.61). The coefficient of genetic similarity (0.59 to 0.88) obtained in the present study indicates a relatively low level of genetic diversity among the 22 snapmelon accessions. In contrast, SSR analysis revealed a wide range (0.14-0.83) of genetic similarity among 31 Spanish melon accessions (Escribano et al., 2012). The marker analyses also revealed VRSM-58 and EC-428160 as the most divergent accessions, which is in agreement to significant differences in the fruit characteristics in these two accessions (Table 1). Therefore, these two accessions along with VRSM-41, VRSM-25 and VRSM-10 could be considered as diverse genotypes for breeding programs, especially for improving fruit characteristics. In the dendrogram, all the 22 accessions grouped in to two main clusters consisting of 8 and 14 genotypes, respectively (Fig. 3). Cluster I consisted of 8 genotypes (DR/KPS-22, VRSM-58, DR/KPS-10, DR/KPS-39, VRSM-25, VRSM-10, VRSM-21 and EC-428157); 4 from UP and 3 from Bihar states of India, and one accession from USA. Cluster II could be further subdivided into two sub-clusters, *i.e.*, sub-cluster IIA consisting of 13 accessions, including 5 from UP (DR/KPS-21, DR/KPS-48, DR/KPS-08, DR/KPS-45 and DR/KPS-24), 4 from Bihar (VRSM-41, VRSM-60, VRSM-73 and VRSM-32), and 4 from USA (EC-424817, EC-428452, EC-424820 and EC-428423). The USA accession EC-4281160 did not group with any other accession (Fig. 3). PCA revealed that principal components, PC1, PC2 and PC3 accounted for 21.1, 18.9 and 8.7% of the total variation, respectively. Thus, the first three PCs together accounted for 48.7% of the total variation. Two-dimensional plot was prepared by using the first two PCs, in which, all the accessions appear to group in two major clusters (Fig. 4). The result of PCA was almost similar to that of UPGMA (unweighted pair group method with arithmetic mean) –based cluster analysis, except for the few differences. For example, the accession DR/KPS-10 from cluster I of UPGMA-based dendrogram grouped into sub-cluster IIA in PCA. Sub-cluster IIA of UPGMA was divided into two sub-clusters, sub-cluster IIA and sub-cluster IIB in PCA consisted of 8 and 6 accessions, respectively. In PCA also, similar to UPGMA dendrogram, the accession EC-428160 did not cluster to any group. Thus, overall, both the hierarchical and the ordination methods resulted in a similar type of grouping pattern. In order to exploit the maximum information from molecular marker data, it has been suggested to use ordination methods

Table 1. Description of six fruit-related traits of 22 snapmelon accessions.

S. No.	Accession	Country of origin	Fruit cracking and skin peeling pattern*	Fruit shape	Fruit skin predominant primary colour	Fruit skin predominant secondary colour	Design due to secondary skin colour	Flesh colour
1.	DR/KPS -22	India (U.P.)	BEC[1]	Pyriform	Light green	Yellow	Stripped	Cream
2.	VRSM-10	India (Bihar)	RSP[2]	Globular	Green	White	No	Orange
3.	DR/KPS -10	India (U.P.)	RC[3]	Cylindrical	Dark green	Yellow	No	Cream
4.	EC-424817	U.S.A.	BEC	Globular	Dark green	Brown Red	Stripped	Cream
5.	VRSM-25	India (U.P.)	BEC	Oval	Dark green	Brown red	Streaked	Cream
6.	VRSM-73	India (Bihar)	BEC	Globular	Green	Orange	Stripped	White
7.	DR/KPS-45	India (U.P.)	LC[4]	Elongate	Light green	Cream	No	White
8.	VRSM-21	India (Bihar)	BEC	Oblong	Light green	Yellow	No	Cream
9.	EC-428160	U.S.A.	Absent	Oval	Green	Yellow	No	White
10	EC-424820	U.S.A.	BEC	Oval	Dark green	Brown Red	Speckled	Orange
11	EC-428452	U.S.A.	Absent	Oblong	Dark green	Yellow	No	Cream
12	VRSM-60	India (Bihar)	LC	Elongate	Light green	Yellow	No	Orange
13	DR/KPS-21	India (U.P.)	RSP	Elongate	Light green	Yellow	Stripped	Cream
14	EC-428423	U.S.A.	Absent	Oval	Green	Brown	Streaked	White
15	EC-428157	U.S.A.	Absent	Elongate	Light green	Yellow	No	Cream
16	DR/KPS-24	India (U.P.)	LC	Elongate	Dark green	Cream	No	Cream
17	VRSM-58	India (Bihar)	RSP	Elongate	Green	Orange	No	Cream
18	VRSM-41	India (Bihar)	LC	Elongate	Dark green	Green/Red	Speckled	Cream
19	VRSM-32	India (Bihar)	RSP	Elongate	Dark green	Yellow	No	White
20	DR/KPS-48	India (U.P.)	Absent	Elongate	Green	Yellow	No	Cream
21	DR/KPS-08	India (U.P.)	Absent	Elongate	Green	Yellow	No	Cream
22	DR/KPS-39	India (U.P.)	LC	Elongate	Light green	Cream	No	Orange

*Fruit cracking and skin peeling pattern: [1]BEC, blossom end cracking; [2]RSP, random skin peeling; [3]RC, random cracking, [4]LC, longitudinal cracking.

Fig 1. Representative snapmelon (*Cucumis melo* var. *momordica*) accessions showing different fruit characteristics.

(PCA and PCoA) in combination with cluster analysis, especially when the first two or three PCs explain >25% of the original variation (Messmer et al., 1993). In the case of 63 *C. melo* genotypes, PCA on pooled data from several molecular markers (RAPD, ISSR and SRAP) and UPGMA classifications resolved the snapmelon genotypes according to their end-uses (Yildiz et al., 2011). Therefore, it is suggested that both UPGMA and PCA should be performed for genetic diversity analyses. In general, the results obtained from PCA were in agreement with UPGMA clustering (Staub et al., 2004; Sensoy et al., 2007; Lopez-Sese et al., 2002).

The genetic variability in the melons found in secondary centres of diversity like eastern Asia, and western Mediterranean area (Monforte et al., 2003), and distal parts of primary centre of diversity like Turkey (Sensoy et al., 2007) is continuously decreasing. Performance-driven selection and grower/consumer preferences create a narrow germplasm base, which is evident by the dominance of only a few melon cultivars worldwide (Duvick, 2005). This has resulted in high genetic uniformity predisposing the crop to biotic and abiotic stresses and restricting the long-term yield improvements. This narrowing of the melon genetic base is underway in India as well, which was also confirmed in present study. Some of the Indian snapmelon accessions have been used as a reference accession due to possessing resistance to downy (e.g., PI124111 by Harwood and Markarian, 1968) and powdery mildew (e.g., PI79376 by Thomas et al., 1988), Fusarium wilt, and Zuccini yellow mosaic virus (PI124112, PI134192, and PI414723 by Pitrat et al., 2000). These genetic stocks have been utilised by breeders worldwide to develop disease resistant lines in others melons. Several other Indian snapmelon accessions have been used for developing mapping populations, and establishing phylogenetic relationships with other melons (Monforte et al., 2003).

Extensive collection, evaluation, and preservation of Indian snapmelon accessions is vital to prevent further genetic erosion in this primary centre of melon diversity. In a previous study, a high degree of variability was reported among the 36 accessions from Punjab, Rajasthan and Haryana states of India using RAPD and morphological data (Dhillon et al., 2007). However, the reproducibility of RAPD markers has often been questioned. Present analysis using ISSR markers revealed a narrow diversity in snapmelon accessions from U.P. and Bihar states of India. Development of melon varieties for higher yield and T.S.S. (total soluble solids) has resulted to improved productivity and fruit quality. Such developments might also result to narrowing of genetic diversity as observed in this study. In this context, variation for agri-horticultural traits among the relatives of melon (C. *melo* var. *momordica*) needs to be analysed and conserved, which may ultimately reduce the risk of genetic erosion. Previously, ISSR markers, e.g., in ash gourd (Verma et al., 2007), bitter gourd (Behera et al., 2008) and water melon (Solmaz et al., 2010) have been applied to assess genetic diversity. ISSRs have proved their greater potential to determine intra- and inter-genomic diversity compared to RAPDs (Zietkiwwicz et al., 1994). As far as the number of markers is concerned, Staub et al. (1997) suggested that in the case of melons, 35 should be the minimum number of markers and 80 could be an adequate number of markers for genetic studies. In this context, the number of ISSR markers analysed in this study was sufficient. The ISSR marker analyses revealed a narrow genetic diversity among the snapmelon accessions, although they possess a great diversity in terms of fruit and fruit-related characters. Four different fruit cracking and peeling patterns were recorded within Indian snapmelon accessions, and different fruit shapes and colour patterns, and flesh colour exists in these accessions.

Table 2. Sequence and details of amplification profile of 20 ISSR primers that generated polymorphic products among the 22 snapmelon accessions.

Primer	Sequence (5'-3')	Number of amplified products	Number of polymorphic products	Product size range (bp)	PIC value
UBC-808	$(AG)_8C$	8	3	470-1100	0.31
UBC-809	$(AG)_8G$	8	4	325-850	0.50
UBC -810	$(GA)_8T$	8	4	500-1100	0.49
UBC -811	$(GA)_8C$	8	4	500-1150	0.50
UBC -812	$(GA)_8A$	8	3	475-1600	0.42
UBC-825	$(AC)_8T$	4	4	420-950	0.59
UBC-826	$(AC)_8C$	5	3	400-1000	0.51
UBC-828	$(TG)_8A$	6	3	700-1350	0.27
UBC -834	$(AG)_8TT$	7	3	320-1200	0.31
UBC -834	$(AG)_8TC$	8	3	600-1550	0.22
UBC -836	$(AG)_8TA$	8	4	300-1250	0.71
UBC -840	$(GA)_8CT$	4	2	600-1270	0.31
UBC-843	$(GA)_8TC$	4	2	850-1500	0.27
UBC-843	$(GA)_8CC$	6	2	300-2100	0.19
UBC -846	$(CA)_8GT$	5	4	350-1800	0.70
UBC-848	$(CA)_8GG$	6	6	375-1400	0.79
UBC-848	$(CA)_8AG$	5	5	350-1100	0.56
UBC-855	$(AC)_8TT$	5	5	480-1550	0.75
UBC-855	$(AC)_8CT$	7	6	500-1500	0.61
UBC-857	$(AC)_8CG$	7	5	500-1100	0.55

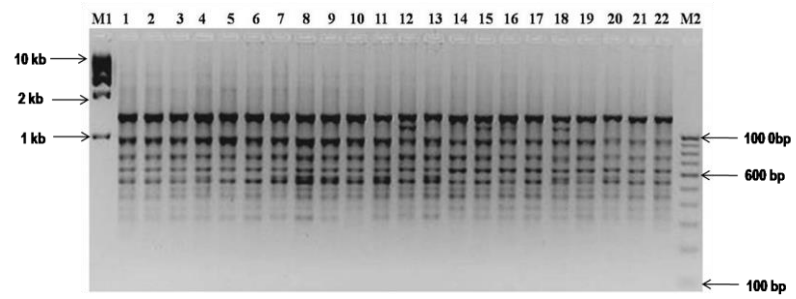

Fig 2. Agarose gel image showing amplification profile generated by ISSR primer UBC-834 [$(AG)_8TC$] with 22 snapmelon accessions: lanes 1-22 correspond to the snapmelon accessions listed in Table 1; lane M1 is 1 kb DNA size marker, and M2 is 100 bp DNA size marker.

Table 3. A summary of the amplification product profile generated by ISSR primers.

Particular	ISSR
Total number of primers used in screening	32
Number of primers failed to produce any amplification product	09
Number of primers that produced monomorphic amplification products	03
Number of primers that produced polymorphic amplification products	20
Total number of amplification products generated by the primers	127
Total number of polymorphic products	74
Average number of polymorphic products per primer	3.7
Percentage of polymorphic products	58.3
Size range (bp) of the polymorphic fragments	300-2100
Average polymorphic information content (PIC) of markers	0.48

This study could be a start of further investigations using more powerful marker system like sequence related amplified polymorphism (SRAP). The information generated could be utilized in germplasm management activities, and to enrich genetically diverse snapmelon accessions, or to develop a core collection to strengthen the breeding programmes. Introduction of unique genetic variability from Indian germplasm to the secondary centres of diversity will increase the genetic variability for solving the current and future breeding problems and introduce new traits into other popular melon cultivars.

Materials and Methods

Plant materials

A total of 22 snapmelon accessions including 16 Indian accessions (collected from Uttar Pradesh and Bihar states) and six exotic collections from USA were selected on the basis of fruit related morphological traits (Table 1). All the genotypes were grown at the research farm of Indian Institute of Vegetable Research (IIVR), Varanasi and fruit related morphological data were recorded on fruit cracking and peel-

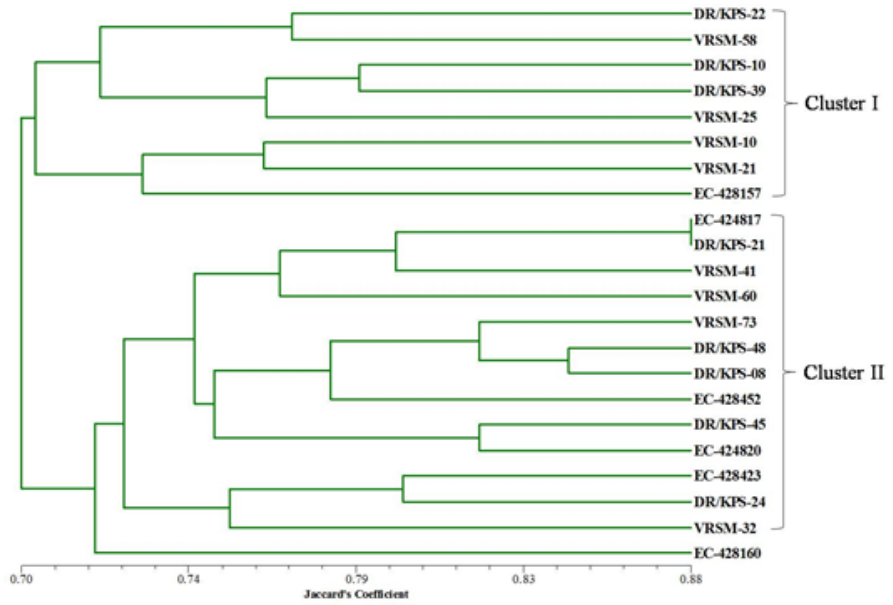

Fig 3. UPGMA Dendrogram of 22 snapmelon accessions using UPGMA cluster analysis based on Jaccard's similarity coefficient. Cluster I comprised of 8 accessions and cluster II consisted of 13 accessions.

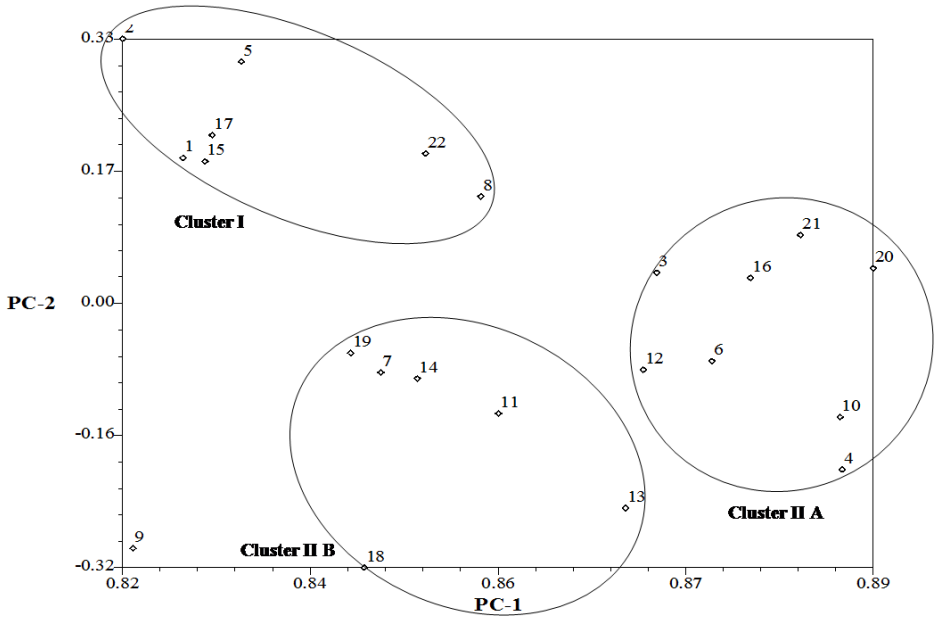

Fig 4. Two-dimensional plot of principal components 1 and 2 based on data from ISSR markers. Numbers 1-22 correspond to the snapmelon accession as listed in Table 1. Cluster I consisted of 8 accessions while cluster II is subdivided into two subclusters. Cluster II A and II B comprised with 8 and 6 accessions, respectively.

ing patterns, shape, predominant peel colour, design due to secondary colour on peel, and flesh colour.

ISSR analysis

Seeds of each snapmelon accession were germinated aseptically on semisolid water-agar (1% agar), and ~100 mg (fresh weight) leaf sample was taken for genomic DNA extraction, using DNA extraction kit (Qiagen, Germany) as per the manufacturer's procedure. DNA quality was ascertained by gel electrophoresis in 0.8% agarose and its quantity was determined using a NanoDrop spectrophotometer (ND1000, Nano Drop Technologies, Wilmington, Denmark). ISSR markers were synthesized from Operon (Operon Technologies, California, USA). The details of markers which produced polymorphic products are presented in Table 2. Amplification reaction was carried out in a 25 µL reaction volume containing 25 ng genomic DNA, 2.5 µL PCR buffer (MBI Fermentas, Hanover, USA), 1.5 mM $MgCl_2$, 200 µM dNTPs (Bangalore Genei, Bangalore, India), 1.25 U *Taq* DNA polymerase (MBI Fermentas), and 1.25 µM marker using a thermal cycler (Touchgene Gradient, Techne, UK). The first amplification cycle consisted of denaturation at 94°C, annealing at 55°C for 30 sec, and extension at 72°C. The duration of denaturation step was 5 min in the first cycle, and 30 sec in the remaining 39 cycles.

Similarly, extension period was 1 min in the first 39 cycles, and 5 min in the final cycle. The amplified DNA fragments were resolved by electrophoresis in 1.5% agarose gel prepared in TBE buffer [54.0 g Tris-base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0) in 1000 ml volume] and visualized in a gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, California, USA). The 1 kb and 100 bp DNA ladders (MBI Fermentas) were used as molecular size markers. The amplification was repeated twice to ensure that the amplification obtained with the markers was reproducible and consistent.

Statistical analyses

ISSR markers generated clear and unambiguous amplification products of various sizes that were scored for their presence (1) and absence (0) among the genotypes and the binary data matrix was analysed using NTSYS-pc ver. 2.11W (Rohlf, 1997). The SIMQUAL program was used to calculate the Jaccard's coefficient (Jaccard, 1908). A dendrogram was constructed using the UPGMA cluster algorithm using SAHN module of the software. Polymorphic information content (PIC) for molecular markers was calculated as per the formula, $PIC_i = 1- \Sigma P_{ij}^2$, where PIC_i is the polymorphic information content of marker *i*; P*ij* is the frequency of the j^{th} pattern for marker *i* and the summation extends over *n* patterns. Principal component analysis (PCA) was also done to check the results of UPGMA-based clustering using EIGEN module of NTSYSpc.

Conclusion

Twenty-two Snapmelon (*Cucumis melo* var. *momordica*) accessions belonging to India and USA were evaluated using 20 polymorphic ISSR markers to find the genetic diversity. Pair-wise Jaccord's similarity coefficient ranged from 0.59 to 0.88, revealing a narrow diversity in the studied samples owing to dominant nature of the ISSR markers. UPGMA based dendrogram grouped these 22 snapmelon accessions into two main clusters, consisting of 8 and 14 accessions, respectively. UPGMA clustering was also supported by principal components analysis (PCA). The first three principal components together contributed for 48.7% variation among the 22 accessions. The present study could provide useful information for management of Indian snapmelon germplasm, and to enrich genetically diverse snapmelon accessions, or to develop a core collection to strengthen the breeding programmes.

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