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Genetic diversity analysis of cucumber (*Cucumis sativus* L.) genotypes based on ISSR markers

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Abstract
Molecular characterization and genetic diversity among fourteen cucumber genotypes were carried out by using eleven Inter Simple Sequence Repeat (ISSR) markers. All the eleven ISSR markers used were found polymorphic having 97.72% polymorphism among fourteen cucumber genotypes comprising pathenocarpic, gynoecious and monoecious cucumbers. These eleven polymorphic markers produced a total of 69 alleles, of which 4 were unique alleles. The allele number for each ISSR locus varied between 4 to 10 with an average of 6.27 alleles per marker. Polymorphic information content values of ISSRs ranged from 0.43 to 0.84 with an average of 0.68. Jaccard's similarity coefficient was employed to study the molecular diversity of 14 cucumber genotypes. The pair wise genetic similarity among 14 cucumber genotypes varied from 0.36 to 0.83. The dendrogram constructed based on genetic similarities among 14 cucumber genotypes identified two major clusters. The four major parthenocarpic genotypes *viz.*, PCUCP-2, PCUCP-4, PCUCP-6 and PCUCP-8 having high genetic resemblance (80-83%) and two major gynoecious genotypes *viz.*, PGYC-3 and PGYC-2 with 68% similarity value were grouped in a single cluster and three monoecious genotypes *viz*., PCUC-8, PCUC-25 and Pant Khira-1 were grouped in another cluster with 76-78% similarity value. This study provides information about diversity among pathenocarpic, gynoecious and monoecious cucumber genotypes and remains helpful in future exploration and utilization of diverse germplasm for developing cultivars and hybrids.

Keywords: Cucumber, *Cucumis* spp, genetic diversity, ISSR markers, morphological

Introduction
Cucumber (*Cucumis sativus* L., 2n=2x=14) is one of the most important member of the family *cucurbitaceae* including several crops of economic importance. About 30 species of cucumber are distributed across the southeast Himalayas with basic chromosome number 7 and across Africa with basic chromosome number 12. Cucumber is a unique crop and many important features of cultivated crops are not associated with discrete mendelian traits, but are of a continuous or quantitative nature. Mostly cucumber is monoceious in nature. But, Parthenocarpy has long been known to occur within the species of *Cucumis sativus* L. (Sturtevant, 1890) [22]. Parthenocarpy is regarded as the ability to develop fruits without pollination. Fruits with developing seeds inhibit the growth of later fruits, however, to a lesser extent if fruits are grown parthenocarpically (Denna, 1973) [5] Gynoecious varieties have mostly or only female flowers those need to be pollinated by male flowers for fruit set. India, the native place of cucumber, possesses vast genetic variability for vegetative and fruit characters, but the genetic diversity of the species in India is relatively unexplored (Sebastian *et al*., 2010; Tiwari, 2015; Naegele and Wehner, 2016) [29, 23, 14]. Assessment of genetic diversity based on phenotypes has certain limitations, since most of the morphological characters are greatly influenced by developmental stage of the plant and certain environmental factors (De Ponti, 1976; Golabadi *et al*., 2012) [4, 8]. Along with the traditional method of genetic studies of the crop, DNA markers are becoming more popular and effective to study the genetic diversity and choosing right parents. DNA markers have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity (Solanki and Seth, 1980; Huang *et al*., 2009) [21, 10]. Advent of polymerase chain reaction (PCR) has revolutionized the DNA based diagnostics. Inter single sequence repeat or ISSR is a PCR based DNA diagnostic assay (Danin-Poleg *et al.*, 2000) [3]. It involves PCR amplification of genomic DNA segments with a primer of arbitrary sequence followed by Agarose Gel Electrophoresis. The main advantage of the technique lies in the unlimited polymorphism, it is able to detect even among closely related genotypes (Singh *et al*., 2016) [20].
To date, the degree of genetic diversity in cucumber has been assessed with a number of DNA markers (Zhuang *et al*., 2008) [24] which provide useful information for cucumber cross

breeding. Molecular markers such as RAPD and SSRs have been employed for determination of genetic diversity in African cucumber (*Cucumis sativus* L.) (Mliki *et al.*, 2003) [13]. There are many approaches used to quantify the diversity at intra as well as interspecies level, however, molecular markers are considered to have enormous potential to explore genetic diversity by detecting polymorphisms at DNA level. ISSR (Inter simple sequence repeat) is a type of molecular marker, proposed by Zietkiewicz *et al*., 1994 [25] for finger printing. ISSR is one of the simplest and widely used techniques, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. Though ISSR markers are dominant like RAPD, they are more stable and reproducible. Because of these properties ISSR markers have recently been found using extensively for finger printing, pohylogenetic analysis, population structure analysis, varietal/line identification, genetic mapping, marker-assisted selection, etc. Keeping this in view, the present study was formulated to understand the molecular diversity among the *Cucumis* genotypes.

Materials and Methods

In the present study Fourteen genotypes of cucumber genotypes consisting of eight parthenocarpic cucumber lines *viz*., PCUCP-1, PCUCP-2, PCUCP-3, PCUCP-4, PCUCP-5, PCUCP-6, PCUCP-7, PCUCP-8 and three gynoecious cucumber lines *viz.,* PGYC-1, PGYC-2, PGYC-3 and three monoecious cucumber lines *viz.*,PCUC-8, PCUC-25, and Pant Khira-1 were grown under greenhouse conditions in G. B. Pant University of Agriculture and Technology, Pantnagar (Table 1). Fresh and young leaves were collected from each genotype. The leaves were cut into small bits and ground to powder using liquid nitrogen. Genomic DNA was then isolated using modified CTAB method, (Doyle and Doyle (1987) [7]. Eleven ISSR primers were used for PCR analysis to detect polymorphism among the *Cucumis* spp. (Table 2). Different parameters were tested to determine optimal concentrations of template DNA, $MgCl_2$, dNTPs, *Taq* DNA polymerase, primer and time intervals during denaturation, annealing and elongation steps which affect amplification, banding pattern and reproducibility. Reproducible and clear banding patterns were obtained in a reaction mixture of 20 l containing 1x reaction buffer, 1 unit of *Taq* DNA polymerase, 200 µM each of dNTPs mix, 0.5 µM/reaction of primer's and 50 ng of template DNA. The Polymerase Chain Reaction was performed in the following cycling parameters: an initial denaturation (94 ^{0}C) for 5 minutes, denaturation (95 ^{0}C) for 1 minutes, Primer annealing (28.9 ^{0}C-55.5 ^{0}C) for 1 minute, Primer Extension (72 ^{0}C) for 2 minutes (40 cycles), followed by final primer extension (72 ^{0}C) for 5 minutes and a hold temperature of 4 ºC.

After PCR reaction, the amplified products were separated on 1.2% agarose gel in 1x TAE buffer using ethidium bromide (EtBr) staining dye. Standard markers of 100 bp and 1 kb DNA ladders (Bangalore Genie, India) were used to determine the size of the amplified DNA fragments. DNA fragments were visualized under UV-trans-illuminator and photographed using gel documentation system. On the basis of presence of bands for each primer, scoring of amplicons obtained was done. Only clear and unambiguous bands were scored for banding pattern for each primer. Comparison of band position was done with molecular weight of standard DNA ladders. Accordingly, a rectangular binary matrix was obtained and statistical analysis was performed using the NTSYS pc version 2.02e (Rohlf, 1993) [18]. A pair wise similarity matrix was generated and the cluster analysis was performed via Un-weighted Pair Group Method with Arithmetic averages (UPGMA) to develop a dendrogram.

Results and Discussion

Eleven ISSR primers were used in the present investigation, and all eleven primers showed amplification. A total of 69 bands were generated of which 67 were found polymorphic *i.e.* 97.72% polymorphism. The total number of amplified bands ranged from 3 (UBC-807) to 10 (UBC-813) with an average of 6.27 per primer (Table 3). The polymorphism percentage ranged from as low as 75.00% (UBC-834) to as high as 100% (UBC-842, UBC- 815, UBC-812, UBC-807, UBC-840, UBC-825, UBC-813, UBC-835, UBC-80, and UBC-858) and the average polymorphism was 97.72% (Table 3). Polymorphic information content values of ISSRs ranged from 0.43 to 0.84 with an average of 0.68. Parvathaneni *et al*. (2011) [17] observed that 15 ISSR primers generated 109 polymorphic alleles with 87.20% polymorphism in cucumber and the average number of ISSR alleles generated was 8.3 per primer. Innark *et al.* (2014) [11] recorded polymorphic information content (PIC) ranged from 0.12 to 0.45 with mean of 0.25. DNA banding profile of individual plant consists of 14 genotypes of cucumber along and its amplification with ISSR primers are depicted in Fig.1, 2, 3 & 4. Four unique bands were detected in four genotypes *viz.*, PGYC-2, PCUC-25, PCUCP-3 and PCUCP-5 with 4 ISSR primers v*iz*., UBC-815 with PGYC-2; UBC-858 with PCUC-25; UBC-812 with PCUCP-3 and UBC-840 with PCUCP-5. Presence of unique alleles may serve as indicators of genome specificity to a particular region, of a particular trait of horticultural importance. The genotypes carrying unique alleles may prove useful for introducing diversity in the future cucumber breeding programme. Therefore, the presence of unique band with specific genotype could be helpful in identification of specific trait of importance in cucumber. All these 4 genotypes gave single number of distinct bands. The size of these unique bands ranged from 200-1800 bp. The data obtained by using ISSR markers were further used to construct similarity matrix using 'Simqual' sub-programme of software NTSYS-pc. Based on ISSR similarity matrix data, the values of similarity coefficient ranged from 0.36 to 0.83 *i.e.* 36-83% or genetic diversity ranged from 17-64% (Table 4).The average similarity coefficient across all the genotypes was 0.59, showing that the genotypes were highly diverse from each other. Manohar *et al*. (2013) [12] revealed the average number of polymorphic bands was of 3.58 per primer and the Jaccard's similarity coefficient ranged from 0.36-0.84 in cucumber. In the present study, maximum similarity value of 0.83 was observed between genotypes of PCUCP-8 and PCUCP-4 followed by PCUCP-6 and PCUCP-2 & PGYC-2 and PCUCP-4 with a similarity coefficient of 0.80 and 0.79 respectively. Genotypes PGYC-2 and PCUCP-5 were found to be genetically diverse with a minimum similarity value of 0.36 followed by PCUCP-5 and PCUCP-7, PCUCP-5 and PCUCP-4, PCUCP-5 and PCUCP-3 having similarity values of 0.41, 0.42 and 0.45 respectively. Pandey *et al.* (2013) [16] and Normohamadi *et al.* (2017) [15] revealed high genetic variability with Jaccard's similarity coefficient ranged between 0.25-0.85 and 0.51-0.92 respectively in the study of genetic diversity in cucumber.

The ISSR cluster tree analysis showed that they could be divided into 2 major groups at a similarity coefficient of 0.51 (Fig. 4). The group A included 13 genotypes *viz.*, PCUCP-1,

PCUCP-2, PCUCP-3, PCUCP-4, PCUCP-6, PCUCP-7, PCUCP-8, PGYC-1, PGYC-2, PGYC-3, PCUC-8, PCUC-25 and Pant Khira-1 and group B included 1 genotype *viz*. PCUCP-5.Group B bifurcated into two Clusters *viz*., Cluster 1 and Cluster 2. Cluster 1 consisted of Cluster 1A and Cluster 1B with similarity coefficient of 0.67. Cluster 1A was again forked into two Clusters with similarity coefficient of 0.73, comprising of genotypes PGYC-1, PCUCP-7 and Pant khira-1. It was believed that PCUCP-7 and Pant Khira-1 were having common ancestor and both were related at 0.78 similarity coefficient. So, it can be assumed that PGYC-1 is distantly related to PCUCP-7 and Pant Khira-1. Similarly, Cluster 1B was further bifurcated into one Cluster at 0.76 similarity coefficient consisting of genotypes PCUC-8 and PCUC-25 and believed that both PCUC-8 and PCUC-25 were having one common ancestor (Fig. 5). Cluster 2 was further subdivided into Cluster 2A and Cluster 2B with 0.66 similarity coefficient. Cluster 2A forked into two Clusters comprised of genotypes PGYC-3 in one Cluster and PCUCP-3, PGYC-2, PCUCP-4, PCUCP-8 in another Cluster. Genotypes PCUCP-4 and PCUCP-8 were related at 0.83 similarity coefficient with each other having one common ancestor. Similarly genotypes PCUCP-3 and PGYC-2 both were having one common ancestor with 0.78 similarity coefficient (Fig. 4). Cluster 2B subdivided into two Clusters with 0.69 similarity coefficient comprised of genotypes PCUCP-1 in one cluster and PCUCP-2, PCUCP-6 in another cluster. Both PCUCP-2 and PCUCP-6 were having one common ancestor with similarity coefficient. 0.80. So, it could be said that these above two genotypes were closely related with each other. It indicates wide molecular diversity present in cucumber genotypes. The present findings are in consistence with the earlier reports (Dijkhuizen *et al*., 1996; Horejsi *et al*., 1999; Choudhary *et al*., 2011) [6, 9, 2]. All workers found high genetic diversity among cucumber germplasms. Bisht *et al.* (2004) [1] reported high diversity for RAPD markers in 29 accessions of cucumber collected from different parts of India.

The marker analysis discriminated between 8 parthenocarpic, 3 gynoecious and 3 monoecious genotypes of cucumber. Now, it is clear from the dendrogram (Fig. 5) that broadly all genotypes were grouped into 2 clusters. Here, 6 parthenocarpic cucumber genotypes (PCUCP-1, PCUCP-2, PCUCP-3, PCUCP-4, PCUCP-6, PCUCP-8) and 2 gynoecious genotypes (PGYC-2, PGYC-3) were grouped into one cluster and all monoecious cucumber genotypes (PCUC-8, PCUC-25 and Pant Khira-1) were grouped in another cluster (Fig. 5).This implies that monoecious and gynoecious genotypes have distinct genetic constitution. As there was wide diversity observed between genotype PCUCP-5 and PGYC-2 (64%) and PCUC-5 and PCUCP-7 (59%) these may play certain important role in carry out new combinations. Now, it has been observed new unique bands or alleles in genotypes like PGYC-2, PCUCP-3, PCUCP-5 and PUCU-25 which can explore new dimensions in cucumber breeding bringing new hybrid combinations of suitable horticultural trait. The study has confirmed, ISSR markers are rapid, reliable, simple and effective method of detecting polymorphism for assessment of genetic diversity among cucumber genotypes. Assessment of genetic variability in cucumber genotypes through ISSR markers can be done better as compared to morphological as well as isozyme and RAPD markers

Table 1: List of cucumber genotypes used for diversity analysis

Sl. No.	Genotypes	Nature	Source
1.	PCUCP-1	Parthenocarpic	Pantnagar
2.	PCUCP-2(Pant Parthenocarpic Cucumber-2)	Parthenocarpic	Pantnagar
3.	PCUCP-3(Pant Parthenocarpic Cucumber-3)	Parthenocarpic	Pantnagar
4.	PCUCP-4	Parthenocarpic	Pantnagar
5	PCUCP-5	Parthenocarpic	Pantnagar
6	PCUCP-6	Parthenocarpic	Pantnagar
7	PCUCP-7	Parthenocarpic	Pantnagar
8	PCUCP-8	Parthenocarpic	Pantnagar
9	PGYC-1	Gynoecious	Pantnagar
10	PGYC-2	Gynoecious	Pantnagar
11	PGYC-3	Gynoecious	Pantnagar
12	PCUC-8	Monoecious	Pantnagar
13	PCUC-25	Monoecious	Pantnagar
14	Pant Khira-1	Monoecious	Pantnagar

Table 2: Characteristics of ISSR Primers

Sl. No.	Oligo reference code	Sequence 5'-3'	GC content (%)	TM (°C)
1	UBC-842	GAGAGAGAGAGAGAGAYG	52.8	48.8
2	UBC-834	AGAGAGAGAGAGAGAGYT	47.2	49.2
3	UBC-815	CTCTCTCTCTCTCTCTG	52.9	46.8
4	UBC-812	GAGAGAGAGAGAGAGAA	47.1	45.7
5	UBC-807	AGAGAGAGAGAGAGAGT	47.1	47.0
6	UBC-840	GAGAGAGAGAGAGAGAYT	47.2	47.4
7	UBC-825	ACACACACACACACACT	47.1	51.4
8	UBC-813	CTCTCTCTCTCTCTCTT	47.1	45.7
9	UBC-808	AGAGAGAGAGAGAGAGC	52.9	48.8
10	UBC-835	AGAGAGAGAGAGAGAGYC	52.8	50.2
11	UBC-858	TGTGTGTGTGTGTGTGRT	47.2	53.1

Table 3: Level of polymorphism revealed by ISSR primers in 14 genotypes of cucumber

Sl. No.	Primer	No. of amplified alleles	Polymorphic band(s)	Monomorphic band (s)	Percent (%) polymorphism	PIC value
1.	UBC-842	6	6	0	100.00	0.81
2.	UBC-834	8	6	2	75.00	0.84
3.	UBC-815	7	7	0	100.00	0.52
4.	UBC-812	9	9	0	100.00	0.79
5.	UBC-807	3	3	0	100.00	0.59
6.	UBC-840	8	8	0	100.00	0.84
7.	UBC-825	4	4	0	100.00	0.69
8.	UBC-813	10	10	0	100.00	0.80
9.	UBC-808	5	5	0	100.00	0.43
10.	UBC-835	5	5	0	100.00	0.61
11.	UBC-858	4	4	0	100.00	0.52
Total		69	67	2	97.72	0.68

Table 4: Pair wise Jaccard's similarity coefficient among 14 genotypes of parthenocarpic, gynoecious and monoecious cucumber

Genotypes	PCUCP-1	PCUCP-2	PCUCP-3	PCUCP-4	PCUCP-5	PCUCP-6	PCUCP-7	PCUCP-8	PGYC-1	PGYC-2	PGYC-3	PCUC-8	PCUC-25	Pant Khira-1
PCUCP-1	1.00													
PCUCP-2	0.76	1.00												
PCUCP-3	0.62	0.65	1.00											
PCUCP-4	0.71	0.65	0.76	1.00										
PCUCP-5	0.56	0.56	0.45	0.42	1.00									
PCUCP-6	0.65	0.80	0.64	0.55	0.70	1.00								
PCUCP-7	0.55	0.61	0.50	0.62	0.41	0.59	1.00							
PCUCP-8	0.73	0.73	0.77	0.83	0.50	0.71	0.61	1.00						
PGYC-1	0.67	0.61	0.53	0.74	0.47	0.53	0.70	0.76	1.00					
PGYC-2	0.53	0.65	0.79	0.79	0.36	0.58	0.62	0.77	0.62	1.00				
PGYC-3	0.64	0.67	0.71	0.62	0.62	0.71	0.58	0.79	0.73	0.68	1.00			
PCUC-8	0.65	0.77	0.55	0.64	0.58	0.73	0.68	0.62	0.62	0.61	0.65	1.00		
PCUC-25	0.50	0.59	0.58	0.64	0.48	0.58	0.65	0.53	0.56	0.67	0.50	0.76	1.00	
Pant Khira-1	0.64	0.76	0.62	0.74	0.53	0.71	0.79	0.79	0.76	0.74	0.76	0.77	0.74	1.00

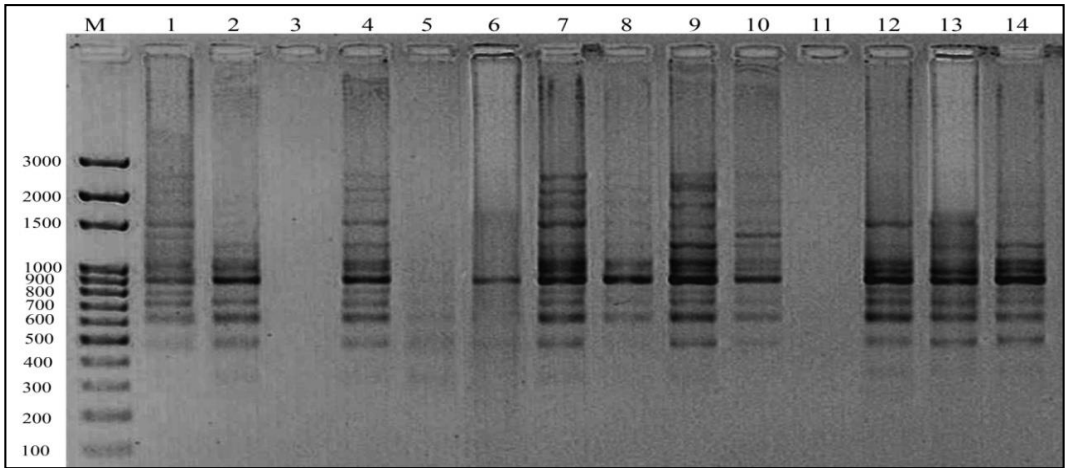

Fig 1: ISSR profile of parthenocarpic, gynoecious and monoecious cucumber genotypes with primer UBC-813

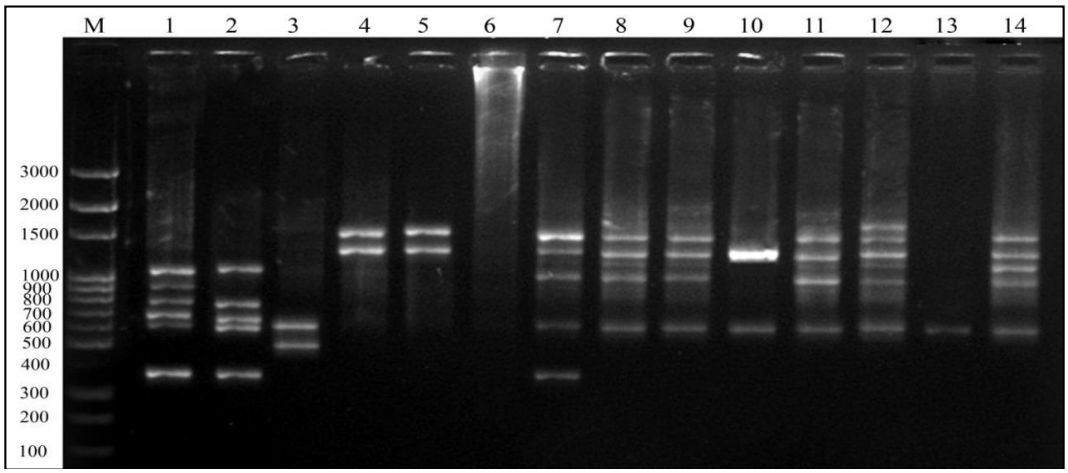

Fig 2: ISSR profile of parthenocarpic, gynoecious and monoecious cucumber genotypes with primer UBC-812

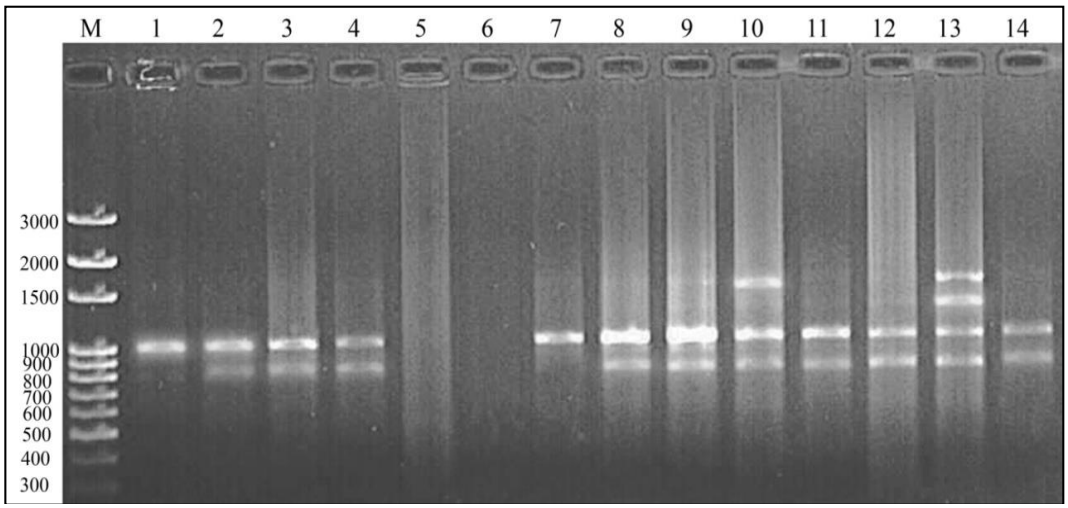

Fig 3: ISSR profile of parthenocarpic, gynoecious and monoecious cucumber genotypes with primer UBC-858

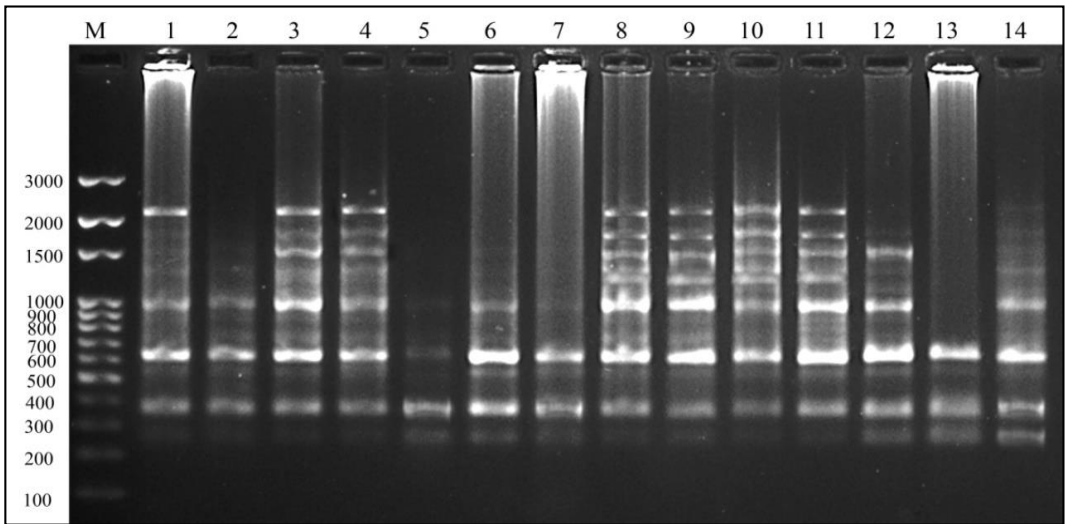

Fig 4: ISSR profile of parthenocarpic, gynoecious and monoecious cucumber genotypes with primer UBC-834

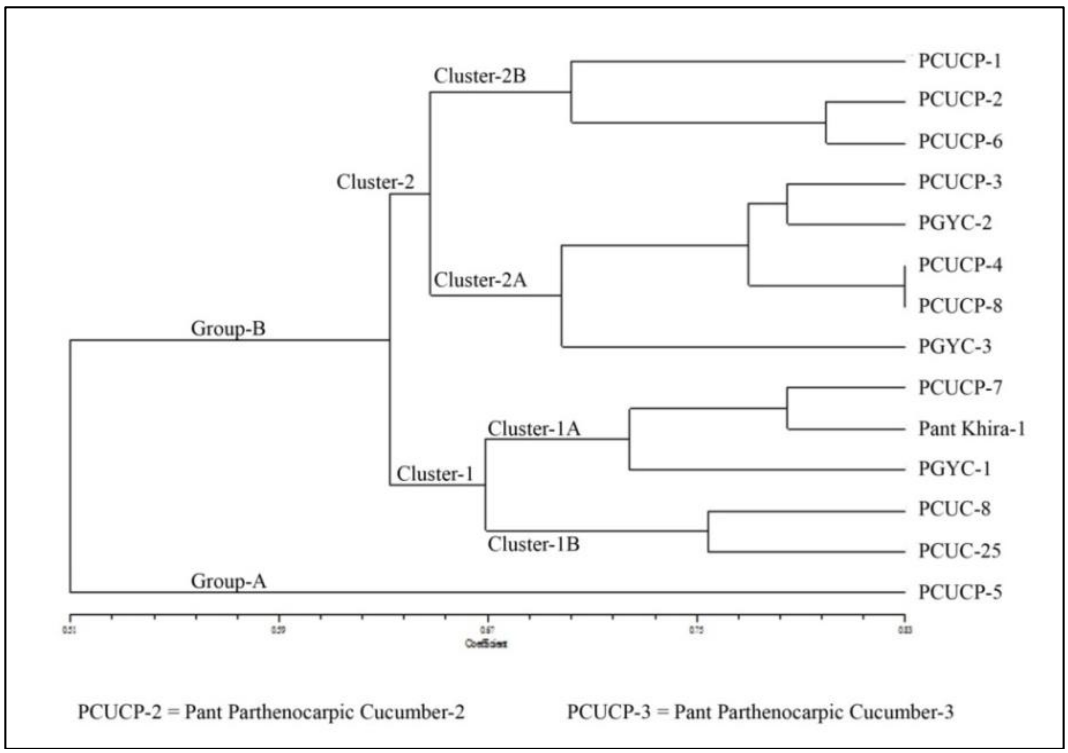

Fig 5: Dendrogram of 14 cucumber genotypes based on morphological data

Conclusion

All the eleven ISSR markers used were found polymorphic having 97.72% polymorphism, produced a total of 69 alleles, of which 4 were unique alleles. The allele number for each ISSR locus varied between 4 to 10 with an average of 6.27 alleles per marker. Polymorphic information content values of ISSRs ranged from 0.43 to 0.84 with an average of 0.68. Jaccard's similarity coefficient was employed to study the molecular diversity of 14 cucumber genotypes. The pair wise genetic similarity among 14 cucumber genotypes varied from 0.36 to 0.83.Hence, it can be concluded that PCUCP-4 and PCUCP-8 are most closely related (0.83) whereas PCUCP-5 and PGYC-2 are most diverse (0.36) genotypes found in the above study. The four major parthenocarpic genotypes *viz.*, PCUCP-2, PCUCP-4, PCUCP-6 and PCUCP-8 having high genetic resemblance (80-83%) and two major gynoecious genotypes *viz.*, PGYC-3 and PGYC-2 with 68% similarity value were grouped in a single cluster and three monoecious genotypes *viz*., PCUC-8, PCUC-25 and Pant Khira-1 were grouped in another cluster with a value of 76-78% similarity. The studied genotypes were characterized for diversity analysis of parthenocarpic, gynoecious and monoecious cucumbers. Hence, it can be useful for advanced cucumber breeding programme in future.

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