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Morphological and molecular diversity analysis in bitter gourd

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Abstract

A study was carried out to assess the morphological and molecular diversity in bitter gourd by using twenty genotypes. Multivariate analysis based on morphological traits grouped all the genotypes into 6 major clusters. Cluster I was having 8 genotypes and cluster II was having seven genotypes out of total genotypes used in this study. The other clusters were monogenotypic. The highest inter cluster distance was found between clusters V and VI. Moreover, cluster V was found superior on the basis of cluster means for maximum yield related traits. For molecular analysis, out of 30 SSR markers used, 6 were found to be polymorphic. PIC values varied from 0.22 to 0.94. From molecular characterization, two major clusters were formed in which economic genotypes were grouped into sub-cluster II A. The genotypes namely, CO-1, BRBT-1, Katahi and Jhalari were found to be in the same cluster in both multivariate analysis and molecular characterization.

Keywords: Bitter gourd, SSR, PIC, morphological marker, molecular marker

Abbreviations: PIC: Polymorphic Information Content, SSR: Simple Sequence Repeat

1. Introduction

Bitter gourd (*Momordica charantia* L.) is an important cucurbit vegetable having immense medicinal and nutritional properties. It is widely cultivated in India, China, Malaysia, Africa and South America. The primary centre of origin is probably India, while secondary centre considered to be China [1]. *Momordica charantia* var. *abbreviata* is considered as the progenitor of present cultivated bitter gourd (*Momordica charantia* L.). Bitterness of fruit is due to Momordicin. Bitter gourd is grown as underexploited crop but having immense nutritional and medicinal properties. It contains various chemicals (charantin, vicine, glycosides, polypeptide p plant insulin) which have hypoglycemic activity. It is known to have anti-lipolytic, anti-inflammatory and astringent properties [2]. Bitter gourd is excellent source of vitamins B₁, B₂, B₃, Vitamin C, magnesium, folic acid, zinc, phosphorus, manganese, and has high dietary fibers. Fresh juice of leaves of bitter gourd is also a useful medicine in early stages of Cholera and other types of diarrhea [3].

Analysis of genetic diversity and relatedness between species and among genotypes is useful in plant breeding programs because it provides a tool for accurate organization of germplasm and efficient parent selection. Identification of genetic diversity based on phenotypic characters is very limiting as environmental factors and plant development stage will affect the morphological characters of plant. Molecular marker can be used in DNA polymorphism and are independent of environmental conditions which exhibits a high level of polymorphism. Previously genetic diversity analysis in bitter gourd was done. But no detailed molecular and morphological analysis of the accessions found in the Eastern and North eastern part of India is available. Diversity study based on morphological characters may not alone suffice because morphological characters are influenced by the environment. Nowadays, molecular methods are a common procedure for identifying and classifying genotypes which very well supports the classical methods (morphological traits). Bitter gourd. Microsatellite or simple sequence repeat (SSR) markers have gained considerable importance due to many desirable attributes like their multi-allelic nature, co-dominant transmission, extensive genome coverage, small amount of starting DNA, and ease of detection by polymerase chain reaction (PCR) [4]. SSRs are known to have high heterozygosity values and are more informative than dominant DNA markers. analyses, microsatellite markers provide accurate results with a minimum number of loci/alleles employed in the study and give way to evolutionary study [5].

2. Materials and Methods

2.1 Field evaluation

The experiment was conducted at the Vegetable Research Farm of the Department of Horticulture (Vegetable and Floriculture), Bihar Agricultural University, Sabour during Rabi season of 2019. The number of genotypes were used in this experiment were 20. Seeds were sown on both sides of the channel with a spacing of 2 m between channels and 0.5 m between hills. The fruits were harvested at marketable stage. Five plants were selected for the measurements after discarding the border plants at both ends. The present investigation was based on 18 quantitative characters as well. The observations were recorded on node to first male flower, node to first female flower, days to first male flower, days to first female flower, days to first fruit set, fruit length(cm), fruit girth(cm), flesh thickness(cm), number of seed at harvestable maturity, number of primary branches, intermodal length(cm), number of pickings, vine length(cm), average fruit weight(g), number of fruits per vine, fruit yield per vine(kg), total soluble solids (°brix) and ascorbic acid (mg/100g).

2.2 DNA extraction and PCR amplification

Genomic DNA from individual single plant was extracted from leaf of all 20 genotypes using CTAB method [6]. 100 mg leaf tissues were cut into tiny pieces, homogenized and digested with 2 ml DNA extraction buffer [CTAB], and 30µl of mercapthethanol. 750µl of leaf extracts was placed in waterbath at 65 °C for 45 minutes and was shaken at regular interval. Following incubation, chloroform and isoamyl alcohol (24:1) was added, mixed well and centrifuged for 5 minutes at 10,000 rpm. The supernatant was collected and 750 µl of isopropanol was added to the supernatant, mixed and centrifuged for 10 minutes at 10,000 rpm. Supernatant was discarded leaving a pellet at the bottom of the tube. The DNA pellet was washed with 70% chilled ethanol and air dried. The pellet was dissolved in 15 µl of 10:1 TE buffer. Quality and quantity of DNA sample was examined under visualization of 0.8% Agarose gel in UV spectrophotometer and it was further diluted to a uniform concentration of 50 ng/µl. The extracted DNA samples, along with the diluted samples were stored at -40°C freezer. Diluted DNA samples were subjected to PCR amplification, using the selected SSR primers in automated thermal cycler (Applied Biosystems model veriti). PCR reaction was carried out in 10µl reaction volume containing 1µl (100ng) of extracted genomic DNA, 1.2µl 10X PCR buffer, 2.5mM Mgcl, 0.1mM of dNTPs, 0.5µM of forward, 0.5µM of reverse primer and 0.35U of Taq DNA Polymerase. Template DNA was initially denatured at 94°C for 4 minutes followed by 35 cycles (30 sec denaturation at 94°C, 40 sec annealing at 52°C, 30 sec of primer extension at 72°C) of PCR amplification, and final extension of 72°C for 10 min followed by hold at 4°C. On completion of reaction 2 µl 6X gel loading buffer (Genei) was added. Agrose gel was prepared, for electrophoresis and then cooled and ethidium bromide (4µl/100 ml of TBE) was incorporated in it. imaged under gel documentation system(UV transilluminator).

2.3 Data analysis

The data were subjected to analysis of variance as per [7]. The

genetic diversity was analyzed by Mahalanobis D^2 analysis and genotypes were grouped into various cluster groupings following Tocher's method. UPGMA based cluster analysis was used to calculate Jaccard similarity coefficient between genotypes. The similarity matrix thus obtained was subjected to cluster analysis based on UPGMA and dendrogram was obtained on basis of genotypic differences using NTSYS pc version software. The polymorphic information content(PIC) was calculated for each primer using formula, $PIC = 1 - \sum p_i^2$ where p_i is the frequency of the i th allele of the j th marker [8].

3. Result

3.1 Morphological analysis

The mean values of eighteen quantitative characters recorded for twenty four genotypes of bitter gourd and there is high morphological diversity which showed diversity among the genotypes. minimum days to first male flower appearance was recorded in genotype Katahi (33.05) while maximum was obtained in Bitter gourd white long (52.45). First female flower were appeared early in Khaelgoan Local(42.55) while Vivek (64.85) flowered late. Among the eighteen genotypes minimum days to first fruit set were observed in Kahelgoan Local(52.40) and maximum in Chinese Long heirloom(72.90). Fruit length was observed maximum in CO-1(19.55) and minimum in Small bitter gourd(3.56). Highest fruit Yield per vine was obtained in BRBT-1(3.21kg) and minimum was observed in small bitter gourd(0.50). Number of fruit per vine was obtained highest in Karela white(40.09) and lowest in CO-1(22.71). TSS and ascorbic acid both found maximum in BRBT-1(8.17 and 105.84 respectively) and minimum in Chinese long heirloom(4.05 and 74.38 respectively) (Table 5)

3.2 Mahalanobis D^2 analysis

Cluster I having maximum number of genotypes. Similar results were found in [9] and [10] Cluster II have seven number of genotypes. Cluster III, IV, V, and VI contains single genotypes. Similar result was reported earlier [11]. The highest inter cluster genetic divergence (Table 2 and figure 2) was noticed between V and VI (267906.60) and was followed by II and V(138645.60) and III and V(108837.10). The intra cluster distance (Table 2 and figure 2) range varied from 8235.36 to 9804.83 with highest in cluster II (9804.33) succeeded by cluster I (8235.36)

From the mean values of clusters, cluster I having high mean values for characters fruit girth and total soluble solids. For characters like days to first female flower, cluster II was found superior. For characters like fruit length, number of seed at harvestable maturity and intermodal length, cluster III was found superior. For characters like number of primary branches, cluster IV was found superior. For characters like flesh thickness, number of pickings, vine length, average fruit weight, fruit yield per vine and ascorbic acid, V was found superior. Similar results obtained by some of previous workers [12]. For characters like node to first male flower, node to first female flower, days to first female flower, number of fruit per vine, days to first fruit set cluster and number of days to first male flower, cluster VI was found superior (Table 3).

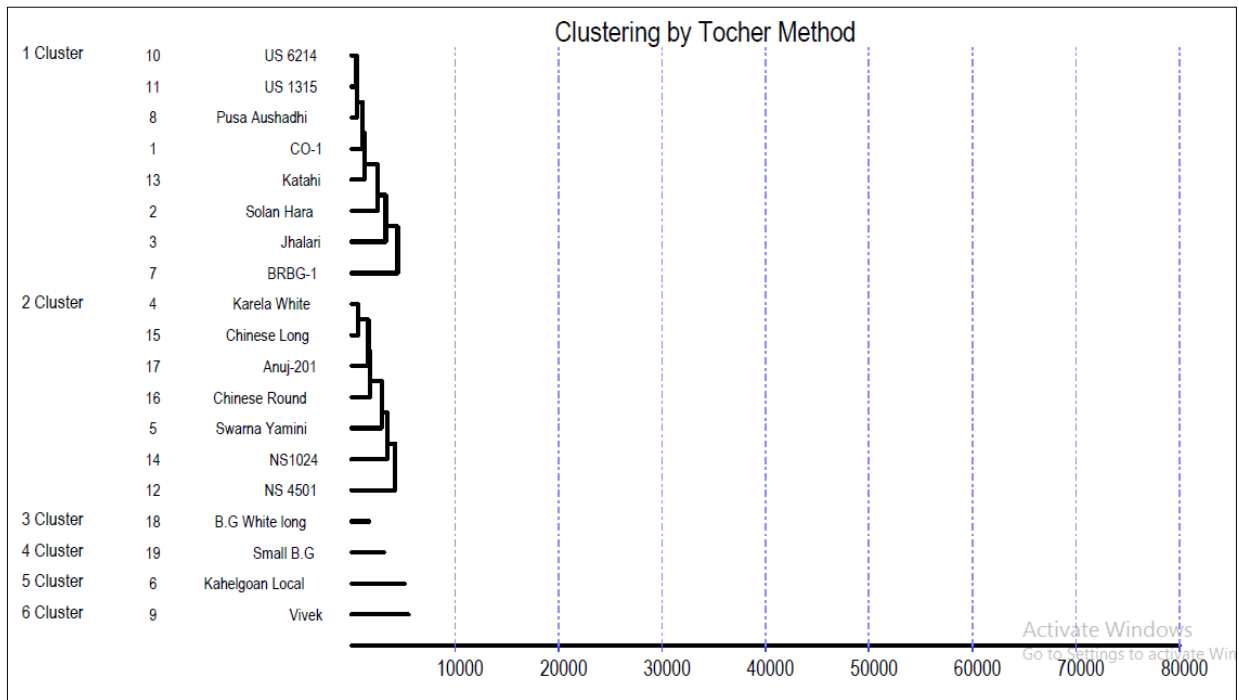


Fig 1: Cluster diagram obtained from Mahalanobis D² Analysis

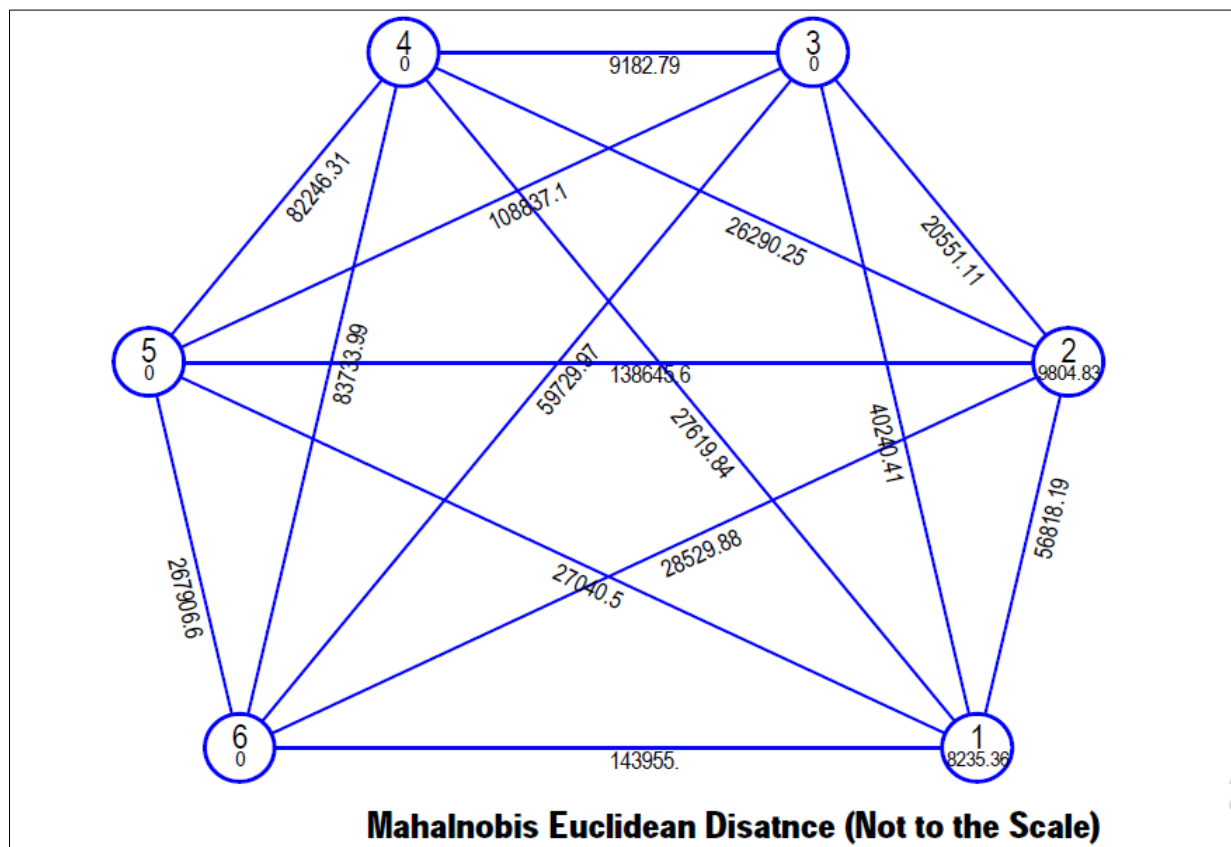


Fig 2: Cluster disatnce obtained from Mahalanobis D² Analysis

Table 1: Cluster composition of 19 bittergourd genotypes by following multivariate analysis

Cluster Number	Number of Genotypes	Genotypes
I	8	US 6214, US 1315, Pusa Aushadhi, CO-1, Katahi, Solan Hara, Jhalari, BRBG-1
II	7	Karela White, Chinese long, Anuj-201, Chinese Round, Swarna Yamini, NS 1024, NS 4501
III	1	B.G White long
IV	1	Small B.G
V	1	Kahelgoan Local
VI	1	Vivek

Table 2: Inter and Intra-cluster distances among six cluster obtained from 18 characters among 19 genotypes

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI
Cluster I	8235.36	56818.19	40240.41	27619.84	27040.50	143955.00
Cluster II		9804.83	20551.11	26290.25	138645.60	28529.88
Cluster III			0.00	9182.79	108837.10	59729.97
Cluster IV				0.00	82246.31	83733.99
Cluster V					0.00	267906.60
Cluster VI						0.00

Table 3: Cluster means for 18 characters in Mahalanobis D² Analysis

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI	Mean	Max	Min
Node to First male flower	7.93	10.75	13.15	10.6	7.45	13.2	12.616	13.20	7.45
Node to First female flower	12.3	16.36	16.05	15.55	10.05	16.75	17.412	16.75	10.05
Days to First male flower	38.75	46.19	52.45	43.75	37.65	53.05	54.368	53.05	38.75
Days to First female flower	45.69	56.68	55.35	47.4	42.55	64.85	62.504	64.85	42.55
Days to First Fruit set	57.93	69.61	67.9	63.45	52.60	71.60	76.618	71.60	52.6
Fruit length(cm)	16.27	13.85	16.46	3.56	14.46	14.90	15.9	16.46	3.56
Fruit Girth(cm)	15.32	11.4	10.07	3.37	13.71	10.53	12.88	15.32	3.37
Flesh Thickness(cm)	0.54	0.44	0.52	0.35	0.66	0.45	0.592	0.66	0.44
Numbers of seed at harvestable maturity	6.91	7.04	9.6	2.1	5.65	8.50	7.96	9.60	2.1
Number of primary branches	7.89	11.96	15.1	16.5	3.9	7.1	12.49	16.50	7.1
Internodal Length(cm)	6.99	6.16	7.43	4.94	7.09	6.69	7.86	7.43	4.94
Number of pickings	3.9	2.84	3.5	2.9	4.9	3.1	4.228	4.90	2.84
Vine Length(cm)	239.38	214.74	260.05	140.63	281.14	195.42	266.272	281.4	140.63
Average Fruit Weight(g)	71.25	47.44	65.8	17.4	102.2	58.8	72.578	102.2	17.4
Number of fruit per vine	32.61	33.95	34.53	29.28	25.37	36.87	38.522	36.87	25.37
TSS (° brix)	6.07	5.17	5.06	5.51	4.42	4.26	6.098	6.07	4.26
Ascorbic acid (mg/100g)	97.24	78.09	89.26	93.52	107.85	63.07	105.806	107.85	63.07
Fruit Yield per vine(Kg)	2.52	1.63	2.29	0.51	2.62	2.16	2.346	2.62	0.51

Table 3: List of polymorphic primer, no. alleles amplified, amplicon size (bp) and their PIC values

Primer Name	No. alleles amplified	Amplicon size (Approx. bp)	PIC
McSSR 20	3	220, 210, 200	0.32
McSSR 47	3	250, 240, 220	0.79
McSSR 54	1	190	0.34
McSSR 55	2	190, 90	0.22
McSSR 56	3	240, 220, 200	0.57
McSSR 115	2	250, 240	0.94

Table 4: List of SSR markers

Primer Id	Left primer sequence	Right primer sequence	Product size	Tm °C
McSSR 20 *	GGAATTCAGGTGAACCTGACG	CCAGGAGGAAGAGGAACCTGC	214	60
McSSR 47 *	TTGATTTTGAATCAGCGTTGT	ATTTTGCACAAGGCCTACCA	246	51
McSSR 54 *	CCATCCATATCCCAATCCCA	TCATCACAACCTCCCTTTTTC	173	46
McSSR 55 *	ATCCAACCAATAACCGGAAG	CTACCATTTTGGGGACGAGA	179	46
McSSR 56 *	TGCCATACTCCCAGGAAAAG	CGGAGACCTGTGTTTTTGGT	222	47
McSSR 115	GCAATGACCCTGTTTGTCT	CAAAGGAAGAGTGCACCTTGTGT	250	55

Table 5: Mean performance of 19 bitter melon genotypes

Genotypes	NTFMF	NTFFF	DTFMF	DTFFF	DTFFrS	FL	FG	FT	N Seed	NPB	In L	No P	VL	AFW	NFrPV	FYPV	TSS	Asc Acid
CO-1	7.85	12.85	47.25	51.85	64.70	19.55	16.64	0.49	7.40	13.90	7.07	2.80	276.32	77.70	22.71	1.78	6.06	101.47
Solan Hara	9.75	11.65	40.05	48.15	59.80	16.77	13.97	0.52	6.65	7.80	6.10	3.00	240.39	72.30	30.11	2.19	4.70	95.93
Jhalari	8.35	12.55	33.85	42.95	55.50	13.74	15.99	0.53	6.50	9.70	5.66	3.30	213.38	64.00	36.29	2.34	5.79	93.30
Karela White	6.65	18.25	42.95	53.75	72.00	14.91	14.63	0.45	7.50	11.60	6.06	2.60	202.54	45.50	40.09	1.83	5.61	72.78
Swarna Yamini	8.55	13.05	45.85	53.05	66.10	17.64	13.22	0.48	5.30	6.70	6.25	2.90	224.60	49.50	36.81	1.82	5.34	82.89
Kahelgoan Local	7.45	10.05	37.65	42.55	52.60	14.46	13.71	0.66	5.65	3.90	7.09	4.90	281.14	102.20	25.37	2.62	4.42	107.85
BRBG-1	7.05	10.85	39.05	43.95	54.30	18.21	17.53	0.65	5.11	4.00	6.62	5.10	294.68	103.30	30.84	3.21	8.17	105.84
Pusa Aushadhi	9.35	11.75	38.05	44.05	55.80	16.27	14.48	0.57	4.10	6.20	7.59	4.30	242.69	72.00	33.19	2.41	6.01	93.14
Vivek	13.20	16.75	53.05	64.85	71.60	14.90	10.53	0.45	8.50	7.10	6.68	3.10	195.42	58.80	36.87	2.16	4.26	63.07
US 6214	6.70	12.05	38.75	43.75	55.80	16.26	15.70	0.53	7.70	5.60	8.05	4.80	228.37	54.86	35.97	3.08	5.50	93.37
US 1315	7.45	11.55	39.95	44.15	55.70	15.99	15.29	0.56	6.85	5.90	7.66	4.20	220.66	62.26	38.29	3.02	5.52	97.50
NS 4501	8.25	19.75	41.45	61.45	67.20	12.52	13.90	0.38	8.60	11.60	6.82	3.05	204.21	60.20	33.06	2.00	5.62	75.38
Katahi	6.95	15.15	33.05	47.20	61.85	13.37	12.99	0.48	11.00	10.00	7.19	3.70	198.59	63.60	33.43	2.14	6.80	97.40
NS1024	9.55	17.65	50.80	60.70	68.35	13.71	10.31	0.54	8.90	11.00	6.03	3.40	159.85	59.20	30.21	1.79	5.92	86.30
Chinese Long	13.80	14.85	49.05	58.05	72.90	16.87	13.74	0.38	7.20	15.10	6.32	2.60	226.41	50.40	30.87	1.57	4.05	74.38

Chinese Round	13.20	17.15	41.85	52.60	69.75	6.12	4.44	0.38	2.90	14.20	5.60	2.80	230.78	20.80	27.87	0.59	5.15	76.66
Anuj-201	15.25	13.85	51.35	57.15	71.00	15.20	9.55	0.45	8.90	13.50	6.04	2.50	254.82	46.50	38.70	1.81	4.47	78.21
B.G White long	13.15	16.05	52.45	55.35	67.90	16.46	10.07	0.51	9.60	15.10	7.43	3.50	260.05	65.80	34.52	2.29	5.06	89.26
Small B.G	10.15	15.55	43.75	47.40	63.45	3.56	3.37	0.35	2.10	16.50	4.94	2.90	140.63	17.40	29.27	0.50	5.51	93.52
SE(m) ±	0.81	1.09	1.60	2.08	2.29	0.82	0.57	0.01	0.57	0.59	0.14	0.23	9.60	1.65	2.07	0.07	0.10	1.16
SED	1.15	1.54	2.26	2.94	3.24	1.15	0.80	0.02	0.81	0.84	0.20	0.32	13.57	2.33	2.93	0.10	0.14	1.64
CD ($P \leq 0.05$)	2.41	3.24	4.75	6.18	6.81	2.43	1.69	0.03	1.71	1.76	0.42	0.67	28.51	4.90	6.15	0.20	0.29	3.44
CV(%)	11.93	10.81	5.24	5.75	5.11	7.94	6.36	3.23	11.83	8.42	3.04	9.24	6.00	3.87	8.90	4.73	2.56	1.85

3.3 Molecular Characterization

A total of 30 SSR primers were used for analyzing genetic diversity present (Table 3). Out of these, three primers did not show any amplification, only 6 produced polymorphic bands and rest produced monomorphic bands. Polymorphic primers

identified based on preliminary screening were McSSR 20, McSSR 47, McSSR 54, McSSR55, McSSR56 and McSSR 115 with their PIC values 0.32, 0.79, 0.34, 0.22, 0.57 and 0.94 respectively (Table3) and found contradictory as reported by some of the workers [13].

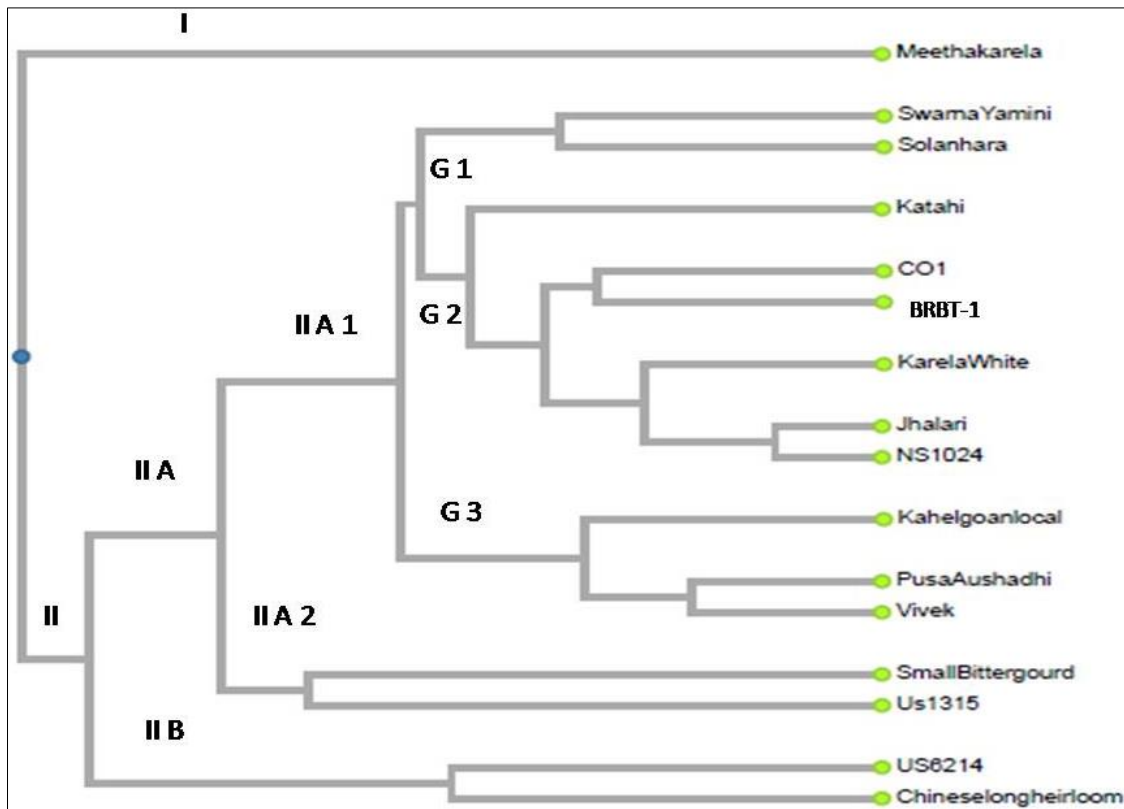


Fig 3: Cluster analysis based on molecular characterization

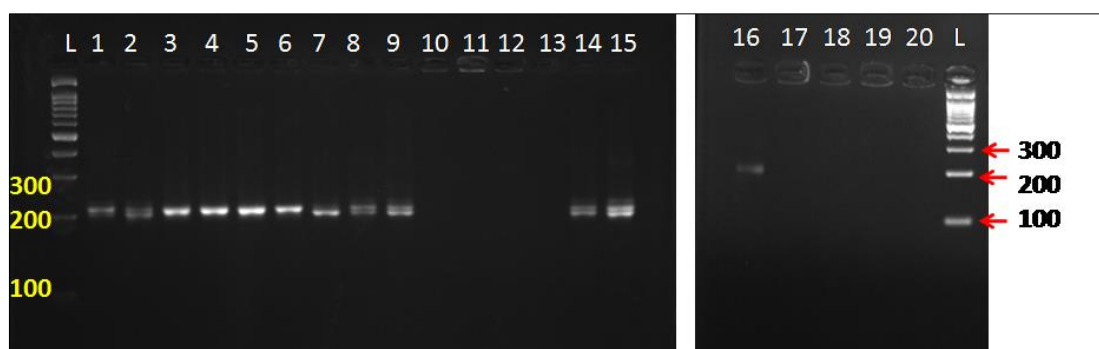


Fig 4: SSR profile of bitter melon genotypes with McSSR 20

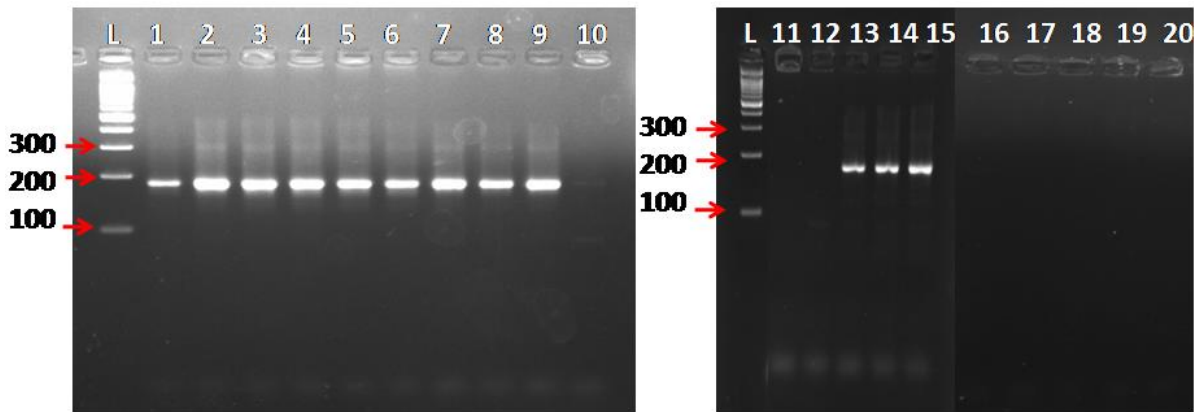


Fig 5: SSR profile of bitter gourd genotypes with McSSR 55

Note: 1. Solan Hara, 2. Swarna Yamini, 3. Kahelgoan Local, 4. Vivek, 5. Katahi, 6. Pusa Aushadhi, 7. BRBT-1, 8. NS 1024, 9.CO-1, 10. Chinese Long heirloom, 11. Meetha Karela, 12. US 6214, 13. US 1315, 14. Jhalari, 15. Karela White, 16. Small bitter gourd, 17. Chinese Round heirloom, 18. NS 4501, 19. Anuj-201, 20. Bitter gourd white long.

UPGMA based cluster analysis (http://genomes.urv.cat/UPGMA/UPGMAboot_v12.cgi; Fig. 2) showed that the grouping of 20 genotypes in two major clusters based on similarity index obtained from SSR marker. Two major clusters were observed by analyzing the SSR data and constructing phylogenetic tree. First major cluster had one genotype i.e. Meetha Karela. Second major cluster is further divided into two subgroups i.e. II A and II B. The cluster II A having 13 genotypes in which Swarna Yamini and Solan Hara, Katahi, CO-1 and BRBG-1, Karela white, Jhalari and NS 1024 were closely related and Kahelgoan Local, Pusa Aushadhi and Vivek were closely related while three genotypes in which Small Bittergourd and US 1315 were found to be closely related. Subgroup II B comprises of 2 genotypes in which US 6214 and Chinese long heirloom is closely related.

Similarity coefficients based on thirty markers were found to be ranged from 0.111 to 0.875. The similarity index was maximum for 0.875 (NS 1024 and Karela White) and minimum for 0.111 (Pusa Aushadhi and Meetha Karela).

4. Discussion

CO-1 and BRBT-1 and Katahi and Jhalari found to be in same cluster in both multivariate analysis and Molecular characterization. While in case of Jhalari and NA 1024, Swarna Yamini and Solan Hara, and Kahelgoan Local and Pusa Aushadhi were in same cluster in molecular characterization, while they were different in multivariate analysis. Small bitter gourd and US 1315 were similar cluster and morphologically they were same morphologically in case of small fruit obtained. Co-1 and BRBT-1 were in same cluster and similar to each other and having high amount of ascorbic acid 101.47 and 105.84 mg/100g respectively while in case of fruit length 19.55 cm and 17.64 cm respectively and for fruit girth 16.64cm and 17.53cm respectively. Katahi and Jhalari were observed to have minimum days taken to male flowering 33.05 and 33.85 respectively. Kahelgoan Local and Pusa Aushadhi were similar to each other and having minimum days to female flower opening 42.55 and 44.05 respectively while in case of days to first fruit set 52.60 and 55.80 respectively.

Meetha Karela is found to be genetically different from all the genotypes and placed singly in major cluster I. Similar type of result obtained by work done earlier^[14]. While Subgroup II B comprises of US 6214 and Chinese long heirloom seemed to

be most divergent and IIB contained all the remaining genotypes. All the genotypes were not seen to produce bands during gel run. This is because either alleles were absent which were present in those genotypes which showed amplifications or the binding site of the primers and alleles were mismatched.

5. Acknowledgment

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