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Genetic diversity analysis of garlic (*Allium sativum* L.) genotypes using ISSR markers

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Abstract
A total of 10 ISSR primers were used among fifty five genotypes of garlic out of which 9 primers gave amplification and generated 35 fragments. The numbers of polymorphic bands were 32 having 31 shared and one unique band with an average of 3.56 bands and 90.37% polymorphism per primer. The PIC values for ISSR marker were ranged from 0.43 (UBC-836) to 0.8 (UBC-849) with an average value of 0.56 per primer and ISSR primer index (IPI) differed from 0.96 (UBC-825) to 4.00 (UBC-849) with an average value of 2.28 per primer. The dendogram generated by ISSR markers revealed two major groups, Cluster-I and cluster-II. Genotypes RGP 429, RGP 117, RGP 445, RGP 495 and RGP 609 showed maximum similarity in cluster-I, whereas RGP 235 and G 282 showed maximum similarity in cluster-II. Genotype GAG-6 and RGP-26 were found as most diverse genotypes among the genotypes.

Keywords: garlic, molecular markers, genetic diversity, ISSR

Introduction
Garlic (*Allium sativum* L.) is a monocotyledonous vegetable belongs to family Alliaceae. "*Allium*" is the largest and the most important representative genus of the Alliaceae family that comprises 700 species, widely distributed in the Northern hemisphere, North America, North Africa, Europe and Asia. Garlic is a diploid species with chromosome number of 2n=2x=16, originated in Central Asia (Kazakhstan), with secondary centers of diversification in China and the Mediterranean area (Etoh and Simon, 2002) [1]. Garlic bulb is an important seasoning ingredient in many of the world's cuisines, and the green flower stalks and young leaves are eaten fresh or cooked. Garlic cloves are used as flavoring agent in many processed food as garlic paste, powder, pearls, pharmaceutical etc.

In India, Garlic is cultivated in about 2.62 lakh hectare area with total production of 14.25 lakh tones and productivity of 5.439 tons per hectare (Anonymous, 2015) [2]. To increase the production and productivity of this crop it is quite essential to screen the germplasm to select and improve cultivars for quantitative traits.

The knowledge about genetic diversity of a crop species is prerequisite for its improvement. Various techniques such as morphological, biochemical and DNA based molecular markers are used to study the genetic diversity of crop plants. A comparison of plant phenotypes is the simplest approach to the characterization of genotypes and the assessment of genetic diversity; however, phenotypic evaluation is influenced by environment and may not distinguish between closely related accessions (Rodriguez *et al*., 1999) [3]. Further, for morphological characterization, the plant must be grown up to flowering or fruiting stage, which is space and time consuming. Therefore, it is desirable to develop cultivar identification tests based on molecular techniques.

Molecular markers have been applied to assess genetic diversity in many crops because they are unlimited in number, not affected by the environment and can be organized into linkage maps. Now-a-days very powerful PCR-based techniques such as RAPD, AFLP, SSR, ISSR etc have also emerged which are very fast, reliable and require minimal amount of tissue for investigation. Inter Simple Sequence Repeat (ISSR) primers target simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and evolve rapidly, but they do not require prior knowledge of DNA sequence for primer design (Fang and Roose, 1997) [4]. ISSR can rapidly differentiate closely related individuals (Zietkiewicz *et al.*, 1994) [5] and have been successfully used to assess genetic diversity among closely related cultivars.

The present study was conducted to assess the genetic diversity among fifty five garlic genotypes on the basis of Inter Simple Sequence Repeats (ISSR) markers which will helpful in improvement of this crop in further plant breeding programmes.

Materials and Methods

Plant Material

The experimental material comprised of 55 garlic accessions obtained from Vegetable Research Station, J.A.U., Junagadh were used in present study (Table 1). All the molecular work was carried out at Department of Biotechnology, College of Agriculture, J.A.U., Junagadh.

Table 1: List of garlic genotypes

S. No.	Genotypes	S. No.	Genotypes
1.	GAG-6	29.	RGP-509
2.	RGP-601	30.	RGP-429
3.	GG-4	31.	RGP-562
4.	RGP-216	32.	RGP-610
5.	RGP-559	33.	RGP-117
6.	RGP-219	34.	RGP-445
7.	RGP-504	35.	RGP-182
8.	RGP-616	36.	RGP-240
9.	RGP-614	37.	RGP-238
10.	RGP-247	38.	RGP-162
11.	RGP-272	39.	RGP-511
12.	RGP-160	40.	GJG-5
13.	RGP-99	41.	RGP-396
14.	RGP-252	42.	RGP-465
15.	RGP-327	43.	RGP-108
16.	RGP-235	44.	RGP-52
17.	RGP-222	45.	RGP-495
18.	RGP-581	46.	RGP-609
19.	RGP-48	47.	RGP-291
20.	G-282	48.	RGP-619
21.	RGP-488	49.	RGP-134
22.	RGP-329	50.	RGP-55
23.	RGP-591	51.	RGP-449
24.	RGP-281	52.	RGP-122
25.	RGP-474	53.	RGP-171
26.	RGP-26	54.	RGP-127
27.	RGP-278	55.	RGP-87
28.	RGP-257		

DNA extraction

Total genomic DNA was isolated from young leaves of all the fifty five accessions of garlic separately which were grown in field of Vegetable Research Station, J.A.U., Junagadh. DNA extraction was carried out by CTAB method as described by Doyle and Doyle (1990) [6] with minor modifications. The reagents and buffers used for DNA isolation were prepared as per Sambrook *et al.* (1989) [7].

For DNA extraction, 1 g of leaf tissues were grinded in liquid N_2 with the help of mortar pestle. Then, 1.5 ml pre-warmed (65^0C) DNA extraction buffer (CTAB DNA extraction Buffer) was added in homogenized leaf material followed by transfer in capped polypropylene tubes. Tubes were incubated for 1 hr at 65^0C in water bath with gentle swirling. After incubation tubes were removed and spun at 10000 rpm for 10 minutes at room temperature (25^0C). One ml aqueous phase was transferred to another fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion for 15 minutes to ensure emulsification of the phase. Tubes were then spun at 10000 rpm for 10 minutes at room temperature (25^0C). Aqueous phase was transferred to another fresh tube. This steps was repeated for two times. Equal volume of Ice-cold Iso-propanol was added to aqueous phase to precipitate DNA. DNA-CTAB complex was precipitated as a fibrous network. It was lifted by auto-pipette and was transformed to polypropylene tubes and centrifuged at 12000 rpm for 10 minutes at 4^0C. After removing supernatant 1.5 ml of 80% alcohol was added in pellet and was kept for 10 minutes with gentle agitation. It was centrifuged at 10,000 rpm for 10 minutes at 20^0C. The tubes were inverted and drained on a paper towel. The pellet was air dried for 30 minutes. Each pellet was re-dissolved in 200 µl of TE buffer by keeping 30 minutes at room temperature without agitation.

Estimation of Quality of DNA

Quality of extracted DNA was checked on a 0.8% agarose gel. The gel was buffered with 1x TAE, stained with ethidium bromide and visualized under UV transilluminator and photographed using Syngene Gel documentation system (Software: Genesys V1.4.1.0) using UV light.

Dilution of DNA for PCR

The quantified DNA was diluted to final concentration of 50 $ng.\mu l^{-1}$ in TE buffer for further research work.

DNA amplification using ISSR marker

The genomic DNA was amplified using ISSR primers of UBC series as listed in Table 2. The PCR reactions for ISSR were carried out according to the method given by Al-Otayk *et al.* (2009) [8] with some modifications. The reactions for PCR-ISSR were carried out in a final reaction volume of 20 µl (Table 3). To avoid pipetting error in measuring small volumes, a cocktail was prepared where constituents common to all the reactions were combines in one tube multiplying volume for one reaction with total number of samples.

Table 2: List of ISSR primers used in present study

Sr. No.	Markers	Sequence 5' – 3'	GC (%)	Tm (^{0}C)
1.	UBC-812	GAGAGAGAGAGAGAGAA	47	44.3
2.	UBC-818	CACACACACACACACAG	53	52.1
3.	UBC-823	TCTCTCTCTCTCTCTCC	53	47.5
4.	UBC-825	ACACACACACACACACT	47	49.2
5.	UBC-836	AGAGAGAGAGAGAGAGYA	44.4	40.8
6.	UBC-840	GAGAGAGAGAGAGAGAYT	44.4	52.9
7.	UBC-846	CACACACACACACACART	44.4	53.7
8.	UBC-848	CACACACACACACACARG	50	50.6
9.	UBC-849	GTGTGTGTGTGTGTGTYA	44.4	42.9
10.	UBC-878	GGATGGATGGATGGAT	50	55.9

Table 3: Preparation of reaction mixture for ISSR primers

Sr. No.	Reagent	Quantity
1.	Milli Q Water	14.74 µl
2.	PCR buffer with $MgCl_2$	2.0 µl
3.	dNTPs mix (0.2 mM each)	0.06 µl
4.	Primer	2.0 µl
5.	Taq polymerase (3 U/µl)	0.2 µl
6.	Template DNA	1.0 µl
Total		20.0 µl

All the PCR reactions were carried out in 0.2 ml capacity thin walled PCR tubes. As per the above cocktail, Millipore sterilized water was added first followed by addition of PCR mastermix, primer in sequence and finally the template DNA. The reagents were mixed gently by tapping against the tube followed by a short spinning (~3,000 rpm for 30 seconds).

The tubes were then placed in the Thermal Cycler for cyclic amplification (Table 4).

Table 4: PCR conditions for ISSR

Sr. No.	Steps	Temperature (ºC)	Duration	Cycle
1.	Initial denaturation	94	4.0 min	1
2.	Denaturation	94	1.0 min	40
3.	Annealing	48	45 sec	
4.	Extension	72	2.0 min	
5.	Final extension	72	10.0 min	1
6.	Hold	4	∞	∞

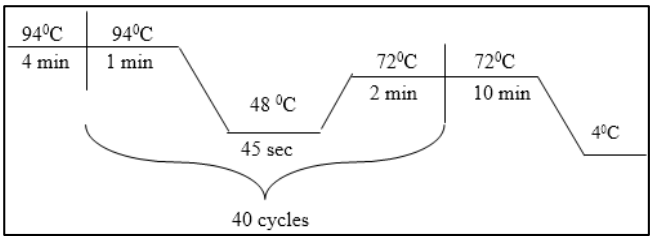

Fig 1: Steps in ISSR PCR amplification

Electrophoresis of amplified product

The amplified products of ISSR were analyzed using 1.5% agarose gel. The gel was buffered with 1x TAE, stained with ethidium bromide and visualized under UV transilluminator and photographed using Syngene Gel documentation system (Software: Genesys V1.4.1.0) using UV light.

Molecular data analysis

Polymorphic information content (PIC) for ISSR was calculated on the basis of allele frequency (Anderson *et al*., 1993) [9]. PIC values were used to calculate ISSR primer indexes (IPI) which were generated by multiplying the PIC values of all the markers amplified by the same primer.

Clear and distinct bands amplified by ISSR primers were scored for the presence (1) and absence (0) for the corresponding band among the Genotypes. The data were entered in to MS-Excel data sheet and subsequently analyzed using NTSYS pc version 2.02 (Rohlf, 1994) [10]. Similarity matrices, generated according to the Jaccard similarity coeeficient and were used to perform cluster analysis using the unweighted pair group method with arithmetic average (UPGMA). Dendrogram, indicating the estimated similarity among the garlic genotypes, was constructed with the TREE programme of NTSY Spc.

Results and Discussion

Out of 10 ISSR primers screened, 9 primers amplified a total of 35 bands out of which 32 bands/alleles were polymorphic with an average 3.56 bands per primer and out of 32 polymorphic bands, total 31 bands were shared polymorphic and one unique-polymorphic band was observed while 3 bands were found monomorphic (Table 5).

The average percentage of polymorphism was about 90.37% for the 9 ISSR primers. The amplified fragments were in range of 192-1340 bp. The smallest fragment of 192 bp was amplified by UBC-840 primer demonstrated a shortest distance between two adjacent microsatellites and the largest fragment of 1340 bp was amplified by UBC-823 primer represented the longest distance between two microsatellites in the present study. UBC-818 primer produced highest 6 bands (alleles) while UBC-825 primer produced lowest 2 bands.

The polymorphic information content (PIC) was calculated for each primer. The highest PIC value calculated was 0.8 which was with UBC-849, while lowest PIC value was 0.43 recorded with primer UBC-836, with an average of 0.56 per primer. ISSR primer index differed from 0.96 to 4.00 with an average of 2.28 per primer. The lowest IPI value was with UBC-825 primer and highest value was observed with primer UBC-849 (Table 5).

Table 5: Size, number of amplified bands, percent polymorphism and PIC obtained by ISSR primers

Sr. No.	ISSR primer	Allele/Band Size (bp)	Total no. of Allele or Bands	Polymorphic Bands			Monomorphic Bands	% Polymorphism	PIC	IPI
				S	U	T				
1.	UBC 812	271-464	3	2	0	2	1	66.67	0.53	1.59
2.	UBC 818	439-1308	6	6	0	6	0	100	0.59	3.54
3.	UBC 823	696-1340	3	2	0	2	1	66.67	0.54	1.62
4.	UBC 825	308-649	2	2	0	2	0	100	0.48	0.96
5.	UBC 836	209-628	3	2	1	3	0	100	0.43	1.29
6.	UBC 840	192-749	5	5	0	5	0	100	0.72	3.6
7.	UBC 846	301-970	4	4	0	4	0	100	0.54	2.16
8.	UBC 848	376-1038	4	4	0	4	0	100	0.44	1.76
9.	UBC 849	311-1004	5	4	0	4	1	80.00	0.8	4
Total			35	31	1	32	3	-	-	-
Average			-	-	-	3.56	-	90.37	0.56	2.28

S = Shared; **U** = Unique; **T** = Total Polymorphic bands; **PIC** = Polymorphism Information Content; **SPI** = SSR Primer Index = Number of Bands × PIC

The present study results are quiet supported by the following research done by several scientists. The average polymorphism percentage of our study is 90.37%. It is very nearer with earlier studies such as 90% in 16 genotypes of onion by Kesralikar *et al.* (2017) [11], 92.90% by Anwar *et al* (2016) [12] and is higher than earlier studies such as 50.10% in 131 garlic accessions by Sharma *et al*. (2016) [13], 79.48% in 32 onion germplasm by Qijiang *et al.* (2007) [14] and 75% in 39 garlic accessions by Chen *et al*. (2014) [15].

Nikhil *et al*. (2015) [16] applied Inter Simple Sequence Repeat (ISSR) markers on 22 genotypes of onion (*Allium cepa* L.) in order to assess the degree of polymorphism. Considering all the amplified primers a total of 81 scorable amplification products were obtained out of which 69 were polymorphic bands and 12 bands were monomorphic. These findings supported the present research in garlic.

Genetic similarity

All bands generated from Nine ISSR primers were further subjected to genetic similarity (GS) assessment by using Jaccard's similarity index which revealed that similarity between 55 garlic genotypes ranged from 32.10% to 100%. The lowest similarity of 32.10% was noticed between genotypes RGP 26 and RGP 559, while highest similarity of

100% was noticed between genotypes RGP 235, G 282, RGP 429, RGP 117, RGP 429, RGP 445, RGP 117, RGP 445, RGP 495 and RGP 609. The mean genetic similarity coefficient was 0.715.

Cluster analysis of ISSR

The dendrogram was constructed using UPGMA based on Jaccard's similarity coefficient through NTSYSSpc-2.02i software for SSR data of 55 garlic genotypes (Fig. 2). The genotypes were grouped into two main clusters, cluster-I and cluster-II sharing 41% similarity. Cluster-I comprised of two subclusters A and B with 58% likeness. Subcluster A consisted of 2 genotypes GAG 6 and RGP 601 with near about 88% similarity. Subcluster B was further divided into group B1 and B2 sharing near about 60% likeness. Group B1 further bifurcated into two subgroups B1 (a) and B1 (b) with near about 64% similarity. Subgroup B1 (a) further divided into two groups B1(ai) and B1(aii) with near about 71% similarity. Subgroup B1(ai) consisted of 44 genotypes namely RGP 216, RGP 616, RGP 247, RGP 272, RGP 99, RGP 160, RGP 252, RGP 327, RGP 504, RGP 559, RGP 614, RGP 449, RGP 240, RGP 52, RGP 108, RGP 429, RGP 117, RGP 445, RGP 610, RGP 238, RGP 562, RGP 162, RGP 495, RGP 609, GJG 5, RGP 396, RGP 134, RGP 122, RGP 511, RGP 171, RGP 509, RGP 219, RGP 291, RGP 488, RGP 465, RGP 222, RGP 581, RGP 329, RGP 591, RGP 278, RGP 257, RGP 281, RGP 474 and RGP 48 sharing near about 73% likeness. In this cluster genotypes RGP 429, RGP 117, RGP 445, RGP 495 and RGP 609 shared maximum 100% similarity. Subgroup B1 (aii) consisted of two genotypes RGP 619 and RGP 55 sharing 92% similarity. Subgroup B1 (b) consisted of two genotypes RGP 182 and RGP 127 with near about 78% likeness. Group B2 consisted of only one genotype RGP 87.

Main cluster II consisted of 4 genotypes namely GG 4, RGP 235, G 282 and RGP 26 which were shared more than 72% similarity. In this cluster genotype RGP 235 and G 282 shared maximum 100% similarity. The cluster analysis showed the highest (100%) similarity between the genotypes RGP 429, RGP 117 and RGP 445; RGP 495 and RGP 609 and between RGP 235 and G 282.

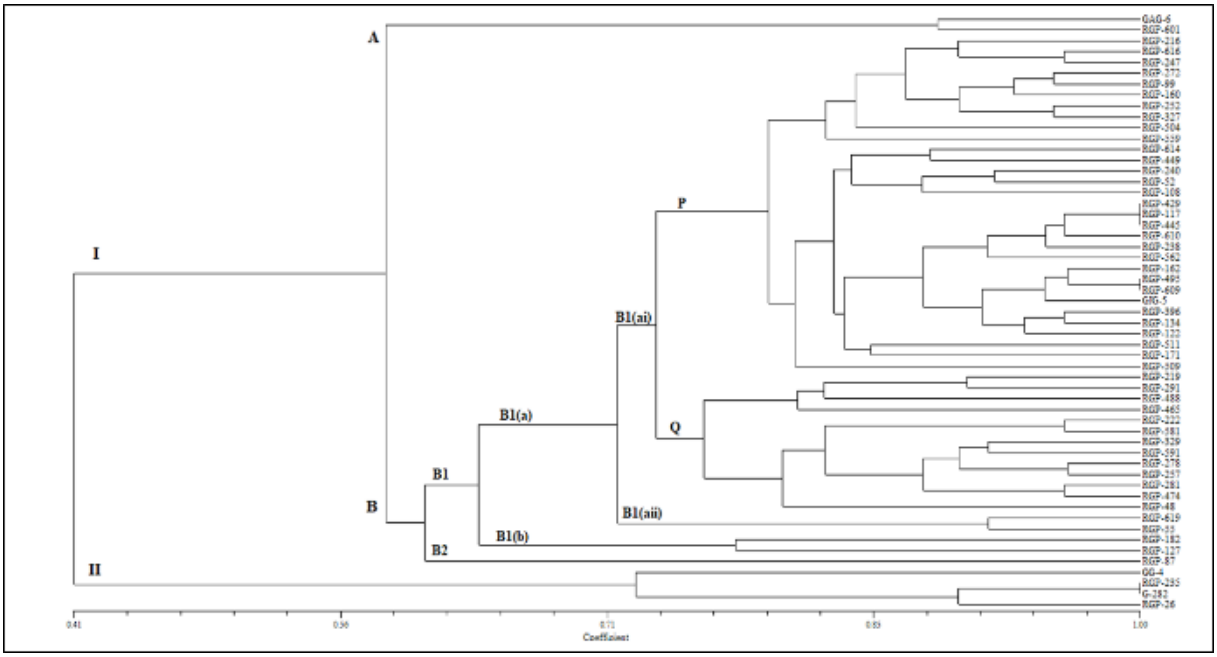

Fig 2: Dendrogram depicting the genetic relationship among 55 garlic genotypes based on data of ISSR marker

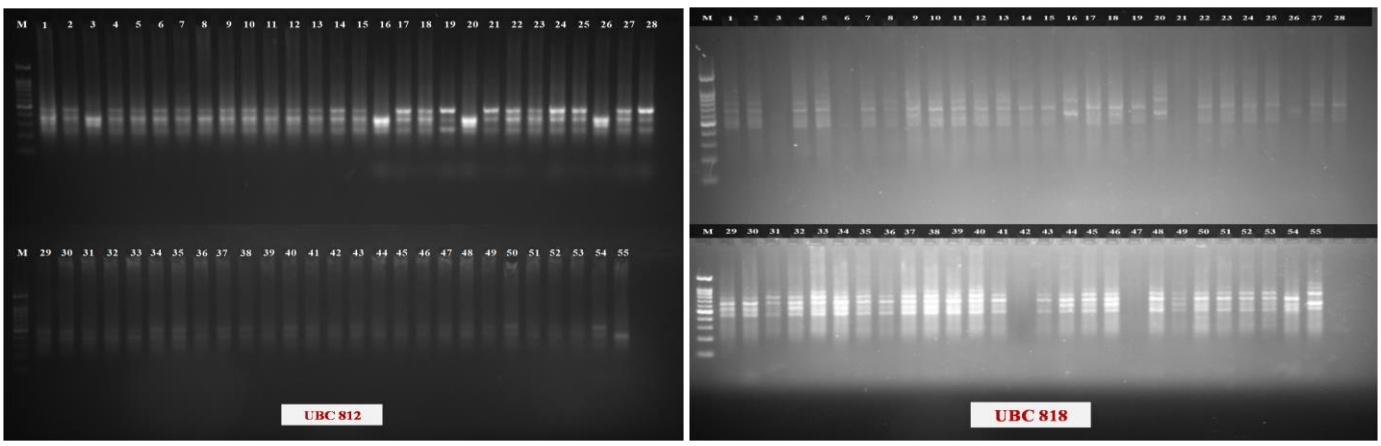

Plate 4.23: Agarose Gel Electrophoresis of amplified products obtained with ISSR Primers UBC-812 and UBC-818 compared with 1500 bp DNA Ladder; [M: Marker (DNA Ladder of 100-1500 bp), 1 to 55: Garlic genotypes in same order as mentioned earlier in Table 3.1]

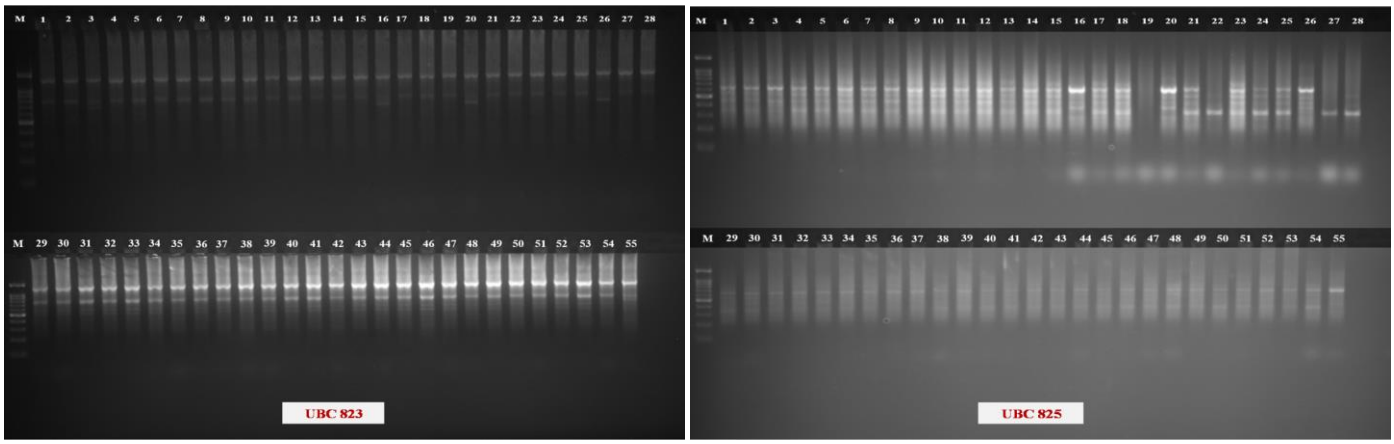

Plate 4.24: Agarose Gel Electrophoresis of amplified products obtained with ISSR Primers UBC-823 and UBC-825 compared with 1500 bp DNA Ladder; [M: Marker (DNA Ladder of 100-1500 bp), 1 to 55: Garlic genotypes in same order as mentioned earlier in Table 3.1]

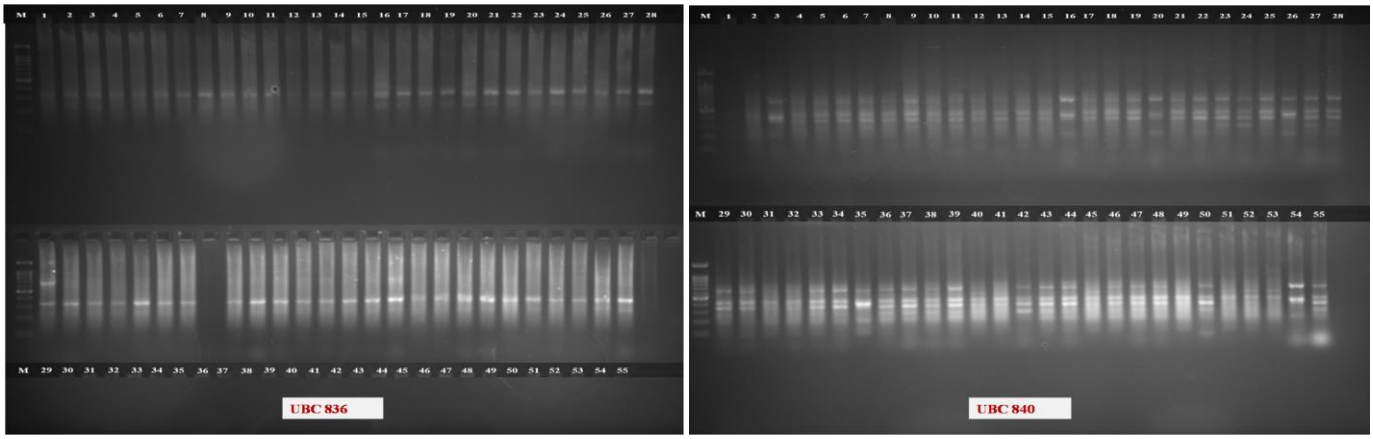

Plate 4.25: Agarose Gel Electrophoresis of amplified products obtained with ISSR Primers UBC-836 and UBC-840 compared with 1500 bp DNA Ladder; [M: Marker (DNA Ladder of 100-1500 bp), 1 to 55: Garlic genotypes in same order as mentioned earlier in Table 3.1]

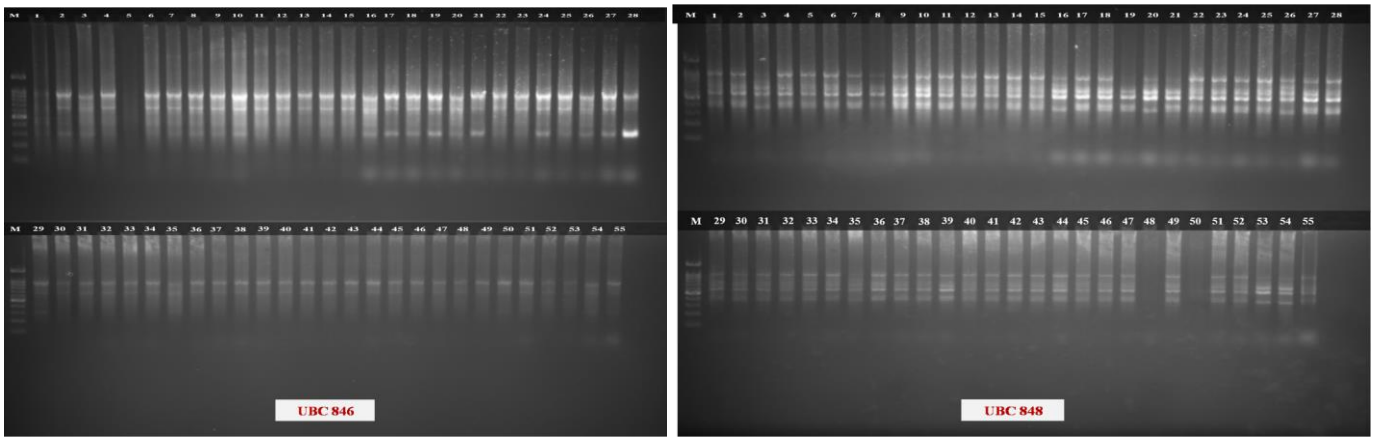

Plate 4.26: Agarose Gel Electrophoresis of amplified products obtained with ISSR Primers UBC-846 and UBC-848 compared with 1500 bp DNA Ladder; [M: Marker (DNA Ladder of 100-1500 bp), 1 to 55: Garlic genotypes in same order as mentioned earlier in Table 3.1]

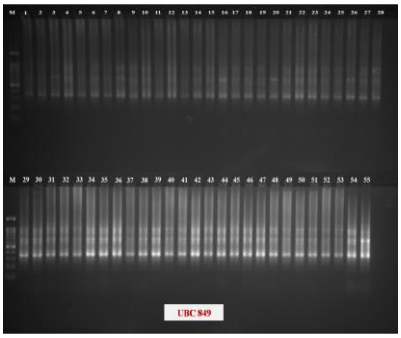

Plate 4.27: Agarose Gel Electrophoresis of amplified products obtained with ISSR Primer UBC-849 compared with 1500 bp DNA Ladder; [M: Marker (DNA Ladder of 100-1500 bp), 1 to 55: Garlic genotypes in same order as mentioned earlier in Table 3.1]

Summary

Total 9 ISSR primers were used which generated 35 fragments in which 32 bands were polymorphic having 31 shared and 1 unique band with an average of 3.56 bands per primer and 3 bands were monomorphic. The percent polymorphism obtained for ISSR primers ranged from 66.67-100% with an average value of 90.37% per primer. The amplified fragments were in range of 192-1340 bp. The smallest fragment of 192 bp amplified by UBC-840 primer and the largest fragment of 1340 bp amplified by UBC-823 primer. The Polymorphism Information Content (PIC) values for ISSR marker ranged from 0.43 to 0.8 with an average value of 0.56 per primer. ISSR primer index (IPI) differed from 0.96 to 4.00 with an average value of 2.28 (Table 4.3). Out of 9 primers, only one primer (UBC-836) was able to produce genotype specific unique band. The phylogenetic tree constructed by UPGMA method generated two main clusters with 41% similarity and similarity coefficient ranged from 32.10% to 100%. The lowest similarity was noticed between genotypes RGP 26 and RGP 559, while highest similarity noticed between genotypes RGP 235, G 282, RGP 429, RGP 117, RGP 429, RGP 445, RGP 117, RGP 445, RGP 495 and RGP 609.

India holds an immense resource of garlic cultivars that are great significance not only for breeders, but also for farmers. Therefore, from the present experiment, the data generated might be useful to some extent for the identification of the genetic diversity among genotypes which could be useful for marker assisted selection for further crop improvement programmes.

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