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Molecular characterization of garlic (*Allium sativum* L.) genotypes differ in total soluble solid content

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Abstract
As garlic (*Allium sativum* L.) as an important role as medicinal and spices, quality of garlic plays major contribution in the fulfilling of its role. Now a day use of molecular markers for the evaluation of molecular diversity is receiving much attention than morphological characterization. Total 15 RAPD primers generated 84 bands in which all 84 bands were polymorphic having 82 shared and 2 unique bands with an average of 5.6 bands and 100% polymorphism per primer. Eighteen ISSR primers engendered 87 bands in which all 87 bands were polymorphic with 84 shared and 3 unique bands having 100% polymorphism with an average of 4.83 bands per primers. Total 12 SSR primers generated the 13 fragments in which all bands were polymorphic shared except primer SSR-13, which gave only one monomorphic band with 91.66% polymorphism with an average of 1 band per primer. An average polymorphism information content (PIC) value for RAPD primer, ISSR primer and SSR primer were 0.77, 0.69 and 0.04, respectively. Primer indices for RAPD, ISSR and SSR were 4.35, 3.52 and 0.08 respectively. The similarity coefficient of clusters analysis was ranged from 21 to 49 % for RAPD, 44 to 60% for ISSR and 49 to 75% for SSR.

Keywords: Garlic, genetic diversity, molecular markers, RAPD, ISSR, SSR

Introduction
Garlic (*Allium sativum* L.) is one of the important cultivated species of the genus A*llium belong* to the *Alliaceae* family. The chromosome number is 2n=16. It is probably native of Central Asia and Southern Europe, especially Mediterranean region. (Thompson and Kelly, 1957) [9]. Garlic is the second most important bulb vegetable grown and used as spice and flavoring agentfor many foods, (Velisek *et al*., 1997) [11]. "*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 (Tsiaganis *et al*., 2006) [10]. The garlic genome has a low GC base content of 36.9% in the composition and a high amount of repetitive DNA (Kirk *et al*., 1970) [5]. At present keeping quality is a burning problem in storage of garlic. Apart from SSR and AFLP, Random Amplification of Polymorphic DNA (RAPD), (Maab and Klaas, 1995; Al-Zahim *et al*., 1997) [6, 2] and Inter Simple Sequence Repeat (ISSR) has also been used to fingerprint the different garlic species and cultivars (Al-Otayk *et al*., 2008) [1]. This study was planned for molecular characterization of garlic based onsoluble solid content. The information on genotypes with higher total soluble solid will facilitate in the selection of genotypes as parents and by involving these genotypes in breeding programme for crop improvement which may helpful can plan to develop new garlic variety with higher total soluble solid content increase shelf life of garlic. An experiment was arranged with 21 genotypes of garlic, among which ten found with high total soluble solid (TSS) content and 11 had low TSS.

Materials and methods Sample collection
Total 150 genotypes of Garlic were screened and out of them, 21 genotypes were selected (Differ in total soluble solid content) which are listed in Table 1. The genotypes were collected from Vegetable Research Station, Junagadh Agricultural University, Junagadh.

Table 1: List of garlic genotypes with high and low total soluble solid content (TSS)

Sr. No.	Name of genotypes with High TSS	Represented as	Sr. No.	Name of genotypes with Low TSS	Represented as
1.	RGP-270	H1	1	RGP-474	L1
2.	RGP-245	H2	2	RGP-66	L2
3.	RGP-182	H3	3	RGP-7	L3
4.	RGP-278	H4	4	RGP-581	L4
5.	RGP-276	H5	5	RGP-602	L5
6.	RGP-1	H6	6	RGP-585	L6
7.	RGP-3	H7	7	RGP-513	L7
8.	RGP-501	H8	8	RGP-77	L8
9	RGP-114	H9	9	RGP-224	L9
10	RGP-491	H10	10	RGP-560	L10
			11	RGP-607	L11

DNA Extraction

Total genomic DNA extraction was carried out by CTAB method as described by Doyle and Doyle (1987) [3] with minor modifications.

Molecular marker analysis

Various molecular marker techniques such as Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSRs), Simple Sequence Repeats (SSRs) were used.

Table 2: Preparation of reaction mixture for RAPD, ISSR, SSR

Sr. No.	Reagent	Quantity
1	PCR buffer (10X) (without MgCl2)	1.5 µl
2	Taq polymerase (3 U/µl)	0.15 µl
3	dNTPs mix (2.5 mM each)	1.2 µl
4	Primer (25 pMoles/µl)	1.2 µl
5	Template DNA (50 ng/µl)	1.2 µl
6	Millipore sterile distilled water	9.75 µl
Total		15.0µl

Randomly Amplified Polymorphic DNA (RAPD)

The genomic DNA was amplified using 20 primers of OPA, OPB, OPD, OPE and OPM, OPN, OPO, OPR, OPQ series. The PCR reactions for RAPD were carried out according to the method given by Khar *et al.* (2008) [4] with some modifications. RAPD reactions were performed in Thermal cycler programmed for 40 cycles of 1 min at 92°C, 1 min at 35°C and 2 min at 72°C. Reaction products were separated by electrophoresis on 1.2% agarose gels, using Tris-borate-EDTA buffer system. The gel was stained with ethidium bromide and visualized under UV trans illuminator and photographed using Syn gene Gel documentation system using UV light.

Inter Simple Sequence Repeats (ISSR)

The PCR reactions for ISSR were carried according to method given by Al-Otayk *et al*. (2008) [1] with required modifications. The genomic DNA was amplified using UBC (University of British Columbia, Canada) primers. ISSR reactions were performed in Thermal cycler programmed for 40 cycles of 1 min at 94°C, 1 min at Tm±2 and 2 min at 72°C Electrophoresis of amplified product were analyzed using 1.5 % agarose gel.

Simple Sequence Repeat (SSR)

The genomic DNA was amplified using 20 primers. PCR reactions for SSR were carried out as per method stated by Mahajan *et al*. (2009) [7] with some modifications. The components for amplification of SSR primers were same as RAPD and ISSR except the primers. In SSR the amplification of reverse and forward primers were carried out. PCR conditions for SSR were initial denaturation at 94°C for 4 min, denaturation at 94°C for 45 sec., annealing at Tm ± 2 for 45 sec. and extension at 72ºC for 1.30 min. Electrophoresis of amplified product The amplified products of SSR were analyzed using 2.5 % agarose gel.

Molecular data analysis

Polymorphic information content (PIC) for RAPD, ISSR, SSR was calculated on the basis of allele frequency Clear and distinct bands amplified by RAPD, ISSR, SSR 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, 1998) [8]. 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 discussions

Among 20 RAPD primers, 15 primers gave amplification with a totalof 84 bands. Primer which amplified are OPA-01, OPA-02, OPA-07, OPB-04, OPB-06, OPD-06, OPE-01, OPE-11, OPE-14, OPN-02, OPO-07, OPP-05, OPQ-10, OPR-01 and OPR-05, Out of 84 bands, all thebands were polymorphic with an average of 5.6 bands per primer. The Polymorphism Information Content (PIC) values for RAPD marker were ranged from 0.66 to 0.86 with an average value of 0.77 per primer and RAPD primer index (RPI) differed from 2.91 to 6 with an average value of 4.35 (Table 3). Among the screened primers, Primer OPO-07 produced one unique band which was 1440bp for L7 (RGP-513). Primer OPE-14 produced one unique band which was 600bp for L10 (RGP-560).

Out of 20 primers, 18 primers gave amplification with a total of 87 bands, out of which 84 bands were polymorphic with an average of 4.83 bands per primer. Out of 87 polymorphic bands 84 bands were polymorphic and shared and 3 bands were polymorphic and unique. The PIC value was recorded with an average of 0.69 per primer. ISSR primer index (IPI) differed from 1 to 7.79 withan average of 3.52 per primer (Table 4). Among all the ISSR primers, UBC- 840 Primer produced one specific band which mol. wt. 908bp in H1 (RGP-270). UBC-857 Primer produced one specific bands of which mol. wt. 359bp in H1 (RGP-270). UBC-879 Primer produced one specific bands of which mol. wt. 2700bp in L10 (RGP-560).

Out of 20 SSR primers, 12 primers gave amplification of the DNA. All the 12 SSRs primers were amplified a total of 13 bands. Out of 13 bands, all 12 bands were polymorphic with anaverage of 1 band per primer and 1 band was monomorphic. The percent polymorphism obtained for SSR primers were ranged from 0 % to 100% with an average value of 91.66% per primer (Table 5).

Genetic Similarity based on pooled data of molecular markers

Genetic similarity of all three molecular markers were determined for each pair of 21 garlic genotypes which revealed that the lowest similarity of 29% was noticed between L10 (RGP-560) and L5 (RGP-602), while highest of 75% was noticed between H7 (RGP-3) and H10 (RGP-491) as given in Table 6.

Table 3: Size, number of amplified bands, percent polymorphism and PIC obtained by RAPD primers in garlic genotypes

Sr. No.	RAPD Primer	Band Size (bp)	Total No. of Bands (A)	Polymorphic Bands (B)			Mono- Mor phic Band	% Poly- Mor Phism (B/A)	PIC*	RPI
				S	U	T				
1	OPA-01	504-1584	6	6	0	6	0	100	0.74	4.44
2	OPA-02	310-993	7	7	0	7	0	100	0.82	5.75
3	OPA-07	197-625	6	6	0	6	0	100	0.78	4.69
4	OPB-04	336-2594	4	4	0	4	0	100	0.72	2.91
5	OPB-06	541-938	4	4	0	4	0	100	0.74	2.96
6	OPD-06	168-2503	6	6	0	6	0	100	0.86	5.16
7	OPE-01	382-765	7	7	0	7	0	100	0.85	6.00
8	OPE-11	194-1752	5	5	0	5	0	100	0.66	3.33
9	OPE-14	151-1353	8	7	1	8	0	100	0.83	5.87
10	OPN-02	204-868	5	5	0	5	0	100	0.76	3.80
11	OPO-07	734-3781	5	5	0	5	0	100	0.74	3.71
12	OPP-05	128-984	6	5	1	5	0	100	0.79	3.98
13	OPQ-10	259-1703	6	6	0	6	0	100	0.67	4.04
14	OPR-01	237-957	6	6	0	6	0	100	0.79	4.76
15	OPR-05	194-1865	5	5	0	5	0	100	0.76	3.83
Total			84	83	2	84	0	-	-	-
Average			-	-	-	5.6	-	-	0.77	4.35

Cluster Analysis Based on Pooled Data of Molecular Markers

Cluster-I divided into two subclusters. A and B with near about 47% similarity. subcluster A further bifurcated in two group A1 and A2 with near about 50% likeness. Group A1 consisted of eight genotypes H1 (RGP-270), H2 (RGP-245),H3 (RGP-182), H4 (RGP-278), H5 (RGP-276), H7 (RGP-3), H9 (RGP-114), H10(RGP-491), while, group A2 consisted of eight genotypes H6 (RGP-1), H8 (RGP-501), L1 (RGP-474), L2 (RGP-66), L3 (RGP-7), L4 (RGP-581), L9 (RGP-224), L8(RGP-77). Group B consisted of four genotype which was L6 (RGP-585), L7 (RGP-513), L10 (RGP-560), L11 (RGP-607). The clusters-II consisted of only one genotype L5 (RGP-602) that was mostdiversified among all the 21 genotypes.

Conclusions

Based on the molecular markers associated with garlic genotypes, it was concluded that molecular markers like RAPD and ISSR are most reliable to distinguish garlic genotypes. breeders can identify the diverse genotypes and by involving these genotypes in breeding programme for crop improvement based on higher total soluble solid content. They can plan to develop new garlic variety with higher total soluble solid content.

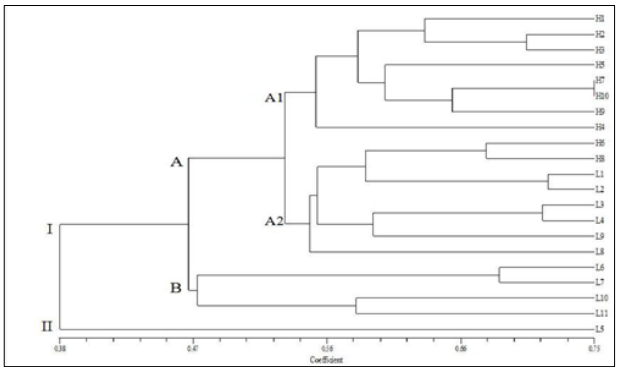

Fig 1: Dendrogram Depicting the Genetic Relationship Among 21 Garlic Genotypes Based on Pooled Data of Molecular Markers.

Table 4: Size, Number of Amplified Bands, Percent Polymorphism and PIC Obtained by ISSR Primers in the 21 garlic Genotypes

Sr. No.	ISSR Primer	Band Size (bp)	Total No. of Bands (A)	Polymorphic Bands (B)			Mono- Mor phic Band	% Poly- Mor Phism (B/A)	PIC*	IPI
				S	U	T				
1	UBC-810	580-2323	5	5	0	5	0	100	0.77	3.88
2	UBC-814	197-2107	6	6	0	6	0	100	0.82	4.94
3	UBC-824	378-4757	9	9	0	9	0	100	0.86	7.79
4	UBC-826	371-1849	5	5	0	5	0	100	0.64	3.24
5	UBC-829	485-3164	7	7	0	7	0	100	0.70	4.96
6	UBC-830	447-2143	3	3	0	3	0	100	0.56	1.68
7	UBC-834	152-1360	7	7	0	7	0	100	0.83	5.82
8	UBC-840	456-1437	4	3	1	4	0	100	0.61	2.45
9	UBC-841	204-1164	4	4	0	4	0	100	0.76	3.07
10	UBC-845	862-1680	2	2	0	2	0	100	0.50	1.00
11	UBC-852	304-1196	4	4	0	4	0	100	0.74	2.98
12	UBC-857	216-1843	6	5	1	6	0	100	0.71	4.31
13	UBC-864	267-1563	3	3	0	3	0	100	0.63	1.89
14	UBC-872	158-813	6	6	0	6	0	100	0.74	4.49

15	UBC-873	365-1168	4	4	0	4	0	100	0.66	2.65
16	UBC-876	334-768	2	2	0	2	0	100	0.50	1.00
17	UBC-879	645-2700	4	3	1	4	0	100	0.64	2.59
18	UBC-880	228-1018	6	6	0	6	0	100	0.76	4.56
Total			87	84	3	87	0	-	-	-
average			-	-	-	4.83	-	100	0.69	3.52

Table 5: Size, number of amplified bands, percent polymorphism and PIC obtained by SSR primers in garlic genotypes

Sr. No.	SSR Primer	Band Size (bp)	Total No. of Bands (A)	Polymorphic Bands (B)			Mono- Morphic Band	% Poly- Morphism (B/A)	PIC*	SPI
				S	U	T				
1	SSR-2	202	1	1	0	1	0	100	0	0
2	SSR-5	146	1	1	0	1	0	100	0	0
3	SSR-6	141	1	1	0	1	0	100	0	0
4	SSR-7	166	1	1	0	1	0	100	0	0
5	SSR-8	293	1	1	0	1	0	100	0	0
6	SSR-9	172	1	1	0	1	0	100	0	0
7	SSR-13	140	1	0	0	0	1	0	0	0
8	SSR-14	413-616	2	2	0	2	0	100	0.5	1
9	SSR-15	155	1	1	0	1	0	100	0	0
10	SSR-17	165	1	1	0	1	0	100	0	0
11	SSR-19	161	1	1	0	1	0	100	0	0
12	SSR-20	415	1	1	0	1	0	100	0	0
Total			13	12	-	12	1	-	-	-
Average			-	-	-	1	-	91.66	0.0416	0.0833

Table 6: Jaccard's Similarity coefficient of garlic genotypes based on pooled data of molecular markers

	H 1	H 2	H 3	H 4	H 5	H 6	H 7	H 8	H 9	H 10	L 1	L 2	L 3	L 4	L 5	L 6	L 7	L 8	L 9	L 10	L 11
H 1	1.00																				
H 2	0.65	1.00																			
H 3	0.62	0.70	1.00																		
H 4	0.55	0.50	0.63	1.00																	
H 5	0.51	0.57	0.65	0.55	1.00																
H 6	0.49	0.55	0.54	0.44	0.54	1.00															
H 7	0.56	0.57	0.62	0.52	0.65	0.59	1.00														
H 8	0.53	0.63	0.60	0.46	0.53	0.68	0.68	1.00													
H 9	0.56	0.53	0.64	0.57	0.56	0.56	0.62	0.59	1.00												
H 10	0.57	0.59	0.67	0.59	0.60	0.53	0.75	0.60	0.68	1.00											
L 1	0.50	0.61	0.61	0.46	0.56	0.57	0.58	0.61	0.46	0.52	1.00										
L 2	0.44	0.57	0.58	0.41	0.56	0.58	0.59	0.60	0.48	0.55	0.72	1.00									
L 3	0.49	0.60	0.59	0.46	0.59	0.63	0.64	0.64	0.56	0.60	0.61	0.62	1.00								
L 4	0.46	0.58	0.56	0.47	0.54	0.54	0.58	0.55	0.51	0.50	0.52	0.56	0.72	1.00							
L 5	0.33	0.40	0.38	0.37	0.42	0.41	0.37	0.39	0.33	0.34	0.34	0.33	0.46	0.46	1.00						
L 6	0.45	0.45	0.52	0.52	0.50	0.39	0.49	0.39	0.51	0.50	0.36	0.33	0.48	0.49	0.39	1.00					
L 7	0.47	0.45	0.54	0.52	0.50	0.46	0.55	0.46	0.57	0.60	0.38	0.38	0.49	0.43	0.40	0.69	1.00				
L 8	0.43	0.58	0.57	0.44	0.55	0.50	0.54	0.56	0.48	0.51	0.55	0.60	0.58	0.55	0.42	0.41	0.48	1.00			
L 9	0.44	0.51	0.53	0.45	0.50	0.45	0.58	0.52	0.50	0.57	0.53	0.53	0.63	0.57	0.36	0.45	0.49	0.52	1.00		
L 10	0.42	0.42	0.49	0.48	0.48	0.38	0.52	0.43	0.57	0.54	0.39	0.41	0.52	0.44	0.29	0.43	0.54	0.41	0.55	1.00	
L 11	0.41	0.41	0.54	0.47	0.50	0.43	0.47	0.44	0.52	0.52	0.39	0.41	0.51	0.43	0.36	0.42	0.50	0.46	0.57	0.58	1.00

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