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Assessment of genetic diversity in garlic (*Allium sativum* L.) genotypes based on ISSR markers

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Abstract
Genetic diversity among one hundred and thirty-one accessions of garlic was analyzed using Inter-simple sequence repeat (ISSR) markers. A total of twelve polymorphic bands were detected with four primers. Genetic similarity result showed genetic variation among the garlic accessions ranged from (0.00) to (1.00) with a mean of 0.501. All 131 accessions were clustered into two major groups. The smallest genetic similarity value was observed between PG-20 and all genotypes except K1 (0.500) and W6-12840 (0.125) genotypes, which appear as the most dissimilar accessions and distantly related. The maximum genetic similarity value of 1.00 was obtained between PG-32 with F4, F5, BG-108, F2, CGF-2 genotypes. The genotypes PG-20, K1, WG-35698, Single Kale, GG-4, RG-77, CFG-3, G-335, and AC-200 are identified as more diverse among the genotypes under study could be used for the genetic improvement of garlic cultivars.

Keywords: diversity, genotypes, ISSR, *Allium sativum*

Introduction
Garlic (*Allium sativum* L.) is a monocotyledonous vegetable grown and used as spice and flavoring agent for foods (Velisek *et al.,* 1997) [23] and its origin in Central Asia (Kazakhstan) with secondary centers of diversification in China and the Mediterranean area (Vavilov, 1951; Etoh and Simon, 2002) [22, 6]. It is a diploid species (2n = 2x = 16) of obligated apomixes, therefore, its reproduction is vegetative (Mc Collum, 1987; Figliuolo *et al.,* 2001; Ipek *et al.,* 2003 & 2005) [14, 7, 8, 9]. Garlic has a very large genome 33.5 pg/2C (Ranjekar *et al.,* 1978) [19].
Garlic is grown in the world over 12.25 lakh ha with 242.55 lakh tons production which translates into 12.80 tons/ha productivity. China is the world leader in production (192.33 lakh tons) contributing to 77.07 % of world tonnage followed by India (Anonymous, 2014). In our country, the average productivity of 5.4 tons/ha which is quite low as compared to the other garlic growing countries (Singh *et al.,* 2012) [21]. To increase the production and productivity of this crop for domestic and international market, there is urgent need to screen the germplasm to select and improve cultivars for quantitative traits.
For centuries, garlic has been clonally propagated, which may be speculated to result in a bottleneck for genetic variation in garlic. In India, the effort of germplasm collection and evaluation has been made but the information for higher yield and yield contributing characters is limited. Therefore, it is essential to estimate the genetic variation among the germplasm for selection of diverse parents which may be useful as donors to complement various breeding methods. Presently, such assessment is mainly based on a small number of phenotypic traits. However, environmental conditions may affect their expression and so assessing only morphological traits may not reflect the genetic diversity available.
In recent years, molecular markers such as random amplified polymorphic DNA (RAPD) (Ipek *et al*., 2003) [8, 9] amplified fragment length polymorphisms (AFLP) (Morales *et al*., 2013) [15], SSR (Cunha *et al*., 2012; Ma *et al*., 2009) [4, 13], sequence-related amplified polymorphism (SRAP) (Chen *et al*., 2013) [2], inter-simple sequence repeat (ISSR) (Jabbes *et al*., 2011) [10] have been used to assess genetic diversity and the relationships among garlic varieties, as they are not affected by environmental conditions (Jo *et al*., 2012) [11]. These markers are extremely sensitive and are capable of identifying allelic germplasm collected from the different geographical regions of the world.
The objective of this study was to assess genetic diversity of one hundred and thirty-one garlic genotypes collected from different parts of India based on ISSR markers will make us understand the variation between accessions and select out those with our interested character for various breeding programs.

Materials and Methods

Plant material

The experimental material comprised of 131 garlic accessions representing landraces, released varieties, cultivars and breeding lines obtained from different breeding centers of India and source of the collection are present in Table 1. The experimental trail was laid out in Randomized Block Design (RBD) with three replication each during the 2014-Rabi season. Standard agronomic practices were followed during the course of the investigation. All the field experiments were conducted at Horticultural Research Centre and molecular work was performed at Molecular Biology Laboratory (MBL), Department of Genetics and Plant Breeding, College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (U.P), India ($29^{o}01$ latitude N and $77^{o}43$ longitude E & 219.75 MSL).

Table 1: List of 131 garlic genotypes used and their source of collection for present study

S/N Germplasm	Source
1. F-4	SVPUAT, Meerut
2. KANT GOLA	SVPUAT, Meerut
3. F-5	SVPUAT, Meerut
4. PG-32	SVPUAT, Meerut
5. GODAVARI	SVPUAT, Meerut
6. CFG-3	SVPUAT, Meerut
7. BG-108	SVPUAT, Meerut
8. GG-1	SVPUAT, Meerut
9. G-50	SVPUAT, Meerut
10. F-2	SVPUAT, Meerut
11. CFG-2	SVPUAT, Meerut
12. PHULE BASANT	SVPUAT, Meerut
13. PG-35	SVPUAT, Meerut
14. CL LAMBA	SVPUAT, Meerut
15. SINGLE KALE	SVPUAT, Meerut
16. CFG-4	SVPUAT, Meerut
17. G-335	SVPUAT, Meerut
18. F-3	SVPUAT, Meerut
19. CHACHENA MOTA	SVPUAT, Meerut
20. AVT G-4	SVPUAT, Meerut
21. F-13	SVPUAT, Meerut
22. GG-4	SVPUAT, Meerut
23. BHIMA OMKAR	SVPUAT, Meerut
24. CGG-8	SVPUAT, Meerut
25. BHIMA PURPLE	SVPUAT, Meerut
26. DESI LASHUN	SVPUAT, Meerut
27. PALAMPUR SELECTION	Palampur, H.P.
28. TG-F1	Telangana
29. GHC-1	Palampur, H.P.
30. K1	J & K
31. TG-F2	Telangana
32. TG-F3	Telangana
33.CHAMLOA LOCAL	Palampur, H.P.
34. TG-F4	Telangana
35. TG-F5	Telangana
36. K2	J & K
37. TG-F6	Telangana
38. TG-F7	Telangana
39. PUNNUR LOCAL	Palampur, H.P.
40. KADARI-4	SVPUAT, Meerut
41. CFG-6	SVPUAT, Meerut
42. CFG-5	SVPUAT, Meerut
43. CFG-7	SVPUAT, Meerut
44. CFG-1	SVPUAT, Meerut
45. F-6 SF	SVPUAT, Meerut
46. AVTG-1	SVPUAT, Meerut
47. G-282	SVPUAT, Meerut
48. PG-17	SVPUAT, Meerut
49. G-282	SVPUAT, Meerut
50. UP CHATTA	SVPUAT, Meerut
51. PG-24	SVPUAT, Meerut
52. CFG-8	SVPUAT, Meerut
53. PG-9	SVPUAT, Meerut
54. JAWA	SVPUAT, Meerut
55. ROHINI-2	SVPUAT, Meerut
56. CHEENIA	SVPUAT, Meerut
57. HARI RANI	SVPUAT, Meerut
58. INDIAN GARLIC	SVPUAT, Meerut
59. G-323	SVPUAT, Meerut
60. F-2 SF	SVPUAT, Meerut
61. F-1	SVPUAT, Meerut
62. SAKHA-44	SVPUAT, Meerut
63. F-3-1	SVPUAT, Meerut
64. GG-2	SVPUAT, Meerut
65. AVTG-5	PAU, Ludhiana
66. GRS-1330	PAU, Ludhiana
67. W6-12840	PAU, Ludhiana
68. PG-31	PAU, Ludhiana
69. F-2013-10	PAU, Ludhiana
70. BGSD-1228	PAU, Ludhiana
71. BGSD-1217	PAU, Ludhiana
72. F-2013-7	PAU, Ludhiana
73. PG-24	PAU, Ludhiana
74. AVTG-2	PAU, Ludhiana
75. IETG-5	PAU, Ludhiana
76. BGSD-1230	PAU, Ludhiana
77. AL-50	PAU, Ludhiana
78. BGSD-1219	PAU, Ludhiana
79. IETG-9	PAU, Ludhiana
80. BGSD-1225	PAU, Ludhiana
81. F-2013-16	PAU, Ludhiana
82. GRS-1337	PAU, Ludhiana
83. INGD-216	PAU, Ludhiana
84. F-2013-11	PAU, Ludhiana
85. GRS-1345	PAU, Ludhiana
86. F-2013-13	PAU, Ludhiana
87. F-2013-12	PAU, Ludhiana
88. GRS-1332	PAU, Ludhiana
89. F-2013-18	PAU, Ludhiana
90. W6-35698	PAU, Ludhiana
91. PG-18	PAU, Ludhiana
92. AVTG-4	PAU, Ludhiana
93. F-II-SF	PAU, Ludhiana
94. IETG-6	PAU, Ludhiana
95. BG-117	PAU, Ludhiana
96. F-2013-17	PAU, Ludhiana
97. F-2013-4	PAU, Ludhiana
98. PG-20	PAU, Ludhiana
99. GRS-1338	PAU, Ludhiana
100. BGSD-1232	PAU, Ludhiana
101. F-2013-3	PAU, Ludhiana
102. 581	DOGR, Pune
103. M-162	DOGR, Pune
104. IC-372944	DOGR, Pune
105. IC-370510	DOGR, Pune
106. M-352	DOGR, Pune
107. WG-34	DOGR, Pune
108. 646	DOGR, Pune
109. IC-374981	DOGR, Pune
110. IC-372907	DOGR, Pune
111. 606	DOGR, Pune
112. 650	DOGR, Pune
113. WG-418	DOGR, Pune
14. IC-372930	DOGR, Pune
115. 681	DOGR, Pune
116. IC-175327	DOGR, Pune
117. 486	DOGR, Pune
118. WG-13	DOGR, Pune
119. IC-372954	DOGR, Pune
120. AC-200	DOGR, Pune
121. RG-338	DOGR, Pune
122. 604	DOGR, Pune
123. WG-29	DOGR, Pune
124. IC-87880	DOGR, Pune
125. 599	DOGR, Pune
126. IC-64363	DOGR, Pune
127. 678	DOGR, Pune
128. 652	DOGR, Pune
129. IC-372905	DOGR, Pune
130. RG-77	DOGR, Pune
131. WG-48	DOGR, Pune

DOGR-Directorate of Onion and Garlic Research, Pune;
H.P-Palampur, Himachal Pradesh;
J & K-Jammu & Kashmir;
SVPUAT-Sardar Vallabhabhai Patel University of Agri. & Tech, Meerut,
PAU-Punjab Agriculture University, Ludhiana.

DNA extraction and ISSR amplification

The total DNA was isolated from and young leaf tissues following CTAB method (Doyle and Doyle, 1987) [5]. The quality of DNA was checked on 0.8% agarose gel and DNA concentrate was determined using Bio-Rad's Spec^{TM} Plus spectrophotometer. The DNA concentration was adjusted to 25ng/ul. A total of 10 ISSR markers were used for diversity analysis (Table 2). The primers were custom synthesized by Macflow Engineering Pvt. Ltd. The PCR reaction was setup and DNA amplification was performed in final volume of 20μl of ISSR reaction mixture included 6μl DNA(25ng/μl), 2μl Taq buffer 10x with $MgCl_2$, 2μl primers (5μM), 4μl dNTPs Mix (1mM), 1μl Taq DNA polymerase (U/μl) and 5μl Milli Pore water. The amplification reactions were performed

in BIO-RAD MyCycler^{TM}: Initial denaturation 94ºC for 5min followed by 38 cycles of denaturation (94ºC for 1min),annealing (35-37ºC for 1 min), extension (72ºC for 2 min) and a final extension 72ºC for 7 min. PCR product were separated via Type 1 agarose gel (2%) electrophoresis in 1x TAE buffer for 1½ h and for size marker we used a 100 bp DNA ladder (Bangalore Genei Pvt. Ltd., Bangalore, India) and the ethidium bromide stained gels were photographed and documented using Alpha Imager 1200^{TM} (Alpha Innotech Corporation, USA).

Table 2: List of ISSR primers, sequences, and annealing temperatures

		ISSR PRIMERS	
S. No	**Primer Code**	**Sequence 5'-3'**	**Annealing Temperature (º C)**
1.	ISSR-05	GGAGAGGAGAGGAGA	48
2.	UBC 808	AGAGAGAGAGAGAGAGC	52
3.	UBC 810	GAGAGAGAGAGAGAGAT	50
4.	UBC 824	TCTCTCTCTCTCTCTCG	52

Molecular data analysis

Distinct and reproducible bands produced by ISSR were scored as either present (1) or absent (0) on the gels. ISSR bands of individual genotypes were recorded. The data of two matrices were then used for the following statistical analysis. PIC value was calculated using the formula PIC = $1-\sum p_i^2$, where p_i is the frequency of the ith allele (Kumar *et al*., 2011) [12]. Marker index (MI) is the statistic used to calculate the overall utility of a marker system, it is the product of polymorphic information content (or expected heterozygosity) and the effective multiplex ratio (MI = PIC x EMR). EMR effective multiplex ratio is the product of the total number of alleles per primer (n). The higher the value of MI better with the technique. (Powell *et al.,* (1996) [18] and Nagaraju *et al*., (2001) [16]. The power of each primer to distinguish among the studied genotypes was evaluated by the Resolving Power (RP) (Prevost and Wilkinson, 1999) [17]. i.e., Rp= Σ Ib, where, Ib is the band informativeness, that takes the values of 1-(2 x [0.5-p]), being p the proportion of the garlic genotypes containing the band.

Results and Discussion

A total of twelve bands were obtained from four primers, showing an average of three bands per primer. All the amplified bands were observed to be polymorphic and a number of bands produced per primer ranged from one (UBC 824 and ISSR-05) to six (UBC 810) with the average number of polymorphic bands was three per primer. Earlier Chen *et al.* (2014) [3] screened 39 garlic genotypes using 17 ISSR primers and Shaaf *et al.* (2014) [20] analyzed 31 garlic genotypes with 6 ISSR primers. Furthermore, the size of fragments ranged from 240 bp to 800 bp.

Moreover, PIC values ranged from 0.045 (ISSR-05) to 0.99 (UBC 824) with an average PIC of 0.650 (Table 3 and Fig 1) which was observed to be low when compared with earlier reports of Chen *et al.* (2014) [3] with average PIC value of 0.72. Resolving power (RP) varies between 0.198 (UBC 824) and 4.062 (UBC 810) with an average value of 2.410 suggesting that the set of ISSR primers used was not sufficient to distinguish the genotypes. Whereas Marker index (MI) values varied from 0.045 (ISSR-05) to 4.803 (UBC-810) with an average value of 2.225.

Values of PIC and MI parameters to compare the informative content of polymorphic ISSR markers and use of RP to select the most informative ISSR marker to distinguish between the different genotypes. Based on values of PIC, RP, and MI it may be concluded that ISSR primer UBC 810 having above average PIC, MI and more efficient in the present analysis.

All bands generated from four ISSR primers were further subjected to genetic similarity (GS) assessment by using Jaccard's similarity index (Table 4 and Fig 2.). Genetic similarity result showed significant genetic variation among all garlic accessions that was assessed. GS ranged from (0.00) to (1.00) with a mean of 0.501. Out of the 131 pairwise combinations generated, the smallest GS value was observed between PG-20 and all genotypes except K1 (0.500) and W6-12840 (0.125) genotypes, which indicates they are the most dissimilar accessions and distantly related. The maximum genetic similarity value of 1.00 was obtained between PG-32 with F4, F5, BG-108, F2, CGF-2, *etc.,* genotypes.

Cluster analysis based on ISSR molecular markers can be presented in a dendrogram (Fig. 2) to indicate the estimated relations between different genotypes. In this study, cluster analysis based on unweighted paired group method of arithmetic means (UPGMA) in XLSTAT version 2015.1.01 software with 4 ISSR primers was used for the classification of cultivars and based on clustering, 131 garlic genotypes were clustered into two main groups Group I and Group II. While, Groups I, includes only two genotypes *viz.,* K1 (from Jammu and Kashmir) and PG-20 (from Punjab), Group II, includes 129 genotypes and was further divided into three main Clusters (GI, GII and GIII). Meanwhile, GII-1 & GII-2 includes forty-two and eleven genotypes. Furthermore, GII-3 which is a complex cluster includes seventy-six genotypes. Similar findings were earlier reported by Jabbes *et al.* (2011) [10] screened as many as 35 garlic genotypes using 7 ISSR markers and Shaaf *et al.* (2014) [20] evaluated 31 garlic genotypes using 6 ISSR markers and Chen *et al.* (2014) [3] screened 39 genotypes using ISSR primers.

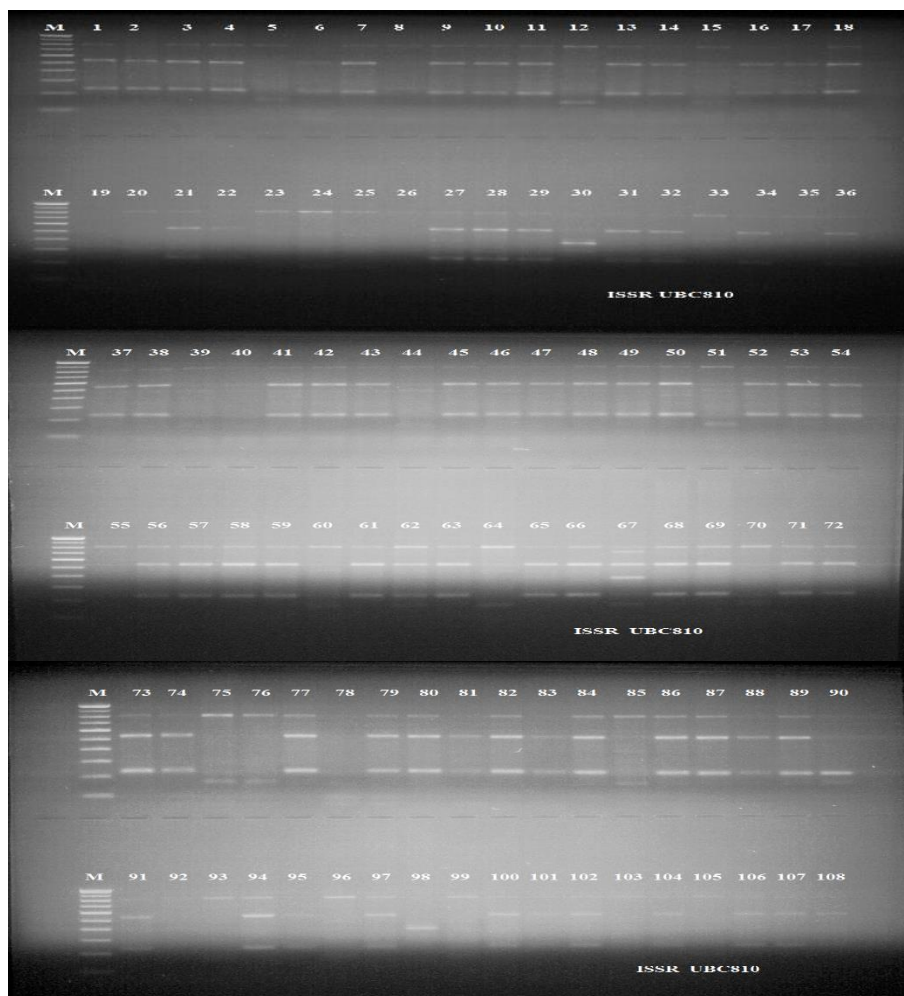
India holds an immense resource of garlic cultivars that are great significance not only for breeders but also for farmers. The result of the present study proved the utility of ISSR markers in genetic diversity at random regions of the genome of garlic genotypes. All the genotypes analyzed were not distinct from each other at the molecular level this may be due to insufficient availability of primers to distinguish the genotypes. The genotypes PG-20, K1, WG-35698, Single Kale, GG-4, RG-77, CFG-3, G-335, and AC-200 are identified as more diverse in contrast to other genotypes. So these genotypes could be used for the genetic improvement of garlic cultivars.

Table 3: Primer name, Primer sequences, Molecular weight range, PIC, RP, MI, Monomorphic bands, Polymorphic bands and % Polymorphism values in ISSR analysis

S. N.	Primer Name	Molecular weight Range (bp)	PIC	RP	MI	Monomorphic Band	Polymorphic Band	Polymorphic %
1	ISSR-05	570	0.045	1.954	0.045	0	1	100
2	UBC808	400-720	0.766	3.390	3.064	0	4	100
3	UBC810	240-800	0.801	4.062	4.803	0	6	100
4	UBC824	600	0.990	0.198	0.990	0	1	100
	Average		0.650	2.401	2.22	Average- 0 Total-0	Average- 3 Total-12	

Table 4: Cluster analysis based on genetic distance and UPGMA dendrogram on Jaccard's similarity coefficient obtained from ISSR analysis of 131 garlic genotypes.

	Groups	Main cluster	Cluster		Genotypes
ISSR	I	1	---	2	K1, PG-20
	II	3	GI	42	'652, 678, WG-29, IC-878880, IC-64363, WG-48, W6-35698, 650, BGSD-1219, AVTG-4, RG-338, BHIMA PURPLE, ROHINI-2, 604, BHIMA ONKAR, F-II SF, IC-370510, M162, F-2013-17, GRS-1338, GRS-1345, PHULE BASANT, CGG-8, 486, IC-175327, IC372930, IC372907, WG-418, CFG-1, PUNNUR LOCAL, TG-F5, CHAMLOA LOCAL, GG-1, AVTG-4, SINGLE KALE, BGSD-1230, IETG-5, BGSD-1228, GG-2, F-2 SF, GODAVARI and PG-24;
			GII	11	'GRS-1332, GG-4, F-2013-3, INDG-216, CHACHENA, F-2013-16, CFG-3, BG-117, AC-200, DESI LASHUN and KADARI-4.
			GIII	76	'W6-12480, RG-77, SAKHA-44, IC-374981, 606, KANT GOLA, PG-18, IC-372954, CHEENA, WG-13, F-2013-4, G-335, 646, 599, 681, AVTG-2, PG-24, AVTG-5, CFG-8, G-282, K2, TG-F4, TG-F3, TG-F2, CL LAMBA, CFG-4, IC-372905, BGSD-1232, IETG-6, F-2013-18, F-2013-12, F-2013-13, F-2013-11, GRS-1337, BGSD-1225, IETG-9, AL-50, BGSD-1217, F-2013-10, PG-31, GRS-1330, F-3, F-1, G-323, INDIAN GARLIC, HARI RANI, JAWA, PG-9, UP CHATTA, G-282, PF-17, AVTG-1, F-6 SF, CFG-7, CFG-5, CFG-6, TG-F7, TG-F6, GHC-1, TG-F1, PALAMPUR LOCAL, F-13, F-3, PG-35, CFG-2, F-2, G-50, BG-108, PG-32, F-4, F-5, WG-34, M-352, IC-372944, F-2013-7 and 581.
			Total	131	



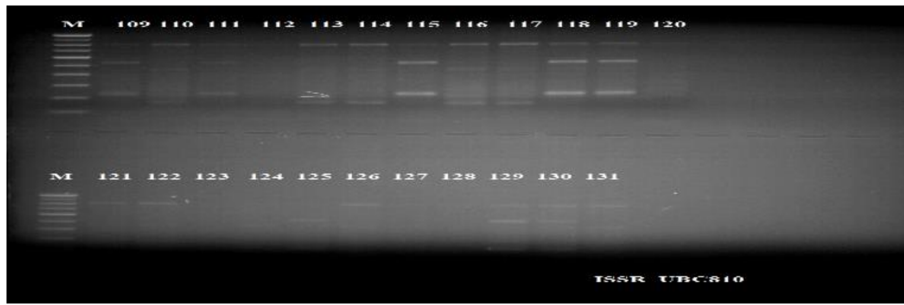


Fig 1: ISSR profiling pattern of 131 garlic genotypes with UBC810 primer (M=100bp molecular marker)

Fig 2: Dendrogram showing clustering of 131 garlic genotypes constructed using UPGMA based on Jaccard's similarity coefficient obtained from ISSR analysis

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