Unravelling Genetic Diversity of Garlic (*Allium Sativum* L.) Genotypes through RAPD Molecular Markers

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Abstract

Garlic (*Allium sativum* L.) is a multipurpose crop of high nutritional and economic value used as vegetable, spice and medicinal plant. The purpose of this research was to assess the genetic variability between indigenous and exotic garlic germplasm using 50 Randomly Amplified Polymorphic DNA (RAPD) markers. After initial screening 25 RAPD markers were selected for further evaluation of garlic genotypes. All the markers produced 621 polymorphic RAPD amplicons depicting considerable genetic variation for the studied loci. Markers presence/ absence data was used to calculate Euclidean distances among seven garlic genotypes. The highest genetic similarity was observed between MJ-84 & Italian genotype (88%) followed by Lehson Gulabi & GTS-001 (73%) and then Chinese & Italian genotypes (72 %). Moreover,

genotypes Iranian, NARC-09 and Chinese were among the most diverse genotypes on the basis of RAPD markers. Similarity coefficient matrix based dendrogram was constructed which distributed all the genotypes into two major clusters. Six out of the seven genotypes were included in the cluster A (Iranian, Italian, MJ-84, GTS-001, Lehson Gulabi and Chinese) while only 1 genotype (NARC-09) was presented in cluster B. NARC-09 showed more divergence in genetic pattern as compared to other genotypes. The present study will pave a way forward for the breeders to efficiently use the available genetic variability in any crop improvement program. In continuation to the experiment conducted, further genomic studies may be carried out to formulate effective marker assisted selection in the crop.

Keywords: *Genetic Variability, Polymorphism, Cluster Analysis, Marker Assisted Selection,*

Introduction

Garlic is a bulbous perennial herb which belongs to the family *Liliaceae* and genus *Allium*. It is a, diploid species (2n = 2x = 16) of obligated apomixis, therefore its reproduction is vegetative (Sun et al., 2012, Ipek et al. 2003; 2006). Although it is clonally propagated yet considerable genetic variation has been reported in garlic germplasm (Ata, 2005, Buso et al., 2008). Garlic plant shows wide range of variations in color and size of bulb, plant height, flowering, number and size of the cloves, days to harvesting, resistance to storage capacity, dormancy and adaptation to changing agro climatic situations (Mario et al. 2007, Osman et al., 2007, Wang et al., 2014 and Mahmoud et al., 2017). It is a multipurpose crop widely grown due to its high nutritional qualities and medicinal importance (Ipek et al., 2005). Garlic produces a chemical called allicin which is used as dietary supplement for anti-oxidant and anti-inflammatory properties (Rahman, 2007, Bozin et al., 2008 and Block, 2010). In traditional medicine, oil of the garlic is used to treat fungal infections of skin or nails, warts, and corns. It is also applied to the skin for hair loss and thrush. Garlic is injected into the body for chest pain (Mikaili et al., 2013, Zhang et al., 2020).

In foods and beverages, fresh garlic, garlic powder, and garlic oil are used to add flavor (Kik and Gebhardt, 2001, Bongiorno et al., 2008). Its extract has also been used as a remedy for the prevention and treatment of cardiovascular diseases (Zhou et al., 2016). Garlic is used to lower blood pressure, cure parasitic infections, food poisoning tumors and acts as mild anticoagulant (Timba et al., 2019). However, due to its growing demand for food and medicinal purposes there is strong need to develop high yielding, biotic and abiotic stress resistance garlic cultivars. Studies of crop diversity illustrate that present levels of diversity are the outcome of farmer's needs and preferences as well as the increasing availability of a limited number of high yielding

varieties (Jiffinvir et al., 2016). Therefore, as a first step of crop improvement, researchers and breeders need to explore the genetic diversity among the available garlic germplasm. In addition, the maintenance of a high level of genetic diversity is assumed as an indispensable prerequisite for the conservation of viable populations (Reed and Frankham, 2003; Kik, 2008; Petropoulos et al. 2015)

Garlic displays a great morphological diversity in terms of bulb and leaf parameters as size, color and shape, in addition to this scape presence, height, flower color, fertility and bulbils (topset) development in inflorescence (Pooler and Simon, 1993). Genetic characterization of garlic clones maintained in germplasm collections has been accomplished using various molecular markers, such as isozymes (Ipek and Simon, 2002), AFLPs (Lee et al., 2002; Volk et al., 2004; Ipek et al., 2006; Wang et al., 2016), SSRs (Anwar et al., 2016; Kumar et al., 2019 and randomly amplified polymorphic DNA (RAPD) markers (Bradley et al., 1996; Ipek et al., 2003; Khar et al., 2008; Abdoli et al., 2009; Anwar et al., 2020). RAPDs have been used extensively for estimating genetic variations at the population level and have ability to describe better comparison among closely related species owing to its speedy process and simplicity (Pinky et al., 2017; Irshad et al., 2019; Sudha et al., 2019). Isozyme and RAPD markers have been utilized extensively to categorize wide range garlic germplasm (Pooler and Simon, 1993; Al-Zahim et al. 1997; Haksoon et al. 2003).

The RAPD markers are abundantly found in genomes and considered as a powerful technique for detecting DNA polymorphism among cultivars or clones belonging to plant kingdom (Micheli and Bova, 1997). In Pakistan, there is a diverse collection of indigenous and exotic garlic species which differ in qualitative and quantitative traits. However, molecular basis of the genetic diversity in garlic germplasm has not been explored. The present study aims to

evaluate genetic diversity between indigenous and exotic garlic varieties using RAPD markers along with determination of relatedness of various species with each other depending on the hierarchal information.

Material and Methods

Studies on molecular characterization of Garlic genotypes were carried out at Plant Genetic Resources Program at National Agricultural Research Centre, Islamabad. Seven promising cultivars i.e. namely " NARC-001, Italian, MJ- 84, Chinese, GTS-001 Lehsongulabi and Iranian line obtained from the Gene bank of Bio-Resources Conservation Institute (BCI) were characterized to assess the genetic diversity among these genotypes.

Total nucleic acid isolation

All the seven garlic cultivars were grown in pots and 50 g of fresh leaf of each genotype was used to isolate total genomic DNA following CTAB extraction method (Doyle and Doyle, 1987). Upon extraction, genomic DNA was dissolved in desired concentration of the TE buffer with 1µL of RNAase to remove RNA. After extraction the DNA confirmation was done by running it through1% agarose gel that was prepared by adding one gram agarose powder in 100 mL 1X TBE buffer stained with 5 µL ethidium bromide. DNA quantification was done using nanodrop and all the DNA samples were diluted to 50 ng/µL before using it for PCR.

PCR amplification

Garlic varieties were discriminated by decamer oligonucleotides developed by Operon Technologies (Alameda, California, USA) on the basis of DNA polymorphism. After initial screening of RAPD markers, primers producing unambiguous and reliable bands were selected to amplify the genomic DNA of each garlic genotype. PCR amplification reactions were performed in 20 µL PCR tubes where reaction mixture contained PCR buffer (10mM Tris-HCl,

50mM KCl, pH 8.4), 0.25 mM dNTPs, 2 mM $MgCl_2$ and 0.4 mM of forward and reverse primer, 1 unit *Taq* DNA polymerase and approximately 50 ng of template DNA. Reactions were carried out in Veriti Thermal cycler (The Applied Biosystems Inc. USA) by programming for 1 cycle of initial melting for 5 min at 94 °C followed by 50 cycles of denaturation at 94°C for 30 seconds. Annealing was maintained in a range of 32-37 ^{o}C for 30 seconds followed by extension at 72°C for 5 minutes. The final extension was carried out at 72°C for 5 minutes. PCR was performed two times to authenticate the amplification. Amplified products of PCR were resolved on 1.3% agarose gel prepared with 1xTBE buffer (10mM Tris-Borate and 1mM EDTA). A DNA ladder of 1kb was used as standard to measure the amplified fragment size. Gels were stained with Ethidum Bromide dye for 40 minutes and illuminated bands under UV were photographed by Gel documentation apparatus.

Data analysis

Molecular data was recorded on the basis of presence or absence of DNA fragments. Only unambiguous and score able bands were considered for genetic diversity analysis. The binary data obtained was used to estimate the similarity on the basis of the number of shared amplified products to combine the data of all the accessions as expressed in Table 1. Genetic similarity matrices were generated on the basis of Nei and Li's (1979) coefficients equation;

Similarity (F) = 2Nxy/ (Nx+Ny)

Where Nx = the number of scored fragments of individual 'x'

Ny = the number of scored fragments detected in individual 'y' and

Nxy = the number of shared fragments between 'x' and 'y'

NTSyspc 2.0 software package was used for dendrogram construction using unweighted pair group method of arithmetic means (UPGMA).

Table 1: Information of the primers used for diversity studies for seven Garlic Genotypes

Sr. No	Primer name	Sequence 5`- 3`	Bands/ primer	Polymorphic bands	Polymorphism %age
1	**OPA-02**	TGCCGAGCTG	19	12	63.16
2	**OPA-04**	AATCGGGCTG	20	20	100
3	**OPA-16**	AGCCAGCGAA	25	13	52
4	**OPA-17**	GACCGCAGAA	22	22	100
5	**OPA-20**	GTTGCGATCC	20	4	20
6	**OPB-11**	GTAGACCCGT	15	8	53.33
7	**OPB-12**	CCTTGACGCA	18	7	38.89
8	**OPB-15**	GGAGGGTGTT	35	35	100
9	**OPB-18**	CCACAGCAGT	26	17	65.38
10	**OPC-07**	GTCCCGACGT	17	10	58.82
11	**OPC-08**	AGGACCGGTG	39	39	100
12	**OPC-10**	TGTCTGGGTG	26	26	100
13	**OPC-14**	TGCGAGCTAG	33	33	100
14	**OPC-15**	GACGGATCAG	23	23	100
15	**OPC-16**	TGTCATCCCC	26	26	100
16	**OPC-20**	ACTTCGCCAG	9	2	25
17	**OPN-08**	ACCTCAGCTC	15	15	100
18	**OPN-09**	TGCCGGCTTG	20	20	100
19	**OPN-10**	ACAACTGGGG	20	20	100
20	**OPN-11**	TCGCCGCAAA	31	31	100
21	**OPN-12**	CACAGACACC	25	25	100
22	**OPN-13**	AGCGTCACTC	16	10	62.5
23	**OPN-15**	CAGCGACTGT	26	19	73.08
24	**OPN-17**	CATTGGGGAG	18	18	100
25	**OPN-20**	GGTGCTCCGT	9	9	100

Results and Discussion

DNA samples from all the genotypes were preliminarily tested with 50 primers to find out polymorphic bands. From 50 randomly tested primers only 25 were chosen (Table 1) based on the detected polymorphic bands and were used to analyze 7 genotypes. These primers produced 621 unambiguous, readable and reproducible markers which were used to analyze genetic diversity among these cultivars. The number of amplification products generated by each primer varied from 09 (OPC-20 and OPN 20) to 39 (OPC-08) with an average of 25 bands per primer. The size of amplified fragment ranged from 250-2500 bp. All primers produced polymorphic bands where polymorphism was 100% in case of 15 primers while some variation ranging in between 20 % to 73 % was also observed for other primers. Two primers namely OPA-20 and OPC-20 were found to have minimum level of polymorphism as they could demonstrate only 25% and 20% polymorphism (Table 1).

Due to the large number of bands which detected polymorphism in this analysis, it can be said that the RAPDs gave a good approximation of the genetic relationship existing among the evaluated clones. It is assumed that the amplified DNA fragments (amplicons) that co-migrate in the different accessions are similar in their sequences. Shaaf et al. (2014) isolated and analyzed several common RAPD garlic bands and analyzed their homology by means of a DNA hybridization process under two types of astringency, low and medium-high. The results obtained indicated that all the common RAPD bands isolated of similar intensity showed high homology. However, in the gel some of these common bands showed signals of hybridization of different strength, which would indicate the existence of some differences in sequences homology. Another study, carried out on garlic locus specific DNA markers, pointed out that 95.4% of the fragments analyzed had a high homology rate at DNA sequences level (Ipek et al.,

2008). The analysis of genetic diversity plays an important role in breeding programs. In this order, molecular techniques could be used to evaluate diversity between genotypes. The RAPD technique could be effective in detecting the genetic variation in Garlic (Adesoye et al. 2012). In this study RAPD markers were generated from 7 genotypes of Garlic with the objective to evaluate genetic differences. Twenty five random decamer primers were selected from initially tried on the seven genotypes of Garlic.

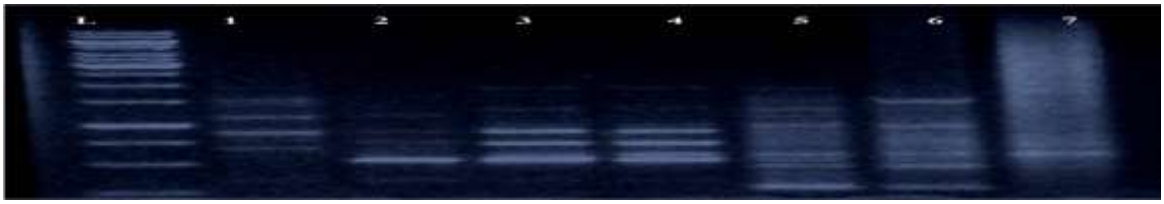

Fig-1: RAPD fragments with primer OPC-08

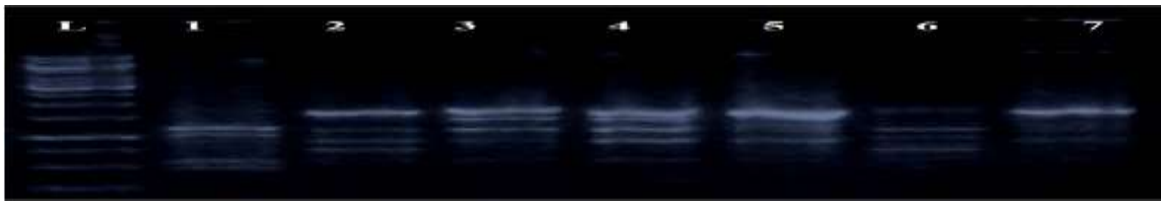

Fig-2: RAPD fragments with primer OPB-15

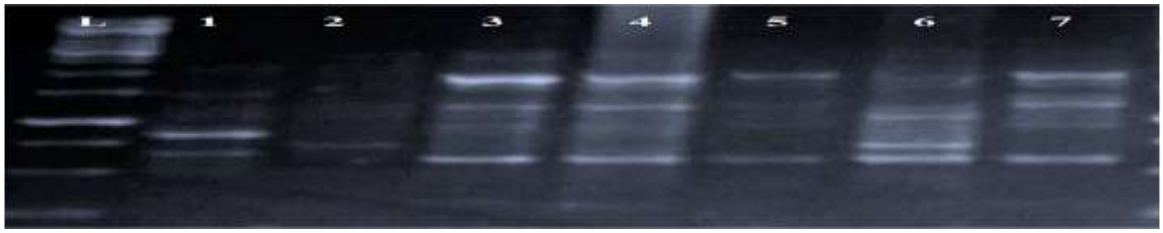

Fig-3: RAPD fragments with primer OPN-11

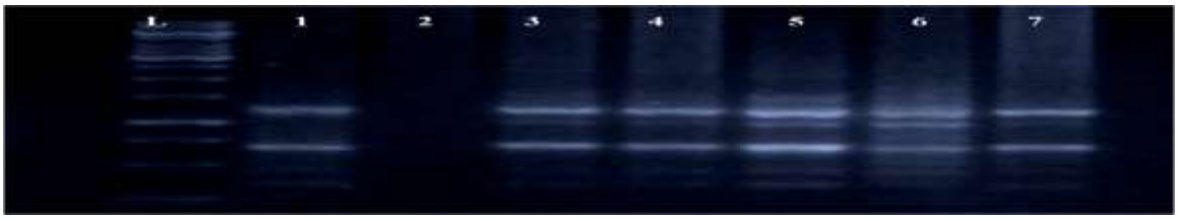

Fig-4: RAPD fragments with primer OPA-16

A similarity matrix based on the proportion of shared RAPD fragments were used to establish the level of relatedness between Garlic genotypes. The number of bands varied in different samples with the level of similarity between samples ranging 0.88 to 0.45 (Table 2). Higher the value of similarity coefficient, closer will be the relation of two genotypes and a value above 0.5 known to be representing the significant relatedness of the concerned genotypes. From the case studied MJ-84 and Italian garlic were the closest genotypes with highest average similarity index value of 0.88. This was followed by 73 and 72% similarity between Lehson Gulabi and GTS-001 and Chinese and Italian genotypes respectively. Among all the available garlic genotypes the lowest values form each pair were noted in case of NARC-09 being the part of that group. It is clear from Table-2 that it could not gain higher value of similarity for any other genotype as compared to rest of the genotypes. The lowest level of similarity at 45% was obtained between NARC-09 and Chinese garlic varieties. In addition to the NARC-09, Iranian genotype was found to be less similar with Chinese and GTS-001 having 0.52 and 0.5 index value.

Table 2: RAPD Similarity matrix based on similarity coefficient of the amplified bands of the garlic genotypes

	NARC-09	**Iranian**	**MJ-84**	**Italian**	**Chinese**	**Lehson Gulabi**	**GTS-001**
NARC-09	1						
Iranian	0.54	1					
MJ-84	0.52	0.64	1				
Italian	0.51	0.61	0.88	1			
Chinese	0.45	0.52	0.7	0.72	1		
Lesson G	0.55	0.69	0.7	0.66	0.66	1	
GTS-001	0.46	0.5	0.67	0.7	0.61	0.73	1

The cluster analysis was portrayed as a dendrogram indicating the estimated relations between the Garlic genotypes. Cluster analysis divides genotypes into smaller number groups at different levels of similarity index where variation within a group is non-significant while among the groups is significant. A dendrogram was created by PAST, using seven genotypes that divided them into two major groups, six out of the seven genotypes were included in the cluster A (Iranian, Italian, MJ-84, GTS-001, Lehson Gulabi and Chinese) while only 1 genotype (NARC-09) was presented in cluster B. The results revealed by the dendrogram were also supported by the similarity matrix as expressed in Table 2. The study of Rosales-Longo et al. (2007) was in strong conformity with our results. In the present study polymorphism among garlic genotypes were detected by RAPD-PCR. Polymorphism among different Garlic genotypes using PCR based RAPD markers profiles along with dendrogram are presented in Fig 5. Hierarchal banding pattern based on electrophoretic data grouped the seven genotypes into two main clusters at 50 % homology (Fig 1). Present results revealed a moderate genetic diversity in the tested germplasm based on RAPD analysis. Among the tested genotypes NARC-09 was most divergent as compared to the others. It was also observed that the major cluster (Cluster B) was further splitted into two groups at 60 % similarity index where Iranian genotype got separated. As we move on towards narrowing down the similarity the genotypes get more divergent as aove 90% all represented separate group.

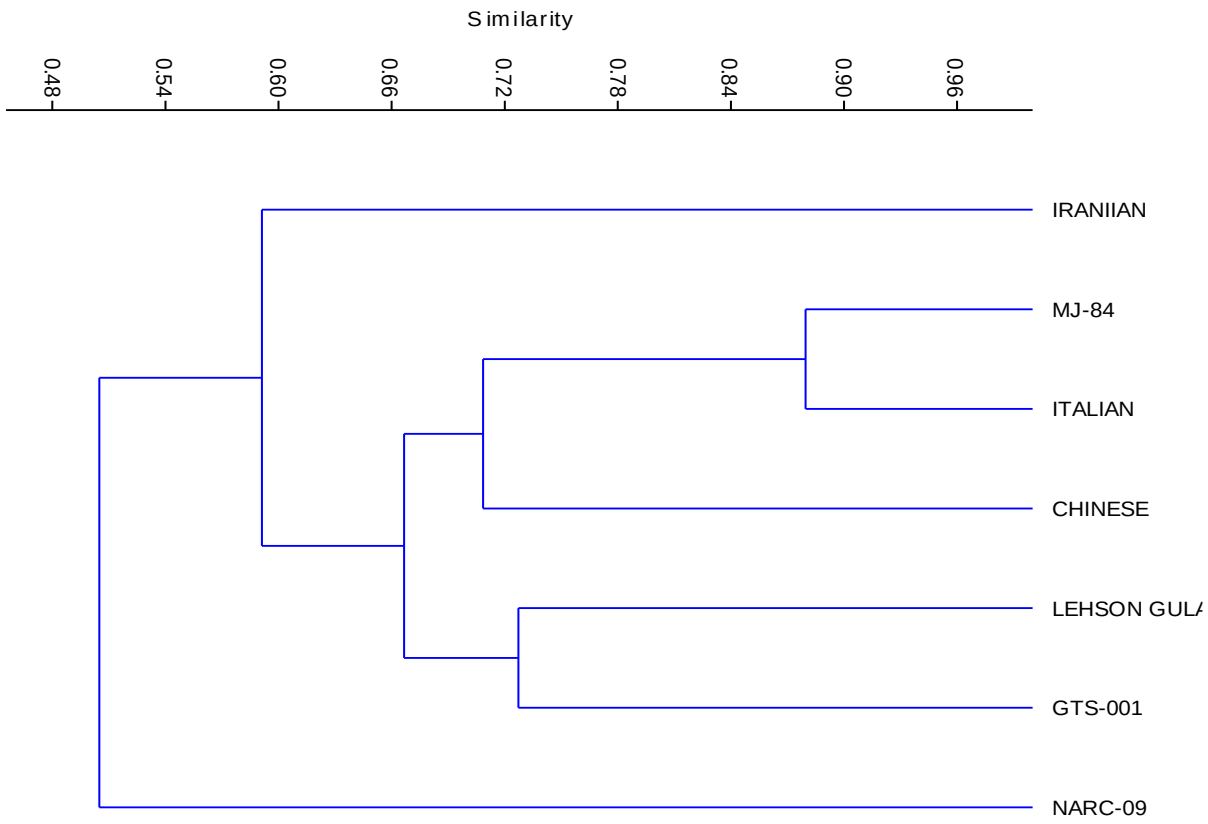

Figure 1:Dendrogram showing the genetic relationship (similarity coefficient) between seven Garlic genotypes using 25 RAPD markers

Conclusion

The objective of this study was to find out inherent genetic diversity between varieties of garlic. In this research, RAPD markers were employed to evaluate the molecular marker based variations among 7 genotypes of garlic. Fifty RAPD primers were tested, out of which 25 showed polymorphism among garlic genotypes. On the basis of molecular data, NARC-09 was found to be the most diverse genotypes among all the genotypes. Results of dendrogram generated on the information of 25 markers revealed that high genetic variability was present between cultivars and can be used in an effective crop improvement program.

Authors Contribution

Muhammad Jalal Hassan and Madiha Urooj conducted the research activity and drafted the manuscript. Rana Arsalan Javaid and Hafiz Muhammad Jhanzaib participated in data recording, statistical analysis and paper write up. Humaira Iqbal and Qurat ul Ain Sani performed the lab activities including DNA Extraction and PCR performance. Haris Khurshid and Faiza Siddique helped in field activities and data recording. Abid Majeed and Muhammad Arshad supervised the research and did proof reading of the paper.

Conflict of Interest

"The authors declare no conflict of interest"

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