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Research Article

Genetic Diversity Analysis among Garlic (*Allium sativum* L.) Genotypes through Molecular Markers

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Abstract

An experimental research was conducted among the 16 garlic genotypes for analysis of genetic diversity. The present investigation on "Genetic diversity analysis among garlic (*Allium sativum* L.) genotypes through molecular markers" was planned to conduct with objective of DNA fingerprinting through RAPD, ISSR and SSR primers. RAPD ISSR and SSR markers are used for DNA fingerprinting. Among all markers ISSR and SSR markers gave diversified results than RAPD.

Keywords: Allium sativum L; Genetic Diversity Analysis; Genotypes; Molecular Markers

Introduction

Garlic (*Allium sativum* L.) belongs to the family Alliaceae. The chromosome number is 2n = 16. It is probably native of central Asia and southern Europe, especially Mediterranean region. It is one of the important underground bulb crops of tropical and subtropical countries [1]. Garlic has a large and complex genome with two pairs of satellite chromosomes in the basic karyotype. The knowledge of genetic diversity in a crop species is fundamental to its improvement. Various techniques such as morphological, biochemical and DNA based molecular markers are used to study the genetic diversity of crop plants. Morphological traits have many limitations, including low polymorphism, low heritability and late expression. The biochemical methods like protein profiling, isoenzyme are useful for varietal identification but they are influenced by the environmental factors. The methods have some disadvantages e.g. that they are also profoundly influenced by tissue specificity and developmental stage. Isozymes are suitable as genetic markers because they segregate as expected in mendelian inheritance [2].

The use of molecular markers for the evaluation of genetic diversity is receiving much attention than morphological characterization. The various marker tools are Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR). DNA-based assays are useful in marker-assisted selection because they provide more polymorphisms. The technique offers significant advantages of the species identification in that they are rapid, relatively cheap, eliminate the need to grow plants up to maturity. Random amplified polymorphic DNA (RAPD) is another type of DNA- based assay which was developed by Williams., *et al* [3]. In the RAPD technique, DNA polymorphisms are produced using a single arbitrary primer that binds to the opposite strands of the genomic DNA template [4]. Compared to RFLPs, RAPDs are faster and easier to generate because of the fewer numbers of steps involved namely, extraction, amplification, and separation. The main advantages of RAPDs are the utility of universal primers [3] and DNA sequence information or radioactive chemicals are not required [5]. Microsatellite sequences are especially suited to distinguish closely related genotypes; because of their high degree of variability, they are, therefore, favoured in population studies [6] and for the identification of closely related cultivars [7]. ISSRs are DNA fragments of about 100 - 3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16 - 18 bp).

Materials and Methods

The experimental material comprised of 16 Genotypes of garlic. The 2X CTAB extraction buffer was prepared by using components (2%CTAB, 5M NaCl, 1M Tris, 0.5M EDTA). and adjusting to 100 ml with ultrapure H_2O. Just before use, enough volume to be used was aliquoted into a clean 50-ml tube and adds 40 ml of β-mercaptoethanol per 20 ml solution and the buffer pre-warm to

65°C. Total genomic DNA was isolated from young leaves of different garlic plants which were grown in pot. The DNA extraction was carried out by CTAB method as described by Doyle and Doyle [8] with minor modifications. Purification of DNA was carried out with RNAase treatment and DNA was analysed by agarose gel electrophoresis. In order to perform PCR based analysis, the DNA concentration was determined by Picodrop PET01 using software v2.08 (Picodrop Ltd., Cambridge U.K). Two microlitres of DNA was held in UV transparent tip attached to micropipette used for measurement of quality at A260/A280 ratio which was indicated between 1.6 and 1.9. The concentration of DNA was adjusted to 50 ngµl^{-1} for further work.

Dilution of DNA for PCR

The quantified DNA was diluted to final concentration of 50 ng/µl in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Molecular markers

For the fingerprinting of garlic genotypes, various molecular marker techniques such as Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) were used. Primers required for the above techniques were synthesized from Banglore Genei, Banglore. All primers for RAPD, ISSR and SSR were diluted by adding equal amount of deionized sterile distilled water equal to its concentration.

Randomly amplified polymorphic DNA (RAPD)

Amplification of RAPD fragments was performed according to Ganesh., *et al.* (2007) with some modifications using decamer arbitrary primers of OPA, OPE, OPC, OPG, OPB series. The reagents used for RAPD-PCR amplification of DNA were as follows. PCR buffer (10X) Taq DNA polymerase - Bangalore Genei dNTPs (dATP, dCTP, dGTP and dTTP) - Bangalore Genei Primer (25 p moles/µl) synthesised from Bangalore Genei. PCR reactions for RAPD were carried out in a final reaction volume of 25 µl.

Preparation of reaction mixture of RAPD marker

The reaction mixture for RAPD-PCR was consisted of following reagents. PCR buffer (10X) (2.5 µl), Taq polymerase (3 U/l) (0.5 µl), dNTP mix (2 µl), primer (2 µl),Template DNA (2 µl) Millipore sterilized distilled water (16 µl) total volume becomes 25 µl. As per the given information, Millipore sterilized water was added first followed by addition of PCR master mix (Bangalore Genei Pvt. Ltd.), primer in sequence and finally the template DNA.

PCR conditions for RAPD

The thermal cycler was set to following cyclic condition for RAPD analysis. Denaturation at 94ºC, annealing 37ºC and extension at 72ºC.

Electrophoresis of amplified product

The reagents used for electrophoresis of amplified RAPD products were analysed by agarose gel electrophoresis. The concentration of agarose was 1.5%.

Inter simple sequence repeat (ISSR)

The genomic DNA was amplified using UBC (University of British Columbia, Canada) primers. The PCR reactions for ISSR were carried out according to the method given by Al-Otayk., *et al*. [9] with some modifications.

Electrophoresis of amplified product

The amplified products of ISSR were analyzed using 1.5% agarose gel.

Simple sequence repeat (SSR)

The genomic DNA was amplified using primers. PCR reactions for SSR were carried out in a reaction volume of 25 µl using method given by Mahajan., *et al*. [10] with some minor modifications. The reagents used for SSR-PCR amplification of DNA were as follows. PCR buffer (10X) - Bangalore Genei, Taq DNA polymerase - Bangalore Genei, dNTPs (dATP, dCTP, dGTP and dTTP) - Bangalore Genei, Primer - Bangalore Genei. The reactions for PCR-SSR were carried out in a final reaction volume of 25 µl. Reverse and forward SSR primers were ACM and AFS series.

Preparation of reaction mixture

The reaction mixture for SSR-PCR was consisted of following reagents. PCR conditions for SSR were denaturation at 94ºC, annealing at 92ºC and extension at 72ºC.

Electrophoresis of amplified product

The amplified products of SSR were analyzed using 2.5% agarose gel.

Statistical analysis

In order to score and preserve banding pattern photograph of the gel was taken by a Gel Documentation System, under UV transilluminator. RAPD, ISSR and SSR bands were designated on the basis of their molecular size (length of polynucleotide amplified). 100bp DNA ladder for PCR product loaded simultaneously with primer products in the gel was used to estimate the molecular size. The data was entered into MS-Excel data sheet and subsequently analyzed using NTSYS pc version 2.02 [11].

Figure 1: RAPD markers of garlic genotypes generated by OPE-11.

Figure 2: ISSR markers of garlic genotypes generated by D-14.

Figure 3: SSR markers of garlic genotypes generated by AFS-039.

Figure 4: Dendrogram depicting the genetic relationship among 16 garlic genotypes based on the ISSR data.

Figure 5: Dendrogram depicting the genetic relationship among 16 garlic genotypes based on the SSR data.

Figure 6: Dendrogram depicting the genetic relationship among 16 garlic genotypes based on the RAPD data.

Preparation of reaction mixture for RAPD

The reaction mixture for RAPD-PCR was consisted of following reagents.

Sr. No.	Reagent	Quantity
1	PCR buffer (10X)	2.5 µl
2	Taq polymerase (3 U/µl)	0.5µl
3	dNTPs mix (2.5 mM each)	2.0 µl
4	Primer (25 pmoles/µl)	1.0 µl
5	Template DNA (50 ng/µl)	1.0 µl
6	Millipore Sterile distilled water	18.0 µl
Total		25.0 µl

Table 1

PCR conditions for RAPD

Sr. No	Step	Temperature (ºC)	Duration
1	Initial Denaturation	94	4.0 min
2	Denaturation	94	1.0 min
3	Annealing	37	1.0 min
4	Extension	72	2.0 min
Repeat the steps 2 to 4 for 40 times			
5	Final extension	72	10.0 min
6	Hold	4	--

Table 2

PCR conditions for ISSR

The thermal cycler was set to following cyclic condition for ISSR analysis.

Sr. No.	Step	Temperature (ºC)	Duration
1	Initial Denaturation	94	4.0 min
2	Denaturation	94	45 sec
3	Annealing	As per Tm ± 2	1 min
4	Extension	72	1.30 min
5	Final extension	72	10.0 min
6	Hold	4	--

Table 3

PCR conditions for SSR

The thermal cycler was set to following cyclic condition for SSR analysis.

Sr. No.	Steps	Temperature (ºC)	Duration
1	Initial Denaturation	94	4.0 min
2	Denaturation	94	45 sec
3	Annealing	Tm ± 2	45 sec
4	Extension	72	1.30 sec
Repeat the steps 2 to 4 for 35 times			
5	Final extension	72	8.0 min
6	Hold	4	--

Table 4

Results and Discussion

The RAPD was used for characterization of garlic genotypes for studying genetic diversity and its relationships between different genotypes.

In present investigation, 16 garlic genotypes were subjected to RAPD analysis using 20 different random primers of Operon series such as OPA, OPD, OPE and OPG. Out of 20 RAPD primers screened, 16 primers amplified a total of 87 bands/alleles out of which 82 bands were polymorphic with an average of 5.43 bands per primer while remaining five bands were monomorphic and out of 82 polymorphic bands 72 were polymorphic and shared between at least two individuals and 10 bands were polymorphic and unique. The percentage of polymorphic markers varied from 50 to 100% with an average of 92.85% polymorphism per primer. Twelve primers resulted in 100% polymorphism, while four primers OPD-03, OPD-05, OPE-03 and OPE-14 gave 83.33%, 50.0%, 66.66% and 85.71% polymorphism respectively.

Inter simple sequence repeat (ISSR) analysis

The 16 garlic genotypes were subjected to ISSR analysis using 15 different primers series such as, D, HB and ISSR containing GA, CAC, CA, GAC, CTC, GTC and GT repeats. The part of genomic DNA that represents microsatellite repeats of a given primer were produced a highly polymorphic map [9]. Out of 15 ISSR primers screened, 10 primers amplified a total of 62 bands out of which 57 bands/alleles were polymorphic with an average 6.2 bands per primer and out of 57 polymorphic bands 54 were polymorphic and shared between at least two individuals and 3 bands were polymorphic and unique bands. The average percentage of polymorphism about 90.57% was recorded for all the ten ISSR primers.

Simple sequence repeat (SSR) analysis

The 16 garlic genotypes were subjected to SSR analysis using 15 different primers series such as ACM and AFS. The part of genomic DNA that represents microsatellite repeats of a given primer were produced a highly polymorphic map [10]. Out of 15 SSR primers screened, 11 primers amplified a total of 18 bands out of which 14 bands/alleles were polymorphic with an average 1.6 bands per primer and out of 13 polymorphic bands total 13 bands were polymorphic and there was no unique bands were observed. The average percentage of polymorphism about 30.30% was recorded for all the eleven SSR primers.

Conclusion

Based on molecular marker study through RAPD, ISSR and SSRs one can differentiate genotypes and genotypes from one another to some extent. The reliability of one particular marker does not fulfill the goal of identification of genotypes [9].

Jaccard's similarity coefficient and UPGMA method developed a dendrogram showed the highest similarity of 74.4% between JG-0704 and JG-0805 and the lowest of 33.8% between JG-0615 and JG-2003-263. The dendrogram consist two main clusters-I and II with an average similarity of 45%, consisted of 16 garlic genotypes which was not reported in earlier study, except RAPD. The cluster-I consist of only one genotypes viz. JG-2003-263 and these was separated from the other genotypes and these genotypes also reported in ISSR marker. The cluster-II split into two subcluster-A and B consisted of fifteen genotypes. The subcluster-A divided into two group-A1 and A2. The group- A1 consist of eight genotypes. The genotype JG-0805 and JG-0805 shared an equal and highest similarity of 74.4%. The genotypes JG-0704 were found to be most similar genotypes in all molecular marker from earlier study. The genotypes GG-1 was put aside in group-A1 and these genotypes were stable and different from all the genotypes in present study of RAPD, ISSR and SSR data. The group-A2 consists of three genotypes and shared a similarity around the 54%. The subcluster-b formed with four genotypes and the greater similarity 60.7% between the G-496 and JG-0612 was obtained. The genotypes JG-0704 was found to be most similar genotypes in all molecular marker from earlier study. The genotypes GG-1 was put aside in group-A1 and these genotypes were stable and different from all the genotypes in present study of RAPD, ISSR and SSR data. Study of molecular marker through RAPD, ISSR and SSR used to conform the differences and similarity between the garlic genotypes. The Similarity coefficient ranged from 0.338 to 0.744 and showed the highest relationship of 74.4% between JG-0704 and JG-0805 and lowest resemblance of 33.8% between JG-0615 and JG-2003-263. The dendrogram consist of two main clusters with an average of 45% similarity and in ISSR the similarity were recorded in 41% [12-14].

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