RAPID GENOMIC DNA EXTRACTION IN BULB CROPS AND ESTIMATION OF GENETIC DIVERSITY IN GARLIC (*Allium sativum*L.) USING RAPD MARKER

A THESIS

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CERTIFICATE

This is to certify that the thesis entitled ***"RAPID GENOMIC DNA EXTRACTION IN BULB CROPS AND ESTIMATION OF GENETIC DIVERSITY IN GARLIC (Allium sativum L.) USING RAPD MARKER"*** *submitted to the* ***Faculty of Agriculture,*** *Sher-e-Bangla Agricultural University (SAU), Dhaka in partial fulfillment of the requirements for the degree of* ***Masters of Science (MS) in Biotechnology,*** *embodies the results of a piece of bona fide research work carried out by* ***KAMRUN NAHAR SHEULY, Registration no. 10-04047*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: JUNE 2016
Place: Dhaka, Bangladesh

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DEDICATED TO
MY
BELOVED FAMILY

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ABSTRACT

An experiment was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh to develop low cost and rapid genomic DNA isolation technique and to study the DNA fingerprinting and genetic diversity of nine garlic genotypes. Conventional CTAB/SDS method of DNA extraction required more time and cost. In contrast, the newly developed method to low cost and very fast method for extraction of genomic DNA in bulb crops. The garlic germplasms were BARI-G1, GC001, GC008, GC0012, GC0013, GC0017, GC0024, GC0027 and GC0028. Genomic DNA was extracted in both methods to perform PCR reaction with seven RAPD primers. Sum of 53 distinct and differential amplified DNA bands were generated from PCR amplification. Out of them, 40 bands (75.88%) were polymorphic and 13 bands (24.12%) were monomorphic. The Nei's genetic identity among 9 garlic genotypes ranged from 0.8053 to 0.9966 and genetic distance ranged from 0.0053 to 0.2166. The UPGMA Dendrogram segregated the 9 garlic genotypes into two main clusters. The first cluster contained 1 genotypes and the second cluster had 8 genotypes. The Dendrogram also indicated that BARI-G1 vs GC0027 varietal pair showed highest Nei's genetic distance (0.2166) and GC0012 vs GC0017 varietal pair showed lowest genetic distance (0.0053). The study revealed a significant amount of relationship and genetic diversity among the studied 9 garlic genotypes. The RAPD markers were found to be useful tool for molecular characterization and polymorphism study in garlic genotypes.

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LIST OF ABBREVIATIONS AND ACRONYMES

AFLP	: Amplified Fragment Length Polymorphism
BBS	: Bangladesh Bureau of Statistics
bp	: Base pair
CTAB	: Cetyltrymethyl ammonium bromide
ddH_2O	: Double distilled water
dH_2O	: Distilled water
DHA	: Docosahexaenoic Acid
DNA	: Deoxyribo Nucleic Acid
dNTPs	: Deoxynucleoside triphosphate set
EDTA	: Ethylene diamine tetra acetic acid
EPA	: Eicosapentaenoic acid
FAO	Food and Agricultural Organization
Kb	: Kilo base pair
LNA	: Alpha – Linolenic Acid
M	: Molar
mM	: Milimolar
PCR	: Polymerase Chain Reaction
RAPD	: Random Amplified Polymorphic DNA
RCBD	: Randomized Complete Block Design
RFLP	: Restriction Fragment Length Polymorphism
rpm	: Rotation per minute
SDS	: Sodium Dodecyl Sulfate
SNP	: Single Nucleotide Polymorphism
SSR	: Simple Sequence Repeats
STS	: Sequence Tagged Sites
TBE	: Tris-boric acid EDTA
TE	: Tris-EDTA
TVP	: Textured vegetable protein
UNDP	: United Nation Development Programme
UPGMA	: Unweighted Pair Group Method with Arithmetic Means
UV	: Ultra violate
VNTRs	: Variable Number Tandem Repeats
µl	: Micro liter
10-mer	: Decamer

CHAPTER I
INTRODUCTION

Garlic (*Allium sativum*), belongs to the family Alliaceae, which possesses more than 500 species. It is an herbaceous annual aromatic bulb and spice crop containing pungent smell. It is the second most widely cultivated *Allium* species and hold second position in terms of production followed by onion (Bose and Som, 1990). The word garlic comes from Old English garleac, meaning "spear leek". It is one of the oldest vegetables which have positive effect on human has been known for thousand years. It was believed that, garlic originated from Central Asia from where its culture spread towards North-East to the Pamir-Alai and Tien shen regions of China (Katyal and Chandha, 1996). Presently, the major Garlic producing countries are China, India, South Korea, Egypt and Russia(FAO, 2015).

Garlic are used all over the world as a valuable spice for various culinary purposes like chutneys, pickles, curry powders, curried vegetables, tomato ketchup etc. Fresh garlic cloves are widely used in cooking because of its ability to improve the taste of food and dehydrated products are quite commonly used as a condiment in the food industry (Takagi, 1990). It is also possessed medicinal value because of its biochemical activities that include antibiotic, antitumor, cholesterol lowering and anti-thrombic effects on animal cells (Fujiwara and Natata, 1967). Garlic contains Allicin, a sulphur containing component, which has a hypocholesterolaemic action and reduces the cholesterol concentration in human blood (Conn *et al.*, 1987).Garlic extract is also used in seed treatment and as botanical pesticide.

Garlic is grown in all parts of the Bangladesh but for commercial purposes it is cultivated in the Faridpur, Pabna, Comilla, Rangpur and Jessore. The average yield in Bangladesh comes to only 2.86 mt ha^{-1} whereas the world average yield is of about 11.99 mt ha^{-1} (BBS-2015). Because of deficit situation in garlic production in Bangladesh, a huge quantity of garlic is imported annually.

In addition, the size of the local bulb of this crop is one third of the exotic one indicating the venue of development either in bulb size or in number of cloves per bulb.

Improvement of garlic production can be achieved through chromosomal manipulation and selecting the agronomically desirable variants e. g. large bulb size, no. of cloves/bulb etc. For such chromosomal manipulation activities, individual chromosome identification is a primary need. This background prompted us to apply RAPD for the identification of common garlic cultivars.

Among different molecular markers, Random Amplified Polymorphic DNA are increasingly being employed in genetic research owing to their speed and simplicity (Welsh and McClelland, 1990; Williams *et al.,* 1990). RAPD is one of the widely used molecular markers of which DNA fragment amplified by polymerease chain reaction (PCR) using short synthetic primers of random sequence. RAPD markers tend to estimate intra or inter genetic distances more distantly in related individual. Recent developments of molecular genetics resulted in several procedures based on DNA for detecting genetic polymorphism.

RAPD technique is being used successfully to identify, characterize and estimate genetic divergence of garlic cultivars. It is also being used for detection of somaclonal variation in many crops including garlic (Al-Zahim *et al.,* 1999). Molecular diversity study is prerequisite for any crop improvement program. It helps scientist to select divergence material for breeding purpose. Very few reports are available on molecular diversity analysis in garlic. DNA profiling data are not enough under Bangladesh condition. Genomic DNA extraction is the pre -requisite of any molecular biology work. Extra pure high quality DNA is essential for PCR based molecular diversity study. Conventional CTAB /SDS method of DNA extraction required more time and money. Dozens of high price chemicals are required in CTAB method. More than 15 hours are needed to complete the hole process. Hence, scientists are looking for any alternative approaches. Which can shorter the time and cost

effective for large amount of DNA sample preparation. With an idea of rapid extraction technique, we try to develop “Low Cost Very Fast (LCVF)” DNA isolation protocol in bulbous crop like ginger, onion etc.

Considering the aspects mentioned above the present research programme was undertaken with the following objectives:

1. To develop low cost and very fast DNA extraction protocol development in bulbous crop.
2. To estimate genetic diversity among garlic genotypes using RAPD markers.

CHAPTER II
REVIEW OF LITERATURE

Garlic (*Allium sativum* L.) is an important crop that has been used for centuries as a spice and medicine. Because all known cultivated garlic cultivars are sterile, they can only be propagated vegetatively, this has prevented the production of new cultivars by plant breeding. Much of the tissue culture work on garlic has been directed towards molecular characterization, using as explants shoot meristems or isolated flower heads and basal plates (Novák and Ddezel, 1986). The PCR-based RAPD (Random Amplified Polymorphic DNA) technique (Williams *et al*., 1990) is used for diversity analysis of garlic by several workshops. Many researchers worked in this area and many researchers are conducting experiments in a running manner. The following section describes some of the findings observed by other researchers in relation to the mentioned points.

2.1 Marker or Genetic marker

A distinguishing feature that can be used to identify a particular part or region of agenome, chromosome or genetic linkage group is called marker. Molecular markers arethe molecules that could be used to trace a desired gene in test genotypes. In fact, a pieceof DNA or a protein can be used as a marker.

Datta *et al*. (2011) defined genetic marker as a readily recognizable genetic trait, gene, DNA segment, or gene product used for identification purposes especially when closely linked to traitsor to genetic material that is difficult to identify.Genetic markers can be classified into three broad categories i.e. Morphological marker, Biochemical marker and Molecular marker or DNA marker.

2.1.1 Morphological markers

Morphological markers are specific and distinct morphological traits that are scored visually (Datta *et al.*, 2011; Bhat *et al.*, 2010). These are related to shape, size, color and surface of various plant parts. Such characters are used

for the varietal identification. Morphological markers differ among species, genus and varieties of plants and animals (Bagali *et al.,* 2010). These traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation. However, morphological markers availability is limited, affected by environment, show low level of polymorphism, and many of these markers are not associated with important economic traits (e.g. yield and quality) and even have undesirable effects on the development and growth of plants (Jiang, 2013).

These types of markers are still having advantage and they are mandatory for distinguishing the adult plants from their genetic contamination in the field, for example, spiny seeds, bristled panicle, and flower/leaf color variants (Mondini *et al.*, 2009).

2.1.2 Molecular markers or DNA markers

Most widely used genetic marker type is molecular markers, comprising a large variety of DNA. Datta *et al.* (2011) defined molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome.

A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes (Amin, *et al*., 2010).

Choudhary *et al.* (2008) stated that molecular markers are phenotypically neutral and it should not be considered as normal genes as they usually do not have any biological effect. Instead, they can be thought of as constant landmarks in the genome.There are mainly two types of molecular markers i.e. (i) Hybridization based or Non-PCR based marker for example Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al*., 1980) and (ii) Polymerase chain reaction (PCR) based markers, e.g. Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Sequence characterized amplified regions (SCARs) (Michelmore *et al*., 1991) and Amplified Fragment

Length Polymorphism (AFLP) (Vos *et al*., 1995).

Molecular markers are used for development of saturated genetic maps, DNA fingerprinting, phylogenetic and evolutionary studies, heterotic breeding, gene tagging and marker assisted selection (MAS). They have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species (Madhumati, 2014).

Amin *et al*. (2010) reported the use of molecular markers in identification of vegetables crop varieties like tomato, potato, onion, garlic and related species and linked to major disease resistance in tomato like *Meloidogyne incognita* and tomato mosaic virus. In CTAB method is time consuming and depends on environmental conditions. Breeding a new variety takes 8 to 12 years. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in plant breeding (Gosal *et al*., 2010; Choudhary *et al.*, 2008)

2.1.3 Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA involves the use of single short primers of arbitrary nucleotide sequence to reproducibly amplify segments of target genomic DNA. These short primers referred to as genetic markers are used to reveal polymorphisms among the amplification products, which are seen as visible bands with the aid of ethidium bromide-stained agarose gel electrophoresis (Williams *et al.*, 1990).

Technically, RAPD has been described as the simplest version of PCR with arbitrary primers used for detecting DNA variation and for convenience, all RAPD variants are commonly referred to as RAPD (Weising *et al*., 2005). Besides providing an efficient technique for polymorphism that allows rapid identification and isolation of chromosome-specific DNA fragments, RAPD markers are also useful for genetic mapping, DNA fingerprinting, plant and animal breeding (Venkatachalam *et al*., 2008).

The use of RAPD markers are especially beneficial to discriminate between materials that are genetically similar, to evaluate genetic variability within a collection and to choose the components of the core collection (Piola *et al*., 1999). RAPD techniques have been successfully used to assess genetic relationship in many plants, for example, sugar cane (Devarumath *et al*., 2007), sorghum (Singh *et al*., 2006) and apple (Bernardo and Itoiz, 2004)

Despite all these limitation, RAPD have remained attractive to researchers when financial investment is limited because the input cost is cheaper than other molecular markers such as AFLP and microsatellites (Weising *et al*., 2005). In addition, the problem of RAPD reliability and transferability among laboratories could be minimized and eliminated by following a standard protocol, replication of amplification reactions and a conservative criterion of band selection (Belaj *et al*., 2003).

RAPD analysis is the appropriate marker for the genetic analysis due to its simplicity and its automated procedure as compared to RFLP. It also requires very small amount of template. Among molecular markers Random amplified polymorphic DNA are increasingly being employed in genetic research owing to their speed and simplicity (Williams' *et al*., 1993; Welsh and McCelland, 1990).

Madhumati (2014) mentioned in a review article that, several factors have been reported to influence the reproducibility of RAPD reactions i.e. quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, Taq DNA polymerase brand or source, and thermal cycler brand. The concern about reproducibility of RAPD markers, however, could be overcome through choice of an appropriate DNA extraction protocol to remove any contaminants.

Main advantages of the RAPD technology include suitability for work on anonymous genomes, involves no blotting or hybridization steps, hence, it is quick, simple and efficient, applicability to problems where only limited quantities of DNA are available and Unit costs per assay are low compared

toother marker technologies (Kumari and Thakur, 2014; Kumar and Gurusubramanian, 2011).

The major drawback of RAPD method is that the profiling is dependent on reaction conditions which can vary between laboratories and even a difference of a degree in temperature is sufficient to produce different patterns. Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Madhumati, 2014; Bardakci, 2001).

2.2 RAPD for Garlic

In order to apply new emerging biotechnologies as tools for the improvement of local varieties and production of new breeds, introduction of molecular marker technology is needed. Moreover, precise genetic analysis of *in vitro* regenerated garlic is also required for the evaluation of the regeneration protocols and for the detection of somaclonal variations. Background prompted us to apply RAPD for the identification of common garlic cultivars distributed in Egypt and screening of *in vitro* regenerated plants for somaclonal variations (Saker and Wagdy, 1998).

RAPD analysis of genomic DNA isolated from *in vitro* regenerated garlic plants and in vivo control seedling using arbitrary selected oligonucleotide primers (OPA2 and OPC2) resulted in the resolution of 14 polymorphic bands. Genetic changes as revealed by RAPD analysis were detected in 20 % of the examined plants and 80 % having RAPD fingerprints identical to those of the control seedling (field-grown plants). It also requires very small amount of template DNA (Williams *et al.,* 1990). This feature allows the wide applications of RAPD for cultivar identification and detection of somaclonal variations. In the previous study many researchers used RAPD markers in different crop for cultivar identification. (Wang *et al.,* 1994; Castiglione *et al.,* 1993; Rani *et al.,* 1995; Taylor *et al.,* 1995 and Corniquel and Mercier, 1994).

Although the origin of Chinese cultivar is different from the Egyptian cultivars, some markers among the scored polymorphic RAPD marker were similar to that of the Egyptian cultivars. Such observation suggests that the Chinese cultivar is exposed to breeding or selection programs (Saker and Wagdy, 1998).

The molecular mechanism underlying somaclonal variations have been attributed to chromosome breakage, single base changes, changes in copy number of repeated sequences and alteration in DNA methylation patterns (Kaeppler and Philips, 1993; Landsmann and Uhring, 1985; Munthali *et al.*, 1996).

Genetic variations among regenerated garlic plants were also reported by Ali and Metwally (1992)by using RAPD marker.

RAPD can be successfully used to detect somaclonal variations among *in vitro* regenerated garlic plants. Numerous researches proved that the sensitivity of RAPD was sufficient enough to detect genetic changes in many of tissue culture derived plants; for instance, Brown *et al.*, 1993), Taylor *et al.*, (1995) and Rani *et al.*, (1995). RAPD is used for detection of somaclonal variation in many crops including garlic (Al-Zahim *et al*., 1997; Maas and Klass, 1995).

CHAPTER III
MATERIALS AND METHODS

The details of materials and methodologies applied for the study have been described in this chapter.

3.1 Experimental site and time duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh. The period of the experiment was July, 2014 to June, 2015.

3.2 Name and source of study materials

Nine garlic germplasm were used in the study. The sources of germplasm were presented in Table 1.

Table 1. Name and sources of 9 garlic germplasms

Sl. No.	Name of the Genotypes	Source
1.	BARI-G1	Spice Research Center(SRC), BARI, Gazipur
2.	GC001	Spice Research Center(SRC), BARI, Gazipur
3.	GC008	Spice Research Center(SRC), BARI, Gazipur
4.	GC0012	Spice Research Center(SRC), BARI, Gazipur
5.	GC0013	Spice Research Center(SRC), BARI, Gazipur
6.	GC0017	Spice Research Center(SRC), BARI, Gazipur
7.	GC0024	Spice Research Center(SRC), BARI, Gazipur
8.	GC0027	Spice Research Center(SRC), BARI, Gazipur
9.	GC0028	Spice Research Center(SRC), BARI, Gazipur

BARI=Bangladesh Agricultural Research Institute

3.3 Genomic DNA extraction from garlic

The genomic DNA of garlic was extracted by two different methods. One is well established method known as CTAB method which follow the protocol of (Doyle and Doyle, 1987).Another method of DNA extraction is very fast and low cost technique.The idea of this new method was generated from different journals and internet.Each of the methodology are given bellow:

3.3.1 Method 1: Low cost very fast DNA extraction technique

It is a new method, which is invented in Department of Biotechnology, Sher E Bangla agricultural University, Dhaka, Bangladesh.

The details procedures are given below

3.3.1.1 Required chemicals and small instrument

1.Table salts (NaCl)

2.100% absolute ethanol

3. Vim liquid

4.Bunsen burner

5.Tripod stand

6.Mortar and pester

7. Distilled water

8.Petri dish

9.Small beaker

3.3.1.2 Sequential steps for DNA extraction procedure

1. Garlic/cloves (2-3) were peeled.

2. The cloves were chopped into small pieces with knife.

3. The pieces were grinded with mortar and pester.

4. A pinch of salt (200-300mg) were added for good grinding.

5. In small beaker 5ml water and 2 drops of vim liquid was added.

6. The grinded mixture of garlic was added to the beaker.

7. It was heated with Bunsen burner with the help of tripod stand about 2-3 minute.

8. The mixture was filtered gently and the liquid was taken in a petri dish which has 5-10 ml of 100% cold ethanol.

9. Within short period of time genomic DNA of garlic strand was visible as cotton thread like structure in the petri dish.

10. Then the DNA solution was centrifuged at 13000 rpm for 15 minutes and it was taken in the eppendorf tube.

11. The liquid was removed completely.

12.500µl 70% ethanol was added to the solution and tapped for washing the DNA.

13. Again the solution was centrifuged at 13000 rpm for 10 minutes.

14. The liquid was remove carefully.

15. The DNA plates were dried completely for 45-50 minutes.

17. The DNA pellet was dissolve in 100-150µl of TE buffer.

18. RNAse treatment was given by 20-30µl of solution to remove the RNA in the sample.

19. The solution was incubated at 37^0c for 20-25 minutes in hot water bath and finger tapping was done after 10-minute interval.

20. The solution was spinned for 4-5 seconds.

21. Finally the DNA sample was frizzed at -20^0c.

3.3.2 Method-2: Extraction through CTAB/SDS methods

Total genomic DNA was isolated from garlic cloves.

3.3.2.1 Reagents used

1. Extraction buffer, pH= 8.0

   Composition of extraction buffer are as follows:

   - 1M Tris-HCl
   - 0.5M EDTA(Ethylene diamine tetra-acetic Acid) (pH= 8.0)
   - 5M NaCl
   - D.H_2O
   - 1% SDS **(**Sodium Dodecyl Sulphate)

2. Phenol: Chloroform: Isoamyl Alcohol= 25: 24: 1
3. TE (Tris-EDTA) buffer, pH=8.0

   Composition of extraction TE buffer are as follows:

   - 1M Tris-HCl
   - 0.5M EDTA
   - Sterilized distil water

4. Isopropanol
5. 0.3M Sodium Acetate
6. Absulate (100%) ethanol
7. Ethanol (70%)
8. RNAase
9. Ethidium Bromide solution

3.3.2.2 Reagent preparation for DNA extraction (Stock solution)

Extraction buffer (1000 mL)

For the preparation of 1000 ml DNA extraction buffer, 100 ml 1M Tris-HCl (pH=8.0) was mixed with 40 mL of 0.5M EDTA and added to 100 ml 5M NaCl in a 1000 ml measuring cylinder. Finally, Sterilized ddH_2O was added to make the

volume up to 1000 mL, then mixed well and autoclaved.

1M Tris-HCl (pH 8.0) (250 ml)

At first 30.28 g Tris was taken in a volumetric flask (5000 mL). 100 m dd.H2O was added and pH was adjusted to 8.0 by adding HCl. Then sterilized dd.H2O was added to make the volume up to 250 ml. The solution was autoclaved.

0.5M EDTA (pH 8.0) (250 ml)

At first 46.53 g EDTA.2H2O was added in a volumetric flask (5000 ml) and100 mL dd.H_2O was added. Then 4 g NaOH was added. Then pH was adjusted to 8.0 with NaOH. Then sterilized dd.H_2O was added to make the volume up to 250 ml. Then the solution was autoclaved.

5M NaCl (250 ml)

For the preparation of 5M NaCI, 73.05g of NaCI was added in 250 ml dd.H_2O in a 500 ml volumetric flask, mixed well and autoclaved.

1% SDS (100 ml)

One gram of SDS was added in 100 ml dd.H_2O in a 250ml beaker. As SDS is hazardous, the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.

1X TE Buffer (100 ml)

One ml Tris (pH 8.0) was taken in a volumetric flask (250 ml). Then 0.2 ml EDTA (pH 8.0) was added. Sterilized dd.H_2O was added to make the volume up to 100 ml.

5X TBE buffer (1 litter)

At first 54 g Tris-HCl was taken in a volumetric flask (1000 mL) containing about 200 mL dH_2O. Then 27.5 g of Boric acid and 4.65 g of EDTA was added separately. The volume was made 1L adding D.H_2O up to the mark. At last pH was adjusted at 8.3.

Phenol: Chloroform: Isoamyl Alcohol= 25: 24: 1(100 ml)

At first 50 ml Phenol was taken in a volumetric flask (250 ml). Then, 48 mL Chloroform was added. Then, 2 ml Isoamyl alcohol was also added and mixed well. The solution was stored at 4°C.

RNase

10 mg of RNase was added to 1 ml of sterilized H_2O. Then it was dissolved completely with the help of necessary heat (at 50℃ in water bath for 30 minutes).

3.4 Sequential steps of CTAB method for DNA extraction

The following steps were followed for DNA extraction

1. For isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues should be collect from 15 days plant. Total DNA is isolated by using phenol: chloroform: isoamyl alcohol purification and ethanol precipitation method.
2. The youngest leaves are select in order to make the tissue grinding process easy. Initially, healthy youngest leaves were washed thoroughly by tap water followed by deionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 200 mg of young leaves are cut into small pieces and then taken in morter. 300 µl of extraction buffer are added and then leaf tissues are ground manually with the help of a pestle.
4. The sample is then taken into eppendorf tube and 300 µl of extraction buffer is added to it. The ground sample is then vortexed for 20 seconds in a vortex mixture and then incubated at 65°C for 20 minutes in hot water bath.

5. Equal volume (500 µl) of Chloroform: Isoamyl alcohol (24:1) is added to the tube and then it was vortexted for 10 seconds.
6. The solution is then centrifuged for 10 minutes at 13000 rpm. The supernatant is recovered using a pipette tip without disturbing the lower portion and transferred into a new eppendorf tube. Then, 250 µl of Isopropanol (0.6 volume of the liquid) is added to it. It is then tapped by finger for 20/30 seconds (The genomic DNA was visible as cotton like structure).
7. After tapping the sample is again centrifuged at 13000 rpm for 15 minutes. The liquid is discarded completely and re-precipitation of the DNA solution is done by adding 500 µl of absolute (100%) cold ethanol plus 20 µl 0.3M sodium acetate.
8. It is shaken gently. Tapping is done to separate pellet. The sample was centrifuged at 13000 rpm for 15 mins. The liquid is removed completely by pouring and blotting the open tube end on fresh tissue paper.
9. The DNA pellet is then air dried for 2 to 3 hours. It is then dissolved in an appropriate volume (30 to 40 µl) of TE buffer and treated with 3 µl of RNAase for removing RNA. Then it is spinned for 4-5 seconds.
10. Finally, the DNA samples are stored in freezer at -20°C.

3.5 Confirmation of DNA preparation

1% agarose gels were used for assessing the quality of the genomic DNA and the amount of RNA present.

3.5.1 Preparation of 1 % agarose gel

One gram of agarose powder was taken in a 500 ml Erlenmeyer flask containing 100 ml electrophoresis buffer (1x TBE buffer) prepared by adding 20 ml of 5X TBE buffer in 80 ml of sterile deionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. It was melted for about 5

minutes into a microwave oven with occasional swirling until complete disappearance of agarose particles to generate homogeneous and crystal clear suspension. The agarose solution was cooled to about 50^0C (flask was cool enough to hold comfortably with bare hand) and 0.75 µl (10 mg/mL) ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (15 x 15 x 2 cm^3; in size), that was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2mm above the plate. Air bubbles were removed by pushing away to the side using a disposable tip. After 45 minutes to one hour gel became completely cooled at room temperature and solidified and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.5.2 Preparation of DNA samples for electrophoresis

The samples were all in the same concentration of buffer. For each sample, 6 µl x TBE buffer was placed on a piece of aluminum foil paper and 2 µL loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA) was added to it using 0.5-10 µl adjustable micropipette. Loading buffer was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 2 µl extracted DNA was added to it and mixed well using same micropipette. The samples were then added slowly to allow them to sink to the bottom of the wells.

The gel was placed in the gel chamber (Continental Lab product Inc.) containing 1X TBE buffer. The final level of buffer was 5 mm above the gel. The power supply (EPS-301) was then connected and turned on to move the DNA from negative to positive electrode (black to red). Electrophoresis was carried out at 80V for about 75 minutes. After the bromophenol blue dye had reached three-fourths of the gel length, the electrophoresis was stopped and the power supply was disconnected.

3.5.3 Documentation of the DNA samples

The gel was taken from the gel chamber and was placed on an ultraviolet light box (UV transilluminator) to examine and photographed by a Gel Cam Polaroid camera. Better quality band showing DNA samples were taken for quantification and working solution preparation.

3.6 Quantification of DNA by spectrophotometer

The concentration of DNA is one of the important variables for PCR amplification. It is necessary to stay above this critical concentration. Moreover, excessive DNA concentration is likely to produce poor resolution or "smears" resulting in a lack of clearly defined bands in the gel. Therefore, quantification of DNA in each sample was determined spectrophotometrically.

Concentration of genomic DNA was examined by calculating the ratio of the optical density measured at 260 nm using a spectrophotometer (Spectronic^{R} Genesis^{Tm}). Spectrophotometer UV-lamp was turned on and after it had warmed up, the wavelength was set at 260 nm. One cuvette (the "Zero" or "blank" cuvette) was filled with 2 µl sterile distilled water and placed in spectrophotometer. The test samples were prepared by taking 2 µl of each sample in a cuvette containing 2 µl sterile distilled water. The sample was uniformly mixed and placed in spectrophotometer and the absorbance reading was taken at 260 nm. Then the cuvette was rinsed out with sterile water, for measuring the absorbance of every sample. DNA concentration in each sample was then determined according to the following formula:

$$\text{Concentration of DNA (µl)} = \text{Absorbance} \times \frac{\text{Volume of distilled water}}{\text{Amount of DNA (µl)}} \times \text{Conversion factor (0.05)} \times 100$$

Where,

Absorbance	=	Spectrophotometer reading
Volume of distilled water	=	2000 µl
Amount of DNA	=	2 µl
Conversion factor	=	0.05

3.7 Preparation of working solution of DNA samples

DNA concentration was adjusted to 25 ng/µl for conducting PCR using the following formula:

$$\mathbf{V_1 \times S_1 = V_2 \times S_2}$$

Where,

V_1= Initial volume of DNA solution (µl)

S_1= Initial DNA concentration (ng/µl)

V_2= Finial volume of DNA solution (µl)

S_2= Final DNA concentration (ng/µl)

3.8 PCR amplification of genomic DNA through RAPD markers

3.8.1 Principle of the amplification of RAPD

For performing amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo-stable DNA polymerase and a suitable buffer and then it is subjected to temperature cycling conditions typical to the polymerase chain reaction (PCR). The product of the reaction depends on the sequence and length of the oligonucleotide, as well as the reaction conditions. At an appropriate annealing temperature, the single primer binds to sites on opposite strands of the genomic DNA that are within an

amplifiable distance of each other and a discrete DNA segment is produced. The presence or absence of this specific product, will be diagnostic for the amplified with an arbitrary primer binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Often a single primer can be used to identify several polymorphisms, each of which matches to a different locus.

3.8.2 Primer selection

Seven decamer RAPD primers *viz.*OPJ-13, OPA-20, OPBD-16, OPP-12, OPB-06, OPBX-02 and OPBM-05 (Operon Technologies, Inc., Alameda, California, USA) were screened for PCR reaction in 9 garlic germplasm. List of RAPD primers are given in Table 2.

Table 2. Name, sequence and GC content of RAPD primers

Sl. No.	Primer name	Sequence (5´ to 3´)	(G+C) %
1	OPJ-13	AGGTGACCGT	60
2	OPBX-02	GTTGCGATCC	60
3	OPB-06	TGCTCTGCCC	70
4	OPP-12	GTCCACACGG	70
5	OPBM-05	TTCGAGCCAG	60
6	OPBD-16	GGACCCAACC	70
7	OPA-20	GTTGCGATCC	70

3.8.3 PCR amplification

PCR reactions were performed following two methods.

(1) Using individual PCR chemicals (each reagent is measured separately and prepared a mixture) and
(2) Using 2X Taq Mastermix (GeneON, Germany)

3.8.4 Using individual PCR chemicals

DNA amplification was performed inthermal cycler (Esco Technologies Swift™ Mini Thermal Cyclers, Singapore). The reaction mix was preheated for 4 mins at 94°C for pre-denaturation and then 33 cycles consisted of 1 min denaturation at 94°C, 50 second at 35°C for annealing and 1 min at 72°C for elongation or extension. After the last cycle, a final step of 7 min at 72°C was added to allow complete extension of all amplified fragments.

PCR reactions were performed on each DNA sample in a 25 µL reaction mix containing following reagents (Table 3).

Table 3. Reaction mixture composition for PCR

Sl. No.	Reagents	Amount
1	Taq DNA polymerase buffer (10x)	4 µl
2	Primer (10 µM)	2.0 µl
3	dNTPs (250 µM)	2 µl
4	Taq DNA polymerase	0.5 µl
5	Genomic DNA (25 ng/µL)	2 µl
6	Sterile de-ionized water	13.5 µl
7	**Total reaction volume**	**25 µl**

From frozen stocks the PCR buffer, dNTPs, primer and DNA samples solutions were taken and thawed, mixed by vortexing and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermo cycler used (0.5 mL). A pre-mixture was then prepared in the course of the following order: reaction buffer, dNTPs, DNA template and sterile distilled water. Taq polymerase enzyme was then added to the pre-mixture. The pre-mixture was then mixed up well and aliquoted into the tubes that already containing primer.The tubes were then covered in a thermo cycle and the cycling was started immediately.

3.8.5 PCR reaction with 2X Taq Master Mix (Geneon, Germany)

Ready mix Taq DNA polymerase and other composition were used for PCR reaction. The ratio of each chemical was given below (Table 4).

Table 4. Ready mix PCR mixture for amplification of Garlic genotypes

Sl. No.	Reagents	Amount (µL)
1	2X Taq Master Mix	5.0
2	RAPD primer	1.5
3	Sterile de-ionize water	1.5
4	Genomic DNA (25ng/ µl)	2.0
5	**Total reaction volume**	**10**

From the frozen stocks the Master Mix, primer and DNA samples solutions were taken and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermo cycler used. The required amount of 2X Taq Master Mix, RAPD primer and sterile de-ionized water were then added into it. The mixture was then mixed up well. The tubes were then covered in a thermo cycle and the cycling was started immediately.

3.8.6 Thermal profile

DNA amplification was performed in Q cycler (Hain brand, UK). The reaction mixture was pre-denatured at 95^{o} C for 5 min followed by 33 cycles of 45 sec denaturation at 95^{o} C, 30 sec annealing at 35^{o} C and elongation or extension at 72^{o}C for 1 minute. After the last cycle, a final step of 5 minutes at 72 ^{o} C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4 ^{o} C.

3.8.7 Electrophoresis of the amplified products

From each sample, PCR products were confirmed by running 2% agarose gel containing 0.75 µl ethidium bromide in 1x TBE buffer at 80V for 75 minutes. Loading dye (5.0 µl) was added to the PCR products and loaded in the wells. Two molecular weight markers 100 bp (BIONEER, Cat. No. D-1030, South Korea) and 1kb (BIONEER, Cat. No. D-1040, South Korea) DNA ladder were also loaded on left and right side of the gel respectively.

3.8.8 Documentation of the DNA samples

After completion of electrophoresis process, the gel was taken out carefully from the gel chamber and was placed on high performance ultraviolet light box (UV trans-illuminator) of gel documentation for checking the DNA was observed band and photographed by a Gel Cam Polaroid camera.

3.9 RAPD data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *el at.*, 1990). One molecular weight marker, 100 bp (BIONEER, Cat. No. D-1030, South Korea) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given

identification numbers according to their on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The size of the band length was calculated using a computer program DNAfrag, version 3.03.

The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. This was used to estimate polymorphic loci. Nei's(1972) gene diversity, Genetic distance (GD), frequencies of polymorphism and to construct a UPGMA (Un weighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program POPGENE (version 1.31) (Yeh *el al*., 1999). The same program was used to perform test of homogeneity in different locus between population pairs.

Estimation of gene frequencies of RAPD loci were based on the assumption of a two-allele system. Of the two alleles, only one is capable of amplification of a RAPD band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer annealing site. The two-allele assumption was in most cases acceptable, because co-dominant loci showing band shifts are few (Welsh and McClelland, 1990; Elo *et al.*, 1997). In this system only a null homozygote is detectable as negative for the RAPD-band of interest.

Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be $(N/n)^{½}$ where N and n are the number of band negative individuals observed and the sample size respectively.

The frequency of the other allele is 1-q, the assumption of the two-allele system enables us to calculate the Nei's genetic distance (Nei's, 1972) from the RAPD pattern.

Genetic similarity values defined as the fraction of shared bands between the RAPD profiles of any two individuals on same gel were calculated manually RAPD markers of the molecular weight on the data matrix according to the following formula:

$$\textbf{Similarity Index}(\textbf{SI}) = \frac{2\mathrm{Nxy}}{\mathrm{Nx} + \mathrm{Ny}}$$

Where, Nxy is the number of RAPD bands shared by individuals x and y respectively, and Nx and Ny are the number of bands in individuals x and y respectively (Wilde *et al.*, 1992).

The SI value ranges from 0 to 1. When SI=1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles. Within population similarity (Si) was calculated as the average of SI across all possible comparisons between individuals within a population. Between population similarity (Sij) was calculated as the average similarity between each paired individuals of population i and j (Lynch and Milligan, 1994).

Nei's genetic distance and genetic identity values were computed from frequencies of polymorphic markers to estimate genetic relationship among the studied nine garlic genotypes using the Unweighted Pair Group Method of Arithmetic Means (UPGMA) (Sneath and Sokal, 1973). The dendrogram was constructed using a computer program, POPGENE; (Version 1.31) (Yeh *et al*., 1999).

Precautions

1. To avoid DNAase contamination, all glassware, micropipette tips, eppendorf tubes, glass pipettes, de-ionized water and buffer solutions were properly autoclaved. Scissors, forceps were sterilized with absolute ethanol.
2. Since Ethidium Bromide (Et-Br) is a powerful mutagen and carcinogenic in nature, hand gloves were used when handling anything that has been exposed to Et-Br.
3. Always power pack was kept turn off and the leads was unplugged before opening the electrophoresis unit to avoid electrical hazard.
4. A trans-illuminator produces UV radiation of 254 nm range. The weave length can cause eye damage. Thus, eye protector was used while working with it.

5. The usual laboratory precautions were maintained when performing PCR reactions. All the disposable such as PCR tubes, tips, eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn during handling of PCR components. Contamination of PCR components was avoided.

CHAPTER IV
RESULTS AND DISCUSSION

Isolation of genomic DNA of any crops is essential for its molecular biology research. The quality DNA and quick extraction technique can save time, money and energy. Hence new method of DNA extraction in bulbous crop like garlic and its molecular diversity analysis was done in the chapter.

4.1 Comparison of Rapid DNA extraction method over CTAB method

Though conventional method of DNA extraction procedure needs more times and costlier as it need more chemicals and can be extract from young plant parts or leaves but rapid DNA extraction method is less time consuming and ecofriendly.

Now some comparison between CTAB and Rapid DNA extraction method are shown below.

1. Amount of DNA: In Rapid DNA extraction method, huge amount of DNA can be extracted which can easily visible in naked eye.

Plate 1. Huge amount of DNA is visible in low cost very fast method of DNA extraction

Plate 2. The visibility of DNA is poor in conventional CTAB method

Whereas in CTAB method the amount is very low. Sometimes DNA is not found in the eppendorf tube.

2. Cost effective: Rapid DNA extraction method is highly cost effective in compared to conventional DNA extraction method.
3. Chemical requirement: Only ethanol and table salt is required per sample need less than one taka. Whereas in CTAB method, minimum 5-10 taka is required as chemical cost. A comparison is given in Table 5.

Table 5.Comparison between CTAB method and Rapid DNA extraction method in respect of chemical requirement

Chemical	Volume	CTAB Method Cost	LCVF Method Cost
EDTA	500 ml	7,000	N/A
NaCl	500 gm AG	400	400
SDS	500 ml	4,000	N/A
Sodium acetate	100 gm	2,000	N/A
Phenol	1 L	5,000	N/A
Chloroform	1 L	5,000	N/A
Isoamyl alcohol	500 ml	3,000	N/A
Vim Liquid	1 Btl	N/A	70
100% absolute ethanol	1 L	3,000	3,000
Total		29400	3470

4. Time duration: Rapid DNA extraction method need comparatively less time than CTAB method. CTAB methods require 14 hours whereas rapid DNA extraction method only 5 hours.

4.2 DNA confirmation and quantification

The extracted 9 genomic DNA samples were loaded on 1% agarose gel for conformation and quantification of DNA. There was no smear and sharing of DNA with RNA. The quantification of DNA was also done in spectrophotometer (Table 6). It revealed that the highest concentration (450 ng/µl) of genomic DNA was observed in the sample BARI-G2, GC006, GC009, GC0013, GC0024

andGC0028.On the other hand the lowest (250 ng/µL) was in the sample BARI-G1, GC005,GC008,GC0017 and GC0027. It is recommended that only 25-50 ng/µL DNA is required for PCR amplification. Hence, the genomic DNA of each sample was diluted based on concentration. The working DNA sample was prepared for PCR works.

Table 6. Absorbance reading and concentrations of different DNA samples collected from 9 garlic genotypes

SL No.	Garlic Genotypes	**Absorbance Reading(260nm)**	**DNA Concentration(ng/µL)**
1.	BARI-G1	0.005	250
2.	GC001	0.007	350
3.	GC008	0.005	250
4.	GC0012	0.006	300
5.	GC0013	0.009	450
6.	GC0017	0.005	250
7.	GC0024	0.009	450
8.	GC0027	0.005	250
9.	GC0028	0.009	450

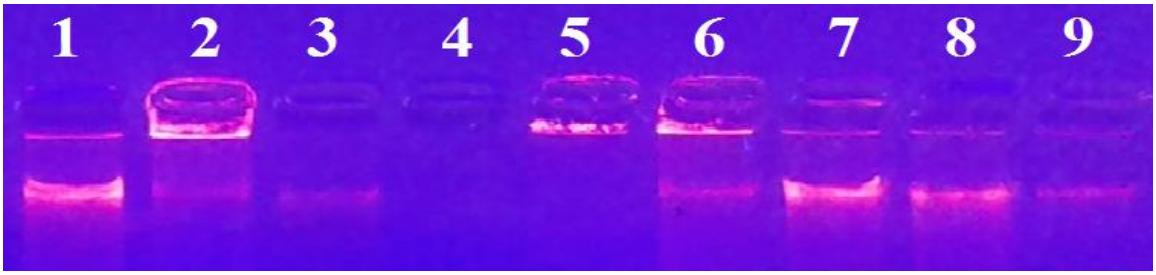

Plate 3. Confirmation of genomic DNA samples of 9 garlic genotypes (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028) by CTAB DNA extraction method.

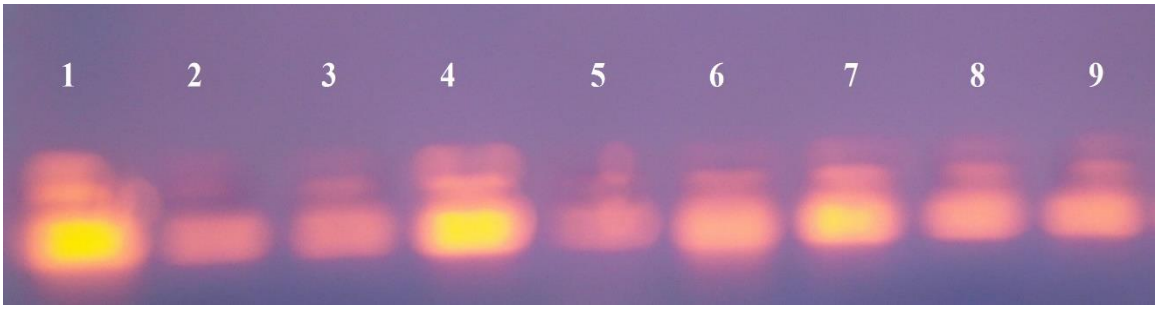

Plate 4. Confirmation of genomic DNA samples of 9 garlic genotypes (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028) by low cost very fast DNA extraction method.

4.3RAPD Primer selection for PCR amplification

Fifteen primers were initially screened on nine garlic genotypes to produce polymorphic patterns, seven primers (OPJ-13, OPA-20, OPBD-16, OPP-12, OPB-06, OPBX-02 and OPBM-05) were primarily selected of which seven primes that gave reproducible and distinct polymorphic amplified products were finally selected for the study. DNA amplification from all the primers tested in this study was not consistently reproducible, a very common feature of RAPD technique. The present findings agree with those of Hadrys *et al.,* (1992) and Williams *et al.,* (1993). Many authors (Schierwater and Ender, 1993; Lynch and Milligan, 1994) have also reported technical problems from amplification of the RAPD technique in the field of genetic population research.

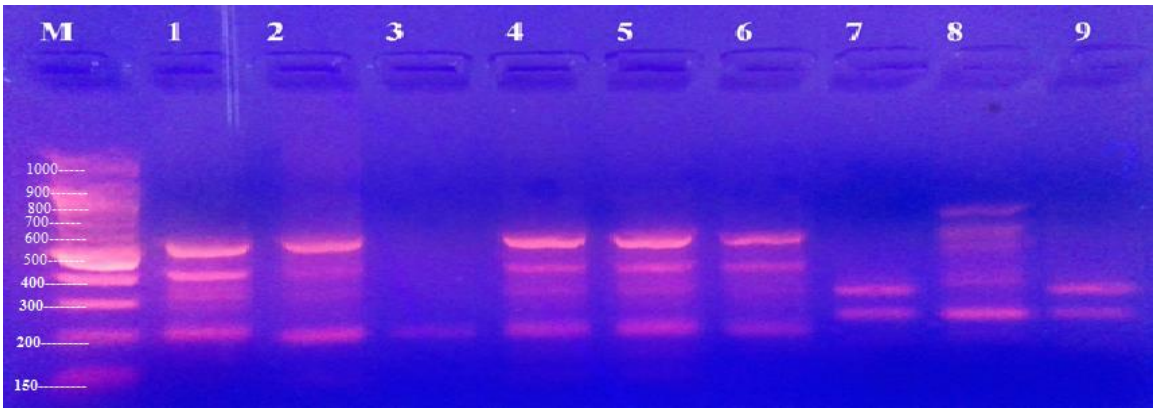

Plate 5. Primer test: PCR amplification products by 7 different decamer RAPD primers using DNA from nine different genotypes. (Lane 1-2: OPJ-13; Lane 3-4: OPA-20; Lane 5: OPBD-16; Lane 6: OPP-12; Lane 7: OPB-06; Lane 8: OPB-X2, Lane 9: OPB-M5. M1= 100bp DNA ladder)

4.4 RAPD banding pattern and DNA polymorphism

The name of the selected seven primers were OPJ-13, OPA-20, OPBD-16, OPP-12, OPB-06, OPBX-02 and OPBM-05. Each of the primers produced separate RAPD patterns (bands) in 9 garlic genotypes. The RAPD amplifications of individual primer were given in plate (6-12) and Table 7.

Selected 7 primers generated a total of 53 distinct and differential amplified bands. The average was 7.57 bands per primer and 5.88 bands per garlic genotypes. Out of total 53 bands, 40 bands (75.88%) were polymorphic DNA bands and 13 bands (24.12%) were monomorphic. The result gave an average 5.71 polymorphic and 1.86 monomorphic bands per primer. The seven different primers produced various banding patterns. The primer OPBX-02 produced maximum number of bands (10) followed by OPBM-05 and OPA-20 produced same number (8) of bands, OPJ-13 (07), OPP-12, OPBD-16 produced same number (07) of bands and OPB-06 produced the least number of (06) bands (Plates 3 to 9). On the other hand, OPP-12 and OPBD-16 amplified the highest percentage of (85.71%) polymorphic bands, followed by OPB-06 (83.33%), OPBX-02 (80.00%), OPJ-13 (71.42%) and least polymorphic bands was produced by OPBM-05 and OPA-20(62.50%). It revealed that, OPP-12 and OPBD-16 showed the highest level of polymorphism and OPBM-05 and OPA-20 showed the lowest level of polymorphism among these 7 primers. The amplification of DNA bands numbers per primer and polymorphism percentage were given in Table 7.

Al-Zahim *et al*.(1997),found on average of 2.42 polymorphic bands per primer and 21% band were polymorphic band.

Table 7. RAPD primers with corresponding bands scored and their size together with polymorphic bands observed in 9 garlic genotypes

Primer codes	Sequences (5′- 3′)	(G+C)%	Total no of Bands scored	Number of polymorphic bands	Proportion of polymorphic loci (%)
OPJ-13	AGGTGACCGT	60	07	05	71.42
OPBX-02	GTTGCGATCC	60	10	08	80.00
OPB-06	TGCTCTGCCC	70	06	05	83.33
OPP-12	GTCCACACGG	70	07	06	85.71
OPBM-05	TTCGAGCCAG	60	08	05	62.50
OPBD-16	GGACCCAACC	70	07	06	85.71
OPA-20	GTTGCGATCC	70	08	05	62.50
Total	**-**	**460**	**53**	**40**	**531.17**
Average	**-**	**65.71**	**7.57**	**5.71**	**75.88**

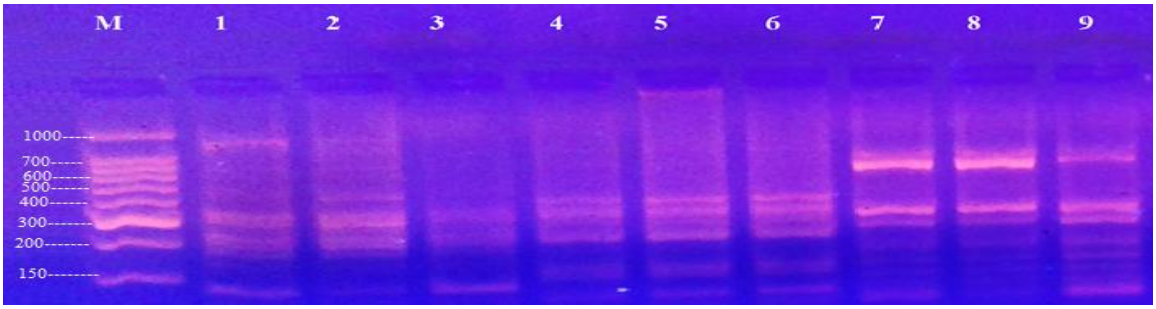

Plate 6. RAPD profiles of 9 garlic genotypes using primer **OPP-12** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

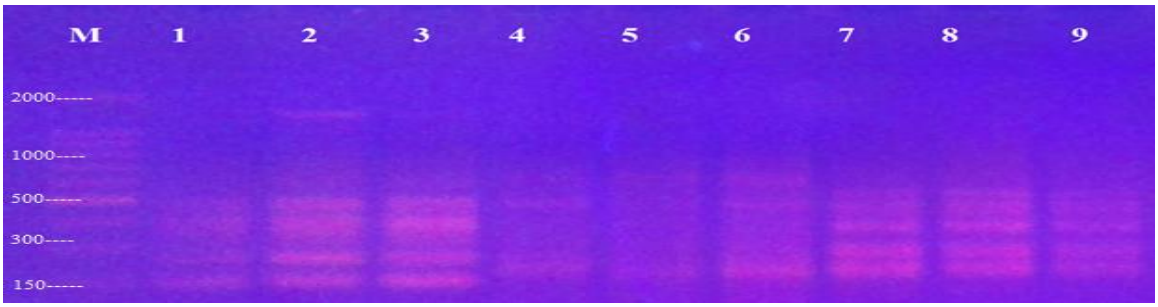

Plate 7. RAPD profiles of 9 garlic genotypes using primer **OPJ-13** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

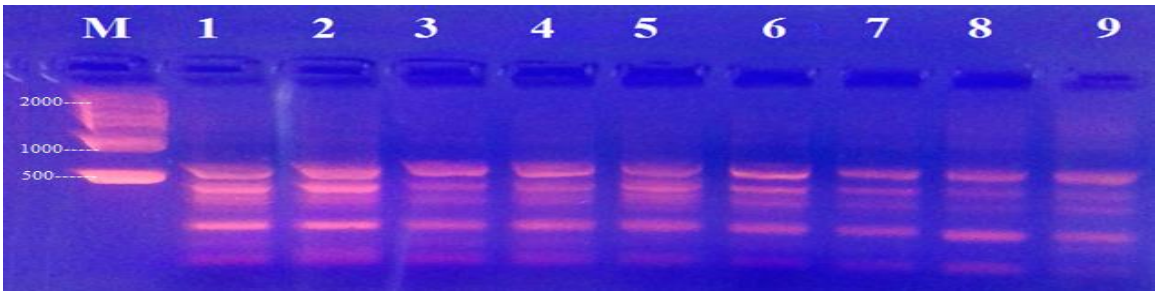

Plate 8. RAPD profiles of 9 garlic genotypes using primer **OPBM-05** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

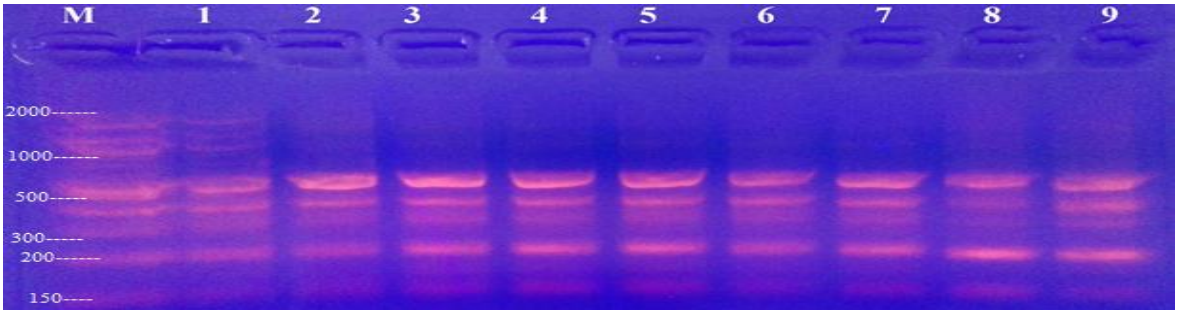

Plate 9. RAPD profiles of 9 garlic genotypes using primer **OPBX-02** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

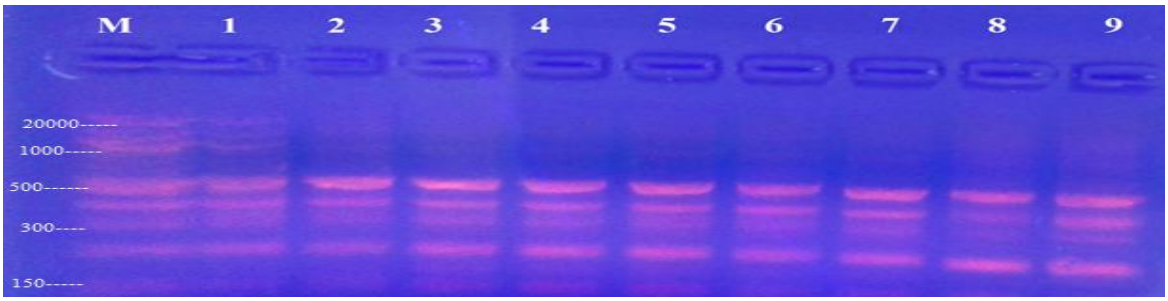

Plate 10. RAPD profiles of 9 garlic genotypes using primer **OPA-20** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

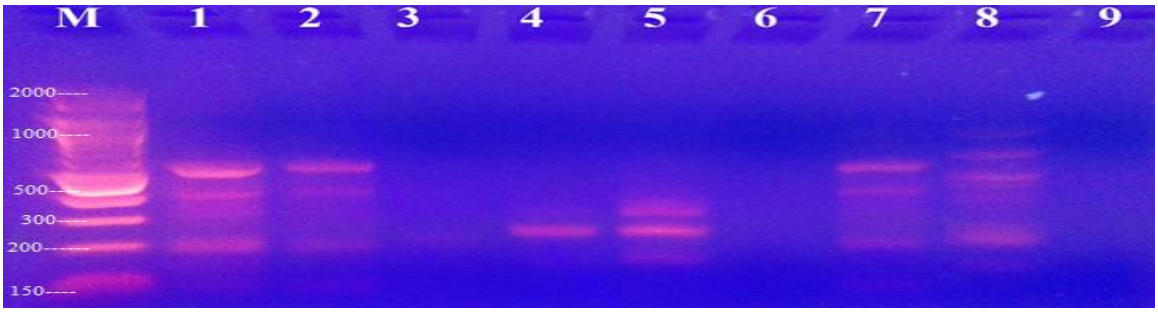

Plate 11. RAPD profiles of 9 garlic genotypes using primer **OPB-06** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

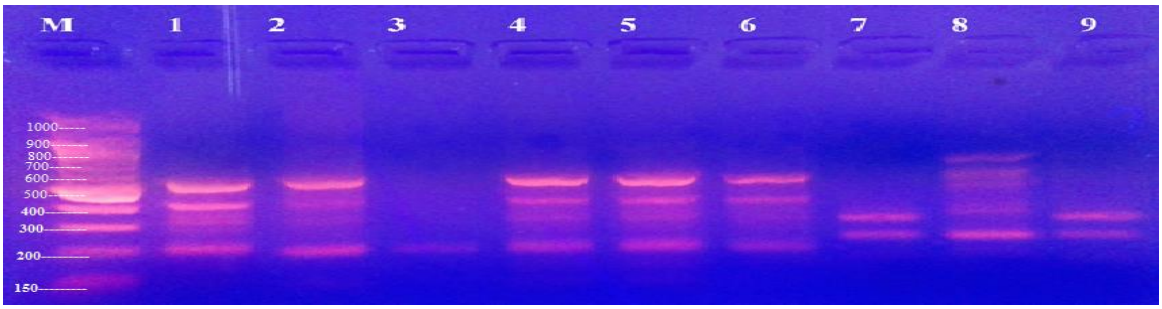

Plate 12. RAPD profiles of 9 garlic genotypes using primer **OPBD-16** (Lane 1: BARI-G1, Lane 2: GC001, Lane 3: GC008, Lane 4: GC0012, Lane 5: GC0013, Lane 6: GC0017, Lane 7: GC0024, Lane 8: GC0027, Lane 9: GC0028,M= 100bp DNA ladder)

4.5 Gene frequency and frequency of polymorphic loci

The DNA polymorphisms were detected according to band presence or absence. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between primer sites (Clark and Lanigan,1993).

The seven primers used in the present study showed different levels of gene frequency and different frequency of polymorphic loci (Table 8). The highest gene frequency shown by the primer OPA-12 (i.e. in allele 0 was 0.7984 and in allele 1 was 0.2016).

On the other hand, the lowest gene frequency showed by the primer OPB-X2 (in allele 0 was 0.5268 and in allele 1 was 0.4732).

Table 8. Overall Gene Frequency by RAPD markers in garlic genotypes

Locus/ Allele	Allele 0	Allele 1
OPJ-13	0.7338	0.2662
OPA-20	0.6286	0.3714
OPBD-16	0.7895	0.2105
OPP-12	0.7984	0.2016
OPB-06	0.7063	0.2937
OPB-X2	0.5268	0.4732
OPB-M5	0.5516	0.4484

Khatab and El-Banna (2011) conducted a study and found that the gene frequency ranged from 0.056 to 1.00 and frequency of polymorphic loci ranged from 0 to 0.944.

4.6 Genetic differentiation and rate of migration among subdivided population

The highest gene flow (Nm*) was obtained in primer OPJ-13 (15.9375) and lowest in primer OPBD-16 (2.0717) with an average estimated gene flow 5.9059. The highest co-efficient of gene differentiation (Gst) was obtained in primer OPBD-16 (0.1944) and the lowest in primer OPB-06 (0.0054) with a mean value 0.0781across all loci (Table 9). The highest Hs was obtained in primer OPBX-02 (0.4758) and lowest in primer OPBD-16(0.2678) with an average Hs value0.3846. The highest Ht was obtained in primer OPBX-02(0.4986) and lowest in primer OPP-12(0.322) with an average Ht 0.4172.

Table 9. Gene flow (Nm) and the proportion of co-efficient of gene differentiation (Gst) across different RAPD markers of studied Garlic genotypes.

Locus	Sample Size	Ht	Hs	Gst	Nm*
OPJ-13	90	0.3906	0.3788	0.0304	15.9374
OPA-20	90	0.4669	0.4349	0.0685	6.7975
OPBD-16	90	0.3324	0.2678	0.1944	2.0717
OPP-12	90	0.322	0.3078	0.0439	10.8831
OPB-06	90	0.4149	0.4126	0.0054	91.721
OPB-X2	90	0.4986	0.4758	0.0457	10.4512
OPB-M5	90	0.4947	0.4144	0.1622	2.5817
Mean	90	0.4172	0.3846	0.0781	5.9059
St. Dev		0.0053	0.0053		

Ht = Hardy-Weinberg average heterozygosity expected in sub-population

Hs = Hardy-Weinberg average heterozygosity obtained in sub-population

Gst = Co-efficient of gene differentiation

Nm* = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst;

SD=Standard deviation

The number of polymorphic loci is: 7

The percentage of polymorphic loci is: 100.00

4.7Gene diversity for the RAPD Primer

Genetic diversity values of nine garlic genotypes for 7 primers are given in Table 10. Average gene diversity **(**h) and Shannon's Information index (I) across all primer against genotypes for all loci was found 0.4172and 0.6059 respectively. High level of gene diversity value and Shannon's Information index found in locus OPB-X2 which was 0.4986 and0.6917 respectively. Lowest level of gene diversity value and Shannon's Information index found in locus OPP-12 which was 0.322 and 0.5027 respectively (Table 10).

Table 10. Genetic diversity for 7 primers in 9 garlic genotypes

Locus	Sample Size	na*	ne*	h*	I*
OPJ-13	90	2	1.6411	0.3906	0.5794
OPA-20	90	2	1.8759	0.4669	0.6597
OPBD-16	90	2	1.498	0.3324	0.5147
OPP-12	90	2	1.4749	0.322	0.5027
OPB-06	90	2	1.709	0.4149	0.6054
OPB-X2	90	2	1.9943	0.4986	0.6917
OPB-M5	90	2	1.9789	0.4947	0.6878
Mean	90	2	1.7389	0.4172	0.6059
St. Dev		0	0.216	0.0731	0.0782

* na = Observed number of alleles

* ne = Effective number of alleles [Kimura and Crow (1964)]

* h = Nei's (1973) gene diversity

* I = Shannon's Information index [Lewontin (1972)]

4.8 Nei's Genetic identity and genetic distance

Pair wise comparison of Nei's(1972) genetic identity between 9 garlic cultivars were calculated from the combined data of the seven primers it was presented in Table 11. The value ranges from 0.8053 to 0.9966.The highest Nei's genetic identity (0.9966) was observed in GC0028 vs GC0017 varietal pair whereas the lowest genetic identity (0.8053) was estimated in GC0027 vs BARI-G1 varietal pair (Table 11).The differences between highest and lowest genetic identity indicates the presence of variability among 9 accession of garlic.

Nei's(1972) genetic distance between 9 garlic cultivars ranges from 0.0053 to 0.2166.The highest Nei's genetic distance(0.2166) was observed in BARI-G1 vs GC0027 varietal pair whereas the lowest genetic distance(0.0053) was observed in GC0012 vs GC0017 varietal pair.

Table 11. Summary of Genetic identity (above diagonal) and Nei's (1972) genetic distance (below diagonal) values among 9 garlic genotypes

Genotype	BARI-G1	GC001	GC008	GC0012	GC0013	GC0017	GC0024	GC0027	GC0028
BARI-G1	****	0.8412	0.8701	0.8555	0.8534	0.8263	0.8652	0.8053	0.8184
GC001	0.1729	****	0.9727	0.9871	0.9808	0.991	0.9859	0.9576	0.9847
GC008	0.1391	0.0277	****	0.9839	0.9684	0.9814	0.9649	0.8873	0.9872
GC0012	0.156	0.013	0.0163	****	0.9936	0.9947	0.972	0.933	0.9904
GC0013	0.1585	0.0194	0.0321	0.0064	****	0.9878	0.9795	0.9484	0.9822
GC0017	0.1908	0.0091	0.0188	0.0053	0.0123	****	0.9767	0.9307	0.9966
GC0024	0.1448	0.0142	0.0358	0.0284	0.0207	0.0236	****	0.9596	0.9724
GC0027	0.2166	0.0433	0.1196	0.0694	0.053	0.0718	0.0413	****	0.9104
GC0028	0.2005	0.0154	0.0128	0.0096	0.018	0.0034	0.028	0.0939	****

4.9 UPGMA Dendrogram

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 9 genotypes of garlic into two main clusters: A and B. The first major cluster (A) had 1 genotypes i.e. BARI-G1. The second major cluster (B) had 8 genotypes i.e. GC001, GC008, GC0012, GC0017, GC0028, GC0013, GC0024 and GC0027.

The first major cluster A had no sub-cluster.

Again, the second major cluster B was divided into two sub-cluster: BI and BII. Sub-cluster BII had only one genotype (GC0027), and Sub-cluster BI had 7 genotypes (GC001, GC008, GC0012, GC0017, GC0028, GC0013 and GC0024). Sub-cluster BI was divided into two groups: CI (GC001, GC008, GC0012, GC0017 and GC0028) and CII (GC0013and GC0024). The group CI was divided into two sub-groups EI had 4 genotypes (GC001, GC008, GC0012 and GC0017) and EII had 1 genotype (GC0028). The group EI divided into 2 sub group FI had 3 genotypes (GC001, GC008 and GC0012) and FII had 1 genotype (GC0017). Again, group FI divided into 2 subgroups G1 which had 2 genotypes (GC001 and GC008) and GII had 1 genotype (GC0012). Group G1 divided into two groups HI(GC001) and HII(GC008).The above results were shown in Figure 1.

The result indicates that the low or high level genetic distance exists between varieties with their same or different origins.BARI-G1 vs GC0027 varietal pair showed highest Nei's geneticdistance (0.2166) as they are released from different parental origin. On the other hand, GC0012 vs GC0017 varietal pair showed lowest genetic distance (0.0053) as they are released from same parental origin. This variation can be created by geographical origin. The result also reveal that the genetic base among these garlic varieties is rather narrow. Collection of diverse germplasm from centers of diversity may borden the genetic base. RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management.

The above results are shown in Figure 1. The results indicate that, different level of genetic identity and distance present between the studied 9 garlic genotypes.

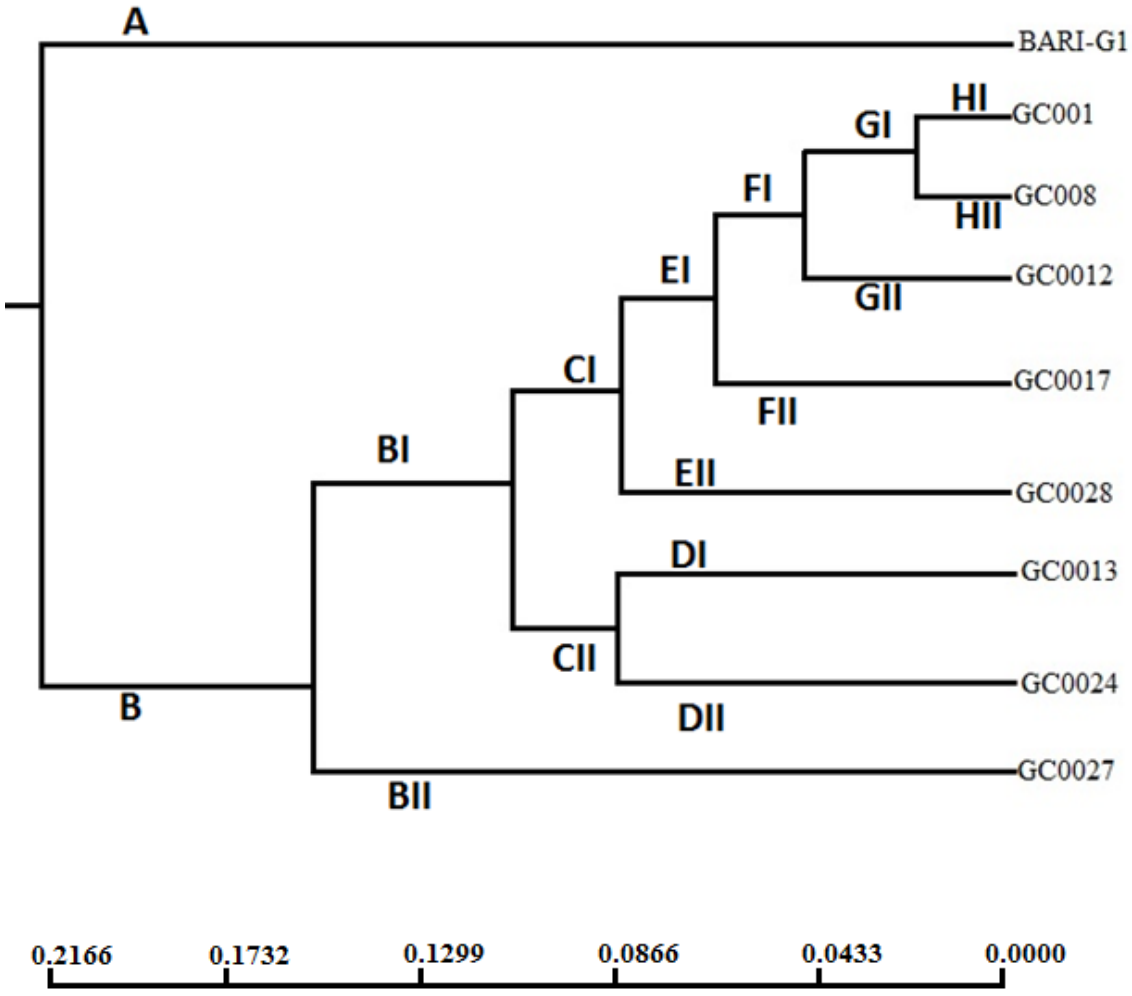

Figure 1. UPGMA dendrogram based on Nei's (1978) genetic distance summarizing the data on differentiation between 9 garlic genotypes according to RAPD analysis.

CHAPTER V
SUMMERY AND CONCLUSION

Garlic (*Allium sativum* L.) is an important vegetable and spice crop all over the world. It is also possessed medicinal value because of its biochemical activities that include antibiotic, antitumor, cholesterol lowering and anti-thrombic effects on animal cells. The agricultural traits of garlic germplasms have normally shown wide variations in morphological traits such as bulb weight, coat layer, leaf length and growth habit. Developing of new variety through molecular characterization and diversity analysis is important for garlic improvement.The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka - 1207, during the period from July, 2014 to June, 2015 to study the genomic DNA isolation procedure development and molecular diversity analysis of nine garlic genotypes through RAPD primers. Conventional CTAB /SDS method of DNA extraction required more time and money. Dozens of high price chemicals are required in CTAB method. More than 15 hours are needed to complete the hole process.Hence, the present study revealed a protocol which is "Low Cost and Very Fast technique for genomic DNA extraction.

Nine (BARI-G1, GC001, GC008, GC0012, GC0013, GC0017, GC0024, GC0027 and GC0028) popular garlic genotypes and seven RAPD primers *viz* OPJ-13, OPA-20, OPBD-16, OPP-12, OPB-06, OPBX-02 and OPBM-05 were used for PCR amplification.

Selected 7 primers generated a total of 53 distinct and differential amplified bands. The average was 7.57 bands per primer and 5.88 bands per garlic genotypes. Out of total 53 bands, 40 bands (75.88%) were polymorphic DNA bands. The primer OPBX-02 produced maximum number of bands (10) and OPB-06 produced the

least number of (06) bands. OPP-12 and OPBD-16 amplified the highest percentage of (85.71%) polymorphic bands and least polymorphic bands was produced by OPBM-05 and OPA-20 (62.50%).

The highest gene frequency shown by the primer OPA-12 (i.e. in allele 0 was 0.7984 and in allele 1 was 0.2016). On the other hand, the lowest gene frequency showed by the primer OPB-X2 (in allele 0 was 0.5268 and in allele 1 was 0.4732).

Nei's (1972) genetic distance between 9 garlic cultivars ranges from 0.0053 to 0.2166. The highest Nei's genetic distance (0.2166) was observed in BARI-G1 vs GC0027 varietal pair whereas the lowest genetic distance (0.0053) was observed in GC0012 vs GC0017 varietal pair.

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 9 genotypes of garlic into two main clusters: A and B. The first major cluster (A) had 1 genotypes i.e. BARI-G1. The second major cluster (B) had 8 genotypes i.e. GC001, GC008, GC0012, GC0017, GC0028, GC0013, GC0024 and GC0027.

From the study, it is concluded that RAPD markers can be used for genetic diversity analysis among and within nine garlic genotypes and effectively trace their genetic relationship. However, larger number of genotypes and higher number of primers would be necessary to construct an appropriate relationship and diversity but present type of study is widely acceptable in all concerns. The present study can be used as a guideline for future fingerprinting and genetic diversity study of garlic varieties.

RECOMMENDATION

This study recommended some points for future consideration while starting DNA fingerprinting and diversity analysis in garlic varieties:

1. To obtain more precious result large number of RAPD primers are needed to be amplified.

2. Large number of varieties and their variant genotypes should be studied.

3. The genotypes and primers can be used in replication method.

4. Other molecular markers such as SSR, AFLP, SNP etc. should be used for diversity analysis in garlic

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