Molecular Characterization of Lentil by RAPD Markers

BY

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CERTIFICATE

This is to certify that the thesis entitled, **"MOLECULAR CHARACTERIZATION OF LENTIL BY RAPD MARKERS"** submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN GENETICS AND PLANT BREEDING,** embodies the result of a piece of bona fide research work carried out by **Md. Mahmudul Hasan, Registration No. 04-01233,** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.

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

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Dedicated To

MY BELOVED

PARENTS

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The Author

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ABBREVIATIONS

AFLP	**: Amplified Fragment Length Polymorphism**
BARI	**: Bangladesh Agriculture Research Institute**
BBS	**: Bangladesh Bureau of Statistics**
BINA	**: Bangladesh Institute of Nuclear Agriculture**
bp	**: Base Pair**
cM	**: Centi Morgan**
CTAB	**: Cetyl Trimethyl Ammonium Bromide**
ddH_2O	**: Double Distilled Water**
DNA	**: Deoxyribo Nucleic Acid**
dNTP	**: Ethelene Diamine Tetra acetic Acid**
EtBr	**: Ethidium Bromide**
FAO	**: Food and Agriculture Organization**
GD	**: Genetic Distance**
ISSR	**: Intersimple Sequence Repeat**
Na_2EDTA	**: Sodium salt of ferric Ethelene Diamine Tetra Acetate**
PCR	**: Polymerase Chain Reaction**
QTL	**: Quantitative Trait Loci**
RAPD	**: Random Amplified Polymorphic DNA**
RIL	**: Recombinant Inbreed Lines**
rpm	**: Rotation Per Minute**
SDS	**: Sodium Dodecyle Sulphate**
STS	**: Sequence Tagged Site**
Taq	**: *Thermophilus aquaticus***
TBE	**: Tris Boric Acid EDTA**
TE	**: Tris - EDTA**
UPGMA	**: Un-weighted Pair Group Method of Arithmetic Mean**
UV	**: Ultra Violet**

Abstract

Random Amplified Polymorphic DNA (RAPD) technique was used to assess genetic diversity and relationship among six lentil varieties, namely BARImasur-1, BARImasur-2, BARImasur-3, BARImasur-4, BARImasur-5 and BARImasur-6. Genomic DNA was extracted form young leaves using SDS method. Three individual from each of the six varieties was analyzed using seven random primers. Amplification with seven (out of 10) random primers generated 53 RAPD markers of which 32 (60.37%) were considered as polymorphic. The highest proportion of polymorphic loci was 11.32% for BARImasur-3. Nei's gene diversity value (0.0552) was the highest in case of BARImasur-3. Results of this investigation indicated relatively high level of genetic variation in BARImasur-3 compared to other varieties. High level of variety differentiation (G_{st} = 1.00) and low level of gene flow (Nm= 0.1328) estimates across all loci reflected that the level of genetic variation existed among all the six lentil varieties. The similarity indices for individual cultivars (S_i) was higher (average 98.99%) than inter varietal similarity (S_{ij}) (average 95.15%). The inter varietal similarity (S_{ij}) for BARImasur-1 Vs BARImasur-2 was higher (95.11%) whereas S_{ij} value was lowest for BARImasur-1 Vs BARImasur-5 (69.63%). The un-weighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's genetci distance, grouped the six cultivars into two main clusters. BARImasur-1, BARImasur-2 and BARImasur-3 in cluster I and BARImasur-4, BARImasur-5 and BARImasur-6 ware group in cluster II. The cultivar BARImasur-4 was close to the cultivar BARImasur-6 with the lowest genetic distance (0.0972) and the highest genetic distance (0.5002) was found between BARImasur-1 and BARImasur-5, The RAPD markers were found to be useful tool in molecular characterization of lentil varieties which could be utilized by the breeders for the improvement of lentil cultivars.

Chapter I

INTRODUCTION

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INTRODUCTION

Lentil *(Lens culinaris* ssp Medik*)* is a diploid (2n=2x= 14), auto-gamous species and is one of the oldest crops in the world. It originated in the Near East (Zohary, 1972). Lentil (*Lens culinaris* Medik) belongs to the family Fabaceae, sub family papilionaceae is one of the major legume crops in Bangladesh. The genus '*lens'* is represented by five species, namely *Lens culinaris* (Medik), *Lens orientalis (*Bioss*), Lens nigricans* (Bieb Godr), *Lens ervoides* (Brign granlo), *Lens montbretti* (Fisch and may). *Lens culinaris* is the only cultivated species which is further divided into two major groups; macrosperma with bold seeds and microsperma with small seeds. In Bangladesh all the indigenous landraces and varieties are micro-sperma with red cotyledons. The exotic macro-sperma varieties possess yellow cotyledons. Lentil is still traditionally cultivated in the Mediterranean Basin, Asia and has a certain diffusion in the America. The major lentil producing regions are Asia and the West Asia-North Africa region. Lentil is the most important pulse crop in Bangladesh India and Nepal, where it significantly contributes to the diet. Farmers also grow lentils in Pakistan, Iran, and Turkey. Other significant producers in the developing world include Argentina, China, Ethiopia, Morocco and Syria. Production is expanding due to the rising demand of increasing population.

Lentil ranks seventh among grain legumes and is grown on over 3.5 million hectares in over 48 countries with a total production of over 3 million metric tons. In Bangladesh it stands after *lathyrus*. Among the various pulses, Lentil *(Lens culinaris* ssp. *culinaris)* is one of the four major pulse crops of Bangladesh and ranks second in respect of yield and production of seed protein (Ahmed. 1996). About 179354 acres of land was under its cultivation and its production was 71535 metric tones (BBS, 2008). It has been reported that the average yield of lentil is-about 985.53 kg /ha which is lower than that of other countries of the world (BBS, 2008).

Lentil is an important source of dietary protein including the essential ammo acids isoleucine and lysine for both human and animal diet, second only to soybeans as a source of usable protein (CGIAR). Lentils are deficient in two essential ammo acids, methionine and cystine but sprouted lentils contain sufficient levels of all essential amino acids, including methionine and cystine (Bejiga, 2006). It is an excellent source of vitamin A. lentils also provides fiber, potassium, vitamin B, and iron.

Many abiotic stresses are important constraints for lentil improvement such as drought, heat, salt susceptibility and iron deficiency. Plant breeders and geneticists have addressed these stresses by identifying resistant/tolerant germplasm, determining the genetics involved and the genetic map positions of the resistant genes. Comparative genomics and synteny analysis with closely related legumes promises to further advance the knowledge of the lentil genome and provide lentil breeders with additional genes and selectable markers for use in marker assisted selection. Genomic tools such as macro and micro arrays, reversed

genetics and genetic transformation are emerging technologies that may eventually be available for use in lentil improvement (Muehlbauer *et al,* 2006).

Several markers may be used to identify and assess the genetic diversity and phylogenic relationships in plant genetic resources. The traditional method based on morphological traits requires extensive observation of mature plants but cannot serve as unambiguous markers because of environmental influences. Protein and isozyme electrophoresis may be used, but the major limitation of these techniques is insufficient polymorphism among the closely related cultivars. With the development of a wide range of molecular techniques, marker assisted breeding is now used to enhance traditional breeding programs to improve crops (Frey *et al.,* 2004). These include restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) (Karp et al., 1997) and the amplified fragment length polymorphism (AFLP) (Vos *et al,* 1995). Among them, randomly amplified polymorphic DNA (RAPD) technique developed by Williams *et al.* (1990) is reliable, faster and easier for exploiting genetic polymorphism within and among species and populations (Gustine and Huff, 1999).

Specific amplified products segregate in Mendelian fashion therefore, these markers can be effectively used as genetic markers. RAPD analysis can be used to characterize DNA variation patterns within species and among closely related taxa. Within grain legume crops alone, RAPD markers have been widely used for the identification of genetic relationships (a) among cultivars (Afzal *et al.,* 2004; Sonnante and Pignone, 2001; Tosti and Negri, 2002), (b) among wild forms (Freyre *et*

al., 1996; Cattan-Tou-pance *et al,* 1998) and (c) between cultivars and wild forms (Ba *et al.*, 2004; Xu *et al.*, 2000).

RAPD analysis can efficiently be used to reveal genetic relationships among different varieties of a species. Knowledge of the genetic relationships among landraces is useful to gene bank managers because it permits a better organization of the crop's gene pool management, more efficient sampling of the available germplasm resources and better access to useful genetic variation for breeders. Since yields of lentil have remained low across Subtropical and Tropical Asia, it is important to estimate genetic diversity in existing cultivars in order to see if the lack of genetic variability might be a constraining factor.

The aim of this work to provide genetic variation and relatedness of lentil cultivars by PCR based RAPD technique as it is important particularly for variety selection for breeding purpose, hybridization, evaluation and conservation of their diverse gene pool. To attain this aim, the present study was carried out with the following objectives:

1. To study the polymorphism among the different released lentil varieties;
2. To characterize lentil germplasms at molecular level through RAPD markers;
3. Diversity analysis of lentil at DNA level.

Chapter II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Among the legume crops of Bangladesh, lentil is important one. For the improvement of this crop through breeding, the knowledge of genetic diversity is a pre-requisite. RAPD marker has been used in analysis of genetic diversity, gene mapping and cultivars identification in different countries of the world.

However, in spite of the importance the molecular characterization of *Lens* using marker such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR), and Amplified Fragment Length Polymorphism (AFLP) etc., has yet not been developed extensively in Bangladesh. Some works based on polymorphic markers has been performed only. Several authors have demonstrated that the RAPD-PCR based method is a powerful tool in the assessment of genetic diversity, which are capable of discriminating between species and sub-species in a wide range of plant genome including lentil. Despite an important crop, little information is available on genetic structure of lentil cultivars in Bangladesh. However, in these aspects a very little work has been done in Bangladesh. The most relevant literature about the present study has been reviewed here under following subheadings:

2.1 Molecular Marker

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

2. 2 Morphological Markers

The traits which are visible such as shoot and stalk number, leaf and leaf blade color, bud shape, seed color and seed size etc. are known as morphological markers. The limitations of visible markers are relatively few in number, incapable to discriminate individuals in many cases. So in order to have sufficient number of visible markers, many progenies of cross have to be screened.

2. 3 Biochemical Markers

It can be assays of enzymes from tissues such as isozymes are the measurement of protein or gene products. The isozyme process involves extraction of the enzymatic proteins and detection of the activity of isozymes using starch, polyacrylamide or cellulose acetate gel electrophoresis and staining. Isozyme expression is determined by number of polypeptide chains and number of alleles.

Each isozyme has one or more distinct polypeptide chains made from amino acids sequences. Isozymes have limitations in protein expression which is affected by changes in environment and plant development. They also lack specificity and sensitivity to detect some genomic changes such-as allelic variation in introns or small amino acid substitutions.

2. 4 Molecular Markers

These are traits that reflect specific sequences of DNA from genomic DNA of plants or animal which include chromosomal, mitochondrial, nuclear and ribosomal DNA. Living cells are to replicate their DNA with high accuracy about many mechanisms are operative which cause changes in DNA. Simple base pair changes may occur, or larger scale changes as a result of inversion, translocation, deletion or transposition. There is large amount of DNA variation present on natural populations and the DNA based markers offer an opportunity to utilize this variation in modern genetics.

Molecular markers can be considered as additional characters of traits-for the evaluation of genetic differences. The advantage of molecular markers is that it is not influenced by the environment (Henry, 1997).

Different molecular markers are Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Micro satellite or Simple Sequence Repeat (SSR), Sequence Tagged Site (STS). Different genetic markers have different scopes (fine-grained vs. coarse-grained analyses) and different advantages and disadvantages (e.g. specificity, cost, ease of analytical interpretation of the resulting data).

Conventional breeding is time consuming and depends on environmental conditions. Breeding a new variety takes 8-12 years. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in plant breeding.

Molecular markers are rapidly being adopted for crop improvement research globally as an effective and appropriate tool for basic and applied studies addressing biological components in agricultural production systems (Jones *et al,* 1997).

Molecular analysis of genetic variation in populations of rare and endangered plants allows management of plant populations so as to preserve genetic diversity and may help to ensure the long-term survival of the species. The diversity of species in an environment has been shown to contribute to the sustainability and productivity of the ecosystem (Tilman *et al.,* 1996). Molecular methods offer options for assessment of biodiversity and may be the key to the development of appropriate conservation strategies (Szmidt, 1994).

For an effective breeding program, information concerning the extent and nature of genetic diversity or variation within a crop species is essential. It is particularly useful for characterizing individual accessions and cultivars and as a general guide in the selection of the parents for hybridization.

2. 5 Concepts of RAPD markers

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. RAPD is a widely applied approach for characterization of DNA from plants and other organisms using PCR with short oligo-nucleotide primers of arbitrary (random) sequence to generate to RAPD-PCR primers are not designed to amplify a specific target sequence, 'the amplified loci are anonymous and

presumably scattered throughout the genome (Williams et al., 1990; Tinker *et al.*, 1993).

The RAPD-PCR technique has provided a relatively simple and inexpensive method for examining variation in the total genome (Hadrys *et al.*, 1992). RAPD analysis is advantageous over isozyme electrophoresis because it generates much greater numbers of loci required for genetic analysis (Kimbeling *et al.*, 1996). RAPD markers can be used as supposedly unbiased; and neutral markers for genetic mapping applications (Michelmore. *et al.*, 1991), in population genetics (Haig *et al.*, 1994), taxonomy (Chapco *et al*, 1992) as well as for genetic diagnostics.

DNA-based markers provide powerful tools for discerning variations within crop germplasm and for studying evolutionary relationships (Gepts *et-al*, 1993).

RAPD have been used to construct genetic maps and for the molecular tagging of various agronomic traits in various crop species (O' Brien, 1990; Williams *et al.*, 1993). It permits the identification of texa and the determination of phylogenetic relationships and intra-specific diversity at a molecular genetic level (Williams *et al.*, 1990).

2.6 RAPD markers in genetic diversity analysis

Genetic Diversity is generally defined as the amount of genotypic variability in a population, or the number of different alleles for loci and the proportion of loci with more than one alleles in a species or population. Knowledge about germplasm diversity and genetic relationship among breeding materials are highly valuable tools in

currently available for analysis of genetic diversity in germplasm accessions, breeding lines and populations.

Rana *et al.* (2007) studied molecular analysis of 29 lentil *(Lens culinaris)* cultivars and landraces of Indian origin using twenty RAPD and ten cross-species STMS primers. A total of 97 markers (72 RAPD and 25 STMS) were amplified of which 42.3% were polymorphic. Genetic similarity among the cultivars and landraces was 89.7%. The observed results indicated low level of genetic diversity in the studied material. UPGMA cluster analysis for the combined data of RAPD and STMS revealed two broad clusters- Cluster I with three landraces and Cluster II containing all remaining landraces and cultivars except Precoz. Germplasm line Precoz was found to be the most distinct in individual as well as combined analyses. All cultivars and landraces except K-75 and L-4076 could be discriminated from one another-using combined data for the two techniques Germplasm lines. Precoz, L-830-and cultivars L-4147 and JL-3 were quite distinct and could be potential germplasm resource.

Karuppanapandian *et al.* (2006) studied random amplified polymorphic DNA (RAPD) analysis of green gram *(Vigna rddiata* L.) landraces from various localities of Southern Tamil Nadu, India to determine the extent of genetic diversity at DNA level using 20 decamer primers. All the primers produced polymorphic amplification products with some extent of variation. A total of 200 bands were generated with an average of 10 per primer and exhibited 83.0% polymorphism. Jaccard's similarity coefficient ranged from 0.64 to 0.93 and concentrated mostly between 0.76 to 0.93, this indicated narrow genetic base of tested green gram landraces. Clustering of green gram landraces into two groups showed reasonable variability.

Ba *et al.* (2004) undertaken a study using RAPD analysis to characterize genetic variation in domesticated cowpea and its wild progenitor as well as their relationships. The materials used consisted of 26 domesticated accessions, including accessions from each of the five cultivar group and 30 wild weedy accessions which including accessions from West, East and southern Africa. A total of 28 primers generated 202 RAPD bands. One hundred and eight bands were polymorphic among the domesticated compared to 191 among wild weedy cowpea accessions. Wild accessions were more diverse in East Africa which is the likely area of origin of *V. unguiculata* var. *spontanea.* Var. *spontanea* is supposed to have spread westward and southward, with a Joss of variability, loss counterbalanced in southern Africa by introgressions with local perennial sub-species. The result also suggested that primitive cultivars were more diverse than evolved cultivars.

Souframanien & Gopalakrishna (2003) studied the DNA polymorphism in elite black gram genotypes using RAPD and ISSR markers. Amplification of genomic DNA of the 18 genotypes, using 25 RAPD primers yielded 104 fragments of which 44 were polymorphic with an average of 1.8 polymorphic fragments per primer. Number of amplified fragments with random primers ranged from two to nine and varied in size from 200 bp to 2,500 bp. Percentage polymorphism ranged from 16.6% to a maximum of 66.6% and with an average of 42.7%.

2. 7 Analysis of genetic variation and relationship

Duran *et al.,* (2004) assessed the genetic variation of a collection of twenty two *Lens* accessions using random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. The collection included accessions of the cultivated lentil, *Lens culinaris* subsp. *culinaris* and its wild ancestor *L. culinaris* subsp. *orientalis,* and the other wild species of the genus *L. odemensis, L. ervoides, ,L. nigricans, L. tomentosus* and *L. famottei.* Both types of markers produced a relatively high number of polymorphic band in the whole collection, Although the degree of variation within the cultivated materials was lower. ISSR markers produced on average more bands and useful polymorphisms than RAPD markers. The Fitch-Margoliash dendrogram, based on Jaccard indices taking jointly RAPD and ISSR into account, showed that the cultivated materials were grouped according to their macro and micro-sperma type and their geographical origin and was compatible with the hypothesis of the existence of six different species in the genus *Lens*, with *L. tomentosus* being the closest species to *L. culinaris.*

Sonnante *el al.* (2001) conducted an experiment where a collection of 46 lentil landraces, mostly from Italy, including Mediterranean and foreign germplasm as reference was evaluated using RAPDs, micro satellite-primed PCR and ISSRs. A low level of useful polymorphic bands was detected with the former two markers whereas ISSRs revealed a higher degree of variation. The UPGMA trees based on Jaccard similarity index matrices obtained with RAPD and ISSR respectively, did not produce similar clusters. ISSR markers proved to be useful for distinguishing closely related genotypes and possibly for substantiating the genetic

peculiarity of ecotypes applying for the obtainment of origin and quality marks.

Betal *et al.* (2004) isolated DNA from 14 cultivars of *Vigna radiata* L. Wilczek and subjected to RAPD analysis using 14 random decamer primers. These cultivars revealed polymorphism with respect to RAPD markers. Analysis of banding patterns confirmed that two strongly aromatic cultivars $1C_1$, $1C_4$, were closely linked. But another aromatic cultivar B_1 formed a separate cluster. The high yielding cultivars were closely related to B_1. The phylogenetic tree that RAPD results were co-related with morphological characters like plant height, leaf and seed size, seed color, etc.

Hoque *et al.* (2002) studied inheritance and linkage relationship between eight morphological and RAPD markers in lentil based on the analysis of F_2 population of the cross $L_{6163} \times L_{330}$. One hundred and forty RAPD decamer primers were screened for detection of polymorphism in the two parents viz., L 6163 and L 830. Only nine primers showed reproducible polymorphism among the parents. Out of nine polymorphic primers only two primer gave expected monogenic mode of inheritance. All morphological traits showed monogenic mode of inheritance. Joint segregation analysis between morphological markers revealed that the traits leaf, stem and pod pigmentation were linked to each other.

2.8 Analysis of RAPD marker

Subhojit *ei al.* (2007) conducted an experiment with RAPD analysis of 16 varieties representing eight different pulse crops namely, pea, lentil, lathyrus, chickpea, pigeon pea, French bean, urdbean and mung bean and he reported that RAPD primers can effectively be used to establish genomic relationship among the pulse crops.

Subhojit *et al.* (2007) surveyed 40 random decamer primers used in RAPD analysis among 16 varieties of eight different pulse crops (viz., pea, lentil, lathyrus, chickpea, pigeon pea, French bean, urdbean and mung bean,) 30 resulted in polymorphic amplification products in the range of 4 to ll with amplicon size ranging from 0.2 to 3.5 kb. Cluster analysis using NTSYS showed distinct genotypic group for each crop i.e., no variety of a particular crop was grouped with varieties of other crops. At the species level, chickpea and lentil were grouped together whereas pea and lathyrus were close to each other. The two crops of the genus Vigna mung bean and urdbean were grouped together with their varieties forming separate sub-clusters, French bean and pigeon pea were grouped in distinct clusters. The analysis resulted that with appropriate primer choice and RAPD can be a very useful tool for phylogenetic analysis of pulse crops.

Tullu *et al.* (2006) investigated the genetics of resistance to ascochyta blight using a population of recombinant inbred lines (RILs) developed in Saskatchewan from a cross between the susceptible *Lens culinaris* 'Eston' and resistant germplasm line (PI 320937). To identify markers associated with resistance to ascochyta blight, using a quantitative-trait loci (QTLs) analysis. The RILs were spray-inoculated

with an isolate of A. lentis (3C-2) in a replicated greenhouse test. The RILs scores for reaction to ascochyta blight showed a continuous distribution suggesting a polygenic inheritance of resistance to ascochyta blight in lentil. QTL that explained 41% of the variation in the reaction to ascochyta blight was identified on the linkage group 6. This QTL was localized between an amplified fragment length polymorphism (AFLP) marker and LCt2, a gene for resistance to anthracnose *[Colletotrichum truncatum]*. This is the first evidence in lentil for linkage between genes conferring resistance to two different foliar pathogens. The result was further verified by co-segregation of a marker locus (OP-P4) in mapping population which was also previously identified to be linked to a major QTL for resistance to ascochyta blight in the lentil germplasm line ILL-5588. The AFLP and random amplified polymorphic DNA (RAPD) markers localized around the resistance region will be useful tools in marker assisted selection.

Eujayl *et al.* (2006) investigated the inheritance of resistance to lentil *(Lens culinaris* Medik.) vascular wilt caused by *Fusarium oxysporum* f.sp. lentil in a cross between resistant (1LL5588) and susceptible (L692-I6-l(s)) lines. F2:4 progenies and F6:8, F6:9 recombinant inbred line (RIL) populations were assessed for their wilt reaction for three seasons in a well established wilt sick plot. Resistance to wilt was conditioned by a single dominant gene in the populations studied. The map location of the *Fw* locus was identified for the first time through linkage to a random amplified polymorphic DNA (RAPD) marker (OPK-15900) at 10.8 cM. Two other RAPD markers (OP-BH800 an OP-DI5500) identified by bulked segregant analysis were associated in coupling phase with the resistance trait and another marker (OP-C0465) associated with repulsion.

The DNA markers reported here will provide a starting point in marker assisted selection for vascular wilt resistance in lentil.

Afzal *et al* (2004) studied the genetic diversity of mung bean *[vigna radiata(L.* Wilczek] cultivars using DNA markers. Twenty one mungbean cultivars were subjected to RAPD analysis using 34 decamer primers in this study. All the primers produced polymorphic amplification products with some extent of variation. A total of 204 bands were generated with an average of. 6.0 per primer and exhibited 75.0% polymorphism. This indicated a rather broad genetic base of tested Cultivars. Genetic similarity obtained in this study may be used for selecting parents for breeding purposes. Clustering of cultivars into four groups showed reasonable variability that may be exploited for yield improvement.

Anil *et al.,* (2004) used rust resistant lentil genotype precoz and the susceptible PL-639 to develop the mapping (F_2 population) which was pathologically evaluated in the field in New Delhi, India. DNA of the parental lines and 42 F_2 individuals was extracted during the early seedling stage. Aliquots of the same DNA preparations were used both for RAPD and AFLP which were performed using standard procedures. For RAPD a large number of Operon 10mer primers were used to screen the parental polymorphism and polymorphic primers were used on mapping population of 42 individual F_2 plants with rust resistance gene. RAPD marker OPX-15 and OPX-17 were found to be linked with rust (*Uromyces fabae* f.sp. lentis [U. *viciae-fabae*]) resistance locus with a distance of 11.4 and 16.5 cM respectively. Whereas none of the AFLP markers was found to be linked with the rust resistance. Ford *et al.* (2004) assessed Canadian isolates of *C. truncatum* from lentil for

morphological and molecular differences against Australian and international isolates of *C. truncatum* from hosts other than lentil and other *Colletotrichum* species. Although similar in dimension, conidio-spores from lentil *C. truncatum* were ellipsoidal and those from *C truncatum* originating from Australian *Glycine max, Xanthium occidentalis* and *Arachis hypogaea* were falcate in shape. Conidio-spores of Australian *C. trifolii, C. gloeosporioides and C. destructivum* were cylindrical. Molecular analysis using both RAPD markers and 18-25S rDNA sequences, demonstrated the genetic relatedness of *C. truncatum* isolates from the same host species and discriminated among isolates from lentil and other host species.

Janila and sharma (2004) studied to identify molecular markers linked to the *er* gene which offers resistance to powdery mildew caused by *Erysiphe pisi* in pea. Screening of the powdery mildew-resistant cultivar 'DMR11' and its susceptible near isogenic line for polymorphism revealed linkage of two RAPD Primers (OPO-02 and OPU-17) to the *er* gene and a sequence characterized polymorphic region (SCAR) primer. ScOPD-lO_{650} with *er* in a population of $83F_2$ plants in the order: OPU-17-cr - ScOPD-10_{650} -OPO-02. The markers ScOPD-lOeso and OPU-17 being coupled with the allele causing resistance would substantially increase the efficiency of marker-assisted selection in pea breeding for powdery mildew.

Tullu *et al.* (2003) conducted an experiment to examine the resistance to anthracnose in P.I 320937 lentil and to identify molecular markers linked to the resistance gene in a recombinant inbred line (RIL) developed from a cross of Eston lentil, the susceptible parent, and PI 320937, the resistant parent. A total of 147 F (5:6) RILs were evaluated for resistance to

anthracnose in the greenhouse using isolate 95B36 of *C. truncatum*. Bulked segregant analysis (BSA) strategy was employed and two contrasting DNA bulks were constructed based on greenhouse inoculation of F (5)-derived F (6) RILs. From the parents and bulks were screened with 700 RAPD primers and seven AFLP primer combinations. Analysis of segregation data indicated that a major dominant gene was responsible for resistance to anthracnose while variations in the resistance level among RILs could be the influences of minor genes.

Tosti and Negri (2002) investigated the efficiency of RAPD, AFLP, and SAMPL marker systems in detecting genetic polymorphism in cowpea landraces *(Vigna unguiculata* subsp, *unguiculata* L. *Walp.).* A second objective was to determine the level of diversity among landraces from a restricted area, to define the most appropriate strategy of on-farm conservation. Each marker system was able to discriminate among the materials analyzed. The average diversity index was quite similar for each marker system, but owing to the differences in the effective multiplex ratio values the marker index was. Higher for the AFLP and SAMPL systems than for the RAPD system. The study detected low genetic similarity among landraces.

Chowdhury *et al.* (2001) reported that resistance to ascochyta blight of lentil *(Lens culinaris* Medikus) caused by the fungus *Ascochyta lentis* is determined by a single recessive gene, *ra/2,* in the lentil cultivar Indian head. Sixty F_2 individuals from a cross between Eston (susceptible) and India head . Resistant genotyps were analyzed for the presence of random amplified polymorphic DNA (RAPD) markers linked to the *rail* gene using bulked segregant analysis (BSA). Out of 800 deca nucleotide primers screened two produced polymorphic markers that co-segregated

with the resistance locus. These two RAPD markers, UBC2271290 and OPD-10870 flanked and were linked in repulsion phase to the gene ra/2 at 12 cm and 16 cm, respectively. The RAPD fragments were convened to SCAR markers. The SCAR marker developed from UBC2271290 could not detect any polymorphism between the two parents or in the F2. The SCAR marker developed from OPD-10870 retained its polymorphism. The polymorphic RAPD marker UBC2271290 and the SCAR marker developed from OPD-10870 can be used together in marker assisted selection program for ascochyta blight resistance in lentil.

Lakhanpaul *et al.* (2000) investigated the genetic polymorphism among 32 Indian cultivars of mung bean through random amplified polymorphic DMA (RAPD) analysis using 21 decamer primers, a total of 267 amplification products were formed at an average of 12.71 per primer with an overall polymorphism of 64%. The extent of polymorphism was moderate to low. The result revealed greater homology between cultivars released from the same source.

2.9 Estimation of genetic distance

Saini *et al.* (2004) investigated the comparison in efficiency of long primers (ranging from 18 to 22 base in length) and 10 base primers in detecting RAPDs in mung bean and evaluated some selected long primers for their ability to discriminate 46 mung bean genotypes and to study the genetic relationships among them. Both the groups of primers were evaluated for the total number of discrete and detectable amplified fragments and polymorphic bands detected between two mung bean genotypes. The long primers yielded significantly higher number of discrete and detectable bands as well as polymorphic bands than 10 base

primers. A set of eight long primers was used for AP-PCR: analysis of 46 mung bean genotypes. A total of 173 fragments were amplified of which 39.08 % were polymorphic. A high degree of genetic variation was observed among different genotypes, whereas, those originating from the same source were highly related. The results show that long primers can be used for efficiently analyzing genetic diversity and the relationships in a large mung bean germplasm collection.

Tayyaba Sultana (2003) reported genetic diversity due to seed proteins was significantly associated with genetic diversity due to RAPD markers, qualitative and quantitative traits, whereas it had no association with isozymes. Twenty clusters were observed for RAPD that indicated the worth of this technique to investigate even intra-specific diversity and for fingerprinting of lentil germplasm. The RAPD markers exhibited significant association with there parameters (SDS-PAGE, isozymes and qualitative trait). All the five parameters facilitated to resolve issues related to genetic diversity in lentil, anyhow total seed proteins and isozyme (six in present case) gave low level of genetic diversity that suggested to incorporate more isozymes for investigation due to its precise fingerprinting and wider use. The RAPD could be enhanced including more primers including more germplasm, as 50% primers in general were polymorphic in nature.

2.10 Variety identification and classification

Souframanien *et al.* (2002) studied the DNA polymorphism in 12 gamma ray induced morphological mutants of blackgram *(Vigna mungo* L. Hepper) using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. Of the 35 random and eight ISSR primers used, 15 random and five ISSR primers detected polymorphism among the mutants. Total number of bands varied from 2 to 9 for RAPD and from 6 to 10 for ISSR. The number of polymorphic bands varied from 1 to 3 for RAPD and from 1 to 6 for ISSR. Percentage of polymorphism ranged from 12.5 to 50.0 for RAPD and. 12.5 to 44.4 for ISSR. Significant DNA polymorphism among the mutants were observed using RAPD (25.8%) and ISSR (33.3%) markers. An young leaf chlorina mutant and a smooth pod mutant showed more DNA polymorphism as compared to the parent. DNA polymorphism data revealed by RAPD and ISSR could facilitate selection of mutants to be involved in cross breeding and genome mapping.

Taylor *et at.* (2002) have been applied a number of molecular marker techniques to improve the quality of cultivated lentil, aiding in the breeding and release of better-adapted cultivars and in quality assurance of export products. Continued increase in lentil production is dependent on the identification of present and potential limiting factors to the progression of lentil breeding.

From the above mentioned review of literature, it is clearly found that RAPD analysis is very efficient in molecular genetic variation studies within and/or among the populations of lentil cultivars or wild species. The present study is aimed to determine genetic variation and relatedness between six varieties of lentil of Bangladesh using RAPD markers, so that, the level of genetic distance and similarities between the varieties may be estimated which can help in selective breeding techniques for variety improvement using that six varieties.

Chapter III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The experiment was carried out at the Biotechnology laboratory of pulse Research Centre (PRC), Bangladesh Agriculture Research Institute (BARI), Joydebpur, Gazipur. The details of different materials used and methodologies followed for the study have been described below.

3.1 Study Materials

The plant materials for this study consisted of six varieties of lentil viz. BARImasur-1, BARImasur-2, BARImasur-3, BARImasur-4, BARImasur-5 and BARImasur-6. All the varieties were collected from Bangladesh Agricultural Research Institute (PRC) and (BARD) Gazipur.

3.2 Collection of Leaf Sample

Seed germination was performed and germinated seeds were sown in the pot at field laboratory of PRC, BARI (Bangladesh Agriculture Research Institute). Important morphological traits of each materials were given in table 1. In order to carry out RAPD analysis, fresh and young leaf samples were collected from 28 days old seedlings of each genotype and used as the source of genomic DNA.

Initially, each sample was washed carefully in running tap water and preserved in airtight polythene packet separately. Finally, the samples were brought to the laboratory, wrapped by aluminum foil and stored at -20°c freezer.

Table 1. Some salient features of the six lentil varieties used in the study

Name of variety	Days of maturity	Leaf color	Flower color	Seed size	100 Seed Weight(g)	Yield (kg/ha)	Released year
BARImasur-1	105-110	Dark Green	White	Large	14.4	1700-1800	1991
BARImasur-2	105-110	Dark Green	White	Medium	12.5	1500-1700	1993
BARImasur-3	100-105	Dark Green	White	Large	23.8	1500-1700	1996
BARImasur-4	110-115	Light Green	White	Large	19.84	1600-1700	1996
BARImasur-5	110-115	Green	Reddish Violet	Large	19.00	1700-1800	2006
BARImasur-6	105-110	Dark Green	White	Large	19.8	2200-2300	2006

Source: Pulse Research Center of Bangladesh Agriculture Research Institute (BARI), Joydebpur, Gazipur.

3.3 Genomic DNA isolation

According to Doyle and Doyle (1987) following reagents and methods were used with few modifications for the isolation of total genomic DNA.

3.3.1 Reagents used:

i. Extraction buffer, pH=8.0
   - 50mM Tris- base
   - 25mM EDTA (Ethylene di-amine tetra-Acetic Acid)
   - 300 mM NaCl, and
   - 20% SDS (Sodium Dodecyl Sulphate)

ii. Phenol: Chloroform: Isoamyl alcohol = 25: 24: 1, equilibrated to pH near 8.0

iii. TE buffer, pH 8.0
   - Tris- base 10 mM,
   - 1mM EDTA,

iv. Sodium acetate (3M), pH =5.2

v. Absolute (100%) ethanol

vi. Ethanol (70%)

3.3.2 Equipments required

1. Mortar and pestle
2. Water bath
3. Centrifuge
4. Vortex mixture
5. Ice maker

3.3.3 Reagent preparation for DNA extraction (Stock solution)

Extraction buffer (1000 ml)

For the preparation of 1000 ml DNA extraction buffer, 100 ml 1M Tris (pH=8.0) was mixed with 40 ml of 0.5 ml EDTA and added to100ml 5M NaCl in a 1000ml measuring cylinder. Finally, Sterilized ddH_2O was added to make the volume up to 1000ml, then mixed well and autoclaved.

20% SDS (100 ml)

To prepare 100 ml 20% SDS, 20 g SDS was mixed with 100 ml ddH_2O in a 250 ml beaker. SDS is hazardous, so the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.

5M NaCL (250 ml)

For the preparation of 5M NaCl, 73.05 g of NaCl was added in 250 ml ddH_2O in a 500 ml volumetric flask, mixed well and autoclaved.

Phenol: chloroform: isoamyl alcohol =25:24:1(100 ml)

To prepare 100 ml of phenol: chloroform: isoamyl alcohol (25:24:1); 2 ml of Isoamyl alcohol and 48 ml Chloroform were added in 50 ml of phenol and mixed well

70% Ethanol (300ml)

90ml ddH_2O was added in 210 ml absolute alcohol

100 ml 1X TE

For preparation of 100 ml 1X TE, 10 ml 1M Tris-base (pH 8.0) was mixed with 0.2 EDTA(pH 8.0) in a 250 ml beaker. Finally water (ddH_2O) was added to make volume up to 100ml and autoclaved.

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

To prepare 250ml 1M Tris base (pH 8.0),30.28g Tris base was added to 200ml ddH2O in a 500ml volumetric flask while pH was adjusted to 8.0 by adding HCl. Then sterilized water was added to make the volume up to 250ml. Finally the solution was autoclaved.

0.5 M EDTA (pH 8.0)(250 ml)

For the preparation of 250ml EDTA (pH 8.0), 200ml ddH2O was taken in a 500ml beaker and 46.33 g EDTA.2H2O was added to it and stirred vigorously. Then 4g NaOH was added for pH adjustment. Sterilized ddH2O was added to make the volume up to 250ml and then the solution was autoclaved.

3.4 Methods for DNA extraction

The following steps were followed for DNA extraction:

- For isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from six lentil varieties. Total DNA was isolated by using phenol: chloroform: isoamyl alcohol purification and ethanol precipitation method.
- The youngest leaves were selected in order to make the tissue grinding process easy. Initially, healthy youngest leaves (16 to 24days aged) were taken out and washed consequently in sterile distilled water and ethanol and dried on fresh tissue paper to remove spore of microorganisms and any other sources foreign DNA.
- Approximately 0.1g of leaves was taken into centrifuge tube (1.5 ml). Digestion was done by adding 800 µl extraction buffer into the tube in two installments; first leaf tissues were ground manually with the help pestle and mortar with 400 µl extraction buffer then

the remaining 400 µl extraction buffer was added into eppendorf tube (1.5 ml).

- The ground sample was vortexed for 20 seconds in vortex mixture (IUCHI Automatic Lobo mixture, Japan) and incubated at 65°C for 5 min in hot water bath (JP Selecta(R)). Following further mixing with a vortex mixture for about 20 seconds, then sample was again incubated at the same temperature for approximately 10 minutes.

- The extract was centrifuged for 10 min at 14000 rpm with a micro centrifuge to allow precipitation of the cell debris.

- The upper aqueous phase of about 600 µl was transferred to another eppendorf tube. Then 4 µl of RNAase was added to remove RNA and incubated at 55°C for 30 min in hot water bath.

- Then the upper aqueous of about 600 µl was transferred to another eppendorf tube. For purification, equal volume 600 µl of Phenol: Chloroform: Isoamyl alcohol (v: v: v=25: 24: 1) was added to the tube and vortexed for few seconds.

- The solution was centrifuged for 10 min at 14000 rpm. The upper aqueous phase was recovered using a pipette tip without disturbing the lower portion and transferred into a new eppendorf tube.

- DNA was precipitated with 800 µl of absolute ethanol (100%). At this point, DNA became visible as white strands by flicking the tube several times with fingers.

- DNA was pelleted by centrifugation for 3 min at 14000 rpm.

- Then the liquid was discarded completely and re-precipitation of the DNA solution was done by adding 400 µl of 70% ethanol with 20 µl 3 M sodium acetate and pelleted by centrifugation for 3 min at 14000 rpm.

- The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper. The pellets were then air dried and dissolved in an appropriate volume (20 µl-50 µl) of TE buffer, pH 8.0.

- Finally, the DNA samples were stored at -20°C.

3.5 Confirmation of DNA preparation

Sometimes isolated genomic DNA contains a large amount of RNA and pigments which usually cause over estimation of DNA concentration on a spectrophotometer. To confirm 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 100m1 electrophoresis buffer (1X TBE buffer) prepared by adding 20 ml of 5 X TBE buffer in 80 m1of sterile de-ionized 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°C (flask was cool enough to hold comfortably with bare hand) and 1µ1 (10mg/mi) 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 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.

Composition of 5 × TBE buffer (1 liter)

- 54 g of Tris base
- 27.5 g of Boric acid
- 4.65g of EDTA
- pH = 8.3

3.5.2 Preparation of DNA samples for electrophoresis

The samples were all in the same concentration of buffer. For each sample, 6 µl × 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 1mM 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 (Blue Marine Scrva) containing 1 × 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 90V for about 45 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

The concentration of DNA is one of the important variables for PCR amplification. Because different DNA extraction methods produced DNA of widely different purity it may be necessary to optimize the amount of DNA used in the RAPD assay to achieve reproducibility and strong signal. Below a certain critical concentration of genomic DNA, RAPD amplification is no longer reproducible (Williams *et al.*, 1993). So, 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™) and stored in freezer. In the beginning of the quantification of DNA samples, spectrophotometer UV-lamp was turned on and after it had warmed up, the wavelength was set at 260nm. One cuvette (the "Zero" or "blank" cuvette) was filled with 2 ml sterile distilled water and placed in spectrophotometer. The test samples were prepared by taking 2 µl of each sample in a cuvette containing 2ml sterile distilled water. The sample was uniformly mixed and placed in spec trop ho to meter and the absorbance reading was taken at 260nm. 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:

DNA concentration (ng/µl)

$$= \text{Absorbance} \times \frac{\text{Volume of distilled water (µl)}}{\text{Amount of DNA (µl)}} \times \text{Conversion factor (0.05)} \times 1000$$

Where,

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

Table 2 Absorbance reading and concentration of DNA samples collected from six lentil germplasm

Lentil varieties	Serial No. (Replication)	Absorbance reading (260 nm)	DNA concentration (ng/µl)
BARImasur-	V1-1	.068	3400
	V1-2	.079	3450
	V1-3	.077	3850
BARImasur-	V2-1	.075	3750
	V2-2	.085	4250
	V2-3	.087	4350
BARImasur-	V3-1	.078	3900
	V3-2	.078	3900
	V3-3	.071	3550
BARImasur-	V4-1	.081	4050
	V4-2	.078	3900
	V4-3	.086	4300
BARImasur-	V5-1	.069	3450
	V5-2	.071	3550
	V5-3	.068	3400
BARImasur-	V6-1	.088	4400
	V6-2	.183	4150
	V6-3	.088	3400

3.7 Preparation of working solution of DNA samples

DNA concentrations were adjusted to 25ng/µl for doing PCR and using the following formula:

$S_1V_1 = S_2V_2$

Where,

S_1 = Initial strength (ng/µl)

V_1 = initial volume of DNA solution (µl)

S_2 = Final strength (ng/µl)

V_2 = Final volume of DNA solution (µl)

3.8 Amplification of RAPD markers by PCR

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 is subjected to temperature cycling conditions typical to the polymerase chain reaction. The products of the reaction depend 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 (e.g., within a few thousand nucleotides), and a discrete DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligo-nucleotide 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. Polymorphic bands are noted and polymorphisms can be mapped in a segregating population. Often a

single primer can be used to identify several polymorphisms, each of which matches to a different locus.

3.8.2 Primer selection

Initially, ten decamer primers (2 from kit A, 1 from kit B. 1 from kit C, 3 from kit D, 2 from kit W and 1 from kit Y. Operon Technologies, Inc., Alameda, California, USA) (Table 3.5) of random sequence were screened on a sub sample of two randomly chosen lentil accessions, to test their suitability for amplifying lentil RAPDs that could be accurately scored. Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers, consistency within individuals and potentiality for discriminating different accessions. A final subset of seven primers (Table 3.6) exhibiting better quality banding patterns was selected for the analysis of whole sample set of accessions. To confirm the reproducibility of RAPD markers, the selected seven primers were screened three times on the same samples.

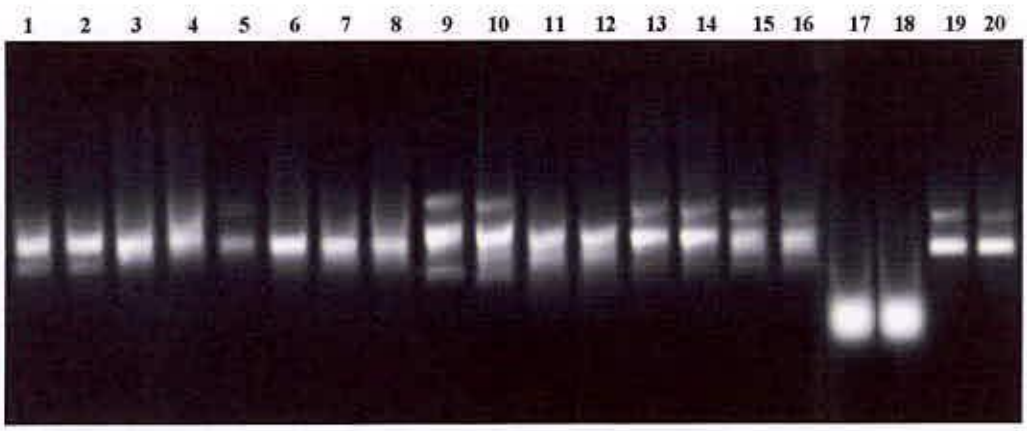

Fig.1: Primer test: PCR amplification products by10 different decamer random primers using DNA from different varieties. Lane 1-2: ; Lane 3-4: ; Lane 5-6: ; Lane 7-8: ; Lane 9-10: ; Lane 11-12: ; Lane 13-14: ; Lane 15-16: ; Lane 17-18: and Lane 19-20 .

3.8.3 PCR amplification

The amplification conditions were based on Williams *et al.* (1990) with some modifications. PCR reactions were performed on each DNA sample in a 10 µl reaction mix containing 1.5 µl of 10 × Ampli Taq polymerase buffer, 1 µl of 10 µM primer, 1 µl of 250 µM dNTPs, 0.5 unit of Ampli Taq DNA polymerase (Bangalore Genei, India) and 4 µl (100 ng) of genomic DNA and suitable amount of sterile deionized water. DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94°C for pre-de-naturation and then 40 cycles consisted of: 1 min de-naturation at 94°C, 1 min at 36°C for annealing and 2 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.

Table 3: Primer codes and sequences used for the detection of polymorphism in lentil accessions

Primer codes	Sequence (5′ to 3′)	(G +C) %
OPA01	TGGACCGGTG	70%
OPA18	CAATCGCCGT	60%
OPB07	GAGGGTCGGA	70%
OPC08	CCGCATCTAC	60%
OPD02	GTGTGCCCCA	70%
OPD03	GGTGACGCAG	70%
OPD18	GGCATCCACT	60%
OPW01	ACCACGCACC	70%
OPW05	AAGCCTCGTC	60%
OPY11	CCAGCAGCTT	60%

Primer code	Sequences (5′-3′)	(G +C) %
OPA01	TGGACCGGTG	70%
OPA18	CAATCGCCGT	60%
OPC08	CCGCATCTAC	60%
OPD02	GTGTGCCCCA	70%
OPD03	GGTGACGCAG	70%
OPD18	GGCATCCACT	60%
OPW01	ACCACGCACC	70%

3.8.4 Reaction mix preparation to perform Polymerase Chain Reaction (PCR)

William *et al.* (1990) mentioned the conditions for RAPD amplifications reactions with some modifications. PCR reactions were performed on each DNA sample in a 10 µl reaction mix containing following reagents:

Reagents	Amount
Taq DNA polymerase buffer (10X)	1.5 µl
Primer (10 µM)	2 µl
dNTPs (250 µM)	1
Taq DNA polymerase	0.5µl
Genomic DNA (25 ng/µl)	3 µl
Sterile de-ionized water	2 µl
. Total reaction volume	10 µl

From frozen stocks the PCR buffer, dNTPs, primer and DNA samples solutions were thawed, mixed by vortexing and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermo

cycler used (0.2 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 sealed and placed in a thermo cycle and the cycling was started immediately.

3.8.5 Thermal Profile

DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf, Germany). The PCR was programmed as per Fall et al. (2003) used for cowpea DNA amplification. According to them the reaction mixture was preheated at 94^0C for 3 minutes followed by 38 cycles of 1 minute de-naturation at 94^0C, 1 minute annealing at 42^0C and elongation or extension at 72^0C for 1.5 minute. After the last cycle, a final step of 5 minutes at 72^0C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4^0C.

3.8.6 Electrophoresis of the amplified products

From each sample PCR products were confirmed by running 0.8% agarose gel containing 1-µl ethidium bromide in 1X TBE buffer at 90V for 55 minutes. Loading dye (2.0µl) was added to the PCR products and loaded in the wells. Molecular weight marker 100 bp DNA ladder were also loaded on either side of the gel. Under Ultra Violet (UV) light on a transilluminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.8.7 Documentation of the DNA samples

After staining, the gel was taken out carefully from the staining tray and placed on high performance ultraviolet light box (UV transilluminator) of gel documentation for checking the DNA was observed as band and saved the records.

Precautions:

The usual 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 especially for Taq polymerase. Hand-gloves were worn during handling of PCR components. Contamination of PCR components was avoided.

3.9 RAPD Data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *et al.*, 1990). One molecular weight marker, 100 bp 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 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, Nie's(1973) gene diversity, population differentiation

(Gst), gene flow(Nm). Genetic distance (GD) and to construct a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program, POPGENE (version 1.31) (Yeh *et 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 was 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 by mutation. The two-allele assumption was in most cases acceptable, because co-dominant loci showing band shifts are few (Elo *et al.*, 1997; Welsh and McClelland 1990). 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 $(N/n)^{1}/_{2}$ 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 Nel's genetic distance (Nel'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:

Similarity index (SI) : $2N_{xy}/ (N_x + N_y)$

Where , N_{xy} is the number of RAPD bands shared by individuals x and y respectively, and N_x and N_y are the number of bands in individuals x and y respectively, (Chapco *et al.*, 1992, Wilde *et al.*, 1992, Lynch, 1990).

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, 1991)

Geneflow (N_m) was estimated according to the following formula: gene flow , $N_m = 0.5 (1-G_{st}) / G_{st}$

Where G_{st} is the proportion of total genetic diversity attributable to subpopulation. It is also known as coefficient of gene differentiation. The G_{st} values were calculated by using the following formula:

$G_{st} = 1 - H_s/H_t$

Where, H_s is the mean average heterozygosity of the total population and H_t is the mean of Hardy- Weinberg expectation of heterozygosity obtained with population average allele frequencies.

Nel's genetic distance and Nel's genetic identity values were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied six lentil germplasm using the unweighted pair-group method of Arithmetic means(UPGMA)(Sneath and Sokal, 1973). The dendrogram was constructed using the POPGENE; (Version1.31) computer program (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 wave length can cause eye damage. Thus eye protector was used while working with it.

Chapter IV

RESULT AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

This chapter comprise the presentation and discussion of the results of the experiment. The results were obtained from the experiment using RAPD markers on lentil varieties. In the RAPD analysis significant genetic variation and polymorphisms for characterization of different lentil cultivars were identified. The present study indicate the effectiveness of RAPD analysis in detecting substantial amount of polymorphisms among different cultivars of lentil. Some of the data have been presented and expressed in Table-4-11 and others in Figures 2-6 for ease of understanding.

4.1 primer selection and RAPD pattern

Ten primers were initially screened on six lentil varieties for their ability to produce polymorphic patterns and seven primers (OPA01 OPA18 OPC08 OPD02 OPD03 OPD18 andOPW01) which gave reproducible and distinct polymorphic amplified products were selected. DNA amplification from all the primers tested in this study was not consistently reproducible, is very common feature of RAPD technique. The present findings agree with those of Hardy *et al.* (1992), and Williams *et al.* (1993).

Technical problems from amplification of the RAPD techniques in the field of genetic population research have also been reported by many authors (Schierwater and Ender, 1993; Lynch and Milligan, 1994). A total of 53 bands were scored of which 32(60.37%) polymorphic amplification products were obtained by using these arbitrary primers the size of the amplification products ranged from 120-1000 bp (Table-5). The selected seven primers produced comparatively maximum number of high intensity band with minimal smearing, good technical resolution and sufficient variation among different cultivars. The highest numbers of bands were generated by primer OPD 03 whereas; the least number of bands were produced by primer OPD 02(Table). Besides the primer OPA18 OPW01 and OPD18 generated the Maximum number of polymorphic bands (19%) while the primer OPA08 generated the least (7%) polymorphic bands which were minimal in number. The polymorphic amplification bands ranged from (2-5) and were amounted to be 4 on average (Table 5). The banding pattern of six lentil varieties using OPA01 OPA18 OPC08 OPD02 OPD03 OPD18 and OPW01 are shown in figs. 2, 3, 4, 5, 6and 7 respectively. No. of RAPD markers scored for each individual six varieties for each primer are presented in Table 5.

Table 5: RAPD primers with corresponding bands score and their size range together with polymorphic bands observed in six lentil varieties

Primer code	Sequences (5′-3′)	Total number of bands scored	Size ranges (bp)	Number of polymorphic bands
OPA01	TGGACCGGTG	8	380-1440	5
OPA18	CAATCGCCGT	9	380-920	6
OPC08	CCGCATCTAC	6	580-980	2
OPD02	GTGTGCCCCA	5	400-1000	3
OPD03	GGTGACGCAG	10	400-1000	4
OPD18	GGCATCTACT	8	350-1000	6
OPW01	ACCACCCACC	7	320-980	6
Total		**53**		**32**

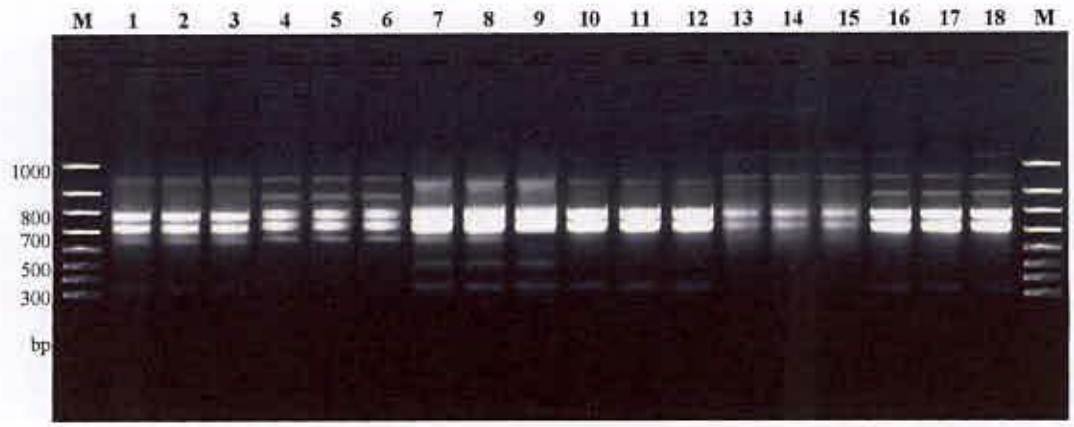

Fig.1 RAPD profiles of 6 lentil germplasm using primer OPA01. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

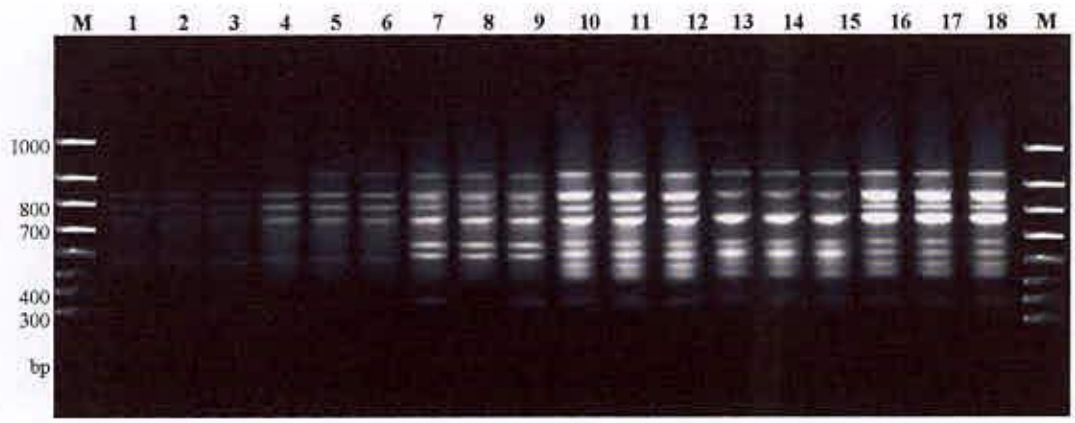

Fig.2 RAPD profiles of 6 lentil germplasm using primer OPA18. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

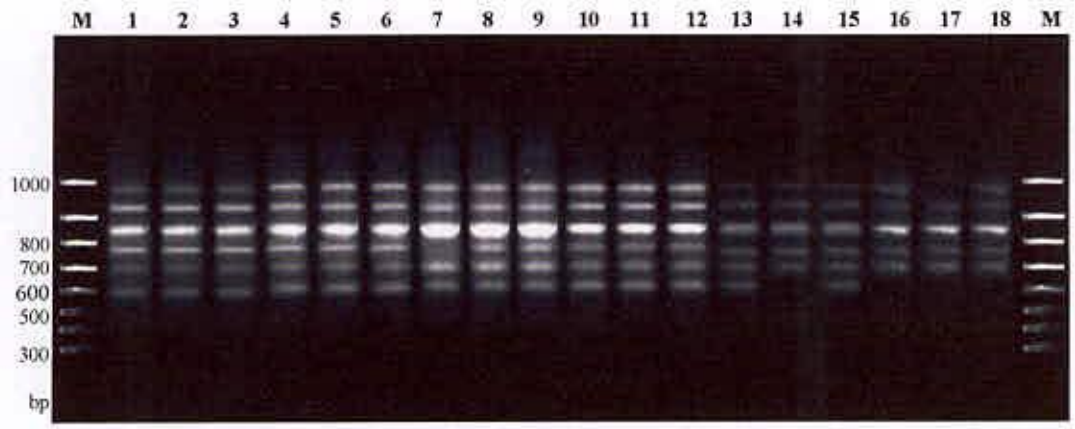

Fig.3 RAPD profiles of 6 lentil germplasm using primer OPC08. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

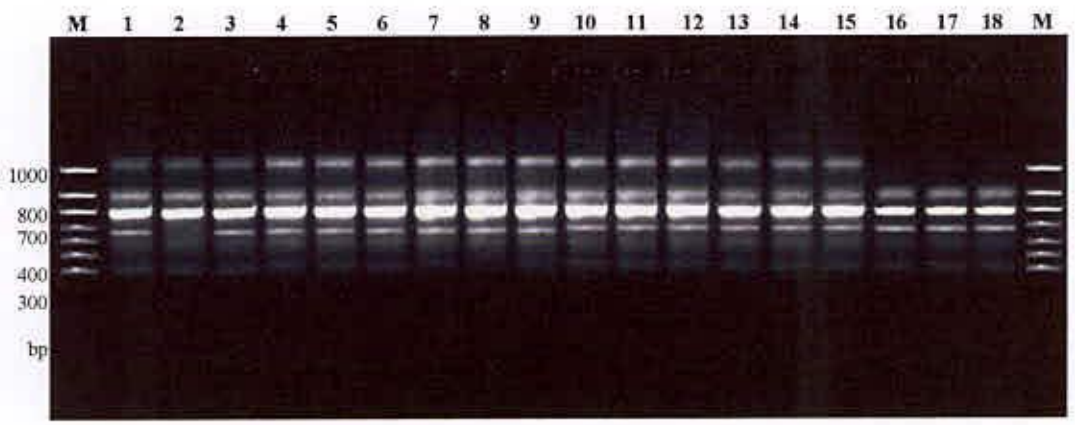

Fig.4 RAPD profiles of 6 lentil germplasm using primer OPD02. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side).

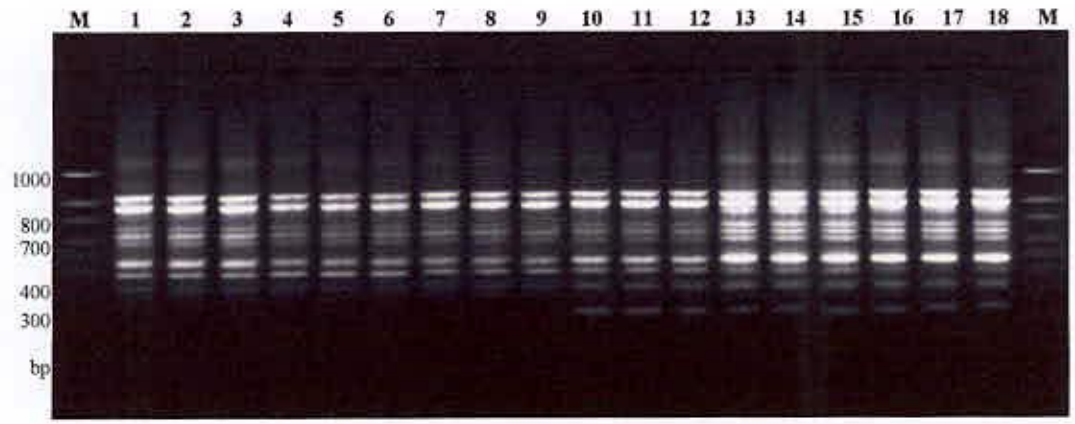

Fig.5 RAPD profiles of 6 lentil germplasm using primer OPD03. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

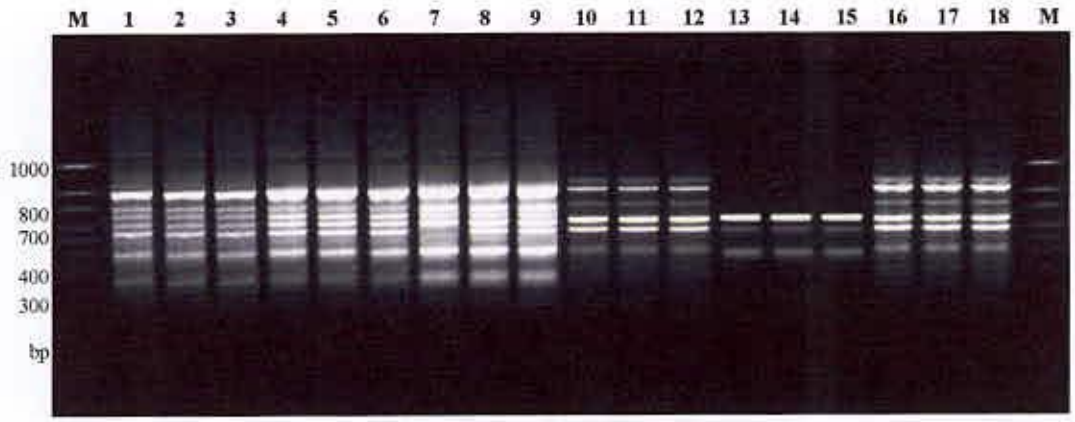

Fig.6. RAPD profiles of 6 lentil germplasm using primer OPD18. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

Fig.7. RAPD profiles of 6 lentil germplasm using primer OPW01. Lane 1-3: BARI musur-1; Lane 4-6: BARI musur-2; Lane 7-9: BARI musur-3; Lane 10-12: BARI musur-4; Lane 13-15: BARI musur-5 and Lane 16-18: BARI musur-6. M: Molecular weight marker (100 bp DNA ladder in left side and PUC in right side)

Table 6: Range of RAPD bands for each primer shown in 3 Replication of 6 lentil varieties

Lentil varieties	Primers						
	OPA01	OPA18	OPC08	OPD02	OPD03	OPD18	OPW01
BARI musur-1	5-5	4-4	6-6	4-5	7-8	7-7	4-4
BARI musur-2	5-5	5-6	6-6	5-5	8-8	7-7	4-4
BARI musur-3	5-6	6-7	6-6	4-5	7-8	6-7	3-4
BARI musur-4	4-4	9-9	6-6	5-5	9-9	6-6	4-4
BARI musur-5	4-4	8-8	5-6	5-5	10-10	3-3	3-3
BARI musur-6	5-6	9-9	4-6	4-4	9-10	6-6	5-5

This proportion is higher compared to some previous molecular analysis I 29 lentil (*Lens culinaris*) cultivar and landraces of 97 markers (72RAPD and 25 STMS) were amplified of which 42.3% were polymorphic (Rana *et al.*, 2007), 50% were polymorphic in lentil germplasm (Tayeba Sultana, 2003). This difference can be attributed to the primers used and the genotypes evaluated.

The present experiment produced 7.5 scorable bands per primer and 4 polymorphic RAPD markers per primer. The reasons of the considerable number of average scorable and polymorphic bands could be that the primers used in this study, consist of 60-70% GC content. Fukuoka et al. (1992) observed an increase in the number of bands with increasing GC content of the primer. The explanation for this correlation between the GC content of the primer and the number of bands is that the stability of base complementation is higher when G is pairing with C by three hydrogen bonds than that of the complementation of A with T by two hydrogen bonds.

Table 7. Frequencies of polymorphic RAPD markers in 6 lentil varieties

Locus	BARI masur-1	BARI masur-2	BARI masur-3	BARI masur-4	BARI masur-5	BARI masur-6
OPA01-1	0.0000	0.0000	0.0000	0.0000	1.0000	0.4226
OPA01-2	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA01-3	0.0000	1.0000	0.0000	0.0000	0.0000	1.0000
OPA01-4	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA01-5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA01-6	1.0000	1.0000	0.4226	0.0000	0.0000	0.0000
OPA01-7	0.0000	0.0000	1.0000	0.0000	0.0000	0.0000
OPA01-8	1.0000	0.0000	1.0000	1.0000	0.0000	1.0000
OPA18-1	0.0000	0.4226	1.0000	1.0000	1.0000	1.0000
OPA18-2	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA18-3	1.0000	1.0000	1.0000	1.0000	0.0000	1.0000
OPA18-4	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA18-5	0.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA18-6	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPA18-7	0.0000	0.0000	0.0000	1.0000	1.0000	1.0000
OPA18-8	0.0000	0.0000	0.0000	1.0000	1.0000	1.0000
OPA18-9	0.0000	0.0000	0.4226	1.0000	1.0000	1.0000
OPC08-1	1.0000	1.0000	1.0000	1.0000	1.0000	0.4226
OPC08-2	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPC08-3	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPC08-4	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPC08-5	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPC08-6	1.0000	1.0000	1.0000	1.0000	0.4226	0.0000
OPD02-1	1.0000	1.0000	1.0000	1.0000	1.0000	0.0000
OPD02-2	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPD02-3	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
OPD02-4	0.4226	1.0000	1.0000	1.0000	1.0000	1.0000
OPD02-5	1.0000	1.0000	0.4226	1.0000	1.0000	1.0000

4.2 Frequency of polymorphic loci

The DNA polymorphisms were detected according to band presence and 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).

Table 8: Summary of band sharing based similarity indices within and between individuals of 6 different lentil varieties.

8. A. Intra-varietal similarity indices (Si)

Lentil varieties	Band sharing values (%)							
	OPA01	OPA18	OPC08	OPD02	OPD03	OPD18	OPW01	Average
BARI musur-1	100.00	100.00	100.00	93.94	100.00	100.00	100.00	99.13
BARI musur-2	100.00	93.94	100.00	100.00	100.00	100.00	100.00	99.13
BARI musur-3	93.94	94.87	100.00	93.94	93.94	93.94	93.94	94.94
BARI musur-4	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100
BARI musur-5	100.00	100.00	93.94	100.00	100.00	100.00	100.00	99.13
BARI musur-6	93.94	100.00	93.94	100.00	93.94	100.00	100.00	97.40
Average	97.98	98.135	98.99	97.98	97.98	98.79	97.98	98.29

4.3 Intra and inter – varietal similarity indices

Table 8 (A and B) showed that intra- variety similarity indices (Si) were higher (ranged from 94.94%-100% with an average of 98.28%) than inter variety similarity indices (Sij) (ranging from 69.63%-94.18% with an average of 82.53%). This implies that individuals within each variety are genetically more similar into each other, as it expected to be, than to individuals from all other varieties.

For the population of intra-varietal similarity indices were founded to be higher in BARI masur-4(100%) varieties than the other five lentil varieties. On the other hand individuals of BARImasur-3 variety showed lowest similarity indices (94.94%) (Table 8A)

This highest Si value reflects lowest genetic variability within the varieties. From this study, it is likely that individuals of BARImasur-4 varieties were the most homogeneous while those of BARImasur-3 were found to be the lowest. The less degree of similarity (Si=94.945) between the RAPD profiles of BARImasur-3 indicated highest genetic variability among individuals of the population. This is possibly because of its self pollinating behavior. In this study, it is also found that the genetic variability of other four varieties namely BARImasur-1, BARImasur-2, BARImasur-5 and BARImasur-6 were higher than BARImasur-3 and lower than BARImasur-4.

8. B. Inter-varietal similarity indices (Sij)

Varietals pair	Band sharing values (%)							
	OPA01	OPA18	OPC08	OPD02	OPD03	OPD18	OPW01	Average
BARI musur-1 Vs BARI musur-2	80.00	82.96	100.00	96.30	100.00	100.00	100.00	94.18
BARI musur-1 Vs BARI musur-3	93.94	69.09	100.00	91.67	97.78	97.64	52.38	86.07
BARI musur-1 Vs BARI musur-4	88.89	61.54	100.00	96.30	94.12	76.92	44.44	80.32
BARI musur-1 Vs BARI musur-5	66.67	50.00	96.97	96.30	88.89	60.00	28.57	69.63
BARI musur-1 Vs BARI musur-6	75.15	61.54	87.27	84.26	83.01	76.92	44.44	73.23
BARI musur-2 Vs BARI musur-3	78.49	91.88	100.00	96.30	97.78	97.44	52.38	87.75
BARI musur-2 Vs BARI musur-4	66.67	77.14	100.00	100.00	94.12	76.92	44.44	79.90
BARI musur-2 Vs BARI musur-5	66.67	68.13	96.97	100.00	88.89	60.00	28.57	72.75
BARI musur-2 Vs BARI musur-6	75.15	77.14	87.27	88.89	83.01	92.31	44.44	78.31
BARI musur-3 Vs BARI musur-4	82.96	82.50	100.00	96.30	91.91	73.50	84.26	87.35
BARI musur-3 Vs BARI musur-5	62.22	74.29	96.97	96.30	86.71	54.89	79.36	78.68
BARI musur-3 Vs BARI musur-6	70.70	85.00	87.27	88.89	84.53	73.50	84.26	82.02
BARI musur-4 Vs BARI musur-5	75.00	94.14	96.97	100.00	94.74	66.67	75.00	86.07
BARI musur-4 Vs BARI musur-6	82.96	100.00	87.27	88.89	92.79	100.00	100.00	93.13
BARI musur-5 Vs BARI musur-6	75.56	94.12	90.24	88.89	96.49	100.00	75.00	88.61
Average	76.07	77.96	95.15	93.95	91.65	80.45	62.5	82.53

Inter-varietal means of the pair wise similarity indices(Sij) ranged from (69.63-94.18). The highest similarity indices of 94.18% were found between BARImasur-1 and BARImasur-2. Genetic distance was lower between that pair than rest of the varietal pairs except BARImasur-4 and BARImasur-6 varietal pairs. BARImasur-1 and BARImasur-5 showed least inter-variety similarity indices 69.63%. and genetic distance was higher between that pair than rest of the varietal pairs.

Band sharing intra- varietal similarity indices were higher than inter-varietal similarity indices. Among the seven primers, OPC08 showed highest intra-variety (98.99%) and inter-variety similarity indices (95.15%) while OPW01 showed lowest intra-variety (97.98%) and inter-variety similarity indices (62.5%) Table8.

All the 15 varietal pairs were not homogenous at different number of loci. Therefore, this study clearly indicates that there was a high level of genetic variation among different varieties.

4. 4 polymorphic loci and gene diversity

The highest number of polymorphic loci (6) was found in the variety BARimasur-3. The highest proportion of polymorphic loci was found in the variety BARimasur-3 which was 11.32%, whereas the lowest was recorded in the variety BARimasur-4 which was 0.00% (table 9). However, the highest and lowest Nei's gene diversity analysis values were found in BARimasur-3 and BARimasur-4 respectively.

In this study, RAPD technique was found in discriminating six lentil varieties. Varieties showing higher intra- varietal similarity and lower frequency polymorphic loci are likely to have less homozygosity as compared to those lower intra- varietal similarity . In other words, populations having higher similarity are more homogeneous groups.

Table 9. Number and proportion of polymorphic bands, gene diversity obtained in different six lentil varieties

Name of variety	No. of polymorphic loci	Proportion of polymorphic loci (%)	Gene diversity
BARI musur-1	1	1.89	0.0092
BARI musur-2	1	1.89	0.0092
BARI musur-3	6	11.32	0.0552
BARI musur-4	0	0.00	0.0000
BARI musur-5	1	1.89	0.0092
BARI musur-6	3	5.66	0.0276

Table 10. Summary of genetic variation statistics across all loci

Locus	Sample Size	Ht	Hs	Gst	Nm*
OPA01-1	18	0.3618	0.0813	0.7752	0.1450
OPA01-2	18	0.0000	0.0000	****	****
OPA01-3	18	0.4444	0.0000	1.0000	0.0000
OPA01-4	18	0.0000	0.0000	****	****
OPA01-5	18	0.0000	0.0000	****	****
OPA01-6	18	0.4815	0.0813	0.8311	0.1016
OPA01-7	18	0.2778	0.0000	1.0000	0.0000
OPA01-8	18	0.4444	0.0000	1.0000	0.0000
OPA18-1	18	0.3876	0.0813	0.7901	0.1328
OPA18-2	18	0.0000	0.0000	****	****
OPA18-3	18	0.2778	0.0000	1.0000	0.0000
OPA18-4	18	0.0000	0.0000	****	****
OPA18-5	18	0.2778	0.0000	1.0000	0.0000
OPA18-6	18	0.0000	0.0000	****	****
OPA18-7	18	0.5000	0.0000	1.0000	0.0000
OPA18-8	18	0.5000	0.0000	1.0000	0.0000
OPA18-9	18	0.4901	0.0813	0.8340	0.0995
OPC08-1	18	0.1739	0.0813	0.5324	0.4392
OPC08-2	18	0.0000	0.0000	****	****
OPC08-3	18	0.0000	0.0000	****	****
OPC08-4	18	0.0000	0.0000	****	****
OPC08-5	18	0.0000	0.0000	****	****
OPC08-6	18	0.3876	0.0813	0.7901	0.1328
OPD02-1	18	0.2778	0.0000	1.0000	0.0000
OPD02-2	18	0.0000	0.0000	****	****
OPD02-3	18	0.0000	0.0000	****	****
OPD02-4	18	0.1739	0.0813	0.5324	0.4392
OPD02-5	18	0.1739	0.0813	0.5324	0.4392

Table 10 (Cont'd). Summary of genetic variation statistics across all loci

Locus	Sample Size	Ht	Hs	Gst	Nm*
OPD03-1	18	0.0000	0.0000	****	****
OPD03-2	18	0.0000	0.0000	****	****
OPD03-3	18	0.4444	0.0000	1.0000	0.0000
OPD03-4	18	0.1739	0.0813	0.5324	0.4392
OPD03-5	18	0.0000	0.0000	****	****
OPD03-6	18	0.0000	0.0000	****	****
OPD03-7	18	0.0000	0.0000	****	****
OPD03-8	18	0.1739	0.0813	0.5324	0.4392
OPD03-9	18	0.0000	0.0000	****	****
OPD03-10	18	0.5000	0.0000	1.0000	0.0000
OPD18-1	18	0.4444	0.0000	1.0000	0.0000
OPD18-2	18	0.2778	0.0000	1.0000	0.0000
OPD18-3	18	0.2778	0.0000	1.0000	0.0000
OPD18-4	18	0.5000	0.0000	1.0000	0.0000
OPD18-5	18	0.0000	0.0000	****	****
OPD18-6	18	0.1739	0.0813	0.5324	0.4392
OPD18-7	18	0.0000	0.0000	****	****
OPD18-8	18	0.5000	0.0000	1.0000	0.0000
OPW01-1	18	0.4444	0.0000	1.0000	0.0000
OPW01-2	18	0.4444	0.0000	1.0000	0.0000
OPW01-3	18	0.4901	0.0813	0.8340	0.0995
OPW01-4	18	0.2778	0.0000	1.0000	0.0000
OPW01-5	18	0.0000	0.0000	****	****
OPW01-6	18	0.4444	0.0000	1.0000	0.0000
OPW01-7	18	0.4444	0.0000	1.0000	0.0000
Mean		0.2197	0.0184	0.9162	0.0458
St. Dev		0.0411	0.0012	-	-

H_t = Hardy-Weinberg average heterozygoity expected in sub-population
H_s = Hardy-Weinberg average heterozygosity obtained in sub-population
G_{st} = Co-efficient of gene differentiation
Nm = Estimate of gene flow from Gst or Gcs. e.g., $N_m = 0.5(1-G_{st})/G_{st}$

4.5 Nei's (1972) genetic identity and genetic distance

The highest Nei's genetic identity (**0.9331**) was observed in BARImasur-2 vs. BARImasur-1 varietals pair's whereas; the lowest genetic identity (0.6064) was estimated in BARImasur-5 vs. BARImasur-1 varietals pair (Table 11).

The highest Nei's (1972) genetic distance (0.5002) was observed in BARImasur-5 vs. BARImasur-4 whereas; the lowest genetic distance (0.0692) was found in BARImasur-2 vs. BARImasur-1 varietals pair (Table 11). Furthermore, high level of genetic distance was found in BARImasur-6 vs. BARImasur-4(0.4908); BARImasur-5 vs. BARImasur-2(0.4392) varietals pair and low level of genetic distance was observed in BARImasur-6 vs. BARImasur-4 (0.0972); BARImasur-3 vs. BARImasur-1and BARImasur-2(0.16988) (Table 11)

Table 11. Summary of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values between 10 lentil varieties

Acc. No.	BARI musur-1	BARI musur-2	BARI musur-3	BARI musur-4	BARI musur-5	BARI musur-6
BARI musur-1	****	**0.9331**	0.8438	0.7094	0.6064	0.6122
BARI musur-2	0.0692	****	0.8438	0.7094	0.6445	0.6506
BARI musur-3	0.1698	0.1698	****	0.8093	0.7043	0.7109
BARI musur-4	0.3434	0.3434	0.2116	****	0.8231	0.9074
BARI musur-5	0.5002	0.4392	0.3506	0.1947	****	0.8044
BARI musur-6	0.4908	0.4299	0.3412	0.0972	0.2177	****

Table 12. Gene flow value (Nm) in different varietals pairs (below diagonal)

Acc. No.	BARI musur-1	BARI musur-2	BARI musur-3	BARI musur-4	BARI musur-5
BARI musur-2	0.0692				
BARI musur-3	0.1698	0.1698			
BARI musur-4	0.3434	0.3434	0.2116		
BARI musur-5	0.5002	0.4392	0.3506	0.1947	
BARI musur-6	0.4908	0.4299	0.3412	0.0972	0.2177

4.6 Dendrogram

A dendrogram based on Nei's (1972) genetic distance using un-weighted Pair Group Method of Arithmetic Mean (UPGMA), indicates segregation of six varieties of lentil into two main clusters; BARImasur-1, BARImasur-2 and BARImasur-3 were grouped in cluster I and BARImasur-4; BARImasur-5 and BARImasur-6 were grouped in cluster II. In cluster I BARImasur-1 and BARImasur-2 formed sub-cluster I and BARImasur-3 formed sub-cluster II. In cluster II BARImasur-4 and BARImasur-6 were grouped as sub-cluster I and BARImasur-5 formed sub-cluster II.

The results indicate that the low or high level genetic distance exists between varieties with their same or different origins. BARImasur-1 vs. BARImasur-5varietals pair showed the highest Nei's (1972) genetic distance (0.5002), as they are released from different parental origin. On the other hand, BARImasur-2 vs. BARImasur-1 varietals pair showed the lowest genetic distance (0.0692) as they are released from same parental origin. The present findings agree with those of Lakhanpaul *et al.* (2000) and Saini *et al.* (2004).

This study indicates the highest genetic variation between BARImasur-1 vs. BARImasur-5 and lowest genetic variation between BARImasur-1 vs. BARImasur-2 that can be used for breeding programs that aim to improve lentil varieties. The results also revealed that the genetic base among these lentil varieties is rather narrow. Collection of diverse germplasm from centers of diversity and acquired other sources may broaden the genetic base.

RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resources management. However, the results are consistent with band-sharing based similarity indices, pair wise gene flow and Nei's genetic distance.

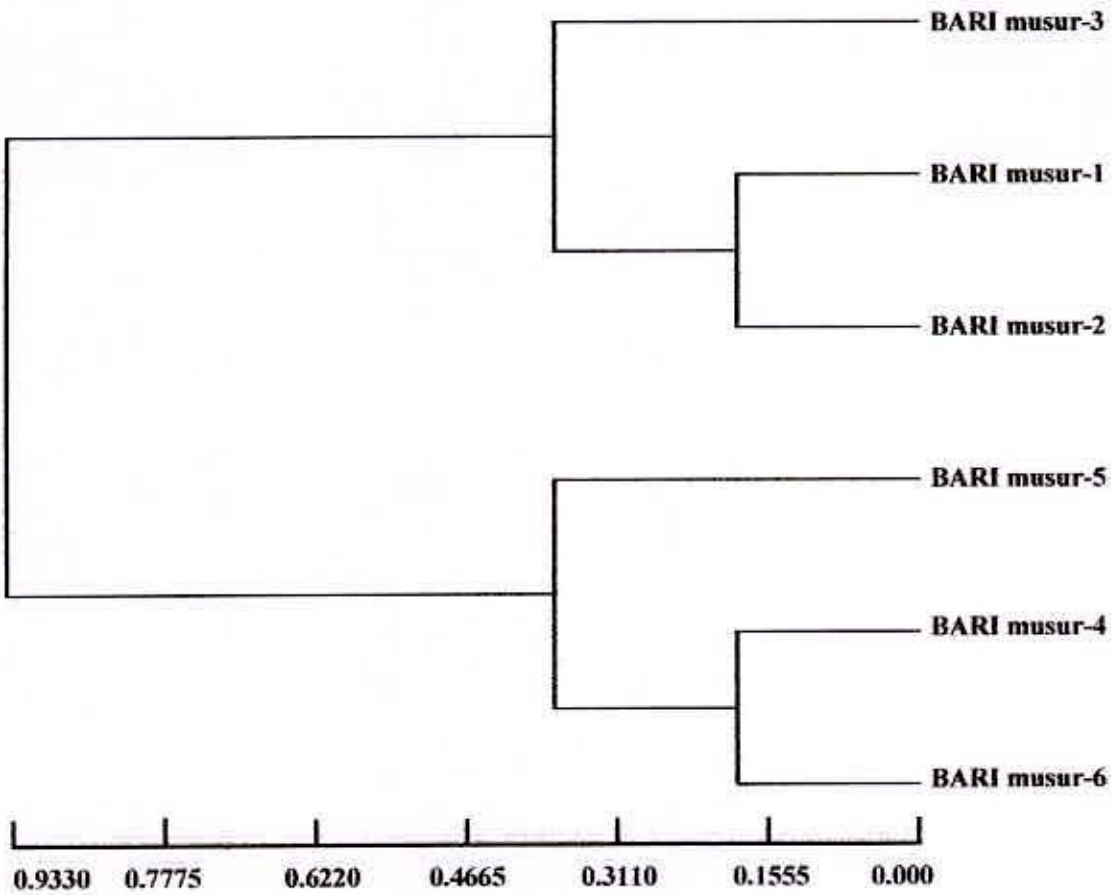

Fig. 8 Un-weighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in 6 lentil accession according to RAPD analysis.

This study indicates the highest genetic variation between BARImasur-1 vs. BARImasur-5 and lowest genetic variation between BARImasur-1 vs. BARImasur-2 that can be used for breeding programs that aim to improve lentil varieties. The results also revealed that the genetic base among these lentil varieties is rather narrow. Collection of diverse germplasm from centers of diversity and acquired other sources may broaden the genetic base.

RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resources management. However, the results are consistent with band-sharing based similarity indices, pair wise gene flow and Nei's genetic distance.

Chapter V

SUMMARY AND CONCLUSION

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Among the legume crops of Bangladesh, lentil is very important. In Bangladesh it stands after khesari. Different varieties of Lentil are developed in different agricultural research institutes of Bangladesh for sustainable production. The genetic characterization of these varieties is yet to be done using molecular markers. The objectives of present study were to characterization and to reveal DNA level variation of the six varieties using RAPD analysis.

In this study, the genetic variation of six lentil varieties (BARImasur-1, BARImasur-2, BARI masur-3, BARIinasur-4, BARImasur-5 and BARImasur-6,) were analyzed and amplified the RAPD markers by polymerase chain reaction (PCR) using seven primers (OPA01 OPA18 OPC08 OPD02 OPD03 OPD18 OPW01). Ten RAPD markers were amplified of which nine were found to be polymorphic. Seven primers generated an average of 4 polymorphic bands per primer. The maximum number of polymorphic loci (6) and the highest proportion of polymorphic loci (11.32) were found in BARImasur-3. The lowest proportion of polymorphic loci (0.00%) was found in BARImasur-4. Nei's (1973) gene diversity values were 0.092, 0.092, 0.0552, 0.000, 0.0092 and 0.0276 for BARImasur-1, BARImasur-2, BARImasur-3, BARImasur-4, BARImasur-5 -and BARImasur-6, respectively. The highest intra-varietal similarity indices (Si) value was found in BARImasur-4 (100%) and the lowest in BARImasur-3 (94.94%). The lowest intra-varietals similarity indices and the highest proportion of polymorphic loci and gene diversity in BARImasur-3 clearly indicate the

maximum level of genetic variation for this variety, whereas BARImasur-4 exhibited the lowest genetic variability. On the other hand, the highest inter-varietal similarity indices (Sij) (94.18%) was observed between BARImasur-1 Vs BARImasur-2 varietal pair and the lowest (69.63%) in between BARImasur-1 Vs BARImasur-5. The co-efficient of gene differentiation (Gst) and gene flow (Nm) values were 0.0731 and 0.5002 respectively. Those values were reflecting the high level of genetic variation among 10 lentii varieties.

The UPGMA dendrogram based on Nei's (1972) genetic distance between different pairs was correlated with their sources of origin. The dendrogram indicated segregation of six varieties of lentil into 2 main clusters. BARImasur-1, BARImasur-2 and BARImasur-3 were grouped in cluster I and BARImasur-4; BARImasur-5 and BARImasur-6 were grouped in cluster II. In cluster I BARImasur-1 and BARImasur-2 formed sub-cluster I and BARImasur-3 formed sub-cluster II. In cluster II BARImasur-4 and BARImasur-6 were grouped as sub-cluster I and BARImasur-5 formed sub-cluster II. The variety BARImasur-1 was close to the variety BARImasur-2 with the least genetic distance (0.0692). Both of these two varieties were released from the same source. On the other hand, BARImasur-I showed the highest genetic distance (0.5002) with BARImasur-5. Both of these two varieties were released from different sources.

Though, larger number of samples and higher number of primers would be necessary to generate and construct an appropriate genetic relationship, sample identification and analysis of genetic variation among different varieties and cultivars is widely acceptable by all concern. Using larger number of samples and higher number of primers could be useful in future study.

In conclusion, RAPD markers have been proved to be a powerful tool for molecular genetic analysis of lentil cultivars for plant breeding programs to assess genetic diversity for the development of improved varieties that are able to withstand biotic and a-biotic stresses. The results of this study can be used as a baseline for the future diversity assessment and genetic analysis of lentil varieties of Bangladesh.

The present work was the preliminary study to assess genetic variation of lentil varieties and it had some limitations in terms of limited number of individual's and. varieties as well as number of primers used. The results indicate that BARImasur-1 and BARImasur-5 are maintaining higher genetic variation than other varieties. The present study might be used as a guideline for further study and the following points might be considered for sustaining the genetic qualities of lentil in Bangladesh.

- Detail survey work should be conducted using more molecular markers for obtaining diagnostic loci for lentil varieties.
- Other molecular markers such as SNP, micro-satellite, etc. should be developed for lentil varieties of Bangladesh.

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