MOLECULAR DIVERSITY ANALYSIS AND DNA FINGERPRINTING OF DIFFERENT TURMERIC (*Curcuma longa L.*) GENOTYPES USING RAPD MARKERS

SHAMIM ARA SUMI

DEPARTMENT OF BIOTECHNOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

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SHAMIM ARA SUMI

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Approved by:

(Dr. Md. Ekramul Hoque)	**(Homayra Huq)**
Professor	Professor
Department of Biotechnology	Department of Biotechnology
Sher-e-Bangla Agricultural University	Sher-e-Bangla Agricultural University
Dhaka-1207	Dhaka-1207
Supervisor	**Co-Supervisor**

(Dr. Md. Ekramul Hoque)
Chairman
Examination committee

DEPARTMENT OF BIOTECHNOLOGY
SHER-e-BANGLA AGRICULTURAL UNIVERSITY
SHER-E-BANGLANAGAR, DHAKA-1207

Ref :
Date :

CERTIFICATE

This is to certify that thesis entitled, ***" MOLECULAR DIVERSITY ANALYSIS AND DNA FINGERPRINTING OF DIFFERENT TURMERIC (Curcuma longa) GENOTYPES USING 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 (MS) in BIOTECHNOLOGY,*** *embodies the result of a piece of bona fide research work carried out by* ***SHAMIM ARA SUMI****, Registration No.* ***11-04313*** *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, 2018

Dhaka, Bangladesh

.....
Prof. Dr. Md. Ekramul Hoque
Department of Biotechnology
Sher-e-Bangla Agricultural University
Supervisor

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SAU, Dhaka

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ABSTRACT

Turmeric (*Curcuma longa*) has been widely used as a spice and flavouring agent in food. This study was done to analyze the molecular diversity among 8 turmeric genotypes using Random Amplified Polymorphic (RAPD) DNA primers. A total 12 RAPD primers were used to determine polymorphism among the turmeric genotypes to produce scorable DNA bands. Some total of 34 DNA bands were amplified and among them 23 were polymorphic bands. The range of DNA amplification varied from 100 to 900 bp. The rate of polymorphism was obtained about 63.88%. Genetic diversity ranged from 0.531 to 0.750 and the frequency of major allele ranged from 0.375 to 0.625. Nei's genetic distance ranging from 0.0930 to 0.2013. The PIC value ranged from 0.468 to 0.712 with the average value was 0.589. The PIC value indicates that most of the studied turmeric genotypes was highly diversified and homogenous as well as no heterozygosity found. A dendogram indicating the relative genetic similarity of the turmeric genotypes was constructed which followed two major clusters (A and B) among the studied material. The cluster B was further subdivided. The results also showed that the genotypes can be separated from each other at the molecular level by taking advantage of some of the RAPD markers. The prevalence level of polymorphism in the local genotypes of turmeric will help breeders for the improvement of turmeric breeding program. This study can be used as a baseline for the future molecular research work on turmeric genotypes in Bangladesh.

ABBREVIATIONS

Full word	Abbreviation
2,4-Dichlorophenoxy acetic acid	2,4-D
Agricultural	Agril.
Agriculture	Agric.
American	Am
Amplified Fragment Length Polymorphism AFLP	AFLP
And others (at elli)	*et al.*
As for example	e.g.
Bangladesh Agricultural Rsearch Instution	BARI
Bangladesh Bureau of Statistics	B.B.S
Base pair	Bp
Biology	Biol.
Biotechnology	Biotech.
Botany	Bot.
Breeding	Breed.
Cetyl Trimethyl Ammonium Bromide	CTAB
Continued	Cont.
Cultivar	cv.
Culture	Cult.
Degree celsius	°C
Deoxyribonucleic acid	DNA
Distilled deionized water	ddH20
Etcetera	etc.
Ethidium Bromide	Et-Br
Ethylene Diamine Tetra Acetic Acid	EDTA
Genetic Distance	GD
Genetics	*Genet.*
Gram per Liter	g/L
Hectare	ha
Horticulture	*Hort.*
International	*Int.*
Inter simple sequence repeat	ISSR
Journal	*J.*
Marker assisted breeding	MAS
Metric ton	mt
Micro liter	*µl*
Mili liter	mL
Mili metre	mm

ABBREVIATIONS

Full word	Abbreviation
Molecular	Mol
Namely	*viz.*
Percent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Principle component analysis	PCA
Publication	*Pub.*
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment length Polymorphism	RFLP
Research	Res.
Ribonucleic Acid	RNA
Rotation per minute	Rpm
Science	*Sci.*
Sequence Tagged Site	STS
Single nucleotide polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium chloride	NaCl
Sodium Dodecyl sulphate	SDS
Species (plural)	Spp.
That is	i.e.
Tris Boric Acid EDTA	TBE
Tris-EDTA	TE
Un-weighted Pair Group Method of Arithmetic Mean	UPGMA
Ultra violet	UV
Volt	V

p

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CHAPTER I

INTRODUCTION

Turmeric (*Curcuma longa* L.) belonging to the Zingiberaceae family is an important spice, well known as condiment and colouring agent. *Curcuma longa* L.(turmeric) is widely cultivated and extremely marketable spice in Asian country, having distinctive chemical and physical properties. The plant has wide industrial applications and finds extensive use in the indigenous system of medicine (Ravindran *et al.,* 2007 and Das *et al.,* 2011). Turmeric is popularly known as "Indian saffron". There are about 100 species of genus *Curcuma* found in the tropics of Asia which extends from Africa to Australia. The highest diversity is noticed in India and Thailand, with at least 40 species in each area, followed by Burma, Bangladesh, Indonesia and Vietnam (Jan *et al*., 2011).

Turmeric is a triploid [2n = 3x = 63; x = 21] (Islam *et al*., 2007) species with nuclear DNA content (2C) of 2.71 pg (Leong-Skornickova *et al.*,2007). However, recent data on chromosome counts and flow cytometry-based studies revealed a new ploidy status (9x) with a basic chromosome number (x = 7) (Leong-Skornickova *et al*., 2007). The ecology of the species varied so much that their habitat ranges from sea level (sandy coastal habitat) to high altitude such as above 2000 m in the Himalayas. Daod and Aslam, (1996) have suggested that *C. longa* has been widely cultivated at the mass level in Pakistan in the regions of Kasur, Sahiwal, Okara, Bannu, Pubbi and Haripur (Jan *et al*.,2012).

The Turmeric rhizome contains most importent chemical compound called curcuminoids which include curcumin (diferuloyl methane), demethoxy curcumin and bisdemethoxy curcumin. The best studied compound is curcumin, which constitutes 3.14% (on average) of powdered turmeric. It has high nutritional value (per 100gram turmeric) contain vitamin B_6 (5%), vitamin C (3%), magnesium (3%), potassium (4%), calcium (1%) and protein (1%). (Wikipedia).In the world, the total number of cultivated spicecs is 109 according to International Standardization

Organization. But in Bangladesh only 30 genotypes of turmeric are cultivated. Normally turmeric is imported to our country but 55 lakh 45 thousand turemeric is exported in the of 2015– 2016 according to Bangladesh Bank.The farmers of Bangladesh have been following conventional practices for turmeric cultivation since long back as a result of which the crop used to exhibit low yield per unit area of land. Just before development and introduction of improved varieties of turmeric in Bangladesh, commercial yield per hectare was very low. Due to development of some improved varieties like- BARI holud 1, BARI holud 2, BARI holud 3, BARI holud 4 and BARI holud 5; the production rate is increased from earlier condition. Turmeric occupies with an area of about 63549 acres with the puoduction of about 149985 MT and per acre yield is 2360 kg (BBS, 2017-18). Despite the economic and nutritional importance of turmeric, breeding efforts in this spice crop has been limited.

It has been used in the Indian and Chinese systems of medicines to treat wounds and sprains, and gastrointestinal, pulmonary, and liver disorders (Aggarwal *et al*., 2007). Many pharmacological studies have been conducted to describe multiple biological actions of curcumin. These studies have demonstrated that curcumin possesses antioxidant (Sreejayan and Rao, 1994), anti-inflammatory (Ammon and Wahl, 1991), anticarcinogenic (Johnson *et al*., 2009), antimicrobial (Limtrakul *et al.*, 1997), hepatoprotective (Kiso *et al*., 1983), hypoglycemic (Babu and Srinivasan 1995), thrombosuppressive (Srivastava *et al.*, 1985), and antiarthritic (Deodhar *et al*., 1980) activities.

In crop improvement programme, genetic diversity has been considered as an important tool, essential to meet the diverse goal in the field of plant breeding such as producing cultivars with increased yields (Joshi and Dhawan , 1966), wider adaptation , desirable quality and pest resistance (Nevo *et al*., 1982).Furthermore, knowledge of genetic diversity is essential for plant genetic resource conservation. Different genotypes with diversified patterns are pre- requisite for hybridization to establish desirable character. Information on genetic diversity among the plant materials is vital to a plant breeder for an efficient choice of parents for hybridization. It is an established fact that genetically diverse parents are likely to contribute desirable segregate to produce high heterotic F_1. Genetic diversity

analysis is used for estimating and establishing of genetic relationship in germplasm collection, identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection and introgressing desirable genes from diverse germplasm into the available genetic base (Islam *et al*., 2012).

Molecular markers are a useful tool for the characterization of genetic diversity available in natural population and germplasm collection. Molecular markers can be used to detect relationships between the various accessions of an individual taxon (Dehmer, 2003). Molecular markers have been developed based on the detailed knowledge of genome structure and considerable emphasis has been laid on the use of molecular markers in practical breeding and genotype identification (Ovesna *et.al*., 2002).1 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 programme to improve crops (Frey *et al.,* 2004). These include Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR), Randomly Amplified Polymorphic DNA (RAPD) (Karp *et al*., 1997) and 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 population. To have technical simplicity and speed of RAPD methodology (Gepts,1993), its level of genetic resolution is equivalent to restriction length polymorphism for determining genetic relationships (Dos Santos *et al*.,1994).

Genetic diversity analysis in the turmeric germplasm is essential to increase the efficiency of selection in breeding programs, as well as to direct conservation strategies in germplasm collections. So, the present study was aimed to evaluate

genetic diversity and relationships among natural populations of *C. longa* on the basis of RAPD markers. Molecular markers (RAPD and ISSR) are useful for the discrimination of turmeric cultivars with similar morphological and biochemical characters. The usage of RAPD and ISSR markers is advantageous since no prior knowledge of the genome sequence is required. Systematic understanding of the genus *Curcuma* has encountered problems owing to different mode of reproduction (asexual and sexual) resulting in very less morphological variation between and within the taxa (Leong-Skornickova *et al.*, 2007; Das *et al*., 2011 and Zaveska *et al.*, 2012).

RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide sequences of arbitrary nature as primers (Chen *et al*., 2007). RAPD does not need any prior knowledge of DNA sequence, however, still reveals a high level of polymorphism. RAPD-PCR is currently used as a tool for the assessment of genetic variability between genotypes in breeding programs. Keeping in view the role of RAPD markers in the determination of genetic diversity, the present study was carried out to determine genetic diversity among different genotypes of turmeric using RAPD markers and selection of genetically diverse genotypes for future breeding programs. RAPD analysis also showed promise as an effective tool in estimating genetic polymorphism in different turmeric genotypes.

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

- Molecular diversity analysis of different turmeric genotypes;
- DNA fingerprinting of turmeric genotypes under Bangladesh condition;
- Polymorphism study among different turmeric germplasm.
- Establishment of Dendogram and phylogenetic relationship among the genotypes.

CHAPTER II

REVIEW OF LITERATURE

Turmeric (*Curcuma longa*) is a plant of the family Zingiberaceae commonly known as the ginger family. It is important horticultural crop in Bangladesh which is valued all over the world as spices and medicinal activities. Several researchers throughout the world have performed research activities on turmeric genetic diversity and relationship, phylogenetic study and characterization through molecular markers like Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) etc. Some of the research works have also been done in Bangladesh but genetic data on turmeric is not rich enough. So genetic status of this important spice crop is needed to be established and documented by using DNA markers which may provide valuable information for further breeding program. The most relevant literatures about the present study have been reviewed and some of the relevant literatures are cited below.

2.1 Different Types of Molecular Markers

Molecular markers are powerful tools to analyze genetic relationship and genetic diversity.

Genetic markers can be grouped into three broad categories i.e. Morphological marker, Biochemical marker and Molecular marker or DNA marker.

According to Oxford Advanced Learner's Dictionary, marker is a distinctive feature or characteristic indicative of a particular quality or condition. Markers are any trait of an organism that can be identified with confidence and relative easy way (Bhat *et al*., 2010).

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 a trait or to genetic material that is difficult to identify.

Morphological markers are specific and distinct. These are related to shape, size, colour 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 (Jiang, 2013 and 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 are 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 (Bagali *et al*., 2010).

The use of Biochemical markers involves the analysis of seed storage proteins and isozymes. Isozymes are alternative forms or structural variants of an enzyme that have different molecular weights and electrophoretic mobility but have the same catalytic activity or function. Isozymes reflect the products of different alleles rather than different genes because the difference in electrophoretic mobility is caused by point mutation as a result of amino acid substitution (Xu, 2010). Isozyme markers can be genetically mapped onto chromosomes and then used as genetic markers to map other genes. They are also used in seed purity test and occasionally in plant breeding (Jiang, 2013).

Major advantages of Biochemical markers consist in assessing co-dominance, absence of epistatic and pleiotrophic effects, ease of use, and low costs (Mondini *et al*., 2009). But there are only a small number of isozymes in most crop species and some of them can be identified only with a specific strain. Therefore, the use of enzyme markers is limited (Jiang, 2013). 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 (Jiang, 2013 and Bagali *et al.*, 2010).

A molecular marker with the advent of molecular biology techniques, molecular markers are effective because they identify an abundance of genetic linkage between identifiable locations within a chromosome and are able to be repeated for verification. A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. Amin *et al*. (2010) stated that, the use of molecular

markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes.

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).

Molecular markers can be used for molecular characterization and detecting genetic variation and relationship of plants. These markers can detect the variation that arises from deletion, duplication, inversion, and/or insertion in the chromosomes. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or linked to genes controlling the traits (Mondini *et al.,* 2009).

Conventional breeding 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 and Choudhary *et al.,* 2008).

2.2 The concept of RAPD markers

Random amplified polymorphic DNA (RAPD) is a PCR-based marker system. 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 used approach for characterization of DNA from plants and other organisms. Short oligo-

nucleotide primers of arbitrary (random) sequence are used in PCR reaction to generate amplified products. 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 and 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 diagnostic 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 relationship and intra-specific diversity at a molecular genetics level (Williams *et al.,* 1990).The amplified products are visualized by separation on agarose gel and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, there by detecting diversity within them (Tommercup *et al.,* 1998).

Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics.In spite of having many usefulness of RAPD marker it have some limitation. Because of random nature of genome sampling, the RAPD assay is not an appropriate technique when the difference between the two genomes is being compared is limited to an extremely small genomic fraction. The most unavoidable problem is dominancė of RAPD marker because the presence of given RAPD band does not distinguish whether its respect locus is homozygous or heterozygous or co-dominance which is possible when SSR marker is used (Rahman *et al.,* 2006).

Though having such weakness, the relative ease and speed the high degree of polymorphisms and virtually inexhaustible pool of possible genetic marker makes the

RAPD technique advantageous over other molecular technique (Clark and Lanigan, 1993, Fristsch and Rieseberg, 1996). RAPD markers, in particular, have been successfully employed for determination of intra-species diversity in several plants, whereas fewer reports are available on determination of inter-species diversity (Goswami and Ranade, 1999).

2.3 RAPD markers in genetic diversity of turmeric

Basak *et al.* (2017) studied the genetic structure and genome size of nineteen commercial cultivars of turmeric of northeast (NE) India was assessed by molecular markers and their genome size. The average polymorphism, polymorphic information content, marker index were found to be 98.14%, 0.34, 33.66, and 86.48%, 0.22, 19.57, for RAPD- and ISSR-based markers, respectively. Based on RAPD and ISSR markers, at the interpopulation level, effective number of alleles, Nei's gene diversity and Shannon's information index values were 1.46, 0.28, 0.44 and 1.50, 0.28, 0.41, respectively. As inferred using flow cytometry, the genome size varied between 2.59 ± 0.03 pg (cultivar of Assam) to 2.95 ± 0.04 pg (cultivar of Meghalaya) with a 1.14-fold variation. Terminal positioning of higher genome size containing turmeric cultivars on the dendrogram represents geographical isolation leading to evolutionary younger origin.

Prashanth *et al.* (2015) observed that molecular markers (RAPD, ISSR, SSR, etc.) unlike morphological and biochemical markers are not prone to environmental influence and accurately characterize the plants portraying the extent of genetic diversity among taxa. Of the different molecular markers RAPD and ISSR has been widely used. A marker system called Inter-Simple Sequence Repeats (ISSRs) has only recently been developed. Extremely high variability and high mapping density as compared with RFLP and RAPD data make these new dominant, microsatellite-based molecular markers ideal for producing genetic maps of individual species. PCR amplification of the genomic DNA was carried out using ISSR PCR performed by using a set of 20 primers namely UBC 801-UBC 82. Initially, 20 primers (UBC 801-UBC 820) were screened with a subset of 18 samples. 8 primers which gave scorable banding pattern were used for analysis of all the samples. Majority of amplification products are in the form of strong and well-defined bands in the range of 70 bp to 2.0 kb. ISSR

profile of eighteen popular cultivated Turmeric genotypes analyzed showed the polymorphic index value of 87.27% across all the genotypes examined in the current study. The primers 809, 810, 811, and 816 exhibited higher polymorphism percentage among these 809 were exhibited 100.00 polymorphism.

Curcuma longa.,commonly known as turmeric, is one of the economically and medicinally important plant species. It is predominantly cultivated in the tropical and subtropical countries. India is the largest producer, and exporter of turmeric in the world, followed by China, Indonesia, Bangladesh and Thailand. In the present study, Directed Amplification of Minisatellite DNA (DAMD) and Inter Simple Sequence Repeats (ISSR), methods were used to estimate the genetic variability in indigenous turmeric germplasm. Cumulative data analysis for DAMD (15) and ISSR (13) markers resulted into 478 fragments, out of which 392 fragments were polymorphic, revealing 82 % polymorphism across the turmeric genotypes. Wide range of pairwise genetic distances (0.03–0.59) across the genotypes revealed that these genotypes are genetically quite diverse.

The UPGMA dendrogram generated using cumulative data showed significant relationships amongst the genotypes. All 29 genotypes studied grouped into two clusters irrespective of their geographical affiliations with 100 % bootstrap value except few genotypes, suggesting considerable diversity amongst the genotypes. These results suggested that the current collection of turmeric genotypes preserve the vast majority of natural variations. The results further demonstrate the efficiency and reliability of DAMD and ISSR markers in determining the genetic diversity and relationships among the indigenous turmeric germplasm. DAMD and ISSR profiling have identified diverse turmeric genotypes, which could be further utilized in various genetic improvement programmes including conventional as well as marker assisted breeding towards development of new and desirable turmeric genotypes (Verma *et al*. 2015)

Genetic fingerprinting of turmeric (*Curcuma longa L*.) genotypes collected from 5 different regions of Bannu including Ismail Khel, Kaki, Raakh Sarkar, Michan Khel and Mandan was performed using Randomly Amplified Polymorphic DNA (RAPD). A total of 22 primer were revaluated for genetic diversity studies, of which 3 (OPE-07, OPC-01 and OPA-03) were found suitable. In total these generate 141 fragments, of

which 40 fragments were polymorphic with 28.36% of polymorphism. The number of amplification products generated by each primer varied from 6 (OPC-01) to 17 (OPA-03). The polymorphism of turmeric genotypes using OPA-03 (38.3%) was found highest followed by OPE-07 (25.0%) and OPC01 (21.74%). It was observed that OPA-03 was better to discriminate genotype as compared to other markers. Un-weighed Pair Group Arithmetic Averages (UPGMA) analysis has clustered 50 turmeric genotypes into 6 groups showing their differentiation on the basis of their locality (Khan *et al.* 2013).

Pasuvaraji *et al.* (2013) conducted about twenty isolates of *Colletotrichum capsici* causing leaf spot of turmeric were evaluated for their morphological, pathogenic, virulence and genetic characterization using random amplified polymorphic DNA (RAPD-PCR). The isolates were categorized into seven groups, based on the morphological characteristics, produced cottony colonies with zigzag to ring or circular pattern of growth. However, differences were obtained in colony colour, shape and size of conidia. Isolates were classified into four groups designated as highly resistant, moderately resistant, moderately sensitive and highly sensitive group based on the effect of propiconzole. The 5.8 S rDNA of ITS region was amplified which confirmed the specific amplicon size of 590 bp. The molecular polymorphism among isolates were analysed by means of RAPD-PCR and the genetic coefficient matrix derived from the scores of RAPD profile showed that minimum and maximum per cent similarities among isolates were in the range of 70 to 96 percent respectively. The cluster analysis by unweighted pair-group method with arithmetic average (UPGMA), separated the isolates into four clusters which confirming the genetic diversity among isolates. However, morphological, virulence and RAPD grouping of isolates suggested no correlation among the test isolates.

Jan *et al.* (2011) observed that molecular genetic fingerprints of indigenous turmeric (*Curcuma longa L.)* genotypes were developed using Randomly Amplified Polymorphic DNA (RAPD) marker to elucidate the genetic diversity among the genotypes. DNA was isolated using CTAB method. The amplification was accomplished by using 10 primers and the specific PCR working program. Ten decamer-primers generated 95 RAPD fragments, of which 92 fragments were

polymorphic with 96.84% of polymorphism. Some of the RAPD markers were useful for genotypes discrimination and identification. Most of the RAPD markers studied showed different level of genetic polymorphism. Amplified fragment sizes ranged from 200 to 3640 bp. Pair-wise Nei and Li's similarity coefficient value ranged from 0.00 to 0.71 for 20 genotypes of turmeric. A dendrogram was constructed based on the unweighted pair group method using arithmetic averages. Cluster analysis of data using UPGMA algorithm placed the 20 genotypes of turmeric into four groups that are somewhat congruent with classification based on morphological characters proposed by earlier works.

Das *et al.* (2011) studied that molecular genetic fingerprints of nine *Curcuma* sp. from Northeast India were developed using PCR based markers. The aim involves elucidating there intra and inter-specific genetic diversity important for utilization, management, and conservation. Twelve random amplified polymorphic DNA (RAPD), 19 Inter simple sequence repeats (ISSRs), and four amplified fragment length polymorphism (AFLP) primers produced 266 polymorphic fragments. ISSR confirmed maximum polymorphism of 98.55% whereas RAPD and AFLP showed 93.22 and 97.27%, respectively. Marker index and polymorphic information content varied in the range of 8.64–48.1, 19.75–48.14, and 25–28 and 0.17–0.48, 0.19–0.48, and 0.25–0.29 for RAPD, ISSR, and AFLP markers, respectively. The average value of number of observed alleles, number of effective alleles, mean Nei's gene diversity, and Shannon's information index were 1.93–1.98, 1.37–1.62, 0.23–0.36, and 0.38–0.50, respectively, for three DNA markers used. Dendrograms based on three molecular data using unweighted pair group method with arithmetic mean (UPGMA) was congruent and classified the *Curcuma* sp. into two major clusters.

Thaikert *et al.* (2009) conducted an experiment to develop as an antiulcer drug and as a mosquito repellant on a commercial scale. However, the material supplied to the factory has not been uniform and has contained lower amounts of active compound than specified in the Thai Herbal Pharmacopoeia. The objective of this study was to reveal the genetic diversity and variation in active compounds and the bioactivity of turmeric collected from different parts of Thailand. The total curcuminoids content and antioxidant activity of 67 samples of *Curcuma longa L*. and one sample of *C. mangga* rhizome from various locations were analyzed before and after planting. The highest total curcuminoids content was found in the samples from the central region while the lowest content was found in the samples from the Lao PDR The antioxidant activities of extracts were assayed with DPPH and compared with ascorbic acid. The highest antioxidant activity (EC50 8.04±3.77 mg/ml) was found in the samples from the central region, which was highly correlated with the curcuminoids content. The antioxidant activity of turmeric from all regions decreased after planting for six months. The RAPD technique was performed to detect genetic diversity in the turmeric samples. Nineteen RAPD primers yielded 184 bands of which 166 were polymorphic. At 68.4% genetic similarity, the samples were separated into four groups. The results indicated the possibility of selecting high quality clones for large-scale production.

Angel *et al*. (2008) performed that the genetic variability in starchy *Curcuma* sp. was assessed using Random Amplified Polymorphic DNA (RAPD) technique. The RAPD pattern generated by 20 primers revealed a high degree of polymorphism. A total of 274 bands were generated of which 264 were polymorphic. All the species were separated into 3 clusters using UPGMA. *C. aromatica, C. leucorrhiza*, and *C. brog* formed a cluster within which *C. longa* and *C. zedoaria* formed a subgroup. *C. harita* was genetically distinct from all the other *Curcuma* sp. Since it is difficult to distinguish different species by leaf morphology, the RAPD pattern has high utility in identification of starchy Curcuma species.

Hussain *et al.*(2008) conducted an experiment about a set of 30 accessions of five *Curcuma* species - *C. latifolia, C. malabarica, C. manga and C. raktakanta* and 13

morphotypes (identified on the basis of morphological markers) of C. longa conserved in the In Vitro Genebank at National Bureau of Plant Genetic Resources, New Delhi, were subjected to RAPD analysis. Of the 200 RAPD primers screened, 21 polymorphic primers were selected for further study. Mean genetic similarities based on Jaccard's similarity coefficient ranged from 0.18 to 0.86 in accessions of cultivated species, i.e., C. longa and from 0.25 to 0.86 in wild species. The dendrogram derived from the RAPD data corroborated the morphological classification of the morphotypes. The efficiency of individual RAPD primers was also compared; primers OPC-20, OPO-06, OPC-01 and OPL-03 were adjudged highly informative in discriminating the germplasm of *Curcuma sp*.

Nayak *et al.* (2005) reported an experiment to detection and evaluation of genetic variation in 17 promising cultivars of turmeric (*Curcuma longa L*.) Using 4C Nuclear DNA Content and RAPD markers. The rhizome yield per plant varied significantly from 77.66 to 350g among 17 cultivars of *Curcuma longa*. Significant variation of 4C DNA content was recorded at the intraspecific level with values ranging from 4.30 to 8.84pg. The differential DNA content observed among 17 different cultivars of Curcuma longa comprising same (2n = 48) chromosome number could be attributed to the loss or addition of highly repetitive sequences in the genome. Random amplified polymorphic DNA (RAPD) analysis clearly revealed genetic variation among 17 cultivars of turmeric showing differential polymorphism using 20 primers. The amplification fragments per primer ranged from 4 to 17 in 17 cultivars with fragment size ranging from 0.4kb to 3kb. Intraspecific polymorphism ranged from 35.6% to 98.6% among 17 cultivars studied. The RAPD primers OPN06 and OPA04 having strong resolving power were able to distinguish all 17 cultivars. The extent of genetic diversity among 17 cultivars was computed through Nei's genetic similarity and genetic distances. The genetic variations detected through 4C nuclear DNA content and RAPD analysis have significance for turmeric improvement programmes.

Sasikumar *et al.* 2005 conducted an experiment for the detection of extraneous *Curcuma* Sp. contamination in the powdered market samples of turmeric using molecular markers

(RAPD), which are not easily discriminated by other analytical techniques routinely used for the identification of adulterants in powdered market samples of turmeric. Three market samples of turmeric powder studied revealed the presence of more *Curcuma zedoaria* (wild species) powder than *Curcuma longa* (the common culinary turmeric) powder, though the curcumin levels of the samples tallied with the quality standards prescribed for the commodity.

Vijayalatha et al. (2005) performed an experiment about genetic diversity of 30 accessions of turmeric was assessed at the molecular level and compared to morphological traits for degree of divergence. The pattern of clustering of quantitative data based on D^2, K means and UPGMA revealed discrepancy among them. The cluster profile, based on quantitative data and RAPD markers exhibited considerable levels of congruence between them. Accessions studied for degree of divergence by RAPD profiles revealed 68.50% polymorphism for 21 primers. The highest number of fragments (10) was obtained with primer OPG 19 while OPC 18 was completely monomorphic. Primers OPB 08, OPC 20, OPE 09 and OPG 19 detected a high level of polymorphism (> 90%). Discrepancy observed at both morphological and molecular levels in the accessions emphasizes the need for specific morphological and molecular markers for discriminating these accessions.

CHAPTER III

MATERIALS AND METHODS

This chapter constitutes on the materials and methods of the experiment. The details of materials and methodologies followed for the study have been expressed in this chapter.

3.1 Experimental location and duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh. The period of the experiment was May 2017 to November 2017.

3.2 Source of study materials

Eight turmeric genotypes were used in the study. The sources of turmeric genotypes are presented in Table 1.

Table 1: List of turmeric genotypes and their sources

Sl. No.	Genotypes Name	Source of collection area
1	BARI holud 1	Spices Research Centre , Bogra
2	BARI holud 2	Spices Research Centre , Bogra
3	BARI holud 3	Spices Research Centre , Bogra
4	BARI holud 4	Spices Research Centre , Bogra
5	BARI holud 5	Spices Research Centre , Bogra
6	Mohisha (local variety)	Bandorban
7	Binni (local variety)	Bandorban
8	Patna (local variety)	Bandorban

3.3 Rhizome germination and collection of leaf sample

Good quality, disease free, healthy turmeric rhizome were collected from Spices Research Centre, Bogra and Bandorban district. Soil was collected from nearby nursery and rhizome were sown in nine different pots of the research farm of Department of Biotechnology of Sher-e-Bangla Agricultural University on May 15, 2018. All

management practices were done for raising good quality seedlings from those materials. After raising seedling, in order to carry out RAPD based PCR amplification of turmeric genome, fresh and young leaf samples were collected at 3 to 4 leaf stage after 30 days of germination and used as the source of genomic DNA.

3.4 Extraction of genomic DNA

Genomic DNA was extracted from the leaf sample using appropriate protocol of Phenol- Chloroform- Isoamyl alcohol method described by Islam *et al.* (2013) with some modifications. The following reagents and methods were used for the isolation of total genomic DNA.

3.4.1 Equipments required

1 Morter and pestle

2. Water bath

3. Centrifuge

4. Vortex mixture

5. Ice maker

6. Micropippet

7. PCR machine

8. Electrophoresis system

9. Gel documentation system

10. Micro oven

11. Ultraviolet light box

3.4.2 Reagents used

1. Extraction buffer, pH= 8.0

Composition of extraction buffer are as follows:

- 1M Tris-HCl((pH= 8.0)
- 0.5 M EDTA (Ethylene diamine tetra-acetic Acid) (pH= 8.0)
- 5M NaCl
- D. H_2O
- SDS (Sodium Dodecyl Sulphate)
- Marcapto -ethanol
- PVP (polyvinyl pyrrolidone)

2. Phenol: Chloroform: Isoamyl Alcohol ratio = 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
- D.H_2O

4. Isopropanol

5. 0.3M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)

8. RNase

9. Ethidium Bromide solution

3.4.3 Reagents preparation

Stock solution of Extraction buffer for 1000 ml

- 100 ml 1M Tris HCl (p^H 8.0) was taken in a measuring cylinder.
- Then 40 ml of 0.5M EDTA was added.
- 100 ml 5M NaCl was mixed with the mixture.
- Finally sterilized dd.H_2O was added to make the volume upto 1000 ml.
- Then the mixture was mixed well and autoclaved

Stock solution of 1M Tris-HCl pH 8.0 for 250 ml

- At first 30.28 g Tris was taken in a volumetric flask (500 ml)
- 100 ml dd. H_2O was added.
- pH was adjusted to 8.0 by adding HCl.
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.

Stock solution of 0.5M EDTA. pH= 8.0 for 250 ml

- At first 46.53 g EDTA.2 H_2O was added in a volumetric flask (500 ml)
- 100 ml dd. H_2O was added.
- Then 4 g NaOH was added.
- pH was adjusted to 8.0 with NaOH
- Then sterilized dd H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.
- The solution was autoclaved.

Stock solution of 5M NaCl for 250 ml

- Firstly 73.05 g of NaCl was added in 250 ml dd. H_2O.
- It was then mixed well and autoclaved.
- The solution was autoclaved.

2% SDS Stock solution for 100 ml

- 10 g of SDS was added in 100 ml of extraction buffer solution in a 250 ml beaker.
- As SDS is hazardous, so the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.
- 20 ml of 10% that solution added in 80 ml dd.H_2O in a 250 ml beaker.

Stock solution of TE Buffer for 100 ml

- 1ml Tris HCL (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.

Composition of 5x TBE buffer (1 litter)

- 54 g Tris-HCl
- 27.5 g of Boric acid
- 4.65 g of EDTA
- pH= 8.3
- Added 1000 ml of dd. H_2O and pH was adjusted at 8.3.

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

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

RNase A/H

- 10 g of RNase was added to 1 ml of dd. H_2O.
- Then it was dissolved completely with the help of necessary heat (at 65℃ in water bath for 30 minutes).

70% Ethanol (1000 ml)

- 700 ml absolute ethanol was mixed with 300 ml dd H_2O.

0.3 M Sodium Acetate

- 2.05 gm of Na acetate dissolved in 50 ml sterilized dd.H_20 then we get 0.3 M Na acetate.

3.5 Sequential steps for DNA extraction

1. For Isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from 8 different turmeric germplasm.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by de-ionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 100 mg of young leaves were cut into small pieces and then taken in mortar. 500 µl of extraction buffer and 100 ml of marcapto-ethanol with near about 30 mg of pvp chemical was added to it. The grind samples were taken into the 1.5 ml eppendorf tube and then it was vortexed for 20 seconds in a vortex mixture and incubated at 65^0C for 20 minutes in hot water bath.
4. Equal volume (500 µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the tube. Then it was vortexd for 20 seconds.
5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a micro pipette tip without disturbing the lower portion and transferred into a new eppendorf tube. Approximately 400-450 µl was taken and then equal volume of Chloroform: Isoamyl Alcohol (24: 1) was added to it. The solution was vortexed for 10 seconds.
6. Again the solution was centrifuged at 13000 rpm for 10 minutes.
7. The supernatant was taken in a separate eppendorf tube and the lower layer was discarded.
8. The amount of the solution was multiplied with 0.6 and then same volume of Isopropanol (0.6 volume of the liquid) was added.

9. It was then tapped by finger for 20-30 seconds (the genomic DNA was visible as cotton like structure).

10. After tapping the sample was again centrifuged at 13000 rpm for 15 minutes. The liquid was discarded completely and re-precipitation of the DNA solution was done by adding 500 µl of absolute (100%) cold ethanol plus 20 µl 0.3 M Sodium acetate.

11. It was shaken gently. Tapping was done to separate pellet. The sample was centrifuged at 13000 rpm for 15 minutes. The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper.

12. The DNA pellet was then air dried for 2-3 hours. It was then dissolved in an appropriate volume (30 to 40 µl) of TE buffer and treated with RNAse at 37 ^{o}C in hot water bath for 15-20 minutes. Then it was spinned for 4-5 seconds.

13. Finally, the DNA samples were stored in freezer at -20^{o}C.

3.6 Confirmation of DNA preparation

The isolated genomic DNA contain large amount of RNA and protein as contaminant and was hence purified by treatment with RNase and proteinase further precipitated. To confirm DNA preparation, 1% agarose gel was used for assessing the quality of the genomic DNA.

3.6.1 Preparation of 1 % agarose gel

Reagents:

1. Agarose powder
2. 1x TBE Buffer (pH = 8.0)
3. Ethidium Bromide

Preparation of 1 % agarose gel

600 mg of agarose powder was taken in a 500 mL Erlenmeyer flask containing 60 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 2 to 3 minutes into a microwave oven with occasional swirling until complete disappearance of agarose particles to generate homogeneous and crystal clear suspension. Then the agarose solution was cooled to about 45-50°C (flask was cool enough to hold comfortably with bare hand) and 0.75 µl (10 mg/mL) ethidium bromide 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 tray (10 x 7 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 2 mm above the plate. Air bubbles were removed by pushing away to the side using a disposable tip. After 30-45 minutes 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.

3.6.2 Preparation of DNA samples for electrophoresis

3 µl dd. H_2O and 2 µl loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) and 3.0 µl of sample DNA was taken in an eppendorf tube using 0.5-10 µl adjustable micropipette. Loading dye 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. The sample was then loaded into the well of the gel and allowed them to sink to the bottom of the wells. The gel was placed in the electrophoresis chamber (Continental Lab product. Inc**.)** Keeping the gel horizontal and submerged in 1x TBE buffer (running buffer). The final level of buffer was about 5 mm above the gel. The gel tank was covered and the electrophoresis power supply was connected and turned on to move DNA from negative to positive electrode (Black to Red) through the gel. Electrophoresis was carried out at 80V for about 55 minutes.

Table 2: Amount of DNA confirmation reagents

Components	Amount (µl)
Working DNA sample	3.0
D. H_2O	3.0
2x loading dye	2.0
Total volume	8.0

3.7 Working solution of DNA samples preparation

DNA concentration were adjusted to 20-25ng/µl for doing PCR using the following formula: V1 × S1 = V2 × S2

Where,

V1 = Initial volume of DNA solution (µl)

S1 = Initial DNA concentration (ng/µl)

V2 = Final volume of DNA solution (µl)

S2 = Final DNA concentration (ng/µl)

Documentation of the DNA sample

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

3.8 Amplification of RAPD markers by PCR

3.8.1 Principle of RAPD primer amplification

For amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermo-stable Taq DNA polymerase and a suitable buffer and then it is subjected to temperature cycling conditions typical to the Polymerase Chain Reaction (PCR). 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 oligonucleotide 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 Selection of primers:

15 decamer RAPD primers were tested, they resulting in faint or irreproducible DNA fragments. From them 12 primers were selected for this study. 12 decamer RAPD primers were OPA 03, OPB 17, OPBB 09, OPBC 14, OPBD 12, OPBD 18, OPBD 20 ,OPG 17, OPX10, OPY 17, OPW 05, OPW 10 (Operon Technologies, Inc., Alameda, California, USA) and it was screened for PCR reaction in 8 genotypes of turmeric. The detail of RAPD primers are given in Table 3.

Table 3: Name of RAPD primers with GC content and sequence information

Sl. No.	Primer name	Sequence(5´to3´)	(G+C) %
1	OPA 03	AGTCAGCCAC	60
2	OPBB 09	AGGCCGGTCA	70
3	OPB 17	AGGGAACGAG	60
4	OPBC 14	GGTCCGACGA	70
5	OPBD 12	GGGAACCGTC	70
6	OPBD 18	ACGCACACTC	60
7	OPBD 20	AGGCGGCACA	60
8	OPG 17	ACGACCGACA	60
9	OPX 10	CCCTAGACTG	60
10	OPY 17	TCCGGCCGGA	80
11	OPW 05	GGCGGATAAG	60
12	OPZ 01	CCGACAACC	60

3.8.3 PCR amplification

PCR reactions were performed on each DNA sample. 2x Taq ready Master Mix was used. DNA amplification was performed in oil-free thermal cycler (Esco Technologies swift^{TM} Mini Thermal Cycler and Q-cycler, (Germany). To prepare a 10.0 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 4.

Table 4: PCR mixture composition for each turmeric genotype

Reagents	Amount(µl)
2x Taq Master Mix	5.00
RAPD primer	1.50
De-ionized water	1.50
Sample DNA	2.00
Total Reaction volume	10.00

From frozen stocks of the PCR reagents i.e., 2x Taq Master Mix, primer and DNA working samples were melt, mixed by vortexing and kept on ice for maintain good quality. DNA samples 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 mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.8.4 Thermal profile for PCR

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Q-cycler). The PCR tubes were kept in the thermal cycler and the following programs were run:

Step- 1: 95 ˚C for 4 minutes	– Initial denaturation	
Step- 2: 95˚ C for 45 seconds	– Denaturation	31 cycles
Step- 3: 35˚ C for 40 seconds	– Primer annealing	
Step- 4: 72˚ C for 1 minutes	– Extension	
Step- 5: 72˚ C for 5 minutes	– Final extension	
Step- 6: 4˚ C for 10 minutes	– Hold	

3.8.5 Electrophoresis of the amplified products

After completion of thermal cycler reaction, each sample of PCR products were confirmed by running 2.0% agarose gel containing 1.0 µl ethidium bromide in 1x TBE buffer at 90 V for 75 minutes. Loading dye (3.0 µl) was added to the PCR products and loaded in the wells. Two Molecular weight markers 100 bp (Bio-Basic, Cat. No. M-1070-1, Canada) was loaded on left side of the gel. Under Ultra Violet (UV) light on a trans-illuminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.9 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 (Bio- Basic, Cat. No. M-1070 (Canada) 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 band-size for each of the markers was scored using the Alpha Ease FC 4.0 software.

The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. The individual fragments were assigned as alleles of the appropriate loci. This was used to estimate polymorphic loci. Using Power Marker version 3.25 software (Liu, K. J., 2005). The summary statistics that were determined included the following: the number of alleles, the major allele size and its frequency, gene diversity, and the polymorphism information content (PIC) value**.** The allele frequency data from POWER MARKER was used to export the data in binary format (presence of allele as "1" and absence of allele as "0"). Binary data form of allele frequency used for dendogram construction by NTSYS-pc software (Rholf, F., 2002). The unweighted pair grouping method, using arithmetic average (UPGMA), was used to determine similarity matrix following Dice coefficient with SAHN subprogram.

Polymorphic Information Content (PIC) value of a primer is

calculated as: $PIC = 1-\sum pi^2$

Where *pi* is the frequency of the i^{th} allele. Polymorphic Information Content was used to confirm the suitability of the primers selected for DNA fingerprinting.

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

$$\text{Similarity index(SI)} = \frac{2Nxy}{Nx+Ny}$$

Where,

Nxy = Number of RAPD bands shared by individuals x and y respectively,

Nx and Ny = Number of bands in individuals x and y respectively (Chapco *et al*., 1992; Wilde *et al*., 1992 and 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.

3.10 Precautions

To maintain a strategic distance from all types of contaminations and keep DNA pure, all dishes, micropipette tips, eppendorf tubes, glass pipettes, de-ionized water and buffer solutions were legitimately autoclaved. Metal supplies i.e., scissors, forceps were cleaned with absolute ethanol.

- ❖ Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et - Br.
- ❖ Always power pack was kept turn off and the leads were unplugged before opening the electrophoresis unit to avoid electrical hazard.
- ❖ Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.
- ❖ The common safety measures were kept up when performing PCR responses. All the disposables 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 amid treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

CHAPTER IV

RESULT AND DISCUSION

This chapter comprises the presentation and discussion of the results of the experiment. The results were obtained from 8 genotypes of turmeric using 12 RAPD primers. In the RAPD analysis significant genetic variation and polymorphisms for characterization of different turmeric genotypes were identified. The results of the experiment are presented and expressed in Table 5 to 8, Figure 1 to 3 and Plate 1 to 13 for ease of understanding.

4.1 Extraction of DNA and Confirmation

The genomic DNA extraction of 8 turmeric genotypes was done by using the phenol-chloroform method with minor modification. RNA sharing was removed by applying RNase treatment. The extracted genomic DNA of 8 samples was loaded on 1% agarose gel for confirmation and quantification of DNA sample. It revealed that, all the samples showed clear DNA band in each well (Plate 1). Hence, the genomic DNA of each sample was diluted on the basis of concentration. The working DNA sample was prepared for PCR works.

DNA confirmation

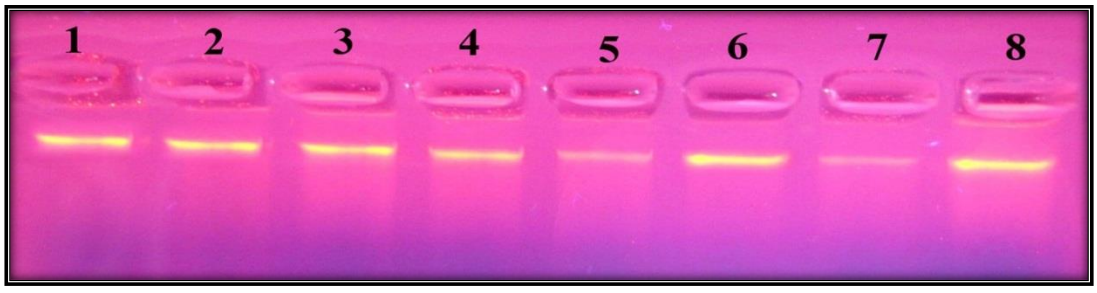

Plate 1: Isolation of genomic DNA in 8 turmeric genotypes

(Lane 1 = BARI holud 1, Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4, Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

4.2 DNA Banding pattern and polymorphism survey of turmeric (*Curcuma longa L.*) genotypes

Initially fifteen DNA decamer RAPD primers were screened on randomly chosen six genotypes from eight turmeric genotypes to evaluate their suitability for amplification of the turmeric DNA fragments. The primers, which gave minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands were selected. It revealed that, out of fifteen RAPD primers, twelve decamer RAPD primers viz. OPA 03, OPBB 09, OPB 17, OPBC 14, OPBD 12, OPBD 18, OPBD 20, OPG 17, OPX 10, OPY 11, OPW 05 and OPZ 01 showed reproducible amplified DNA fragments.

The amplifications of each RAPD primers are presented in Table 5 and Plate 2 to 13.

The RAPD primer OPA 03 produced different DNA fragments in different turmeric genotypes. It produced total two DNA fragments which were ranged from 150 bp to 300 bp and one band was polymorphic (Plate-2). The primer OPBB 09 was able to produce 5 DNA fragments in total and ranged from 130 to 900 bp. Two bands out of four bands were polymorphic (Plate-3). Three DNA fragments were amplified by the primer OPB 17 in different turmeric genotypes which ranged from 100 bp to 650 bp and one of them was polymorphic (Plate-4). The primer OPBC 14 was showed total no. of four bands. Three of bands were polymorphic and other was monomorphic band which ranged from 250 to 600 bp (Plate-5). Four DNA fragments amplification were noticed by the primer OPBD 12 in 8 turmeric genotypes which were range from 200 to 800 bp in which three were polymorphic in nature and another band was monomorphic (Plate-6).

The RAPD primer OPBD 18 was able to amplify one DNA fragments among all the individuals. The DNA fragments produced 180 bp and no band was polymorphic (Plate-7). Two DNA fragments amplification were noticed by the primer OPBD 20 in 8 turmeric genotypes which were range from 380 to 600 bp which one band was polymorphic in nature. Rest of the band was monomorphic (Plate -8).

The RAPD primer OPG 17 produced different DNA fragments in different turmeric genotypes. It produced total three DNA fragments which were ranged from 250 bp to 600 bp and all bands were polymorphic (Plate-9). The primer OPW 05 amplified among the different turmeric genotypes scored three DNA fragments which ranged

from 250 to 600 bp (Plate-10). It was noticed that all of them were polymorphic band and no monomorphic band was found. The primer OPX 10 was able to produced three DNA fragments in total and ranged from 400 to 600 bp, all of the bands were polymorphic (Plate-11). Two DNA fragments were amplified by the primer OPY 11 in different turmeric genotypes which ranged from 400 bp to 500 bp and all the bands were polymorphic (Plate-12). The primer OPZ 01 was able to produce two DNA fragments in total which ranged from 400 to 700 bp and one band was polymorphic (Plate-13).

The 12 primers regenerated total 34 DNA fragments with an average 2.83 per primer among the turmeric germplasm. Out of 34 DNA bands, 23 DNA fragments were polymorphic and the average percent of polymorphism was 64.44. The highest (3) number of polymorphic band was produced by the primer OPBC 14, OPBD 12, OPX 10, OPY 11, OPG 17 and OPW 05 and lowest (1) number of polymorphic band by the primer OPA 03, OPB 17, OPBD 20 and OPZ 01. No polymorphic band was found in the primer OPBD 18.

Maximum 100% of polymorphism was recorded in the primer OPX 10, OPY 11, OPG 17 and OPW 05. OPBC 14 and OPBD 12 produced 75 % polymorphism. 50% polymorphism was recorded in the primer OPA 03, OPBD 20 and OPZ 01.

OPBB 09 and OPB 17 primers produced 40% and 33.33% polymorphism respectively. Individual scerning twelve of RAPD markers each individual of eight turmeric genotypes is presented in Table 5.

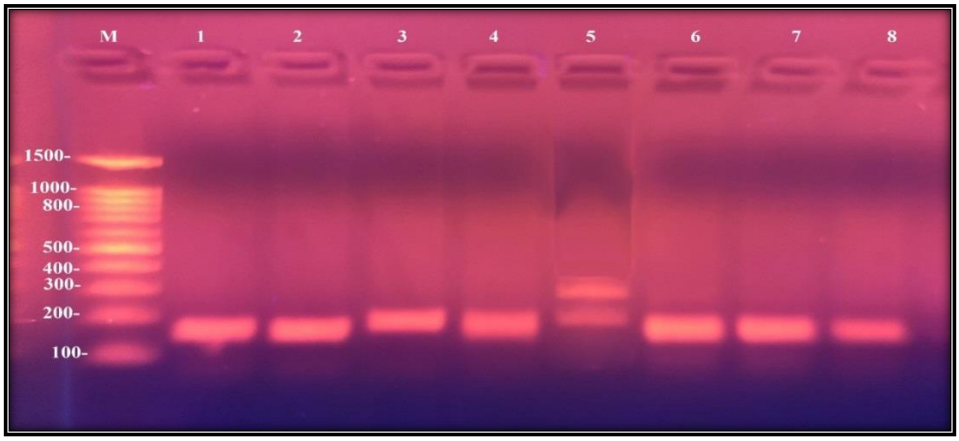

Plate 2: PCR amplification with RAPD primer OPA 03

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

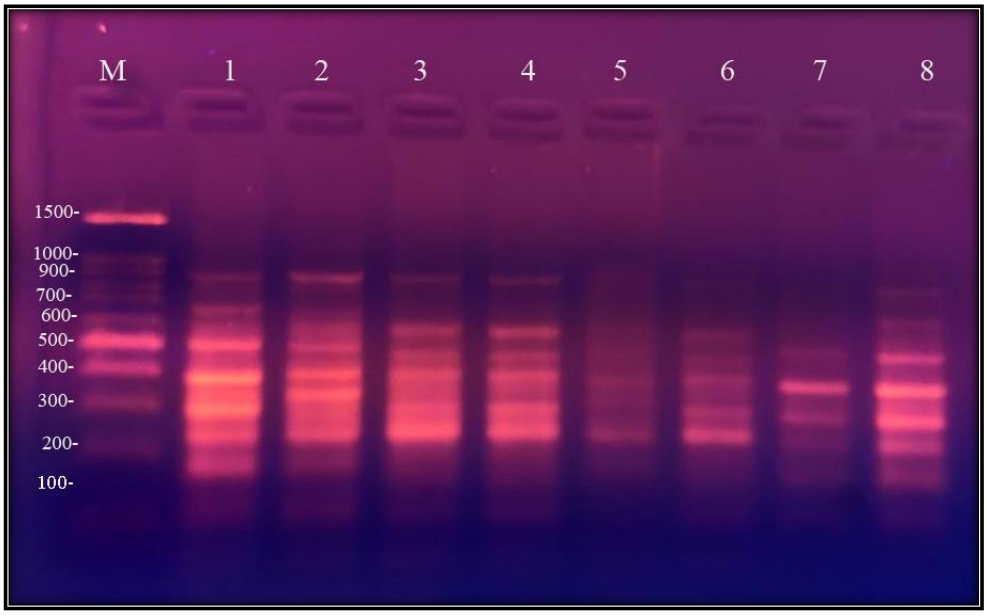

Plate 3: PCR amplification with RAPD primer OPBB 09

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

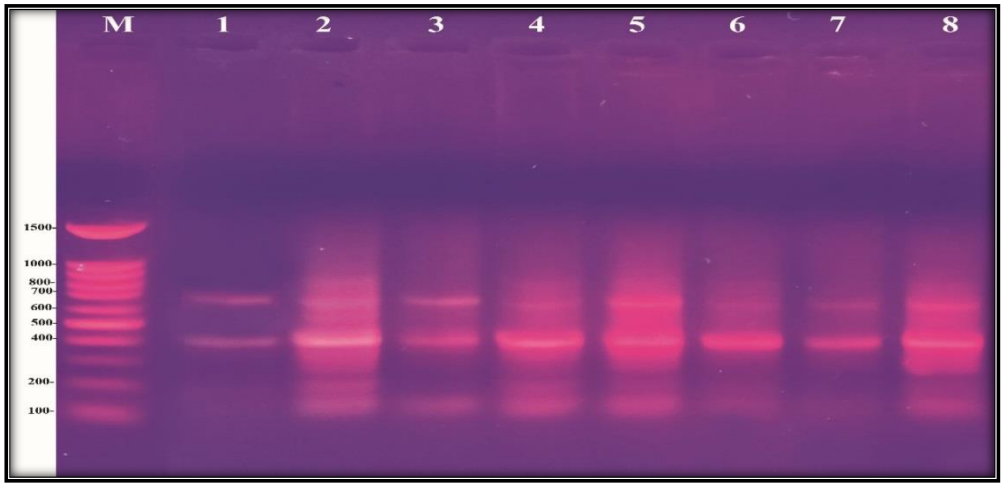

Plate 4: PCR amplification with RAPD primer OPB 17

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

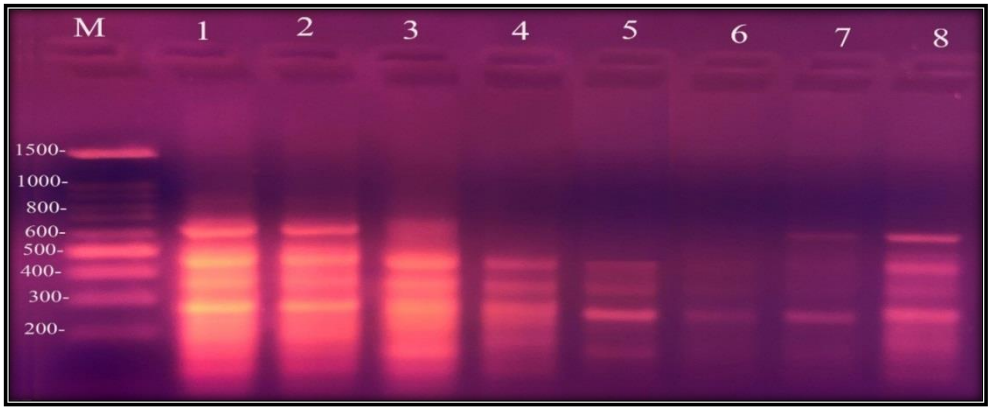

Plate 5: PCR amplification with RAPD primer OPBC 14

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

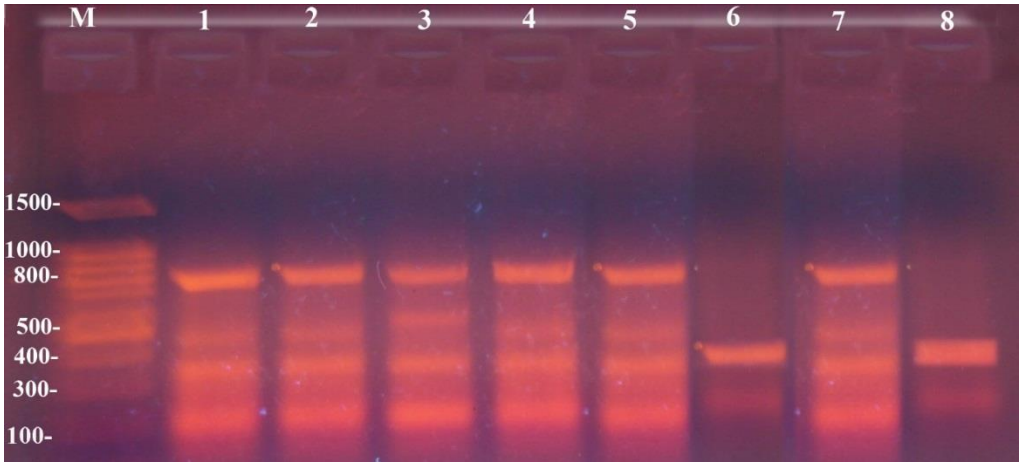

Plate 6: PCR amplification with RAPD primer OPBD 12

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

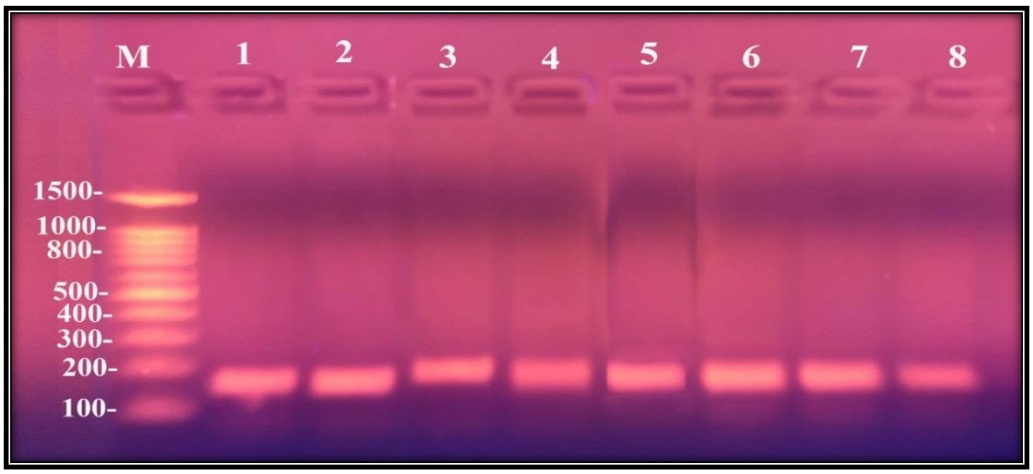

Plate 7: PCR amplification with RAPD primer OPBD 18

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

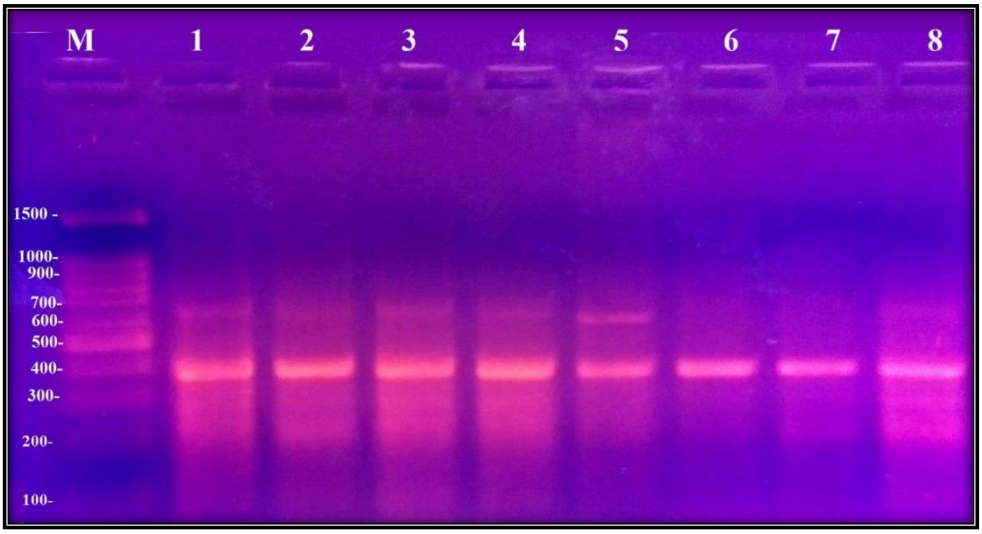

Plate 8: PCR amplification with RAPD primer OPBD 20

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

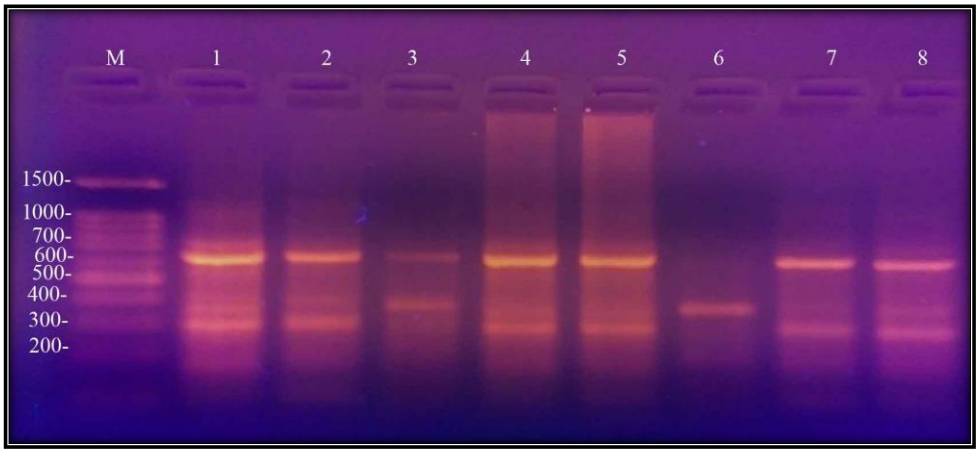

Plate 9: PCR amplification with RAPD primer OPG 17

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

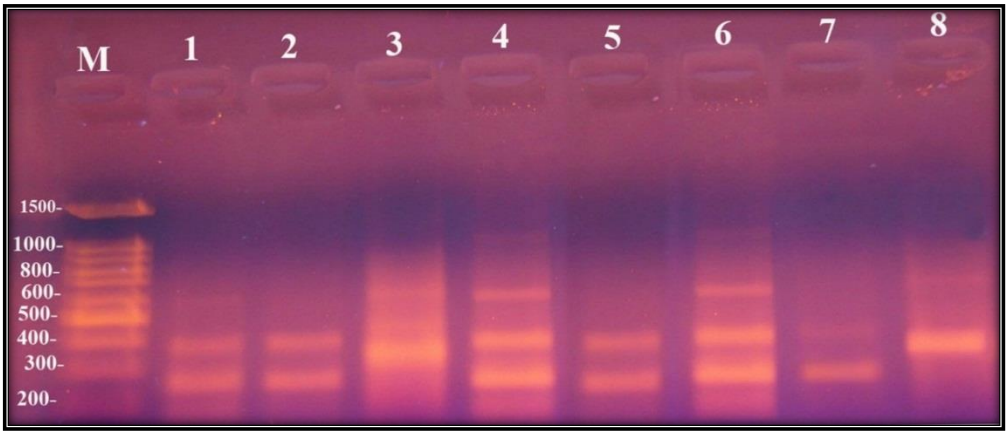

Plate 10: PCR amplification with RAPD primer OPW 05

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

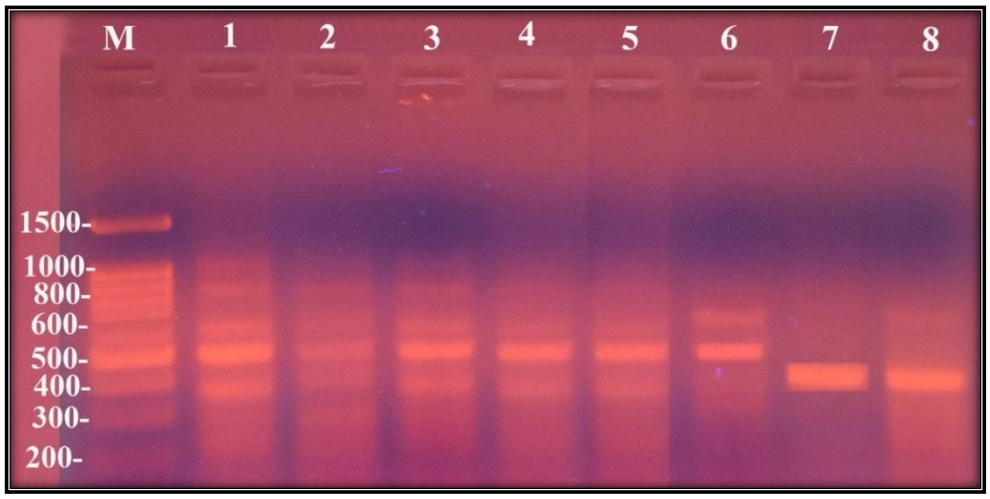

Plate 11: PCR amplification with RAPD primer OPX 10

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

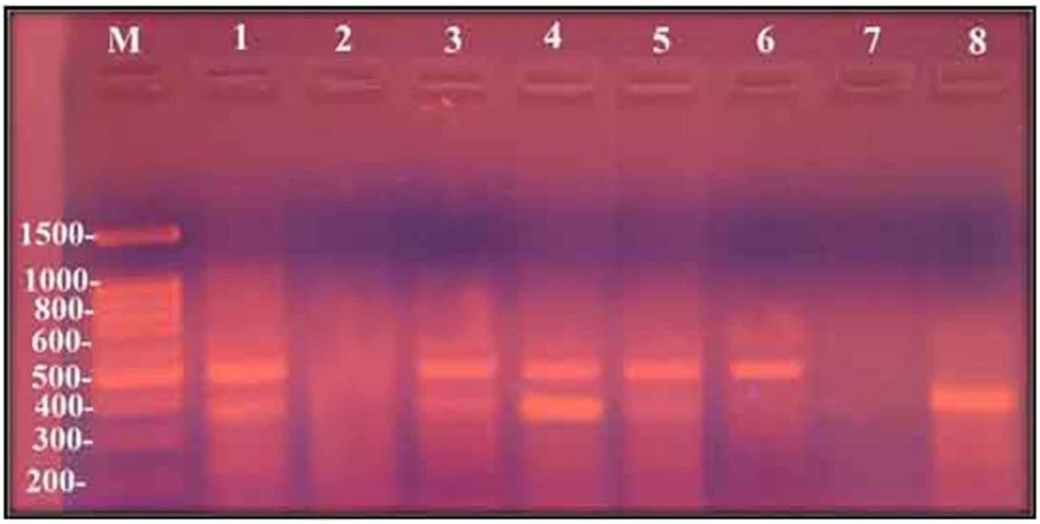

Plate 12: PCR amplification with RAPD primer OPY 11

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

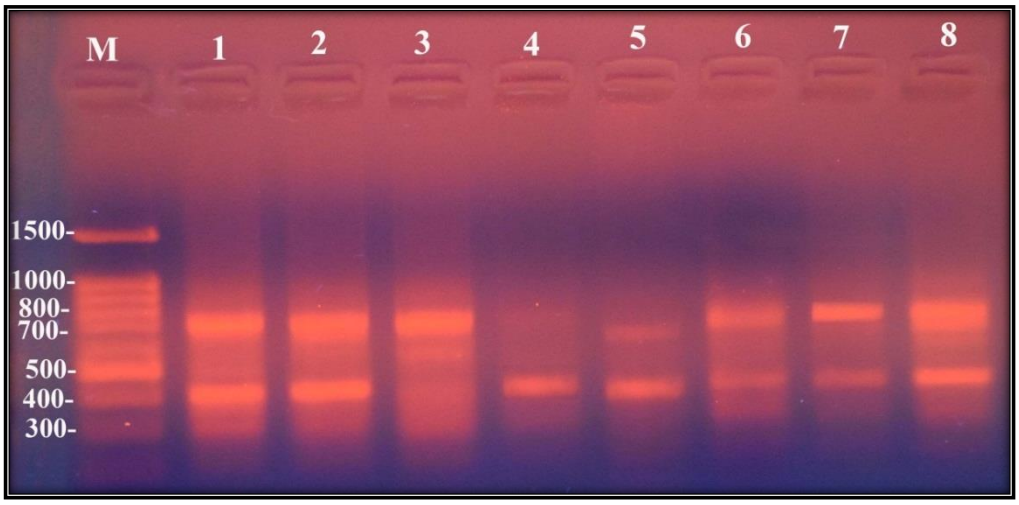

Plate 13: PCR amplification with RAPD primer OPZ 01

(M=100 bp DNA ladder (Bio-Basic, Canada), Lane 1 = BARI holud-1 , Lane 2 = BARI holud 2, Lane 3 = BARI holud 3, Lane 4 = BARI holud 4 , Lane 5 = BARI holud 5, Lane 6 = Mohisha , Lane 7 = Binni , Lane 8 = Patna)

Table 5: RAPD primers with corresponding banding pattern and polymorphism observed in turmeric genotypes.

SL. No.	Name of Primer	Primer Sequence (5′ to 3′)	(G+C) %	Band Scored	No. of Polymorphic Band	% of Polymorphism	Size Range
1	OPA 03	AGTCAGCCAC	60	2	1	50	150-300
2	OPBB 09	AGGCCGGTCA	70	5	2	40	130 - 900
3	OPB 17	AGGGAACGAG	60	3	1	33.33	100 - 650
4	OPBC 14	GGTCCGACGA	70	4	3	75	250 - 600
5	OPBD 12	GGGAACCGTC	70	4	3	75	150 - 850
6	OPBD 18	ACGCACACTC	70	1	0	0	180 - 200
7	OPBD 20	AGGCGGCACA	60	2	1	50	380 - 600
8	OPG 17	GGCGGATAAG	60	3	3	100	250 - 600
9	OPW 05	ACGACCGACA	60	3	3	100	250 - 600
10	OPX 10	CCCTAGACTG	80	3	3	100	400 - 600
11	OPY 11	TCCGGCCGGA	60	2	2	100	400 - 500
12	OPZ 01	CCGACAACC	60	2	1	50	400 - 700
	Total			34	23	773.33	
	Average			2.83	1.91	64.44	

Several scientists reported their observations on turmeric with RAPD primers. Basak *et al.* (2017) analyzed the genetic structure of 19 commercial cultivars of turmeric of northeast india with 20 RAPD primers used, only 9 gave successful amplification for 19 cultivars with a total of 51 bands of which 50 were polymorphic. The amplification product was 5.66 per primer, the maximum was 12 (OPA11), whereas the minimum was 2 (OPA19). The percentage of polymorphic fragments for 9 RAPD primers ranged from 83.3 to 100.0%. The minimum (47.06%) and maximum (64.71%) polymorphism was detected in Manipur and Assam cultivars, respectively.

Singh *et al.* (2012) made an experiment for the evaluation of genetic diversity of turmeric (*Curcuma longa*) found 94 fragments ranging 230 to 3000 bp of which 75

were polymorphic with an average of 6.83 % polymorphic fragment per primer .The polymorphism ranged from 45 to 100 with an average of 91.4%.

Das *et al*. (2011) analyzed the nine different Curcuma species by 12 selected primers which yielded species specific DNA profiles and proved to be informative. A total of 55 mappable RAPD markers were generated by 12 primers. The amplicons ranged between 0.3 and 1.8 kb in size. Amplicon number per primer ranged from 2 (OPAM 20) to 8 (OPC 07) with an average of 4.92.

Jan *et al.* (2011) made an experiment by 10 primers across 20 indigenous turmeric (*Curcuma longa*) genotypes out of which 92 (96.84%) fragments were polymorphic. The number of amplification products generated by each primer varied from 6 (OPA-07 and OPA-08) to 14 (OPC-07). The size of the amplified fragments ranged from 200 (OPA-10) to 3640 bp by (OPD-08).

Panda *et al.* (2007) observed 20 selected RAPD with turmeric (*Curcuma sp.)* And analyzed 86 scorable band ranging from 300 bp to 2700 bp in size. The number of band for each primer varied from 1 to 9 with an average of 3.95 band per RAPD primer.

Nayak *et al*. (2006) analyzed 17 turmeric cultivars using 20 RAPD primers. The amplification fragments per primer ranged from 4 to 17 in 17 cultivars with fragment size range from 0.4kb to 3kb. Polymorphism was found to be highest in ACC31 showing 98.6% polymorphic bands and lowest in PTS51 showing 35.6% polymorphic bands.

Polymorphism in turmeric in different studies could be attributed to the nature of the genetic material under investigation. The low degree of polymorphism in our study compared to other reports, could be due to the less diverse material which belonged to same cultivated varieties of turmeric.

4.3 Allele scoring and diversity analysis

The binary matrix representing different alleles of the 12 markers which were scored as binary data whether present (1) or absent (0) was used for estimation of genetic distance and similarity coefficients. The summary statistics including major allele frequency, gene diversity and polymorphism information content (PIC) values are given in Table No 6.

Table 6: Major allelic frequency, gene diversity and PIC value of different turmeric genotypes

Marker	Observation No.	Allele No.	Major Allele Frequency	Genetic Diversity	PIC Values
OPA O3	8	3	0.625	0.531	0.468
OPBB 09	8	5	0.375	0.750	0.712
OPB 17	8	3	0.625	0.594	0.468
OPBC 14	8	3	0.500	0.625	0.555
OPBD 12	8	4	0.500	0.531	0.605
OPBD 18	8	3	0.625	0.531	0.468
OPBD 20	8	3	0.500	0.656	0.511
OPG 17	8	3	0.500	0.612	0.544
OPW 05	8	3	0.375	0.656	0.582
OPX 10	8	3	0.375	0.656	0.582
OPY 11	8	3	0.500	0.594	0.511
OPZ 01	8	3	0.500	0.625	0.555
Maximum	_	_	0.625	0.750	0.712
Minimum	_	_	0.375	0.531	0.468
Mean	_	_	0.500	0.612	0.589

The frequency of the major allele ranged between 0.375 (OPBB 09, OPW 05, OPX 10) to 0.625 (OPA 03, OPB 17, OPBD 18) with the average value of 0.500. Polymorphic Information Content (PIC) value for 12 markers ranged from 0.468 to

0.712 and the average PIC value was 0.589. The highest PIC value (0.712) was obtained for OPBB 09 and the lowest value PIC value (0.468) was obtained for OPA 03, OPB 17 and OPBD 18. PIC value revealed that OPBB 09 was considered as the best marker in turmeric genotypes followed by OPBD 12, OPW 05, OPX 10 respectively. Gene diversity ranged between 0.750 (OPBB 09) to o.531 (OPA O3, OPBD 12 and OPBD 18) with an average of 0.612. The results indicate that the 12 local turmeric genotypes present a high degree of homozygosity and also considerable intra-varietal group diversity, and a certain degree of genetic differentiation and polymorphism.

The PIC was a good index for genetic diversity evaluation. Botstein *et al.* (1980) reported that PIC value more than 0.5 indicates high diversity and less than 0.25 indicates low diversity. When PIC value lies between 0.25 and 0.5 indicates intermediate diversity. Data in that table show all the studied primers produced polymorphic amplification products with intermediate diversity.

In turmeric, Basak *et al.* (2017) reported intermediate gene diversity value 0.34 in 20 RAPD primers, which indicate that some RAPD markers are useful for differentiating between closely related genotypes. Das *et al.* (2011) found the average value of number of observed alleles and number of effective alleles are 1.93-1.98 and 1.37-1.62 respectively.

4.4 Principal component analysis (PCA)

Principal Component Analysis (PCA**)** is a statistical method capable of reducing the dimensionality of multivariate data. Data reduction is done to clarify the relationship between two or more characters and to divide the total variance of the original characters into a limited number of uncorrelated new variables called principal components.

Principal component analysis (PCA) is a technique used to emphasize variation and bring out strong patterns in a data set. It's often used to visualize genetic distance and relatedness between populations. PCA was invented in 1901 by Karl Pearson. Principal component analysis (PCA) was performed to confirm similarity or dissimilarity among the studied genotypes.

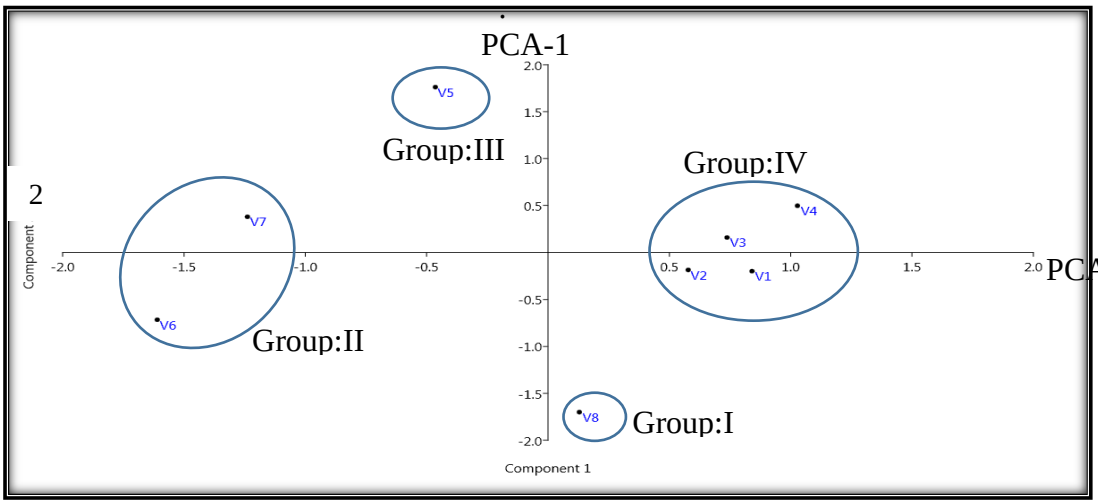

Figure 1: PCA analysis of 8 local Turmeric genotypes based on 12 markers. PCA 1 and PCA 2 represent first and second components, respectively.

Principal components analysis (PCA) analysis was conducted also using the PAST software. All 8 turmeric genotypes were classified into four groups and showed in two dimensional scatter plot. All the groups were separated from each other (Fig. 1). Group-I was formed by only one turmeric genotype V8 (Patna). Group- III represented V5 (BARI holud 5).Group –II was formed by V6 (Mohisha) and V7 (Binni). The highest number of genotypes V1 (BARI holud 1) , V2 (BARI holud 2) , V3 (BARI holud 3) and V4 (BARI holud 4) were present in Group- IV . Highest distance was showed between Group I and Group II. Basically highest distance was noticed between patna (Group I) and Mohisha (Group II). It indicates that, material selection for turmeric improvement program should be taken from this group.

This present study agreed with Ratchadaporn Thaikert and Yingyong Paisook santivatana (2009) investigation, where Principle component analysis (PCA) shows three dimensional scatter plot clustering pattern of 45 accessions of *Curcuma longa.*

4.5 Nei’s genetic distance and genetic identity

Genetic distance refers to the genetic deviation between species or between populations within a species. It is measured by a variety of parameters like Nei's standard genetic distance. This distance assumes that genetic differences arise due to mutations and genetic drift, but this distance measure is known to give more reliable population trees than other distances particularly for DNA data. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similarity between two individuals. There is a variety of alternative measures for expressing similarity, like Jaccard’s coefficient of similarity which can be used for binary data and often is applied in RAPD-based studies. This coefficient is based on number of positive matches between two individuals whereas joint absences are excluded. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Genetic distance can be used to compare the genetic similarity between different species. Genetic diversity studies help in formulating proper conservation, preservation and selection of material for breeding program.

The value of pair-wise comparisons Neiṡ (1972) genetic distance between 8 turmeric genotypes were computed from combined data through 12 primers, ranging from 0.0930 to 0.2013 . The highest genetic distance 0.2013 was observed in Mohisha vs. Patna local varietal pair whereas lowest value (0.0930) was observed in BARI holud 1 vs. BARI holud 2.

Nei's analysis on turmeric genotypes was also performed by several scientists. The study conducted by Basak *et al*. (2017) observed highest Nei's gene diversity (0.25) in assam cultivar and lowest in Monipur cultivar (0.16). Jan *et al.* (2011) analized Nei's genetic diversity ranged from 0.00 to 0.71 for 20 turmeric germplasm.

Das *et al.* (2011) observed Nei's gene diversity ranged from 0.23 to 0.36.

Table 7: Summary of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values among 8 turmeric genotypes

	BARI holud 1	BARI holud 2	BARI holud 3	BARI holud 4	BARI holud 5	Mohisha	Binni	Patna
BARI holud 1	0	0.0930	0.0964	0.0992	0.1536	0.1932	0.1678	0.1353
BARI holud 2	0.0930	0	0.1323	0.1343	0.1362	0.2004	0.1535	0.1152
BARI holud 3	0.0964	0.1323	0	0.0962	0.1565	0.1743	0.1927	0.1648
BARI holud 4	0.0992	0.1343	0.0962	0	0.1583	0.1991	0.1941	0.1664
BARI holud 5	0.1536	0.1362	0.1565	0.1583	0	0.1864	0.1398	0.1680
Mohisha	0.1932	0.2004	0.1743	0.1991	0.1864	0	0.1807	0.2013
Binni	0.1678	0.1535	0.1927	0.1941	0.1398	0.1807	0	0.1594
Patna	0.1353	0.1152	0.1648	0.1664	0.1680	0.2013	0.1594	0

The similarity indices (Sij) for different accession pairs with six different primers and their average re shown in Table 8.

In this experiment, inter varietal means of the pair wise similarity indices (Sij) of ranged from 77.2 to 385.8. The highest similarity indices of 385.8 was observed in Mohishs vs. BARI holud 4 or Patna and Binni vs. BARI holud 4 varietal pair. So, genetic distance was lower between that pair than rest of the varietal pairs. On the other hand, BARI holud 3 vs. BARI holud 4 pair showed least Inter-variety similarity indices of 77.2 and genetic distance was higher between that pair than rest of the varietal pairs.

Rest of all the varietal pairs were not homogenous at different number of loci and with different primers. Therefore, this study clearly indicates that there was a high level of genetic variation among different varieties same parental origin.

Table 8: Similarity and distance indices of turmeric genotypes by RAPD primers

	BARI holud 1	BARI holud 2	BARI holud 3	BARI holud 4	BARI holud 5	Mohisha	Binni	Patna
BARI holud 1	0.0000	102.9	205.8	180.1	180.1	308.7	257.2	180.1
BARI holud 2	102.9	0.0000	257.2	231.5	205.8	360.1	257.2	128.6
BARI holud 3	205.8	257.2	0.0000	77.2	257.2	308.7	411.6	334.4
BARI holud 4	180.1	231. 5	77.2	0.0000	231.5	385.8	385.8	308.7
BARI holud 5	257.2	205.8	257.2	231.5	0.0000	360.1	257.2	283.0
Mohisha	308.7	360.1	308.7	385.8	360.1	0.0000	308.7	385.8
Binni	257.2	257.2	411.6	385.8	257.2	308.7	0.0000	231.5
Patna	180.1	128.6	334.4	308.7	283.0	385.8	231.5	0.0000

This variation can be created by geographical origin. The result also reveals that the genetic base among these turmeric genotypes 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 results indicate that, different level of genetic identity and distance present between the studied 8 turmeric genotypes.

4.6 Cluster analysis

Dendogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 8 genotypes of turmeric into two main clusters: A and B. The first major cluster 'A' had only one genotype V6 (Mohisha) and the second major cluster 'B' had rest of seven genotypes.

The second major cluster (B) was subdivided into two minor clusters (C and D) in which one cluster (C) was subdivided into two minor cluster E and F. In which cluster (F) is divided into two sub clusters (G and H) .The sub cluster (H) which included V1 (BARI holud 1) and V2 (BARI holud 2). V3 (BARI holud 3) and V4 (BARI holud 4) formed in cluster (G). The sub cluster (E) included only one local variety V8 (Patna). The rest variety V5 (BARI holud 5) and V7 (Binni) were grouped cluster (D). (Figure 2).

The result indicates that the low or high level genetic distance exists between varieties with their same or different origins. Mohisha (V6) vs. Patna (v8) showed highest Nei's genetic distance (0.2013) as they are released from different parental origin. On the other hand, BARI holud 1 vs. BARI holud 2 varietal pair showed lowest genetic distance (0.0930) as they are released from BARI.

Cluster analysis on turmeric genotypes was also performed by several scientists. The study conducted by Basak *et al*. (2017) on genetic distance by UPGMA dendrogram segregated the 19 turmeric cultivars into two main clusters, Cluster-I with one cultivar (CLME18) and cluster- II with the rest of 18 cultivars.

Adhipathi *et al*. (2013) conducted Unweighted Pair-Group Method of Arithmetic Means (UPGMA) dendrogram from genetic distance clearly separated the isolates into 4 clusters (I , II, III and IV). Cluster I consisted of two isolates (Cg2TNAU and Cg3TNAU). Cluster II consisted of (Cg1TNAU and Cc8TNAU). Cluster III consisted of Cc5TNAU and CcTNAU . All the remaining isolates belonged to cluster IV.

Khan *et al.,* (2012) constructed an Unweighted Pair-Group Method of Arithmetic Means (UPGMA) dendrogram from genetic distance and 5 turmeric cultivars from 5 regions were grouped into two main clusters (cluster I and cluster II). Cluster I consisted with four populations that is P1, P2, P4 and P5. On the other hand, cluster II comprised of the rest of turmeric population.

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RAPD and other discontinuous markers can serve as means of genetic distances to establish phylogenetic relationships among taxa (Rodriguez *et al*.,1999). Estimation of genetic differences and discrimination of genetic relationship between *Curcuma* spp. are for utilization of plant genetic resources.

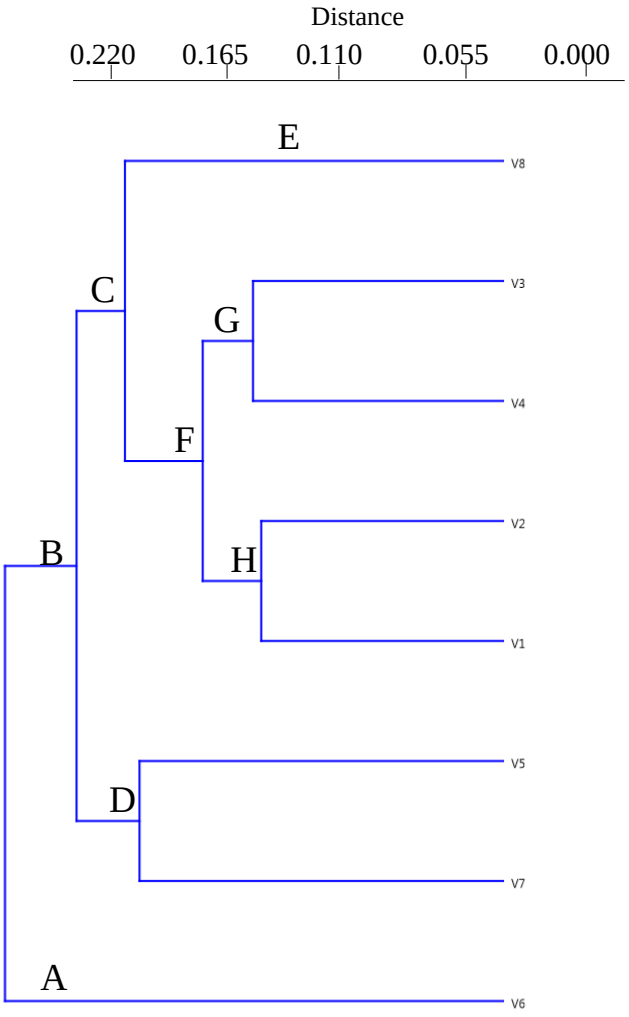

Figure 2: UPGMA method based similarity matrix Dendogram of 8 turmeric genotypes by RAPD markers with PAST software

4.7 Simpson genetic diversity of turmeric germplasm

Diversity of the germplasm was calculated using Simpson diversity index (Simpson 1949) based on the allelic variations of RAPD markers. Simpson index, varies from 0 to 1; where 0 represents no diversity and 1 for maximum diversity. The diversity indexes of turmeric germplasm BARI holud 1, BARI holud 2, BARI holud 3, BARI holud 4, BARI holud 5, Mohisha, Binni and Patna were 0.89, 0.89, 0.90, 0.90, 0.91, 0.89, 0.90 and 0.89, respectively (Fig.3). It indicated that, BARI holud 5 was comparatively more diversified followed by BARI holud 3, BARI holud 4, Binni, BARI holud 1, BARI holud 2, Mohisha and Patna.

The highest genetic diversity was found in BARI holud 5. Diversity indexes was calculated using Simpson diversity index (Simpson, 1949) based on the allelic variations of RAPD markers.

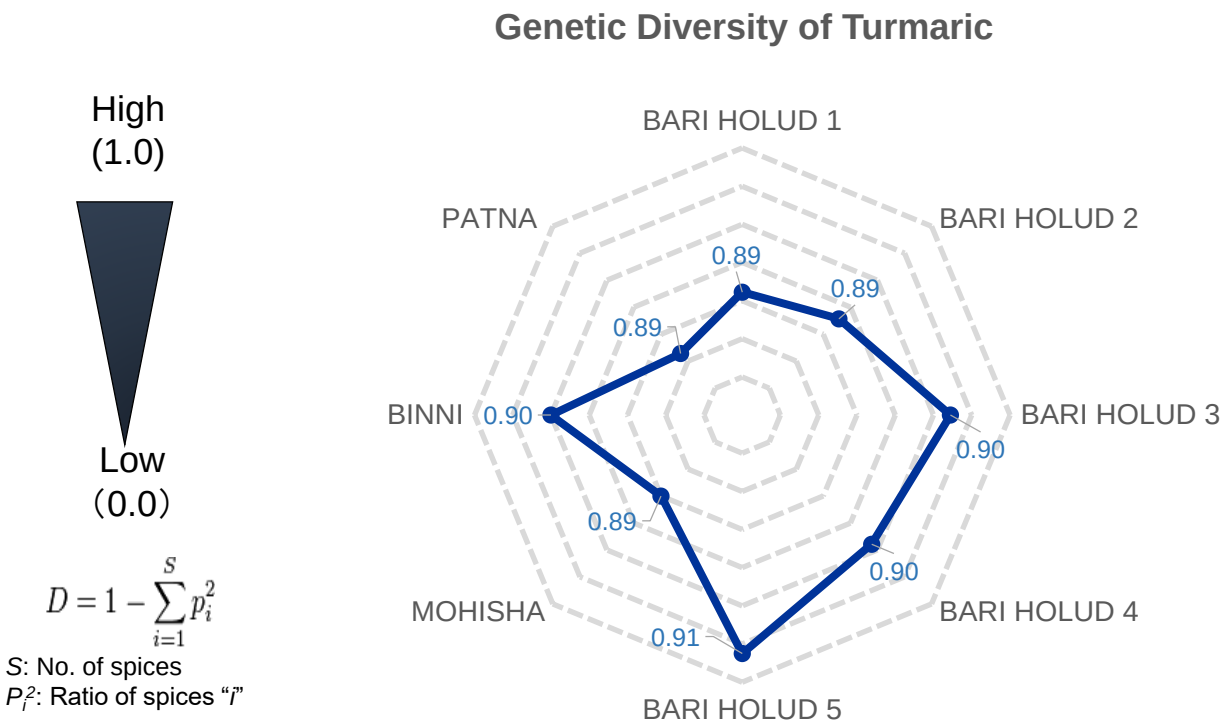

Figure 3: Simpson diversity index of turmeric gerplasm based on the allelic variation of RAPD markers.

CHAPTER V

SUMMARY AND CONCLUSION

Turmeric (*Curcuma longa*) is an essential spice crop in Bangladesh as well as in the world contest. Molecular based research in turmeric for its improvement is limited. For the reason, there is a basic need to study genetic variability of turmeric for improvement, breeding and conservation of turmeric. The experiment presented mainly the molecular characterization of 8 turmeric genotypes with 12 RAPD primer. The present experiment was conducted at Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

The 12 primers regenerated total 34 DNA fragments with an average 2.83 per primer among the turmeric germplasm. Out of 34 DNA fragments, 23 DNA fragments were polymorphic and the average percent of polymorphism was 64.44. The highest (3) number of polymorphic band was produced by the primer OPBC 14, OPBD 12, OPX 10, OPY 11, OPG 17 and OPW 05 and lowest (1) number of polymorphic band by the primer OPA 03, OPB 17, OPBD 20 and OPZ 01. No polymorphic band was found in the primer OPBD 18.

Maximum 100% of polymorphism was recorded in the primer OPX 10, OPY 11, OPG 17 and OPW 05 and OPBC 14 and OPBD 12 produced 75 % polymorphism. 50% polymorphism was recorded in the primer OPA 03, OPBD 20 and OPZ 01. OPBB 09 and OPB 17 primers produced40% and 33.33% polymorphism respectively.

The frequency of the major allele ranged between 0.375 (OPBB 09, OPW 05, OPX 10) to 0.625 (OPA 03, OPB 17, OPBD 18) with the average value of 0.500. Polymorphic Information Content (PIC) value for 12 markers ranged from 0.468 to 0.712 and the average PIC value was 0.589. The highest PIC value (0.712) was obtained for OPBB 09 and the lowest value PIC value (0.468) was obtained for OPA 03, OPB 17 and OPBD 18. PIC value revealed that OPBB 09 was considered as the best marker in turmeric genotypes followed by OPBD 12, OPW 05, OPX 10 respectively. Gene diversity ranged between 0.750 (OPBB 09) to 0.531 (OPA O3, OPBD 12 and OPBD 18) with an average of 0.612. The results indicate that the 8 local turmeric genotypes

present a high degree of homozygosity and also considerable intra-varietal group diversity, and a certain degree of genetic differentiation and polymorphism.

Principal components analysis (PCA) was conducted on 8 turmeric genotypes and classified into four groups and showed in two dimensional scatter plot. All the groups were separated from each other. Highest distance was noticed between patna (Group I) and Mohisha (Group II) that indicates selection of materials for further turmeric improvement. Nei's genetic distance ranging from 0.0930 to 0.2013. A dendogram (UPMGA) indicating the relative genetic similarity of the turmeric genotypes was constructed which followed in two major clusters (A and B) among the studied material. The cluster B was further subdivided .The results also showed that the genotypes can be separated from each other at the molecular level by taking advantage of some of the RAPD markers. This study can be helpful for breeding programme of turmeric.

RECOMMENDATION

Higher number of samples and large number of molecular markers would be essential to establish a proper genetic relationship, sample identification and analysis of genetic diversity among various genotypes of turmeric. There were some limitations in term of the number of samples and molecular markers used in the present study. The present experiment was the initial study to determine the genetic diversity among different turmeric genotypes. So, large number of samples and molecular markers should be used in further study. The present study can be used as a guideline for future experiment and the following points might be considered to develop the genetic characteristics of turmeric in Bangladesh.

1. Large number of genotypes and higher number of molecular markers should be used.

2. Details survey work should be conducted using more molecular markers for obtaining desirable loci for turmeric germplasm.

3. Other molecular markers such as SSR, SNP, AFLP, micro-satellite etc should be developed for turmeric genotypes of Bangladesh.

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