DNA FINGERPRINTING AND MOLECULAR DIVERSITY ANALYSIS OF GINGER (*Zingiber Officinale*) GENOTYPES USING RAPD MARKERS

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CERTIFICATE

This is to certify that thesis entitled, ***"DNA FINGERPRINTING AND MOLECULAR DIVERSITY ANALYSIS OF GINGER (Zingiber Officinale) 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* ***FATEMA- TUZ- ZOHURA****, Registration No.* ***11-04275*** *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, 2017
Place: Dhaka, Bangladesh

.....
(Prof. Dr. Md. Ekramul Hoque)
Supervisor


DEDICATED TO
MY
BELOVED PARENTS

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	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

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The Author

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ABSTRACT

Ginger (*Zingiber officinale*) rhizomes have been widely used as a spice and flavoring agent in foods and beverages in Bangladesh as well as in all over the world for its economical and medicinal values. Identification and characterization of germplasm is an important link between the conservation and utilization of plant genetic resources. The present investigation was undertaken for the assessment of 13 local genotypes of *Zingiber officinale* collected from different region of Bangladesh by 7 RAPD random decamer primers. Good quality genomic DNA was extracted from ginger genotypes using CTAB method. Nine RAPD primers were screened for amplification of genomic DNA and seven primers were selected based on the amplification pattern. A total of 34 distinct and differential amplification bands ranging from 150-1200 kb were observed with an average of 1.14 polymorphic bands per primer. The overall gene diversity was detected 0.8052 and the value of PIC was detected 0.7532. Primers could generate enough polymorphism for possible use in diversity studies, based on provisional multivariate analyses such as cluster analysis and principal component analysis (PCA). PCA classified 13 ginger genotypes into four groups and showed in two dimensional scatter plot. The genetic similarity coefficients among genotypes ranged from 0.103 to 0.654. Cluster analysis based on Jaccard's similarity-coefficient using UPGMA grouped the genotypes into two clusters: Cluster A and Cluster B. The cluster 'A' had only one genotype Kaptai local and the second cluster 'B' had rest of twelve genotypes. Further cluster B subdivided into another cluster. The prevalence level of polymorphism in the local genotypes of ginger will help to breeders for ginger development breeding program.

Chapter I

Introduction

CHAPTER I

INTRODUCTION

Ginger *(Zingiber officinale)* a herbaceous perennial ancient spices belonging to the family Zingiberaceae is also known as canton ginger, cooking stem, common ginger, true ginger, garden ginger (List of Plants in the Colony, 1803). In Bangladesh, it is known as ada. It is valued as medicinal crop and has been used as a spice for over 2000 years (Bartley and Jacobs, 2000). Zingiberaceae is a family of flowering plants made up of about 50 genera with a total of about 1600 species of perennial tropical herbs (Christenhusz and Byng, 2016). Taxonomically, the two main groups can be named: *Z. officinale* cv. group *Officinale*, which is cultivated throughout the tropics, and *Z. officinale* cv. group *Rubrum* ('haliya padi'), grown on a small scale in South-East Asia for medicinal use and as a spice. The latter differs from the former by having smaller, red rhizomes with a stronger and more pungent odour, the red colouring of the basal parts of leafy stems and petioles, larger leaves and the presence of a larger, scarlet-red mottled labellum. Among them *Zingiber officinale* is used worldwide for its various utilization.

Most of the *Zingiber* species are diploid (2n=22) except *Z. mioga* (2n=55) and set seed. In cultivated ginger (*Z. officinale*) it is reported that the normal diploid number of 2n=22 (Ravindran *et al*., 2005).

It is widely used as a spice or herbal medicine because of its aromatic, pungent and hot taste. The chemistry of ginger is well documented with respect to the secondary metabolites oleoresin and volatile oil (Barnes *et al*., 2002; Sweetman, 2007). More than 400 chemicals have been identified in ginger rhizome (Garner-Wizard *et al*., 2006). The relative proportions of chemicals were determined by geographical location and age of rhizome during harvesting and extraction method. Chemical constituents of ginger categorized to volatile oils which constitutes (1–3%) mainly of zingeberene, non-volatile pungent compounds oleoresin constitute (4–7.5%) mainly gingerols % of starch (Robbers *et al*., 1996) which turn out a 'hot' sensation within the mouth.

It is also valued as herbal medicine for a number of conditions including those affecting the digestive tract, headache, nausea, vomiting and motion sickness (Dedov *et al*., 2002; Borrelli *et al.*, 2004). Ginger has been demonstrated to have various pharmacological activities, such

as antiemetic, antiulcer, anti-inflammatory, antioxidant, antiplatelet, and anticancer activities. It also exerts anti-tumorigenic and immunomodulatory effects and is an effective anti-microbial and anti-viral agent (Reddy and Lokesh, 1992; Vimala *et al*., 1999; Akoachere *et al*., 2002).

The history of Ginger goes back over 5000 years when the Indians and ancient Chinese considered it as a tonic root for all ailments. Ginger have known to originated in the tropical rainforests from the Indian subcontinent to Southern Asia where ginger plants show considerable genetic variation (Thomas, 1982). At an early date it was exported to Ancient Rome from India. The Arabs introduced ginger to East Africa in the 13th century and the Portugese spread it to West Africa and the Pacific islands for commercial cultivation (Ravindran *et al.,* 2005). Additional introductions have been made as countries attempted to cultivate ginger on a larger scale (Ravindran and Babu, 2005). Ginger is the most widely cultivated spice (Lawrence, 1984). The major ginger growing countries include Australia, Brazil, Bangladesh, Cameroon, China, Costa Rica, Fiji, Ghana, Guatemala, Hawaii, India, Indonesia, Jamaica, Mauritius, Malaysia, Nepal, New Zealand, Nigeria, Philippines, Sierra Leone, Sri Lanka, Taiwan, Thailand, Trinidad and Uganda. Among them India and China produce the best quality ginger.

Ginger is one of the important cash crops in Bangladesh. In Bangladesh, ginger grows well in Rangpur, Nilphamari, Tangail, Rangamati, Bandarban, Khagrachari and Chittagong district (Choudhury *et al.,* 1998). Ginger is a shade loving monocotyledonous perennial crop and it grows well in warm and humid climate. It is grown easily in homestead and under the forest tree species.

Though ginger is completely sterile (Ravindran and Babu, 2005) and other species in the genus Zingiber are wild so it is propagated exclusively by vegetative means using rhizome known as seed rhizome (Peter *et al*. 2007). Carefully preserved seed rhizomes are cut into small pieces of 2.5-5.0 cm length weighing 20-25 g each having one or two good buds. The seed rate varies from region to region and with the method of cultivation adopted. The seed rate varies from 1500 to 1800 kg/ha. At higher altitudes the seed rate may vary from 2000 to 2500 kg/ha. The seed rhizomes are treated with mancozeb 0.3% (3 g/L of water) for 30 minutes, shade dried for 3-4 hours and planted at a spacing of 20-25 cm along the rows and 20-25 cm between the rows. It is extensively grown during kharif season. Planting is done between mid-April to May. Ginger cultivation requires sandy silt soils and temperature range from 28˚-35˚ C (Paulose, 1973) and average annual rainfall 2500-3500 mm. Ginger attains full maturity in 210-240 days

after planting. But harvesting can be done from 6th month onwards for marketing known as green ginger. For preparing the dry ginger, the crop is harvested between 245 to 260 days. When the leaves turn yellow and start gradually drying up, the clumps are lifted carefully with a spade or digging fork and the adhering soil removed. The average yield per ha varies from 15 to 25 tons. India and China are the world's largest producers and exporters of ginger. Other important producers are Jamaica, Nigeria, Sierra Leone, Thailand, and Australia (Jansen, 1981; Yiljep *et al.*, 2005).

The farmers of Bangladesh have been following conventional practices for ginger 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 ginger in Bangladesh, commercial yield per hectare was very low. Due to development of some improved varieties like- BARI Ada-1, BARI Ada-2 and BARI Ada-3, production rate is increased from earlier condition. In Bangladesh, it occupies an area of about 22,998 acres with the production of 77,478 metric tons (Yearbook of Agricultural Statistics-2017). Bangladesh ranked in 8th position among the Ginger producing countries (FAOSTAT- 2016). Ginger plays a vital role in our national economy.

The yield potential of ginger in Bangladesh is very low compared to other countries due to the incidence of insect pests and diseases, which greatly hampered the production of ginger. Ginger is affected by many diseases like fungal, bacterial, viral and mycoplasma origin (Iyer, 1988). Of them, rhizome rot, bacterial wilt, yellows, Phyllosticta leaf spot, root knot nematode and storage rot are major diseases which cause economic loss. Although there are no serious pests due to the pungent nature of the crop, cut worms, aphids, root knot nematodes, stem borer, African black beetles and rodents sometimes attack the shoots or roots. Bangladesh has no clear idea about appropriate control measure of rhizome rot. Due to this reason, the farmers generally fail to harvest healthy crop.

Crop genetic resources with a broad genetic base and high variability are vital to crop improvement program. Due to low genetic variability, ginger faced many problem in breeding for crop improvement. Because ginger propagates vegetatively because of poor flowering and seed setting that's why hybridization is not effective since its floral biology has not been properly observed yet (Simmonds, 1986). Besides this, most of the crop improvement programs of ginger are restricted to the assessment and selection of naturally occurring clonal variations (Rout *et al.,* 1998; Palai and Rout, 2007). Assessment and characterization of the state of the

existing genetic diversity within the taxon is critical for planning a meaningful breeding strategy (Cooper *et al*., 2001).

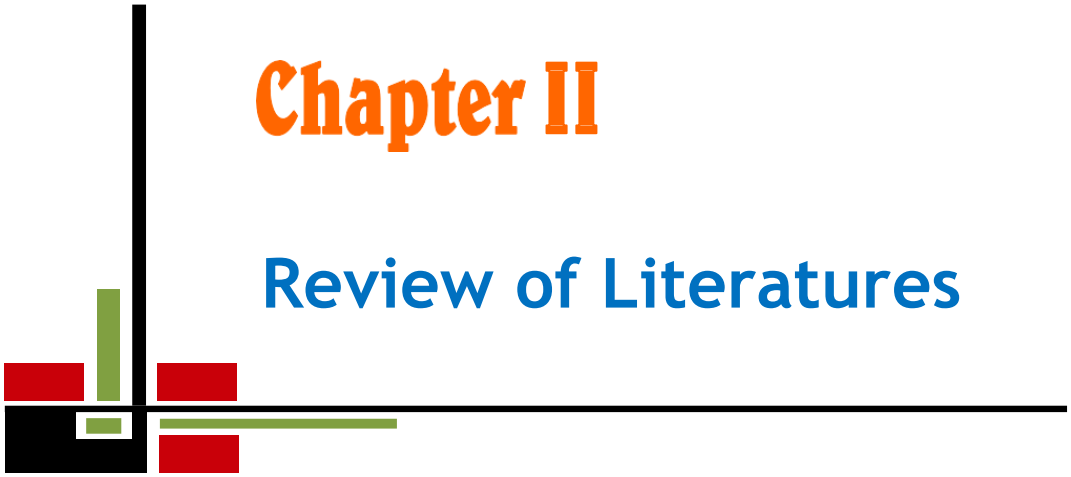
A number of techniques are available for studying the variability of crop germplasm like morphological traits, total seed protein, isozymes and various types of molecular markers. However, molecular markers provide powerful and reliable tools for assessing variations within crop germplasm and for studying evolutionary relationships (Gepts, 1993) as well as molecular markers are not prone to environmental influences and accurately characterize the plants portraying the extent of genetic diversity among taxa (Bennett and Smith, 1991; Rodriguez *et al*., 1999; Das *et al*., 2001). During the past decades, use of molecular markers is gaining attention to reveal polymorphisms at the DNA level. The molecular approach for identification of plant varieties/genotypes seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relationships between plants (Williams *et al*. 1990; Paterson *et al*. 1991). PCR-based molecular markers have been widely used in many plant species for identification, phylogenetic analysis, population studies and genetic linkage mapping (Williams *et al*., 1990).

The most popular molecular markers used in ginger species studies include isozyme, allozyme, RAPD, AFLP, Microsatellite (SSR), and ISSR markers. Among them, the random amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR) has widely been using to assess genetic variation at gene level (Welsh and McClelland, 1990; William *et al.,* 1990). The technique of RAPD gained importance due to its simplicity, efficiency and non-requirement of sequence information (Karp *et al.,* 1997). The intention of this investigation was to assess genetic diversity and relatedness of the ginger genotypes by PCR based RAPD technique as it is very much important for further improvement of ginger.

Till date very few studies have been reported on genetic diversity study of different ginger varieties in Bangladesh. Since ginger is a very poorly studied crop and its molecular information is limited, hence it is imperative to know the genetic diversity among different accessions from different regions of Bangladesh.

By considering the above facts the present study has been undertaken to provide DNA fingerprinting and molecular diversity of different ginger genotypes by PCR amplification technique. Thus the present study was conducted with the following objectives:

1. To assess the molecular diversity of some local ginger genotypes of Bangladesh.
2. To perform DNA fingerprinting and polymorphism among collected ginger germplasm.
3. To identify the most diverse genotypes for its future improvement program.



Chapter II

Review of Literatures

CHAPTER II

REVIEW OF LITERATURE

Zingiber officinale (ginger) belongs to the family Zingiberaceae 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 ginger genetic diversity and relationship, phylogenetic study and characterization through molecular markers like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) etc. Some of the research works also done in Bangladesh, genetic data on ginger 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 The concept of molecular marker

Molecular markers are reliable tools to characterize the DNA profile of plant genotypes to study the genetic diversity. According to Datta *et al*., (2011) Molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome.

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.

Molecular marker able to identify particular locations on a chromosome, allowing creation of physical maps. Molecular marker can identify many alleles an organism for a particular trait (bi allelic or poly allelic).

Molecular marker can identify small changes within the mapping population enabling distinction between a mapping species, allowing for segregation of traits and identity. Some studies which were conducted during the last decade of the 20th century reported numerous DNA markers that have been utilized in plant breeding programs. Apart from the application of molecular markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the genetic variations within cultivars and germplasms (Henry, 1997).

The most interesting application of molecular markers is 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).

Suitable DNA markers should be polymorphic in the DNA level and can be expressed in all tissues, organs, and various developmental stages. The molecular approach for identification of plant genotypes seems to be more effective as it allows direct access to the hereditary material (Paterson *et al.,* 1991) unlike the morphological markers.

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).

Amin *et al.,* (2010) reported the use of molecular markers in identification of vegetables crop varieties like tomato, potato, onion, garlic and related species and linked to major disease resistance in tomato like *Meloidogyne incognita* and tomato mosaic virus.

These markers are selectively neutral because they are usually located in non-coding regions of DNA. Usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors. Apart from the use of DNA markers in construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within cultivars and fingerprinting the germplasms. DNA markers are accepted widely as potentially valuable tools for crop breeding such as rice (Mackill *et al.,* 1999 and McCouch *et al*., 1988), wheat and forage species(Jahufer *et al.,* 2003).

According to Krap and Edward (1997), DNA based marker classified into three categories depending on technique used. Hybridization based DNA markers, arbitrarily primed polymerase chain reaction (PCR)-base markers, and sequence targeted and single locus DNA marker. Restriction Fragment Length Polymorphism (RFLP) is a hybridization based marker in which DNA polymorphism is detected by digesting DNA with restriction enzyme followed by DNA blotting and hybridizations with probes. Arbitrarily primed PCR based markers are employed in organisms for which no genome sequence is available like RAPD and AFLP. Sequence Tagged Sites (STS), Sequence Repeat (SSRs), Single Nucleotide Polymorphism (SNPs) markers belongs to sequence targeted and single locus PCR based DNA markers. Of these, RFLP and micro satellites are codominant markers, while RAPD and AFLP markers are largely dominant markers.

2.2 Concept of RAPD marker

Random Amplified Polymorphic DNA is a PCR-based technique discovered by Williams *et al.,* (1990) and generated by the use of short (10-mer) synthetic oligonucleotides in single strand primer. Kumar and Gurusubramanian (2011) pointed that there is no need to know DNA sequence information for targeted gene. In this technique, a decamer primer of arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome (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.

There are several advantages of RAPDs compared to other DNA based techniques. RAPD. Non-radioactive detection, multiple luci detection in single reaction, requirement of small quantity of DNA, no requirement of prior sequence information, quick and technically simple (Karp *et al*., 1997).

Hadrys *et al.,* 1992 stated that RAPD-PCR technique used for examining variation in the total genome. 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.

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 'dominance of RAPD marker' because the presence of given RAPD band does not distinguish whether its respect locus is homozygous or heterozygous or codominance 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 analysis of ginger

Genetic diversity is the total number of genetic characteristics in the genetic makeup of a species. It is distinguished from genetic variability, which describes the tendency of genetic characteristics to vary. Genetic diversity plays an important role in the survival and adaptability of a species. It is usually refers to the variation or differences between organisms at the DNA sequence level. This can be affected by natural or artificial (i.e. human) selection, mutation, recombination and other mechanisms. This genetic diversity can be successfully analyzed by using RAPD markers. 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 (Mohammadi *et al.,* 2003).

Kamaruddin and Abdullah (2017) assessed genetic variability using molecular marker technique, RAPD by the effect of different gamma doses on the crude fiber composition of irradiated two Malaysian ginger variety: Bentong and Tanjung Sepat. A total of 9 different arbitrary decamers were used as primers to amplify DNA from mutant plant material to assess their polymorphism level of ginger mutant lines. A total of 125 reproducible RAPD bands were scored. Indicating 3 monomorphic and 125 polymorphic. The overall polymorphism was 97.6% and the rest monomorphic bands. The number of DNA fragments per primer ranged from 6 (OPA-12) (OPA-27) to 23 (RN-08), the average number of bands per primer being 13.56 polymorphic bands. Polymorphism of all mutant lines was 97.62% indicating that there were significant changes in genetic sequences in irradiated ginger genotypes. The genetic distance of Bentong ginger found range from 0.69 to 0.32. Two main clusters were delineated from the dendrogram. For Tanjung Sepat ginger, the genetic distance was found (0.64-0.32). Two main clusters were grouped from the dendrogram. The genetic variation analysis through molecular markers provides a strong evidence for the existence of high level of variability among mutant lines of ginger.

Ismail *et al.,* (2016) reported that RAPD markers can differentiate ginger populations based on their collection sources. 32% genetic diversity observed by using RAPD marker. This marker has proven to be an effective tool for detecting genetic diversity at the interspecific and intraspecific levels. Thus, it has been shown that RAPD can be used for identification as well as classification of ginger species. The genetic variation of *Zingiber zerumbet* was determined by a RAPD technique.

Subudhi *et al*., (2016) preformed an investigation with 48 ginger germplasm from 10 agro-climatic zones of Odisha using 20 RAPD primers. Out of 20 RAPD primers selected primers 12 produced distinct reproducible amplifications. A total of 68 amplifications were found whereas only 5 were monomorphic. Highest nine amplified bands were observed with primer OPD20 and lowest one band with primer OPD-08. No unique band was found in OPD series of primers. Nei's genetic distance analysis from RAPD revealed minimum genetic distance was 0.06 and maximum 0.69. All the samples correlated with each other with an average genetic distance of 0.39. SAHN clustering generated a dendrogram that divided the 48 samples into two major clusters. The percent of polymorphic loci among 10 populations ranged from 16.18 % to 75 % and the average PPL was 37.06 %. Highest Nei's gene diversity (H) and Shannon's index (I) were found ($H = 0.265 \pm 0.208$ and $I = 0.386 \pm 0.296$). AMOVA of RAPD data showed 24 % genetic variability among 10 agro-climate zones of Odisha. Principle

component analysis (PCA) showing two dimensional scatter plot clustering pattern of 48 accessions of Zingiber officinal. A further study on the behavior of this estimator is needed.

Mia *et al.*, (2014) investigated the genetic diversity in ginger using random amplified polymorphic DNA (RAPD). Eight Bangladeshi ginger germplasm and twelve primers were used for random polymorphic DNA assay. Three out of twelve primer produced a total of 16 bands, out of which 10 (62.50%) were polymorphic. Each primer generated 3 to 6 amplified fragments with an average of 5.33 fragments per primer. The highest genetic distance (0.5531) was observed between Indian vs. Syedpuri and the lowest genetic identity (0.0302) was found in China vs. Sherpuri genotypic pair. The unweighted pair-group method of arithmetic means (UPGMA) dendrogram was constructed from genetic distance and all ginger cultivars were grouped into two main clusters. The genetic diversity of ginger cultivars reported in this study will be useful guideline for future diversity assessment and genetic analysis of ginger genotypes.

Ashraf *et al.,* (2014) analyzed 12 accessions of *Z. officinale* from subcontinent of India by the means of 20 RAPD primers for their genetic diversity study. Thirteen out of twenty primers screened were informative and produced 275 amplification products, among which 261 products (94.90%) were found to be polymorphic. Most of the RAPD markers studied showed different levels of genetic polymorphism. The data of 275 RAPD bands were used to generate Jaccard's similarity coefficients to construct a dendrogram by means of UPGMA. Pair-wise genetic similarities ranged from 0.21 to 0.39 in all accessions with a mean value of 0.30. Based on the dendrogram, the 12 accessions were grouped into two main clusters (cluster I and cluster II). This investigation was an understanding of genetic variation within the accessions. This investigation will provide an important input into determining resourceful management strategies and help to breeders for ginger improvement program.

Pradhan *et al*., (2014) performed an investigation which was carried out for analysis of genetic diversity among 5 ginger varieties of Sikkim Himalaya through 63 RAPD markers. A total of 104 clear, reproducible and scorable fragments which were ranging from 150-13000 bp were generated from 21 primers and 99% were found polymorphic. Cluster analysis using UPGMA algorithm placed the 40 accessions into three main cluster. The genetic dissimilarity matrix between genotypes ranged from (0.74994-5.33209) between the 5 cultivars studied. Cluster analysis of data using 40 accessions into four main clusters the genetic with a range (0.35-0.87), the principal component analysis (PCA) placed into four groups in three dimensional

scatter plot. The prevalence of a relatively high level of polymorphism in the cultivars of ginger will help to breeders for ginger development breeding program.

Pujaita *et al.,* (2014) studied to assess DNA fingerprinting of Indian ginger varieties through PCR based RAPD technique. Thirty five RAPD primers were screened for amplification of genomic DNA and ten primers were selected based on the amplification pattern. The size of amplicons ranged from 400 bp to 2000 bp. The number of total 194 fragments were found whereas clear and distinct 68 bands and faint 125 bands produced by RAPD primers. The fingerprints developed were unique and specific for the varieties /somaclones and source parent cultivars. This study will be powerful tool for specific parent variety selection.

Sajeev *et al.,* (2011) conducted a study to assess genetic diversity using a set of forty-nine ginger clones cultivated in North-East India with 30 random amplified polymorphic DNA (RAPD) markers. Out of the 30 primers 18 showed reproducible polymorphic bands. The 18 RAPD primers amplified 109 distinct fragments. Number of fragments per primer ranged from 1 to 13 in the size range of 0.3–2.5 kb. Of the 109 fragments amplified, 101 (91.87%) were polymorphic and an average of 6.05 amplified fragments were produced per primer. Dendrogram based on UPGMA analysis separated the forty-nine clones into five major clusters with a Jaccard's similarity coefficient of 0.57–0.96 indicating high genetic variability among the clones. Average gene diversity in the six populations ranged from 0.158 to 0.226. PCA grouped the populations on the three dimensional scatter plot. The contribution of the first three principal components was 37.98%, 24.88% and 18.00%, respectively, leading to a cumulative contribution of 80.87%. This study helped to present a reliable and true picture of the genetic diversity in the studied germplasm. This study has been an attempt to understand the genetic diversity of *Z. officinale* prevailing in the NE India.

Ghosh (2011) performed a study to analyze the genetic diversity of fifteen wild landraces of *Zingiber officinale* Roscoe using 16 random decamer primers (RAPD). RAPD markers were used to evaluate the genetic stability of micropropagated plants of *Zingiber officinale.* Fifteen primers were used to amplify DNA from *in vivo* and *in vitro* plant material to assess the genetic fidelity. Out of 117 amplified products, 97 bands showed polymorphism (82.90%) and only 20 were found to be monomorphic bands. an average of 7.5 bands was amplified per primer. The genetic similarity coefficients among accessions ranged from 0.673 to 0.912 with an average of 0.792. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped the landraces into three clusters: cluster I consisting of 5 landraces, Cluster II consisting of 6

landraces, and cluster III of 4 landrace. The extent of genetic relatedness among *Zingiber officinale* Roscoe genotypes varied considerably (0.703 to 0.912). This study will be helpful for further medicinal investigations.

Kizhakkayil *et al.,* (2010) performed a study to analyze the genetic diversity of ginger using inter-simple sequence repeat (ISSR) and RAPD primers. Though RAPD markers are reported to be more suitable for genetic diversity analysis of clonal organisms the ISSR markers are more reproducible than RAPD markers. The study conducted with 46 ginger accessions in which 30 decamer and 17 ISSR were used. RAPD and ISSR products bands between 250 and 2500 bp. Out of 60 RAPD primers screened, the 30 which gave consistent pattern were used for further amplification. A total of 269 scorable bands were produced by 30 RAPD, out of which 126 were polymorphic. Seventeen ISSR primers produced 160 scorable bands out of which 76 were polymorphic. The genetic similarity coefficients (Jaccard's) obtained by the RAPD and ISSR markers were in the range of 0.76–0.97. The cluster analysis using the combined data of RAPD and ISSR has revealed four clusters in Jaccard's, Sorensen and Simple Matching coefficient dendrogram.

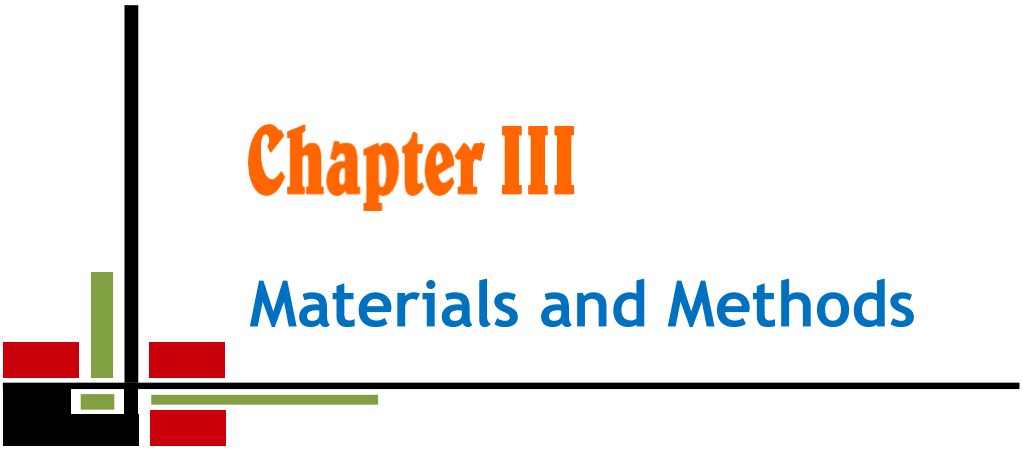
Harisaranraj *et al*., (2009) studied to assess genetic diversity among diverse genotypes of ginger using RAPD technique. Twenty RAPD primers were used to amplify genomic DNA from 8 ginger genotypes. A total of 55 distinct DNA fragments ranging from 0.5-2.4 Kb were amplified by using twelve selected primers. The twenty-decamer primers produced good amplification of RAPD fragment ranging from 500 to 2400 base pairs. Out of 55 fragments obtained, only 25 fragments (45.5%) were polymorphic and rest are monomorphic. Genetic distance (GD) ranged from 0.34 to 0.74. The similarity matrix obtained in the present study was used to construct a dendrogram with the unweighted UPGMA method and resulted in their distant clustering in the dendrogram. The cluster analysis indicated that the eight varieties formed two major clusters. The present study showed the distant variation within the varieties. This investigation will help to breeders for ginger improvement program.

Jatoi *et al*. (2008) conducted the study of diversity assessment of a set of 55 genotypes representing three *Zingiber* species of Asian countries through RAPD markers. A considerable number of band types (184) were amplified by eight primer sets. In *Z. officinale* collected from Myanmar, 84 band types were amplified, ranging from 5 to 15, leading to an average of 10.5 fragments per primer set. Sixty fragments were polymorphic with an average of 7.5 polymorphic bands, leading to an average polymorphism rate of 71%. Sixty-seven band types

were observed in *Z. officinale* collected from Asian markets, with an average of 8.4 bands per primer set. Among the amplified bands, 36 were polymorphic and the average number of polymorphic bands were 4.5; however, RM171 yielded just monomorphic bands. The number of alleles amplified in different collection sources was 81, 70 and 69 in farms, a gene bank and markets, respectively The genetic distance in the three collection sources based on similarity coefficients was 0.61, 0.66 and 0.70 in farms, a gene bank and markets, respectively. Similarly, gene diversity in the farm collection was highest (0.26), followed by the gene bank (0.18) and market collections. The PCA reflected the diverse distribution of the ginger on two dimensional scatter plot. Genetic resources of *Zingiber* species, particularly available on farms, can be a useful source to capture and utilize diversity for conservation as well as further improvement in gingers.

Siddhartha *et al.,* (2007) conducted the study of identification and genetic variation within eight high yielding varieties of ginger through 20 RAPD markers. A total number of 55 amplified fragments was scored across eight varieties of ginger for the selected 12 primers ranging from 0.5–2.4Kb. The twenty-decamer primers produced good amplification of RAPD fragment ranging from 500 to 2400 base pairs. Out of 55 fragment obtained, only 25 fragments (45.5%) were polymorphic rest are monomorphic. The matrix value was ranged from 0.34 to 0.74, with a mean value of 0.54. The high matrix values indicated that there varieties were distantly related to each other. The similarity matrix obtained in the present study was used to construct a dendrogram with the unweighted UPGMA method and resulted in their distant clustering in the dendrogram. The dendrogram shows two major clusters. This investigation will help to breeders for ginger improvement program.

Nayak *et al.,* (2005) conducted an experiment in which 20 RAPD primers were used to analyze the genetic variation in 16 Promising Cultivars of Ginger. A total of 145 RAPD loci were scored out of which 101 (69.66%) were polymorphic and only 44 (30.34%) were monomorphic bands. Out of 44 monomorphic bands 7 (15.91%) fragments were cultivar specific fragments. An average of 7.25 bands were produced per primer. Gene diversity computed among different groups of cultivars was recorded in between 0.079 to 0.196. The effective number of alleles varied from 0.320 to 1.348 across the cultivars collected from different regions. The same order of genetic heterogeneity was discerned through Shannon's information index, which varied from 0.110 to 0.283. The dendrogram obtained using the similarity matrix coefficient presents two main clusters.



Chapter III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The chapter focused on the materials and methods of the experiment. The details of different materials and methodologies followed for the study have been described in this chapter.

3.1 Experimental site and time duration

To fulfill the objectives 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 March 2016 to February 2017.

3.2 Name and source of study materials

Thirteen ginger genotypes were used in the study. The sources of rhizome of the collected cultivars and genotypes are presented in Table 1.

Table 1: Source of study materials

Sl. No.	Genotypes name	Collected area
1.	Kurigram local	Kurigram District
2.	Tangail local	Tangail District
3.	Jamalpur local	Jamalpur District
4.	Modupur local	Tangail District
5.	B. Baria local	B. Baria District
6.	Rangamati local	Rangamati District
7.	Thakurgoan local-1	Thakurgoan District
8.	Thakurgoan local-2	Thakurgoan District
9.	Lalmonirhat local-1	Lalmonirhat District
10.	Lalmonirhat local-2	Lalmonirhat District
11.	Lalmonirhat local-3	Lalmonirhat District
12.	Rangpur local	Rangpur District
13	Kaptai local	Rangamati District

3.3 Seedling raising and collection of leaf sample

Good quality, disease free, healthy rhizomes were collected from different local areas. Soil was collected from nearby nursery and rhizomes were sown in a pot of the research farm of Sher-e-Bangla Agricultural University on April 10, 2016. All management practices were done for raising good quality seedlings from those materials. After raising seedling, in order to carry out PCR amplification of ginger genome, fresh and young leaf samples were collected at 3 to 4 leaf stage of each genotype and used as the source of genomic DNA.

3.4 Extraction of genomic DNA

Total genomic DNA extraction was performed from fresh ginger plant leaves following Doyle and Doyle (1990) method by using Cetyl Trimethyl Ammonium Bromide (CTAB) protocol with little modification.

3.4.1 Reagents used

1. Extraction buffer, pH= 8.0
   Composition of extraction buffer are as follows:
   - 1M Tris-HCl
   - 0.5 M EDTA (Ethylene diamine tetra-acetic Acid) (pH= 8.0)
   - 5 M NaCl
   - D.H_2O
   - 10% CTAB
2. Phenol: Chloroform: Isoamyl Alcohol= 25: 24: 1
3. TE (Tris-EDTA) buffer, pH=8.0

   Composition of extraction TE buffer are as follows:

   - 1M Tris-HCl
   - 0.5M EDTA
   - D.H_2O
4. Isopropanol
5. 0.3M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)

8. RNAase

9. Ethidium Bromide solution

3.4.2 Reagent preparation for DNA extraction (Stock solution)

Extraction buffer (1000 mL)

For the preparation of 1000 mL DNA extraction buffer, 100 mL 1M Tris-HCl (pH=8.0) was mixed with 40 mL of 0.5M EDTA and added to 100 mL 5M NaCl in a 1000 mL measuring cylinder. Finally, sterilized d.H_2O was added to make the volume up to 1000 mL, then mixed well and autoclaved.

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

At first 30.28 g Tris HCl was taken in a volumetric flask (5000 mL). 100 mL d.H_2O was added and pH was adjusted to 8.0 by adding HCl. Then sterilized d.H_2O was added to make the volume up to 250 mL. The solution was autoclaved.

0.5M EDTA (pH 8.0) (250 mL)

46.53 g EDTA.2H_2O was mixed with 100 mL of sterilized d.H_2O in a volumetric flask (5000 mL). Then 4 g NaOH was added. After that pH was adjusted to 8.0 with NaOH. Finally sterilized d.H_2O was added to make the volume up to 250 ml. Then the solution was autoclaved.

5M NaCl (250 mL)

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

10% CTAB (100 ml)

Ten gram of CTAB was added in 100 ml d.H_2O in a 250 ml beaker.

1X TE Buffer (100 ml)

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

5X TBE buffer (1 litter)

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

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

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

RNase

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

70% Ethanol (1000 mL)

700 absolute ethanol was mixed with 300 ml d.H_2O

0.3M Sodium Acetate

2.05 gm of Na acetate dissolved in 50 ml sterilized d.H_20 then we get 0.3M 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 13 different ginger germplasm.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by deionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 200mg of young leaves were cut into small pieces and then taken in mortar. 600µl of extraction buffer was added to it. The ground samples were taken into the 1.5ml 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 (600 µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the tube. Then it was vortexed for 20 seconds.

5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a 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 3 µl of RNAse for removing RNA. 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 pigments as contaminant and was hence purified by treatment with RNase and further precipitated (Sambrook *et al*., 1989). To confirm DNA preparation, 2% agarose gel was used for assessing the quality of the genomic DNA and the amount of RNA present.

3.6.1 Preparation of 2 % agarose gel

Reagents:

1. Agarose powder
2. 5X TBE Buffer (pH 8.3)
3. Ethidium Bromide

Procedure

Two gram of agarose powder was taken in a 500 mL Erlenmeyer flask containing 100 mL electrophoresis buffer (1x TBE buffer) prepared by adding 20 mL of 5X TBE buffer in 80 mL of sterile deionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. It was melted for about 4 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 1 µL (10 mg/mL) ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (15 x 15 x 2 cm^3; in size), that was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2mm above the plate. Air bubbles were removed by pushing away to the side using a disposable tip. After 25-30 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

The samples were all in the same concentration in buffer. For each sample, 3 µl dd.H2O and 3 µl loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) 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. Finally, 2 µl extracted DNA was added to it and mixed 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 90V for about 45 minutes.

Table 2: DNA confirmation reagents with amount

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

3.7 Working solution of DNA samples preparation

DNA concentration were adjusted to 10 ng/µl for doing PCR using the following formula: $V_1 \times S_1 = V_2 \times S_2$

Where,

V_1 = Initial volume of DNA solution (µl)

S_1 = Initial DNA concentration (ng/µl)

V_2 = Final volume of DNA solution (µl)

S_2 = Final DNA concentration (ng/µl)

3.7.1 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 working solution preparation. 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 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 the amplification of RAPD

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

Eleven RAPD primers were tested, they resulting in faint or irreproducible DNA fragments. From them seven primers were selected for this study. Seven decamer RAPD primers viz. OPG 19, OPJ 13, OPM 07, OPM 05, OPP13, OPW03, OPX04 (Operon Technologies, Inc., Alameda, California, USA) were screened for PCR reaction in 13 genotypes of gingers. List of RAPD primers are given in Table 3.

Table 3. Name, sequence and GC content of RAPD primers

Sl. No.	Primer name	Primer Sequence (5´ to 3´)	(G+C) %
1.	OPG 19	GTCAGGGCAA	60
2.	OPP 13	GGAGTGCCTC	70
3.	OPJ 13	CCACACTACC	60
4.	OPM 07	CCGTGACTCA	60
5.	OPX 04	CCGCTACCGA	70
6..	OPM 05	AAGGGCGAGT	60
7.	OPW 03	GAGCGAGGCT	70

3.8.3 PCR amplification

PCR reactions were performed on each DNA sample. 2X Taq ready Master Mix was used. DNA amplification was per formed in oil-free thermal cycler (Esco Technologies swift^{TM} Mini Thermal Cyclers). To prepare a 10 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 4.

Table 4. Reaction mixture composition for PCR for each ginger genotype

Reagents	Amount(µl)
2X Taq Master Mix	5.00
RAPD primer	1.0
De-ionized water	2.00
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 (0.5 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

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Swift™ Mini Thermal Cyclers). 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: 94˚ C for 45 seconds – Denaturation
Step 3: 35˚ C for 30 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

} 31 cycles

The amplified products were loaded on two per cent agarose gel using 50X TAE buffer stained with ethidium bromide along with marker (100bp Invitrogen). Electrophoresis was performed at 70V for two hours. The profile was visualized under UV transilluminator and documented using gel documentation system (BIORAD, USA). The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb/bp of bases were recorded in comparison with marker and using the software Quantity One.

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 5.0 µL ethidium bromide in 1x TBE buffer at 95V for 55 minutes. Loading dye (5.0 µl) was added to the PCR products and loaded in the wells. Two

molecular weight markers 100 bp (BIONEER, Cat. No. D-1030 South Korea) and 1kb (BIONEER, Cat. No. D-1040, South Korea) DNA ladder were also loaded on left and right side of the gel respectively. Under Ultra Violet (UV) light on a trans-illuminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.8.6 Documentation of the DNA samples

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

3.9 Statistical analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *el at.,* 1990). One molecular weight marker, 100 bp (BIONEER, Cat. No. D-1030, South Korea) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The band-size for each of the markers was scored using the AlphaEaseFC 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 POWERMARKER 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 dendrogram 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.

Population structures for germplasm were determined using STRUCTURE, version 2.3.4 (Pritchard, J.K. *et. al.,* 2000 and Falush, D. *et al.*, 2003). Principal components analysis (PCA) analysis was conducted also using the PAST software.

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:

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

Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted to characterize 13 ginger genotypes using seven RAPD primers. This chapter comprises the presentation and discussion of the results of the experiment. In the RAPD analysis significant genetic variation and polymorphisms for characterization of different ginger cultivars were identified. The results of the experiment were presented and expressed in Table 4 to 8, Figure 1 and Plate 1 to 8 for ease of understanding.

4.1 DNA extraction

The genomic DNA extraction of 13 local ginger genotypes were done by using the CTAB method with minor modification. RNA sharing was removed by applying RNAse treatment. Finally, the purified DNA was stored at - 20^{o}C freezer for further use.

4.2 DNA confirmation

The extracted genomic DNA of 13 samples were loaded on 2% agarose gel for conformation 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

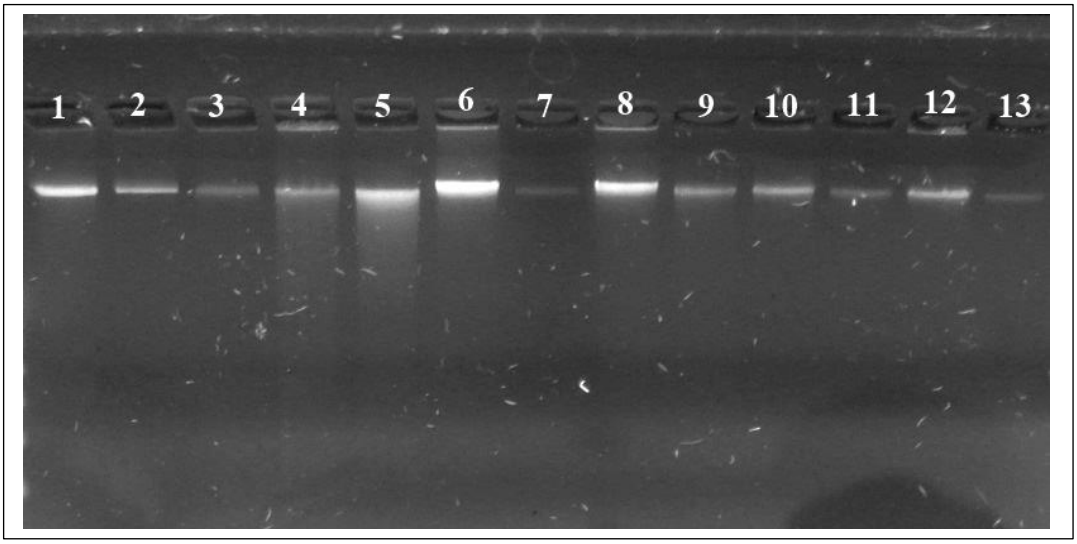


Plate 1: Genomic DNA extraction from different ginger genotypes

(Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12= Rangpur local, Lane 13= Kaptai local)

4.3 Polymorphism survey, band size and banding pattern of *Zingiber* species

Initially eleven decamer RAPD primers were screened on randomly chosen six genotypes from 13 ginger genotypes to evaluate their suitability for amplification of the ginger 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 eleven RAPD primers, seven decamer RAPD primers viz. OPG 19, OPJ 13, OPM 07, OPM 05, OPP 13, OPW 03, and OPX 04 showed reproducible amplified bands.

Seven RAPD primers *viz.* OPG 19, OPJ 13, OPM 05, OPM 07, OPP 13, OPX 04 and OPW 03 showed different banding pattern separately with in 13 ginger genotypes. The amplifications of each RAPD primers are presented in Table 5 and Plate 2 to 8.

The RAPD primer OPG 19 produced different DNA fragments in 13 ginger genotypes. The RAPD primer OPG 19 produced total 6 amplification in all ginger genotypes which was ranged from 150 bp to 1023 bp and all were monomorphic band. Whereas Kurigram local, Tangail

local, Jamalpur local, Modhupur local, B. baria local, Rangamati local, Thakurgoan local-1, Thakurgoan local-2, Lalmonirhat local-1, Lalmonirhat local-2, Lalmonirhat local-3 and Rangpur local were able to produce amplification at 1023 bp is polymorphic band. Six DNA fragments amplification were noticed by the primer OPP 13 in 13 ginger genotypes which were range from 200 - 1000 bp. The band were monomorphic DNA and common in all the genotypes. It was noticed that Kurigram local, Tangail local, Jamalpur local, Modhupur local, B. baria local, Rangamati local, Thakurgoan local-1, Thakurgoan local-2, Lalmonirhat local-1, Lalmonirhat local-2 and Lalmonirhat local-3 produce amplification at 1000 bp, which was polymorphic in nature. Another polymorphic amplification was found in 700 bp in Kurigram local genotype. Genotypes B. baria local, Rangamati local, Thakurgoan local-1, Thakurgoan local-2, Lalmonirhat local-1, Lalmonirhat local-2 and Lalmonirhat local-3 were able to produce polymorphic band at 200 bp. Kaptai local could not able to show any amplification. The RAPD primer OPJ 13 was able to amplify 7 DNA fragments among all the individuals. The DNA fragments ranged from 225 to 1180 bp. It was noticed that all were monomorphic band. The germplasm Rangamati local, Thakurgoan local-1, Thakurgoan local-2, Lalmonirhat local-1 and Lalmonirhat local-3 showed second amplification of DNA band at 350 bp is polymorphic in nature. Another amplification was observed in Tangail local, Modhupur local, Rangamati local, Thakurgoan local-1, Thakurgoan local-2, Lalmonirhat local-1, Lalmonirhat local-3 and Rangpur local at 500 bp is polymorphic amplification. The RAPD primer OPM 07 produced only one DNA fragment among all the genotypes. The approximate fragment size is 550 bp. It was monomorphic DNA band which was common in all the genotypes. A second amplification was noticed at 700 bp in Tangail local, Jamalpur local, Modhupur local, B. baria local, Rangamati local, Thakurgoan local-1, Lalmonirhat local-1, Lalmonirhat local-2, Lalmonirhat local-3, Rangpur local and Kaptai local genotypes is polymorphic band. The RAPD primer OPX 04 has the ability to five fragments of DNA among all the experimental materials. The band size ranged from 200 bp to 600 bp which indicated a monomorphic band. Thakurgoan local-2 was able to regenerate one additional DNA band at 700 bp is polymorphic in nature. Three DNA fragments were amplified by the primer OPM 05 in all the ginger genotypes which ranged from 500 bp to 1200 bp. All the amplification is monomorphic in nature. No polymorphic band was found by this primer. The RAPD primer OPW 03 has the ability to show 4 DNA fragments which ranged from 242 bp to 500 bp. The DNA band which were common in all the genotypes and all are monomorphic DNA band. No polymorphism was detected by this primer.

The 7 primers regenerated total 34 DNA fragments with an average 4.9 per primer among the 13 ginger germplasm. Out of 34 DNA bands, 8 DNA fragments were polymorphic and the average percent of polymorphism was 23.50. The highest (3) number of polymorphic band was produced by the primer OPP-13. The primer OPM-05 and OPW-03 were not able to regenerate any polymorphic band. Maximum 50% of polymorphism were recorded in the primer OPP- 13 and OPM- 07 and it was followed by primer OPJ-13 (28.57%) and OPX- 04 (20%) and OPG-19 (16.67%). No. of RAPD markers scored for each individual of 13 genotypes for each primer is presented in Table 5.

Table 5. RAPD primers with corresponding band score and their size range together with number and percentage of polymorphic loci observed in 13 ginger genotypes

Sl. No.	Primer	Primer Sequence (5′ to 3′)	(G+C) %	Total no of band scored	Number of polymer-phic bands	% of Polymo-rphism	Size ranges
1.	OPG 19	GTCAGGGCAA	60	6	1	16.67	150- 1023
2.	OPP 13	GGAGTGCCTC	70	6	3	50	200-1000
3.	OPJ 13	CCACACTACC	60	7	2	28.57	225-1180
4.	OPM 07	CCGTGACTCA	60	2	1	50	550-700
5.	OPX 04	CCGCTACCGA	70	5	1	20	200-700
6..	OPM 05	AAGGGCGAGT	60	3	0	0	500-1200
7.	OPW 03	GAGCGAGGCT	70	4	0	0	242-500
Total			-	34	8		
Mean			-	4.9	1.14	23.50	

Several scientists reported their observations on ginger with RAPD primers. Kamaruddin and Abdullah. (2017) analyzed two Malaysian popular ginger variety with 9 RAPD markers and obtained 13.89 bands per primer. The overall polymorphism was 97.6% and the rest were monomorphic bands. Mia *et al.,* (2014) analyzed 8 ginger genotypes with 12 RAPD primers, which resulted in average of 5.33 fragments per primer. Ashraf *et al.,* (2014) analyzed 12

accessions of *Z. officinale* from subcontinent of India by the means of 20 RAPD primers and showed 21.15 bands per primer. Pujaita *et al.*, (2014) studied to assess 8 ginger genotypes with 35 RAPD primers and obtained 5.22 bands per primer. Thus, the results from previous studies are similar with the present study.

In this study, the level of polymorphism (23.50%) indicated the effectiveness of RAPD technique to study substantial amount of polymorphisms or diversity among the different genotypes of ginger and this is justified by previous studies. Harisaranraj *et al*., (2009) found 45.5% polymorphism using 20 RAPD primers. Mia *et al*. (2014) investigated 62.50% polymorphism with 12 RAPD primers.

Relatively higher level of polymorphism is also found in various experiments. Subhabrata Ghosh (2011) found 82.90% polymorphism with 15 RAPD primers in 15 wild landraces of *Zingiber officinale*. Kamaruddin and Abdullah. (2017) found 97.6% polymorphism by using 9 RAPD primers in two local Malaysian varieties. Ashraf *et al.*, (2014) found 94.90% polymorphism with 20 RAPD primers in 12 accessions of *Z. officinale* from subcontinent of India.

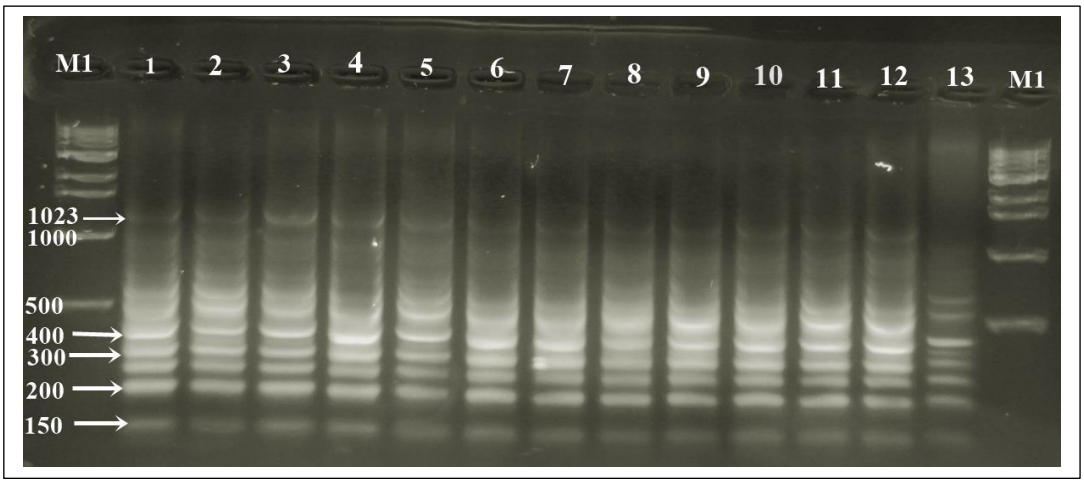


Plate 2: PCR amplification with RAPD primer OPG 19

M1 & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local.

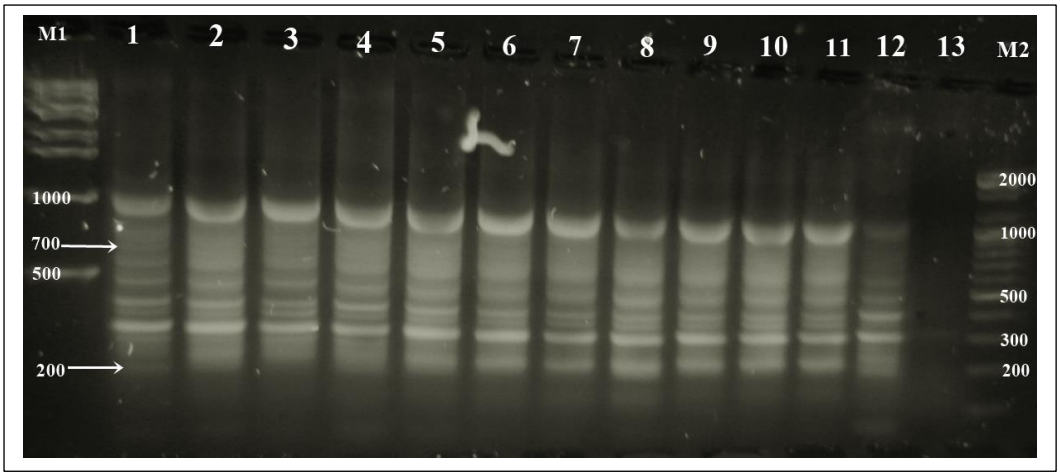


Plate 3: PCR amplification with RAPD primer OPP 13

M1 & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local.

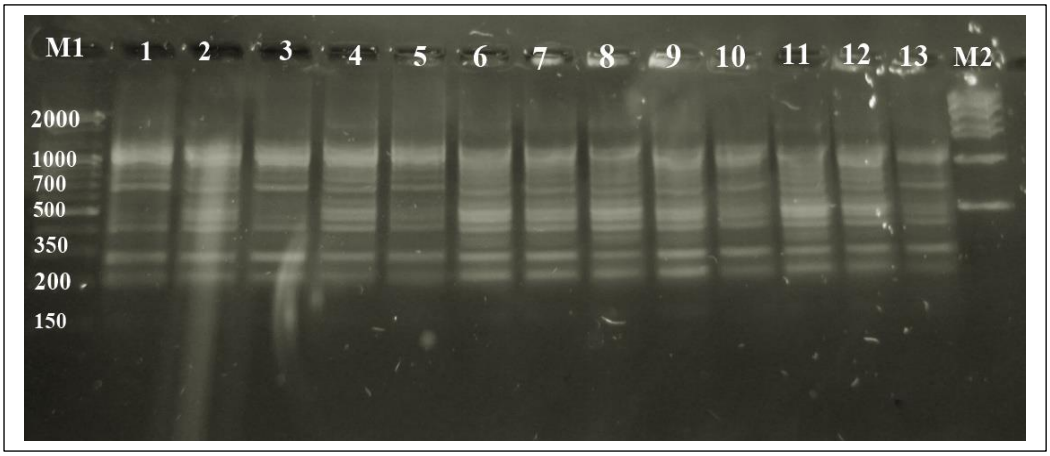


Plate 4: PCR amplification with RAPD primer OPJ 13

M1 & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local.

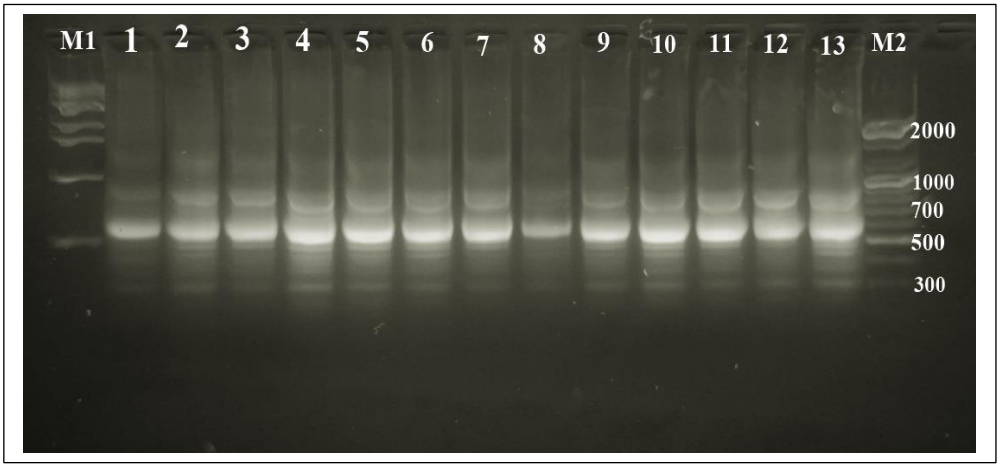


Plate 5: PCR amplification with RAPD primer OPM 07

M1 & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local.

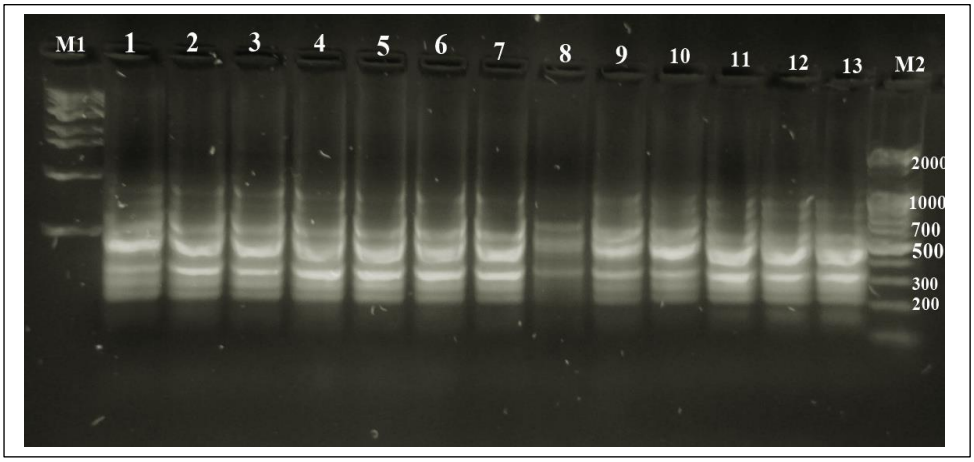


Plate 6: PCR amplification with RAPD primer OPX 04

M1 & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local.

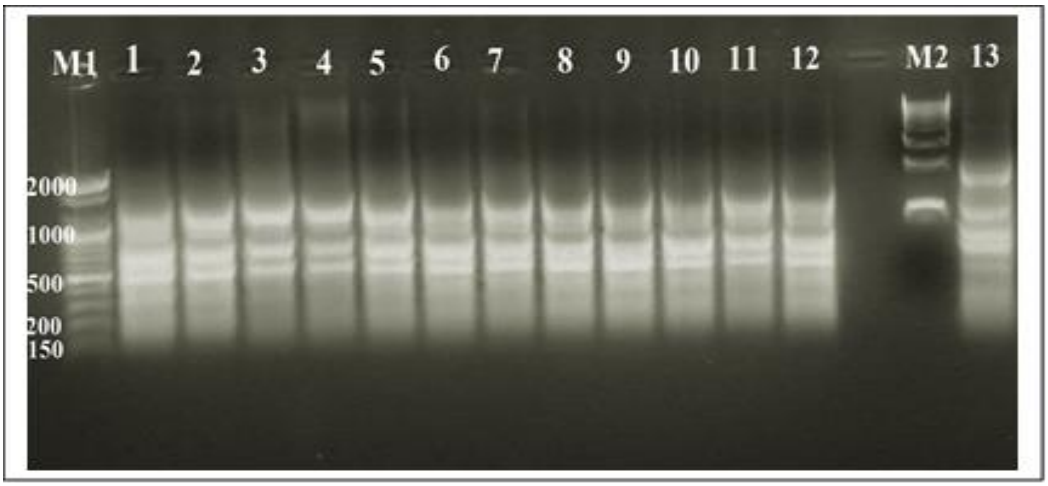


Plate 7: PCR amplification with RAPD primer OPM 05

(M1= 100 bp & M2 = 1 kb DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 = Lalmonirhat local-3, Lane 12 = Rangpur local and Lane13 = Kaptai local).

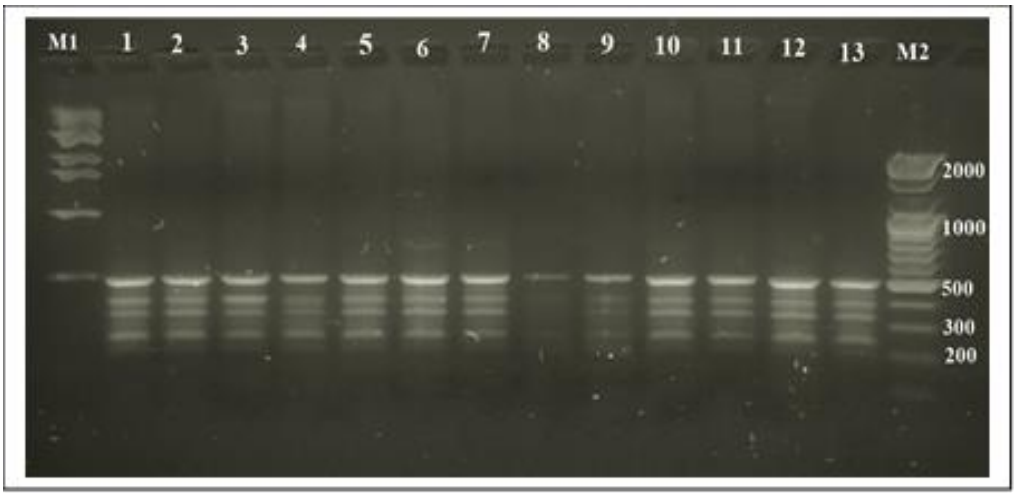


Plate 8: PCR amplification with RAPD primer OPW 03

(M1= 1 kb & M2 = 100 bp DNA ladder, Lane 1 = Kurigram local, Lane 2 = Tangail local, Lane 3 = Jamalpur local, Lane 4 = Modupur local, Lane 5 = B. Baria local, Lane 6 = Rangamati local, Lane 7 = Thakurgoan local-1, Lane 8 = Thakurgoan local-2, Lane 9 = Lalmonirhat local-1, Lane 10 = Lalmonirhat local-2, Lane 11 =

4.4 Allele scoring and diversity analysis

The binary matrix representing different alleles of the seven 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 ginger genotypes

Marker	Observations No.	Major Allele Frequency	Genetic Diversity	PIC Values
OPG 19	13	0.3077	0.8402	0.7176
OPJ 13	13	0.4615	0.8439	0.8496
OPM 05	13	0.2308	0.8402	0.6560
OPM 07	13	0.2308	0.7574	0.8059
OPP 13	13	0.4615	0.8639	0.8245
OPW 03	13	0.2308	0.6982	0.6560
OPX 04	13	0.3077	0.7929	0.7630
Mean		**0.3187**	**0.8052**	**0.7532**

The frequency of the major allele ranged between 0.2308 (OPM 05, OPM 08, OPW 03) to 0.4615 (OPP 13, OPJ 13) with an average value of 0.3187. Polymorphic Information Content (PIC) value for the 7 markers ranged from 0.6560 to 0. 8496 and the average PIC value was 0.7532. The highest PIC value (0.8496) was obtained for OPJ 13 and the lowest PIC value 0.6560 was obtained for OPW 03 and OPM 05. PIC value revealed that OPJ 13 and OPP 13 was considered as the best marker for 13 ginger genotypes. Gene diversity ranged between

0.6982 (OPW 03) to 0.8496 (OPP 13, OPJ 13) with an average of 0.8052. The results indicate that the 13 local ginger genotypes present a high degree of homozygosity and also considerable intra-varietal group diversity, and a certain degree of genetic differentiation and polymorphism.

This study agreed with Mia *et al*. (2014) investigation on 8 Bangladeshi ginger genotypes whereas the highest genetic distance (0.5531) was observed between Indian vs. Syedpuri and the lowest genetic identity (0.0302) was found in China vs. Sherpuri genotypic pair. The lowest genetic distance (0.1525) was observed in several cultivars. Subudhi *et al*., (2016) showed genetic distance range from 0.06-0.69. Kamaruddin and Abdullah. (2017) analyzed two Malaysian ginger variety. The genetic distance of Bentong ginger ranged from 0.69 to 0.32. For Tanjung Sepat ginger, the genetic distance was found 0.64-0.32.

4.5 Population Structure Analysis

Population structures for germplasm were determined using STRUCTURE, version 2.3.4 (Pritchard *et al.,* 2000 and Falush *et al.,* 2003). The number of clusters (K) investigated, in this study, ranged one to nine. The analysis was conducted through the use of five replications for each K value. The model following admixture and correlated allele frequency with a 50000 burn period and a run length of 100000 was used for conducting model based structure analysis. Output of analysis was collected using the STRUCTURE harvester (Earl , 2012) to identify 2 as the best K value based on the LnP (D) and Evanno's ΔK (Evanno *et al.,* 2005). The value of K= 2 means, the total population divided into two sub groups.

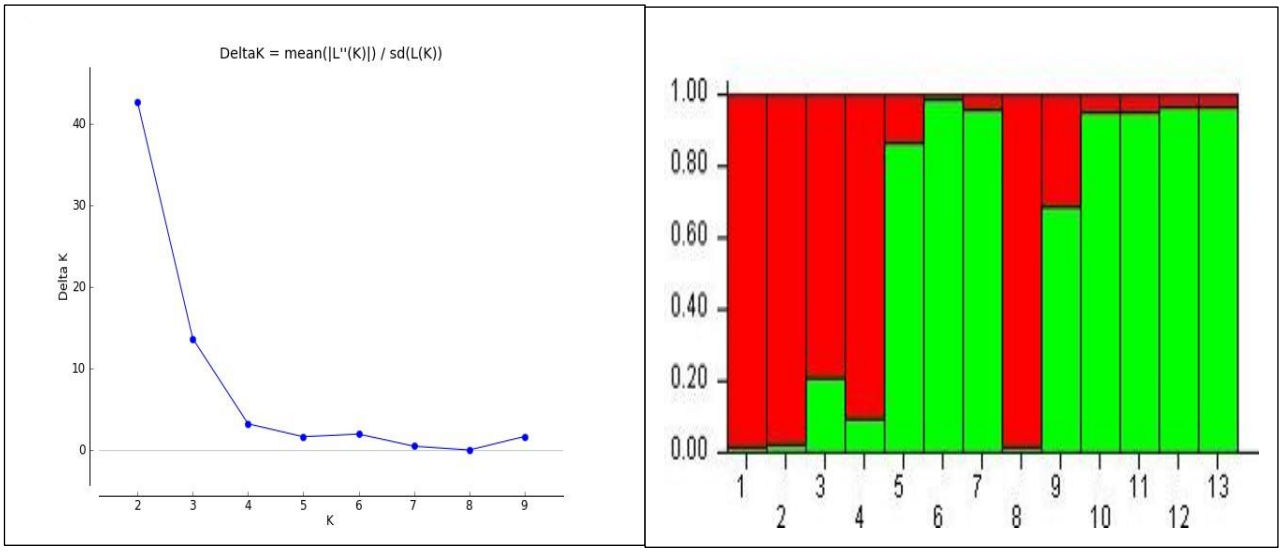


Figure 1: Population structure of 13 local ginger genotypes and 7 RAPD marker (K = 2) and graph of estimated membership fraction for K = 2. The maximum of adhoc measure ΔK determined by structure harvester was found to be K = 2, which indicated that the entire population can be grouped into two subgroups. Different colour within group indicates the proportion of shared ancestry with other group which has the same colour with the admixture.

Bayesian analysis using STRUCTURE characterized the population structure for tested local ginger germplasm. Here only green and only red were pure variety. Both color indicate admixture of character. Kurigram local, Tangail local, Thakurgoan local-2 (red color) and Rangamati local, Thakurgoan local-1, Lalmonirhat local-2, Lalmonirhat local-3, Rangpur local, Kaptai local (green color) showed pure character. The LnP (D) as well Evanno's ΔK values identified two genetically distinct populations (i.e., K = 2; Fig. 1), P_1 (Red colour) and P_2 (green colour) (Fig. 1), representing 40.6%, and 59.4%, of ginger varieties, respectively. Membership fractions were used to classify these populations as pure or an admixture: population P_1 showed three pure (60%) and two admixed (40%) individuals, P_2 showed six pure (75%) and two (25%) admixed individuals.

4.6 Principal Component Analysis (PCA)

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 dataset. 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.

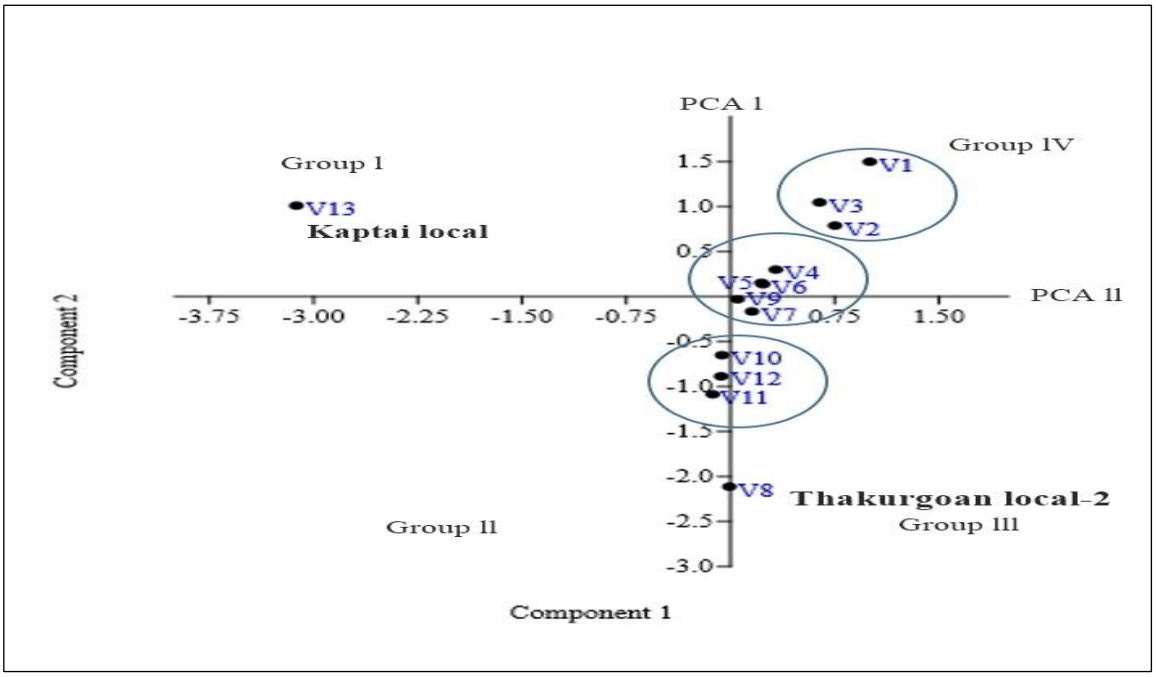


Figure 2: PCA analysis of 13 local ginger genotypes based on 7 markers. PCA 1 and PCA ll represent first and second components, respectively.

Principal components analysis (PCA) analysis was conducted also using the PAST software. All 13 ginger genotypes were classified into four groups and showed in two dimensional scatter plot. All the groups were separated from each other (Fig. 2). Highest distance was showed between Group l and Group lll. Group l represents Kaptai local and Group lll represents Thakurgoan local-2. It indicates that, material selection for ginger improvement program should be taken from this group.

This present study agreed with Subudhi *et al.,* (2016) investigation, where Principle component analysis (PCA) shows two dimensional scatter plot clustering pattern of 48 accessions of *Zingiber officinal*. Another investigation was found in Pradhan *et al*., (2014) analysis where PCA placed four groups in three dimensional scatter plot among five ginger varieties of Sikkim Himalaya. Sajeev *et al.,* (2011) performed a study in which principal component analysis of the molecular data supported grouping of the clones into six hypothetical populations based on

their source or location of collection. PCA grouped the population on the three dimensional scatter plot. The contribution of the first three principal components were 37.98%, 24.88% and 18.00%, respectively, leading to a cumulative contribution of 80.87%.

4.7 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 Nei's (1972) genetic distance between 13 ginger genotypes were computed from combined data through 7 primers, ranging from 0.103 to 0.654. The highest genetic distance 0.654 was observed in Kaptai local vs. Kurigram local varietal pair whereas lowest value was observed in B. Baria local and Modhupur local varietal pair (Table 7).

Several scientists reported similar types of observations in ginger genotypes. Mia *et al*. (2014) studied 8 Bangladeshi ginger genotypes and the highest genetic distance (0.5531) was observed between Indian vs. Syedpuri whereas the lowest genetic identity (0.0302) was found in China vs. Sherpuri genotypic pair. The genetic distance (0.1525) was observed in several cultivars. Pradhan. *et al.,* (2014) observed the genetic similarity coefficients among accessions ranged from (0.35-0.87) using 63 RAPD markers. Pair-wise genetic similarities ranged from 0.21 to 0.39 in all Indian 12 accessions of *Z. officinale* with a mean value of 0.30 was found in Ashraf *et al.,* (2014) assessment.

Table 7: Summary of Nei's genetic similarity and distance indices among the 13 ginger genotype

	Kurigram local	Tangail local	Jamalpur local	Modupur local	B. Baria local	Rangamati local	Thakurgoan local-1	Thakurgoan local-2	Lalmonirhat local-1	Lalmonirhat local-2	Lalmonirhat local-3	Rangpur local	Kaptai Local
Kurigram local	0.000												
Tangail local	0.163	0.000											
Jamalpur local	0.169	0.123	0.000										
Modupur local	0.299	0.229	0.191	0.000									
B. Baria local	0.283	0.225	0.183	0.**103**	0.000								
Rangamati local	0.350	0.273	0.265	0.177	0.167	0.000							
Thakurgoan local-1	0.406	0.303	0.276	0.183	0.191	0.116	0.000						
Thakurgoan local-2	0.411	0.348	0.303	0.221	0.243	0.237	0.210	0.000					
Lalmonirhat local-1	0.441	0.354	0.337	0.250	0.284	0.201	0.191	0.225	0.000				
Lalmonirhat local-2	0.392	0.320	0.313	0.212	0.253	0.164	0.219	0.233	0.196	0.000			
Lalmonirhat local-3	0.402	0.380	0.356	0.250	0.254	0.206	0.258	0.226	0.227	0.139	0.000		
Rangpur local	0.498	0.430	0.407	0.320	0.365	0.316	0.314	0.265	0.252	0.240	0.184	0.000	
Kaptai Local	**0.654**	0.630	0.606	0.505	0.520	0.513	0.553	0.466	0.483	0.434	0.383	0.340	0.000

4.8 Cluster analysis

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 13 genotypes of ginger into two main clusters: A and B. The first major cluster 'A' had only one genotype (Kaptai local) and the second major cluster 'B' had rest of twelve genotypes. The second major cluster was subdivided into two minor clusters (C & D) in which one cluster (D) was subdivided into two minor cluster which included Kurigram local, Tangail local and Jamalpur Local. The other cluster (C) had rest of the nine. This minor cluster was also subdivided into two clusters (E & F). In which cluster (E) is divided into two sub clusters (G and H). Modhpur Local, B. Baria Local, Rangamati Local and Thakurgoan Local-1 were grouped in Cluster (G) and Thakurgoan Local-2 formed in cluster (H). Cluster (I) and (J) were the subdivision of cluster (F). Lalmonirhat Local-1, Lalmonirhat Local-2 and Lalmonirhat Local-3 grouped in cluster (I) and Rangpur Local formed in cluster (J) (Figure 3).

The result indicates that the low or high level genetic distance exists between varieties with their same or different origins. Kaptai local vs. Kurigram local showed highest Nei's genetic distance (0.654) as they are released from different parental origin. On the other hand B.Baria local vs. Modhupur local varietal pair showed lowest genetic distance (0.103) as they are released from same parental origin. This variation can be created by geographical origin. The result also reveals that the genetic base among these ginger 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 13 ginger genotypes.

Cluster analysis on ginger genotypes was also performed by several scientists. The study conducted by Mia *et al*. (2014) on genetic distance by UPGMA dendrogram segregated the Eight Bangladeshi ginger germplasm into two main clusters.

The first major cluster had only one genotype -Indian. Second major cluster had the rest seven genotypes (Wild, Chittagong, Fulbaria, Syedpuri, BARI ada-1, China and Sherpuri). The clusters also divided into sub-clusters and sub-clusters further into groups.

Ashraf *et al.*, (2014) constructed an Unweighted Pair-Group Method of Arithmetic Means (UPGMA) dendrogram from genetic distance and all the 12 accessions of *Z. officinale* from subcontinent of India were grouped into two main clusters (cluster I and cluster II). Sajeev et al., (2011) conducted a study to assess genetic diversity using a set of forty-nine ginger clones cultivated in North-East India using polymorphic RAPD marker. The dendrogram based on UPGMA analysis separated the forty-nine clones into five major clusters.

RAPD and other discontinuous markers can serve as means of genetic distances to establish phylogenetic relationships among taxa (Karihaloo and Gottieb, 1995; Rodriguez et al., 1999; Rabey *et al.*, 2002). Estimation of genetic differences and discrimination of genetic relationship between *Zingiber* spp. are for utilization of plant genetic resources.

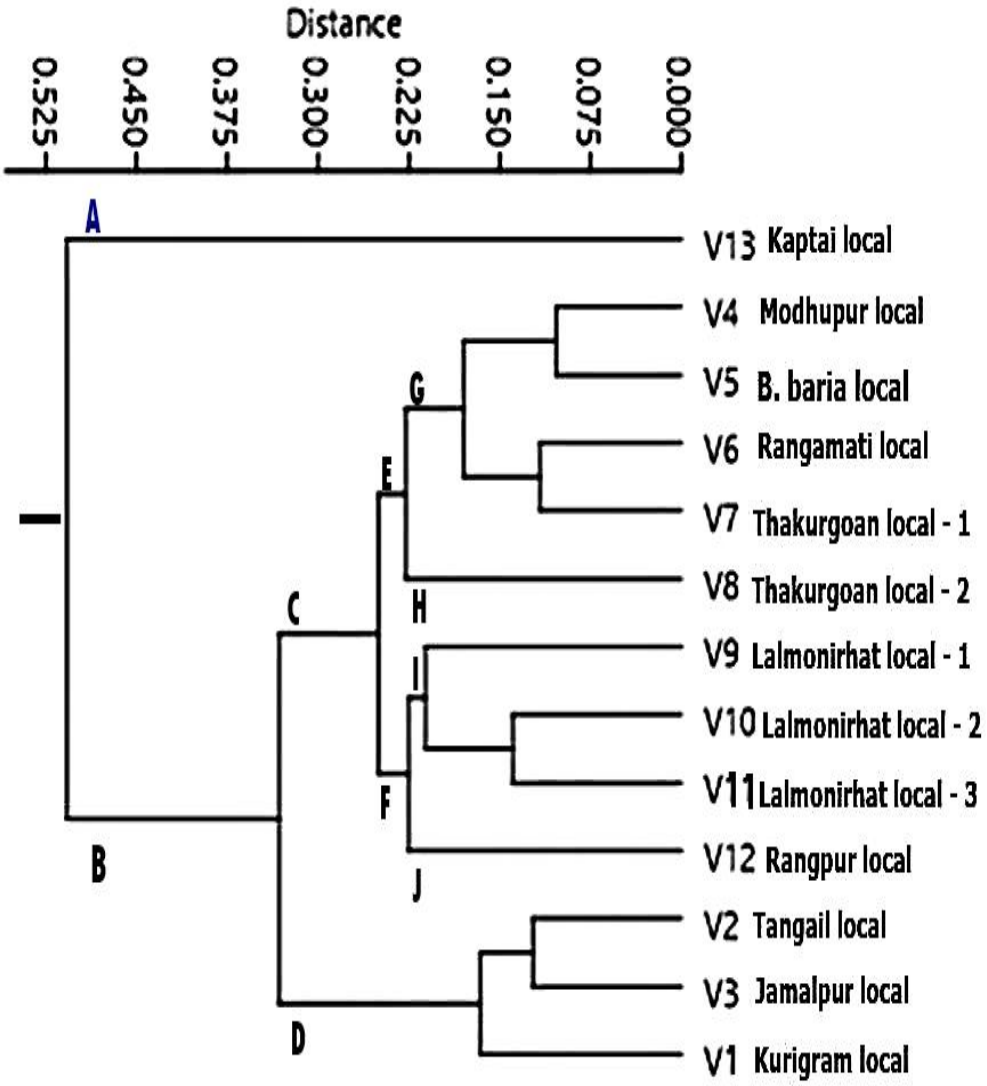
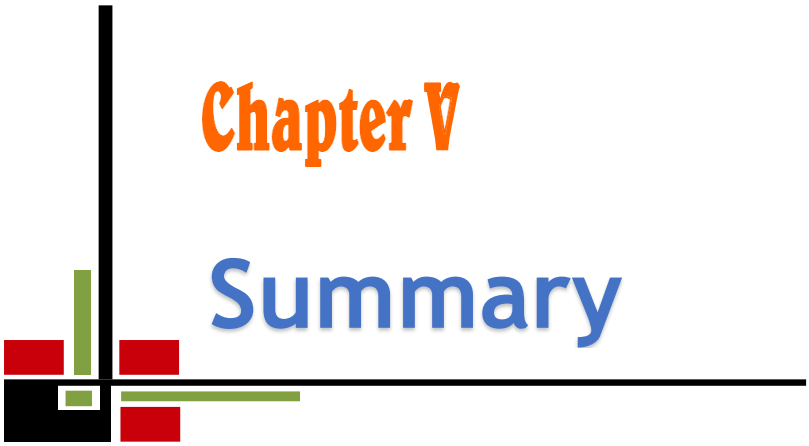


Figure 3: Dendogram of 13 ginger variety was based on RAPD marker, according to the un-weighted pair group mean algorithm (UPGMA) method based on a similarity matrix by PAST software



Chapter V

Summary

CHAPTER V

SUMMARY

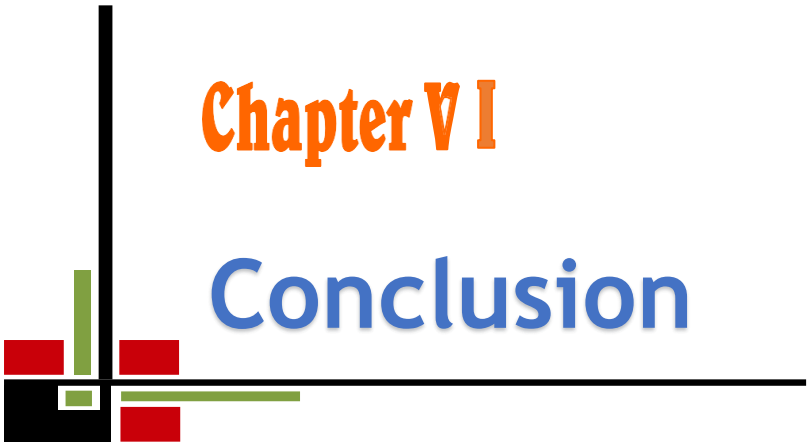
Ginger (*Zingiber officinale*) is an important spice crop in Bangladesh as well as in all over the world for its economical and medicinal values. Being a poorly studied genome, little information is available on the molecular characterization of ginger. This is why, there is a need to conduct studies to evaluate the genetic diversity of ginger for breeding and conservation purposes. This research investigation presented mainly the molecular characterization of 13 local ginger genotypes. The present experiment was conducted at Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207.

Seven RAPD primers generated total 34 distinct and differential amplification bands with an average of 4.9 bands per primer. The highest no. of bands (7) generated by OPJ 13 primer and lowest no. of bands (2) generated by primer OPM 07. The highest percentage of polymorphic bands (50%) was generated by primer OPP 13 and OPM 07 while no polymorphic bands generated by primer OPM 05 and OPW 03.

The frequency of the major allele ranged between 0.2308 to 0.4615 with an average value of 0.3187. Polymorphic Information Content (PIC) value for the 7 markers ranged from 0.6560 to 0.8496 and the average PIC value was 0.7532. The overall gene diversity ranged between 0.6982 (OPW03) to 0. 8639 (OPP 13) with an average of 0.8052. PIC value revealed that OPJ 13 and OPP 13 was considered as the best marker for 13 ginger genotypes followed by OPM 07, OPX 04 and OPG 19 could be considered as the least powerful marker.

In principal component analysis, all 13 ginger genotypes were classified into four groups and showed in two dimensional scatter plot. Highest distance showed between Group l (Kaptai local) and Group lll (Thakurgoan local-2) that means, highest genetic gain will be found when those material used for ginger improvement program. The value of pair-wise comparisons Nei's (1972) genetic distance between 13 ginger varieties were computed from combined data for the 7 primers, range from 0.103 to 0.654. The highest genetics 0.654 was observed in Kaptai local vs. Kurigram local varietal pair whereas lowest value observed in B.Baria local and

Modhupur local varietal pair. Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Mean (UPMGA) segregated the segregation of 13 genotypes into two major clusters (Cluster A and Cluster B). The first major cluster 'A' had only one genotype (Kaptai local) and the second major cluster 'B' had rest of twelve genotypes. Cluster B was further subdivided into another two clusters.



Chapter V I

Conclusion

CHAPTER VI

CONCLUSION

After above discussion, the result indicates that the low level genetic distance exists between varieties with their same or different origins. Kaptai local vs. Kurigram local showed highest Nei's genetic distance (0.654) as they are exit from different parental origin. On the other hand B.Baria local vs. Modhupur local varietal pair showed lowest genetic distance (0.103) as they are cultivated from same parental origin. This variation can be created by geographical origin. The result also reveals that the genetic base among these ginger genotypes is rather narrow. RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management. Being a poorly studied genome, little information is available on the molecular characterization of ginger. The result of the present study can be used as a guideline for future diversity assessment and genetic analysis of ginger genotypes.


Recommendation

RECOMMENDATION

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 are widely acceptable by all concern. Using larger number of samples and higher number of primers could be useful in future study.

The present work was the preliminary study to assess genetic variation of ginger genotypes and it had some limitations in term of limited number of individuals and varieties as well as number of primers used. The results indicate that 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 ginger in Bangladesh:

1. Large number of varieties and higher number of primers should be studied with adequate replications to increase accuracy.
2. Details survey work should be conducted using more molecular markers for obtaining diagnostic loci for ginger varieties.
3. Other molecular markers such as SNP, AFLP, micro-satellite, etc. should be developed for ginger varieties of Bangladesh.

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