Morphological and Molecular Diversity Analysis among Selective Varieties of Pumpkin (*Cucurbita maxima*) and Bottle Gourd (*Lagenaria siceraria*) Using RAPD Markers

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Sibbir Ahmed

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Session # 2011-2012

BIOTECHNOLOGY AND GENETIC ENGINEERING DISCIPLINE
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KHULNA-9208, BANGLADESH

AUGUST, 2014

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DECLARATION

This thesis report is the original research work of the authors, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor.

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DEDICATED

TO

MY BELOVED PARENTS

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The Author

ABSTRACT

An experiment was carried out to study the morphological and yielding performance of four pumpkin and four bottle gourd genotypes at phenotypic level, to determine the relationship and diversity among the individual genotypes at molecular level through RAPD markers during the period from July 2012 to April 2014. Variation was found among the genotypes for first flowering date, first fruiting date, leaf color, leaf size, fruits per plant, fruit weight, 100-seed weight etc. For molecular characterization, in a total 9 DNA bands were scored by using three selected random primers (A17, A21 and A24) of which 7 (77.78%) bands were proved to be polymorphic. The highest gene frequency of polymorphic loci was 0.8750 for A24-3 and A24-5. The highest gene diversity value was 0.5000 for A21-1 and A24-1. The highest genetic diversity among genotypes found in pumpkin Sweety and the lowest found in Bari lau. The average co-efficient of gene differentiation (Gst), gene flow (Nm) and Shannon's Information Index values were 1.0000, 0.0000 and 0.4252, respectively. Based on pair-wise comparison of RAPD amplification products, genetic similarity was estimated using Nie's (1972) genetic distance and a dendrogram was constructed using unweighted pair group method of arithmetic mean (UPGMA). The results of cluster analysis indicated that eight genotypes were classified into two major groups: pumpkin Sweety (Hybrid), Metal (Hybrid), Alamin (Local), Dewan (Local) and bottle gourd Lalima (Local), Higreen (Hybrid) were included in cluster I while were Bari lau (Local) and Martina (Hybrid) genotypes in cluster II. The pumpkin Alamin (Local) was closer to the bottle gourd variety Martina (Hybrid) (0.0000) and the highest genetic distance was found between pumpkin Alamin (local) and bottle gourd Bari lau (Local) and pumpkin Dewan (local) and bottle gourd Bari lau (Local). Each cluster further divided into two sub clusters. This result indicates that the varieties which formed grouped together, they are less diversed. On the other hand the varieties remain in different clusters or different groups are much diversed. Thus RAPD perform a potentially simple, rapid and reliable method to evaluate genetic diversity and molecular characterization as well.

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ABBREVIATIONS AND SYMBOLS

AFLP	Amplified Fragment Length Polymorphism
CTAB	Cetyl trymethyl ammonium bromide
d H_20	Distilled water
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxynucleoside triphosphate set
EDTA	Ethylenediamine tetraacetic acid
Gel Doc	Gel documentation system
GD	Genetic Distance
GS	Genetic Similarity
GT	Gelatinization temperature
Kb	Kilo base
MAS	Marker assisted selection
mM	Milimole
ml	Mililiter
mg	Miligram
ng	Nanogram
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
PVP	Polyvinyl Pyrilidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
r p m	Rotation per minute
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeat
Taq	Thermophilus aquaticus
TAE	Tris-acid EDTA buffer
TBE	Tris Boric EDTA
TE	Tris EDTA buffer
UPGMA	Unweighted Pair Group Method of Arithmetic Means
µg	Microgram
µl	Microliter

CHAPTER ONE

INTRODUCTION

Pumpkin (*Cucurbita maxima* L) and Bottle Gourd (*Lagenaria siceraria* L) are very familiar vegetables in Bangladesh. Both vegetables are nutritionally and economically important. These are belonged to *Cucurbitaceae* family and *Cucurbitoideae* sub-family (Bates and Robinson, 1995). Pumpkin is originated in North America (Wilson and Doebley, 1992). Also, it is cultivated in Bangladesh, India, Myanmar and other southern Asian countries. Bottle gourd may have been carried from Africa to Asia, Europe and the America in the course of human migration (Erickson *et al.*, 2005). Both vegetables are economically important and consumed as patient food, cooking, boiled, dessert, decoration purposes. Also, these are used as medicine for many diseases such as diarrhea, diabetes, fatigue, hypertension etc (Facciola, 1998). Different pumpkin and bottle gourd varieties have already developed and still trying for further development. In Bangladesh pumpkin and bottle gourd are very popular vegetables. Those are cultivated in different area in Bangladesh at large amount. In 2006-2007 sessions, total cultivation area of pumpkin is 54475 acres and the production is 181435 metric tons (summer and winter). Also, the cultivation area of bottle gourd is 31910 acres and the production is 117120 metric tons. In 2007-2008 sessions, cultivation area of pumpkin is 58340 acres and the production is 189734 metric tons. Also, the cultivation area of bottle gourd is 33021 acres and the production is 125949 metric tons (source: BBS, 2009).

Pumpkin and bottle gourd are helpful to strong our immune system. Both are very rich in carotenoids, which is known for keeping the immune system of an individual strong and healthy. Beta-carotene is found in pumpkin, is a powerful antioxidant as well as an anti-inflammatory agent (Facciola, 1998). It helps prevent build up of cholesterol on the arterial walls, thus reducing chances of strokes. Pumpkins have been known to reduce the risk of a serious eye problem than usually results in blindness. Also, the juice of bottle gourd is a valuable medicine for excessive thirst due to severe diarrhea, diabetes and excessive use of fatty or fried food. The gourd fruit juice is used in the treatment of insanity, epilepsy and other nervous diseases. Bottle gourd is not only rich in essential minerals, iron, protein and trace elements, it is also rich in fiber (Rahman, 2003).

To increase the varieties of pumpkin and bottle gourd utility for breeder and farmers, morphological and molecular characterization is needed. Yield of pumpkin and bottle gourd is correlated with primary yield components and also with other morphological and physiological characters. Therefore, the relationship between yield and different yield contributing characters establishes a complex chain of network and can be further analyzed in more simple way through path coefficient analysis. Therefore, attempts must be made to increase the yield per unit area by applying improved technology and management practices (Afzal *et al.*, 2004).

Characterization and cataloguing of germplasm have been traditionally carried out by using morpho-agronomic traits, while in the past two decades or so the molecular markers have also been used for germplasm characterization (Das *et al.*, 2014). Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at genetic level. To determine whether or not a plant species is closely related to another, it is necessary to specify the criteria for evaluating interrelationships between varieties belonging to different species (Weir, 1990).

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

RAPD analysis is a PCR based molecular marker technique. This technique use a single short oligonucleotide primer (9-12 bp) of arbitrary DNA sequence and polymerase chain reaction (PCR) mediated amplification of random fragment from genomic DNA. So, among the available DNA molecular techniques, RAPD has many advantages over others such as ease and rapidity of analysis, a relatively low cost, availability of large numbers of primers and the requirement of a very small amount of DNA for analysis (Weising *et al.*, 1995).

RAPD technique can be used to determine the taxonomic identity, assess kinships, detect inter-specific gene flow, analyze hybrid speciation, and create specific probes. The RAPD technology has provided a quick and efficient screen for DNA sequence base polymorphism at a very large number of loci. The major advantages are that no prior DNA sequence is required (Afzal *et al.*, 2004). Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species, and replication to ensure that only the reproducible bands are scored. RAPD is favorite marker technique in mapping, the determination of phylogenetic relationships, genetic diversity, and identification of cultivars and parents in a number of plant species (Xu *et al.*, 2000). Knowledge of the genetic relationships among varieties is useful to gene bank because it permits a better organization of the crops gene pool management, more efficient sampling of the available genotypes resources and better access to useful genetic variation for breeders.

Thus this study was conducted with the following objectives:

a) To study morphological characters and yielding performance in pumpkin and bottle gourd genotypes.

b) To study the genetic diversity among studied genotypes using RAPD markers.

c) To determine the genetic relationship between two species for the development of distant hybrid.

CHAPTER TWO

REVIEW OF LITERATURE

The *Cucurbitaceae* is an important plant family consisting of various squashes, melons, and gourds, including crops such as cucumbers, pumpkins, luffas, bottle gourd and watermelons. They show genetic diversity. It is determined by using molecular technique like RAPD, RFLP, AFLP etc.

2.1. Pumpkin

A pumpkin is a gourd-like squash of the genus *Cucurbita* and the family *Cucurbitaceae*. They typically have a thick, orange or yellow shell, creased from the stem to the bottom, containing the seeds and pulp. Pumpkins are widely grown for commercial use and are used both in food and recreation (Bates and Robinson, 1995).

Pumpkins are monoecious, having both male and female flowers on the same plant. The female flower is distinguished by the small ovary at the base of the petals. These bright and colorful flowers have extremely short life spans and may only open for as short a time as one day. The color of pumpkins is derived from the orange pigments abundant in them. The main nutrients are lutein and both alpha and beta carotene, the latter of which generates vitamin A in the body (Susan and Arnum, 1998).

2.1.1. Etymology and Origin

The word pumpkin originates from the word *pepon*, which is Greek for "large melon". The French adapted this word to *pompon*, which the British changed to *pumpion* and later American colonists changed that to the word we use today, "pumpkin". The origin of pumpkins is not definitively known, although they are thought to have originated in North America. The oldest evidence, pumpkin-related seeds dating between 7000 and 5500 BC, were found in Mexico (Michae *et al.*, 2000).

2.1.2. Cultivation

Cultivation of pumpkin and squash has a long history and it can be said that these cucurbits are associated with the origins of agriculture. The genus *Cucurbita* is native to the America. There is convincing evidence from archaeological sites in Central and South America that *C. pepo*, *C. maxima* and *C. moschata* were widely cultivated in pre Colombian times(Whitaker and Roinson, 1986).

2.1.3. Health Benefits of Pumpkin

Pumpkin is very rich in carotenoids, which is known for keeping the immune system of an individual strong and healthy. Beta-carotene, found in pumpkin, is a powerful antioxidant as well as an anti- inflammatory agent. It helps prevent build up of cholesterol on the arterial walls, thus reducing chances of strokes (Yadav *et al.*, 2010).

Pumpkins have been known to reduce the risk of macular degeneration, a serious eye problem than usually results in blindness. The high amount of fiber, present in a pumpkin, is good for the bowel health of an individual. Being rich in alpha-carotene, pumpkin is believed to slow the process of aging and also prevent cataract formation (Susan and Arnum, 1998).

The presence of zinc in pumpkins boosts the immune system and also improves the bone density. Being loaded with potassium, pumpkin is associated with lowering the risk of hypertension (Rayburn *et al.*, 1989; Tatum *et al.*, 2006).

2.1.4. Morphology of Pumpkin

The pumpkin varies greatly in shape, ranging from oblate through oblong. The rind is smooth and usually lightly ribbed. Generally the weight of pumpkin is 3-4 kg but *C. maxima* capable of reaching a weight of over 34kg. Pumpkins are usually orange or yellow, but some fruits are dark green, pale green, white, red etc. The color of pumpkins is derived from the orange pigments abundant in them (Bates and Robinson, 1995).

Pumpkins are very versatile in their uses for cooking and have an advantage over other vegetables as the fruit can be stored for up to 6 months before being consumed and hence can play an important role in maintaining nutritional levels during the long dry seasons (Mendlinger *et al.* 1991).

Fruits of *C. maxima* are medium to medium large. The smaller types have hard and durable shells. The leaves are rounded with irregular lobes. The flowers have a broad corolla. Seeds average 5-8 inch in length and 3-5 inch wide. Skin is regular texture, grey color and variable ribs, fruits shape oval, rounded.

2.1.5. Genetic Diversity of Pumpkin

Genetic diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will possess variations of alleles that are suited for the environment. Those individuals are more likely to survive to produce offspring bearing that allele. The population will continue for more generations because of the success of these individuals (Decker *et al.*, 2001). Changes in cellular parameters that are brought about by variations in genome size are referred to as nucleotypic effects (Bennett, 1972; Rayburn, 1993b). Nucleotypic effects have been observed in various plant species (Rayburn *et al.*, 1989).

Over the past several years, concerns have been raised over the methods used to determine intraspecific genome size variation (Greilhuber, 2005). These concerns have led to doubts of the existence of intraspecific genome size variation and the amount of such variation. With the exception of maize (Rayburn *et al.*, 1989), most reports of high genome size variation have not stood up under closer scrutiny (Greilhuber, 2005). Although it is interesting that the *C. maxima* cultivars have a smaller genome size than most of the *C. pepo* cultivars, this fact sheds no light on existence of intraspecific variation because any differences observed would represent interspecific variation. The miniature fruit types, i.e., Jack-B-Little, that found to have small genome sizes have the smallest fruit type of the pumpkins Plant and fruit size have been hypothesized to correlate with altered genome sizes in plant species (Rayburn *et al.*, 1989; Tatum *et al.*, 2006).

2.2. Bottle Gourd

Bottle gourd (*Lagenaria siceraria*) is a vine grown for its fruit, which can either be harvested young and used as a vegetable or harvested mature, dried, and used as a bottle, utensil or pipe. For this reason, the calabash is widely known as the bottle gourd. It belongs to the gourd family Cucurbitaceae the fresh fruit has a light green smooth skin and a white flesh. Rounder varieties are called calabash gourds. They come in a variety of shapes, they can be huge and rounded, or small and bottle shaped or slim and more than a meter long. The calabash was one of the first cultivated plants in the world, grown not primarily for food, but for use as a water container. The bottle gourd may have been carried from Africa to Asia, Europe and the Americas in the course of human migration (Erickson *et al.*, 2005).

2.2.1. Etymology and Origin of Bottle Gourd

The word comes from the Spanish *calabaza*, possibly from Arabic *qar'a yabisa* "dry gourd", from Persian *kharabuz*, used of various large melons; or from a pre-Roman Iberian *calapaccia*. It is a commonly cultivated plant in tropical and subtropical areas of the world, now believed by some to have spread or originated from wild populations in southern Africa. Stands of *Lagenaria siceraria* that may be source plants, and not merely domesticated stands run wild, were reported recently in Zimbabwe (Adhyaru and Priya, 2010).

2.2.2. Cultivation

Calabash had been cultivated in Asia, Europe and the Americas for thousands of years before Columbus's discovery of America.Recent research indicates some can have an African origin and at least two unrelated domestications: one 8–9 thousand years ago, based on the analysis of archeological samples found in Asia, a second, four thousand years ago, traced from archeological discoveries in Egypt (Walahfrid and Gabathuler, 2000).

Genetic research on archeological samples published by the National Academy of Sciences in December 2005 suggests calabash may have been domesticated earlier than food crops and livestock, and like dogs, were brought into the New World at the end of the ice age by Paleo-Indians. It is supposed that bottle gourds were carried by people in boats or on foot across the

then existing land bridge between Asia and America. Once in Florida and Mexico, bottle gourd seeds could still be viable after long periods of migration (White and Nancy, 2005).

2.2.3. Health Benefits of Bottle Gourd

Bottle gourds are one of the favorite vegetables of Bangladesh and others countries. It has numerous health benefits, especially well for old aged peoples. The juice of bottle gourd is a valuable medicine for excessive thirst due to severe diarrhea, diabetes and excessive use of fatty or fried food. The Gourd fruit juice is used in the treatment of insanity, epilepsy and other nervous diseases. The juice of the fruit is used in the treatment of stomach acidity, indigestion and ulcers (Davies and Stewart, 1990).

Bottle gourd is not only rich in essential minerals, iron, protein and trace elements; it is also rich in fiber. Fiber helps in preventing constipation and other digestive disorders like flatulence and piles. It is very valuable in urinary disorders. It should be given once daily in the treatment of burning sensation in urinary passage due to high acidity of urine. It helps in overcoming jaundice (Davies and Stewart, 1990). Bottle gourd juice is also helpful in losing weight. Cooked Bottle gourd is anti-bilious. It prevents excessive loss of sodium, quenches thirst and helps in preventing fatigue. Its juice is helpful to curb hunger pangs in starvation condition. It is also effective in treating cardiac ailments, alleviating bronchitis, cough, asthma and biligenic ailments (Mendlinger *et al.*, 1991).

2.2.4. Morphology of Bottle Gourd

Bottle gourd is characterized by different shaped fruits that can be used as decorative ornaments, whilst younger juicy fruits are edible and nutritious (Berenji, 1992). It has significant presence in rural life, primarily in household and agricultural uses. More recently, it is used as a vegetable and in art as a decorative ornament (Sivaraj and Pandravada, 2005). Practical consideration bottle gourds have shown that large seeds have better germination capacity and vigor, and will produce more competitive seedlings than smaller seeds, hence high seed quality (Camelia, 2011).

Fruit size varies from 5 to 40 cm wide, and 20 to 90 cm long. Seed forms also differ according to shape, size, presence or absence of frills and seed lines, and seed coat surface texture. The large genetic variability in bottle gourd is a much desirable trait as it also reflects on its wide adaptation it possesses (Sivaraj and Pandravada, 2005).

Seed quality as the standard of excellence in certain characteristics that will determine seed performance when the seed is either stored or sown. Also, it is the physiological (seed germination ability and seed vigor) and genetic quality (Geus *et al.*, 2008). Seed size is considered as one of the least plastic traits of seed morphology and its heritability varies for different species. For example, the heritability in Medicago sativa was 0.14, while variation in seed size and quality between and within plant species, within plants for inflorescences produced at different growth stages, and for seeds developing at different positions within the fruit (Vaughton and Ramsey, 1997)

2.2.5. Genetic Diversity of Bottle Gourd

Genetic diversity can be identifying at all levels of organization in the nucleus, including the amount of DNA per cell, chromosome number and DNA structure (Tasrif *et al.*, 2004).

In spite of the relatively small genome size of bottle gourd, there are only dozens of bottle gourd DNA sequences available in the public DNA database, making it unfeasible to identify bottle gourd genes or to analyze their functions. A limited number of anonymous random amplified polymorphic DNA (RAPD) markers have been described but there has been no locus specific DNA markers such as microsatellite (SSR), sequence tagged site (STS) or single nucleotide polymorphism (SNP) markers available for bottle gourd so far (Decker *et al.*,2001).

The advent of molecular biology techniques has provided another dimension to detect genetic polymorphism based on protein or DNA facilitating in biological research branches such as phylogenetic relationships, taxonomy and genetic diversity (Williams *et al.*, 1990). In this regards, proteins and enzymes polymorphism have been success fully used to identify cultivars in various fruit and crop species, including Bottle gourd (Upadhyay and Ram, 2006).

Characterization of genetic resources is regarded as an important activity for gene banks. It involves determining the expression of highly heritable characters, ranging from morphological features to seed proteins and possibly including molecular markers. Such characters enable easy and quick discrimination among phenotypes and allow simple grouping of the accessions (Engels and Visser, 2003).

In addition to molecular markers, scoring of such characters allows establishment of systematic relationship among accessions and even crops including their evolutionary relationships. This directly facilitates utilization of collections and allows detection of misidentification (Bretting and Widrlechner, 1995).

2.3. DNA Extraction

The isolation and purification of DNA from cells is one of the most common procedures in molecular biology and embodies a transition from cell biology to molecular biology. DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods (Dahm, 2008)

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light (http://en.wikipedia.org/wiki/DNA_extraction, 10.05.2014).

2.4. Molecular markers

A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily monitor. In fact, a piece of DNA or a protein can be used as a marker. However, DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Molecular marker plays a great role to define the distinctiveness of species and their ranking according to the number of close relative and their phylogenic position. A molecular marker very closely linked to the target gene can act as a "tag" which can be used for indirect selection of the gene(s) in a breeding programmed.

DNA-based molecular markers have acted as resourceful tools in various fields like taxonomy, physiology, embryology, plant breeding, ecology, genetic engineering etc. The innovation of polymerase chain reaction was a milestone in the development of DNA markers. This facilitated the development of marker based gene tags, cloning of agronomically important genes, variability studies, phylogenetic analysis, selection of wanted genotypes, etc. Thus DNA markers offer several advantages over conventional phenotypic markers, as they provide data that can be analyzed objectively (Sarma and Bahar, 2006).

The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. No molecular markers are available yet that fulfill all requirements needed by researchers (Semagn *et al.*, 2006).

Marker assisted selection (MAS), which is widely applied to rice genomics because MAS effectively identifies the relationship between phenotype and genotype. Subsequently, a number of rice genome projects were launched, including linkage map construction, genomic library production and full-length cDNA analyses (Ashikari and Matsuoka, 2006).

There are two types of molecular markers:

1. Non-PCR based markers, which involves RFLP, minisatellites, moderately repeated dispersed and highly variable DNA.

2. PCR based markers which involves AFLP, ALP, RAPD, DNA amplification (finger printing), SSR (microsatellite) and SSCP (Single Strand Conformation Polymorphism). Single nucleotide polymorphisms (SNP) are likely to become the next choice of markers.

A wide variety of DNA-based markers have been developed in the past few years. There is large amount of DNA variation present in natural populations and the DNA-based markers offer an opportunity to utilize this variation in modern genetics.

The DNA-based marker techniques are listed below:

- Restriction Fragment Length Polymorphism (RFLP)
- Simple Sequence Repeats (SSR)
- Random Amplified Polymorphic DNA (RAPD)
- Amplified Fragment Length Polymorphism (AFLP)
- Sequenced Characterized Amplified Region (SCAR)
- Sequence Tagged Sites (STS)
- Variable Number Tandem Repeats (VNTRs)
- Single Nucleotide Polymorphism (SNP)

2.4.1. RAPD markers

Random Amplified Polymorphic DNA (RAPD) analysis is one of the novel techniques added to the repertoire of the molecular geneticists. The RAPD technique has provided a relatively simple and inexpensive method for examining variation in the total genome (Hadrys *et al.*, 1992).

RAPD analysis is advantageous over isozyme electrophoresis because it generates much greater numbers of loci required for genetic analyses (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,taxonomy (Chapco *et al.*, 1992) as well as for genetic diagnostics. The standard RAPD technology (Williams *et al.*, 1993) utilizes short synthetic oligonucleotides of random sequence as primers to amplify nanogram amounts of total genomic DNA under low annealing temperature by PCR.

2.4.2. RAPD markers in genetic diversity analysis

The variability among different genotypes of a species is known as genetic diversity. Genetic diversity plays an important role in plant breeding and is a key component of any agricultural production system. This genetic diversity or similarity may be measured through genetic markers. These have been used to determine evolutionary relationship within and between species, genera or higher taxonomic categories (Bonilla *et al.*, 2002).

Genetic diversity is commonly measured by genetic distance (GD) or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Weir, 1990).

Published applications of molecular marker based GD in plant breeding have been limited primarily to pumpkin but results should apply to other species. Molecular marker based GD also has potential for assessing changes in genetic diversity over time protection of intellectual property rights registration of germplasm in countries having ratified the rules of the UPOV convention, and evaluation of new sources of germplasm for their potential to increase genetic diversity (Smith and Smith ,1992). Genetic distance–similarity between two genotypes, populations, or individuals may be calculated by various statistical measures depending on the data set. Discus Clustered on various distance measures are available in the associated literature (Nei, 1987; Weir, 1990). Euclidean or straight-line measure of distance is the most commonly used statistic for estimating genetic distance (GD) between individuals (genotypes or populations) by morphological data.

2.5. Polymerase chain reaction (PCR)

PCR is a technique that allows the selective amplification of any fragment of DNA, provided the DNA sequences flanking the fragment are known. The PCR process relies on the sequence of 'base-pairs' along the length of the two strands that make the complete DNA molecule. The strands or polymers that comprise the DNA molecule are held to each other by Hydrogen bonds between the base pairs.

Fragments of genomic DNA are suitable as genetic markers can be produced by PCR amplification. In the PCR, direct approach can be detected length polymorphism less often will conventional RFLP analysis because a smaller portion of genome was surveyed. The maximum PCR products can be about 5 kb and even smaller fragments of 1 to 2 kb are more efficiently amplified (Kochert, 1994).

Polymerase Chain Reaction (PCR) is an *in vitro* method for producing the large amounts of a specific fragment of DNA necessary for analysis. Basically a reaction that Xeroxes DNA making millions of exact copies of the same fragment. The strands or polymers that comprise the DNA molecule are held to each other by hydrogen bonds between the base pairs. The PCR process relies on the sequence of base pairs along the length of the two strands that make the complete DNA molecule. The purpose of a PCR is to make a huge number of copies of gene. This is necessary to have enough starting template for sequencing. There are three major steps in a PCR which are reported for 30-40 cycles i.e.(i) denaturation at 94°C (ii) annealing at ~54°C and (iii) extension at ~72°C. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time. PCR is a procedure of enzymatic amplification of specific segments of DNA using specific primers. PCR is a method of amplifying DNA of any organism using two specific oligonucleotide primers of about 15 bases pair length, which flank the region of interest. The method was developed by Cetus Corporation, Emeryvilled, California in the mid-1980's and is the extreme value in diagnostic, forensic and general molecular biology (IRRI, 2010). The PCR analysis is a simple, quick and efficient way to screen DNA sequence-based polymorphism in a large number of loci (Kwon *et al.*, 2002).

The versatility of the PCR is that any kind of primers can be chosen, depending on the purpose of the study. On the one extreme, a particular DNA sequence can be amplified by pair of specific primers, the sequences of which are designed based on sequence information. Such a strategy is useful for gene isolation or for the analysis of transferred genes in transgenic organisms. Specific primers based on unique sequences that flank simple tandem repeat (microsatellite) are also increasingly used for mapping purposes and population genetics.

A basic PCR set up requires several components and reagents. These components include DNA template that contains that DNA region (target) to be amplified; one or more primers, which are complementary to the DNA regions at the 5' (five prime) and 3'(prime) ends of the DNA region, a DNA polymerase such as Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C, Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerases synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase, divalent cations, magnesium or manganese ions; generally Mg^{2+} is used , but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.

RAPD analysis of genomic DNA was performed in 10µl reaction containing around 2-3 µl of DNA template, 1.6 µl 10X PCR buffer (containing 200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 µl of 1 mM dNTPs, 0.5-0.7 µl of 5 µM primer, around 0.1-0.5 µl of Taq DNA polymerase(Chen *et al,* 1987).

2.6. Gel Electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge.It is used in clinical chemistry to separate proteins by charge and/or size (IEF agarose, essentially size independent) and in biochemistry and molecular biology to separate a mixed population of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge (Kryndushkin, 2003).

In the case of nucleic acids, the direction of migration, from negative to positive electrodes, is due to the naturally occurring negative charge carried by their sugar-phosphate backbone (Lodish, 2004).

Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications (Schagger, 2006).

2.7. Dendrogram

A dendrogram is a tree diagram frequently used to illustrate the arrangement of the clusters produced by hierarchical clustering. Dendrograms are often used in computational biology to illustrate the clustering of genes or samples (http://en.wikipedia.org/wiki/Dendrogram).

Similarity dendrograms based on morphological traits and DNA markers revealed 2 groups, one of them, with 70% similarity, included all accessions that had the term 'Lageado' in its common name. A dendrogram based on DNA markers further splitted the second group into 2 groups with different protein contents. The results indicated that, in some cases, different accessions may actually represent the same genotype (Areias *et al.* 2006).

One hundred eleven glutinous rice evaluated for their genetic diversity based on Random Amplified Polymorphic DNA (RAPD) analysis, which were collected from 7 countries. Fifteen primers generated 117 bands and 81 bands (69.2%) showed polymorphism. The number of amplified bands per primer ranged from 5 to 11, with an average number of bands of 7.8. With a similarity value of 0.78 in the dendrogram derived from the cluster analysis based on RAPD (Kim *et al.*, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Place of Study

The study was conducted in the experimental field and Plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna.

3.2. Soil

The soil of the experimental site was medium high land and it was highly fertile.

3.3. Experimental Materials

Total eight genotypes (four pumpkins and four bottle gourds) were used for the experiments. The genotypes of pumpkin were Sweety (hybrid), Alamin (local), Metal (hybrid) and Dewan (local). The genotypes of bottle gourd were Bari lau (local), Higreen (hybrid), Martina (hybrid) and Lalima (local).

Table 1. List of genotypes of pumpkin and bottle gourd, their origin and characteristics

Serial No	Name	Origin	Characteristics
1	Sweety	Bangladesh	Hybrid
2	Alamin	Bangladesh	Local
3	Metal	Bangladesh	Local
4	Dewan	Bangladesh	Hybrid
5	Bari lau	Bangladesh	Local
6	Higreen	Bangladesh	Hybrid
7	Lalima	Bangladesh	Local
8	Martina	Bangladesh	Hybrid

3.4. Field Experiment (Data Collection)

i. First flowering date

First flowering date was counted from the date of sowing to the date of first flowering for each variety.

ii. First fruiting date

First fruiting date was counted from the date of sowing to the date of first fruiting date for each variety.

iii. Leaf color

The leaf color was observed when plants were matured.

iv. Leaf size

Leaf size was measured by simple scale when plants were matured.

v. Average fruits per plants

Average fruits per plant were counted manually

vi. Average weight of fruits

Average weight of fruits was measured by weight machine when fruits were matured.

vii. Average fruit length and circumferences

Average fruit length and circumferences were measured by measuring tape.

viii. Fruits shape and color observation

Fruits shape and color were collected by intensive observation.

ix. 100-seed weight (g)

One hundred clean sun dried seeds were counted from the seed stock obtained from the sample plants and weighed by using electronic balance.

3.5. Genotyping of Pumpkin and Bottle Gourd Genotypes

3.5.1. Collection of leaf sample

To extract genomic DNA, young, actively growing fresh leaves were collected. Young leaves from each variety were cut apart with sterilized scissors and washed in distilled water and dried on fresh tissue paper.

3.5.2. Reagents preparation for DNA extraction (Stock solution)

Isolation Buffer (100 ml)

- 10 ml of 0.1M Tris (p^H 8.0) was taken in a measuring cylinder.
- 6.38 gm of 0.35M sorbitol was added to it.
- 10 gm of 10% polyethylene glycol was taken in the measuring cylinder.
- 1 gm o 1% polyvinyl pyrilidone (PVP) was added.
- 0.5 ml merceptoethanol was added.
- Finally sterilized distilled water (dH_20) was added to make the volume upto 100 ml
- The mixture was then autoclaved

Lysis Buffer (100 ml)

- 10 ml of 0.1M Tris (p^H 8.0) was taken in a measuring cylinder.
- 6.38 gm of 0.35M sorbitol was added to it.
- Finally sterilized distilled water (dH_20) was added to make the volume upto 100 ml
- The mixture was then autoclaved

CTAB Buffer (100 ml)

- 2 gm of CTAB was added in 40 ml of distilled water.
- 10 ml of 0.1M Tris (p^H 8.0) was added.
- 4 ml of .02 M EDTA was added.
- 8.2 gm of 1.4 M NaCl was added to it.
- The mixture was then autoclaved

1M Tris P^H 8.0 (100 ml)

- 12.11 g Tris base was added in 60 ml dH_2O and mixed well.
- By adding HCl p^H was adjusted to 8.0.
- Then distilled water (dH_2O) was added to make the volume 100 ml.
- Finally the solution was autoclaved.

TE (100 ml)

- 10 ml 1M Tris (p^H 8.0) was taken in a volumetric flask.
- 0.2 ml EDTA p^H 8.0 was added in it.
- Finally distilled water (dH_2O) was added to make volume upto 100 ml.
- The mixture was then autoclaved.

O.5M EDTA p^H 8.0 (250 ml)

- 9.306 gm EDTA was added to 40 ml dH_2O.
- p^H was adjusted to 8.0 with NaOH.
- Distilled water (dH_2O) was added to make the volume 100 ml.
- The solution was autoclaved.

Chloroform-Isoamyl alcohol (24:1) (100 ml)

- Four milliliter of Isoamyl alcohol was added in 96 ml Chloroform.

70% Ethanol (500 ml)

- 150 ml distilled water (dH_2O) was added in 350m1 absolute ethanol.

50X TAE (100 ml)

- 12.114 gm Tris HCl was taken in 40 ml dH_2O.
- Then the mixture was stirred for 30 minutes
- After stirring 2.186 ml glacial acetic acid was added.
- After that 50 ml 0.5 M EDTA (p^H 8.0) was added.
- Finally distilled water (dH_2O) was added to make the volume upto 100 ml.
- Then the solution was autoclaved.

1X TAE (1000m1)

- 20 ml 50X TAE was added in 980 ml dH_2O.

3.5.3. Extraction of DNA

DNA extraction was done by using the Modified CTAB method. Specific samples were used for each genotype. The following steps were followed for DNA extraction:

- Collected leaf samples were weighted (0.1g) and washed with distilled water and water was soaked by tissue paper. Leaves were ground into fine paste in the presence of glass beads with 2000 µl of isolation buffer using ice cold morter and pistel.
- The paste was transferred into 2ml micro eppendorf tube.
- The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C.
- Supernatant was removed and pellet was suspended into 500 µl of lysis buffer. The mixture was vortexed and incubated at room temperature for 30 minutes.
- An equal volume of pre-heated CTAB Buffer was added, gently mixed and incubated at 65°C for 30 minutes.
- Then Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed.

- The samples were centrifuged at 10,000 rpm at 4°C, for 10 minutes; after centrifugation, the aqueous phase was collected into a fresh micro centrifuge tube. 800 µl of 2-isopropanol was added and mixed gently.
- The samples were centrifuged at 10,000 rpm at 4°C, for 10 minutes
- Supernatant was removed and pellet was air dried. The pellet was then washed with 500µl of 70% ethanol by centrifugation at 10,000 rpm for 10 minutes at 4°c.
- The Supernatant was discarded, pellet was air dried and dissolved in 50µl of TE buffer, 2µl of RNase A solution was added and incubated at 37°C for 30 minutes. Finally, it was kept in freeze at -20°C for further use.

Precaution

- Glassware, micropipette tips, centrifuge tubes, glass pipettes, distilled water and buffer solutions were properly autoclaved to keep away from DNAase contamination.

3.5.4. DNA confirmation using gel electrophoresis

Preparation of 1 % Agarose gel (50 ml)

- 0.5 gm of agarose was added to 50 ml of distilled water.
- 500 µl 50X TAE buffer was taken in a flask and mixed well by shaking.
- The mixture was then kept in microwave oven cooked for 1 min. to dissolve agarose.
- The gel was kept in room temperature for 2-5 min to cool down at 55°- 65°C.
- The gel was then poured into tray carefully.
- Meanwhile comb was placed on the gel.
- Within 30 minutes the gel was solidified.
- The combs were removed from the gel.
- The gel was then ready for loading the DNA samples.

3.5.6. DNA sample preparation and electrophoresis

Reagents required

- Loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA)
- DNA marker (DNA ladder)

Procedure

- 2 µl extracted DNA sample was added to pcr tube.
- 4 µl of loading dye was added to it.
- Then mixed well by vortex and centrifuge this mixture using mini cetrifuge.
- After that samples were loaded in the well of the gel.
- The gel was placed to gel tank and the gel tank was covered and the electrophoresis apparatus were connected to the power supply unit and switched on.
- Electrophoresis was carried out at 100v for 50 min.
- When DNA migrated sufficiently the power supply was switched off

3.5.7. Documentation of the DNA samples

- After electrophoresis, the gel was taken out carefully from the gel chamber and tranferred in ethidium bromide solution (10µg/ml) for staining.
- Staining was done for 45 min. and then the gel was placed on the dark chamber of the Gel Documentation System.
- The UV light of the system was switched on;
- The image was visualized on the monitor and photographs were taken.

Precautions

- As ethidium bromide (EtBr) is a powerful mutagen and carcinogen, hand gloves were used when handling anything that had been exposed to EtBr.
- A transilluminator produces UV radiation in the 254 nm range. This wavelength can cause eye damage (short term burns, long term cataracts and skin cancers). Thus eye protectors were while working with it.

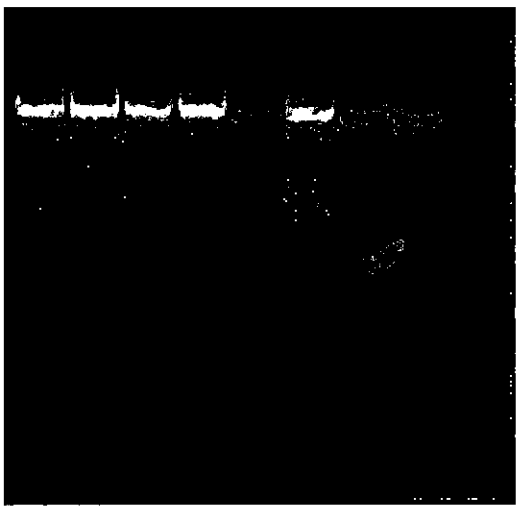

Fig-1: Conformation of DNA extracted from eight samples.

3.5.8. Quantification of DNA concentration

Before PCR amplification it is important to know the concentration of genomic DNA because different DNA extraction methods produced DNA of widely different purity. It is necessary to optimize the amount of DNA achieve reproducibility and strong signal in PCR assay. DNA concentration was measured by spectrophotometer and compared with standard lamda DNA (300ng, 100ng, 50ng, 25ng etc.)

3.5.8.1. Use of spectrophotometer

For more conformation, DNA was also quantified through spectrophotometer. During the quantification of DNA concentration, at first the spectrophotometer (Spectronic® Genesis™) UV-lamp was turned on and after it had warmed up the wave length was set at 260 nm. After washing the cuvette carefully with 1980 µl deionized water and 20 µl TE buffur were taken in the cuvette (the 'zero' or 'blank' cuvette) and the reading was adjusted to zero. In the same way other cuvette (the 'test' cuvette) was filled with 1980 µl of deionized water and 20 µl of each DNA sample was added and uniformly mixed with the help of micropipette. The absorbance of each sample was measured at 260 nm and absorbance reading was recorded. The cuvette was again rinsed with deionized water and wiped with tissue paper and absorbance readings for the other samples were taken in the same way and recorded.

With the absorbance readings, the original sample concentrations were determined according to the following formula:

DNA concentration (ng/µL) =

$$\text{Absorbance} \times \frac{\text{Volume of deionized water (µl)}}{\text{Volume of DNA (µl)}} \times \text{conversion factor}$$

(Here, Volume of deionized water was used 1980 µl and amount of DNA used 20 µl and the conversion factor is 50 because 1 O.D. at 260 nm for doudle-stranded DNA = 50 ng/µl of dsDNA)

3.5.9. Preparation of working solution of DNA samples

Before running the samples in PCR machine/ program the DNA concentrations were adjusted to 1 ng/ µl using the following formula: $S_1 V_1 = S_2 V_2$

$$S_1 \times V_1 = S_2 \times V_2$$

Where,

S_1: Initial DNA concentration (ng/µl)

V_1: Initial volume of DNA solution (µl)

S_2: Final DNA concentration (ng/µl)

V_2: Final volume of DNA solution (µl)

3.6. Amplification of RAPD markers by polymerase chain reaction (PCR)

To perform the amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence was mixed with genomic DNA in the presence of a thermostable DNA polymerase and suitable buffer then subjected to temperature cycling conditions typical to the polymerase chain reaction (PCR).

3.6.1. Preparation of reaction mixture for PCR

The following components were used to prepare PCR master mixture **(Table 2)**. The total volume of PCR cocktail was 9 µl per sample. A total of 10µl reaction mixture was performed (PCR master mixture 9µl + Template 1µl). Then the 0.2ml PCR tube was vortexed and placed in thermocycler.

PCR reactions were performed on each DNA sample in a total 10µl reaction mix containing reagents those are presented in table 2.

Table 2. Reagent used in PCR reaction

Reagents	Stock concentration	Reaction conentration	Amount (per reaction)
Taq polymerase	5U/ µl	1 U/µl	0.2µl
Primer	200µM	100 µM	0.5µl
dNTPs	10mM	300 µM	0.3µl
Taq reaction buffer	10X	10X	1µl
$MgCl_2$	25mM	1.5nM	0.6 µl
Sterile deionized water	-	-	6.4 µl
Genomic DNA		1ng/µl	1µl
Total			10µl

Precautions

- Care was taken during pipetting all the chemicals of the reaction mix.
- Care was taken during use of the enzyme Taq DNA polymerase.
- *Taq* DNA polymerase, primer, reaction mix were always kept on ice.

3.6.2. Thermal profile

The PCR tubes were set on the wells of the thermocycler. Then the machine was run according to the following setup

- Initial denaturation at 94°C for 5 min (step 1, one cycle)
- Denaturation at 94°C for 2 min (step 2)
- Annealing at 35°C and 38° for 30 sec (step 3)
- Elongation or extension at 72°C for 2 min (step 4)
- Step 2 to step 4 reaped 30 cycles. (step 5)
- Final extension at 72°C for 5 min(step 6, one cycle)
- Completion of cycling program, reactions were held at 8°C

3.6.3. Primer test

Six primers of random sequence were screened for amplification of the DNA sequences. The details of the primers are given in Table 3. A final subset of three primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis.

Table 3. Random primers used in the present study for polymorphism

Primer Code	Sequence (5′-3′)	GC Content (%)	Tm value	Annealing
A06	ACT GGC CGA GGG	75	42	38
A17	GGT TCG GGA ATG	58	38	38
A21	GTG ACC GAT CCA	58	38	35
A23	AAG TGG TGG TAT	41	34	38
A24	GAC GGT TCA AGC	58	38	35
A33	GAC TGC TAT ACA	58	34	34

3. 6.4. Electrophoresis of the amplified products

Agarose gel (1%) was prepared and poured into gel mold carefully when the gel solution cooled to about 55°C. After cooling the gel the combs were removed carefully that gel slots were not injured.

Then the PCR products were mixed with 5 µl of gel loading dye. Fifteen micolitres of the mixture was loaded slowly per well on the gel. The molecular weight marker (100 bp DNA ladder) was loaded at the first well on the gel. Then the gel was sunk in the electrophoresis tank. The tank was covered and all connections were checked. Electrophoresis machine was run for 1.5 hrs at 100 volts. The separation process was monitored by the migration of the dyes. When the bromophenol blue dye had reached about three-fourths of the gel width, the electrophoresis was stopped.

3. 6.5. Ethidium bromide staining

After completion of electrophoresis the gel was stained in ethidium bromide (10μg/ ml) solution for 45 min.

3. 6.6. Documentation of the DNA samples

After staining, the gel was taken out carefully from the staining tray and placed gel doc for checking the DNA bands. Amplified products were visualized under UV-light and photographs were taken.

3. 6.7. RAPD data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *et al.*, 1990). One molecular weight marker, 100 bp DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer.

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

Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be (N/n) 1/2 where N and n are the number of band negative individuals observed and the sample size, respectively. The frequency of the other allele (P) is 1-q. The assumption of the two allele system enables us to calculate the Nei's genetic distance (Nei, 1972) from the RAPD pattern.

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

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

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

The SI value ranges from 0 to1. When SI=1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles. Within population similarity (S_i) was calculated as the average of SI across all possible comparisons between individuals within a population. Between population similarity (S_{ij}) was calculated as the average similarity between each paired individuals of population i and j (Lynch and Walsh, 1998)

Geneflow (N_m) was estimated according to the following formula:

Gene flow, $Nm= 0.5 (1 - G_{st})/G_{st}$

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

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

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

Nei's genetic distance and identity values were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied eight maize genotypes using the unweighted pair-group method of arithmetic means (UPGMA) (Sneath and sokal, 1973). The dendrogram was constructed using the POPGENE; (Version 1.31) computer program (Yeh *et al.*, 1999).

CHAPTER FOUR

RESULT AND DISCUSSION

Two experiments were conducted separately for the fulfillment of the objectives and the results related to these experiments are presented and discussed in this chapter:

4.1. Characterization of genotypes through phenotypic marker

4.1.1. Variations and performance of four genotypes of Pumpkin

Among the four genotypes of pumpkin, two genotypes were hybrid and two genotypes were local. Hybrid genotypes were Sweety and Metal; local genotypes were Dewan and Alamin. The important characteristics of four genotypes plants are shown in **Table 4**. Seeds of four genotypes were sowed in same day but they germinated in different days. Sweety and Dewan were germinated after 5 days of sowing and Metal and Alamin were germinated after 4 days of sowing.

Days to opening of first flower varied among the genotypes studied. Among the 4 genotypes, 1st flowering was observed in Alamin genotype which took 47 days after sowing and Metal took maximum days for first flowering, it took 49 days after sowing.

Data indicated that days required for fruiting also varied significantly among the genotypes. Among these genotypes Sweety genotype took 53 days for first fruiting and Dewan genotype took maximum 55 days for first fruiting. On the other hand, Alamin and Metal took 54 days for first fruiting.

Characteristics of leaf were varies in leaf size and leaf color. The leaf colors of Sweety, Dewan and Alamin genotypes were green and Metal genotype was light green. On the other hand, among these genotypes, Sweety produces big leaf (Size (LxW) 8.9 X 7 inch) and Dewan produce small leaf [Size (LxW) 5.85 X 6.4]. Also, Metal and Alamin genotypes could preduce moderate sized leaf.

Characteristics of fruits of four pumpkin genotypes are shown in **Table-4**. The production performance was so good in Sweety genotype. The average fruits production per plant of Sweety genotype was 8 pumpkins. On the other hand, Alamin produced lowest number of fruit per plant. Also, the numbers of fruits produced by Metal and Dewan were 7 and 6 pumpkins respectively. These data proved that the performances of hybrid genotypes were better than local genotypes.

Colors of fruits were varies among four genotypes. Color of fruits in Sweety was yellow, fruits color of Metal fruits was green and the color of Dewan and Alamin fruits were yellowish green. Also, different fruit shapes were found in different genotypes. The fruits of Metal genotype were showed their shapes as oval shape and fruits of other genotypes were showed their shapes as round shape.

Results showed that there were variations of average fruits weight among the studied genotype. The highest average weight showed in Metal genotype (2.7kg) and lowest average weight shoed in Alamin genotype (1.4). Also, the average weights of Sweety and Dewan were 2.6 kg and 2.4 kg respectively. This data proved that hybrid genotypes were performed better than local genotypes. The highest average length of fruits was showed in Metal (11 inch) and lowest average length was showed in Alamin genotype (8 inch). Also, the length of Sweety and Dewan were 10 inchs and 9 inchs respectively. On the other hand, the highest average circumference of fruits was in Metal (17.5) and lowest was in Alamin (11.5). Also, the average circumference of fruits of Sweety and Dewan were 15 inches and 15 inches respectively.

Table-4: Morphological characteristics of four Pumpkin (*Cucurbita maxima*) varieties

Variety Name	Days to germination	Days to first flowering	Days to first fruiting	Leaf characteristies		Fruits characteristics						
				Color	Size(LxW) inch	Average fruits per plant	Color	Shape	Average weight	Average length (inch)	Circum ference (inch)	Weight per 100 seed(g)
Sweety (Hybrid)	5	48	53	Green	8.9 X 7	8	Yellow	Round	2.6	10	15	19.70
Al amin (Local)	4	47	54	Green	6.48 X 3.26	5	Yellowish green	Round	1.4	8	11.5	14.40
Metal (Hybrid)	4	49	54	Light Green	8.2 X 6.4	7	Green	Oval	2.7	11	17.5	17.84
Dewan (Local)	5	48	55	Green	5.85 X 3.46	6	Yellowish green	Round	2.4	9	15	15.30

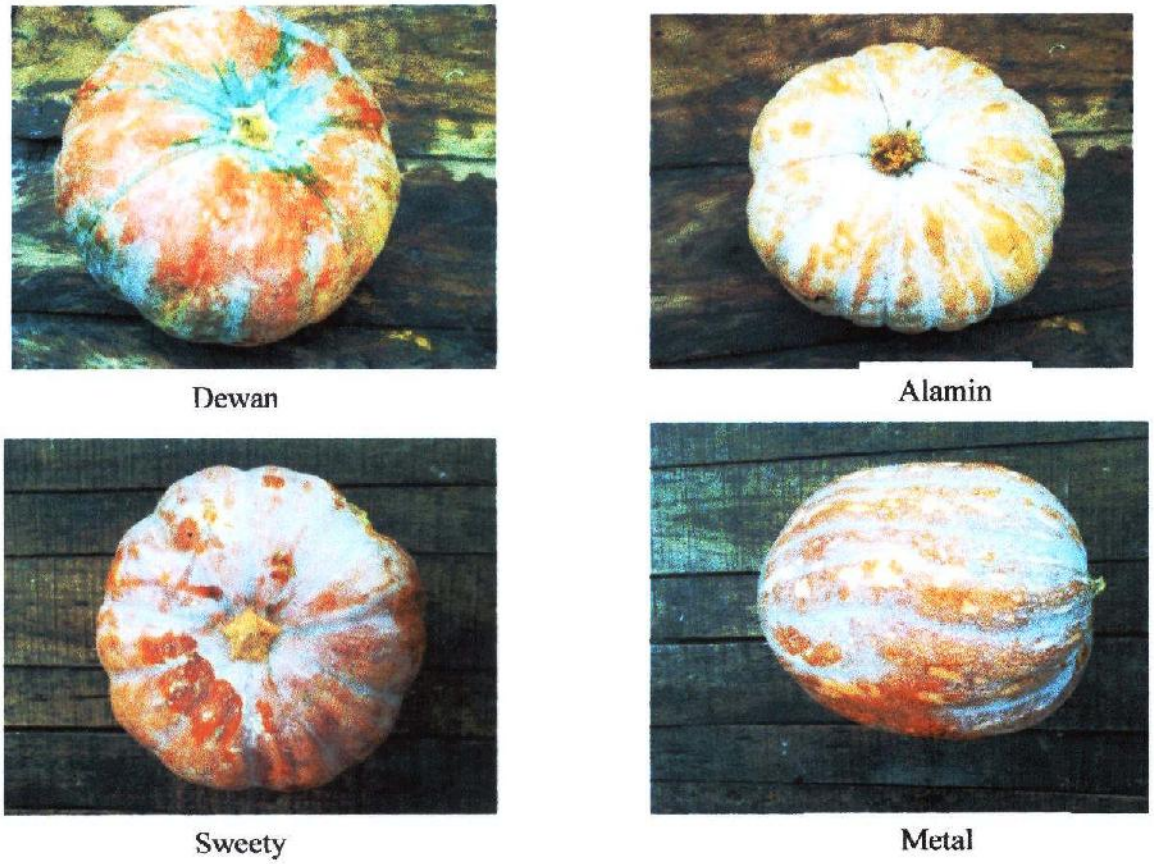

Fig-2: Pictures of the fruits of four pumpkin genotypes

The data showed that there are significant differences in 100 seeds weight among four genotypes. The highest 100-seed weight (19.70 g) was recorded in Sweety genotype and the lowest 100-seed weight (14.40g) was found in the genotype Alamin genotype. On the other hand, the 100 seed weight of Metal and Dewan were 17.84g and 15.30 g respectively.

Fig-3: Weighing of 100 seeds weight of pumpkin

4.1.2. Variations and performance of four genotypes of Bottle Gourd

Among the four genotypes of Bottle Gourd, two genotypes were hybrid and two genotypes were local. Hybrid genotypes were Higreen and Martina; local genotypes were Bari lau and Lalima. The important characteristics of four genotypes plants are shown in **Table 5.** Seeds of four genotypes were sowed in same day but they germinated in different days. Bari lau and Martina were germinated after 5 days of sowing and Higreen and Lalima were germinated after 4 days of sowing.

Days to opening of first flower varied among the genotypes studied. Among the 4 genotypes, 1st flowering was observed in Higreen genotype which took 48 days after sowing and Martina took maximum days for first flowering, it took 54 days after sowing.

Data indicated that days required for fruiting also varied significantly among the genotypes. Among these genotypes Higreen genotype took 55 days for first fruiting and Lalima genotype took maximum days for first fruiting. On the other hand, Martina and Bari lau took 61 and 58 days for first fruiting.

Characteristics of leaf were varies in leaf size but leaf color were same. The leaf colors of four genotypes were light green. On the other hand, among these genotypes Higreen produce big leaf (Size (LxW) 9.2×7 inch) and Bari lau produce small leaf [Size (LxW) 6.5×4]. Also, Martina and Lalima genotypes could produce moderate sized leaf.

Characteristics of fruits of four Bottle Gourd genotypes are shown in table-5. The production performance was so good in Higreen genotype. The average fruits production per plant of Higeen genotype was 9. On the other hand, Bari lau produced lowest number of fruit per plant. Also, the numbers of fruits produced by Martina and Lalima were 8 and 6 respectively. These data proved that the performances of hybrid genotypes were better than local genotypes.

Table-5: Morphological characteristics of four Bottle Guard (*Lagenaria siceraria*) varieties

Variety Name	Days to germination	Days to first flowering	Days to first fruiting	Leaf characteristics		Fruits characteristics						
				Color	Size(LxW) inch	Average fruits per plant	Color	Shape	Average weight	Average length (inch)	Circum ference (inch)	Weight per 100 seed(g)
Bari lau (Local)	5	49	58	Light Green	6.5×4	**5**	Light Green	Long	3.0	12.5	15	25.72
Higreen (Hybrid)	4	48	55	Light Green	9.2×7	**9**	Green	Long	4.5	16	17.5	16.88
Lalima (Local)	4	54	62	Light Green	8.1×5	**6**	Green	Round	2	11	12.5	14.70
Martina (Hybrid)	5	54	61	Light Green	8×5.5	**8**	Green	Long	3.7	15	17	26.44

Colors of fruits were varies among four genotypes. Color of fruits produced in Bari lau was whitish green and fruits color of other genotypes was green. Also, different fruit shapes were found in different genotypes. The fruits of Lalima genotype were showed their shapes as round shape and fruits of other genotypes were showed their shapes as long shape.

Results showed that there was variation of average fruits weight among the studied genotype. The highest average weight showed in Higreen genotype and lowest average weight showed in Lalima genotype. Also, the average weights of Martina and Bari lau were 3.7 kg and 3.0 kg respectively. This data proved that hybrid genotypes were performed better than local genotypes. The highest average length of fruits was showed in Higreen and lowest average length was showed in Lalima genotype. Also, the length of Martina and Bari lau were 15 inches and 12.5 inchs respectively. On the other hand, the highest average circumference of fruits was in Higreen and lowest was in Lalima. Also, the average circumference of fruits of Martina and Bari lau were 17 inches and 15 inches respectively.

Martina

Lalima

Bari lau

Higreen

Fig-4: Pictures of fruits of four genotypes of Bottle Gourd

The data showed that there are significant differences in 100 seeds weight among four genotypes. The highest 100-seed weight (26.44 g) was recorded in Martina. In contrast, the lowest 100-seed weight (16.88g) was found in the genotype Higreen.

Fig-5: Weighing of 100 seeds weight of Bottle Gourd

4.2. Characterization of genotypes through molecular marker

The experiment was conducted at the Plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, to characterize eight genotypes (four pumpkins and four bottle gourd genotypes) at molecular level.

4.2.1. Molecular characterization of pumpkin and bottle gourd using RAPD markers

This section comprises the presentation and discussion of the results of the experiment. The results were obtained from the experiment using RAPD markers on pumpkin and bottle gourd varieties. In this study, the RAPD analysis discovered moderate genetic variation and polymorphisms for characterization of different pumpkin and bottle gourd varieties. The results obtained from the experiment have been presented and discussed under the following heading:

4.2.2. Primer selection and RAPD pattern of four pumpkin and four bottle gourd genotypes

Six primers were initially screened on eight genotypes for their ability to produce polymorphic patterns and three primers viz. A17, A21 and A24 which gave reproducible and distinct polymorphic amplified products were selected. Total of 9 RAPD bands were scored of which 7 (77.77%) polymorphic amplification products were obtained by using these primers. Also, the average number of polymorphic bands is 2.33(66.67%) **(Table 6).** The selected three primers produced comparatively maximum number of high intensity band with minimal smearing, good technical resolution and sufficient variation among different variety. The dissimilar numbers of bands were generated by primer A17, A21 and A24.

Here, the primer A24 amplified maximum number of polymorphic bands (100%) while the primer A17 and A21 generated the least (50%) polymorphic bands which were minimal in number. The banding patterns of 8 genotypes using primers A17, A21 and A24 are shown in Figs. 10, 11 and 12, respectively.

Table-6: RAPD primers with corresponding bands score and polymorphic bands observed in 4 pumpkin and 4 bottle gourd genotypes

Primer code	Sequences (5'-3')	Number of bands scored	Number of polymorphic bands	Polymorphic loci (%)
A 17	GGT TCG GGA ATG	2	1	50
A 21	GTG ACC GAT CCA	2	1	50
A 24	GAC GGT TCA AGC	5	5	100
Total		9	7	200
Average		3	2.33	66.67

4.2.3. Intra genome similarity indices

Higher intra-genotype similarity indices (Si) were 100% with an average of 100%. The highest intra-genotype similarity indices (Si) value was found in pumpkin Alamin (Local) and bottle gourd Martina (Hybrid) for every primer. The average lowest intra-genotype similarity indices (Si) value 60% was found in pumpkin Sweety (Hybrid) **(Table 7).**

Table-7: Summary of the band sharing based on percentage similarity indices between individuals of four pumpkin and four bottle gourd genotypes.

Variety	Band sharing values (%)			
	A 17	A21	A 24	Average S_i (%)
Sweety	**50.00**	**50.00**	**80.00**	**60.00**
Alamin	**100.00**	**100.00**	**100.00**	**100.00**
Metal	100.00	50.00	80.00	76.67
Dewan	50.00	100.00	100.00	83.33
Bari lau	100.00	50.00	0.00	75.00
Higreen	100.00	50.00	100.00	83.33
Lalima	100.00	100.00	40.00	80.00
Martina	**100.00**	**100.00**	**100.00**	**100.00**
Average	87.50	75.00	85.71	82.74

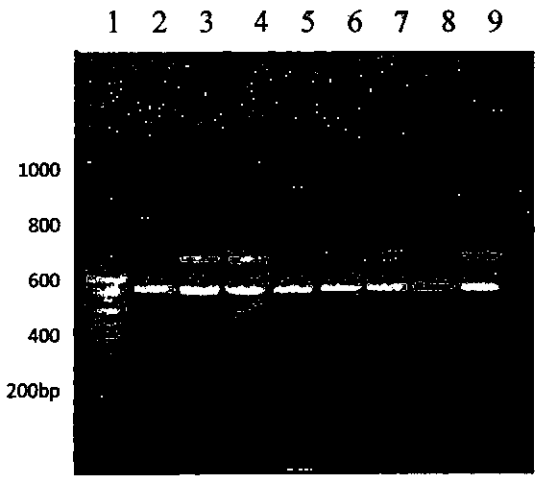

Fig-6: RAPD profiles of 4 pumpkin and 4 bottle gourd genotypes using primer A17. 1: 100 bp DNA ladder, 2: Sweety, 3: Alamin, 4: Metal, 5: Dewan, 6:Bari lau, 6:Higreen, 8:Lalima, 8: Martina

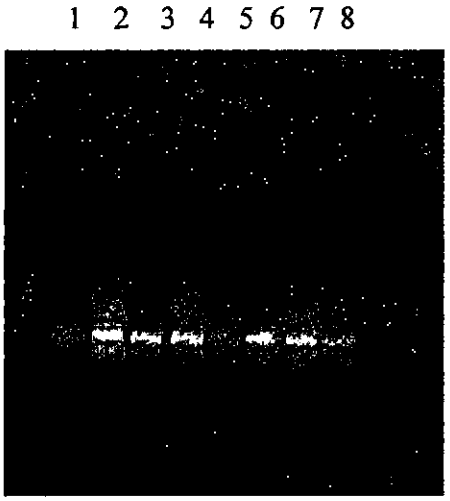

Fig-7: RAPD profiles of 4 pumpkin and 4 bottle gourd genotypes using primer A21. 1: Sweety, 2: Alamin, 3: Metal, 4: Dewan, 5: Bari lau, 6: Higreen 7: Lalima, 8: Martina

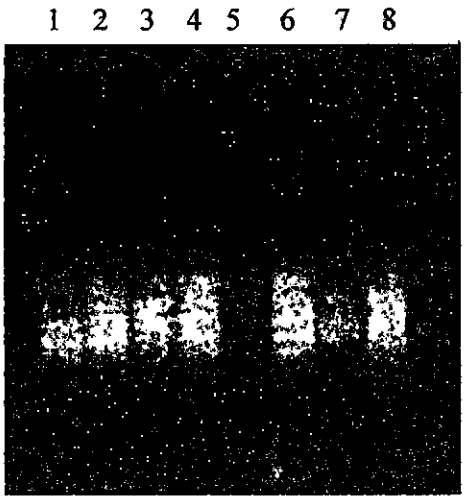

Fig-8: RAPD profiles of 4 pumpkin and 4 bottle gourd genotypes using primer A24. 1: Sweety, 2: Alamin, 3: Metal, 4: Dewan, 5: Bari lau, 6: Higreen, 7: Lalima, 8: Martina

4.2.4. Gene frequencies of polymorphic RAPD markers in four pumpkin and four bottle gourd genotypes

Allele frequency or gene frequency is the proportion of a particular allele among all allele copies being considered. Also, gene frequency is the percentage of all alleles at a given locus in a population gene pool represented by a particular allele (King *et al.*, 2006).

The DNA polymorphisms were detected according to the presence and absence of band. Absence of band may be caused by failure of primer to anneal a site in some individuals due to nucleotide, sequences difference or by insertions or deletions between primer sites (Clark and Lanigan, 1993). Frequencies of maximum number of polymorphic loci were found to be high with the exception of A17-2 and A21-2 (0.000) **(Table 8).**

Table-8: Gene frequencies of polymorphic RAPD markers in four pumpkin and four bottle gourd genotypes

Locus	Gen frequency
A17-1	0.7500
A17-2	0.0000
A21-1	0.5000
A21-2	0.0000
A24-1	0.5000
A24-2	0.7500
A24-3	**0.8750**
A24-4	0.7500
A24-5	**0.8750**

4.2.5. Gene diversity for the RAPD primer

Genetic diversity refers to 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 and biodiversity are dependent upon each other that diversity within a species is necessary to maintain diversity among species, and vice versa. Genotypic and phenotypic diversity have been found in all species at the protein, DNA, and

organismal levels, in nature this diversity is nonrandom, heavily structured, and correlated with environmental variation and stress (Nevo and Eviatar , 2001).

Genetic diversity values of 8 genotypes for 3 primers are given in **Table 9**. Average gene diversity (h) and Shannon's information index (I) across all primer against genotypes for all loci was found 0.2847and 0.4252 respectively.

Table-9: Gene diversity for three primers in all varieties

Locus	Sample size	na*	ne*	h*	I*
A17-1	8	2.0000	1.6000	0.3750	0.5623
A17-2	8	1.0000	1.0000	0.0000	0.0000
A21-1	8	2.0000	2.0000	**0.5000**	**0.6931**
A21-2	8	1.0000	1.0000	0.0000	0.0000
A24-1	8	2.0000	2.0000	**0.5000**	**0.6931**
A24-2	8	2.0000	1.6000	0.3750	0.5623
A24-3	8	2.0000	1.2800	0.2188	0.3768
A24-4	8	2.0000	1.6000	0.3750	0.5623
A24-5	8	2.0000	1.2800	0.2188	0.3768
Mean	8	1.7778	1.4844	0.2847	0.4252
St. Dev.		0.4410	0.3749	0.1897	0.2661

*na = Observed number of alleles

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

*h = Nei's (1973) gene diversity

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

High level of gene diversity value and Shannon's information index was found in locus A21-1 and A24-1 (0.5000 and 0.6931, respectively). Lowest level of gene diversity value and Shannon's information index (0.0000 and 0.0000) was found in locus A17-2 and A21-2 **(Table 9).**

The average number of allele and effective number of allele was 1.7778 and 1.4844 respectively. The highest and lowest number of allele was 2.0000 and 1.0000 respectively. Also, the highest and lowest effective number of allele was 2.0000 and 1.0000 respectively **(Table 9).**

4.2.6. Genetic Diversity among pumpkin and bottle gourd varieties

Genetic diversity among 4 pumpkin genotypes and 4 bottle gourd genotypes were ranging from 0.1400 to 0.5100. The highest genetic diversity was found in pumpkin Sweety (hybrid) and the lowest in bottle gourd Lalima (local). Among the pumpkin genotypes Sweety showed the highest and Dewan showed the lowest genetic diversity. Also, among the bottle gourd genotypes Martina showed the highest and Bari lau showed the lowest genetic diversity.

Table-10: Genetic diversity among varieties

Serial No.	Varieties	Genetic Diversity
1	Sweety (Hybrid)	**0.5100**
2	Alamin (Local)	0.2500
3	Metal (Hybrid)	0.4230
4	Dewan (Local)	0.2130
5	Bari lau (Local)	**0.0600**
6	Higreen (Hybrid)	0.2700
7	Lalima (Local)	0.1400
8	Martina (Hybrid)	0.3500

4.2.7. Genetic distance

Genetic distance is a measure of the genetic divergence between species or between populations within a species. Populations with many similar genes have small genetic distances. This indicates that they are closely related and have a recent common ancestor (Nei, 1987).

Genetic distance is useful for reconstructing the history of populations. For example, evidence from genetic distance suggests that African and Eurasian people diverged about 100,000 years ago (Nei and Roychoudhury, 1974). Genetic distance is also used for understanding the origin of biodiversity. For example, the genetic distances between different breeds of domesticated animals or plants are often investigated in order to determine which breeds should be protected to maintain genetic diversity (Ruane, 1999).

Table-11: Summary of Nei's (1972) genetic distance values among studied 4 pumpkin and 4 bottle gourd genotypes

Geno	Sweety	Alamin	Metal	Dewan	Bari lau	Higreen	Lalima	Martina
Sweety	****							
Alamin	0.4055	****						
Metal	0.1178	0.2513	****					
Dewan	0.2513	0.1178	0.4055	****				
Bari lau	0.8109	**1.0000**	0.5878	**1.0000**	****			
Higreen	0.2513	0.1178	0.1178	0.2513	0.8109	****		
Lalima	0.5878	0.4055	0.4055	0.5878	0.4055	0.5878	****	
Martina	0.4055	**0.0000**	0.2513	0.1178	**1.0000**	0.1178	0.4055	****

The values of pair-wise comparisons of Nei's (1972) genetic distance among 8 genotypes were computed from combined data sets for the three primers ranging from 0.0000 to 1.0000 **(Table 11).** Comparatively higher genetic distance 1.0000 was found between bottle gourd Bari lau (Local) and pumpkin Lalima (Local), Dewan (Local) and Bari lau (Local) and Bari lau (Local) and Martina (Hybrid). The lowest genetic distance 0.0000 was revealed between bottle gourd Martina (Hybrid) and pumpkin Alamin (Local).

4.2.8. Gene flow and population differentiation

Gene flow or gene migration is the transfer of alleles or genes from one population to another. Migration into or out of a population may be responsible for a marked change in allele frequencies. Immigration may also result in the addition of new genetic variants to the established gene pool of a particular species or population (Su *et al.*, 2003).

Table-12: Gene flow (N_m) and the proportion of total genetic diversity (G_{st}) across different RAPD markers of 4 pumpkin and 4 bottle gourd genotypes

Locus	Sample size	Ht	Hs	Gst	Nm
A17-1	8	0.3750	0.0000	1.0000	0.0000
A17-2	8	0.0000	0.0000	****	****
A21-1	8	0.5000	0.0000	1.0000	0.0000
A21-2	8	0.0000	0.0000	****	****
A24-1	8	0.5000	0.0000	1.0000	0.0000
A24-2	8	0.3750	0.0000	1.0000	0.0000
A24-3	8	0.2188	0.0000	1.0000	0.0000
A24-4	8	0.3750	0.0000	1.0000	0.0000
A24-5	8	0.2188	0.0000	1.0000	0.0000
Mean	8	0.2847	0.0000	1.0000	0.0000
St. Dev.		0.0360	0.0000		

H_t = Hardy-Weinberg average heterozygosity expected in subpopulation

H_s = Hardy-Weinberg average heterozygosity obtained in Sub population

G_{st} = Co-efficient of gene differentiation

N_m = Estimation of gene flow from G_{st} $N_m = 0.5\ (I-G_{st})/G_{st}$

The average estimated gene flow (Nm) was 0.0000 and co-efficient of gene differentiation (Gst) was 1.0000 across all loci **(Table 12).** Average Ht (Hardy-Weinberg average heterozygosity expected in subpopulation) was 0.2847and Hs (Hardy-Weinberg average heterozygosity obtained in Sub population) was 0.0000 across all primers. RAPD marker A17-2 and A21-2 revealed low level of differentiation (Gst).

4.2.9. UPGMA Dendrogram

A dendrogram was constructed based on Nei's (1972) genetic distance following the Unweighted Pair Group Method of Arithmetic Means (UPGMA). The 4 pumpkin and 4 bottle gourd genotypes were grouped into 2 main clusters **(Fig-9).** Genotypes Sweety (Hybrid), Metal (Hybrid), Alamin (Local), Lalima (Local), Dewan (Local) and Higreen (Hybrid) were included in cluster I while Bari lau (Local) and Martina (Hybrid) genotypes were included in cluster II.

In Cluster I, Sweety (Hybrid), Metal (Hybrid), Alamin (Local), Lalima (Local) and Dewan (Local) formed sub-cluster I while only Higreen (Hybrid) formed sub-cluster II. Again, among the lines of sub-cluster I, Sweety (Hybrid), Metal (Hybrid) and Alamin (Local) organized as sub-sub cluster I, while Lalima (Local) and Dewan (Local) belongs to sub-sub cluster II. Also, Dewan was separated from sub-sub cluster I. On the other hand, In cluster II, Bari lau (Local) and Martina (Hybrid) were divided into two sub cluster. Genetic relationship was present between two clusters. Genotypic variations based on molecular characterization indicated that genotypes belonging to different clusters depend on their genetic components itself, but not at geographical origin at all.

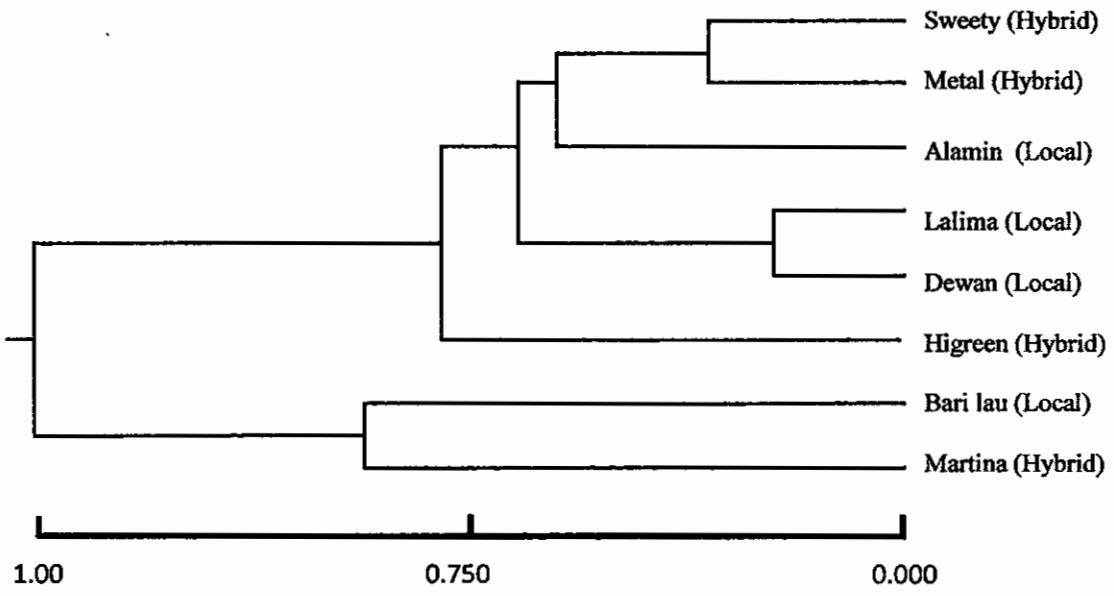

Fig-11: UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation four pumpkin and four bottle gourd genotypes, according to RAPD analysis.

CHAPTER FIVE

SUMMARY AND CONCLUSION

The present study was undertaken with a view to evaluating the performance of four pumpkin and bottle gourd genotypes in respect of parameters of important quantitative characters. The experiments were carried out at the experimental field and Plant Biotechnology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, during the period from July 2012 to April 2014.

The main objectives of the study were, to study the morphological characters in pumpkin and bottle gourd genotypes, to determine the genetic relationship between two species for the development of distant hybrid, to study the genetic diversity using RAPD markers in the selected pumpkin and bottle gourd genotypes. The results obtained using the RAPD technique was moderately satisfactory. Four pumpkin and four bottle gourd genotypes were used for screening their performances and genetic diversity.

Morphological characters were evaluated and there were some variations among the genotypes for the characters first flowering date, first fruiting date, leaf color, leaf size, average fruits per plant, fruit weight, fruit length, fruit circumference and 100-seed weight. Among these pumpkin and bottle gourd genotypes, hybrid genotypes were performed better than local genotypes. Among the pumpkin genotypes, Alamin (local) was early flowering genotype and the Sweety (hybrid) was an early fruiting genotype. On the other hand, Metal was late flowering and Dewan was late fruiting genotype. Also, Sweety produced highest number of fruits and these fruits were moderate weighted. In contrast, Alamin (local) produced lowest number of fruits and fruits were low weighted. The highest 100-seed weight was recorded 18.76 gm in Sweety (hybrid) and the lowest was found in Alamin (local). Among the bottle gourd genotypes, Higreen (hybrid) was both early flowering and early fruiting genotype. On the other hand, Lalima (local) was late flowering and fruiting genotype. Also, Higreen produced highest number of fruits and these fruits were high weighted. In contrast, Bari lau produced lowest number of fruits. The highest 100-seed weight was recorded 26.44 gm in Martina (hybrid) and the lowest was found in Lalima (local).

The RAPD technique was used for assessing genetic variation and relationship among eight selective varieties of pumpkin and bottle gourd. Six random primers were initially screened and finally three primers A17, A21 and A24 were selected for the analysis. RAPD makers were amplified by PCR technique, separated in 1% agarose gel electrophoretically, stained in ethidium bromide solution and visualized on UV trans-illuminator.

A total of 9 RAPD bands were scored of which 7 (77.78%) polymorphic amplification products were obtained by using these primers. Among these primers, A24 amplified maximum number of polymorphic bands (100%) while rest two primers A17 and A21 generated the least (50%) polymorphic bands which were minimal in number.

The highest intra-genotype similarity indices (S_i) value was found in pumpkin Alamin (Local) and bottle gourd Martina (Hybrid). The lowest intra-genotype similarity indices (Si) value 60% was found in pumpkin Sweety (Hybrid). High level of gene diversity value and Shannon's information index was found in locus A21-1 and A24-1 (0.5000 and 0.6931, respectively). Lowest level of gene diversity value and Shannon's information index (0.0000 and 0.0000) was found in locus A17-2 and A21-2. Among the pumpkin genotypes Sweety showed the highest and Dewan showed the lowest genetic diversity. Also, among the bottle gourd genotypes Martina showed the highest and Bari lau showed the lowest genetic diversity.

Pair-wise comparisons of Nei's (1972) genetic distance (GD) among four pumpkin and four bottle gourd genotypes were comparatively higher genetic distance 1.0000 was found between bottle gourd Bari lau (Local) and pumpkin Alamin (Local), pumpkin Dewan (Local) and bottle gourd Bari lau (Local) and bottle gourd Bari lau (Local) and bottle gourd Martina (Hybrid). The lowest genetic distance 0.0000 was revealed between bottle gourd Martina (Hybrid) and pumpkin Alamin (Local). The average estimated gene flow (Nm) was 0.0000 and co-efficient of gene differentiation (Gst) was 1.0000 across all loci. Average Ht (Hardy-Weinberg average heterozygosity expected in subpopulation) was 0.2847and Hs (Hardy-Weinberg average heterozygosity obtained in Sub population) was 0.0000 across all primers. RAPD marker A17-2 and A21-2 revealed low level of differentiation (Gst).

Following the Unweighted Pair Group Method of Arithmetic Means (UPGMA) on Nei's (1972) genetic distance, the eight genotypes of pumpkin and bottle gourd were grouped into 2 main clusters. Genotypes Sweety (Hybrid), Metal (Hybrid), Alamin (Local), Lalima (Local), Dewan (Local) and Higreen (Hybrid) were included in cluster I while were Bari lau (Local) and Martina (Hybrid) genotypes in cluster II. Genotypic variations based on molecular characterization indicated that genotypes belonging to different clusters depend on their genetic components itself, but not at geographical origin at all.

From the study, it is concluded that RAPD markers can be sensitive, simple, efficient and powerful tool for genetic diversity analysis among and within rice genotypes and effectively trace their genetic relationships. The results of this study can be used as a baseline of relationships for future diversity assessment and genetic analysis of pumpkin and bottle gourd varieties in Bangladesh. However, there were a few limitations in the study, 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 diversity among different varieties is widely acceptable by all concern. Using larger number of samples and higher number of primers could be useful in future research. Overall RAPD is a suitable technique for molecular genetic analysis and for reasonable relationship among the germplasm studied if large number of samples and higher number of primers are used.

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