MOLECULAR DIVERSITY AND GENETIC RELATIONSHIPS BETWEEN PARENTAL LINES AND THEIR F_1 OF CULTIVARS *Cucurbita moschata* USING RAPD ANALYSIS

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DECLARATION

This thesis report is the original research work of the author, except as otherwise stared. 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 The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.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 Late Grandmother

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July 2014 **The Author**

Abstract

Pumpkin (*Cucurbita moschata*) is a widely grown and economically important species and nutritious due to high content of vitamin A which can play a vital role in meeting the vegetable shortage and nutritional problem. Present investigation was carried out at the Field Laboratory and Plant Biotechnology Laboratory of Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna. Molecular characteristics were studied by using four RAPD primers to determine the genetic diversity and relationship between parental [Sweety (V_1) and Local (V_2)] and their F_1 hybrids. Morphological characteristics of *Cucurbita* showed a wide range of variation between hybrid and local cultivars. Number of male/female flowers showed significance at $P < 0.01$ which means the desired ratio of flowers for crossing. Among the parents and hybrids the RAPD analysis showed a total of 16 bands of which 14 showed polymorphisms which represents 90.84% polymorphisms. The similarity values based on Jaccard's similarity coefficient between F_1 and the donor parents plant was (0.000-1.000). Dendrogram analysis delineated two major clusters of which 60% F_1 showed similarity like male (V_2) and the rest like female (V_1) donor plant. The coefficient of similarity and UPGMA revealed two main clusters among 2 parents and 15 F_1 genotypes of *Cucurbita moschata* except accession number 12, i.e., cluster A and cluster B at 93.33% overall similarity and 6.66% variation within parents and the F_1. Cophenetic Correlation Coefficient (CP) 0.621 was confirmed the strongest correlation between the two parents and their F_1 progeny. This study demonstrates that RAPD markers are useful in assessing genetic diversity and relationships among cucurbits for further improvement of pumpkin genotypes through breeding program.

CONTENTS

Title	Page No
Title Page	I
Approval Page	III
Declaration Page	IV
Dedication	V
Acknowledgement	VI
Abstract	VII
Contents	VIII
List of Tables	XI
List of Figures	XII
List of Appendices	XIII
CHAPTER ONE	1-3
Introduction	1
CHAPTER TWO	4-19
Review of Literature	4
2.1. Botanical Classifications, Origins and Uses	4
2.2. Domestication	4-5
2.3. Production	5-6
2.4. Cultivation	6-7
2.5. Hybridization and Introgression	7-8
2.6. Genetics	8-9
2.7. Genetic Diversity of Pumpkin	9-11

2.8. Molecular Characterization 11

2.9. DNA Extraction 12-13

2.2.0. Polymerase Chain Reaction 13-14

2.2.1. The Use and Development of Molecular Breeding Tools in *Cucurbita* 14-16

2.2.2. Random Amplified Polymorphic DNA (RAPDs) as a Tool for the Identification of Trait-Linked Markers and Molecular Map Construction 16 -18

2.2.3. Advantages of RAPD Markers 18-19

CHAPTER THREE 20-30

3.1. Plant Materials 20

3.2. Field Experiment for Collecting Morphology of Pumpkin 20-21

3.3 Methods of crossing and generation of F_1 progeny 21

3.4. Mating design and field experiment and F_1 progeny generation 22

3.5. Genotyping of Pumpkin Germplasm 22

3.6. Pumpkin Genomic DNA Extraction 23

3.6.1. Leaf sample collection 23

3.6.2. Reagents preparation for DNA extraction (Stock Solution) (Appendices-1) 23

3.6.3. Extraction of DNA 23- 24

3.6.4. DNA Confirmation Using Gel Electrophoresis 24

3.6.5. DNA sample preparation and electrophoresis 24-25

3.6.6. Staining of the Gel 25

3.6.7. Documentation of the DNA Samples 25

3.6.8. Quantification of DNA concentration 26

3.6.9. Preparation of working solution (25 ng/ µl) of DNA samples 26

3.7. PCR Reaction 27

3.7.1. Primer Selection 27

3.7.2. PCR Amplification 28

3.7.3 Temperature Profile 28

3.7.4. Electrophoresis of the amplified products and documentation 29

3.8. Statistical analyses 29

3.9. Calculating the cophenetic correlation coefficient 29-30

3.2.0. RAPD Analysis 30

CHAPTER FOUR 31-47

Results and Discussion 31

4.1. Morphological Characterization 31-33

4.2. Morphology of flowers produced and corolla length and texture of 4 accessions of *Cucurbita moschata* parents 34-37

4.3. Primers and randomly amplified polymorphic DNA (RAPD) banding patterns. 37-40

4.4. Similarity co-efficient of *Cucurbita moschata* Parents and F_1 genotypes based on RAPD markers 41-42

4.5. Estimation genetic Variability by the Distance matrix based on

Jaccard coefficient 43-44

4.6. Cluster analysis based on coefficient of similarity and dissimilarity 45-46

4.7. P value and statistical significance of Genetic diversity 46-47

CHAPTER FIVE 48-49

Summary and Conclusion 48-49

CHAPTER SIX 50-67

References 50-67

LIST OF TABLES

SL.NO	TITLE OF TABLE	PAGE NO
Table 1:	Parent Plant materials used in morphological and molecular analysis	20
Table 2:	Layout of the half diallel cross design based on the Griffing's second method for Four Cucurbita maxima inbred lines.	22
Table 3:	Random Primer Used in the Present Study for Screening	27
Table 4:	PCR master mixture (10µl)	27
Table 5	Morphological analysis of Pumpkins Leaf and fruit	33
Table 6:	Number of flowers produced and corolla length and texture of 4 accessions of *Cucurbita moschata* parents	35
Table 7:	Number of F_1 by species and percentage of presence by provenance	36
Table 8:	RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 4 pumpkin accessions	38
Table 9:	Similarity matrix based on Jaccard's similarity coefficient	42
Table 10:	Distance matrix based on Jaccard coefficient	44

LIST OF FIGURE

SL.NO.	FIGURE TITLE	PAGE NO
Figure 1:	Electrophoregram of eithidium bromide stained genomic DNA samples extracted from different genotypes of Pumpkin.	25
Figure 2:	Four parents pumpkin *(Cucurbita moschata)* fruit.	32
Figure 3:	F_1 is germinated and transplanted in the field.	36
Figure 4:	RAPD pattern generated by the primer A17 with parents and F_1 genotypes of *Cucurbita moschata.* The number corresponds to the serial number of the F_1 genotypes.	39
Figure 5:	RAPD pattern generated by the primer A21with parents and F_1 genotypes of *Cucurbita moschata* .The number corresponds to the serial number of the F_1 genotypes.	39
Figure 6:	RAPD pattern generated by the primer A23 with parents and F_1 genotypes of *Cucurbita moschata.* The number corresponds to the serial number of the F_1 genotypes.	40
Figure 7:	RAPD pattern generated by the primer A24 with parents and F_1 genotypes of *Cucurbita moschata.* The number corresponds to the serial number of the F_1 genotypes.	40
Figure 8:	Dendrogram produced using UPGMA cluster analysis based on similarity matrix demonstrating the association among 17 accessions; similarity values were calculated according to Jaccard (1907) based on 16 different RAPD polymorphic fragments.	46

LIST OF APPENDICES

Appendices 1: Reagents preparation for DNA extraction (Stock solution) **68-69**

Chapter One

Introduction

Introduction

Cucurbita moschata is a worldwide cultivated fruit and economically important species of the genus *Cucurbita* L. Pumpkin (*Cucurbita* spp.) is one of the cucurbitacous fruit vegetables consumed and relished by most local people in Bangladesh, India, Burma, southern Appalachians of the USA, Nigeria and in the sub-Saharan Africa. In the genus *Cucurbita,* five domesticated species were characterized, including *Cucurbita argyrosperma*, *Cucurbita ficifolia*, *Cucurbita maxima*, *Cucurbita moschata* and *C. pepo*, in addition to ten wild species. (Robinson and Decker-Walters, 1997). Most species originated in Mexico with only the species *C. maxima*, being native to South America. It is important as a good source of minerals, fibres, vitamins, anti-nutrients, antioxidants and phyto-nutrients (Pandey *et al.*, 2003; Pratt and Matthew, 2003; Ward, 2007; Aruah *et al.*, 2010a; Atuonwu and Akobundu, 2010) and this makes the fruit wholesome and healthy for human consumption. Some authors have reported that pumpkin fruits have high medicinal values (Jones, 1996; Keles *et al.*, 2001; Sentu and Debjani, 2007; Mahasneh and El-Oqlah, 1999; Abd El-Aziz and Abd El- Kalek, 2011). Different parts of the plant have also been used as medicine in the developed world and the leaves are haematinic, analgesic, and also used externally for treating burns. Traditionally, the pulp is used to relieve intestinal inflammation or enteritis, dyspepsia and stomach disorders. Pumpkin seed is an excellent source of protein and also has pharmacological activities such as anti-diabetic (Li *et al.*, 2003), antifungal (Wang and Ng, 2003), antibacterial and anti-inflammation activities (Fu *et al.*, 2006) and antioxidant effects (Nkosi *et al,*. 2006).

With current emphasis on consumption of fruits and vegetables to promote good health and longevity, it is expected that consumption and the demand of pumpkins may be increased in developing countries and, this increase must be matched with an increase in pumpkin production. A large number of pumpkin accessions are cultivated in the developing countries but no serious attempts have been made to improve them for higher productivity and acceptability.

Therefore, there is a need to improve the productivity and fruit yield of the crop to meet the nutritional and dietary need of the people most especially the rural populations who are among the poorest and most vulnerable to malnutrition and poverty. This can be achieved through vigorous breeding programs. The success of any crop improvement program depends, to a large extent, on the amount of genetic variability present in the population. Very few research works

relating to variability of pumpkin accessions have been conducted in the developing countries so; intensive research efforts are needed in several areas particularly in selection of superior pumpkin genotypes.

There is wide genetic diversity and relationships among the existing pumpkin accessions (Ferriol *et al.,* 2003, 2004; Aruah *et al.*, 2010b; Aliu *et al.*, 2011) and thus, the utilization of such diversity in the crop's breeding programmes is possible. The breeding programs depend on the knowledge of key traits, genetic systems controlling their inheritance, genetic and the environmental factors that influence their expression.

Characterization based on morphological and horticultural traits have some disadvantages as being influenced by environmental factors (Dey, 1997; Sammour *et al.,* 2007). It is tedious, unreliable and time consuming. Instead, molecular characterization have proven many advantages, being able to distinguish polymorphisms which not produce phenotypic variation, highly polymorphic, easy and fast to detect and not possessing pleiotropic effects. Moreover, they can facilitate rapid screening of large numbers of genotypes for polymorphic loci (Williams *et al.,* 1990). Random amplified polymorphic DNA (RAPD) has established as a good genetic marker system to assay and evaluate the genetic diversity and genetic relationship among species, among populations and furthermore among individuals in the same population (Ding *et al.,* 2009; Mathew *et al.,* 2010). It is an informative marker that screen large in the genome; either the expressed or the non-expressed and even the regulatory sequences (Rakhee *et al.,* 2004; Elena *et al.,* 2010).

Most studies proved RAPD marker reliability in estimating inter-specific genetic relationships. The advantages of RAPD over other DNA-Based methods include a lack of the requirement for sequence information of the species, ease and speed of the assay, little amount of DNA required, no use of radioactivity and the ability to provide markers in genomic regions with repetitive DNA sequences (Dos-Santos *et al.,* 1994; Halden *et al.*, 1994; Thormann *et al.,* 1994).

There are quite few studies concerning the molecular analysis of *Cucurbita* species. The RAPD markers were used to analyze the genetic diversity among *C. moschata* Lanraces from Korea, Southern Africa and other geographical origins (Youn and Chung, 1998; Baranek *et al.,* 2000).

Ferriol *et al.* (2004) studied the genetic diversity among nineteen Spanish accessions of *C. maxima* using two different molecular markers; sequence related amplified polymorphism (SRAP and RAPD. More recently Ferriol *et al.* (2004 a, b) employed the SRAP and RFLP molecular marker for analyzing the diversity among a large number of *C. moschata* and *C. maxima* landraces,

Therefore, In this research work discuss about the genetic relationship and diversity within *C. maxima* and the F_1 using RAPD molecular marker has been focused on for the assessing genetic relationships and diversity in Pumpkin F_1 (*Cucurbita moschata*) germplasm collections for future development and production of pumpkin.

Considering the above-mentioned fact and file, present research work was conducted with the following objectives-

1. To analyze genetic diversity among four commonly growing cultivars of *Cucurbita moschata* using RAPD markers for assisting in selection of parent genotypes for genetic improvement.
2. To compare genetic relationship present between parental genotypes and their F_1 to test the efficacy of RAPD for *Cucurbita moschata* genotype identification.

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Chapter Two

Literature Review

Literature review

This section is followed by a review of the status of genome mapping in *Cucurbita* and the other major cucurbits, and a discussion of the strengths and weaknesses of Random Amplified Polymorphic DNA (RAPD) markers, which were the primary type used in the molecular work presented here. The final section of the literature review covers what is known about the genetic control of the phenotypic traits segregation.

2.1. Botanical Classifications, Origins and Uses

Both pumpkins and gourds are members of the cucurbit family Cucurbitaceae. Other vegetables in this family include watermelons, squash, cucumbers and cantaloupes. The cultivation of both plants predate colonial America. Native Americans cultivated pumpkins before the arrival of European settlers to the Americas. The origins of the pumpkin can be traced to the southern regions of North America and the northern regions of South America. Botanically pumpkins and squash are quite similar since varieties of both can be found in *Cucurbita pepo*, *C. argyrosperma*, and *C. moschata* species.*C. maxima* are also a species of pumpkin generally associated with the larger pumpkins. Mature and immature fruit of the pumpkin are generally edible. However, a large portion of the commercially produced pumpkins are used for decorative purposes.

2.2. Domestication

All of the Cucurbita are native to the Americas. The centres of origin and domestication for cultivated Cucurbita species can be identified as various areas in North and South America (Jeffrey, 1990). At least five species of the genus Cucurbita were domesticated before the European Contact in the late 15th century forming important food sources in native American economies, and some of these species were among the earliest plants taken under cultivation and domestication in the New World (Smith, 2001; Sanjur *et al.*, 2002).

The natural distribution of *C. argyrosperma* ranges from the southwestern USA through Mexico into Central America. The area of domestication for *C. argyrosperma* is considered to be from the southwestern USA to the centre-south region of Mexico (Merrick and Bates, 1989). The natural distribution of *C. ficifolia* ranges from the Mexican highlands south to northern Chile and Argentina. It grows as an annual in temperate climates and can appear to be a perennial in tropical zones. The precise location of the centre of domestication of *Cucurbita ficifolia* is still uncertain. The natural distribution of *Cucurbita maxima* is comprised of Bolivia, Argentina and Chile *C. maxima* was domesticated in South America. Historical chronicles indicate that during the time of the conquest of Río de La Plata (16th century), this species was one of the main crops of the Guaraní people living in what is now northeastern Argentina and Paraguay (Parodi, 1935).

In the 19th century, several cultivars were introduced into the United States from South America (Decker-Walters and Walters, 2000). Secondary centres of diversity include India, Bangladesh, Burma and the southern Appalachians of the USA, e.g., the landrace 'Candy Roaster' was originally developed by the Cherokee people in the southern Appalachians. These findings suggest that for *C. maxima*, in addition to the regions of South America mentioned above, multiple centres of diversity, primarily composed of landraces, exist around the world.

The natural distribution of *Cucurbita moschata* is from the lowlands of Mexico into Central America . *Cucurbita moschata* was domesticated in Latin America (Whitaker, 1947) but there is no consensus as to the precise area where domestication likely occurred. It has been proposed that *C. moschata* was domesticated in Mesoamerica (Whitaker and Davis, 1962) or alternatively in South America, more specifically in what is now Colombia.

The natural distribution of *C. pepo* ranges from the eastern USA north into the State of Illinois through the Mississippi Valley, through the State of Texas and south into Mexico.

2.3. Production

Squash, pumpkins and gourds are major vegetable crops with a combined worldwide production of 210 million tonnes in 2007 (FAOStat, 2009a). Summer squash represent a small portion of this production, but because summer squash production is concentrated in economically

developed countries, the crop value may exceed the other *Cucurbita* varieties in this category (Paris, 2008). In 2007, the United States ranked 4th in production of squash, pumpkins and gourds with 864,180 metric tonnes, of which 284, 220 tonnes were squash (FAOStat, 2009b; USDA, 2009). The value of United States squash production for 2008 was $204 million dollars, with Florida contributing $53 million dollars as the top squash-producing state (USDA, 2008). California is ranked 14 second in terms of production value followed by New York, Georgia and Texas. In the United States, squash ranks in the top 15 vegetables in production, planting acreage and crop value (USDA, 2009). Squash production in the United States has changed greatly in the last two decades with nationwide production reports only becoming available in 2000 as squash production began to exceed other vegetables and melons (Cantliffe *et al.*, 2007).

2.4. Cultivation

Summer squash are short-season crops grown in warm seasons, typically fall and/or spring, when the threat of cold temperature to the herbaceous, frost-sensitive plants is at a minimum (Kemble *et al.*, 2005; Paris, 2001). Summer squash are typically direct-seeded or transplanted in flat ground or raised beds, with or without polyethylenemulch (Kemble *et al.*, 2005). Squash can be planted on plastic mulched beds following crops of other fruit or vegetables, but to avoid potential nematode and soil-borne disease problems, summer squash should not follow other *Cucurbitaceae* crops. Well drained sandy loam soils of pH 6.0 to 6.5 with high levels of organic matter are preferred (Kemble *et al.*, 2005; Peet, 2001b). Summer squash require relatively high fertilization rates, especially before flowering (Peet, 2001b). Fertilization rates are dependent upon soil testing results. Summer squash have palmate leaves and a bush habit (Paris, 1989; Paris, 2001). Because open growth and bush habits facilitate harvesting, vining varieties are rare. Foliage of *C. pepo* is typically rough and spiney, but smooth varieties of summer squash are desired for easier hand harvest and avoidance of scratches to the tender fruit (Paris, 1989). Squash plants are monoecious, producing conspicuous, nectarproducing, orange-yellow, unisexual flowers (Paris, 2001). Squash plants produce male15flowers 3 to 4 days before female flowers are formed and in a typical ratio of 3 male's flowers per female flower (Peet, 2001a). Summer squash crops are highly dependent on effective bee pollination for crop yield and quality (Kemble *et al.*, 2005; Stephens, 2003). Poor pollination results in small, misshapen or aborted squash fruit (Peet, 2001a). For adequate fruit set, one honeybee hive per acre of

production is recommended. Squash require 40 to 50 days from seeding to reach marketable fruit size (Kemble *et al.*, 2005; Stephens, 2003). Market preference is for smaller, glossy fruit with intense color and less stippling (Paris, 2001). Yellow fruit are in demand in the United States, but not in other areas of production.

2.5. Hybridization and Introgression

A wide range of factors that control the incidence and direction of gene flow and introgression within the *Cucurbita* genus has been identified (Merrick, 1990), including spatial and temporal separation, behavior of pollinators, genetic compatibility factors, physiological differences and environmental adaptation. Numerous attempts at interspecific hybridization within *Cucurbita* have been conducted over the years and these have encountered a wide range in the degree of their success (Singh, 1990; Lebeda *et al.*, 2006).

In *Cucurbita*, all attempts at crossing the xerophytic species, those adapted to arid environments (*C. digitata, C. foetidissima, C. pedatifolia* and *C. radicans*), with the mesophytic species, those adapted to moist environments (*C. argyrosperma, C. ecuadorensis, C. ficifolia, C. lundelliana, C. maxima,C. moschata, C. okeechobeensis* and *C. pepo*), have failed to produce fertile hybrids (Lebeda *et al.*, 2006).

The genetic compatibility relations between five cultivated, and with the other mesophytic species of the genus *Cucurbita* have been widely studied (Whitaker, 1951; Whitaker and Bemis, 1965; Merrick, 1990; Lira *et al.*, 1995). In general, the cultivated *Cucurbita* species are reproductively isolated from one another. The primary gene pools of each species are represented by their landraces and commercial cultivars as well as by their intraspecific taxa. Although experimental interspecific crosses can be made among the cultivated species, these frequently result in hybrids that are only partially fertile, while others result in no fruit set (Merrick 1995). Spontaneous crosses between the cultivated *Cucurbita* are uncommon, but have been reported occasionally between certain of the various species' landraces, mostly in Mexico (Decker-Walters *et al.*, 1990; Merrick 1990,). Given the experimental results, these are also likely to be hybrids that are only partially fertile or result in no fruit set. Nevertheless, none of the genus' species is completely reproductively isolated from the others in terms of barriers to

hybridization. The cultivated *Cucurbita* species of interest, *i.e.*, those listed in the leftmost column, cross readily with plants within their primary gene pool. The secondary gene pool includes species that when crossed experimentally with the cultivated species in the leftmost column can yield at least partially fertile F_1 on hybridization. Although genes can be moved in breeding between the cultivated species and plants in their secondary gene pool, the F_1 are usually sterile or sparingly fertile. Species listed as being in the tertiary gene pool of the cultivated species represent the outer limit of potential genetic resources for breeding: Pre-zygotic and post-zygotic barriers can cause partial or complete hybridization failure, inhibiting introgression between the cultivated species and plants in the tertiary gene pool (Lebeda *et al.*, 2006). Crosses between a cultivated *Cucurbita* species and other cultivated species in the secondary or tertiary gene pools, present a more complicated picture; the use of techniques such as embryo culture, which are used to by-pass hybrid sterility barriers, may be required. Hybrids obtained from such crosses are frequently sterile or exhibit reduced fertility (Whitaker and Robinson, 1986). Among the *Cucurbita*, success in crossing frequently depends on the genotypes used as parental.

2.6. Genetics

The basic chromosome number of the *Cucurbita* is 2n = 2x = 40. Karyotypes suggest that these species are of allopolyploid origins. Results from electrophoretic analyses also helped confirm this genus' polyploidy (Kirkpatrick *et al.*, 1985), or more specifically, allotetraploid origin (Weeden 1984). Weeden (1984) and Singh (1990) suggested that the *Cucurbita* are ancestral tetraploid, derived from an ancestor with a haploid chromosome number of 10. Although these authors suggested an apparent homogeneity, Weiling (1959) suggested that the genome in *Cucurbita ficifolia* is AACC (each letter refers to a different ancestral plant genome), whereas in the four remaining domesticated species it is AABB. A recent sequence analysis of an intron from the mitochondrial gene *nad1* indicated that *C. ficifolia* was basal to all other taxa in this group (Sanjur *et al.*, 2002). Wilson *et al.* (1992) determined that the annual *Cucurbita* species evolved from the perennial species.

The morphology of the chromosomes is not well characterized as they are small and not easily differentiated (Weeden, 1984; Weeden and Robinson, 1990). Using flow cytometry,

Arumuganathan and Earle (1991) determined that the haploid genome of zucchini (*C. pepo* ssp. *pepo*) is approximately 500 million base pairs long. A typical nucleus (2n) contains 1.04 – 1.08 picograms of DNA. Most morphological traits appear to be unlinked, and many markers are required to adequately map the genome. Havey *et al.* (1998) used RFLPs to study the transmission of the chloroplast and mitochondrial genomes in cucurbits. They concluded that both organelle genomes were maternally transmitted in *Cucurbita*.

Some work on self-incompatibility and inbreeding depression in the genus has been performed. Some authors have seen little evidence of inbreeding depression in members of the genus (Borghi, 1976). Others, however, have observed indications of inbreeding depression. Mahzabin *et al.* (2008) indicate that *Cucurbita maxima* shows abrupt inbreeding depression after two generations of selfing. Cardoso (2004) observed inbreeding depression affecting certain traits in a *Cucurbita moschata* variety after four successive self-pollination generations. Cardoso (2004) and Hayes *et al.* (2005a, 2005b) have studied inbreeding depression after four successive generations of self-pollination in *Cucurbita pepo* var. *texana*. In general, inbreeding depression seems to be intense, which suggests a level of genetic variation at least as recessive deleterious genes. The selfing rate showed a range from 0.16 to 0.54, but this might vary among characters, years and conditions (Hayes *et al.*, 2005a, 2005b). Whitaker and Robinson (1986) suggest these different observations might represent the response of different species or varieties of *Cucurbita* to inbreeding.

2.7. Genetic Diversity of Pumpkin

Assessment of genetic diversity is important in plant breeding if there is to be improvement by selection. For assessment of genetic diversity, molecular markers have been generally superior to morphological, pedigree, heterosis, and biochemical data (isozymes and chromatography) (Melchinger *et al.*, 1991; Melchinger, 1993). Genetic diversity commonly is measured by genetic distance (GD) or genetic similarity (GS = 1 - GD), 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 (Duvick, 1984) 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).

An equally important application of molecular marker-based GD may be in the selection of parents to cross in a breeding program. This application deserves serious attention because breeders currently rely primarily on pedigree and performance data for choosing parents in breeding programs. Using molecular markers to select parents has the potential to allow simultaneous maintenance of genetic diversity and performance. One application of molecular markers for choosing parents, and additional research is needed in this area. Using molecular markers to choose parents likely will require establishment of a relation between GD and genetic variation, and many of the same conditions necessary for predicting hybrid performance may be required for choosing parents. Using molecular markers as a diagnostic tool to survey new or exotic germplasm for novel genetic diversity also may be possible. It is unlikely, however, that this use will be possible with random genomic or cDNA clones because molecular marker-based genetic diversity will not guarantee genetic diversity for the traits of interest. Screening with probes of expressed genes with known function offers the greatest potential in this area.

Genetic divergence or genetic diversity is the process in which two or more populations of an ancestral species accumulate independent genetic changes (mutations) through time, often after the populations have become reproductively isolated for some period. In some cases, subpopulations living in ecologically distinct peripheral environments can exhibit genetic divergence from the remainder of a population, especially where the range of a population is very large. The genetic differences among divergent populations can involve silent mutations (that have no effect on the phenotype) or rive rise to significant morphology changes. Genetic

divergence will always accompany reproductive isolation, either due to novel adaptations or due to genetic drift, and is the principle mechanism underlying speciation.

The divergence analysis has a definite role to play an efficient choice of divergent parents in any plant breeding program. Its importance in the improvement of a crop has been stressed in both self and cross –pollinated crops (Murty and Anand, 1966; Gaur *et al.*,1978). The quantification of genetic diversity through biometrical procedures (Anderson, 1957) has made it important to know the source of genes for a particular trait within the available germplasm.

2.8. Molecular Characterization

A number of *Cucurbita* genes have been characterized at the molecular level and cloned. However, this work has been done entirely by molecular geneticists interested in the control and functioning of pathways common to many plants. Thus the genes cloned have been of little direct use to squash breeders. Researchers in the laboratory of G.A. Thompson at the University of Arizona have studied and cloned the genes encoding important proteins in the phloem transport system of *C. maxima* (Bostwick *et al.*, 1994; Clark *et al.*, 1997; Leineweber *et al.*, 2000). Other recently cloned genes in *Cucurbita* include a calcium-dependent protein kinase from zucchini (Ellard-Ivey, *et al.*, 1999), a class-3 chitinase (Kim, *et al.*, 1999), a glyoxysomal malate dehydrogenase (Kato, *et al.*, 1998), and an anionic peroxidase (Carpine, *et al.*, 1998). Cloning of these types of pathway controlling genes from *Cucurbita* has been greatly facilitated by the availability of probes and sequence information from other species where genes with the same function have already been cloned It is apparent from the literature that molecular genetics is just beginning in *Cucurbita*. As the genome is mapped, it should be possible to identify markers for many useful traits. Markers could be particularly useful for tagging the complementary virus-resistance genes, such as Zym-2 and Zym-3 (Paris, *et al.*, 2000.) and ensuring that they are transferred during backcrossing. Molecular maps will also allow further investigation into the evolution and species relationships in *Cucurbita,* and between *Cucurbita* and other cucurbits.

2.9. DNA Extraction

DNA extraction and separation by agarose gel electrophoresis is a simple and exciting process that anyone can perform. Here, described a cost effective way of extracting and electrophoresing DNA using products found in local grocery stores.

PCR techniques such as RAPD (Williams *et al.*, 1990), CAPS (Konieczny and Ausubel, 1993), STSs (Mazur *et al.*, 1995), microsatellite (Panaud *et al.*, 1996), and AFLP (Vos *et al.*, 1995), have been used in plants for molecular mapping, identification of genotypes associated with genes of interest, and genetic diversity studies. A unique advantage of these PCR techniques is the rapid DNA analysis of many plant samples using small quantities of DNA. Thus, a simple and rapid DNA extraction method is needed for studies, such as genetic analysis, that require large populations. Several methods for minimizing the DNA extraction steps have been reported (Berthomieu and Meyer, 1991; Edwards *et al.*, 199; Tomas and Tanksley, 1989), but they require a large amount of plant tissue and grinding of plant tissues in liquid nitrogen. In addition, growth and management of plants and storage of the plant samples in freezers is often difficult due to space constraints.

There are many methods proposed for isolation of nuclear DNA from a variety of plant species, plant organs and tissues. Now it is considered that three main factors are critical for extraction and purification of maximally undegraded and pure genomic DNA:

1. Physical forces used to homogenize the plant tissue i.e. disruption of plant cell walls.

2. The composition of the grinding buffer.

3. Elimination of enzyme-inhibiting polysaccharides (Rogers and Bendich, 1985).

The use of liquid nitrogen for grinding of the plant material and homogenization buffers which contain CTAB (Watson and Thompson, 1986) or polyamines like spermine and spermidine (Hewish and Burgoyne, 1973) as well as the addition of EtBr to inhibit the endogenous nuclease activity (Luthe and Quatrano, 1980) allowed obtaining a high molecular weight plant DNA. Most of these methods for extraction and especially for the purification of plant DNA have

employed caesium chloride density gradients to eliminate proteins, RNA and the severe polysaccharides contaminations. This method replaced successfully the classical method of phenol/chloroform purification of DNA (Razin, 1988) but it is not convenient and time consuming since buoyant density centrifugation is required. Recently a rapid and simple method has been proposed for purification of DNA from human cells (Boom *et al.*, 1990). Briefly, this method is based on both the lysing and nuclease-inactivating activities of the chaotropic agent guanidiniumthiocyanate (GuSCN) together with the high binding properties of silica particles or diatoms to DNA in the presence of this agent. In this paper we describe the use of diatoms preparation Celite (the fossilized cell walls of unicellular algae) for purification of crude nuclear lysate DNA or phenol-deproteinized DNA from excised cotyledons of *Cucurbita pepo L.* (zucchini).

Doyel and Doyel (1987) stated in their paper that the standard protocol used in our laboratories is based on the cetylrimethylammonium bromide (CTAB) procedure of total DNA isolation.

According to Doyel and Doyel (1990), DNA can be successfully isolated from plants och in polysaccharides by simply increasing the CTAB concentration in the isolation buffer. CTAB binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction. When choosing a particular protocol, one should also keep in mind those different kinds of experiments demand different levels of DNA purity. For example, DNA fingerprinting analyses based on restriction and hybridization experiments require relatively pure high molecular weight DNA, while polymerase chain reaction (PCR) may also work on rather crude and somewhat degraded DNA sample (Berthomieu and Meyer, 1991).

2.2.0. Polymerase Chain Reaction

PCR-based marker techniques have been employed extensively for confirming genotypes of organisms at the level of species and population. The PCR method requires little biological material and provides a rapid method for screening large sample sizes. PCR markers have been developed using either arbitrary primers or specifically designed primers from known DNA information such as repetitive sequences Multiple arbitrary amplicon profile (MAAP) was developed for the use of single or multiple arbitrary primers (Caetano-Anolles, 1994; Caetano-

Anolles and Gresshoff, 1997; Caetano-Anolles *et al.*, 1991), and includes the techniques of RAPD (Williams *et al.*, 1990), AP-PCR (Welsh and McClelland, 1990; Welsh and McClelland, 1990), and DNA amplification fingerprinting (DAF) (Caetano- Anolles *et al.*, 1992). Because of its simplicity, the RAPD method has been used widely in studies of genetic diversity and in examining phylogenic and taxonomic relationship (Caetano-Anolles and Gresshoff, 1997). However, in PCR techniques using arbitrary primers, low reproducibility that causes variable PCR results depending on PCR condition has been recognized as a disadvantage factor.

The PCR conditions for RAPD analysis can be optimized by varying the many components, namely, type and concentration of thermo stable polymerase, deoxynucleotide triphosphates, Mg2+ ions, primer and DNA template concentration, and other reaction factors such as primer annealing, primer extension, denaturation time and temperature. All these factors can influence a PCR reaction, and this is exacerbated by the fact that not all processes and mechanisms involved in such reactions are as yet understood fully (Wolff *et al.*, 1993).

2.2.1. The Use and Development of Molecular Breeding Tools in *Cucurbita*

The development of molecular tools for use in breeding *Cucurbita* species is still in the early stages, particularly when compared to cereal crops such as com and wheat, and other vegetable crops such as tomatoes and lettuce. Even the *Cucumis* species *C. melo* and *C. sativus* are far ahead of *Cucurbita.* One reason for the late development of molecular tools in *Cucurbita* is that all species have twenty pairs of relatively short chromosomes. Most morphological traits appear to be unlinked, and many markers are required to adequately map the genome. *Cucurbita* species are of limited economic significance in developed countries, and most *Cucurbita* researchers work on several crops. The large size of the plants makes them ill suited to genetics studies, and some types require a very long growing season. No linkage map of any *Cucurbita* species existed prior to the development of molecular mapping. In the 1980s the use of isozymes revealed that *Cucurbita is* an ancient allotetraploid (Weeden, 1984). Isozymes also provided a marker linked to one of the complementary genes responsible for the expression of WMV resistance in crosses between *C maxima* and *C. ecuadorensis* (Weeden and Robinson, 1986). 16 Many of the species in *Cucurbita* can be successfully crossed, particularly if embryo rescue is used. However, the F_1

and later generations of the interspecific crosses are frequently sterile or exhibit reduced fertility (Robinson and Decker-Walters, 1997). Isozymes have been used to determine if the reduced fertility was a result of chromosomal rearrangement in crosses between *C. maxima* and *C. ecuadorensis.* Wall and Whitaker (1971) examined the inheritance of leucineaminopeptidase and esterase isozymes in a set of *C. ecuadorensis* x *C. maxima* crosses and concluded that the chromosomal structure of the two species differed in the region of the esterase locus. Weeden and Robinson (1986) examined the inheritance of twenty isozymes in the cross *C. maxima* x *C. ecuadorensis.* They used their data to build the first map of *Cucurbita.* It was based on the F_2 of the cross *C. maxima* x *C. ecuadorensis,* and contained 11 isozyme loci in five linkage groups. From the isozyme map Weeden and Robinson (1986) were able to determine that the significant decrease in fertility of the F_2 and backcross generations of crosses between *C. maxima* and *C. ecuadorensis* was not a result of minor chromosomal rearrangements. Lee *et al.* (1995) developed a RAPD map of an F_1population of a *C. pepo* x *C. moschata* interspecific hybrid. They screened the parents with 70 10-mer primers from the University of British Columbia (UBC 501-570); 15 of the primers were polymorphic between the parents. These fifteen primers were used to amplify DNA from 40 F_2 individuals, resulting in 58 RAPD bands. Forty-seven reproducible markers were used to build the map; 28 markers were mapped into five linkage groups. No morphological traits or other types of markers were included on the map. The RAPD map of Lee *et al.* (1995) was small, and based on a small number of progeny. In addition, they selected arbitrary identifiers for the markers on their map, rather than following the standard practice of identifying the markers by the primer and the band size in base pairs. This prevents comparisons between their map and other *Cucurbita* maps. The map of Lee *et al.* (1995) is currently the only published molecular map of *Cucurbita.*

Data were collected for a random amplified polymorphic DNA (RAPD) map on a *C. maxima* x *C. ecuadorensis* population. 17 RAPDs and other molecular marker technologies have been used to do DNA fingerprinting analysis within and between *Cucurbita* species. Jeon *et al.* (1994) used RAPDs to distinguish among Korean cultivars of *C. pepo* and *C. moschata,* while Youn *maxima* used RAPDs to study the genetic relationships among South Korean landraces of *C. moschata.* Stachel *et al.* (1998) used RAPDs to estimate genetic diversity among commercial inbred lines of Austrian oilseed pumpkin, *C. pepo*var. *styriaca.* Gwanama *et al.* (2000) used RAPDs to

determine the genetic variability present in the *C. moschata* landraces of south central Africa. Baranek *et al.* (2000) used RAPDs to study the genetic diversity within and between species of *C. pepo, C. moschata,* and *C.maxima.* All the researchers found RAPDs to be effective for determining the relatedness of different *Cucurbita* accessions. Polymorphism levels in *Cucurbita* are moderate. Baranek *et al.* (2000) found 42.5% marker polymorphism among six Austrian *C. pepo* geno types and 55.9% marker polymorphism among a disparate collection of *C maxima.* Published estimates of polymorphism in *C. moschata* range from 18.6% to 64.1% (Youn and Chung, 1998; Baranek *et al.,* 2000). Katzir *et al.* (1998) used microsatellite-anchored sequences as primers (ISSR) to classify cultivars of *C. pepo;* 82% of the ISSR markers were polymorphic. No microsatellites specifically designed for *Cucurbita* have been published, but polymorphisms among *Cucurbitapepo* accessions have been detected using SSRs developed for *Cucumis.* Fifty SSRs were screened, resulting in 14% polymorphism (Katzir *et al.,* 1996, 2000). There are at least 200 publicly available *Cucumis* SSRs. A number of *Cucurbita* genes have been characterized at the molecular level and cloned. However, this work has been done entirely by molecular geneticists interested in the control and functioning of pathways common to many plants. Thus, the genes cloned have been of little direct use to squash breeders. Researchers in the laboratory of G. A. Thompson at the University of Arizona have studied and cloned the genes encoding important proteins in the phloem transport system of *C. maxima* (Leineweber *et al.,* 2000; Clark *et al.,* 1997; Bostwick *et al.,* 1994). Other recently clonedgenes in *Cucurbita* include a calcium-dependent protein kinase from zucchini (Ellard-Ivey *et al.,* 1999), a class-3 chitinase (Kim *et al.,* 1999), a glyoxysomal malate18 dehydrogenase (Kato *et al.,* 1998), and an anionic peroxidase (Carpine *et al.,* 1999).Cloning of these types of pathway-controlling genes from *Cucurbita* has been gready facilitated by the availability of probes and sequence information from other species where genes with the same function have already been cloned.

2.2.2. Random Amplified Polymorphic DNA (RAPDs) as a Tool for the Identification of Trait-Linked Markers and Molecular Map Construction

In the early days of genetic mapping, there were three types of markers: morphological markers, isozymes, and RFLPs. Morphological markers were often the most directly useful to breeders, but only a few segregate in any given cross, so determining the linkages among them is very

difficult. Also, many traits can be affected by growing environment and modifying genes. Isozymes are easier to use, and being biochemical markers they often have not been directly affected by selection. In addition, they are co-dominant, so heterozygotes can be readily detected. However, there are a limited number of isozymes, polymorphism is often quite low, and they were rarely tightly linked to morphological traits of interest to breeders. RFLPs are also co-dominant markers, and they are stable across populations within a species.

However, RFLP polymorphism is often quite low, and the construction of RFLP probes for a species requires large amounts of DNA and is time consuming (Staub *et al.,* 1996). Thus, while RFLPs have been used extensively in well-studied crops such as the cereal grains, lettuce, tomato, and even melon and cucumber, they have been impractical for low-budget crops such as *Cucurbita.* The 1990s saw the introduction of a number of PCR-based marker technologies. One of these was RAPDs (Williams *et al.,* 1990; Welsh and McClelland, 1990), which has been widely used by plant breeders to build maps and identify markers linked to traits of interest. RAPDs are based on the amplification of DNA using a single primer that is constructed arbitrarily without knowledge of the sequence of the genomic DNA to be amplified. These primers are usually ten nucleotides long, and several thousand different sequences are commercially available.

RAPD markers have been used to create or expand genetic maps in many species, including watermelon (Hashizvune *et al.,* 1996), cucumber (Serquen *et al.,* 1997), melon (Liou *et al.,* 1998), bean (Adam-Blondon *et al.,* 1994), tomato (Stevens *et al.,* 1995), maize (Beavunont *et al.,* 1996), and pea (Rameau *et al.,* 1998). They have also been effectively used to identify markers tightly linked to morphological or disease resistance traits of interest by screening near-isogenic lines (NILs) or bulked segregant analysis of a population segregating for the trait of interest. Bulked segregant analysis (BSA) is based on maximizing the randomness at all loci other than that co-segregating with the trait of interest. This is done by using an F_2 or other segregating generation of a cross between parents of contrasting phenotype, and grouping the progeny based on phenotype for the trait of interest (Michelmore *et al.,* 1991). DNA from the individuals in each group is mixed and used as a template for amplification with RAPD primers or other PCR-based marker technologies. The primary advantage of BSA over NILs is that it saves the

considerable time and effort involved in creating NILs. BSA was designed for detecting markers linked to disease resistance, but it has been used for a number of other traits as well, including seedlessness in grapes (Lahoghe *et al.,* 1998), sex expression in asparagus (Jiang and Sink, 1997), and skin color in apple fruit (Cheng *et al.,* 1996). BSA can also be used with quantitatively inherited traits by making the bulks from plants at opposite ends of the bell curve. This has been done to identify markers linked to plant architecture in pea (Rameau *et al,* 1998) and drought resistance in maize (Quarrie *et al.,* 1999).

2.2.3. Advantages of RAPD Markers

While RAPDs have many limitations as a marker system, there are still many reasons to use them for constructing linkage maps. One reason to use RAPDs is expense. Ragot and Hoisington (1993) compared the costs of using RAPDs and RFLPs for genotyping in maize, and found them to be comparable. However, they noted that RFLPs became prohibitively expensive in developing countries such as Mexico, where the radioisotopes needed to visualize the RFLPs cost six times what they do in the United States. The ability to visualize RAPDs without radioactivity or expensive fluorometnc primers can be a real benefit, both in developing countries and in laboratories away from large university or corporate research campuses, where the systems for handling and disposing of radioactive materials may not be available.

Maize researchers such as Ragot and Hoisington benefit from the large library of publicly available maize RFLP probes, which do not exist for many other species. Staub *et al.* (1996) note that probe development is a major expense of using RFLPs. AFLPs would seem to be a more cost-effective alternative to RAPDs, especially if the bands are visualized with silver stain rather than radioactivity. Like RAPDs, AFLPs are co-dominant, but they result in more bands per reaction and are even better suited to automation than are RAPDs. The major drawbacks of AFLPs is that the initial cost of setting up the lab is greater than for RAPDs, and the reagents are more costly. Depending on the cost of labor, the price difference may be offset by the increased number of bands per reaction with AFLPs. It is apparent from the literature that RAPD markers can be successfully used to construct useful and reliable genetic linkage maps if their weaknesses are taken into consideration and compensated for. Optimization of protocols, standardization of

DNA template concentration, use of a single type and supplier of DNA polymerase, attention to actual temperature profiles when programming thermocyclers, and replication can reduce many of the problems with repeatability of RAPD markers (Rafalski *et al.,* 1994). Careful choice of population structure can prevent loss of information caused by the dominant nature of RAPD markers. Secondary sequencing of bands linked within 5 cM of traits of interest permits construction of primers that are more specific and detection and elimination of co-segregating, non-homologous bands, making markers more robust for use in selection. Anchoring RAPD maps with SSR markers or other markers that are stable across populations can increase their utility. Finally, clustering within the linkage group may be a real result of differences in recombination rate, and not an artifact of RAPD markers or any other marker system.

Chapter Three

Materials & Methods

Materials and Methods

3.1. Plant Materials

Four cucurbits genotypes representing the common cultivated varieties of *Cucurbita moschata* (pumpkin) was selected for this investigation. All genotypes exhibit morphological variation in some characters, such as fruit shape, fruit size and fruit color. Cucurbits plants were grown directly field and maintained during winter season of 2012 in the Experimental Station of Biotechnology and Genetic Engineering Field Laboratory, Khulna University, Khulna.

Table 1: Parent Plant materials used in morphological and molecular analysis.

Serial No	Designation	Source/origin	Release centre
1	Sweety	Bangladesh	Lal Teer
2	Local	Bangladesh	Local market
3	Lalima	Bangladesh	Metal Seed
4	Local	Bangladesh	Local market

3.2. Field Experiment for Collecting Morphology of Pumpkin

The experiment was conducted at the field of Biotechnology and Genetic Engineering Discipline, Khulna University, during September 2012. The seeds were sown on 24 September 2012 and 10 days old seedlings were transplanted in the main field on 4 October 2012 .The experiment was laid out in RCB design with three replications. The unit plot size was (11m x 5 m). (1.5m x 1.25m) Spacing was maintained for each plant. The land was fertilized with cow dung kg 30/plot, TSP 35gm/plant, MP 20gm/plant, and

urea was given when plant 7-8 inches long. The rest of urea and MP were applied in different amounts. Sevin powder was used also to protect the pumpkin beetle. The intercultural operations were done as and when needed. Data on days to 1st male flower open, days to 1st female flower open, node order to 1st male flower open, node order to 1st female flower open, fruit length (cm), fruit diameter (cm), single fruit weight (g), fruit number/ plant, harvest (days) and yield/ plant (kg) were recorded from three randomly selected plants per entry per replication.

3.3 Methods of crossing and generation of F_1 progeny:

A cross between the *C. moschata* common name "Sweety" and *C. moschata* "Local". Both were chosen as a parent. Sweety was chosen because it is resistant to powdery mildew, which is a persistent problem in the winter season, because of its bush habit, and because it contrasts with 'Local' for many traits, including fruit color.

The initial cross was *C. moschata* V_1 (Sweety) x *C. moschata* V_2 (Local'). It was made in the experimental field at Biotechnology and Genetic Engineering Discipline in 13 November 2012. The cross was made in both directions to Sweety. A single F_1 fruit was obtained. Seed was collected and kept for growing in the next. The F_1 population used in this experiment was from the cross with sweety as female and the local, as male. It is not known if the reciprocal crosses differ in any way, as the population with the local, as female and the sweety as male.

The F_1 was seeded into 7.5 cm (3 inch) pots on 26 August 2013. The plants were kept in the field for approximately five weeks under healthy conditions. When plants had 1-2 true leaves, the leaves were harvested for DNA extraction. Plants were grown to maturity in the experimental field.

3.4. Mating design and field experiment and F_1 progeny generation

The experiments were carried out for the F_1 progeny of six crosses between the four promising *Cucurbita* inbred lines (Parents). The parents were crossed in half diallel cross design using Griffing's second method (Griffing, 1956; Garretsen and Keuls, 1978; Madry and Ubysz Borucka,1982). Among six crosses $V_1 \times V_2$ is considered for the generation of F_1 progeny. The crossing scheme is presented in Table 2.

Table 2: Layout of the half diallel cross design based on the Griffing's second method for Four *Cucurbita moschata* inbred lines.

Cucurbita moschata			V_1	V_2	V_3	V_4
V_1	----------					
V_2	$V_1 \times V_2$	----------				
V_3	$V_1 \times V_3$	$V_2 \times V_3$	.....			
V_4	$V_1 \times V_4$	$V_2 \times V_4$	$V_3 \times V_4$			

Note: V_1= Sweety, V_2 = Local, V_3 = Metal Seed, V_4= local

3.5. Genotyping of Pumpkin Germplasm

The CTAB method was followed to extract DNA from the plant leaves at Plant Biotechnology Lab of Khulna University, Khulna. The simplified method for DNA isolation in PCR analysis was used that was developed in (Williams *et al.*, 1990) The quality of the isolated DNA through this procedure was satisfactory for PCR analysis The following steps were followed in PCR-based DNA marker analysis.

3.6. Pumpkin Genomic DNA Extraction

3.6.1. Leaf sample collection

Immature, vigorously growing fresh leaf samples were collected from 30-day old seedlings for isolation of genomic DNA. Leaf sample were collected from selected germplasm. Initially, healthy portion of the youngest leaves of the tiller was cut apart with sterilized scissors and washed in distilled water and ethanol and dried on fresh tissue paper to remove spore of microorganisms and any other source of foreign DNA.

3.6.2. Reagents preparation for DNA extraction (Stock Solution)

Reagents preparation for DNA extraction (Stock solution) that table mention in Appendices-1

3.6.3. Extraction of DNA

DNA extraction was done by using the mini preparation CTAB method. Strict hygiene condition was maintained throughout the DNA extraction process by autoclaving all glass wares, micropipette tips, PCR tubes, distilled water and buffer solutions to avoid contamination. Scissors, forceps, glass rods etc were sterilized using 70% ethanol. Collected leaf sample were cut into 2-3 cm pieces and 0.2g of fully expanded young leaves was taken. It was then washed with tap water. Water was removed with tissue paper and the leaves were grinded with isolation buffer volume at 200 µl. The pest was centrifuges at 10,000rpm for 15 mins. Supernatant was removed and 0.5 µl lysis buffer was added. The mixture was votexed and incubated at room temperature 30°C for 30 mins. 0.5 ml CTAB buffer was then added after 10 mins incubated CTAB into the hot water. Then, incubated at 65°C for 30 mins. Equal volume of Choloform:Isoamyl alcohol (24:1) was added and mixed gently. Centrifuged at 10,000 rpm for 15 mins and aqueous phase was collected in another micro fuse tube. Equal volume of 2-propanol was added and mixed gently. It was kept in ice for 20 mins and centrifuged at 10,000 rpm for 10

mins. The pellet was dissolved in 100 µl TE buffer. 5 µl of RNase solution was added and incubated at 37°C for 1hour. Finally Kept it in freeze (-20°C).

3.6.4. DNA Confirmation Using Gel Electrophoresis

Isolated genomic DNA contains a large amount of RNA and pigments, which causes over estimation of DNA concentration. Therefore, the DNA samples were evaluated both quantitatively and qualitatively agarose gel electrophoresis.

Preparation of 2 % agarose gel (80 ml)

80ml distill water was taken in a beaker and 0.8g agarose was mixed with 80 ml distill water then 800 µl 50x TAE buffer was added. Boiled in oven to dissolved agar. Poured on tray and then comb was set on gel solution. Kept at room temperature for 30 mins.

3.6.5. DNA sample preparation and electrophoresis

Reagents required

- Loading dye (25 mg xylene cyanol, 25 mg bromophenol blue, 3 ml glycerol and sterile distill water 7 ml). Firstly, Glycerol and water was mixed gently. Then bromophenol blue & xylene cyanol was added and store at 4°C.
- DNA marker (λ DNA ladder)

Procedure

Loading dye (5 µl) was placed on a piece of para film using micropipette and 3 µl extracted DNA sample was added into it and mixed well and then the mixture was loaded in the well of the gel. Then the known DNA marker was loaded in the first lane of the gel. Then the gel tank was covered and the electrophoresis apparatus were connected to the power supply unit. After switching on, the DNA was migrated from negative to positive electrode (black to red). The separation process was monitored by the migration of the dye in the loading buffer. Electrophoresis was carried out at 100-50 v for 50-60 minutes.

When the bromophenol blue dye had reached 3/4, th of the gel length, the electrophoresis was stopped and the power supply was disconnected.

3.6.6. Staining of the Gel

Gel was stained at the Ethidium Bromide (EtBr) Solution (250 ml Distill Water, 15 µl EtBr) to observe DNA clearly.

3.6.7. Documentation of the DNA Samples

After electrophoresis, the gel was taken out carefully from the gel chamber and placed on the UV transilluminator (364 nm) in the dark chamber of the Gel Documentation System to observe DNA. The UV light of the system was switched on; the image was viewed on the monitor. The electrophoregram of DNA samples of 5 genotypes are shown in Fig.1.

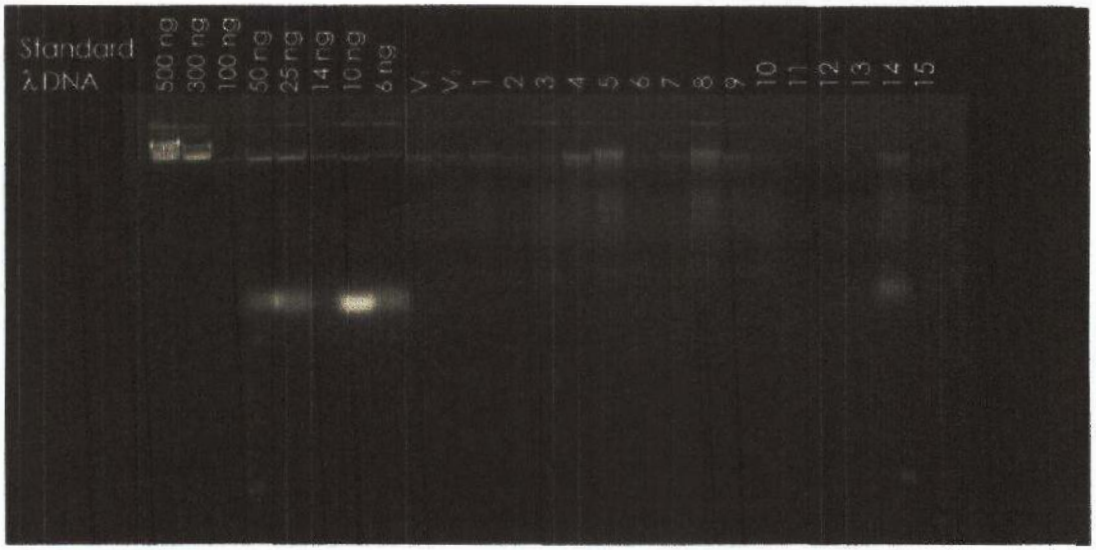

Figure 1: Electrophoregram of eithidium bromide stained genomic DNA samples extracted from different genotypes of Pumpkin.

3.6.8. 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. Thus, it is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay. Excessive genomic DNA may result smears or lack of clearly defined bands in the gel. On the other hand, too little DNA will give non-reproducible patterns (Williams *et al.*, 1990)

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Determination of Quantification by using λ (Lambda) DNA

λ (Lambda) DNA was used for quantification of DNA concentration as 0.5 µl, 1.0 µl, and 2.0 µl. It is to be noted that 0.5 µl λ DNA contains 25 ng/µl DNA. After Gel electrophoresis and staining, the gel was taken out carefully from the electrophoresis chamber and placed on high performance ultraviolet light box (UV transilluminator) for checking the DNA bands. The DNA was visualized as band and photographed using Gel DOC. Few DNA bands of the germplasms were clear, some bands were found hazy and some showed smears. Dilution was done simply by adding sterilized ddH_2O. A concentration of about 25 ng/µl was maintained in the DNA samples.

3.6.9. Preparation of working solution (25 ng/ µl) of DNA samples

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (25 ng/ µl) using the following formula:

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

Where,

S_1= Initial strength (ng/ µl),

V_1= Initial volume (µl),

S_2= Final strength (ng/µl) and

V_2= Final volume (µl).

3.7. PCR Reaction

3.7.1. Primer Selection

Primers were selected based on intensity of bands, presence of smearing, consistency within individuals and potential for population discrimination. A final subset of five primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis.

Table 3: Random Primer Used in the Present Study for Screening.

Primer code	Sequence (5' – 3')	GC content (%)	Tm of Primer	Annealing Temp.(Ta)
A17	GGTTCGGGAATG	58.3	38°C	38
A21	GTGACCGATCCA	58.3	38°C	35
A23	AAGTGGTGGTAT	41.7	34°C	35
A24	GACGGTTCAAGC	58.3	38°C	35

Calculation of Annealing Temperature:

Annealing temperature was calculated by the following formula-

0.3× Tm (Primer) + 0.7×Tm (Product) -25

3.7.2. PCR Amplification

PCR amplification was carried out according to the protocol of Williams *et al.* (1990) with minor modifications. The following components were used to prepare PCR mixture. The total volume of PCR mixture was 8 µl per sample. 2µl genomic DNA was added with 8µl PCR mixture and finally, total volume was made 10 µl.

Table 4: PCR master mixture (10µl).

Sl. No.	Reagents	Amount Per sample
1	Primer (1µM)	0.5µl
2	dNTPs(300µM)	0.3µl
3	*Taq* DNA polymerase(1U)	0.2µl
4	Reaction buffer(10x)	1µl
5	$MgCl_2$ (1.5mM)	0.6µl
6	dH_2O	6.4µl

3.7.3 Temperature Profile

The first amplification cycle consisted of the following steps:

1) Initial denaturation at 94°C for 5 min
2) Denaturation at 94°C for 1 min
3) Annealing at 35°C for 30 Sec
4) Elongation or extension at 72°C for 2 min
5) Cycle to step 2 for 40 more time.
6) Final extraction at 72°C for 5 min.
7) Completion of cycling program (41 cycles), reactions were held at 8°C

3.7.4. Electrophoresis of the amplified products and documentation

Amplified PCR products were electrophoresed on an agarose gel (2%) in TAE buffer and visualized by staining with ethidium bromide solution. Band documentation also calculated and polymorphisms also documented by the observation of UV transilluminator and picture was taken by the GEL DOC.

3.8. Statistical analyses

Statistical data is analyzed by the some one line tools of statistics. Morphological and molecular significance analyzed is done by the following tools. The Chi Square and P value analysis also calculated by the following tools of social statistics.

http://www.socscistatistics.com/tests/chisquare/Default2.aspx

http://easycalculation.com/statistics/chi-square.php

http://www.socscistatistics.com/pvalues/chidistribution.aspx

http://easycalculation.com/statistics/chi-square.php

http://www.fgse.nova.edu/edl/secure/stats/lesson7.htm

3.9. Calculating the cophenetic correlation coefficient

Suppose that the original data {Xi} have been modeled using a cluster method to produce a dendrogram {Ti}; that is, a simplified model in which data that are "close" have been grouped into a hierarchical tree. Define the following distance measures

- $x(i,j) = |X_i - X_j|$, the ordinary Euclidean distance between the ith and jth observations.
- $t(i,j)$ = the dendrogrammatic distance between the model points T_i and T_j. This distance is the height of the node at which these two points are first joined together.

- Then, letting $\bar{x}$ be the average of the $x(i,j)$, and letting $\bar{t}$ be the average of the $t(i,j)$, the cophenetic correlation coefficient c is given by Math works statistics toolbox

$$c = \frac{\sum_{i<j}(x(i,j) - \bar{x})(t(i,j) - \bar{t})}{\sqrt{[\sum_{i<j}(x(i,j) - \bar{x})^2][\sum_{i<j}(t(i,j) - \bar{t})^2]}}.$$

3.2.0. RAPD Analysis

Each accession was scored manually for presence or absence of a particular amplification product. Data was analyzed by the Plygel gel software and Pair-wise distances between the accessions based on (a) Jaccard similarity metrics (Jaccard 1907), (b) Distance matrix based on Jaccard coefficient. The unweighted pair group method with arithmetic averages (UPGMA)-based dendrogram was constructed using the Dendro UPGMA: (created at March 2002, modified at October 2006, Novembre 2008, January 2009 and December 2009 by Dr. Santi Garcia-Vallvé (santi.garcia-vallve AT urv.net) and Dr. Pere Puigbo Biochemistry and Biotechnology Department. Universitat Rovira i Virgili (URV). Tarragona. Spain.A dendrogram construction utility). . Numerical value was considered to construct the dendogram and presence of band (1) and absence of band (0) value consider for these RAPD analysis.

Chapter Four

Results & Discussion

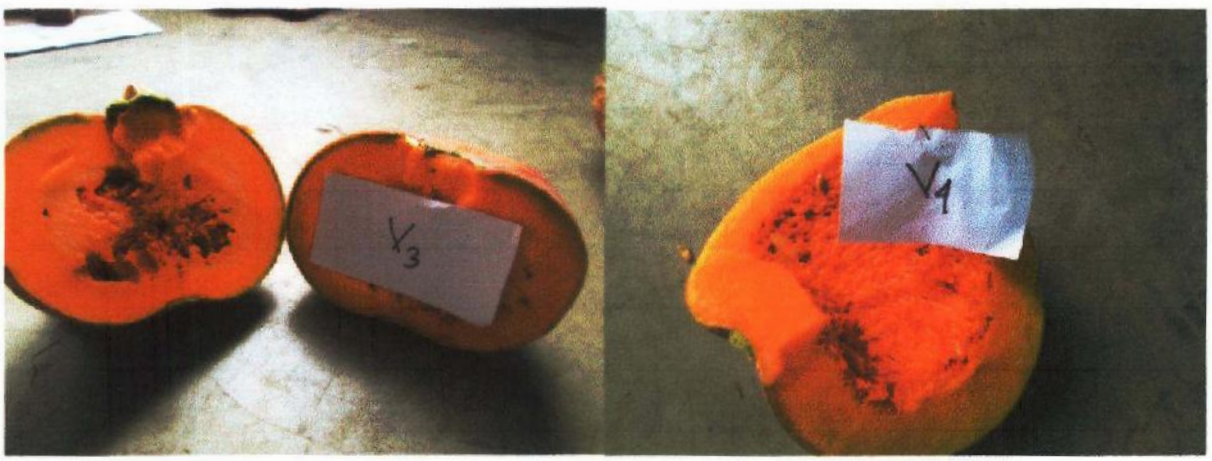
Results and Discussion

Morphological and molecular characterizations were used to the 4 accessions and displayed different levels of variability concerning the morphological traits of pumpkin. Molecular characterization was determined by using the molecular marker. RAPD markers are most useful for understanding the naturally occurring polymorphisms in living organisms.

4.1. Morphological Characterization

Morphological characteristics of four commonly available varieties of pumpkin have been given in (Table 5) such as leaf colour, length, wide, fruit colour, shape, thickness, seed producing rate etc. The varieties were cultivated in 24 September 2012 in the field laboratory of Biotechnology and Genetic Engineering Discipline and showed morphological similarities and dissimilarities. Among the 4 varieties, Variety Sweety (V_1) was performed best by considering the early flowering & early fruiting as well as moderate weight increased the fruits per plant that was well known. It is the best varieties to human consumption and farmers would like to cultivate it for the best performance of the variety. Similarly, Local also formed good performance in contrast of Sweety variety (Table 5).

Sweety (V_1) Local (V_2)



Lalima (V_3) Local (V_4)

Figure 2: Four parental pumpkin *(Cucurbita moschata)* fruit.

Table 5: Morphological analysis of Pumpkins Leaf and fruit.

Variety Name	Leaf Characteristics		First Flowering date	First Fruiting Date	Fruits characteristics				Fruits per plant	Seed producing rate
	Color	Size(LxW)inch			Weight	Thickness	Shape	Color		
Sweety (V_1) (Hybrid)	Green	8.9 X 7	12.11.12	17.11.12	2.6	2	Round	Yellow	8	Low
Al amin (V_2) (Local)	Green	6.48 X 3.26	11.11.12	18.11.12	1.4	1.3	Round	Yellowish green	5	Moderate
Metal (V_3) (Hybrid)	Light Green	8.2 X 6.4	13.11.12	18.11.12	2.7	1.2	Oval	Green	7	Moderate
Dewan(V_4) (Local)	Green	5.85 X 3.46	12.11.12	19.11.12	2.4	1.4	Round	Yellowish green	6	Low

4.2. Morphology of flowers produced and corolla length and texture of 4 accessions of *Cucurbita moschata* parents

It is well-known that *Cucurbita moschata L.* is extremely diverse in fruit characteristics. This diversity has been depicted and described inmany publications and cultivar-groups have been categorized on the basis of fruit shape. In addition to the extreme diversity in fruit size, shape, and color, there is also a great diversity in seed size and relative dimensions in vegetative characteristics and in characteristics of the flowers. Observed a number of characteristics of the flowers that appear to be relevant: number produced per plant of each, male and female, corolla length and corolla texture.

The results in (Table 6) indicate that large differences occurred among the accessions for number of flowers produced over the period of the experiment. Plant sexuality also differed greatly, ranging from accessions producing mostly male flowers and to others producing mostly female flowers. The accessions also differed greatly in the length of the corolla. Furthermore, the flowers, both male and female, of the V_1 is fairly firm and V_2 accessions is nearly firm and V_3 and V_4 accessions were softer. (Table 6)

Table 6: Number of flowers produced and corolla length and texture of 4 accessions of *Cucurbita moschata* parents.

Accession	No of Flowers					Corolla length (cm)		Corolla texture[z]	
	Male	Female	Chi Square (x^2)	P Value	Total	Male	Female	Male	Female
V_1	31	15	2.78	0.095448	54	12	11	1.5	2.0
V_2	25	10	3.21	0.073189	34	9	9	1.5	2.2
V_3	18	11	0.85	0.356552	35	10	8	1.0	1.0
V_4	16	9	0.98	0.322199	29	8	8	1.0	1.0

V_1= The result is significant at $p < 0.10$.

V_2 =The result is significant at $p < 0.10$.

V_3= The result is not significant at $p < 0.10$.

V_4= The result is not significant at $p < 0.10$.

Is not 1% level significant

[z]Scale of 1=soft, 2=fairly firm, 3=firm

Note: V_1= Sweety, V_2 = Local, V_3 = Metal Seed, V_4= local

Table 7: Number of F_1 by species and percentage of presence by provenance.

species	Provenance	Source[b]	Number of plants		χ^2	P Value	Presence % by Provenance
			Presence	Absence			
Cucurbita maxima Sweety (V_1)	Argentina Brasil India Bangladesh	Lal Teer	17	3	4.9	0.026857	85
Cucurbita maxima Local (V_2)	Bangladesh	Local	15	5	2.5	0.113846	75
Total			32	8			Average 80%

[b]= Tal Teer Seed Company, Bangladesh

[b]= Local region, Bangladesh.

V_1=The result is significant at $p < 0.05$.

V_2=The result is not significant at $p < 0.010$.

(a) (b)

Figure 3: F_1 is germinated and transplanted in the field.

This results suggest that the F_1 generation of the wild-crop cross does not present a strong barrier to introgression of crop genes into free-living *Cucurbita* populations. Quesada *et al.* (1991, 1993, 1996) showed that subsequent generations of offspring of such hybrids are viable (Table 07). Allozyme frequency distributions and distinctive patterns of variation in fruit structure, colour, and bitterness within populations of free-living *C. pepo* indicate that past hybridization events have resulted in introgression between cultivated *C. pepo* L and free-living *C. pepo* (Decker and Wilson, 1987; Wilson, 1990).

4.3. Primers and randomly amplified polymorphic DNA (RAPD) banding patterns.

RAPD has been successfully used for the assessment of genetic diversity. *Cucurbita* genotypes. So our work based on screening the parental and F_1genotypes with RAPD (Fig- 4, 5, 6 and 7). The 4 recruited primers yielded a DNA amplification pattern of 16 fragments; flanking primer average was 4.0 fragments/primer (Table 8). The primer A21 had the greatest number of DNA-amplified fragments (6 bands)which 5 of them were polymorphic. While, the primer A23 had the lowest number of fragments regards to amplification (1) and polymorphism (1). The level of polymorphism **90.84%** obtained was higher than some similar studies such as Melo *et al.* (2001) who obtained **61.46%** of polymorphic bands working with hybrids. Also, Lanza *et al.* (1997) obtained 80.6% of polymorphism in studying genetic divergence between inbred lines using RAPD markers. The level of polymorphism to be obtained depends on the degree of divergence between the genotypes under study.

Four primers (Table 3) were selected that used in RAPD analysis of 4 pumpkin accessions amplified 16 different reproducible bands. Four different primers generated various banding patterns ranging from 1 to 6 and the average bands per primer was 4.0 and of the 16 bands scored, 14 **(90.84%)** were found to be polymorphic and 2 bands (0.5%) were found to be common in nature.

Table 8: RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 4 pumpkin accessions

#	Primer code	Sequence (5'-3')	RAPD total bands	Polymorphic bands	Monomorphic bands	Percentage of polymorphic markers
1	A17	GGTTCGGGAATG	5	4	1	80%
2	A21	GTGACCGATCCA	6	5	0	83.34%
3	A23	AAGTGGTGGTAT	1	1	0	100%
4	A24	GACGGTTCAAGC	4	4	1	100%
Total			16	14	2	363.34
Mean			4.0	3.5	0.5	90.84%

The frequencies of polymorphic bands varied from primer to primer. Ferriol *et al.* (2003a) studied the genetic diversity of 19 accessions of *Cucurbita maxima* along with 8 related *Cucurbita* accessions from Spain using Randomly Amplified Polymorphic DNA (RAPD) and Sequence Based Amplified Polymorphisms (SBAP) markers and found 57% and 33 % polymorphisms for these markers, respectively. In the present study using ISSR and SRAP markers, we found 100 % polymorphisms for these markers, respectively.

Though no accession-specific marker was identified, the high level of polymorphism revealed by the proportion of polymorphic loci (**90.84%**) indicated that RAPD markers could be considered as effective tools for estimating genetic diversity in different accessions of pumpkin. Accessions could be distinguished by a combination of fragments and differences between clusters reflected differences in frequencies rather than presence or absence of accession specific fragments.

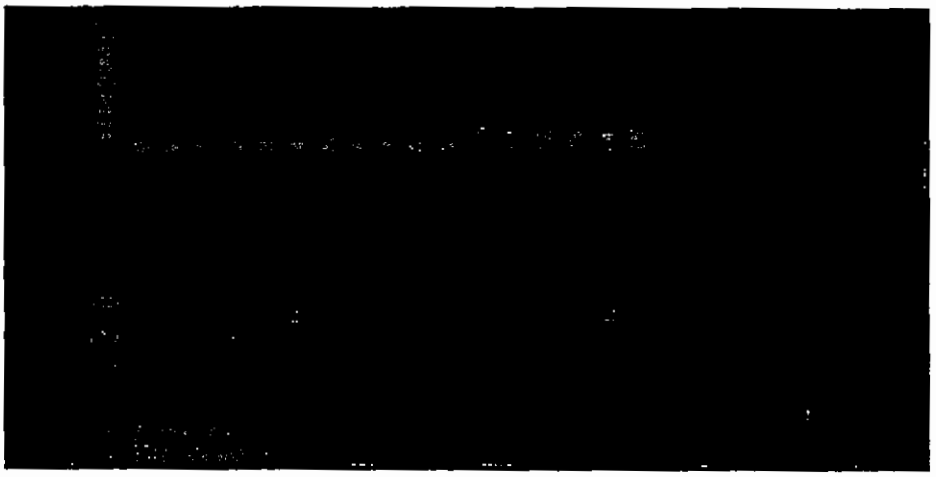


Figure 4: RAPD pattern generated by the primer A17 with parents and F_1 genotypes of *Cucurbita moschata* .The number corresponds to the serial number of the F_1 genotypes.

Figure 5: RAPD pattern generated by the primer A21 with parents and F_1 genotypes of *Cucurbita moschata* .The number corresponds to the serial number of the F_1 genotypes.

Figure 6: RAPD pattern generated by the primer A23 with parents and F_1 genotypes of *Cucurbita moschata.* The number corresponds to the serial number of the F_1 genotypes.

Figure 7: RAPD pattern generated by the primer A24 with parents and F_1 genotypes of *Cucurbita moschata* .The number corresponds to the serial number of the F_1 genotypes.

4.4. Similarity co-efficient of *Cucurbita moschata* Parents and F_1 genotypes based on RAPD markers

The genetic similarity based on polymorphism was found to range from 1.000 to 0.000for parental genotypes (Table 9). The similarity values based on Jaccard's similarity coefficient between the F_1 and the donor parents plant. The dendrogram generated through UPGMA analysis revealed 40 % similarity amongst the regenerants and the donor V_1 parent plant and 60% similarity amongst the V_2 donor parent plant.

The highest genetic similarity was reported among V_1 parent and 2, 4,7,10 F_1 (1.00) as they localized at the same sub-cluster (Fig-8) and V_2 parent and F_1 (1, 9, 13, 14, 15) also shows the highest genetic similarity (1.00) (Table 9). While the lowest genetic similarity was observed between 6 and 12 F_1 (0.000) they were found in different clusters (Fig-8). On the other words, the genetic distance between V_1 and 5 F_1 is 0.400 that is equals to the 2, 4, 7 F_1 and the genetic distance between V_1 and 8 F_1 is 0.357 that is equals to the 2, 4, 7 F_1 so the subcluster of 5 and 8 F_1 is relates to V_1 parents.

Again F_1 (3, 6,11 and 12) is genetically similar to the V_2 parent because the genetic distance between V_2 and 3 (0.400), V_2 and 6 (0.143), V_2 and 11 (0.308) and V_2 and 12 (0.100) (Table 10) that is equals to the F_1 (1and 9) (Fig-8).

Table 9: Similarity matrix based on Jaccard's similarity coefficient.

Similarity Matrix computed with Jaccard coefficient

	V1	V2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
V1	1	0.385	0.385	1	0.278	1	0.4	0.286	1	0.357	0.385	1	0.267	0.083	0.385	0.385	0.385
V2		1	1	0.385	0.4	0.385	0.267	0.143	0.385	0.308	1	0.385	0.308	0.1	1	1	1
1			1	0.385	0.4	0.385	0.267	0.143	0.385	0.308	1	0.385	0.308	0.1	1	1	1
2				1	0.278	1	0.4	0.286	1	0.357	0.385	1	0.267	0.083	0.385	0.385	0.385
3					1	0.278	0.412	0.312	0.278	0.375	0.4	0.278	0.222	0	0.4	0.4	0.4
4						1	0.4	0.286	1	0.357	0.385	1	0.267	0.083	0.385	0.385	0.385
5							1	0.267	0.4	0.538	0.267	0.4	0.429	0.077	0.267	0.267	0.267
6								1	0.286	0.214	0.143	0.286	0.417	0	0.143	0.143	0.143
7									1	0.357	0.385	1	0.267	0.083	0.385	0.385	0.385
8										1	0.308	0.357	0.2	0.091	0.308	0.308	0.308
9											1	0.385	0.308	0.1	1	1	1
10												1	0.267	0.083	0.385	0.385	0.385
11													1	0.2	0.308	0.308	0.308
12														1	0.1	0.1	0.1
13															1	1	1
14																1	1
15																	1

4.5. Estimation genetic Variability by the Distance matrix based on Jaccard coefficient

There was a high level of genetic variation among the studied accessions of pumpkin as indicated by the proportion of polymorphic loci. Estimation of higher level of genetic variation in the pumpkin might be consistent with the fact that it is a highly polymorphic plant. The values of Distance matrix based on Jaccard coefficient between parents and F_1 pumpkin genotypes were computed from combined data sets for the 4 primers ranging from 0.000 to 1.000 (Table 13). Comparatively higher genetic distance (1.000) was found between (3 and 12) and (6 and 12) F_1. The lowest genetic distance **(0.000)** was revealed between V_1 and F_1 (2, 4, 7 and 10) and V_2 and F_1 (1, 13, 14 and 15). The range of genetic distance of 2 parents and 15 F_1 genotypes is (0.000±1.000) and the difference between the highest and lowest genetic distance indicated the presence of variability among the 15 F_1 genotype and 2 parents of pumpkin.

Table 10: Distance matrix based on Jaccard coefficient

Distance matrix based on Jaccard coefficient

	V1	V2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
V1	0	0.615	0.615	0	0.722	0	0.6	0.714	0	0.643	0.615	0	0.733	0.917	0.615	0.615	0.615
V2		0	0	0.615	0.6	0.615	0.733	0.857	0.615	0.692	0	0.615	0.692	0.9	0	0	0
1			0	0.615	0.6	0.615	0.733	0.857	0.615	0.692	0	0.615	0.692	0.9	0	0	0
2				0	0.722	0	0.6	0.714	0	0.643	0.615	0	0.733	0.917	0.615	0.615	0.615
3					0	0.722	0.588	0.688	0.722	0.625	0.6	0.722	0.778	1	0.6	0.6	0.6
4						0	0.6	0.714	0	0.643	0.615	0	0.733	0.917	0.615	0.615	0.615
5							0	0.733	0.6	0.462	0.733	0.6	0.571	0.923	0.733	0.733	0.733
6								0	0.714	0.786	0.857	0.714	0.583	1	0.857	0.857	0.857
7									0	0.643	0.615	0	0.733	0.917	0.615	0.615	0.615
8										0	0.692	0.643	0.8	0.909	0.692	0.692	0.692
9											0	0.615	0.692	0.9	0	0	0
10												0	0.733	0.917	0.615	0.615	0.615
11													0	0.8	0.692	0.692	0.692
12														0	0.9	0.9	0.9
13															0	0	0
14																0	0
15																	0

4.6. Cluster analysis based on coefficient of similarity and dissimilarity

Two different coefficients were employed to contrast pair wise similarity and dissimilarity among accessions. The UPGMA cluster analysis revealed following results. The coefficient of similarity and UPGMA revealed two main clusters among 2 parents and 15 F_1 genotypes of *Cucurbita maxima* except accession number 12, i.e., cluster A and cluster B at 93.33% overall similarity within parents and the F_1, accessions have been grouped into Cluster A was divided into two sub cluster A_1 and A_2 (Fig-08).A_1 is further divided into 2 groups A_{1a} and A_{1b}. There were 5 accessions V_1, 2, 4, 7 and 10 in sub cluster A1a and 2 accessions in 5 and 8 are in sub cluster A_{1b}. A_2 is further divided into two groups A_{2a} and A_{2b}. There were 6 accessions (V_2, 1, 9, 13, 14 and 15) in sub cluster A_{2a}. Accession number 3 in one group (A_{2b}) and B is sub divided into B_1 and B_2. Accession number 6 and 11 in B_1 and B_2.

In the present investigation, 6.66% variation was observed amongst the parents and F_1. This small genetic variation in DNA may be attributed to naturally occurring variation or due to the accumulation of mutation. Genetic variation induced by such genetic and epigenetic mechanism can be reflected in the banding profile developed by different marker system (Phillips *et al.*1994). Therefore, it could be recommended as a reservoir of alleles useful for breeding programs in parental crosses. The multivariate analysis using the principle component analysis separated all the cultivars on the first component indicating the high correlation between them. The strongest Cophenetic Correlation Coefficient (CP) = 0.621254491674655 was confirmed between the two parents and their F_1.

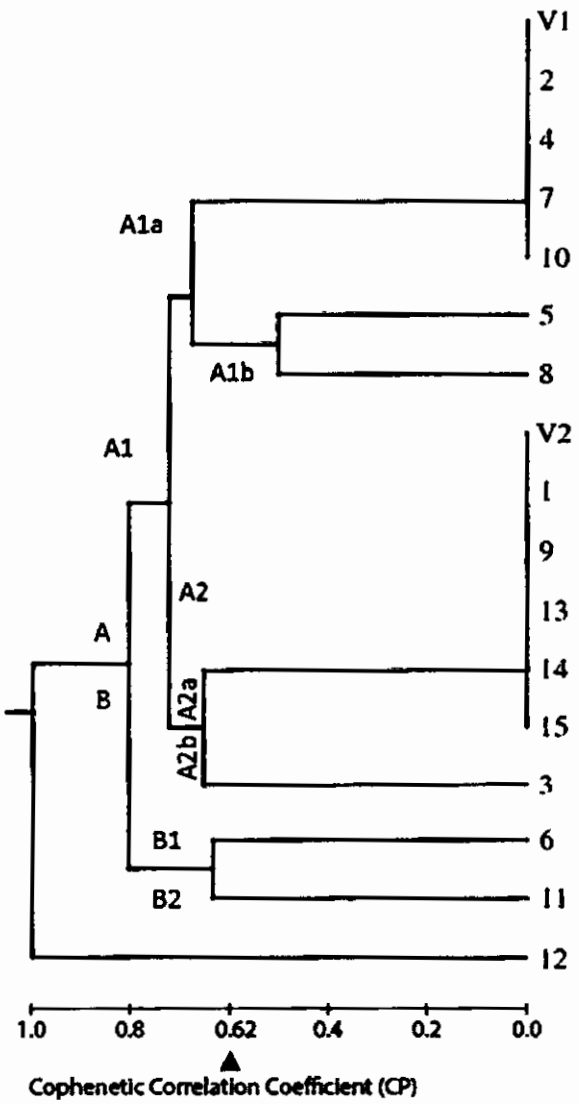


Figure 8: Dendrogram produced using UPGMA cluster analysis based on similarity matrix demonstrating the association among 17 accessions; similarity values were calculated according to Jaccard (1907) based on 16 different RAPD polymorphic fragments

Note: V_1 and V_2 are the parents of (Sweety × Local) and F_1= (1 to 15)

4.7. P value and statistical significance of Genetic diversity

Genotyping the F_1 progeny derived from Sweety and 'Local revealed 40% similarity with V_1 and 60& similarity with the V_1 parents. Polymorphic RAPDs with distorted GD, based on chi-square

goodness of fit. These were eliminated from further analysis. Of the remaining 4 RAPD primers, Chi squared equals 4.000 and the two-tailed P value equals 0.0455 By conventional criteria, this difference is considered to be statistically significant ($p< 0.05$).

Chapter Five

Summary & Conclusion

Summary and Conclusion

The present phenotypic investigation was conducted in Biotechnology and Genetic Engineering field Laboratory, Khulna University, Khulna. Morphological characters analyzed through field experiments and the phenotypic characteristic considered according to the leaf colour, length, wide and fruit colour, shape, thickness etc. Four selective varieties of pumpkin were considered for a successful crossing by the Griffing's second method and evaluate morphological and molecular characterization among the parents and their F_1. Among the four varieties Sweety was performed the best regarding the high yielding performance compared to others and that was well known because it is an established variety by the Lal Teer Seed Company. On the other hands, a local variety was performed better in leu of Sweety, s performance.

With the most genetic diversities (Mohanty and Mishra, 1999) pumpkin usually manifests itself in many ways, such as fruit shape,fruit weight, leaf size, etc. The rich of diversity provide more selection chances in breeding, but brings complexity in classification of germplasm resources and breeding selection. In our study, the diversities of 4 traits were summarized into 3 major factors represented by leaf factor (represented by leaf size), fruit factor(represented by fruit width) and flesh quality factor. These 3 major factors could be as the priority indexes in screening of germplasm and breeding selection.

RAPD analysis was conducted and DNA amplified by four decamer random primers, was used to characterize 2 parents and their 15 F_1 pumpkin genotypes based on genetic variability and relatedness among them. In the RAPD technique, alleles were separated on 2% agarose gel electrophoretically, stained with ethidium bromide, and visualized on UV transilluminator.

Four primers produced 16 scorable bands in this study, of which 14 were considered as polymorphic. Two common band was found and the percentage of polymorphism **(90.84%).** The primer A17, A21 and A24 were amplified maximum number of

polymorphic bands. The genetic similarity based on polymorphism was found to range from 1.000 to 0.000 for parental genotypes. The similarity values based on Jaccard's similarity coefficient between the F_1 and the donor parents plant. The dendrogram generated through UPGMA analysis revealed 40 % similarity amongst the regenerants and the donor V_1 parent plant and 60% similarity amongst the V_2 donor parent plant.

The greater GD between C. maxima accessions and F_1 and firstly separating between interspecies of parents in this study. That were accorded with the previous studies that relationship between interspecies were more distant than that of intraspecies (Li, 2006). None of the classifications obtained clearly grouped the different accessions based on geographic origin in C. moschata, which was accord with previous grouping by DNA markers (Zhang, 2005b; Chu *et al.*, 2007). The same phenomenon seems to occur among farmers from Malawi and Zambia, where 40% of the C. moschata seeds are exchanged (Gwanama *et al.*, 2000) and also in Mexico, where the percentage of C. moschata seed exchange is approximately 62% (Montes-Hernaindez and Eguiarte, 2002).

The RAPD analysis discovered sufficient genetic relationship and diversity between the some cultivars of *Cucurbita moschata* (pumpkin) and their F_1. No information on genetic structure of the pumpkin was available in Bangladesh. Only 4 polymorphic markers were generated which was possibly not sufficient to cover *Cucurbita* spp genome particularly the regions influencing the expression of quantitative traits. Hence, sufficient and more efficient RAPD primers showing maximum number of polymorphic bands or other available markers systems could be utilized for analysis of *Cucurbita*. The result of the present study indicated that RAPD can be, used for identification of the available pumpkin gremplasm. It can also be used to study relationship of pumpkin germplasm among each other for further improvement through cross breeding

Chapter Six

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Appendices-1

Reagents preparation for DNA extraction (Stock solution)

Component	Concentration (100ml)
1. Isolation Buffer	
Polyethylene glycol (8000) ---------- 10%-----	10g
Sorbitol----------0.35M-----	6.38g
Tris ----------0.1M-------	10ml
1% PVP----------	1g
Mercaptoethanol----------	0.5 ml(500µl.)
2. Lysis Buffer	
Sorbital ----------0.35M ----	6.38g
Tris ----------0.1M------	10ml
N-Lauroylsarcosine----------10%--------	10g
3. CTAB buffer	
CTAB---------- 2%--------	2g
Tris---------- 0.1M-------	10ml
EDTA---------- 0.02M-----	4 ml
NaCl---------- 1.4M------	8.2g
4. TE buffer	
Tris ---------- 0.01M -----0.5ml	
EDTA---------- 0.001M-----0.1 ml	
5. Chloroform : Isoamyl alcohol (24:1) ----------50 ml	
Chloroform----------48 ml	
Isoamyl alcohol----------2 ml	