MOLECULAR DIVERSITY STUDY OF DIFFERENT HYBRID VARIETIES OF BOTTLE GOURD (*Lagenaria siceraria* Mol.)

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MOLECULAR DIVERSITY SYUDY OF DIFFERENT HYBRID VARIETIES OF BOTTLE GOURD (*Lagenaria siceraria* Mol.)

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CERTIFICATE

*This is to certify that the thesis entitled "**MOLECULAR DIVERSITY STUDY OF DIFFERENT HYBRID VARIETIES OF BOTTLE GOURD (Lagenaria siceraria Mol.)**" submitted to the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE (MS) IN BIOTECHNOLOGY, embodies the result of a piece of bonafide research work carried out by MAHBUBA HOQUE MUNNI, Registration No. 17-08286 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed during the course of this investigation has been duly acknowledged and style of this thesis has been approved and recommended for submission.

Dated: December, 2018

Place: Dhaka, Bangladesh

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Prof. Dr Md. Ekramul Hoque

Supervisor

ABBREVIATION

FULL WORD	ABBREVIATION
Agriculture	Agril.
American	Am.
Amplified Fragment Length Polymorphism	AFLP
And other (at Elli)	*et al.*
Base pair	bp
Biology	Boil.
Biotechnology	Biotech.
Botany	Bot.
Continued	Cont'd
Degree Celsius	^{0} C
Deoxyribonucleic acid	DNA
Distilled deionized water	ddH_2O
Etcetera	etc.
Ethidium Bromide	Et-Br
Ethylene Diamine Tetra Acetic Acid	EDTA
Genetics	*Genet.*
Government	Govt.
Gram	g
Gram per litre	g/L
International	Intl.
Journal	J.
Marker-assisted breeding	MAS
Micro litre	µl
Milli litre	ml
Mili mole	mM
Molecular	Mol
Namely	viz.
Negative logarithm og hydrogen ion concentration (-log [H^+])	pH
Per cent	%
Polymerase chain reaction	PCR
Polymorphic information content	PIC
Random Amplified Polymorphic DNA	RAPD
Restriction Fragment Length Polymorphism	RELF
Research	Res.
Rotation per minute	rpm
Science	*Sci.*
Single Nucleotide Polymorphism	SNP
Simple Sequence Repeat	SSR
Sodium Dodecyl Sulphate	SDS
Species	Sp.
That is	i.e
Tris Boric Acid EDTA	TBE
Tris- EDTA	TE
Unweighted pair group of arithmetic mean	UPGMA
Ultraviolet	UV
Volt	V

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The Author

LIST OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

MOLECULAR DIVERSITY STUDY OF DIFFERENT HYBRID VARIETIES OF BOTTLE GOURD (*Lagenaria siceraria* Mol.)

ABSTRACT

Bottle gourd (*Lagenaria siceraria* Mol.) is one of the most important Cucurbitaceous vegetables in the world. It is widely grown for leafy and green fleshy vegetables. This study was conducted to determine the molecular diversity among eight bottle gourd hybrid varieties which were collected from three renowned private Seed Companies (Metal Agro Limited, ACI Limited, Lal Teer Seed Limited) of Bangladesh. A total of nine primers (RAPD, ISSR and SSR) were used to determine polymorphism among bottle gourd hybrids to produce scorable DNA bands. Total 26 bands were amplified by nine primers. Among them 18 were polymorphic bands. The range of DNA amplification varied from 1000-100 bp. Average 64.82% polymorphism was obtained among the genotypes. Genetic diversity ranged from 0.2188 to 0.5000 and the frequency of major allele ranged from 0.5000 to 0.8750. Nei's genetic distance ranged from 0.2308 to 0.7308. The PIC value ranged from 0.1948 to 0.3750 with an average value 0.3243. The PIC value indicates that the studied bottle gourd hybrids had medium level of molecular diversity. A dendrogram indicating the relative genetic similarity of the bottle gourd hybrids was constructed which followed three major clusters (A, B and C) among the studied material. The two hybrid H7 and H8 showed more similarity in genetic identity that fall in the same group-B which has collected from same seed company. Likewise, the hybrid H5 and H6 classified in the same group-C were also obtained from same seed company. Group-A contains the two hybrids H3 and H4 which are collected from Lal Teer Seed Ltd. The present investigation revealed that, the bottle gourd hybrid developed by the seed companies has more similarity within their own genotypes but to some extend genetically diverse between different seed companies. These experimental findings can be used for the protection of hybrids in commercial purpose and in managing bottle gourd genetic resource in Bangladesh.

CHAPTER I
INTRODUCTION

Bottle gourd [*Lagenaria siceraria* (Mol.) Standl] is a diploid crop (2n=2x=22), belonging to Cucurbitaceae family. Its estimated genome size is of 334 Mb (Beevy and Kuriachan, 1996; Achigan-Dako *et al*., 2008) and distributed over 11 chromosomes (Decker-Walters *et al*., 2001). Phylogenetic analysis has shown a close genetic relationship of bottle gourd with other economically important cucurbit species such as cucumber, melon and watermelon (Xu *et al*., 2011). Indeed, the partial sequence of bottle gourd genome revealed a 90% sequence similarity to the cucumber genome. A total of six species have been known belonging to the genus *Lagenaria*, out of which only *L. siceraria* is domesticated for human use. It is monoecious and annual in nature, while other five *Lagenaria* species are wild congeners, perennial and dioecious in nature. Bottle gourd is a hairy, rapid growing trailing climbing annual herb, extending 3 to 15 meters in length. It is a highly cross-pollinated crop due to monoecious plant which has resulted in a large variation in shape and size of fruits. Bottle gourd fruits has different shapes resembling as a bottle. The most common shapes are cylindrical, round, oval and oblong. The yellowish-green or creamish coloured fruits are relatively soft in texture with white pulp and large white seeds. Bottle gourd is considered to be originated in Africa and America and it occurs in wild form in South Africa and India. However largest variability among *Lagenaria spp*. is reported from India. It has an ancient pan-tropical distribution and is one of the earliest crops known to have been cultivated in both the old and new continents in the pre-Columbian era. The earliest record of white-flowered gourd utilization comes from Peru dating back to BC 13,000-11,000 (Whitaker 1971). Bottle gourd is an economically important crop cultivated worldwide for vegetable purpose or medicinal practice. Numerous health benefits are reported in bottle gourd including its anti-cancerous, cardioprotective (Fard *et al*. 2008), diuretic, aphrodisiac, general tonic, antidote to certain poisons and scorpion stings, alternative purgative and cooling effects (Badmanaban and Patel, 2010). It can also be used to cure pain, ulcers and fever and is used for pectoral cough, asthma and other bronchial disorders (Upaganlawar and Balaraman, 2010). Fresh bottle gourd juice is used as medicine to cure various diseases including flatulence, diabetes mellitus, hypertension, liver diseases and as a diuretic (Ghule *et al*., 2007).The seeds of this crop are rich in amino acids and oil. Some bottle gourd types are exclusively grown for their seeds (Achigan-Dako *et al*., 2008).

Nutritively, bottle gourd fruit contains 96.1% moisture, 0.2 g protein, 2.5 g carbohydrates, 0.1 g fat, 0.5 g minerals, 0.3 mg thiamine, 0.01 mg riboflavin, 0.2 mg niacin and 12 kcal energy per 100 g fresh weight (Gopalan *et al*., 1982 and Singh *et al*., 2001). It contains higher concentrations of dietary fibre, protein, Vitamin A, Vitamin C, Vitamin K, Vitamin B6, Vitamin E, Folate, Pantothenic acid, Potassium, Manganese, Calcium, Iron, Magnesium, Phosphorus and Selenium. In Bangladesh it is mainly a winter crop but now-a-days it is grown all the year-round as a commercial way in the field as well as homestead in rural areas. Vegetables are grown in Bangladesh at 2.63 per cent of cultivable land (BBS 2014). Total vegetable growing area in the country is about 225,153 hectare; of which 65% are cultivated during winter. People of Bangladesh take 463.9 gm of cereal per capita per day whereas actual cereals need is 372 gm per capita per day (BBS, 2011). On the other hand, people take 166.1 g vegetable per day whereas the requirement is 200 g per capita per day (BBS, 2011). Though rice is the main food item for the people of the country, rice alone cannot solve the demand for balanced diet. A large percentage of people in Bangladesh are suffering from severe malnutrition (Awal, 2013). If enough vegetables are not provided to the nutritional deficiency will be to a greater extent. Due to urbanization and industrialization ensuring food security getting harder because of declining cultivable land. Commercially produced highly nutritious vegetable can play an important role to meet up the demand for vegetables to ensure food security (Amin, 2013).

In this context, commercial production of bottle gourd can be the best solution to fulfil the demand for vegetables on daily basis. Moreover, Bangladesh seemed to have prospects of vegetable export for its high demand to the foreign ethnic market (EPB, 2014). Since seed quality played a significant role on the bottle gourd yield. Both the government and private institutions should take necessary steps to ensure availability of high yielding variety (HYV) at the doorstep of the farmers at reasonable price. Hybrid bottle gourd is the first generation (F1) crop grown from the cross of two distantly related genotypes. Hybrids are often vigorous in growth and yield. Though hybrid seeds are costly, they lose their yield advantage in subsequent generation. For that farmers are bound to buy hybrid seeds every year for continuing their production. During recent years, the interest in hybrid bottle gourd production has increased rapidly because of high yield, good market value, place in the national food and nutritional requirements. Improved production technology especially selection of improved varieties not only increases yield but also ensures earliness and qualities will enhance considerably. Different private seed companies such as Lal Teer Seed Ltd, ACI Agro Industries, Metal Seed Ltd, Supreme Seed Ltd etc. developed hybrid bottle gourd varieties. Those hybrid varieties are very much popular to the Bangladeshi consumer due to taste and nutritional aspect. Analysis of genetic purity, variability and diversity is important for protection and documentation of hybrid

variety. Although some morphological characterization was done those bottle gourd hybrid but molecular characterization or DNA profiling was not done for accurate documentation and purity identification of hybrid bottle gourd. Hence, a study is needed to identify the hybrid bottle gourd through molecular approaches. Bottle gourd exhibits significant genetic variation in quantitative traits (Morimoto *et al*., 2005; Yetisir *et al*., 2008; Koffi *et al*., 2009; Xu *et al*., 2014) which are useful attributes in genetic characterization of diverse genetic resources. Further, the crop exhibits great variation in fruit qualitative traits such as fruit shape, skin colour, presence or absence of fruit necks or skin texture (Morimoto *et al*., 2005; Achigan-Dako *et al*., 2008; Yetisir *et al*., 2008; Mladenovic *et al*., 2013). These variations can be useful for genetic analysis and improvement of the crop. This necessitates the assessment of genetic diversity present in bottle gourd using modern approaches. This would allow for more efficient utilization of plants characters in developing suitable varieties for yield. An assessment of genetic diversity based on phenotypes has limitations since most of the morphological characteristics are greatly influenced by the environmental factors and also depends on the developmental stage of plant. In contrast, molecular markers based on DNA sequence polymorphism are independent of environmental conditions and show a higher level of polymorphism. The discovery of polymerase chain reaction (PCR) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling system. This facilitated the development of marker-based gene, variability studies, phylogenetic analysis, marker-assisted selection of desirable genotypes etc. Thus, PCR bases DNA markers gives new dimensions to concerted efforts of breeding and marker-aided selection that can reduce the time span of developing new and improved varieties/hybrids. The use of molecular markers for the evaluation of genetic diversity receiving much attention than morphological characterization. Various molecular markers have been used to assess genetic variability in bottle gourd, namely randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats or SSRs, inter simple sequence repeats (ISSR) and allozyme markers (Decker-Walters *et al*., 2001; Koffi *et al*., 2009; Saxena *et al*., 2015; Xu *et al*., 2014). Among the different types of molecular markers, randomly amplified polymorphic DNAs (RAPDs), are useful for the assessment of genetic diversity (Williams *et al*., 1990) owing to their simplicity, speed and relatively low cost compared to other types of molecular markers. The major advantages of RAPDs are the utility of universal primers and DNA sequence information or radioactive chemicals are not required (Ragot and Hoisington, 1993). ISSR method is also widely disseminated in genome with high polymorphism. Its implementation does not require prior knowledge of sequence information (Zietkiewicz *et al.*, 1994). Microsatellites (SSRs) are the marker of choice for genetic diversity analysis studies because of their high degree of polymorphism and random distribution across

the genome (Gong *et al*., 2012; Varshney *et al*., 2005). SSR markers are efficient in detecting genetic polymorphism and discriminating among genotypes from germplasms of the various sources even they can detect finer level of variation among closely related breeding lines within the same variety (Lapttan *et al*., 2007). These DNA markers offer several advantages over traditional phenotypic markers as they provide data that can be analysed objectively. The knowledge acquired through this investigation may play a pivotal role in the application of molecular markers in bottle gourd improvement programmes. Therefore, the aim of present study has to analyze genetic diversity with three PCR based marker system viz, RAPD, ISSR and SSR. Keeping all the above facts in view, the present investigation was undertaken with following objectives.

1. Molecular diversity analysis of different hybrid bottle gourd.
2. Polymorphism study among hybrid bottle gourd available in Bangladesh.
3. Establishment of the dendrogram and phylogenetic relationship among the studied varieties.

CHAPTER II
REVIEW OF LITERATURE

Bottle gourd is an important cultivated vegetable in Bangladesh. Though it is a common vegetable, presently production of bottle gourd depends on landraces. This results in unpredictable yields, poor quality and low disease resistant. Hybrid bottle gourd, one of the viable and proven technologies has been considered as a new frontier to increase bottle gourd production for meeting the growing demand for vegetable in Bangladesh.

As this species is poorly characterized it is important at the beginning of a breeding programme to establish the level of genetic diversity and thereby identify the most suitable material for crosses. The selection of genotypes and characterization of existing genetic variability is decisive to improve, the efficiency of the breeding programme. There are no available reports related to Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) on bottle gourd genotypes. The present study could help the researchers in this regard in future. Keeping in view the role of was carried out to determine genetic diversity among different varieties of hybrid bottle gourd using RAPD, ISSR and SSR markers and selection of genetically diverse genotypes for the breeding programme. Efforts have been made to accumulate the findings of different studies genetic diversity of bottle gourd and other crops under the Cucurbitaceae involving molecular markers are stated below.

2.1 The concept of molecular marker

Molecular markers are reliable tools to characterize the DNA profile of plant genotypes to study genetic diversity. According to Datta *et al.* (2011), molecular markers are specific fragments of DNA that can be identified within the whole genome. Molecular markers are found at specific locations of the genome. Molecular markers have been found to be more dependable than the phenotypic observations for evaluating the variations and in the assessment of the genetic stability (Leroy *et al.*, 2000) and provide an efficient means to link phenotypic and genotypic variation (Varshney *et al*., 2005). These methods are being very rapidly adopted researchers all over the world crop improvement. The molecular marker techniques are diverse and vary in principle, application and amount of polymorphism observed and in time requirements. Molecular markers present an efficient tool for fingerprinting of cultivars, and assessment of genetic resemblance and relationships (Vilanova *et al.*, 2012). With the advent of molecular

biology techniques, molecular markers are effective because they identify an abundance of genetic linkage between identifiable locations within a chromosome and are able to be repeated for verification. A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. Amin *et al*. (2010) stated that the use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes. Molecular markers can be used for molecular characterization and detecting genetic variation and relationship of plants. These markers can detect the variation that arises from deletion, duplication, inversion and/or insertion in the chromosomes. Such markers themselves do not affect the phenotype of the traits of interest because they are located only near or linked to genes controlling the traits (Mondini *et al.,* 2009). According to Karp *et al.* (1997), DNA based marker is classified into three categories depending on the technique used. Hybridization based DNA markers, arbitrarily primed polymerase chain reaction (PCR)-base markers, and sequence targeted and single-locus DNA marker. Restriction Fragment Length Polymorphism (RFLP) is a hybridization based marker in which DNA polymorphism is detected by digesting DNA with a restriction enzyme followed by DNA blotting and hybridizations with probes. Sequence Tagged Sites (STS), Sequence Repeat (SSRs), Single Nucleotide Polymorphism (SNPs) markers belong to sequence targeted and single-locus PCR based DNA markers. Of these, RFLP and microsatellites are co-dominant markers, while RAPD and AFLP markers are largely dominant markers. The most interesting application of molecular markers in marker-assisted selection (MAS). They have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species (Madhumati, 2014). These markers are selectively neutral because they are usually located in non-coding regions of DNA. Usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors. Apart from the use of DNA markers in construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within cultivars and fingerprinting the germplasms. Ferriol *et al*. (2003) worked on nineteen accessions of *Cucurbita maxima* and eight related *Cucurbita* accessions and all 27 accessions were included in genetic diversity analysis.

Among the available molecular markers, microsatellites or simple sequence repeats (SSRs) which are tandem repeats of one to six nucleotide long DNA motifs, have gained considerable importance in plant genetics and breeding owing to many desirable genetic attributes including hypervariability, multiallelic nature, codominant inheritance, reproducibility, relative abundance, extensive genome coverage including organellar genomes, chromosome-specific location and amenability to automation and high throughput genotyping (Kalia *et al*., 2011). Molecular

markers have successfully been applied in registration activities like cultivar identification (Mailer *et al*., 1994) or controls of seed purity of hybrid varieties (Marshall *et al*., 1994) and also for the variety identification as a part of seed and grain trade (Bligh *et al*., 1999). A powerful technique for DNA fingerprinting is successful Polymerase Chain Reaction (PCR) amplification of tandem repeat sequences, which have long been known to be polymorphic and widespread in plant genomes referred to as Simple Sequence Repeats (SSR) or Microsatellite polymorphism (Cregan, 1992; Morgante and Olivieri, 1993). Conventional breeding is time-consuming and depends on environmental conditions. Breeding a new variety takes 8 to 12 years. Molecular marker technology offers a possibility by adopting a wide range of novel approaches to improve the selection strategies in plant breeding (Gosal *et al*., 2010; Choudhary *et al*., 2008).

2.2. Concept of RAPD marker

Random Amplified Polymorphic DNA is a PCR-based technique discovered by Williams *et al*. (1990) and generated by the use of short (10-mer) synthetic oligonucleotides in single strand primer. Kumar and Gurusubramanian (2011) pointed out that there is no need to know DNA sequence information for the targeted gene. In this technique, a decamer primer of the arbitrary sequence is allowed to anneal at a relatively low temperature priming the amplification of DNA fragments distributed at random in the genome (Williams *et al*., 1990). The amplified products are visualized by separation on an agarose gel and stained with ethidium bromide. They usually result in DNA fragment patterns that are polymorphic between genotypes, thereby detecting diversity within them (Tommercup *et al*., 1998). Vierling and Nguyen (1992) pointed out that, the polymorphism detected between amplification products of different individuals using the short, random, single primers made RAPD marker studies good for genetic diversity, genetic relationships, genetic mapping, plant breeding, DNA fingerprinting and population genetics. There are several advantages of RAPDs compared to other DNA based techniques. It includes non-radioactive detection, multiple loci detections in a single reaction, the requirement of a small quantity of DNA, no requirement of prior sequence information, quick and technically simple (Karp *et al*., 1997). Main advantages of the RAPD technology include suitability for work on anonymous genomes, involves no blotting or hybridization steps, hence, it is quick, simple and efficient, applicability to problems where only limited quantities of DNA are available and Unit costs per assay are low compared to other marker technologies (Kumari and Thakur, 2014; Madhumati, 2014; Kumar and Gurusubramanian, 2011). Hadrys *et al.* 1992 stated that RAPD-PCR technique used for examining variation in the total genome. RAPD analysis is advantageous over isozyme electrophoresis because it generates much greater numbers of loci

required for genetic analysis (Kimbeling *et al.,* 1996). RAPD markers can be used as supposedly unbiased; and neutral markers for genetic mapping applications (Michelmore *et al.,* 1991), in population genetics (Haig *et al.,* 1994), taxonomy (Chapco *et al.,* 1992) as well as for genetic diagnostics. In spite of having much usefulness of RAPD marker, it has some limitation. Because of the random nature of genome sampling, the RAPD assay is not an appropriate technique when the difference between the two genomes is being compared is limited to an extremely small genomic fraction. The most unavoidable problem is dominancė of RAPD marker because the presence of given RAPD band does not distinguish whether its respect locus is homozygous or heterozygous or co-dominance which is possible when SSR marker is used (Rahman *et al.*, 2006).Though having such weakness, the relative ease and speed the high degree of polymorphisms and a virtually inexhaustible pool of possible genetic marker make the RAPD technique advantageous over other molecular technique (Clark and Lanigan, 1993; Fristsch and Rieseberg, 1996). RAPD markers, in particular, have been successfully employed for the determination of intra-species diversity in several plants, whereas fewer reports are available on the determination of inter-species diversity (Goswami and Ranade, 1999).

2.3 Concept of ISSR Markers

Ever since the molecular markers were developed, they have been utilized to provide us with requisite landmarks for the elucidation of genetic variation. ISSR markers are a variant of microsatellite and involve the amplification of DNA region located between two microsatellites loci using primers with a single SSR motif attached at the 3´ or 5´end by a limited nucleotide (Zietkiewicz *et al*., 1994). ISSR markers are present in both nuclear and organelle genomes and provide a genotyping system with features of stability, reliability and dominancy (Peng *et al*., 2006). Dje *et al*. (2006) applied a molecular approach using inter-simple sequence repeat (ISSR) markers in three African edible seeded cucurbits (*Citrullus lanatus* L. Matsumura and Nakai , *Cucumeropsis mannu* L. Naudin and *Cucumis melo* var. *agrestis* L. Naudin).To obtain clear and reproducible bands on 1.5% agarose gels, they screened 21 ISSR primers and three parameters (annealing temperature, voltage and running time).The resolution of 11 ISSR markers was performed, with optional annealing temperature (Ta) varying from 50 to 52 °C. The best combination to obtain clear and well-distinguished band patterns was 1.5% agarose gel with a 20-lanes tray (6 mm width) at 80 V for 5 hours. Applying the ISSR primers on DNA extracted from an accession of *C. lanatus*, 66 bands with 4 to 11 bands per primer were observed.

Yeboah *et al*. (2007) constructed a genetic map for cucumber (*Cucumis sativus* L.) based on the segregation pattern of SRAP (Sequence-related amplified polymorphism) and ISSR (Inter-simple sequence repeat) markers using 112 F2 plants derived from a cross between two inbred lines (PW 0832 and PW 0832). In the investigation of polymorphisms with 50 ISSR primers and 132 SRAP primer combinations, 13 (26%) ISSR primers and 26 (20%) SRAP primers pairs were polymorphic generating a total of 109 polymorphic markers of which 48 were ISSR and 61 were SRAP. The average polymorphic bands were four for ISSR and two for SRAP. All the 109 polymorphic markers were scored for segregation of which 86 satisfied the Mendelian segregation ratio 3:1. These data were used to construct an integrated linkage map for cucumber consisting of 62 loci. These markers would be a very useful tool for marker-assisted selection, polymorphism study and genetic diversity analysis. Huang *et al*. (2007) selected 27 ISSR markers for identification and genetic diversity of 38 diverse bitter gourd accessions. The 247 amplicons yielded from ISSR-PCR analysis and 115 polymorphic were included. Each primer produced 4.25 polymorphic bands on average. Numbers of the polymorphic amplicon was 0 to 9 and each size of products ranged from 150 to 2700 bp. The lowest and highest genetic similarly coefficients were 0.508 and 0.938, respectively. The results showed that discrimination for the 18 accessions just needs six ISSR primers were effective and convenient for bitter gourd varietal identification. Bhawna *et al*. (2014) undertook 42 geographically distant genotypes accessions of bottle gourd (*Lagenaria siceraria*) from India northeastern (14) and northern region (28) using inter-simple sequence repeat (ISSR) markers. A total of 209 amplified bands were obtained from 20 ISSR primers used in this study, of which 186 were polymorphic with 89.00% band polymorphism. The data provided enough evidence of its high applicability in diversity analysis *of L. siceraria* crop.

2.4 Concept of SSR markers

Microsatellites (SSRs) are short tandem repeats of simple (1–6 nt) motifs. Their value for genetic analysis lies in their multi-allelism, co-dominant inheritance, relative abundance, genome coverage and suitability for high-throughput PCR-based platforms. They can serve as highly informative genetic markers, and in conjunction with the use of polymerase chain reaction (PCR) technology enable the detection of length variation (Powell, 1996). In plants, it has been demonstrated that SSRs are highly informative, locus-specific markers in many species (Akkaya *et al*., 1992; Lagarcrantz *et al*., 1993). Microsatellite primers developed for one species can be used to detect polymorphism at homologous sites in related species. They have become one of the most useful molecular marker systems in plant breeding. The development of SSR

markers from genomic libraries is expensive and inefficient (Squirrell *et al.*, 2003). SSR markers are non-isotype based precise PCR technology. Stretches of DNA, consisting of randomly repeating small nucleotide units and conserved regions flanking the repeats are suitable for designing PCR primer pairs. Used for amplifying the intervening repeat loci were first referred to as Microsatellites by Litt and Luty (1989). SSR is a marker of choice for molecular characterization as it is co-dominant, distributed throughout the genome, highly reproducible, variable, reliable, easily scorable, abundant and multiallelic in nature. SSR marker are highly informative, mostly monolocus, co-dominant, easily analyzed and cost-effective (Gracia *et al*., 2004) and able to detect high level of allelic diversity thus being widely applied in genetic diversity analysis, molecular map construction and gene mapping and analysis of germplasm diversity. The SSR markers are particularly suitable for evaluating genetic diversity and relationships among plant species, populations, or individuals (Kostova *et al.*, 2006).

SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasms of various sources, even they can detect the finer level of variation among closely related breeding lines within the same variety (Lapitan *et al*., 2007). Among various PCR-based markers, microsatellites (SSRs) are more appropriate and successfully used for assessing genetic diversity among closely related bottle gourd cultivars compared to other molecular markers; because it can be simply amplified by PCR reaction, abundant, highly informative, mostly mono locus, co-dominant, easily analyzed, cost-effective and it can identify higher degree polymorphism in bottle gourd. SSRs are the marker of choice for genetic diversity analysis studies because of their high degree of polymorphism and random distribution across the genome (Gong *et al*., 2012; Varhney *et al*., 2005). Sarao *et al.* (2013) fingerprinted 20 accessions of bottle gourd in India using 20 SSR primers and reported the discriminatory power of these markers.

2.5 Genetic diversity studies in bottle gourd genotypes by RAPD, ISSR and SSR markers

Genetic diversity study is of prime importance in the conservation of endangered species and utilization of appropriate plant resources from diverse germplasm. Successful breeding for crop development programmes depends on genetic variability that arises from genetic diversity (Rana and Bhat, 2004). Lack of genetic variability may limit breeding progress and gain from the selection. So, knowledge of the genetic diversity of any germplasm collection provides a basis for improvement of crops and the development of superior cultivars. Morphological features are indicative of the genotype but are represented by only a few loci because they are not large enough. Moreover, they can also be affected by environmental factors and cultural practices. To have an accurate and reliable estimate of genetic relationships and genetic diversity assessment, there is a need for polymorphic

molecular markers. Stepansky *et al*. (1999) studied melon (*Cucumus Melo* L.) genotypes that differed widely in morphological and biochemical traits. A collection of 54 accessions representing diverse genotypes from 23 countries was surveyed. DNA polymorphism among the accessions was assessed using the ISSR and RAPD techniques that detected abundant DNA polymorphism among melon genotypes. Katzir *et al*. (2000) used inter simple sequence repeats (ISSR) multi locus marker system to compare 28 accessions of *Cucurbita pepo*. A total of 90 polymorphic bands were scored and results were subjected to cluster analysis. Preliminary results using simple sequence repeats (SSR) suggested that this marker system also be useful for better understanding of intraspecific relationships within *C. pepo*. Staub *et al*. (2000) compared random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers to characterized genetic relationships among 46 accessions in two *C. melo* L. subsp *melo* (*Cantalupensis, Inodorus*) and subsp. *agrestis* (*Conomon, and Flexuosus*) groups. Genetic distance (GD) estimates were made among and between accessions in four melon market classes. 135 RAPD bands and 54 SSR bands (products of 17 SSR primers) were used to calculate GD. Band polymorphisms observed with 21 RAPD primers and 7 SSR primers were important in the detection of genetic diversity. The results of RAPD marker analysis suggested that 80 marker bands were adequate for assessing the genetic variation in the accessions examined.

Paris *et al*. (2003) determined genetic relationships in *Cucurbita pepo* (pumpkin, squash and gourd) as viewed at the DNA level, through the use of AFLP, ISSR and SSR markers. Forty-five accessions were compared for presence or absence of 448 AFLP, 147 ISSR and 20 SSR bands, their genetic distances (GDs) were estimated. Correlation coefficients were 0.95 between AFLPs and ISSRs, 0.78 between AFLPs and SSRs and 0.77 between ISSRs and SSRs, all three comparisons with $P<0.001$. Overall, clustering and sub-clustering were much in accordance with two highly polygenic characteristics i.e. fruit shape and fruit size. Nakata *et al*. (2005) studied the genetic diversity among 67 melon (*C. Melo* L.) cultivars from five Japanese seed companies using 25 (10-mer RAPD) primers (56 bands) and 9 SSR (36 alleles) markers. Genetic variation among these cultivars was compared with variation observed in reference array (RA) consisting of 34 selected melon accessions from previous studies. Levi *et al*. (2006) studied 71 amplified fragment length polymorphism (AFLPs); 93 sequences related amplified polymorphism (SRAP), and 14 simple sequence repeat (SSR) markers and were used to strengthen an initial genetic linkage map for watermelon. The initial map was based on 151 randomly amplified polymorphic DNA (RAPD) and 30 inter-simple sequence repeat (ISSR) markers. Hadia *et al*. (2008) studied two PCR molecular marker techniques *viz*. random amplified polymorphic DNA (RAPD) and inter simple

sequence repeats (ISSR) to identify the polymorphisms and the relationships between 14 genotypes, which belong to three different *Cucurbita* species (*C. pepo, C. moschata and C. maxima*). In RAPD analysis, six random primers revealed a total of 463 fragments in which 405 (87.5%) were polymorphic. 31 out of 463 RAPD-PCR fragments were found to be useful as genotype-specific markers. Seven ISSR primers gave a total of 263 ISSR amplified fragments in which 243 (92.4%) were polymorphic. 10 genotypes out of 14 were identified by a total of 21 unique markers with the 7 ISSR primers. The information about polymorphism using RAPD and ISSR in a set of genotypes is highly useful in the assessment of genetic diversity and genetic relationships and could be useful in the future breeding programme. A few microsatellites have been available for *Cucurbita*, which were studied by Gong *et al*. (2008). The Australian oil-pumpkin variety 'Gleisdorfer Olkurbis' (*C. pepo subsp. pepo*) and the *C. moschata* cultivar 'Soler' (Puerto Rico) were used for SSR primers. Of these, 405 (81%) amplified polymorphic fragments in a set of 12 genotypes. On average, *C. pepo* and *C. moschata* produced 3.3 alleles per primer pair, showing high inter-species transferability. Sestili *et al*. (2008) studied genetic diversity and relationships among 13 Italian inodorous Melon (*Cucumis melo* L.) populations of different geographic origin by using 90 ISSR primers and 18 SSR primer pairs. Out of 90 primers ISSR used, 39 resulted polymorphic among the landraces revealing a total of 358 polymorphic bands. The preliminary SSR analysis revealed a high level of polymorphism.

CHAPTER III
MATERIALS AND METHODS

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

3.1 Experimental site and time duration

The experiment was carried out at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, Bangladesh. The period of the experiment was from April 2018 to November 2018.

3.2 Name and source of study materials

Eight bottle gourd hybrids were used as experimental materials (Table 1). All the genotypes were collected from three renowned private Seed Company of Bangladesh. They are Metal Agro Limited, ACI Limited, Lal Teer Seed Limited. The list of hybrids is given in Table 1.

Table 1: Name of hybrids of different Bottle gourd genotypes

Sl.No.	Hybrid Name	Source
1	Hi Green Hybrid bottle gourd (H1)	Metal Agro Limited
2	Maina Hybrid bottle gourd (H2)	ACI Limited
3	Diana Hybrid bottle gourd (H3)	Lal Teer Seed Limited
4	Arosh Hybrid bottle gourd (H4)	Lal Teer Seed Limited
5	Marshal Super Hybrid bottle gourd (H5)	ACI Limited
6	Rawnok Hybrid bottle gourd (H6)	ACI Limited
7	Rajkonna Hybrid bottle gourd (H7)	Metal Agro Limited
8	Nandini Hybrid bottle gourd (H8)	Metal Agro Limited

3.3 Collection of leaf sample for DNA extraction

Good quality, disease-free, healthy bottle gourd seeds were collected from the above seed company. Seeds were soaked in water for 3 days. Seeds were then sown in pots. The soil was collected from nearby nursery and different pots were kept in the research farm of Sher-e-Bangla Agricultural University. Leaf samples were collected from 15-day old seedlings. Those leaves were used as a source of genomic DNA extraction.

3.4 Genomic DNA extraction

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

3.4.1 List of equipment required

1. Morter and pestle
2. Water bath
3. Centrifuge machine
4. Vortex mixture
5. Ice maker
6. Micropipet
7. Electrophoresis system
8. Gel documentation system etc.

3.4.2 Reagents required

1. Extraction buffer, pH =8.0

Composition of extraction buffer is as follows:

- 1 M Tris HCl
- 0.5 M EDT A (Ethylene diamine tetra-acetic Acid) (pH =8.0)
- 5 M NaCI
- Distilled H_20
- 1 % SDS (Sodium Dodecyl Sulphate)
- Marcapto-ethanol
- PVP (Polyvinylpyrrolidone)

2. Phenol: Chloroform: Isoamyl Alcohol = 25: 24: 1

3. TE (Tris-EDTA) buffer, pH =8.0

Composition of extraction TE buffers is as follows:

- 1 M Tris HCI
- 0.5 M EDTA
- Distilled H_20

4. Isopropanol

5. 0.3 M Sodium Acetate

6. Absolute (100%) ethanol

7. Ethanol (70%)

8. RNase

9. Ethidium Bromide Solution

3.4.3 Reagent preparation for DNA extraction

Stock solution for 1000 ml extraction buffer

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

Stock solution for 250 ml 1M Tris-HCI pH 8.0

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

Stock solution for 250 ml 0.5 M EDTA pH 8.0

- At first 46.53 g, EDTA.2 .H_2O was added in a volumetric flask (500 ml)
- 100 ml dd.H_2O was added.

- Then 4 g NaOH was added.
- pH was adjusted to 8.0 with NaOH
- Then sterilized dd.H_2O was added to make the volume up to 250 ml.
- The solution was autoclaved.

Stock solution for 250 ml 5 M NaCl

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

2 % SDS Stock solution for 100 ml

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

Stock solution for 100 ml TE buffer

- 1 ml Tris HCl (pH 8.0) was taken in a volumetric flask (250 ml).
- Then 0.2 ml EDTA (pH 8.0) was added.
- Sterilized dd.H_2O was added to make the volume up to 100 ml.

Composition of 5x TBE buffer (1 litre)

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

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

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

RNase

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

70% Ethanol (1000 ml)

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

0.3 M Sodium Acetate

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

3.5 Sequential steps for DNA extraction

1. For isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from eight different bottle gourd hybrids.
2. Initially, healthy youngest leaves were washed thoroughly by tap water followed by washing with deionized water. Then sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
3. Approximately 150 mg of young leaves were cut into small pieces and then taken in morter. 600 µl of extraction buffer was added to it. The ground samples were taken into the 1.5 ml Eppendorf tube and then it was vortexed for 20 seconds in a vortex mixer and then incubated at 65 °C for 20 minutes in hot water bath.
4. An equal volume (600 µl) of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) was added to the tube. Then it was vortexed for 20 seconds.
5. The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a micropipette tip without disturbing the lower portion and transferred into a new Eppendorf tube. Approximately 400-450 µl was taken and then an equal volume of Chloroform: Isoamyl Alcohol (24: 1) was added to it. The solution was vortexed for 10 seconds.
6. Again, the solution was centrifuged at 13000 rpm for 10 minutes.
7. The supernatant was taken in a separate Eppendorf tube and the lower layer was discarded.
8. The amount of the solution was multiplied with 0.6 and then the same volume of Isopropanol (0.6 volume of the liquid) was added.
9. It was then tapped by finger for 20-30 seconds (The genomic DNA was visible as cotton-likestructure).

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

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

12. The DNA pellet was then air-dried for 2-3 hours. It was then dissolved in an appropriate volume (30 to 40 µl) of TE buffer and treated with 3 µl of RNase at 37 ^{0}C in hot water bath for 15-20 minutes for removing RNA. Then it was spun for 4-5 seconds.

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

3.6 Confirmation of extracted DNA

To confirm the extracted DNA sample 1% agarose gel, working sample of each genomic DNA, 2x loading dye and de-ionized H_2O was needed.

3.6.1 Agarose gel preparation (1%)

Reagents

- Agarose powder
- 0.5 x TBE buffer (Ph 8)
- Ethidium Bromide

Gel preparation procedure

Five hundred miligram of agarose powder was taken in 250 ml Erlenmeyer flask containing 50 ml electrophoresis buffer (0.5 x TBE buffer) prepared by adding 25 ml of 1 x TBE buffer in 25 ml of de-ionized water. The flask was enclosed with aluminium foil paper to prevent excessive evaporation. The flask was heated in a microwave oven for about 1 minute with occasional swirling to generate uniform suspension until no agarose particle was seen to generate homogenous and crystal-clear suspension. The agarose solution was cooled to about 45-50 ^{0} C (flask was cool enough to hold comfortably with a bare hand) and 0.75 µl ethidium bromide was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (130×13×59

mm^3 size). That was placed on a level bench and an appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth. After 25 minutes, the gel was completely cooled at room temperature and solidified, and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.6.2 Preparation of DNA samples for electrophoresis and confirmation

DNA sample was prepared by mixing 3 µl de-ionized H_20 and 2 µl 2x loading dye (0.25% xylene ethanol, 0.25% bromophenol blue, 30% glycerol and 1mM EDTA) and 3.0 µl of sample DNA were taken in an Eppendorf tube using 0.5-10 µl adjustable micropipette. Loading dye was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 8.0 µl expected DNA was added to it and mixed well. The sample was, then loaded into the well of the gel and allowed them to sink to the bottom of the well. The gel was placed in the electrophoresis chamber keeping the gel horizontal and submerged in 0.5x TBE buffer (running buffer). The gel tank was covered and the electrophoresis power supply was connected and turned on to move DNA from negative to the positive electrode through the gel. Electrophoresis was carried out at 75 volt for about 60 minutes.

Table 2: DNA confirmation reagents with the amount

Components	**Amount (µl)**
Working a DNA sample	3.0
De-ionized water	3.0
2x loading dye	2.0
Total	**8.0**

3.6.3 Documentation of the DNA samples

The gel was carried out from the gel chamber and was placed on an ultraviolet lightbox (UV trans-illuminator) to examine and photographed. DNA samples showing better quality bands were taken for quantification and working solution preparation for next process.

3.7 Working solution for DNA samples preparation

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

$V_1 \times S_1 = V_2 \times S_2$

Where,

V_1 = Initial volume of DNA solution (µl)

S_1 = Initial DNA concentration (ng/µl)

V_2 = Final volume of DNA solution (µl)

S_2 = Final DNA concentration (ng/µl)

3.8. Amplification of RAPD markers by PCR

3.8.1 Principle of RAPD primer amplification

For amplification of RAPD, a single oligonucleotide of arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermostable DNA polymerase and a suitable buffer and then it is subjected to temperature cycling conditions typical to the Polymerase Chain Reaction (PCR). The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction conditions. At an appropriate annealing temperature the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (e.g., within a few thousand nucleotides) and a discrete DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide binding sites on the genomic DNA. In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Often a single primer can be used to identify several polymorphisms, each of which matches to a different locus.

3.8.2 Selection of primers (RAPD, ISSR, UBC and SSR)

Eight RAPD primers were tested. Out of them, four RAPD primers were OPF 10, OPB O4, OPA 04 and OPD 03 (Operon Technologies, INC., Alameda, California, USA) were used for PCR reaction in 8 hybrid varieties of bottle gourd. The primer which produced faint and irreproducible DNA band were rejected for further amplification. Likewise, ISSR primer were

also selected. The selected ISSR primers were ISSR 05, UBC 834, UBC 855 UBC 854. One SSR primer *viz.* ZJULM 55 also used in this work. The detail of the primers is given in Table 3.

Table 3: Nine of primers (RAPD, ISSR and SSR) with GC content and sequence information

Sl.No.	Primer name	Sequence (5´ to 3´)	(G+C) %
1	OPB 04	GGACTGGAGT	60%
2	OPD 03	GTCGCCGTCA	70%
3	OPA 04	AATCGGGCTG	60%
4	OPF 10	GGAAGCTTGG	60%
5	ISSR 05	GGAGGAGGAGGA	66.67%
6	UBC 834	AGAGAGAGAGAGAGAGYT	44.44%
7	UBC 855	ACACACACACACACACCTT	47.37%
8	UBC 854	TCTCTCTCTCTCTCTCAGG	52.63%
9	ZJULM 55 (SSR)	F: GATAATGGAAATAAACCACCCT	36.36%
		R: GCCACAGACCCTACTTGAGA	

3.8.3 PCR amplification of RAPD marker

PCR reactions were performed on each DNA sample. 2x Taq ready Master Mix was used. DNA amplification was performed in an oil free thermal cycler (Q-cycler II, A Hain Lifescience Brand). To prepare a 10.0 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 4.

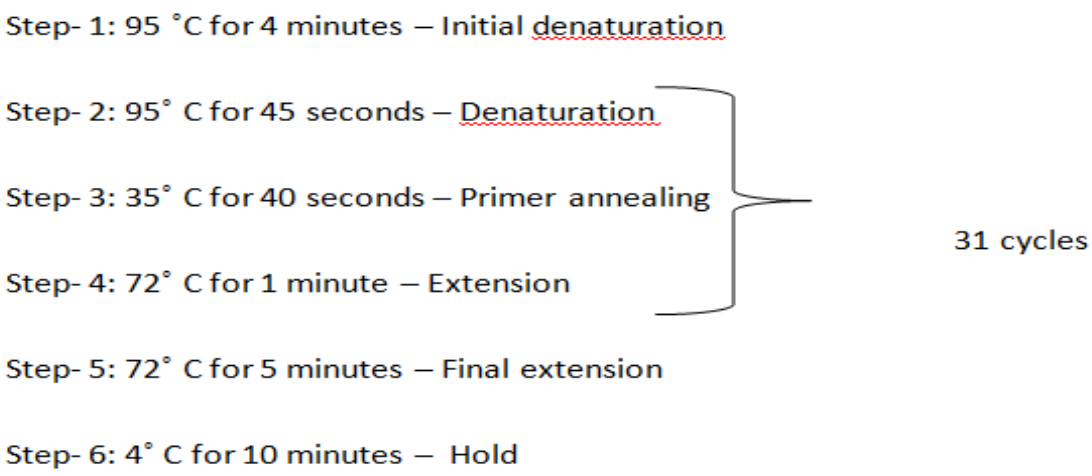
Table 4: PCR mixture composition for each hybrid

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

From frozen stocks of the PCR reagents i.e., 2x Taq Master Mix, primer and DNA working samples were melted, mixed by vortexing and kept on ice for maintaining good quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.2 ml). A pre-mixture was then prepared in the course of the following order: reaction mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.8.4 Thermal profile for PCR

DNA amplification was performed in an oil free thermal cycler (Q-cycler II, A Hain Lifescience Brand). The PCR tubes were kept in the thermal cycler and the following programs were run for RAPD primer.



3.8.5 Electrophoresis of the amplified products and documentation of DNA sample

After completion of thermal cycler reaction, each sample of PCR products was confirmed by running 1% agarose gel containing 0.75 µl ethidium bromide in 0.5x TBE buffer at 75 volt. Loading dye (3.0 µl) was added to the PCR products and loaded in the wells. One molecular weight marker 100 bp DNA ladder (Bio Basic, Cat. No. M-1070-1, Canada) was also loaded on the left side of the gel respectively. Electrophoresis was performed at 75 volt for 60 minutes. The profile was visualized under a UV transilluminator and documented using a gel

documentation system. The documented RAPD profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb/bp of bases were recorded in comparison with the marker.

3.9 Data analysis

Since RAPD markers are dominant, we assumed that each band represented the phenotype at a single allelic locus (Williams *el at.,* 1990). One molecular weight marker, 100 bp DNA ladder (Bio Basic, Cat. No. M-1070, Canada) was used to estimate the size of the amplification products by comparing the distance travelled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The band-size for each of the markers was scored using the Alpha Ease FC 4.0 software. The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. The individual fragments were assigned as alleles of the appropriate loci. This was used to estimate polymorphic loci using Power Marker version 3.25 software (Liu K. J., 2005). The summary statistics that were determined included the following: the number of alleles, the major allele size and its frequency, gene diversity, and the polymorphism information content (PIC) value**.** The allele frequency data from the POWER MARKER was used to export the data in binary format (presence of allele as "1" and absence of allele as "0"). Binary data form of allele frequency used for dendrogram construction by NTSYS-pc software (Rholf F., 2002). The unweighted pair grouping method, using the arithmetic average (UPGMA), was used to determine the similarity matrix following the Dice coefficient with SAHN subprogram.

Polymorphic Information Content (PIC) value of a primer is calculated as:

$$PIC = 1-\sum pi^2$$

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

Nei's genetic distance and genetic identity values were computed from frequencies of polymorphic markers to estimate genetic relationship among the studied four rice hybrids using the Unweighted Pair Group Method of Arithmetic Means (UPGMA) (Sneath and Sokal, 1973). The dendrogram was constructed using a computer program, POPGENE; (Version 1.31) (Yeh *et al*., 1999).

3.10 Amplification of ISSR and UBC markers by PCR

3.10.1 Principle of the amplification of ISSR and UBC marker

The PCR reactions for ISSR were carried according to the method by Bahera *et al*. (2008a) with required modifications. The genomic DNA was amplified using both ISSR and UBC (University of British Columbia, Canada) primers. Thermal profile and electrophoresis system were same as RAPD method.

3.11 Amplification of SSR markers by PCR

3.11.1 Principle of the amplification of SSR marker

Microsatellites or SSR are tandem repeats of 1 - 6 nucleotides. For example, $(A)_n$, $(AT)_n$, $(ATG)_n$, $(GATT)_n$, $(CTACG)_n$, $(TACGAC)_n$, and so on. They are abundant in genomes of all organisms. The sequence of unique flanking regions of SSR can be used to design primers and carry out PCR to amplify SSR containing sequences. The polymorphism can be detected by agarose gel electrophoresis if differences are large enough (agarose gels can detect differences greater than 10 base pair).

3.11.2 PCR amplification for SSR

PCR reactions were performed on each DNA sample. 2x Taq ready Master Mix was used. DNA amplification was performed in an oil-free thermal cycler (Q-cycler II, A Hain Lifescience Brand). To prepare a 10.50 µl reaction mixture containing ready mix Taq DNA polymerase and other compositions were given in Table 5.

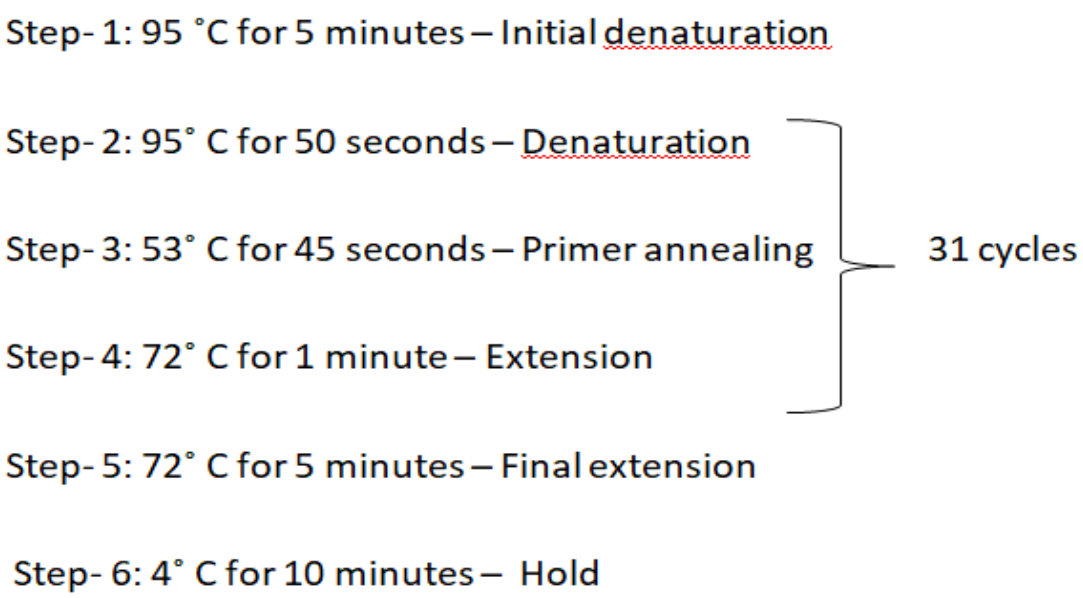
Table 5: Reaction mixture composition for PCR for each hybrid bottle gourd

Reagents	Amount (µl)
2x Taq Master Mix	5.00
SSR Forward primer	1.25
SSR Reverse primer	1.25
Deionized water	1.00
Sample DNA	2.00
Total Reaction volume	**10.50**

From frozen stocks of the PCR reagents i.e., 2x Taq Master Mix, primer and DNA working samples were melted, mixed by vortexing and kept on ice for maintaining good quality. DNA samples were pipetted first into PCR tubes compatible with the thermo-cycler used (0.2 ml). A pre-mixture was then prepared in the course of the following order: reaction mixture, DNA sample and de-ionized water. Then the mixture was mixed up well and aliquoted into the tubes containing primer. The tubes were then sealed and placed in a thermal cycler and the cycling was started immediately.

3.11.3 Thermal profile for PCR

DNA amplification was performed in an oil-free thermal cycler (Q-cycler II, A Hain Lifescience Brand). The PCR tubes were kept in the thermal cycler and the following programs were run:



The amplified products were loaded on one percent agarose gel using 0.5x TAE buffer stained with ethidium bromide along with the marker (100 bp Invitrogen). Electrophoresis was performed 60 minutes. The profile was visualized under a UV transilluminator and documented using a gel documentation system (BIORAD, USA). The documented SSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb/bp of bases were recorded in comparison with the marker.

3.11.4 Electrophoretic separation of the amplified products

PCR products for each sample were confirmed by running it in 1% agarose gel containing 0.75 µl ethidium bromide in 0.5x TBE buffer at one hour. 3 µl loading dye was added to the PCR product and spun them well. Then loaded them in the wells and one molecular weight marker 100 bp DNA ladder (Bio Basic, Cat. No. M-1070-1, Canada) was also placed in the left side of the gel. Under ultra-violet light on a trans-illuminator SSR bands were observed.

3.11.5 Documentation of PCR amplified DNA products

The gel was taken out carefully from the gel chamber and was placed on a high-performance ultra-violet lightbox (UV trans-illuminator) of gel documentation for checking the DNA band and photographed.

Since SSR markers are co-dominant hence, each band represented the phenotype at a single allelic locus (Williams *et al.,* 1990). One molecular weight marker, 100 bp (Bio Basic, Cat. No. M-1070-1, Canada) DNA ladder was used to estimate the size of the amplification products by comparing the distance travelled by each fragment with known sized fragments of molecular weight markers. The band size for each marker was scored. The scores obtained for the SSR primers were then used to assess the polymorphism of different hybrids.

3.12 Precautions

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

- Since Ethidium Bromide (Et-Br) is an intense mutagen and carcinogenic in nature, hand gloves were utilized when taking care of anything that has been presented to Et-Br.

- Always power pack was kept turn off and the leads were unplugged before opening the electrophoresis unit to avoid an electrical hazard.

- Eye protector was used while working with trans-illuminator as it produces UV radiation of 254 nm range which can cause eye damage.

- The common safety measures were kept up when performing PCR responses. All the disposables such as PCR tubes, tips, Eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary. Hand gloves were worn amid the treatment of PCR segments. Contamination of PCR segments was maintained a strategic distance from.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter comprises the presentation and discussion of the results of the experiment. The results were obtained from 8 hybrid varieties of bottle gourd using four RAPD primers, four ISSR primers and one SSR marker. In the RAPD and ISSR analysis, significant genetic variation and polymorphisms were noticed in different hybrid bottle gourd varieties. The results of the experiment were presented and expressed in Table 7 to 9 and Plate 2 to 10 for ease of understanding.

4.1 Extraction of genomic DNA

The genomic DNA extraction of eight hybrids bottle gourd was done by using the phenol-chloroform method with minor modification. RNA sharing was removed by applying RNase treatment. Finally, the purified DNA was stored at – 20 ^{o}C freezer for further use.

4.2 DNA confirmation

The extracted genomic DNA of eight samples was loaded on 1% agarose gel for confirmation and quantification of DNA sample. It revealed that all the samples showed a clear DNA band in each well (Plate 1.). Hence, the genomic DNA of each sample was diluted on the basis of concentration. The working DNA sample was prepared for PCR works.

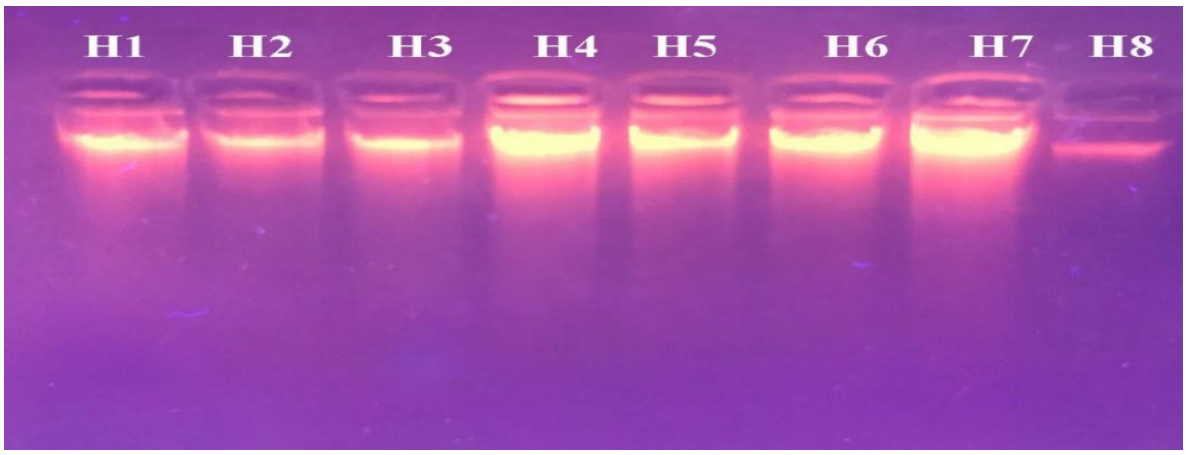


Plate 1: Isolated genomic DNA of 8 bottle gourd hybrids

(Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8)

4.3 Banding pattern and polymorphism survey of eight bottle gourd hybrids by RAPD, ISSR, UBC and SSR primers

Eight DNA decamer RAPD primers, seven ISSR primers and two SSR primers were screened on randomly 8 bottle gourd hybrid varieties to evaluate their suitability for amplification of bottle gourd DNA fragments. The primers, which gave minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands were selected. It revealed that, out of eight RAPD primers, four decamer RAPD *viz*. OPB 04, OPD 03, OPA 04, OPF 10 showed reproducible amplified bands. Out of seven ISSR primers, four ISSR primers *viz*. ISSR 05, UBC 834, UBC 855, UBC 854 produced amplified bands. Among two SSR primers only one SSR primer, ZJLUM 55 give amplified bands.

The RAPD primer OPB 04 produced different DNA fragments in bottle gourd genotypes. It produced a total 4 DNA fragments which ranged from 900 bp to 200 bp. Out of them 3 DNA bands were polymorphic and rest one was monomorphic band (Plate 2). The primer OPD-03 produced 3 DNA fragments which ranged from 600 to 250 bp and all were polymorphic bands (Plate 3). The primer OPA-04 was able to produced 3 DNA fragments in total and ranged from 700 to 200 bp. One bands out of 2 bands was polymorphic (Plate 4). The primer OPF-10 was produced 3 DNA fragments in total which ranged from 900 to 300 bp. Among them 2 bands were polymorphic (Plate 5). Two DNA fragments were amplified by the primer ISSR-05 which ranged from 450 to 200 bp and one band was polymorphic (Plate 6). The primer UBC 834 was showed total number of 4 DNA bands. Three bands were polymorphic and one band was monomorphic which ranged from 750 to 100 bp (Plate 7). Three DNA fragments amplification were noticed by the primer UBC 855 in bottle gourd hybrids which were ranged from 1000 to 300 bp and two were polymorphic in nature and one was monomorphic (Plate 8). The primer UBC 854 was able to amplify two DNA fragments ranged from 600 to 200 bp and one band was polymorphic (Plate 9). The SSR primer ZJULM 55 was produced 2 DNA fragments ranged from 350 to 100 bp and one band was polymorphic in nature (Plate 10). The nine primers generated total 26 DNA fragments with an average 2.88 per primer among eight bottle gourd hybrids. Out of 26 DNA bands, 18 DNA fragments were polymorphic and the average percent of polymorphism was 64.82. The highest number of polymorphic band (3) were produced by the primer OPB 04, OPD 03 and UBC 834, the 2 polymorphic band by the primer OPA 04, OPF 10, UBC 855. The lowest (1) number of polymorphic band produced by the ISSR 05, UBC 854 and SSR primer ZJULM 55. Maximum

100% polymorphism was recorded in the primer OPD 03. The amplification data of the 9 primers were presented in Table 6.

Table 6: RAPD, ISSR, UBC and SSR primers with corresponding banding pattern and - polymorphism observed in eight bottle gourd hybrids

Sl. no.	Primer	Primer sequences (5´-3´)	(G+C) %	Band scored	No. of polymorphic bands	% of polymorphism	Size ranges
1	OPB-04	GGACTGGAGT	60	4	3	75	200-900
2	OPD-03	GTCGCCGTCA	70	3	3	100	250-600
3	OPA-04	AATCGGGCTG	60	3	2	50	200-700
4	OPF-10	GGAAGCTTGG	60	3	2	66.67	300-900
5	ISSR-05	GGAGGAGGAGGA	66.67	2	1	50	200-450
6	UBC 834	AGAGAGAGAGAGAGAGYT	44.44	4	3	75	100-750
7	UBC 855	ACACACACACACACACCTT	47.37	3	2	66.67	300-1000
8	UBC 854	TCTCTCTCTCTCTCTCAGG	52.63	2	1	50	200-600
9	ZJULM 55	F:GATAATGGAAATAAACCACCCT	36.36	2	1	50	100-350
Total				**26**	**18**	**583.34**	
Mean				**2.88**	**2.00**	**64.82**	

In this study, 64.82% polymorphism indicated the effectiveness of the used markers (RAPD, ISSR and SSR). Hadia *et al*. (2008) examined the polymorphism among and within the fourteen *Cucurbita* genotypes by using six RAPD primers which gave 463 total number of reproducible fragments, out of them 405 (87.5%) were polymorphic. The number of specific bands per primer varied (2-8) as with size range of the fragments (133-870 bp). The results indicated that RAPD variation may allow identifying sufficient variation in *Cucurbita* genotypes. Sikdar *et al.* (2010) observed that out of ten (ISSR) primers, eight produced informative data for phylogenetic analysis in *Cucurbita* species, an average of 17.37 bands per primer. Amplified products sizes ranged from 599 to 2399 bp. In the case of SSR maker, similar findings also reported by Watcharawongpaiboon and Chunwongse (2007). They observed that maximum polymorphism information content (PIC) was 0.78 with an average of 0.47 in sixteen *C. sativus* accessions. Polymorphism in bottle gourd in different studies could be attributed to the nature of the genetic material under investigation. Low degree of polymorphism in our study due to the less diverse material among the bottle gourd hybrids.

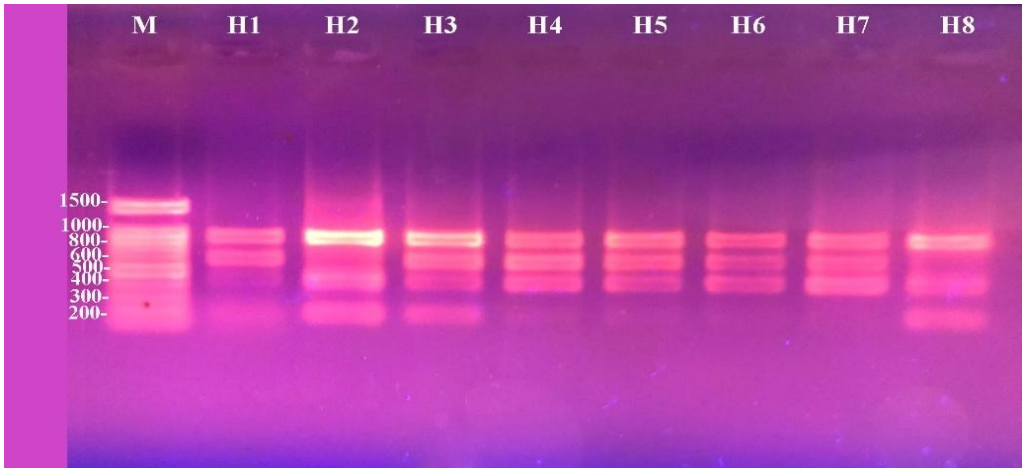


Plate 2: PCR amplification with RAPD primer OPB-04

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

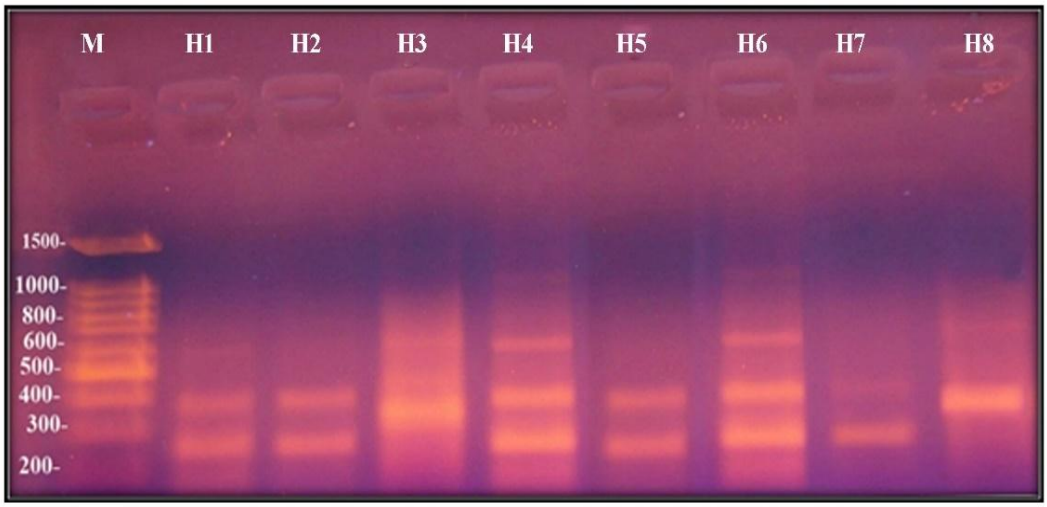


Plate 3: PCR amplification with RAPD primer OPD 03

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

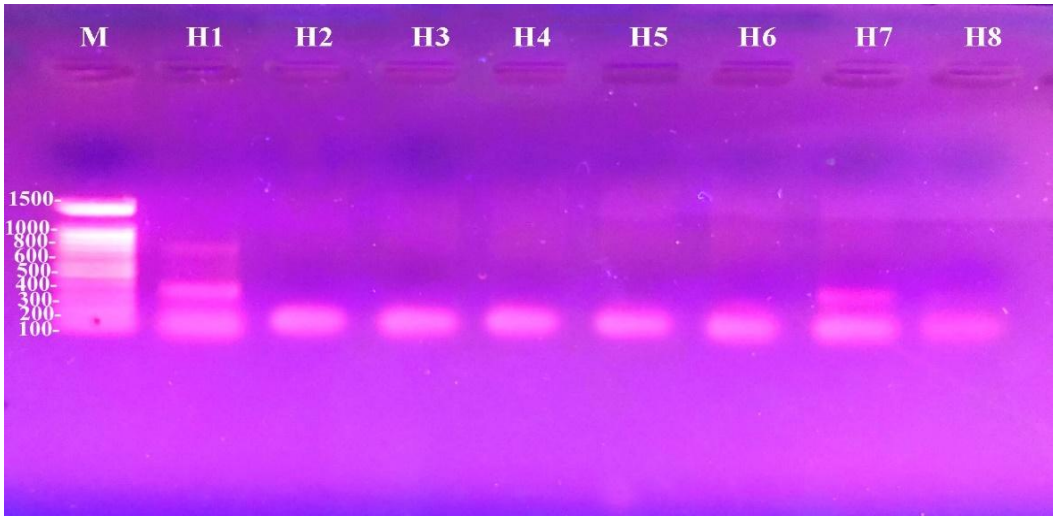


Plate 4: PCR amplification with RAPD primer OPA 04

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

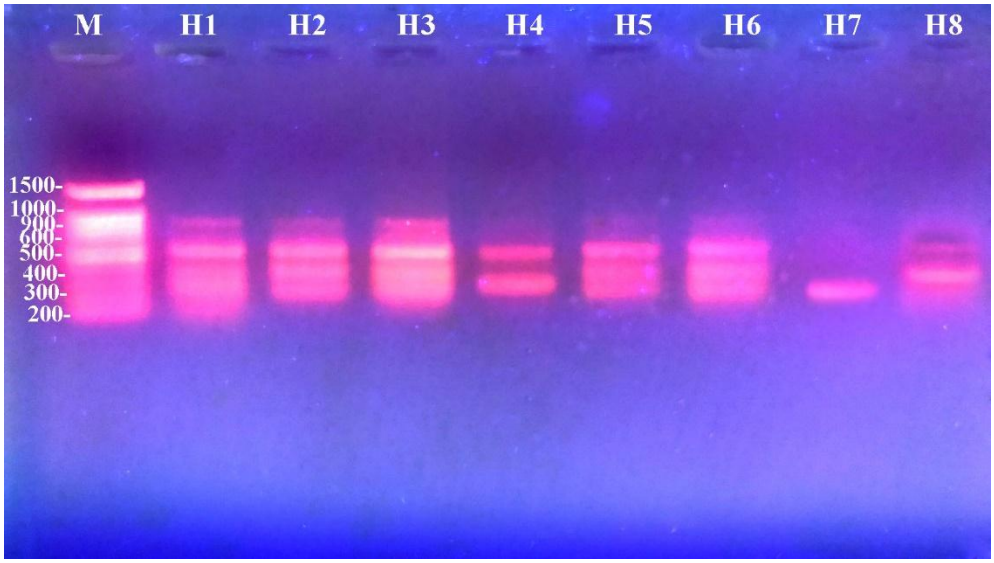


Plate 5: PCR amplification with RAPD primer OPF 10

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

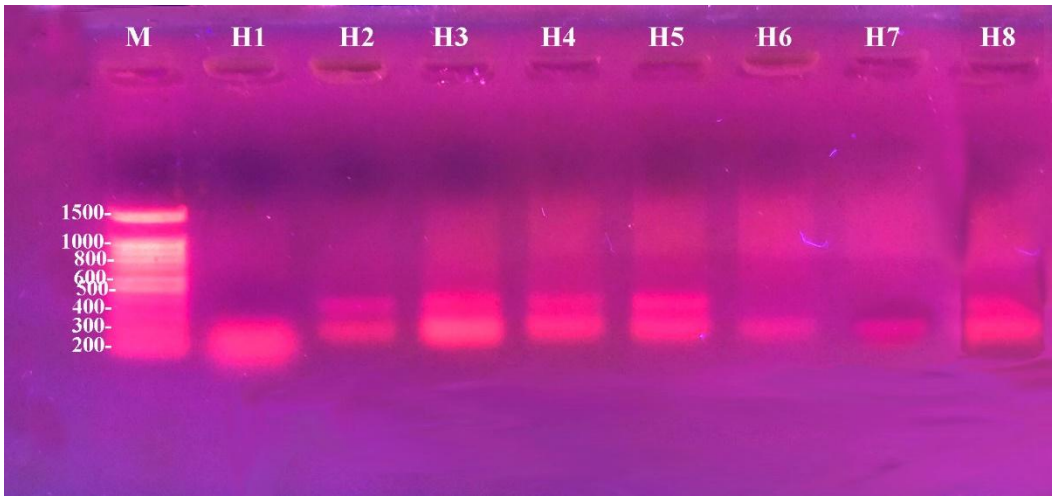


Plate 6: PCR amplification with primer ISSR 05

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

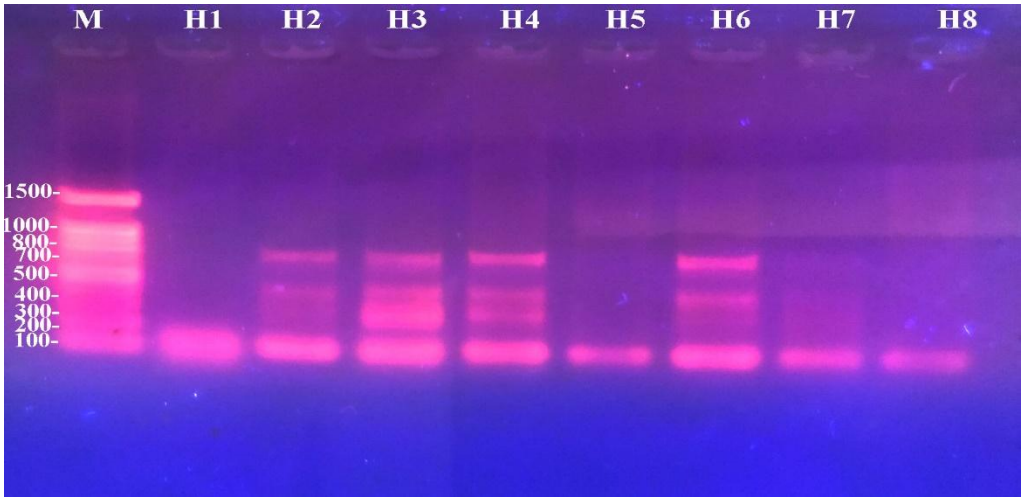


Plate 7: PCR amplification with ISSR primer UBC 834

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

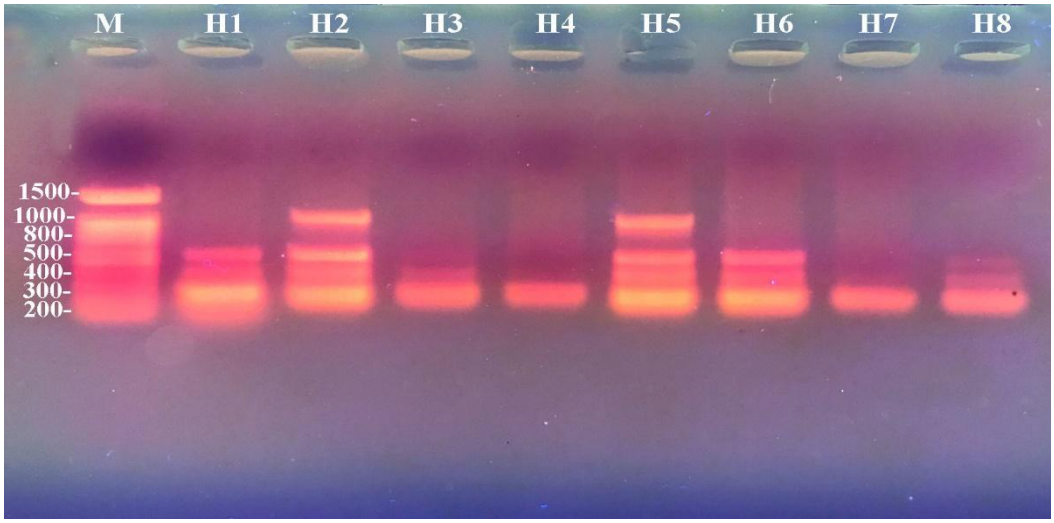


Plate 8: PCR amplification with ISSR primer UBC 855

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

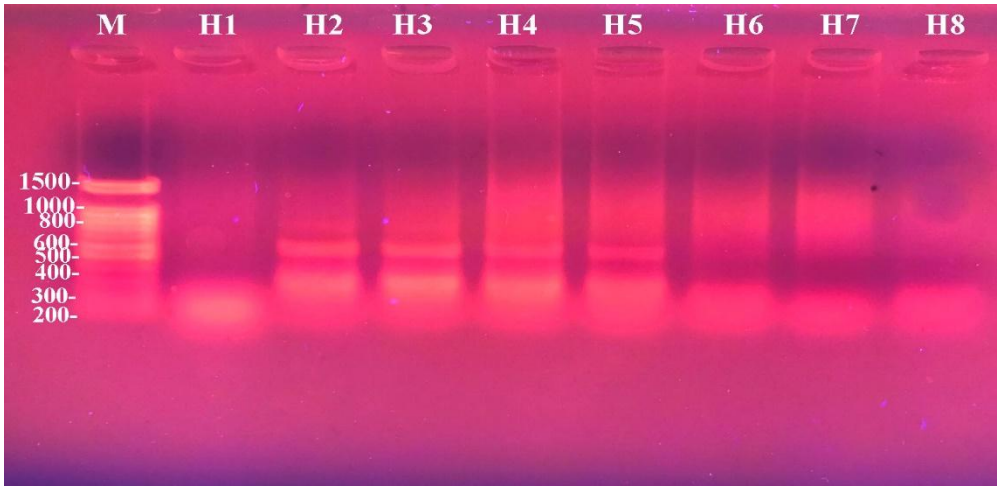


Plate 9: PCR amplification with ISSR primer UBC 854

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

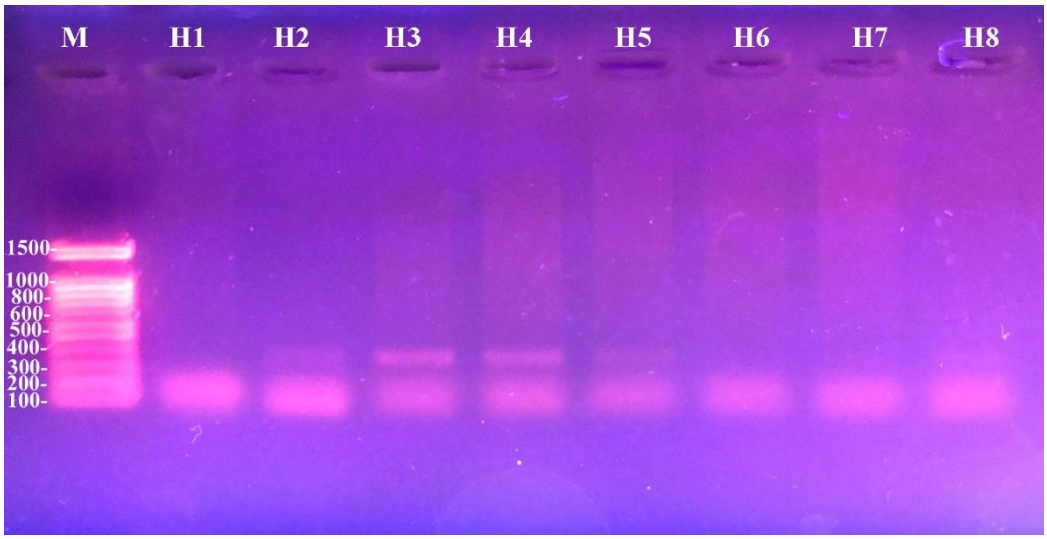


Plate 10: PCR amplification with SSR primer ZJULM 55

M =100 bp DNA ladder (Bio Basic, Canada), Lane 1=Hybrid 1, Lane 2=Hybrid 2, Lane 3=Hybrid 3, Lane 4=Hybrid 4, Lane 5=Hybrid 5, Lane 6=Hybrid 6, Lane 7=Hybrid 7, Lane 8= Hybrid 8

4.4 Gene diversity, gene frequency and PIC values

DNA polymorphisms were detected according to band presence and absence. Absence of bands may be caused by the failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between primer sites. The nine primers used in the present study showed different levels of major allele frequency, gene diversity and polymorphism information content (PIC) values which are given in Table 7.

Table 7: Gene diversity, gene frequency and PIC value of different hybrid varieties of bottle gourd by RAPD, ISSR, UBC and SSR primers

Sl. no.	Primers	Locus no.	Locus size (bp)	Major allele frequency	Gene diversity	PIC value
01	OPB-04	1	900	0.7500	0.3750	0.3047
		2	600	0.8750	0.2188	0.1948
		3	400	0.5000	0.5000	0.3750
		4	200	0.6250	0.4688	0.3589
02	OPD-03	1	600	0.6250	0.4688	0.3589
		2	400	0.7500	0.3750	0.3047
		3	250	0.7500	0.3750	0.3047
03	OPA-04	1	700	0.8750	0.2188	0.1988
		2	350	0.7500	0.3750	0.3047
		3	200	0.6250	0.4688	0.3589
04	OPF-10	1	900	0.6250	0.4688	0.3589
		2	500	0.8750	0.2188	0.1948
		3	300	0.6250	0.4688	0.3589
05	ISSR 05	1	450	0.5000	0.5000	0.3750
		2	200	0.6250	0.4688	0.3589
06	UBC 834	1	750	0.5000	0.5000	0.3750
		2	450	0.5000	0.5000	0.3750
		3	300	0.7500	0.3750	0.3047
		4	100	0.8750	0.2188	0.1984
07	UBC 855	1	1000	0.7500	0.3750	0.3047
		2	500	0.5000	0.5000	0.3750
		3	300	0.5000	0.5000	0.3750

08	UBC 854	1	600	0.5000	0.5000	0.3750
		2	200	0.5000	0.5000	0.3750
09	ZJULM55	1	350	0.7500	0.3750	0.3047
		2	100	0.6250	0.4688	0.3589
Mean				**0.6585**	**0.4147**	**0.3243**

The gene frequency of the major allele ranged between 0.5000 to 0.8750 with an average value of 0.6585 and Nei's (1972) gene diversity ranged from 0 to 0.5000. The highest gene frequency (0.8750) was shown by the primers OPB-04 (600 bp), OPA-04 (700 bp), OPF-10 (500 bp), UBC 834 (100 bp). In OPB-04 (900 bp), OPD-03 (400 and 250 bp), OPA-04 (350 bp), UBC 834 (300 bp), UBC 855(1000 bp) and ZJULM 55 (350 bp) the highest gene frequency (0.7500) were observed. The highest gene diversity was shown by the primer OPB-04 (at 400 bp), ISSR 05 (at 450 bp), UBC 834 (at 750 and 450 bp), UBC 855 (500 and 300 bp) and UBC 854 (at 600 and 200 bp). In the primers OPD-03, OPA-04, OPF-10 and ZJULM-55 highest gene diversity was 0.4688. On the other hand, lowest gene diversity (0.2188) was observed at 600 bp by OPB-04,700 bp by OPA-04, 500 bp by OPF-10, 100 bp by UBC 834.

Polymorphic Information Content (PIC) value for the nine markers ranged from 0.1948 to 0.3750 and the average PIC value was 0.3243. The highest PIC value (0.3750) was obtained for OPB 04 (at 400 bp), ISSR 05 (450 bp), UBC 834 (at 750 bp and 450 bp), UBC 855 (at 500 bp and 300 bp), UBC 854 (at 600 bp and 200 bp). The lowest PIC value (0.1948) was obtained for OPB 04 (at 600 bp), OPA 04 (at 700 bp), OPF 10 (at 500 bp) and UBC 834 (at 100 bp). The PIC value revealed that UBC 834, UBC 855 and UBC 854 were considered as the best marker for nine bottle gourd hybrids followed by ISSR 05 and OPB 04 marker. The results indicated that the eight bottle gourd hybrids present a low degree of homozygosity and a certain degree of genetic differentiation and polymorphism.

The PIC was a good index for genetic diversity evaluation. Botstein *et al*. (1980) reported that PIC index can be used to evaluate the level of gene variation, when PIC>0.5, the locus was of high diversity; when PIC<0.25, the locus was of low diversity and the locus was of intermediate diversity at PIC between 0.25 and 0.5. Lower PIC value indicates that the varieties under study are closely related types, while the higher value of PIC indicates higher diversity of materials, which is better for the development of new varieties.

4.5 Nei's genetic distance and genetic identity

Genetic distance refers to the genetic deviation between species or between populations within a species. It is measured by a variety of parameters like Nei's standard genetic distance. This distance measure is known to give more reliable population trees than other distances, particularly for DNA data. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similarity between two individuals. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. The genetic distance can be used to compare the genetic similarity between different species. Genetic diversity studies help in the selection of material for a breeding program.

The value of pair-wise comparison Nei's (1983) genetic distance between eight bottle gourd hybrids was computed from combined data through nine primers ranging from 0.2308 to 0.7308. The highest Nei's genetic distance (0.7308) was observed in Hybrid 1 vs Hybrid 3, Hybrid 1 vs Hybrid 4 varietal pairs whereas lowest values (0.2308) was observed in Hybrid 2 vs Hybrid 5, Hybrid 7 vs Hybrid 8 varietal pairs (Table 8).

Table 8: Summary of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values among eight bottle gourd hybrids

	Hybrid 1	**Hybrid 2**	**Hybrid 3**	**Hybrid 4**	**Hybrid 5**	**Hybrid 6**	**Hybrid 7**	**Hybrid 8**
Hybrid 1	0.0000	0.5385	0.7308	0.7308	0.5385	0.5000	0.4231	0.5000
Hybrid 2	0.5385	0.0000	0.4231	0.4231	0.2308	0.3462	0.5769	0.4231
Hybrid 3	0.7308	0.4231	0.0000	0.3077	0.5769	0.5385	0.6154	0.6154
Hybrid 4	0.7308	0.4231	0.3077	0.0000	0.3462	0.3077	0.3846	0.4651
Hybrid 5	0.5385	0.2308	0.5769	0.3462	0.0000	0.2692	0.3462	0.3462
Hybrid 6	0.5000	0.3462	0.5385	0.3077	0.2692	0.0000	0.3077	0.3077
Hybrid 7	0.4231	0.5769	0.6154	0.3846	0.3462	0.3077	0.0000	0.2304
Hybrid 8	0.5000	0.4231	0.6154	0.4651	0.3462	0.3077	0.2308	0.0000

This variation can be occurred by geographical origin. The result also reveals that the genetic base among these bottle gourd hybrids is rather narrow. Collection of diverse germplasm from centres of diversity may broaden the genetic base. RAPD, ISSR and SSR markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management.

4.6 UPGMA Dendrogram

Dendrogram based on Nei's (1983) genetic distance using Unweighed Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of eight hybrids of bottle gourd into three main clusters: A, B and C. The first major cluster 'A' had divided only two minority groups, which are also subdivided into 4 Hybrid genotypes (H1, H2, H3 and H4). The second cluster 'B' had 2 Hybrid genotypes (H7 and H8). The third major cluster 'C' had 2 Hybrid genotypes (H5 and H6) (Figure 1).

The main objectives for this study were to study genetic diversity among eight bottle gourd hybrid varieties using RAPD, ISSR and SSR primers. Consideration of estimated genetic distance is important for comparative analysis of diversity levels (Rodriguze *et al*., 1999). The result indicates that the low or high-level genetic distance exists between varieties with their same or different origins. Hybrid 1 vs Hybrid 3 and Hybrid 1 vs Hybrid 4 varietal pairs showed highest Nei's genetic distance (0.7308) as they are released from the different parental origin. On the other hand, Hybrid 2 vs Hybrid 5 and Hybrid 7 vs Hybrid 8 varietal pairs showed lowest genetic distance (0.2308) as they are released from the same parental origin.

Cluster analysis on bottle gourd varieties was also performed by several scientists. The study was conducted by Gbotto *et al*. (2015) on the genetic structure of Oleaginous *Lagenaria siceraria* by UPGMA dendrogram segregated thirty accessions showed clustering into different groups according to geographical location. Koffi *et al*. (2009), the UPGMA cluster analysis of morphological differentiation among cultivars of *Lagenaria siceraria* showed that the used two cultivars were separated into two clusters.

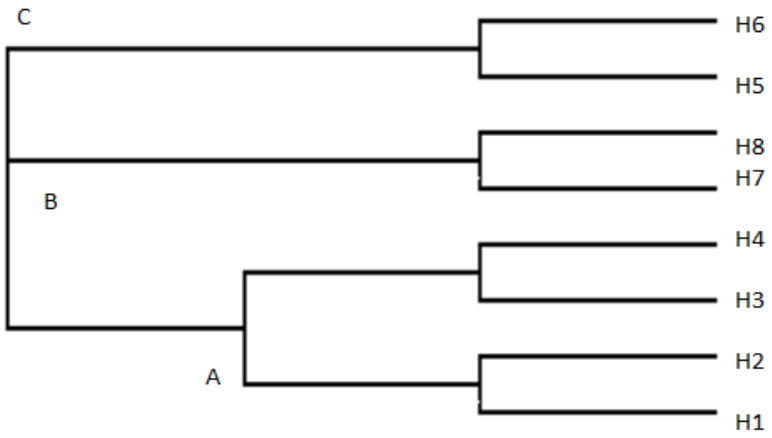


Figure 1. Dendrogram of eight bottle gourd hybrids were based on RAPD, ISSR, UBC and SSR marker, according to the un-weighted pair group method of arithmetic means (UPGMA) method based on a similarity matrix by PAST software.

The bottle gourd hybrids H1, H7, H8 were collected from Metal Seed Limited. It observed that two hybrid H7 and H8 showed more similarity in genetic identity and UPGMA dendrogram. Its reveal that, the parent of this two hybrid are obtained from close genetic distance. Likewise, the hybrid H5 and H6 collected from ACI Seed Ltd. and it is interesting to note that this two hybrid (H5 and H6) also classified in same group C. It is another indication that, the ACI collected experimental material were close in respect of genetic distance. The two hybrid H3 and H4 were collected from Lal Teer Seed Ltd. The DNA fingerprint pattern of this two hybrid fall in the same group A. It is also a good evidence that, within the Lal Teer Seed genotypes are very similar to each other. The present investigation revealed that, the bottle gourd hybrid developed by the seed companies has more similarity within their own genotypes but to some extend genetically diverse between different seed companies.

CHAPTER V

SUMMARY AND CONCLUSION

Bottle gourd is one of the most important vegetables grown all over the country for its diverse uses such as food, medicine, fodder, making household utensils and musical instruments. Farmers are now diverting to bottle gourd cultivation because it has relatively short production cycles, availability of year-round hybrid seeds and proved as a more profitable than some of other staple crops and pulses. Good quality hybrid seeds can accelerate the bottle gourd cultivation and ensure the profit margin. Genetic diversity is one of the most important factors considered in plant breeding and molecular approaches are well accepted and precise to determine the diversity. Despite most commercially important crop in Bangladesh, research findings on genetic analysis of different bottle gourd hybrids using molecular markers are scarce. Hence, it is a need to conduct studies to evaluate the genetic diversity of hybrid bottle gourd for breeding and documentation purposes.

This research investigation presented mainly the molecular characterization of eight bottle gourd hybrids. The present study was conducted at Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207. Nine primers (RAPD, ISSR, UBC and SSR) generated total 26 distinct and differential amplification bands with an average of 2.88 bands per primer. The highest number of bands (3) generated by the primer OPB-04, OPD-03 and UBC 834. The lowest number of bands (2) were generated by the primer ISSR 05, UBC 854 and SSR primer (ZJULM 55). The highest percentage of polymorphic bands (100%) was generated by OPD-03 while lowest percentage of polymorphism (50%) was shown by primer OPA-04, ISSR 05, UBC 854 and SSR primer (ZJULM 55). The frequency of the major allele ranged between 0.5000 to 0.8750 with an average value of 0.6585. Polymorphic Information Content (PIC) value for nine primers ranged from 0.1948 to 0.3750. The overall gene diversity ranged between 0.2188 to 0.5000 with an average of 0.4147. PIC value revealed that UBC 834, UBC 855 and UBC 854 were considered the best markers for eight bottle gourd hybrids followed by ISSR 05 and OPB-04. The OPD -03, OPA-04, OPF-10 and SSR markers (ZJULM 55) were considered the least powerful markers.

The value of pair-wise comparisons Nei's (1972) genetic distance between eight bottle gourd hybrids was computed from combined data for the nine primers, range from 0.2308 to 0.7308. The highest genetic distance (0.7308) was observed in Hybrid 1 vs Hybrid 3 and Hybrid 1 vs Hybrid 4 varietal pairs. On the other hand, Hybrid 2 vs Hybrid 5 and Hybrid 7 vs Hybrid 8 varietal pairs

showed the lowest genetic distance (0.2308). Unweighted Pair Group Method of Arithmetic Mean (UPMGA) segregated eight bottle gourd hybrids into three major clusters (Cluster A, B and C). The first major cluster 'A' was subdivided into two clusters that are further divided into two clusters containing four hybrids (H1, H2, H3 and H4), second major cluster 'B' had two hybrids (H7 and H8) and third major cluster 'C' had two hybrids (H5 and H6).

The result indicates that the low-level genetic distance exists between varieties with their same or different origins. Hybrid 1 vs Hybrid 3 and Hybrid 1 vs Hybrid 4 varietal pairs showed highest Nei's genetic distance (0.7308) as they are released from the different parental origin. On the other hand, Hybrid 2 vs Hybrid 5 and Hybrid 7 vs Hybrid 8 varietal pairs showed lowest genetic distance (0.2308) as they are released from the same parental origin. This variation can be created by geographical origin. The result also reveals that the genetic base among these bottle gourd hybrids is rather narrow. The bottle gourd hybrid H1, H7 and H8 were collected from Metal Seed Limited showed more similarity in genetic identity (UPGMA dendrogram). The hybrid H5 and H6 collected from ACI Seed Ltd. fall in the same group-C because of the they were close in respect of genetic distance. The two hybrid H3 and H4 were collected from Lal Teer Seed Ltd. This two hybrids fall in the same group -A showed that Lal Teer Seed genotypes were very similar to each other. The bottle gourd hybrid developed by the seed companies has more similarity within their own genotypes but to some extent genetically diverse between different seed companies.

RAPD markers provide a fast, efficient technique for variability assessment that complements methods currently being used in genetic resource management. ISSR markers provide a genotyping system with features of stability, reliability, dominancy and high polymorphism. SSR primers showed few polymorphic bands which can be used as evidence of variety protection data. It is essential to submit DNA fingerprint data of a new hybrid for registration and documentation. DNA passport data or DNA blueprint information can help in variety protection of the hybrid varieties. Being a poorly studied genome, little information is available on the molecular characterization of hybrid bottle gourd. The result of the present study can be used as a guideline for future diversity assessment and genetic analysis of hybrid bottle gourd.

RECOMMENDATION

The present work was the preliminary study to assess genetic variation of bottle gourd hybrids and it had some limitations in term of the limited number of individuals and varieties as well as the number of primers used. The results indicate that the present study might be used as a guideline for further study and the following points might be considered for sustaining the genetic qualities of hybrid bottle gourd in Bangladesh:

1. A large number of varieties and the higher number of primers should be studied with adequate replications to increase accuracy.

2. Details survey work should be conducted using more molecular markers for obtaining diagnostic loci for hybrid varieties.

3. Other molecular markers such as SNP, AFLP etc. should be developed for bottle gourd hybrid varieties of Bangladesh.

REFERENCES

Achigan-Dako E. G., Fuchs J., Ahanchede A. and Blattner F. R. (2008). Flow cytometric analysis in *Lagenaria siceraria* (Cucurbitaceae) indicates correlation of genome size with usage types and growing elevation. *Pl. Systm. Evol*. 276: 9–19.

Akkaya M. S., Bhagwat A. A. and Cregan P. B. (1992). Length polymorphism of simple sequence repeat DNA in soybean. *Genet*. 132: 1131-1139.

Amin A., Razvi1 S. M., Dar Z. A., Zafar G., Bhat M. A., Mir M. R., Wani N. and Bhat K. A. (2010). Molecular markers and their application in improvement of vegetable crops. *Intl. J. Curren. Res.* 4: 38-48.

Amin M. R. (2013). Impact of commercial bean farming on livelihood improvement of farmers in Ishwardi upazila of Pabna district. An Unpublished M.S. thesis, Bangladesh Agricultural University, Mymensingh.

Awal A. (2013). An economic analysis of potato and carrot production in selected areas of Mymensingh district. An unpublished M.S. thesis, Bangladesh Agricultural University, Mymensingh.

Badmanaban R. and Patel C. N. (2010). *J. Glbal. Phrma. Tech*. 4: 66–70.

Bahera T. K., Singh A. K. and Staub J. E. (2008 a). Comparative analysis of genetic diversity in Indian bitter gourd (*Momordica charantia* L.) using RAPD and ISSR markers for developing crop improvement strategies. *Scientia Horticultarae*. 115:209-217.

BBS (Bangladesh Bureau of Statistics). 2014. Yearbook of Agricultural Statistics, Statistical Division, Ministry of Planning, Government of the People's Republic of Bangladesh, Dhaka.

BBS (Bangladesh Bureau of Statistics). 2011. Statistical Yearbook of Bangladesh, Statistical Division, Ministry of Planning, Government of the People's Republic of Bangladesh, Dhaka.

Beevy S. S. and Kuriachan P. (1996) Chromosome numbers of south Indian Cucurbitaceae and a note on the cytological evolution in the family. *J. Cytol. Genet*. 31:65–71.

Bhawna A. M. Z., Arya D., Saha A. K., Sureja C., Pandey and Verma M. (2014). Population structure and genetic diversity in bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] germplasm from India assessed by ISSR markers. *Pl. Syst. Evol*. 300:767–773.

Bligh H. F. J., Blackhall N. W., Edwards K. J. and McClung A. M. (1999). Using amplified fragment length polymorphisms and simple sequence length polymorphisms to identify cultivars of brown and white milled rice. *Crop Sci*. 39: 1543-1635.

Botstein D., White R. L., Skolnick M. and Davis R. W. (1980). Construction of genetic linkage map in man using restriction fragment length polymorphisms. *American. J. Hum. Genet*. 32: 314-333.

Chapco W., Ashton N. W., Martel R. K. B. and Antonishyn N. (1992). A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers. 35: 569-574.

Choudhary K., Choudhary O. P. and Shekhawat N. S. (2008). Marker assisted selection: A novel approach for crop improvement. *American Eurasian J. Agron*. 1 (2): 26-30.

Clarck A. G. and Lanigan C. M. S. (1993). Prospects for estimating nucleotide divergence with RAPDs. *Mol. Biol. Evol*. 10: 1069-1111.

Cregan P. B. (1992). Simple sequence repeat DNA length polymorphisms. 2: 18-22.

Datta D., Gupta S., Chaturvedi S. K. and Nadarajan N. (2011). Molecular markers in crop improvement. *Indian Inst. Pulses Res*. pp. 229-233.

Decker-Walters D., Staub J., Lopez-sese A. and Nakata E. (2001). Diversity in landraces and cultivars of bottle gourd (*Lagenaria siceraria* ; Cucurbitaceae) as assessed by random amplified polymorphic DNA. *Genet. Resour. Crop Evol*. 48: 369-380.

Dje Y., Tahi G.C., Zoro B. A., Malice M., Baudoin J. P. and Bertin P. (2006). Optimization of ISSR marker for African edible-seeded Cucurbitaceae species genetic diversity analysis. *African J. Biotech*. 5(2): 83-87.

EPB (2014). Product Export data news, Export Promotion Bureau, Bangladesh Ministry of Commerce, TCB Bhavan, Kawran Bazar, Dhaka.

Fard M. H., Bodhankar S. L. and Dikshit M. (2008). Cardioprotective activity of fruit of *Legenaria siceraria* (Molina) Standl on Doxorubicin induced cardiotoxity in rats. *Intl. J. Phrmclgy*. 6: 466–471.

Ferriol M., Pico B. and Nuez F. (2003). Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. *Theor. Appl. Genet*. 107: 271-282.

Fristsch P. and Rieseberg L. H. (1996). The use of random amplified polymorphic DNA (RAPD) in conservation genetics. In: Smithers T. B. and R. K. Wanye, (eds.). Mol. genetic approaches in Conservation, Oxford University Press, Oxford. pp. 54-73.

Gbotto A. A., Koffi K. K., Baudoin J. P., Bi I. and A. Z. (2015). Determination of the Genetic Structure of the Oleaginous *Lagenaria siceraria* of the Nangui Abrogoua University Germplasm Collection. *American J. Pl. Sci*. 6: 3231-3243.

Ghule B.V., Ghanti M. H., Yeole P. G. and Saoji A. N. (2007). Diuretic activity of *Lagenaria siceraria* fruit extracts in rats. *Indian J. Phrmtcl. Sci*. 69: 817–819.

Gong L., Paris H. S., Nee M. H., Stift G., Pachner S., Vollmann J. and Lelley T. (2012). Genetic relationships and evolution in *Cucurbita pepo* (pumpkin, squash, gourd) as revealed by simple sequence repeat polymorphisms. *Theor. Appl. Genet*. 124: 875–891.

Gong L., Stift G., Kofler R., Pachner M. and Lelley T. (2008). Microsatellites for the genus *Cucurbita* and an SSR-based genetic linkage map of *Cucurbita pepo* L. *Theor. Appl. Genet*. 117:37-48.

Gopalan C., Rama Sastri B.V. and Balasubramaniam S. C. (1982). Nutritive Value of Indian Foods. Indian Council of Medical Research, National Research Institute of Nutrition, Hyderabad.

Gosal S. S., Wani S. H. and Kang M. S. (2010). Biotechnology and crop improvement. *J. Crop Improv.* 24(2): 153-217.

Goswami M. and Ranade S. A. (1999). Analysis of variations in RAPD profiles among accessions of Prosopsis. *J. Genet*. 78: 141-147.

Gracia A. A. F., Benchimol L. L., Antonica M. M., Geraldi I. O. and Deuza A. P. (2004). Comparison of RAPD, RFLP, AFLP and SSR marker for diversity studies in tropical maize inbred lines. *Euphytica*. 108: 53-63.

Hadia H. A., Abdel H.S. and Hafez E. E. (2008). Assessment of Genetic relationships among and within Cucurbita species using RAPD and ISSR markers. *J. Appl. Sci. Res*. 4(5): 515-525.

Hadrys H., Balicki M. and Schierwater B. (1992). Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1: 55-63.

Haig S. M., Rhymer J. M. and Heckel D. G. (1994). Population differentiation in Random Amplified Polymorphic DNA of red-cockaded woodpeckers Picoides borealis. *Mol. Ecol.* 3: 581-595.

Huang C. H., Wang C. J. and Chyuan J. H. (2010). Analysis of the genetic diversity and variety identification of bitter gourd (*Momordica charantia* L.) by ISSR markers. Hualien District Agricultural Improvement Station, Bulletin 28:21-33.

Islam M. N., Molla M. R. and Rahman L. (2013). Genetic characterization of sesame varieties through RAPD fingerprinting technique in Bangladesh. *J. Sher-e-Bangla Agric. Univ*. 7(1): 1-5.

Kalia R. K., Rai M. K., Kalia S., Singh R. and Dhawan A. K. (2011). Microsatellite markers: an overview of the recent progress in plants. *Euphytica*. 177: 309–334.

Karp A., Kresovich S., Bhat K., Ayad W., and Hodgkin T. (1997). Molecular tools in plant genetic resources conservation: a guide to the technologies. IPGRI. Rome, Italy.

Katzir N., Todmor Y., Tzuri G., Leshzeshen E., Mozes-Dauuuuube N., Danin-Poleg Y. and Paris H. S. (2000). Futher ISSR and preliminary SSR analysis of relationships among accessions of *Cucurbita pepo*.*Cucurbitaceae*. 2000: 433-439.

Kimberling D. N., Ferreira A. R., Shuster S. M. and Keim P. (1996). RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the southwestern USA. *Mol. Ecol.* 5 (4): 521-529.

Koffi K. K., Anzera G. K., Malice M., Dje Y., Bertin P., Baudoin J. P. and Zoro-Bi I. A. (2009). Morphological and allozyme variation in a collection of *Lagenaria siceraria* (Molina) Standl from Cote D'Ivoire. *Biotech. Agron. Soc. Environ*. 13: 257-270.

Kostova A., Todorovska E., Christov N., Hristov K. and Atanassov A. (2006). Assessment of Genetic Variability Induced by Chemical Mutagenesis in Elite Maize Germplasm via SSR Markers. *J. Crop Improv*. 16: 37-48.

Kumar N. S. and Gurusubramanian G. (2011). Random amplified polymorphic DNA (RAPD) markers and its applications. *Sci. Vis*. 11: 116-124.

Kumari N. and Thakur S. K. (2014). Randomly Amplified Polymorphic DNA A brief review. *American J. Anim. Vet. Sci*. 9(1): 6-13.

Lagercrantz U., Ellegren H. and Andersson L. (1993). The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. *Nucleic Acids Res*. 21: 1111-1115.

Lapitan V. C., Brar D. S., Abe T. and Redofia E. D. (2007). Assessment of genetic diversity of Philippine rice cultivars carrying good quality traits using SSR markers. *Breed. Sci*. 57: 263-270.

Leroy X. J., Leon K., Charles G. and Branchard M. (2000). Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. *Pl. Cell Rep.* 19: 1102-1107.

Levi A., Thomas C. E., Trebitsh., Salmann A., King J., Karalius J., Newman M., Reddy O. V., Xu Y. and Zhang X. (2006). An extended Linkage map for watermelon based on SRAP, AFLP, ISSR and RAPD markers. *J. American Soc. Hurt. Sci*. 131 (3): 393-402.

Litt M. and Luty J. A. (1989). A hypervariable microsatellite revealed by *in vitro* amplication of a dinucleotide repeat within the cardiac muscle action gene. *American J. Hum. Genet*. 44: 388-396.

Liu K. and Muse S.V. (2005). Power Marker: integrated analysis environment for genetic marker data, Bioinformatics. 21: 2128-2129.

Madhumati B. (2014). Potential and application of molecular markers techniques for plant genome analysis. *Int. J. Pure App. Biosci*. 2(1): 169-188.

Mailer R. J., Scarth R. and Fristensky B. (1994). Discrimination among cultivars of rapeseed (*Brassica napus* L.) using DNA polymorphism amplified from arbitrary primers. *Theor. Appl. Genet.* 87: 697-704.

Marshall P., Marchand M. C., Lisieczko Z. and Landry B. S. (1994). A simple method to estimate the percentage of hybridity in canola (*Brassica napus*) F1 hybrids. *Theor. Appl. Genet.* 89: 853858.

Michelmore R. W., Paran I. and Kesseli R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA*. 88(21): 9828-9832.

Mondini L., Noorani A. and Pagnotta M. A. (2009). Assessing plant genetic diversity by molecular tools. *Diversity*. 1(1): 19-35.

Morgante M. and Olivieri A. (1993). PCR-amplified microsatellites as markers in plant genetics. *Pl. J.* 3:175-182.

Morimoto Y., Maundu P., Fujimaki H. and Morishima H. (2005). Diversity of landraces of the white flowered gourd (*Lagenaria siceraria*) and its wild relatives in Kenya: fruit and seed morphology. *Genet. Resour. Crop Evol*. 52: 737-747.

Nakata E., Staubbb J. E., Lopez A.I. and Katzir N. (2005). Genetic Diversity of Japanese melon cultivars (*Cucumis melo* L.) as assessed by Random Amplified Polymorphic DNA(RAPD) and Simple Sequence Repeats (SSR) markers. *Genet. Resour. Crop Evol.* 52:405-419.

Paris H.S., Portnoy V., Mozes-Daube N., Tzuri G. and Katzir N. (2003). Assessment of genetic relationships in *Cucurbita pepo* (Cucurbitaceae) using DNA markers. *Theor. Appl. Genet*. 106: 971-978.

Peng X., Liu J.J., Xiang Y. and Huang S. (2006). A practical handbook of plant molecular biotechnology. Chemical Industry Press, Beijing.

Powell W., Machray G. C. and Provan J. (1996). Polymorphism revealed by simple sequence repeats. *Trend. Pl. Sci*. 1:215-222.

Ragot M. and Hoisington D. A. (1993). Molecular markers for plant breeding: Comparisons of RFLP and RAPD genotyping costs. *Theor. Appl. Genet*. 86: 965-984.

Rahman S. N., Islam M. S., Alam M. S. and Nasiruddin K. M. (2006). Genetic polymorphism in rice (*Oryza sativa* L.) through RAPD analysis. *Indian J. Biotech*. pp. 224-229.

Rana M. K. and Bhat K. V. (2004). A comparison of AFLP and RAPD markers for genetic diversity and cultivar identification in cotton. *J. Pl. Biochem. Biotech*. 13: 19-24.

Rholf F. (2002). Numerical Taxonomy and Multivariate Analysis System Version 2.2. New York, USA: Department of Ecology and Evolution, State University of New York.

Rodriguez J. M., Berke T., Engle L. and Nienhuis J. (1999). Variation among and within Capsicum species revealed by RAPD markers. *Theor. Appl. Genet*. 99: 147-156.

Sarao N. K., Neha M. P. and Kaur K. (2013). Microsatellite-based DNA fingerprinting and genetic diversity of bottle gourd genotypes. *Pl. Genet. Resour. Charact. Util*. (First View Article):1–4.

Saxena S. A., Singh S., Archak T., Behera J., John S., Meshram A. and Gaikwad. (2015). Development of novel simple sequence repeat markers in bitter gourd (*Momordica charantia* L.) through enriched genomic libraries and their utilization in analysis of genetic diversity and cross-species transferability. *Appl. Biochem. Biotech*. 175:93–118.

Sestili S., Daniele A., Rosa A., Ferrari V., Belisario A. and Ficcandenti N. (2008). Molecular characterization of different Italian *Inodorus* melon populations based on ISSR molecular markers and preliminary SSR analysis. Proceedings of the IXth EUCARPIA meeting on Genetics and Breeding of *Cucurbitaceae* (PitratM, ed), INRA, Avignon (France), May 21-24th, 2008.

Sikdar B., Bhattacharya M., Mukherjee A., Banerjee A., Ghosh E., Gosh B. and Roy S. C. (2010). Genetic diversity in important members of *Cucurbitaceae* using isozyme, RAPD and SSR markers. *Biologia Plantarum.* 54 (1):135-140.

Singh J., Kallo G. and Singh K. P. (2001). Vegetable crops: Nutritional Security: *Indian Inst. Veg. Res*. pp. (14).

Sneath P. H. A. and Sokal R. R. (1973). Numerical taxonomy: the principles and practice of numerical classification. San Francisco: Freeman. pp. 573.

Squirrel J., Hollingsworth P. M., Woodhead M., Russell J., Lowe A. J., Gibby M. and Powell W. (2003). How much effort is required to isolate nuclear microsatellites from plants. *Mol. Ecol*. 12: 1339-1348.

Staub J. E., Danin-Poleg Y., Fazio G., Horejsi T., Noa R. and Katzir N. (2000). Comparative analysis of cultivated melon groups (*Cucumis melo* L.) using Random Amplified Polymorphic DNA and Simple Sequence Repeat markers. *Euphytica*. 115:225-241.

Stepansky A., Kovalski I. and Rafael P. (1999) Intraspecific classification of melons (*Cucumis melo* L), In view of their phenotypic and molecular variation. *Pl. Systm. and Evol*. 217:313-333.

Tommercup I. C., Barton J. E. and Obrein P. A. (1998). Reliability of RAPD fingerprinting of three basidiomycetes fungi, Laccaria, Hydnangium and Rhizoctonia. *Mycol. Res*. 99: 179-186.

Upaganlawar A. and Balaraman R. (2010). Protective effects of *Legenaria siceraria* (Molina) fruit juice in isoproterenol induced myocardial infarction. *Intl. J. Phrmclgy*. 5:645–651.

Varshney R. K., Sigmund R., Borner A., Korzun V., Stein N., Sorrells M. E., Langridge P. and Graner A. (2005). Interspecific transferability and comparative mapping of barley EST–SSR markers in wheat rye and rice. *Pl. Sci*. 168: 195-202.

Varshney R. K., Graner A. and Sorrells M. E. (2005). Genic microsatellite markers in plants: Features and applications. *Trend. Biotech.* 23:48–55.

Vierling R. and Nguyen H. T. (1992). Use of RAPD markers to determine the genetic relationships of diploid wheat genotypes. *Theor. Appl. Genet*. 84: 835-838.

Vilanova S., Manzur J. P. and Prohens J. (2012). Development and characterization of genomic simple sequence repeat markers in eggplant and their application to the study of diversity and relationships in a collection of different cultivar types and origins. *Mol. Breed*. 30**:** 647-660.

Watcharawongpaiboon N. and Chunwongse J. (2007). Development and characterization of microsatellite markers from an enriched genomic library of cucumber (*Cucumis sativus*). *Pl. Breed*. 127(1):74-81.

Whitaker T.W. (1971). Endemism and pre-columbian migration of the bottle gourd, *Lagenaria siceraria* (Mol.) Standl. In: Kelley, J.C., Pennington, C.W., Rands, R.L., (Eds.) Man Across the Sea, University of Texas Press, Austin, pp:320-337.

Williams J. G. K., Kubelik A. E., Levak K. J., Rafalski J. A. and Tingey S. V. (1990). Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymology. 1218: 704-740.

Williams J. GK., Kubelik A. R., Livak K. J., Rafalski J.A. and Tingey S.V. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.

Xu P., Wu X., Luo J., Wang B., Liu Y., Ehlers J. D., Wang S., Lu Z. and Li G. (2011). Partial sequence of the bottle gourd genome reveals markers useful for phylogenetic analysis and breeding. *Genomics.* 12: 467.

Xu P., Xu S., Wu X., Tao Y., Wang B., Wang S., Qin D., Lu Z. and Li G. (2014). Population genomic analysis from low-coverage RAD-seq data: a case study on the non-model cucurbit bottle gourd. *Pl. J.* 77: 430-442.s

Yeboah M. A., Xuehao C., Chen R. F., Liang G. and Minghong G. (2007). A genetic linkage map of cucumber (*Cumus sativus* L.) combining SRAP and ISSR markers. *African J. Biotech*. 6(24):2784-2791.

Yeh F. C., Yang R. C. and Boyle T. (1999). POPGENE VERSION 1.31: Microsoft Window-based free Software for Population Genetic Analysis.

Yetsir H., Sakar M. and Serce S. (2008). Collection and Morphological characterization of *Lagenaria siceraria* germplasm from the Mediterranean region of Turkey. *Genet. Resour. Crop Evol.* 55:1257-1266.

Zietkiewicz E., Rafalski A. and Labuda D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics.* 20:176–183.