MOLECULAR DIVERSITY ANALYSIS OF SOMACLONAL VARIANTS IN POTATO (*Solanum tuberosum* L.) BY RAPD MARKER

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CERTIFICATE

*This is to certify that thesis entitled, "**MOLECULAR DIVERSITY ANALYSIS OF SOMACLONAL VARIANTS IN POTATO (Solanum tuberosum L.) BY RAPD MARKER**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY,** embodies the result of a piece of bona fide research work carried out by **Md Shamsuzzaman, Registration No. 08-02726** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma in any institute.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: December, 2014
Place: Dhaka, Bangladesh

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

DEDICATED TO

MY

BELOVED PARENTS

Some commonly used abbreviation

Full word	Abbreviation	Full word	Abbreviation
2,4-Dichlorophenoxy acetic acid	2,4-D	Namely	viz.
Agricultural	*Agril.*	Negative logarithm of hydrogen ion concentration (-log[H+])	pH
Agriculture	*Agric.*		
American	*Am.*		
And others (at elli)	*et al.*	Newsletter	*Newsl.*
Angstrom	Å	Percent	%
As for example	e.g.	Physiology	*Physiol.*
Bangladesh Bureau of Statistics	BBS	Polymerase chain reaction	PCR
Base pair	bp	Proceeding	*proc.*
Biology	*Biol.*	Publication	*pub.*
Biotechnology	*Biotech.*	Random Amplified Polymorphic DNA	RAPD
Botany	*Bot.*		
Breeding	*Breed.*	Reports	*Rep.*
Continued	Cont'd	Research	*Res.*
Centi Morgan	cM	Ribonucleic Acid	RNA
Cultivar	cv.	Rotation per minute	rpm
Culture	Cult.	Science	*Sci.*
Cetyl Trimethyl Ammonium Bromide	CTAB	Similarity Index	SI
		Sodium chloride	NaCl
Degree celsius	°C	Sodium Dodecyl Sulphate	SDS
Deoxyribonucleic acid	DNA		
Distilled deionized water	ddH2O	Somaclonal variant in potato	SVP
Etcetera	etc.	Somaclone of Indian Potato	SIP
Ethidium Bromide	Et-Br		
Food and Agriculture Organization	FAO	Species (plural)	spp.
		Simple Sequence Repeat	SSR
Genetic Distance	GD		
Genetics	*Genet.*	Sequence Tagged Site	STS
Gram per Liter	g/L	That is	i.e.
Hectare	Ha	*Thermophilus aquaticus*	Taq
Horticulture	*Hort.*		
International	*Int.*	Ultra Violet	UV
Journal	*J.*	Volt	V
Micro mole	µM	Tris Boric Acid EDTA	TBE
Microgram per liter	µg/L	Tris-EDTA	TE
Micro liter	µL	Un-weighted Pair Group Method of Arithmetic Mean	UPGMA
Mili liter	mL		
Mili mole	mM		
Molecular	Mol		

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December, 2014 *The Author*
SAU, Dhaka

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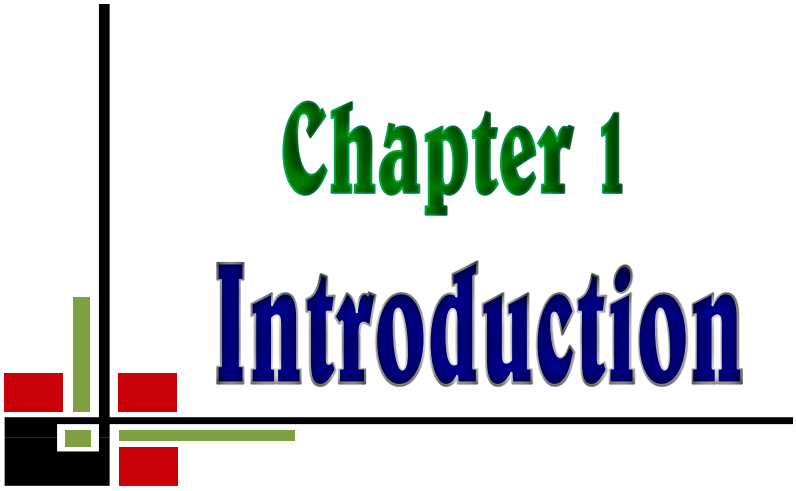
MOLECULAR DIVERSITY ANALYSIS OF SOMACLONAL VARIANTS IN POTATO (*Solanum tuberosum* L.) BY RAPD MARKER

ABSTRACT

An experiment was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh to study the DNA fingerprinting and genetic diversity of nine somaclonal variants of potato (*Solanum tuberosum* L.) and three check varieties (Cardinal, Diamant and Asterix). The somaclonal variants were SIP-3, SIP-5, SVP-6, SVP-18, SVP-19, SVP-25, SVP-55, SVP-56 and SVP-68. Genomic DNA was extracted from young leaves and PCR reaction was performed with six RAPD primers. Sum total of 54 distinct and differential amplified DNA bands were generated from PCR amplification. Out of them, 47 bands (87.04%) were polymorphic and 7 bands (12.96%) were monomorphic. The pair wise inter-variety/genotype similarity indices ranged from 61.59% to 93.55% with an average of 74.31%. The Nei's genetic identity among 12 potato genotypes ranged from 0.5370 to 0.9074 and genetic distance ranged from 0.0972 to 0.6217. The UPGMA Dendrogram segregated the 12 potato genotypes into two main clusters. The first cluster contained 8 genotypes and the second cluster had 4 genotypes. The Dendrogram also indicated the highest genetic distance and the lowest genetic identity between SVP-6 vs SVP-68 genotype pair. The study revealed a significant amount of relationship and genetic diversity among the studied 12 potato genotypes. The RAPD markers were found to be useful tool for DNA fingerprinting, polymorphism study and genetic diversity analysis of potato genotypes with a great potential for the Biotechnologists and Plant breeders for the improvement of potato cultivars.



Chapter 1

Introduction

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) belongs to the family Solanaceae, a large plant family with more than 3000 species and genus *Solanum*, a close relative of tomato, eggplant and pepper (Barrell *et al*., 2013; Spooner and Knapp, 2013; Massa *et al.*, 2011). The genus S*olanum* is very polymorphous and largely tropical and subtropical containing more than 1000 species (Spooner and Knapp, 2013; Fernald, 1970). It is divided into several sections and the sections further divided into several subsections. The section *petota* and subsection *potatoe* are noteworthy among them. The section *Petota* includes approximately 200 tuber-bearing species (Carputo *et al*., 2013; Camadro *et al*., 2012). The subsection *potatoe* is divided into several series, one of which is *tuberosa* which contains about 54 species, both wild and cultivated. One of them is *Solanum tuberosum* (Spooner *et al*., 2007; Hawkes, 1990). *Solanum tuberosum* is divided into two subspecies: *tuberosum* and *andigena* and has over 200 related wild species and 8 closely related cultivar groups (Spooner *et al*., 2007; Hanemnan, 1994; Hawkes, 1990). More than this, there are over 5000 varieties of cultivated potatoes (CIP, 2014).

The genetic and historical testing proved that the origin of potato is in the area of present-day southern Peru and extreme northwestern Bolivia, where they were domesticated approximately 7,000–10,000 years ago (Pasare, 2012; Spooner *et al*., 2005). From there, the potato was introduced to Europe between 1565-1580 AD by the Spaniards (Acquaah, 2007; Hijmans, 2001), and to Virginia, USA in 1621 AD (Acquaah, 2007; Sleper and Poehlman, 2006). Potato was introduced in this subcontinent in the sixteenth century (Acquaah, 2007). Potatoes have been grown in Bangladesh since the 19th century. Nowadays, potato has emerged as a short duration winter crop in Bangladesh and is being cultivated throughout the country (Potato-Banglapedia, 2014; Hossain, 2011). Potato can be grown from latitudes 65ºN to 50ºS, and from sea level to 4,700 metres above sea level (CIP,

2014), wherever average daily temperature ranges 5ºC to 21ºC with adequate water from rain or irrigation and in all types of soil except saline and alkaline soils (Hossain, 2014; Islam, 2012).

Solanum tuberosum is an herbaceous plant that grows to 0.4-1.4 m (meters) tall and may range from erect to fully prostrate (Spooner and Knapp, 2013). Stems range from nearly hairless to densely hairy and may be green, purple, or mottled green and purple. Leaves are pinnate with a single terminal leaflet and flowers are white, purple, pinkish, or bluish, in clusters, fruits are yellowish or green, globose, and less than one inch in diameter (Spooner and Knapp, 2013; Struik, 2007; Hawkes, 1990). The potato plant normally propagates vegetatively by means of planting pieces of tubers that bear two or three eyes (Hossain, 2014; Islam, 2012) or alternatively by true potato seeds or berries (Golmirzaie and Ortiz, 2004).

One hundred and eighty one tuber-bearing species of *Solanum* have known ploidy levels (Spooner *et al*., 2004). All of them possess a basic chromosome number, x = 12 and a haploid genome size of approximately 840 Mbp (840 million base pairs) and may be diploid (2n=2x=24) to hexaploid (2n=6x=72) (Spooner *et al*., 2014; Pasare, 2012; Vellasamy, 2010). Domestic *Solanum tuberosum* are highly heterozygous autotetraploids (2n = 4x = 48) and cultivated worldwide (Hossain, 2014; Andersson and de Vicente 2010; Pandey *et al*., 2010).

Total area of the world under potato crop is 1,93,37,071 ha and total potato production is 37,64,52,524 tons with average yield rate of 19.5 tons per ha (FAO, 2013). In Bangladesh total area under potato crop has been estimated 462032 ha and total potato production has been estimated 89,50,024 metric tons which is 4.03% higher than last year. Average yield rate of potato has been estimated 19.371 metric tons per ha (BBS, 2013-2014).

Potato varieties that are cultivated in Bangladesh are broadly categorized into two groups, local or deshi or indigenous and high yielding or modern. In spite of poor yields and longer vegetative cycles about 27 local varieties are cultivated

in different parts of the country, because of their good taste and cooking qualities (Potato- Banglapedia, 2014; Islam, 2009; Mondol, 2004). Varieties that are introduced here after 1960 AD are designated as modern varieties (Potato- Banglapedia, 2014; Islam, 2009; Mondol, 2004; Hashem, 1990).

Potato is very nutritious tuber vegetables. It contains about 74.7 % moisture, 20.6 % carbohydrates, 2.1% high quality proteins, 0.3 % fat, 1.1 % crude fiber and 0.9 % ash and notably high levels of vitamin C, vitamin B3 (niacin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), potassium, phosphorus, and magnesium (Hossain, 2014; Camire *et al*., 2009). It also contains a good amount of essential amino acids like leucine, tryptophan and isoleucine (Khurana and Naik, 2003); a variety of health promoting phytonutrients that have antioxidant activity such as: carotenoids, flavonoids, caffeic acids and tuber storage proteins such as patatin (Hossain, 2014; Islam, 2012; Karim *et al*., 2010).

Most of the countries consume potato as staple food. Millions of tons of potatoes are processed annually in Europe into starch, potato meal, flour, glucose, dextrose, lactic acid and other products (Potato- Banglapedia, 2014; Ezekiel *et al*., 2013; Bamberg and Rio, 2011). In Bangladesh, potato is mainly consumed as vegetables as complementary food with rice (boiled, fried, mash etc.) but not as staple food. Various other food items (Singara, Samucha, Chop, Chips, French fries, Pan cakes etc.) are also made from potato (Hossain and Miah, 2009; Islam, 2009; Moazzem and Fujita, 2004). Besides its use as food and vegetables it is substantially used in industry for various purposes *viz.* to brew alcoholic beverages, preparing polylactic acid and in the textile industry as adhesives. Moreover potatoes are also use as animal feed (Potato- Banglapedia, 2014; Islam, 2009; Campbell *et al*., 1997)

The yield level of potato in Bangladesh is too low (Hoque *et al*., 2013) when compared with that of other leading countries such as USA (46.6 ton/ha), Netherlands (43.7 ton/ha), and UK (40.1 ton/ha) (Hossain, 2014; FAO, 2013) even in case of high yielding or modern varieties (BBS, 2013-14). Main reasons of that are, varieties differ greatly in respect of time of maturity, yield and

quality, resistance to pests and diseases and rate of degeneration (Hossain, 2014; Rashid *et al*., 1987) and also show differences in certain tuber characteristics (Hossain, 2014; Mondol, 2004; Thompson and Kelly, 1957). These problems can be solved by developing high yielding varieties having others good qualities like resistant to insect and diseases (Hossain, 2014).

Developing high yielding varieties having other good qualities is challenging to potato breeders (Hoque *et al*., 2013; Munir *et al.* 2011; Solmon-Blackburn and Baker, 2001). It has narrow genetic base (Carputo *et al.*, 2013; Afrasiab and Iqbal, 2012a) which hampers its improvement in respect of disease resistance and other agronomic traits (Afrasiab and Iqbal, 2010). Potato is a tetraploid species and using its botanical seeds in commercial cultivation is impossible and it is the great limitation for improvement in conventional method (Tican *et al.*, 2008; Ehsanpour *et al*., 2007; Bordallo *et al*., 2004). Somatic embryogenesis, somaclonal variation and plant regeneration from callus make these challenges easy for the breeders (Ehsanpour *et al*., 2007).

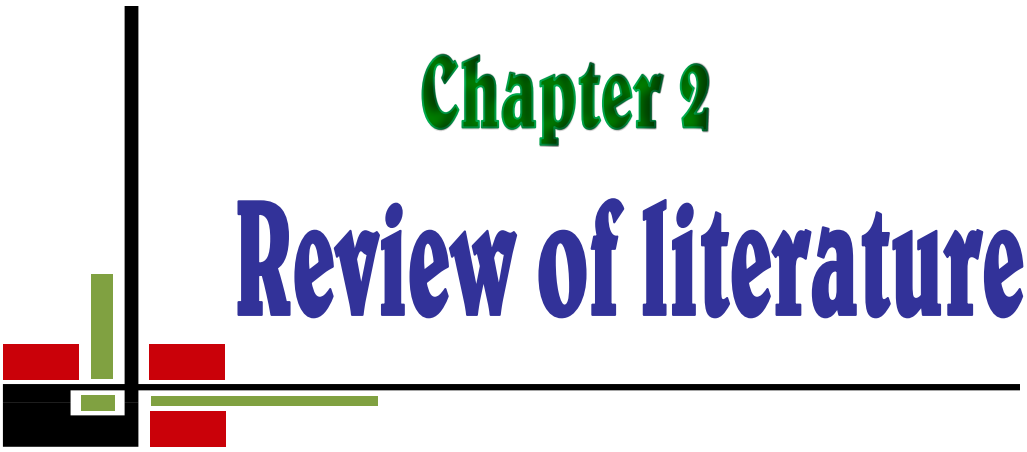
The occurrence of genetic variation among plants regenerated from *in vitro* culture has been referred to as somaclonal variation (Nwauzoma and Jaja, 2013; Rani and Raina, 2000; Leal and Maribona, 1991; Larkin and Scowcroft, 1981). It is also called tissue or culture-induced variation (Ehsanpour *et al.*, 2007; Kaeppler *et al*., 2000). This phenomenon may provide a useful source of variation to the plant breeder (Nwauzoma and Jaja, 2013; Ehsanpour *et al*., 2007). Somaclonal variations are considered to be a good supplement to conventional crop improvement. There is evidence in different crops that the variant characteristics obtained from culture of somatic tissues, is transmitted successfully to the progeny in terms of desirable characteristics (Afrasiab and Iqbal, 2010; Nwauzoma and Jaja, 2013).

There are several methods for detection of somaclonal variation. Scoring of changes in plant morphology can be useful in some studies, but there is limited diversity and trait may be affected by environmental influences. Detection of somaclonal variations using RAPD markers has several advantages, since RAPD

markers are technically simple, quick to perform with small amount of DNA and do not require previous information about genome or radioactive labeling (Michelmore *et al*., 1991). RAPDs are usually dominant and are inherited in a simple Mendelian fashion. The use of the PCR-based RAPD technique to detect somaclonal variations has been applied successfully to several plant species, such as *Lolium* (Wang *et al*., 1993), *Allium sativum* L. (Al-Zahim *et al*., 1999) and *Picea abies* (Heinze and Schmidt, 1995). It has also been applied for tomato (Soniya *et al*., 2001) and potato (Afrasiab and Iqbal, 2010; Ehsanpour *et al*., 2007; Khatab, 2000). Thus RAPD analysis is a useful tool in determining genetic relationships and diversity among regenerated somaclonal variant potato and their original cultivars.

In consideration of the above facts the present study has been undertaken to provide genetic variation and relatedness of some somaclonal potato genotypes by PCR amplification technique, as it is important particularly for variety selection, creation and improvement for breeding purpose. Thus the present study was conducted with the following objectives:

1. To analyze molecular diversity of three popular potato varieties and nine created somaclonal variants in potato.
2. To perform DNA fingerprinting of twelve potato genotypes through RAPD marker.
3. To study Phylogenetic relationship of new potato variants and released potato cultivars.
4. To establish Dendrogram among new potato variants and released potato cultivars.



Chapter 2

Review of literature

CHAPTER II

REVIEW OF LITERATURE

Despite of the importance of potato as crop, genetic data on it in Bangladesh is very limited. Because, in Bangladesh most of the research activities on potato for varietal improvement, variability detection and characterization is performed through traditional breeding methods. On the other hand, several researchers throughout the world have performed a lot of research activities on potato genetic variability and relationship detection, characterization through molecular markers like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) etc. Potato variety improvement through somaclonal variation is a new technique and new variant can be identified with the help of different molecular markers. The most relevant literature about the present study in home and abroad have been reviewed and some of the most relevant literatures are cited below:

2.1 Marker or Genetic marker

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

Datta *et al*. (2011) defined genetic marker as a readily recognizable genetic trait, gene, DNA segment, or gene product used for identification purposes especially when closely linked to a trait or to genetic material that is difficult to identify.

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

2.1.1 Morphological markers

Morphological markers are specific and distinct morphological traits that are scored visually (Datta *et al.*, 2011; Bhat *et al.*, 2010). These are related to shape, size, colour and surface of various plant parts. Such characters are used for the varietal identification. Morphological markers differ among species, genus and varieties of plants and animals (Jiang, 2013; Bagali *et al.*, 2010). These traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation. However, morphological markers availability are limited, affected by environment, show low level of polymorphism, and many of these markers are not associated with important economic traits (e.g. yield and quality) and even have undesirable effects on the development and growth of plants (Jiang, 2013; Bagali *et al.*, 2010).

These types of markers are still having advantage and they are mandatory for distinguishing the adult plants from their genetic contamination in the field, for example, spiny seeds, bristled panicle, and flower/leaf color variants (Mondini *et al*., 2009).

2.1.2 Biochemical markers

Second type of genetic marker is called Biochemical markers. Any specific character that may be detected by a biochemical test, e.g. the presence or absence of a particular enzyme is called Biochemical marker (Oxford Dictionary of Biochemistry and Molecular Biology).

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

used as genetic markers to map other genes. They are also used in seed purity test and occasionally in plant breeding (Jiang, 2013).

Major advantages of Biochemical markers consist in assessing co-dominance, absence of epistatic and pleiotrophic effects, ease of use, and low costs (Mondini *et al*., 2009). But there are only a small number of isozymes in most crop species and some of them can be identified only with a specific strain. Therefore, the use of enzyme markers is limited (Jiang, 2013). Isozymes have limitations in protein expression which is affected by changes in environment and plant development they also lack specificity and sensitivity to detect some genomic changes (Jiang, 2013; Bagali *et al.*, 2010).

2.1.3 Molecular markers or DNA markers

The third and most widely used genetic marker type is molecular markers, comprising a large variety of DNA. Datta *et al.* (2011) defined 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.

A molecular marker is a DNA sequence that can be readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes (Amin, *et al*., 2010).

Choudhary *et al.* (2008) stated that molecular markers are phenotypically neutral and it should not be considered as normal genes as they usually do not have any biological effect. Instead, they can be thought of as constant landmarks in the genome.

There are mainly two types of molecular markers i.e. (i) Hybridization based or Non-PCR based marker for example Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al*., 1980) and (ii) Polymerase chain reaction (PCR) based markers, e.g. Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Sequence characterized amplified regions (SCARs)

(Michelmore *et al*., 1991) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995).

Molecular markers are used for development of saturated genetic maps, DNA fingerprinting, phylogenetic and evolutionary studies, heterotic breeding, gene tagging and marker assisted selection (MAS). They have proved to be excellent tools for assessment of genetic diversity in a wide range of plant species (Madhumati, 2014).

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

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

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

RAPD (pronounced ‘Rapid’), for Random Amplified Polymorphic DNA, is a type of PCR reaction but the segments of DNA that are amplified are random and discovered by Williams *et al.* in 1990. Here no knowledge of the DNA sequence for the targeted gene is required (Kumar and Gurusubramanian, 2011).

Kumar and Gurusubramanian (2011) described the principle of RAPD marker as: a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualised by ethidium bromide staining. The use of a single decamer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and, therefore, cannot be used to identify heterozygotes. The standard RAPD utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

Madhumati (2014) mentioned in a review article that, several factors have been reported to influence the reproducibility of RAPD reactions i.e. quality and quantity of template DNA, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, Taq DNA polymerase brand or source, and thermal cycler brand. The concern about reproducibility of RAPD markers, however, could be overcome through choice of an appropriate DNA extraction protocol to remove any contaminants.

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).

The major drawback of RAPD method is that the profiling is dependent on reaction conditions which can vary between laboratories and even a difference of a degree in temperature is sufficient to produce different patterns. Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Madhumati, 2014; Bardakci, 2001).

Bardakci (2001) reported that, RAPD markers have found a wide range of applications in genetic diversity analysis, gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. RAPD technique can be performed in a moderate laboratory for most of its applications. Despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time and labour.

2.3. Somaclonal variation

Plants regenerated via tissue culture techniques display genetic variations for different characters which have been referred to as somaclonal variations by Larkin and Scowcroft (1981). It is also called tissue or culture-induced variation (Afrasiab and Iqbal, 2012a; Kaeppler *et al*., 2000).

The introduction of variation may also be either problematic or useful for horticulturists, biotechnologists and plant breeders, and may occur in high frequency during adventitious plant regeneration or long-term callus culture variation (Afrasiab and Iqbal, 2012a; Kaeppler *et al*., 2000).

Factors such as source and age of explants, culture duration, number of sub-cultures, culture environment, chemical additives or growth regulators, media composition and the ploidy level influence somaclonal variation (Nwauzoma and Jaja, 2013; Silvarolla, 1992).

2.4 Somaclonal variation in different plants

Sugarcane *(Saccharum officinarum* L.): The potential of somaclonal variation for the genetic improvement of characters of agricultural importance was first demonstrated in *Saccharum officinarum* with the *in vitro* selection of a commercial variety resistant to Fiji disease (Heinz, 1973). There were variations in the morphology, cytogenetics and isoenzyme traits. Liu *et al.* (1972) reported on the morphological variation in stooling and erectness amongst somaclones. In addition, some somaclones were reported to be resistant to Fiji disease virus, downy mildew (Krishnamurthi and Tlaskal, 1974; Krishnamurthi, 1974), eyespot disease (Leal *et al*., 1996) and sugarcane mosaic virus (Nwauzoma and Jaja, 2013). Salt tolerance somaclones have also been generated by a tissue culture cycle (Khan *et al*., 2004).

Potato (*Solanum tuberosum*): Somaclonal variation has been reported in potato plants regenerated from protoplasts of the widely grown variety 'Russet Burbank' (Bannaceur *et al.,* 1991). Some of the somaclones also had greater resistance to *Alternaria solani* toxin than the parents; while others were resistant to late blight caused by *Phytophthora infestans*. These somaclones were stable through a number of vegetative generations (Nwauzoma and Jaja, 2013). Wenzel (1979) observed phenotypic variability from protoplast-derived somaclones of potato diploids after extended periods in culture, which he attributed to culture-induced aneuploidy. Somaclonal variation was used to select potato calli with desirable traits, such as salt tolerance and drought stress (Ehsanpour *et al.,* 2007). Khatab and EL-Banna (2011) employed UV-C radiation to induce somaclonal variation in potato callus cultivar 'Cosima' and its detection using RAPD-PCR. Rosenberg *et al.* (2010) investigated the effect of thermotherapy on the new potato variety Reet clones which differed in yield, number and weight of tubers, late blight resistance and morphological characteristics. Thus, somaclonal variation has for long been used to improve potato cultivars.

Rice (*Oryza sativum*): Several studies on somaclonal variation have been carried out in rice by using cultivars of both subspecies: *indica* and *japonica* (Roy and Mandal, 2005; Kim *et al.*, 2003; Yang *et al.* 1999). Ngezahayo *et al*. (2007) studied the nature of somaclonal variation at the nucleotide sequence level in the cultivar rice Nipponbare using RAPD and ISSR markers and by pairwise sequence analysis. Earlier reports on somaclonal variation in rice include those of Henke *et al.* (1978) and Nishi *et al.* (1968), all from rice callus.

Maize (*Zea mays*): Maize has many other features that make it attractive material for studies of somaclonal variation. Matheka *et al.* (2008) used somaclonal variation to select maize varieties resistant to drought in Kenya. Somaclonal variation in maize has also been shown to affect the mitochondrial genome. Selection for resistance in cultures of T-cytoplasm maize (sensitive to southern corn leaf blight T-toxin of *Drechslera maydis* Race T) by recurrent sub-lethal exposure T-toxin resulted in the recovery of toxin-resistant plants (Nwauzoma and Jaja, 2013).

Plantain (*Musa* spp.): Somaclonal variation is a common phenomenon in both *in vitro* and *in vivo* propagated Musa plants. Certain genotypes are known to exhibit a high variation rate, while others rarely do (Cote *et al.*, 1993; Smith, 1988). Dwarfism in the 'Cavendish' bananas and inflorescence variation in the plantains are the most commonly observed morphological changes. Changes in leaf size, shape and colour of the pseudostem and flower are also common. Some works have been done on the characterization of somaclonal variation in plantain by Nwauzoma *et al.* (2002), Krikorian *et al.* (1993) and Nwauzoma (1999).

2.5 RAPD marker in potato somaclonal variation study

Iuliana and Cerasela (2014) conducted an experiment with several RAPD markers to study the effect of the ultraviolet radiation on the somaclonal variability for *Solanum tuberosum*. After the irradiation with UV-C there were some changes at molecular level by the occurrence in some cases of new DNA bands or the disappearance of DNA bands in other cases which were highlighted using three RAPD primers (OPF-12, OPW-11 and OPX-01). They observed that the polymorphism detected by these primers is not due to the genetic instability or the *in vitro* culture methods because there were a low number of subcultures (two). The ultraviolet mutants can generate mutations at the DNA level, this fact is proved by the extra DNA bands in some cases and the missing ones in other cases.

Khan *et al.* (2014) reported the use of RAPD primers in potato somaclonal variation identification. They analyzed *in vitro* somaclonal variation on plantlets of potato cv. Desiree and investigated the effect of somaclonal variation on the salt tolerance and tuber glycoalkaloid content. Around 38 regenerated plants were selected from tissue culture-induced calli based on their morphological status. Somaclonal variation was confirmed through four RAPD primers.

Ahmad *et al.* (2013) studied genomic DNA of the mutant lines of the three potato cultivars, Cardinal, Diamant and Desiree, with respect to control for polymorphisms by using RAPD markers. Four 10 bp random fragment primers, S-13, S-18, S-19 and R-17 were studied and all of them gave the amplification of genomic DNA. All of the mutant lines gave different banding pattern against different primers with respect to control plants of the three varieties and bands were present at 50 bp to 1500 bp. All these primers with specific banding pattern were unique in their polymorphic behavior.

Tiwari *et al.* (2013) accessed the genetic stability of in vitro propagated potato microtubers using RAPD, ISSR, SSR and AFLP markers. Total 38 (10 RAPD, 11 ISSR, 12 SSR and 5 AFLP) primers produced a total of 407 (58 RAPD, 56

ISSR, 96 SSR and 197 AFLP) clear, distinct and reproducible amplicons. Cluster analysis revealed 100% genetic similarity among the mother plant and its derivatives within the clusters by SSR, ISSR and RAPD analyses, whereas AFLP analysis revealed from 85 to 100% genetic similarity. Dendrogram analysis based on the Jaccard's coefficient classified the genotypes into five clusters (I to V), each cluster consisting of mother plant and its derivatives.

Afrasiab and Iqbal (2012b) showed that RAPD markers were efficient in discriminating somaclonal variants and induced mutants of potato by genetically analyzing somaclonal variants and gamma induced mutants of potato (*Solanum tuberosum* L.) cv. Diamant through RAPD-PCR technique. They selected four somaclonal variants and five gamma induced mutants which were differentiated by banding patterns obtained from 22 primers. The primers revealed 187 clear and easily scorable bands, out of which 140 were polymorphic. The size of the bands ranged from 200 to 3000 bp, but most of the bands were between 300 and 2000 bp. The variant SV5 amplified the maximum number of DNA fragments (129) followed by SV7 (118), SV4 (116), GM7 (115), GM8 and GM9 (108), while variant SV6 amplified minimum number of bands (95). The presence of polymorphic bands in variants and mutants suggest that genetic variation occurred in all the treatments as compared to control. Similarity and clustered analysis were conducted using Jaccard's coefficients and the un-weighted pair group method using arithmetic averages. They summarized results in a dendrogram, which represent genetic diversity among the variants and mutants.

Afrasiab and Iqbal (2012a) conducted an experiment to characterize somaclonal variants and induced mutants of potato. They used both biochemical and molecular methods. Variation at isozymes and random amplified polymorphic DNA (RAPD) were used to detect variability and a total of 24 arbitrary sequence of RAPD primers were evaluated. The RAPD primers produced 123 bands (88 polymorphic), whereas isozymes peroxidase produced 6 bands (5 polymorphic) showing genetic variation as compared to control. The size of the bands that were produced in the RAPD-PCR reactions ranged from 200 to 3,000 bp, but most of

the bands were between 300 to 2,000 bp. The primers were able to amplify the genomic DNA giving both monomorphic and polymorphic bands. About 63% of the bands were polymorphic among the variants and mutants of cv. Desiree. The level of polymorphism was different with different primers among different cultivars and mutants. In the dendrogram two main groups of clusters were identified and Jaccard's coefficient ranged from 5.6 to 8.3 but, the UPGMA analysis did not divide the variants and mutants into strictly separate clusters.

Khatab and El-Banna (2011) detected Somaclonal variations among 14 regenerated plants and their original cultivars using RAPD markers. Out of 10 random primers used, only five successfully produced scoreable RAPD bands (200 bp to 3.05 kb) for all the tested genotypes. Four primers (OPV-02, OPA-12, OPQ-14 and OPE-02) produced polymorphic RAPD profiles and only one primer (OPA05) gave monomorphic bands. All used RAPD primers produced 61 bands in all the tested genotypes, out of them, 23 bands (37.71%) were common in the parental genotypes and the regenerated plants while, the rest were polymorphic (62.29%). Among the primers used, OPE-02 produced the highest number of bands (14) while primers OPA-05 produced the lowest number (7). The highest number (11) and the lowest number (1) of polymorphic bands were observed in Desiree and its regenerates' using primers OPE-02 (78.5%) and OPV-02 (14.3%), respectively. Some new additional bands/loci were present in some regenerates and absent in their originals cultivars.

Munir *et al.* (2011) applied RAPD primers to evaluate somaclonal genetic variability in five different samples of potato calli obtained through *in vitro* propagation. In total 111 reproducible bands were generated with ten primers. The banding pattern of amplified DNA samples ranged from 200 bp to 1500 bp in size. Six primers OPC-02, OPC-03, OPC-05, OPC-07, OPC-08 and OPC-09 resulted in some polymorphism while OPC-01, OPC-04, OPC-06 and OPC-10 primers gave monomorphic bands. The similarity coefficients indicated maximum similarity (0.838) and minimum genetic similarity was observed

(0.419). They reported that different types of growth regulators may be the cause of genetic variability.

Tiwari *et al.* (2010) detected 21 somatic hybrids by RAPD, SSR and Somatic type analysis possessing species-specific diagnostic bands of corresponding parents. Somatic hybrid showed intermediate phenotypes (plants, leaves and floral morphology) to their parents in glass-house grown plants. All the somatic hybrids were male-fertile.

Aghaei *et al.* (2008) studied *in vitro* screening of ten potato cultivars (White Desiree, Russet Burbank, Cosima, Agria, Concord, Diamant, Maradona, Kennebec, Marfona and Main) for salt tolerance at different concentration of NaCl (0, 30, 60, 90 and 120 mM). RAPD analysis was conducted using six decamer primers including OPAA-03, OPAA-19, OPAA-20, OPB-07, FDK1-05 and FPK2-19 for PCR amplification. Dendrogram based on DNA banding pattern showed polymorphism by FPK2-19 primer. Banding pattern of RAPD confirmed a distinct polymorphism between salt sensitive and salt tolerant cultivars. The clustering pattern of the potato cultivars in this study suggested that, the salt tolerance and salt sensitivity of some potato cultivars are due to the genotype variation and possibly not epigenetic adaptation under salt stress condition.

Ehsanpour *et al.* (2007) detected somaclonal variation induced by UV-C radiation by RAPD markers. Evaluation of somaclonal variation by RAPD-PCR showed that 5 out of 28 markers could reveal some polymorphism in the amplified DNA pattern caused by UV-C radiation.

EL-Sawy *et al.* (2007) studied morphological and molecular characterization of potato microtubers produced on coumarin inducing medium using six random oligonucleotide RAPD primers. A total of 61 different DNA bands were reproducibly obtained, 14.8% of which were polymorphic. Each primer produced between 3 to 9 amplification products that ranged in size between approximately 411 and 1200 bp.

Przetakiewicz *et al.* (2006) studied RAPD markers to confirm intraspecific somatic hybrids among 16 different diploid breeding lines of *Solanum tuberosum* which were produced by PEG-induced fusion. The polymorphism in the diploid lines was revealed by at least 13 RAPD and 12 semi-random primers. The DNA amplification pattern obtained with RAPD or semirandom primers confirmed that 6 fusion combinations were hybrids.

Bordallo *et al.* (2004) detected Somaclonal variation on *in vitro* callus culture potato cultivars by RAPD markers. Calli were induced using leaf and stem explants of the commercial potato cultivars Achat, Baraka, Baronesa, Bintje, and Contenda in MS culture media supplemented with 1.65 mM of picloram and 11.5 mM of 2,4-D. Seventy and ninety days after induction, DNA samples of 40 calli were compared concerning the effects of the two explant (leaf and stem) and two growth regulator sources on five potatoes cultivars. A total of 20 arbitrary sequence primers were evaluated. The RAPD pattern generated by these primers suggested a high percentage of polymorphic fragments among the five genotypes, indicating a high level of genetic variation among cultivars. Cultivar Baronesa showed the highest number of polymorphic fragments for all treatments. The cultivar Contenda showed the smallest somaclonal variation, for most of the treatments, except for the treatment which consisted of stem explants, picloram (1.65 mM) application, and a 70-day period of callus formation. 'Contenda' is, therefore, the most suitable cultivar for synthetic seed production.

Badr and Mabrouk (2000) used RAPD markers to characterize and detect somaclonal variations in calli of five commercial potato cultivars (*Solanum tuberosum* L.), commonly cultivated in Egypt. Polymorphism was readily observed among the five potato cultivars with the six primers using the RAPD procedure. The size of the amplified DNA fragments ranged from approximately 158 to 1765 base pairs. Size of DNA fragments produced ranged from 409 to 1765 bp for primers 1 and 3; 223 to 1765 bp for primers 2 and 5; 650 to 1765 bp for primer 4 and 158 to 1176 bp for primer 6. Primer 5 produced two DNA fragments (882 and 409 bp) common to all calli of the five potato cultivars.

Diamond exhibited no somaclonal variations by primers 3 and 6. Also Sponta did not show any somaclonal variations by primer 1. Conversely, Cara showed high somaclonal variations using primers 2 and 5. Diamond showed the least somaclonal variations across all primers used. Moreover, calli cultured for long periods of time showed somaclonal variations. The study provides information on the molecular basis of polymorphism detected as RAPD markers in potato calli.

2.6 Genetic diversity and relationship analysis of potato by RAPD marker

AL-Salihy *et al.* (2014) conducted an experiment with RAPD-PCR to detect DNA profile changes in four potato varieties *viz*. Lusa, Arizona, Ambio and Riviera, to study the relation between the ability of *in vitro* propagation and the genetic variation. Total 6 oligonucleotides primers were used in RAPD and five of them were selected for their stable results. Each primer generated 3 to 11 bands with an average of 6.25 bands per primer. Their results suggested that RAPD markers provided substantial information for the identification of potato genotypes. Among the four potato genotypes, Riviera produced the lowest number of DNA-amplified fragments. They also reported that, RAPD profiles showed significant differences and some primers resulted in alteration of a few amplification products.

Onamu *et al.* (2014) conducted a consensus analysis of RAPD and ISSR to study genetic diversity in fifteen potato cultivars (9 cultivars bred in Europe, N. America and Mexico, and 6 Mexican creole cultivars) grown in Mexico. Amplifications using five decamer RAPD primers and five ISSR primers produced 138 bands, from which 116 (84.4%) were polymorphic. Cultivars Fianna and Armada showed the highest similarity coefficient (0.89). In contrast, Cambray Rose Tollocan and Morelos had the lowest similarity coefficient (0.55). High genetic differentiation among cultivars (GST=0.71) and low gene flow (Nm=0.19) across all loci, indicated high genetic divergence among the 15 cultivars. Analysis of molecular variance (AMOVA) revealed a significant

contribution of differences among regions, among cultivars, among and within populations to the overall diversity of potato cultivars studied. They concluded that consensus data on RAPD and ISSR markers were very useful to study genetic diversity and population structure in potato cultivars.

Hoque *et al.* (2013) detected the molecular diversity of 12 popular potato varieties in Bangladesh. Eight RAPD primers were used to evaluate the genetic diversity of potato varieties. Some total of 36 DNA fragments were amplified and out of them 24 were polymorphic. Those primers generated 61.53% of polymorphic DNA band. The primer OPX-04 produced the highest (9) number of DNA band and out of 9 amplicons 6 were polymorphic. Lowest number (3) of bands was observed in the primer OPA-17. The highest Nei's genetic distance (0.9701) was detected between the variety Granola and Provinto. The highest (0.8205) number of genetic identity/similarity was observed between the varieties Cardinal and Diamant. The unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's genetic distance revealed that the 12 varieties followed into two main clusters. They reported that there was high level of genetic diversity among the varieties which can be used for parental selection in potato breeding program.

Alam *et al.* (2012) studied two native and three high yielding potato varieties available in Bangladesh cytogenetically at molecular level to get a preliminary idea about their genomes and their genetic diversity and relationship. They reported that, these varieties possess $2n = 4x = 48$ chromosomes and each variety had definite centromeric formula. Seven primer combinations were used for RAPD fingerprinting. A few common RAPD bands suggested the sharing of genomic fragments among five varieties. The cluster analysis On the basis of RAPD fingerprinting showed that the variety Granola was totally different from the rest followed by Jaam at linkage distance 19.0 and Lal Sheel at 16.8. Diamant and Cardinal showed close relationship at linkage distance 7.0.

Gauchan *et al.* (2012) studied the genetic diversity of four Nepali local potato cultivars. Ten arbitrary decamer primers produced 29 bands of which 69.0% were polymorphic. The size of the amplified bands ranged between 370 bp and 2500 bp. On average, 17.5 alleles per genotype were amplified using the RAPD primers. The selected primers showed sufficient polymorphism to allow identification of individual genotypes. The dendrogram showed a range of 55.2% to 69.0% relative genetic similarities between the genotypes.

Das *et al.* (2010) analyzed genetic diversity in thirty Indian potatoes employing karyotype, genome size analysis and RAPD markers. RAPD primers revealed 131 amplified DNA fragments (300 to 2200 bp) with 79 unique bands (7 to 71% polymorphism) among the genotypes. Similarity coefficients (ranged from 0.29 to 0.93) and cluster analysis reflected the expected trends in relationships of the full and half-sib potato genotypes. They obtained dendrogram on the basis of the genetic distances radically separated the 30 genotypes into two major clusters each having 15 genotypes. First cluster having all comparatively close genetic affinity which were further divided into two sub groups. In the first cluster K. Badshah and JX- 576 pair was found very closely related. In the second cluster advanced clone 92PT-27 and MTP-I pair was found the closest genetic affinity while advanced breeding line MS/92-209 formed out a group with wide genetic variability from the rest of the genotypes. The advanced breeding lines MS/89-60, MS/92-3146, MTP-II remain close to each other and hence treated as clonal duplications while MS/92-3128 showed some degree of genetic distance.

Rocha *et al.* (2010) evaluated the genetic diversity and identify potato cultivars by RAPD and SSR markers. The genomic DNA of 16 potato cultivars was amplified with 25 RAPD primers that generated 185 bands with an average of 7.4 bands per primer, of which 92 were polymorphic. The dendrograms generated by cluster analysis distinguished the cultivars genetically although the dendrograms were not correlated in the comparison of the two markers used. The PIC values demonstrated the high information content of the primers used and 16 potato varieties were identified based on six RAPD primers and three SSR

primer pairs. Thus, by means of RAPD and SSR markers the genetic diversity was assessed and the 16 commercial potato cultivars analyzed in this study were identified.

Abbas *et al.* (2008) analyzed genetic diversity in Pakistani potato cultivars by using RAPD Primers. A total of 158 DNA fragments were amplified in 6 potato genotypes using 11 RAPD primers, giving an average of 26.3 alleles per genotype. Size of scorable fragments ranged from approximately 250 to >1000 bp. A high level of genetic dissimilarity (GD = 17% to 55%) was estimated among the 6 genotypes. The results of genetic dissimilarity analyses showed that extensive genetic diversity (average G .D. ranging from 17% to 55%) existed in 6 genotypes used. In genetic diversity analyses the comparisons among the 2 genotypes (Appalo and Furika) showed high estimate of genetic distance.

Chakrabarti *et al.* (2006) studied variations in RAPD profiles from leaf, stem, root and tuber tissues in case of two glasshouse grown potato cultivars using 40 decamer primers to suggest possible danger of cultivar mis-identification. Genomic DNA was extracted from the above four tissues of four *in vitro* grown potato cultivars. A significant effect of random primers on fingerprint uniformity was observed in case of both glasshouse and *in vitro* grown samples. They obtained a new concept of stability index for random primers based on homogeneity of RAPD profiles from different tissues of a single plant. They reported that, RAPD analysis of genomic DNA extracted from any tissue of *in vitro* grown potato plants using 14 selected decamer primers could be used to develop RAPD fingerprints for identification of Indian potato cultivars.

Chimote *et al.* (2007) conducted genetic diversity analysis of 32 commercial Indian potato varieties using 4 multi-loci SSR (123 alleles at 12 loci), 14 RAPD (168 alleles) and 21 morphological markers. Fourteen RAPD primers used in this study amplified a total of 168 distinct fragments. The number of bands ranged from 6 (in OPA-20) to 20 (in OPA-16). Of all the RAPD fragments amplified, 28 (20%) were highly polymorphic being present in 40 to 60% of the samples,

while 13 (7.7%) were unique and 29 (17.2%) were monomorphic. RAPD derived dendrogram did not reveal any clear grouping due to narrow range of variation making it difficult to group varieties on the basis of their pedigrees.

Yasmin *et al.* (2006) conducted an experiment to detect the genetic diversity of six potato cultivars with 3 RAPD markers. They generated 35 RAPD bands, of which 33 (94.29%) were polymorphic. The proportion of polymorphic loci and the gene diversity were 14.29% and 0.068, 28.57% and 0.138, 17.14% and 0.075, 51.43% and 0.217 and 54.29% and 0.245 for Cardinal, Diamant, Heera, Raja and TPS, respectively. The results indicated that relatively high level of genetic variation was present in the TPS and Raja cultivars compared to other cultivars studied. No intra-cultivar genetic variation was observed in the Ailsa cultivar. The high level of cultivar differentiation (GsT = 0.634) and low level of gene flow (Nm = 0.289) across all loci reflected that the level of genetic divergence among six cultivars was high. The UPGMA dendrogram based on the Nei's genetic distances segregated the cultivars into three clusters: Ailsa and Heern made one cluster, Cardinal and TPS made another cluster whereas Diamant and Raja grouped into another cluster.

Brenna (2004) reported that DNA fingerprinting is a powerful technique to study the genetic variation between plant varieties and can be used for plant improvement while conducting an experiment to determine the fingerprinting of potato varieties. Ten random primers from Operon Inc. were amplified. It was found that the least number of bands was with the primer OPA-18, which only gave two monomorphic bands in all the potato varieties. Whereas, primers OPA-02 and OPA-14 each produced 14 bands. The Primer OPA02 gave the most polymorphic bands among the potato varieties tested. The data was analyzed and the dendrogram showed the differences among all the varieties. The dendrogram also showed that no potato tested was more than 88% similar.

Fu-cui *et al.* (2004) employed RAPD markers to analyze the genetic diversity of potato cultivars in Yunnan, China. 71 bands were amplified by 11 random

decamer primers in 67 potato cultivars, of which 57 bands were polymorphic. The polymorphism in Yunnan potato cultivars reached 80.3% and the genetic distance between cultivars ranged from 0.033 to 0.900. The results showed that Yunnan potato cultivars had high genetic diversity at the DNA molecular level. Through clustering of RAPD markers by UPGMA, 67 cultivars were separated into 15 groups. The results also showed high variation in genotype and high plasticity in phenotype of potato.

Hong *et al.* (2004) determined the genetic relationship between 17 Neo-tuberosum clones and 7 common potato cultivars with 14 RAPD primers. The Neo-*tuberosum* clones showed highly significant genetic diversity. Total 170 bands were observed, of which 130 were polymorphic and average polymorphism rate was 75.26%. The phylogenetic relationship among 24 genotypes was determined based on a phylogenetic tree developed by UPGMA. The Neo-tuberosum and common cultivars were clustered into different groups.

Jozani *et al.* (2003) conducted an experiment where RAPD procedure was used to evaluate genetic diversity of 28 commercial potato cultivars in Iran. One hundred decamer primers were selected randomly and tested on each sample genotype. Sixteen primers yielded 194 polymorphic DNA fragments (marker), ranging in size from 300 to 2400 bp. A total of 1854 bands were observed among the 28 potato genotypes. Cluster analysis of cultivars was performed based on presence (1) or absence (0) of bands using Jaccards (j.s.c) and simple matching similarity coefficient (S.M.S.C.) by UPGMA method. These analyses indicated that the greatest genetic difference and similarity was present between Vlox and Herta-2, and Herta-1 and Herta-2 (plus Fersco and Ajiba), respectively. Cluster analysis of j.s.c. produced 7 groups and that of S.M.S.C produced 4 groups. Therefore, they concluded that, j.s.c is more sensitive than S.M.C and is more suitable for comparing cultivar bands produced by RAPD. Genetic diversity of potato cultivars was related to their geographical distance and RAPD was useful for classifying germplasm and identifying divergent groups.

2.7 Characterization and fingerprinting of potato by RAPD marker

Sedláková *et al.* (2009) characterized 136 diploid of genus *Solanum* by RAPD marker for the purpose of the differentiation of individual species. The series of OPN and OPG decamers were used from them OPG-08, OPN-03, 5, 8, 11, 15 and 18 primers were selected. They showed that, 9 genotypes primarily classified as *Solanum pinnatisectum,* showed totally different RAPD bands spectrums compared with reference clone *Solanum pinnatisectum* PI275235

Orona-Castro *et al.,* (2006) characterized 13 commercial cultivars and 3 elite potato clones by RAPD and SSR molecular techniques. With the RAPD technique 40 primers were initially tested and 3 were selected (Alpha DNA2, Alpha DNA23 and Alpha DNA30) for the characterization. Alpha DNA2 was used alone, while Alpha DNA23 and Alpha DNA30 were combined. Total 26 bands were produced, of which 69.23% were polymorphic. High genetic similarity was observed, among the potato cultivars as the maximum genetic distance between the groups was 0.19. The highest genetic relationship was observed between the cultivars Sancal and NAU-6 with a value of 88% in the Felsenstein confidence intervals. Groups of genotypes were of two kinds, some with genotypes closely related and other such as cv. Monserrat, genetically distinct.

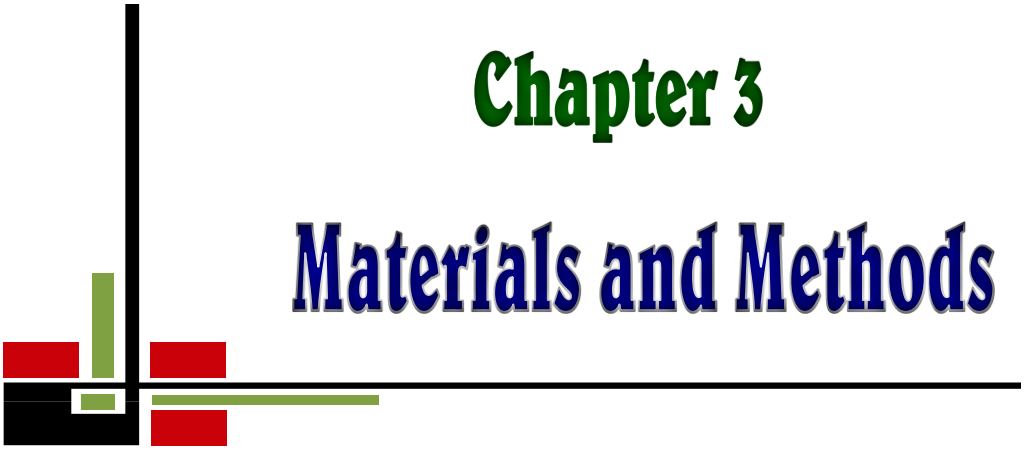
Collares *et al.* (2004) carried out an experiment to characterize 27 potato genotypes using molecular markers. Polyacrylamide gel electrophoresis, RAPD techniques and isozymes of esterase, phosphoglucomutase and soluble proteins were analyzed in tubers, and isocitrate dehydrogenase, aspartate transaminase, phosphoglucomutase and peroxidase in leaves. Eighteen primers were tested and 4 were chosen. The selected primers (OPX-01, OPX-04, OPX-09 and OPY-07) generated 43 amplified products, with fragment numbers varying from 5 (OPX-04) to 14 (OPX-09), averaging 10.75 bands per primer, out of which 39 (90%) were polymorphic and four (10%) were monomorphic. Similarity and cluster analysis were conducted using Jaccard coefficient and the unweighted pair group

method using arithmetic average. Despite the differences detected in the analysis of proteins and isozymes in the tubers, as well as of isozymes in the leaves, the characterization of all genotypes through gel electrophoresis was not possible, while RAPD markers were efficient to characterize all the 27 genotypes.

Pattanayak *et al.* (2004) characterized 22 advanced potato hybrids and 2 clones of a parental line using RAPD markers to assess diversity within the potato lines. Fifteen random decamer primers generated 131 fragments with 87% fragment polymorphism. Pair wise similarity values revealed moderate genetic variability among the 22 advanced lines due to common parentage.

Chakrabarti *et al.* (2001) distinguished and characterized 20 Indian potato cultivars by RAPD marker. A total of 198 storable bands were amplified using 10 random primers. Only 2 of which were monomorphic. They reported that, similarity values among the cultivars ranged from 0.33 to 0.80 and primer having resolving power above 7.4 was sufficient to distinguish all 20 cultivars.

Chakrabarti *et al.* (1999) characterized and detected genetic similarity among 18 commercial Indian potato cultivars. A total of 74 distinct bands ranged from 124 to 4074 bp, were amplified by using 12 random primers. 74 of these bands were polymorphic. Pair-wise genetic similarity analysis revealed a wide range of variability among the cultivars. They reported a wide genetic base of Indian potato cultivars. Kufri swam showed maximum genetic divergence from other cultivars. Similarly, Kufri Badshah and Kufri Alankar were also genetically distinct from other cultivars studied. Remaining fifteen cultivars were grouped in to two closely related clusters.



Chapter 3

Materials and Methods

CHAPTTER III

MATERIALS AND METHODS

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, Dhaka-120, Bangladesh. The period of the experiment was November, 2013 to December, 2014.

3.2 Name and Source of Study Materials

Twelve potato genotypes were used in the study. The sources of seeds tubers of the collected cultivars and genotypes were presented in Table 1.

Table 1. Name and sources of 12 potato genotypes

Sl. No.	Name of the Cultivars/Genotypes	Collected from
1.	Cardinal	BARI
2.	Diamant	BARI
3.	Asterix	BARI
4.	SIP-3	India
5.	SIP-5	India
6.	SVP-6	Department of Biotechnology, SAU
7.	SVP-18	Department of Biotechnology, SAU
8.	SVP-19	Department of Biotechnology, SAU
9.	SVP-25	Department of Biotechnology, SAU
10.	SVP-55	Department of Biotechnology, SAU
11.	SVP-56	Department of Biotechnology, SAU
12.	SVP-68	Department of Biotechnology, SAU

BARI = Bangladesh Agricultural Research Institute, SAU = Sher-e-Bangla Agricultural University

Cardinal, Diamant and Asterix are very popular varieties in Bangladesh and were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur. SIP-3, SIP-5 and SVP series (SVP-6, SVP-18, SVP-19, SVP-25, SVP-55, SVP-56 and SVP-68) are somaclonal variant potato, which were created in the "Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU)", by my research supervisor professor Dr Ekramul Hoque, Department of Biotechnology, SAU, Dhaka. The source materials for the somaclonal variants were Cardinal, Diamant and Asterix.

3.3 Seedling raising and collection of leaf sample

Good quality, disease free, healthy tubers were sown in a plot of the research farm of Sher-e-Bangla Agricultural University. All management practices were done for raising good quality seedlings from those materials. In order to carry out PCR amplification of potato genome, fresh and young leaf samples were collected at 3 to 4 leaf stage of each genotype and used as the source of genomic DNA.

Initially, each sample was washed carefully in running tap water and preserved in airtight polythene packet separately. Finally, the samples were brought to the laboratory, wrapped by aluminum foil and stored at -20^0C freezer.

3. 4 Extraction of genomic DNA

Total genomic DNA was isolated from potato plant leaves following Phenol: Chloroform: Isoamyl alcohol purification and ethanol precipitation method with a few modification.

3.4.1 Reagents used

1. Extraction buffer, pH= 8.0

   Composition of extraction buffer are as follows:

   - 1M Tris-HCl
   - 0.5M EDTA(Ethylene diamine tetra-acetic Acid) (pH= 8.0)
   - 5M NaCl

- D.H_2O
- 1% SDS **(**Sodium Dodecyl Sulphate)

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

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

Composition of extraction TE buffer are as follows:

- 1M Tris-HCl
- 0.5M EDTA
- D.H_2O

4. Isopropanol

5. 0.3M Sodium Acetate

6. Absulate (100%) ethanol

7. Ethanol (70%)

8. RNAase

9. Ethidium Bromide solution

3.4.3 Reagent preparation for DNA extraction (Stock solution)

Extraction buffer (1000 mL)

For the preparation of 1000 mL DNA extraction buffer, 100 mL 1M Tris-HCl (pH=8.0) was mixed with 40 mL of 0.5M EDTA and added to 100 mL 5M NaCl in a 1000 mL measuring cylinder. Finally, Sterilized ddH_2O was added to make the volume up to 1000 mL, then mixed well and autoclaved.

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

At first 30.28 g Tris was taken in a volumetric flask (5000 mL). 100 m dd.H_2O was added and pH was adjusted to 8.0 by adding HCl. Then sterilized dd.H2O was added to make the volume up to 250 mL. The solution was autoclaved.

0.5M EDTA (pH 8.0) (250 mL)

At first 46.53 g EDTA.2H_2O was added in a volumetric flask (5000 mL) and100 mL dd.H2O was added. Then 4 g NaOH was added. Then pH was adjusted to

8.0 with NaOH. Then sterilized dd.H_2O was added to make the volume up to 250 ml. Then the solution was autoclaved.

5M NaCl (250 mL)

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

1% SDS (100 ml)

One gram of SDS was added in 100 ml dd.H_2O in a 250 ml beaker. As SDS is hazardous, the mixture was mixed by a hot top magnetic stirrer well but not autoclaved.

1X TE Buffer (100 ml)

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

5X TBE buffer (1 litter)

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

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

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

RNase A/H

10 mg of RNase was added to 1 ml of dd.H2O. Then it was dissolved completely with the help of necessary heat (at 50℃ in water bath for 30 minutes).

70% Ethanol (1000 mL)

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

3.5 Method of DNA extraction

The following steps were followed for DNA extraction

- For isolation of genomic DNA, vigorous, young, actively growing fresh leaf tissues were collected from 12 potato genotypess. Total DNA was isolated by using phenol: chloroform: isoamyl alcohol purification and ethanol precipitation method.
- The youngest leaves were selected in order to make the tissue grinding process easy. Initially, healthy youngest leaves were washed thoroughly by tap water followed by deionized water. Then Sterilized by ethanol to remove wastes and any source of foreign DNA and leaves are then dried on tissue paper.
- Approximately 200 mg of young leaves were cut into small pieces and then taken in morter. 300 µL of extraction buffer were added and then leaf tissues were ground manually with the help of a pestle.
- The sample was then taken into eppendorf tube and 300 µL of extraction buffer was added to it. The ground sample was then vortexed for 20 seconds in a vortex mixture and then incubated at 65°C for 20 minutes in hot water bath.
- Equal volume (500 µL) of Chloroform: Isoamyl alcohol (24:1) was added to the tube and then it was vortexted for 10 seconds.
- The solution was then centrifuged for 10 minutes at 13000 rpm. The supernatant was recovered using a pipette tip without disturbing the lower portion and transferred into a new eppendorf tube. Then, 250 µL of Isopropanol (0.6 volume of the liquid) was added to it. It was then tapped by finger for 20/30 seconds (The genomic DNA was visible as cotton like structure).

- After tapping the sample was again centrifuged at 13000 rpm for 15 minutes. The liquid was discarded completely and re-precipitation of the DNA solution was done by adding 500 µL of absolute (100%) cold ethanol plus 20 µL 0.3M sodium acetate.
- It was shaken gently. Tapping was done to separate pellet. The sample was centrifuged at 13000 rpm for 15 mins. The liquid was removed completely by pouring and blotting the open tube end on fresh tissue paper.
- The DNA pellet was then air dried for 2 to 3 hours. It was then dissolved in an appropriate volume (30 to 40 µL) of TE buffer and treated with 3 µL of RNAase for removing RNA. Then it was spinned for 4-5 seconds.
- Finally, the DNA samples were stored in freezer at -20°C.

Precautions

i. All glassware, micropipette tips, eppendorf tube, glass pipettes, deionized water and buffer solutions were properly autoclaved to keep away from DNAase contamination. Scissors, forceps were sterilized with absolute ethanol.
ii. As Ethidium Bromide (Et-Br) is a powerful mutagen and carcinogenic. So, hand gloves were used when handling anything that has been exposed to EtBr.
iii. Always power pack was kept turn off and the leads was unplugged before opening the electrophoresis unit to avoid electrical hazard.
iv. A transilluminator produces UV radiation 254 nm ranges. The wave length can cause eye damage (short term = burns, long term cataracts and cancers). Thus eye protector used while working with it.

3.6 Confirmation of DNA preparation

Sometimes isolated genomic DNA contains a large amount of RNA and pigments which usually cause over estimation of DNA concentration on a

spectrophotometer. To confirm DNA preparation. 1% agarose gels were used for assessing the quality of the genomic DNA and the amount of RNA present.

3.6.1 Preparation of 1 % agarose gel

One gram of agarose powder was taken in a 500 mL Erlenmeyer flask containing 100 mL electrophoresis buffer (1x TBE buffer) prepared by adding 20 mL of 5X TBE buffer in 80 mL of sterile deionized water. The flask was enclosed with aluminum foil paper to prevent excessive evaporation. It was melted for about 5 minutes into a microwave oven with occasional swirling until complete disappearance of agarose particles to generate homogeneous and crystal clear suspension. The agarose solution was cooled to about 50^0C (flask was cool enough to hold comfortably with bare hand) and I µL (10 mg/mL) ethidium bromide (DNA stain) was added and mixed well by gentle shaking to make the DNA visible under ultraviolet light box (Trans-illuminator). The molten gel was poured immediately on to a clean gel bed (15 x 15 x 2 cm^3; in size), that was placed on a level bench and appropriate comb was inserted parallel to the plate's edge with the bottom of the teeth about 2mm above the plate. Air bubbles were removed by pushing away to the side using a disposable tip. After 45 minutes to one hour gel became completely cooled at room temperature and solidified and the comb was removed gently. The gel was then ready for loading the DNA samples.

3.6.2 Preparation of DNA samples for electrophoresis

The samples were all in the same concentration of buffer. For each sample, 6 µL x TBE buffer was placed on a piece of aluminum foil paper and 2 µL loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA) was added to it using 0.5-10 µL adjustable micropipette. Loading buffer was used for monitoring loading and the progress of the electrophoresis and to increase the density of the sample so that it stayed in the well. Finally, 2 µL extracted DNA was added to it and mixed well using same micropipette. The samples were then added slowly to allow them to sink to the bottom of the wells.

The gel was placed in the gel chamber (Continental Lab product. Inc.) containing 1X TBE buffer. The final level of buffer was 5 mm above the gel. The power supply (EPS-301) was then connected and turned on to move the DNA from negative to positive electrode (black to red). Electrophoresis was carried out at 90V for about 45 minutes. After the bromophenol blue dye had reached three-fourths of the gel length, the electrophoresis was stopped and the power supply was disconnected.

3.6.3 Documentation of the DNA samples

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

3.7 Quantification of DNA

The concentration of DNA is one of the important variables for PCR amplification. Because different DNA extraction methods produced DNA of widely different purity it may be necessary to optimize the amount of DNA used in the RAND assay to achieve reproducibility and strong signal. Below a certain critical concentration of genomic DNA, RAPD amplification is no longer reproducible (Williams *et al*., 1992). So, it is necessary to stay above this critical concentration. Moreover, excessive DNA concentration is likely to produce poor resolution or "smears" resulting in a lack of clearly defined bands in the gel. Therefore, quantification of DNA in each sample was determined spectrophotometrically.

Concentration of genomic DNA was examined by calculating the ratio of the optical density measured at 260 nm using a spectrophotometer (Spectronic^{R} Genesis^{Tm}) and stored in freezer. In the beginning of the quantification of DNA samples, spectrophotometer UV-lamp was turned on and after it had warmed up, the wavelength was set at 260 nm. One cuvette (the "Zero" or "blank" cuvette)

was filled with 2 mL sterile distilled water and placed in spectrophotometer. The test samples were prepared by taking 2 mL of each sample in a cuvette containing 2 mL sterile distilled water. The sample was uniformly mixed and placed in spectrophotometer and the absorbance reading was taken at 260 nm. Then the cuvette was rinsed out with sterile water, for measuring the absorbance of every sample. DNA concentration in each sample was then determined according to the following formula:

$$\text{Concentration of DNA } (\mu L) = \text{Absorbance} \times \frac{\text{Volume of distilled water}}{\text{Amount of DNA } (\mu L)} \times \text{Conversion factor } (0.05) \times 100$$

Where,

Absorbance	=	Spectrophotometer reading
Volume of distilled water	=	2000 µL
Amount of DNA	=	2 µL
Conversion factor	=	0.05

3.8 Preparation of working solution of DNA samples

DNA concentrations were adjusted to 25 ng/µL for conducting PCR using the following formula:

$$\mathbf{V_1 \times S_1 = V_2 \times S_2}$$

Where,

V_1= Initial volume of DNA solution (µL)

S_1= Initial DNA concentration (ng/µL)

V_2= Finial volume of DNA solution (µL)

S_2= Final DNA concentration (ng/µL)

3.9 Amplification of RAPD markers by PCR

3.9.1 Principle of the amplification of RAPD

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

3.9.2 Primer selection

Nine decamer RAPD primers *viz.* OPA-18, OPA-20, OPB-04, OPB-06, OPB-08, OPC-01, OPD-02, OPF-08 and OPW-01 (Operon Technologies, Inc., Alameda, California, USA) were screened for PCR reaction in 12 genotypes of potato. List of RAPD primers are given in Table 2.

Table 2. Name, sequence and GC content of RAPD primers

Sl. No.	Primer name	Sequence (5′ to 3′)	(G+C) %
1	OPA-18	AGGTGACCGT	60
2	OPA-20	GTTGCGATCC	60
3	OPB-04	GGACTGGAGT	60
4	OPB-06	TGCTCTGCCC	70
5	OPB-08	GTCCACACGG	70
6	OPC-01	TTCGAGCCAG	60
7	OPD-02	GGACCCAACC	70
8	OPF-08	GGGATATCGG	60
9	OPW-01	CTCAGTGTCC	60

3.9.3 PCR amplification

PCR reactions were performed following two methods.

(1) Using individual PCR chemicals (each reagent is measured separately and prepared a mixture) and

(2) Using 2X Taq Mastermix (GeneON, Germany)

3.9.3.1 Using individual PCR chemicals

PCR reactions were performed on each DNA sample in a 25 µL reaction mix containing 4 µL of 10x Ampli Taq polymerase buffer, 2.5 µL of 10 µM primer, 2 µL of 250 µM dNTPs, 1 µL of Ampli Taq DNA polymerase (Bioneer, Korea) and 4 µL (100 ng) of genomic DNA and rest amount of sterile deionized water.

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Swift™ Mini Thermal Cyclers). The reaction mix was preheated for 4 mins at 94°C for pre-denaturation and then 33 cycles consisted of 1 min denaturation at 94°C, 50 sec at 36°C for annealing and 2 min at 72°C for elongation or extension. After the last cycle, a final step of 7 min at 72°C was added to allow complete extension of all amplified fragments.

PCR reactions were performed on each DNA sample in a 25 µL reaction mix containing following reagents (Table 3).

Table 3. Reaction mixture composition for PCR

Sl. No.	Reagents	Amount
1	Taq DNA polymerase buffer (10x)	4 µL
2	Primer (10 µM)	2.5 µL
3	dNTPs (250 µM)	2 µL
4	Taq DNA polymerase	1 µL
5	Genomic DNA (25 ng/µL)	4 µL
6	Sterile de-ionized water	11.5 µL
7	**Total reaction volume**	**25 µL**

From frozen stocks the PCR buffer, dNTPs, primer and DNA samples solutions were taken and thawed, mixed by vortexing and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermo cycler used (0.5 mL). A pre-mixture was then prepared in the course of the following order: reaction buffer, dNTPs, DNA template and sterile distilled water. Taq polymerase enzyme was then added to the pre-mixture. The pre-mixture was then mixed up well and aliquoted into the tubes that already containing primer.

The tubes were then sealed and placed in a thermo cycle and the cycling was started immediately.

3.9.3.2 PCR reaction with 2X Taq Master Mix (Geneon, Germany)

Ready mix Taq DNA polymerase and other composition were used for PCR reaction. The ratio of each chemicals were given below:

Table 4. Ready mix PCR mixture for amplification of potato genotypes

Sl. No.	Reagents	Amount (µL)
1	2X Taq Master Mix	12.5
2	RAPD primer	2.5
3	Sterile de-ionize water	7.5
4	Genomic DNA (25ng/ µl)	2.5
5	**Total reaction volume**	**25**

From the frozen stocks the Master Mix, primer and DNA samples solutions were taken and thawed, mixed by vortexing and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermo cycler used. The required amount of 2X Taq Master Mix, RAPD primer and sterile de-ionized water were then added into it. The mixture was then mixed up well. The tubes were then sealed and placed in a thermo cycle and the cycling was started immediately.

3.9.4 Thermal profile

DNA amplification was performed in an oil-free thermal cycler (Esco Technologies Swift™ Mini Thermal Cyclers). The PCR was programmed with

some modifications of Khatab and El-Banna (2011) and Afrasiab and Iqbal (2012b) used for potato DNA amplification. According to them the reaction mixture was pre-denatured at 95° C for 5 min followed by 33 cycles of 45 sec denaturation at 95° C, 30 sec annealing at 30 ° C and elongation or extension at 72 ° C for 1 minute. After the last cycle, a final step of 5 minutes at 72 ° C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4 ° C.

3.9.5 Electrophoresis of the amplified products

From each sample PCR products were confirmed by running 1.5% agarose gel containing 1 µL ethidium bromide in 1x TBE buffer at 85V for 50 minutes. Loading dye (5.0 µL) was added to the PCR products and loaded in the wells. Two molecular weight markers 100 bp (BIONEER, Cat. No. D-1030, South Korea) and 1kb (BIONEER, Cat. No. D-1040, South Korea) DNA ladder were also loaded on left and right side of the gel respectively. Under Ultra Violet (UV) light on a trans-illuminator RAPD bands were observed and documented by taking photograph using a Gel Cam Polaroid camera.

3.9.6 Documentation of the DNA samples

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

3.9.7 Precautions

The usual precautions were maintained when performing PCR reactions. All the disposable such as PCR tubes, tips, eppendorf tubes and reagents used during preparation of PCR reactions were autoclaved. Freezing condition was maintained when necessary especially for Tag polymerase. Hand-gloves were worn during handling of PCR components. Contamination of PCR components was avoided.

3.10 RAPD 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 (BIONEER, Cat. No. D-1030, South Korea) DNA ladder was used to estimate the size of the amplification products by comparing the distance traveled by each fragment with known sized fragments of molecular weight markers. All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The size of the band length were calculated using a computer program DNAfrag, version 3.03 Nash (1991).

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

Estimation of gene frequencies of RAPD loci was based on the assumption of a two-allele system. Of the two alleles, only one is capable of amplification of a RAPD band by primer annealing at an unknown genomic position (locus). The other is the 'null' allele incapable of amplification, mainly because of loss of the primer annealing site by mutation. The two-allele assumption was in most cases acceptable, because co-dominant loci showing band shifts are few (Welsh and McClelland, 1990; Elo *et al.*, 1997). In this system only a null homozygote is detectable as negative for the RAPD-band of interest.

Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be $(N/n)^{½}$ where N and n are the number of band negative individuals observed and the sample size respectively.

The frequency of the other allele is 1-q, the assumption of the two allele system enables us to calculate the Nei's genetic distance (Nei's, 1972) from the RAPD pattern.

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

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

Where,

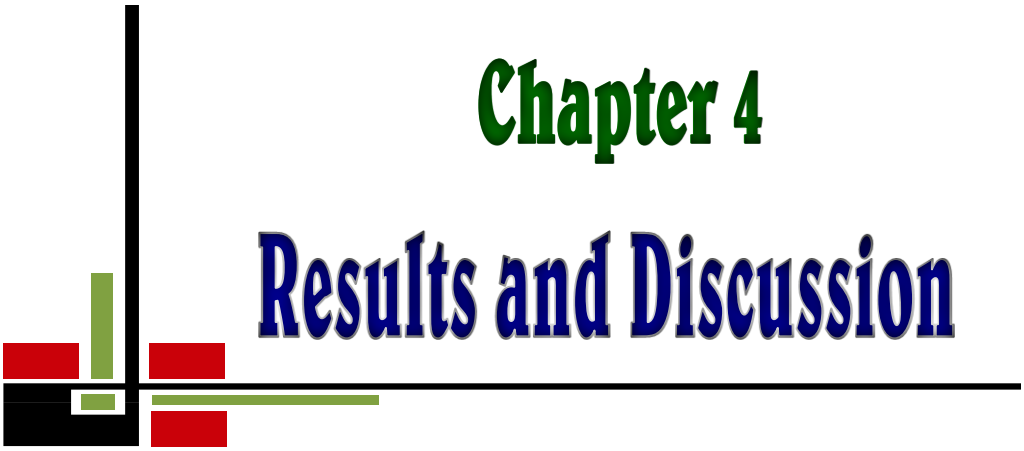
N_{xy} is the number of RAPD bands shared by individuals x and y respectively, and N_x and N_y are the number of bands in individuals x and y respectively (Wilde *et al.*, 1992).

The SI value ranges from 0 to 1. When SI=1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles. Within population similarity (Si) was calculated as the average of SI across all possible comparisons between individuals within a population. Between population similarity (Sij) was calculated as the average similarity between each paired individuals of population i and j (Lynch, 1991).

Nei's genetic distance and genetic identity values were computed from frequencies of polymorphic markers to estimate genetic relationship among the studied twelve potato genotypes 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).

3.11 Precautions

1. To avoid DNAase contamination, all glassware, micropipette tips, eppendorf tubes, glass pipettes, de-ionized water and buffer solutions were properly autoclaved. Scissors, forceps were sterilized with absolute ethanol.
2. Since Ethidium Bromide (Et-Br) is a powerful mutagen and carcinogenic in nature, hand gloves were used when handling anything that has been exposed to Et-Br.
3. Always power pack was kept turn off and the leads was unplugged before opening the electrophoresis unit to avoid electrical hazard.
4. A trans-illuminator produces UV radiation of 254 nm range. The weave length can cause eye damage. Thus eye protector was used while working with it.
5. The usual laboratory precautions were maintained when performing PCR reactions. All the disposable 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 during handling of PCR components. Contamination of PCR components was avoided.



Chapter 4

Results and Discussion

CHAPTER IV

RESULT AND DISCUSSION

This chapter comprises the presentation and discussion of the results of the experiment. The results were obtained from the experiment using RAPD markers on newly created somaclonal variants of potato and three released potato genotypes. In the RAPD analysis significant genetic diversity and polymorphisms of different potato genotypes were identified. The present study indicates the effectiveness of RAPD analysis in detecting substantial amount of genetic variation among different somaclonal variants and three released genotypes of potato. Data have been presented and expressed in Table 6 to 9, Figure 1 and in Plate 1 to 8. The results obtained from the study have been presented and discussed under following headings:

4.1 DNA confirmation and quantification

The extracted 12 genomic DNA samples were loaded on 1% agarose gel for conformation and quantification of DNA. It revealed that all the samples showed clear DNA bands (Plate 1). There was no smear and sharing of DNA with RNA. The quantification of DNA was also done in spectrophotometer (Table 5). It revealed that the highest concentration (4400 ng/µL) of genomic DNA was observed in the sample SVP-6 and the lowest (3250 ng/µL) was in the sample Diamant. It is recommended that only 25-50 ng/µL DNA is required for PCR amplification. Hence, the genomic DNA of each sample was diluted on the basis of concentration. The working DNA sample was prepared for PCR works.

Table 5. Absorbance reading and concentrations of different DNA samples collected from 12 potato genotypes

Sl. No.	Potato Genotypes	Absorbance Reading (260nm)	DNA Concentration (ng/µL)
1	Cardinal	0.086	4300
2	Diamant	0.065	3250
3	Asterix	0.069	3450
4	SIP-3	0.071	3550
5	SIP-5	0.083	4150
6	SVP-6	0.088	4400
7	SVP-18	0.081	4050
8	SVP-19	0.074	3700
9	SVP-25	0.067	3350
10	SVP-55	0.075	3750
11	SVP-56	0.070	3500
12	SVP-68	0.087	4350

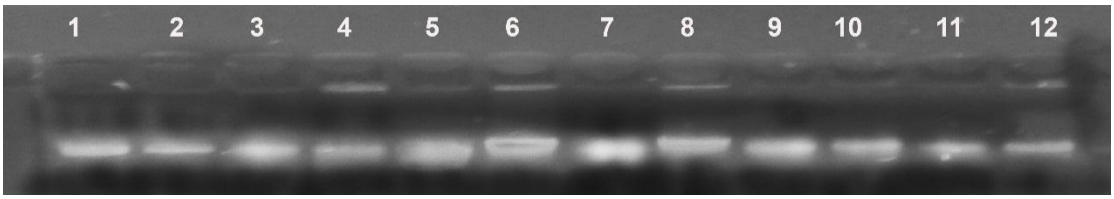


Plate 1. Confirmation of genomic DNA samples of 12 potato genotypes (Lane 1: Cardinal, Lane 2: Diamant, Lane 3: Asterix, Lane 4: SIP-3, Lane 5: SIP-5, Lane 6: SVP-6, Lane 7: SVP-18, Lane 8: SVP-19, Lane 9: SVP-25, Lane 10: SVP-55, Lane 11: SVP-56. Lane 12: SVP-68)

4.2 RAPD Primer selection for PCR amplification

Initially 9 decamer RAPD primers were screened on randomly chosen six genotypes from 12 potato genotypes to evaluate their suitability for amplification of the potato DNA sequences. The primers, which gave minimum smearing, high resolution and maximum reproducible and distinct polymorphic amplified bands were selected. It revealed that, out of 9 RAPD primers 6 primers showed reproducible and polymorphic amplified bands (Plate 2).

The result agreed with AL-Salihy *et al.* (2014), Iuliana and Cerasela (2014), Orona-Castro *et al.* (2006) and Collares *et al.* (2004), where they screened 6 RAPD primers in case 4 of *in vitro* propagated potato, 6 RAPD primers in 6 potato genotypes (3 potato cultivars and their 3 somaclonal variants), 40 RAPD primers in 16 potato cultivars and 18 RAPD primers in 27 potato genotypes respectively. From which they selected clear and distinct polymorphic bands producing 5, 3, 3, and 4 RAPD primers, respectively.

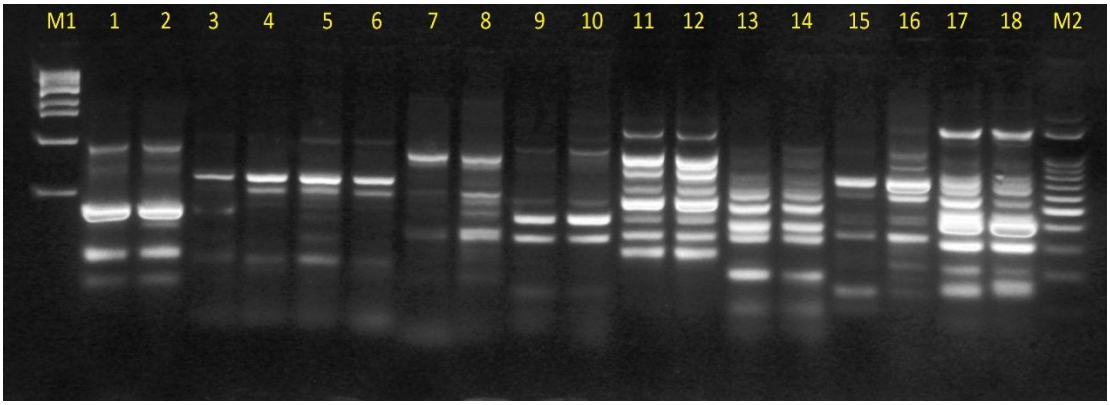


Plate 2. Primer test: PCR amplification products by 9 different decamer RAPD primers using DNA from six different genotypes
(Lane 1-2: OPA-18; Lane 3-4: OPA-20; Lane 5-6: OPB-04; Lane 7-8: OPB-06; Lane 9-10: OPB-08; Lane 11-12: OPC-01, Lane 13-14: OPD-02; Lane 15-16: OPF-08 and Lane 17-18: OPW-01. M1= 1kb ladder and M2= 100bp ladder)

4.3 RAPD banding pattern, band size and DNA polymorphism

The name of the selected six primers were OPA-18, OPB-08, OPC-01, OPD-02, OPF-08 and OPW-01. Each of the primers produced separate RAPD patterns (bands) in 12 potato genotypes. The RAPD amplifications of individual primer were given in plate 3 to 8.

Selected 6 primers generated a total of 54 distinct and differential amplified bands. The average was 9 bands per primer and 4.5 bands per potato genotypes. The size of the bands ranged from 88 to 3265 bp but most of the bands were between 150 to 2000 bp. Out of total 54 bands, 47 bands (87.04%) were polymorphic DNA bands and 7 bands (12.96%) were monomorphic. The result gave an average 7.83 polymorphic and 1.16 monomorphic bands per primer. The six different primers produced various banding patterns. The primer OPF-08 produced maximum number of bands (13) followed by OPB-08 (11), OPD-02 (9), OPC-01 and OPW-01 produced same number (8) of bands and OPA-18 produced the least number of (5) bands (Plates 3 to 8). On the other hand, OPC-01 amplified the highest percentage of (100%) polymorphic bands, followed by OPF-08 (92.31%), OPB-08 (90.91%), OPD-02 (88.89%), OPW-01 (75.0%) and least polymorphic bands was produced by OPA-18 (60%). It revealed that, OPC-01 showed the highest level of polymorphism and OPW-01 showed the lowest level of polymorphism among these 6 primers. The amplification of DNA bands numbers per primer, bands size and polymorphism percentage were given in table 6.

Similar results were found by different scientists. Ahmad *et al.* (2013) studied 4 RAPD primers in mutant lines of the three potato cultivars, Cardinal, Diamant and Desiree and found bands were present at 50 bp to 1500 bp.

On the contray, Afrasiab and Iqbal (2012b) estimated 123 clear and easily storable bands using 24 RAPD primers in 9 potato genotypes (3 somaclonal variant of cultivar Desiree and 6 gamma mutant lines) ranged from 200 to 3000 bp and average 5.12 bands per primers and 13.67 bands per potato cultivar. They detected 63% polymorphic bands and 37% monomorphic bands, which indicated

that their study was almost similar to present study in respect of bands size ranges but higher in case of bands per cultivars and percentage of monomorphic bands and lower in case of percentage of polymorphic bands.

Again, Afrasiab and Iqbal (2012a) estimated 187 clear and easily scorable bands using 22 RAPD primers in 9 potato genotypes (5 gamma irradiant potato and 4 somaclonal variant potato of variety Diamant). They revealed an average 8.5 bands per primer and 20.78 bands per potato cultivar. The size of the bands ranged from 200 to 3000 bp but most of the bands were between 300 to 2000 bp. Out of their estimated 187 bands 140 bands (74.86%) were polymorphic bands and 47 bands (25.14%) were monomorphic bands. The result gave an average 6.36 polymorphic and 2.14 monomorphic bands per primer. The present study was almost similar to their study in respect of average bands per primers and size range of bands but higher in respect of percentage of polymorphic bands and average polymorphic bands per primer and lower in case bands per cultivar, monomorphic bands percentage and average monomorphic bands per primers.

On the other hand, Khatab and El-Banna (2011) used five RAPD primers in 14 somaclonal variants of potato and detected total 61 bands i.e. 12.2 bands per primer and 4.36 bands per cultivar. The size of the bands ranged from 200 to 3050 bp. They revealed 38 (62.29%) polymorphic and 23 (37.71%) monomorphic bands. The result gave an average 7.6 polymorphic and 4.6 monomorphic bands per primer. Among the primers used, OPE-02 produced the highest number (14) of bands while primers OPA-05 produced the lowest number (7) of bands. The highest number (11) of polymorphic bands and polymorphisms (78.5%) and the lowest number (1) of polymorphic bands and polymorphisms (14.3%) were detected using primers OPE-02 and OPV-02 respectively. Their study was almost similar to present study in respect of number of bands per cultivar, average polymorphic bands per primer and size range of the bands but lower in respect of polymorphism percentage, percentage of polymorphic and monomorphic bands and higher than average monomorphic bands per primers.

Munir *et al.* (2011) detected 111 bands with 10 RAPD (OPC-01 to 10) primers in 5 somaclonal variation of potato cultivar Diseree, where OPC-05 and OPC-07 produced the highest number (8) of polymorphic bands and OPC-01, OPC-04 and OPC-10 produced the lowest number (0) of polymorphic bands. They detected the highest polymorphisms (53%) with OPC-03 and the lowest polymorphisms (0%) with OPC-01, OPC-04, OPC-06 and OPC-10 primers.

Table 6. RAPD primers with corresponding bands scored and their size together with polymorphic bands observed in 12 potato genotypes

Primer codes	Sequences (5′- 3′)	(G+C) %	Total no of bands scored	Size ranges (bp)	Number of polymorphic bands	Proportion of polymorphic loci (%)
OPA-18	AGGTGACCGT	60	05	155-1040	03	60.00
OPB-08	GTCCACACGG	70	11	193-1868	10	90.91
OPC-01	TTCGAGCCAG	60	08	201-1172	08	100.00
OPD-02	GGACCCAACC	70	09	194-3265	08	88.89
OPF-08	GGGATATCGG	60	13	120-2007	12	92.31
OPW-01	CTCAGTGTCC	60	08	88-827	06	75.00
Total	**-**	**380**	**54**	**-**	**47**	**-**
Average	**-**	**63.33**	**9.0**	**-**	**7.83**	**87.04**

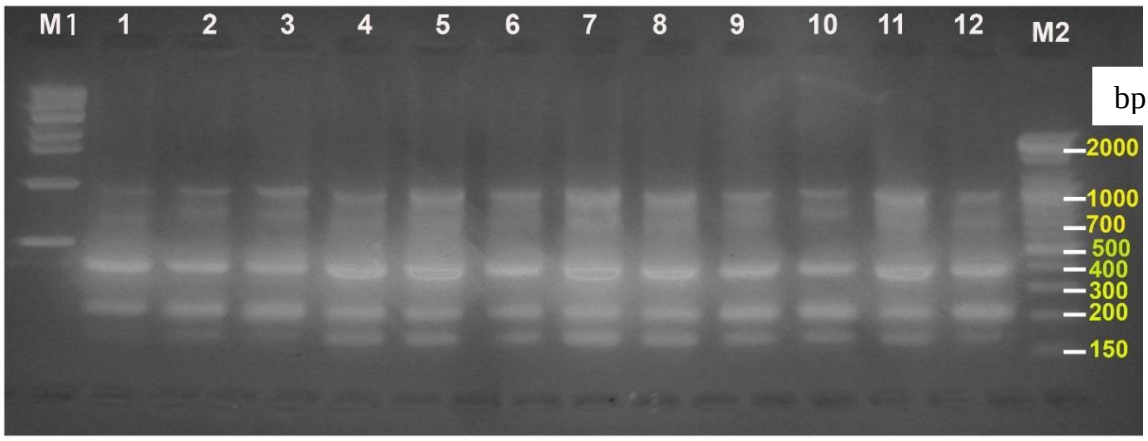


Plate 3. RAPD profiles of 12 potato genotypes using primer **OPA-18**

(Lane 1: SIP-3; Lane 2: SIP-5; Lane 3: SVP-6; Lane 4: SVP-18; Lane 5: SVP-19; Lane 6: SVP-25; Lane 7: SVP-55; Lane 8: SVP-56; Lane 9: SVP-68; Lane 10: Cardinal; Lane 11: Diamant and Lane 12: Asterix. M1 and M2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

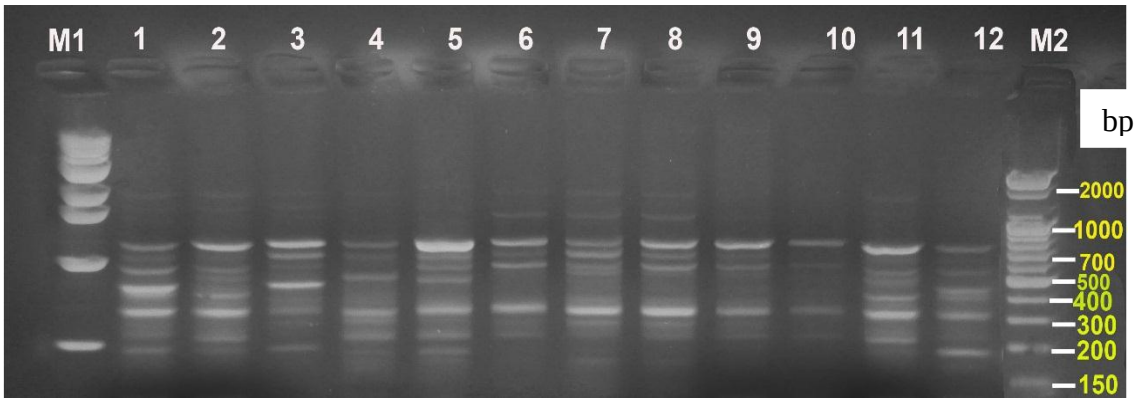


Plate 4. RAPD profiles of 12 potato genotypes using primer **OPB-08**

(Lane 1: SIP-3; Lane 2: SIP-5; Lane 3: SVP-6; Lane 4: SVP-18; Lane 5: SVP-19; Lane 6: SVP-25; Lane 7: SVP-55; Lane 8: SVP-56; Lane 9: SVP-68; Lane 10: Cardinal; Lane 11: Diamant and Lane 12: Asterix. M1 and M2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

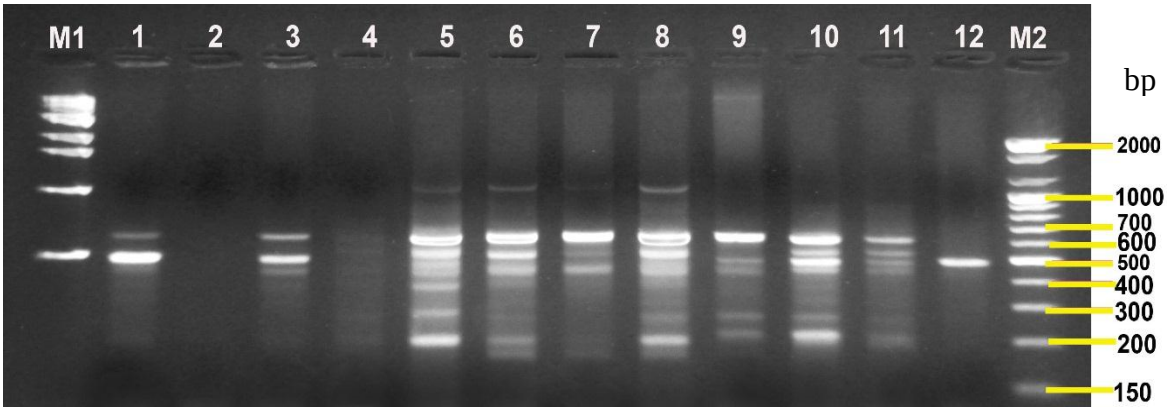


Plate 5. RAPD profiles of 12 potato genotypes using primer **OPC-01**

(Lane 1: Cardinal; Lane 2: Diamant; Lane 3: Asterix; Lane 4: SIP-3; Lane 5: SIP-5. Lane 6: SVP-6; Lane 7: SVP-18; Lane 8: SVP-19; Lane 9: SVP-25; Lane 10: SVP-55; Lane 11: SVP-56 and Lane 12: SVP-68. M1 and M2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

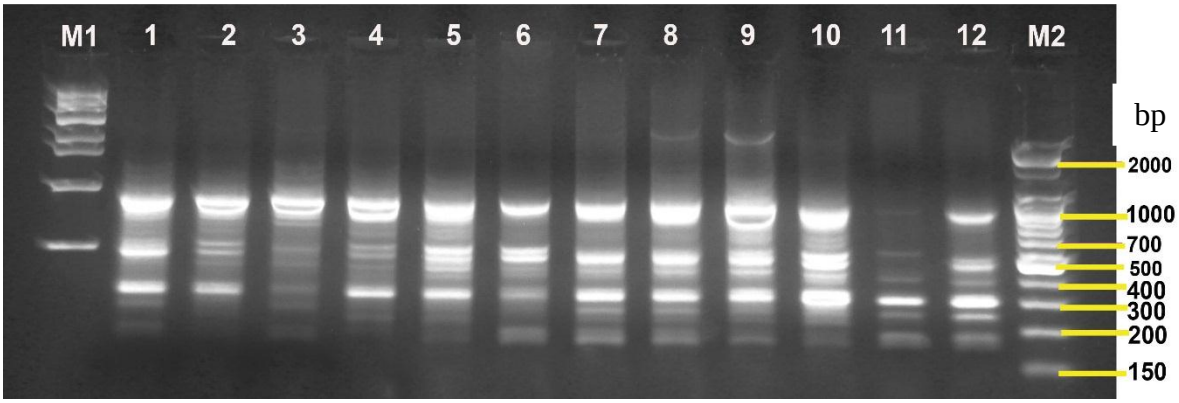


Plate 6. RAPD profiles of 12 potato genotypes using primer **OPD-02**

(Lane 1: Cardinal; Lane 2: Diamant; Lane 3: Asterix; Lane 4: SIP-3; Lane 5: SIP-5. Lane 6: SVP-6; Lane 7: SVP-18; Lane 8: SVP-19; Lane 9: SVP-25; Lane 10: SVP-55; Lane 11: SVP-56 and Lane 12: SVP-68. M1 and M2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

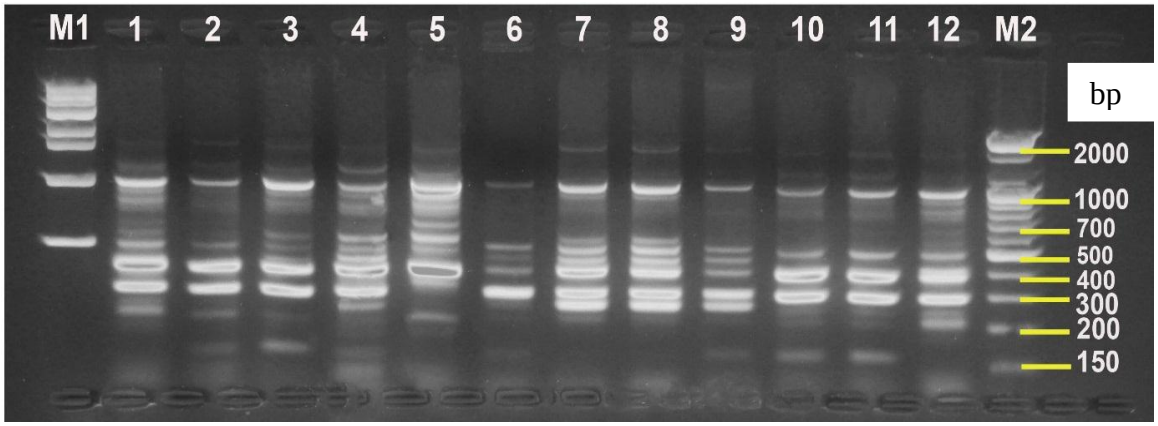


Plate 7. RAPD profiles of 12 potato genotypes using primer **OPF-08**

(Lane 1: Cardinal; Lane 2: Diamant; Lane 3: Asterix; Lane 4: SIP-3; Lane 5: SIP-5. Lane 6: SVP-6; Lane 7: SVP-18; Lane 8: SVP-19; Lane 9: SVP-25; Lane 10: SVP-55; Lane 11: SVP-56 and Lane 12: SVP-68. M_1 and M_2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

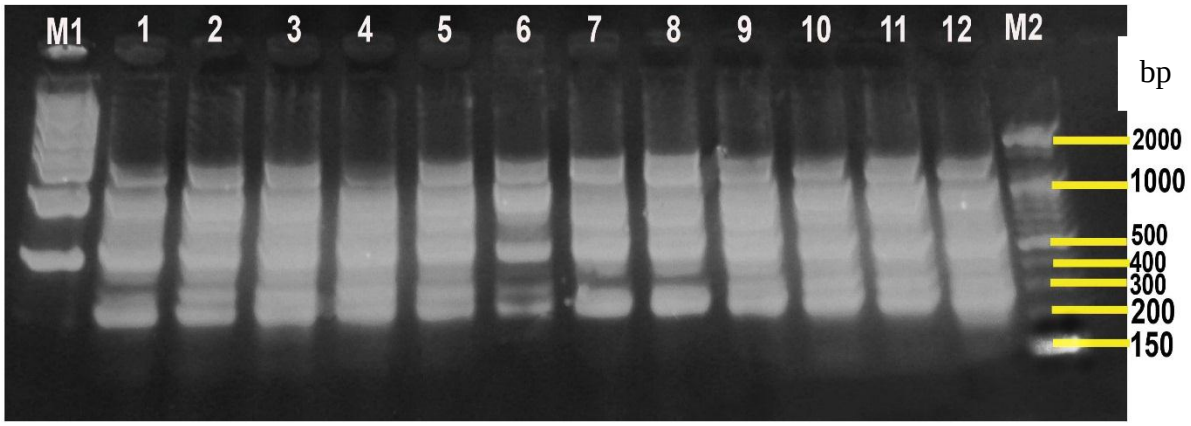


Plate 8. RAPD profiles of 12 potato genotypes using primer **OPW-01**

(Lane 1: Cardinal; Lane 2: Diamant; Lane 3: Asterix; Lane 4: SIP-3; Lane 5: SIP-5. Lane 6: SVP-6; Lane 7: SVP-18; Lane 8: SVP-19; Lane 9: SVP-25; Lane 10: SVP-55; Lane 11: SVP-56 and Lane 12: SVP-68. M1 and M2: Molecular weight marker (1 kb and 100 bp DNA ladder in left side and right side respectively)

4.4 Gene frequency and frequency of polymorphic loci

The DNA polymorphisms were detected according to band presence or absence. Absence of bands may be caused by failure of primers to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between primer sites (Clark and Lanigan. 1993).

The six primers used in the present study showed different levels of gene frequency and different frequency of polymorphic loci (Table 7). The gene frequency ranged from 0.083 to 1.00 and frequency of polymorphic loci ranged from 0 to 0.917. The highest gene frequency (1.00) i .e. the lowest frequency of polymorphic loci (0) was shown by the primer OPA-18 (at 400 and 240 bp), OPB-08 (at 794 bp), OPD-02 (at 340 bp), OPF-08 (at 1047 bp) and OPW-01 (at 521 and 673 bp). On the other hand the lowest gene frequency (0.083) i .e. the highest frequency of polymorphic loci (0.917) is showed by the primer OPF-08 (at 658 bp).

The primer OPA-18 showed the highest gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) at 400 and 240 bp and the lowest gene frequency (0.75) i.e. the highest frequency of polymorphic loci (0.25) at 719 and 155 bp. Primer OPB-08 showed the highest high gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) at 794 bp and the lowest gene frequency (0.33) i.e. the highest frequency of polymorphic loci (0.67) at 391, 335 and 289 bp. The primer OPC-01 showed the highest gene frequency (0.83) i.e. the lowest frequency of polymorphic loci (0.17) at 494 bp and the lowest gene frequency (0.25) i.e. the highest frequency of polymorphic loci (0.75) at 201 bp. Primer OPD-02 showed the highest gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) at 340 bp and the lowest gene frequency (0.167) i.e. the highest frequency of polymorphic loci (0.833) at 3265 bp. The primer OPF-08 showed the highest gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) at 10467 bp and the lowest gene frequency (0.083) i.e. the highest frequency of polymorphic loci (0.917) at 658 bp. The primer OPW-01 showed the highest gene frequency (1.00) i.e. the lowest

frequency of polymorphic loci (0) at 521 and 673 bp and the lowest gene frequency (0.33) i.e. the highest frequency of polymorphic loci (0.67) at 384 bp.

Similar result was shown by Khatab and El-Banna (2011) where they found the gene frequency ranged from 0.056 to 1.00 and frequency of polymorphic loci ranged from 0 to 0.944. The highest gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) was shown by the primer OPA-12 (at 750 bp), and the lowest gene frequency (0.056) i.e. the highest frequency of polymorphic loci (0.944) was shown by the primer OPV-02 (at 240 bp), OPA-12 (at 3050, 3000 and 320 bp), OPQ-14 (at 200 bp), and OPE-02 (at 350 bp).

Table 7. Frequencies of polymorphic loci by RAPD markers in potato genotypes

RAPD Marker	Locus No.	Locus Size (bp)	Gene Frequency	RAPD Marker	Locus No.	Locus Size (bp)	Gene Frequency
OPA-18	1	1040	0.9167	**OPD-02**	4	615	0.9167
	2	719	0.7500		5	532	0.9167
	3	**400**	**1.0000**		6	443	0.9167
	4	**240**	**1.0000**		7	**340**	**1.0000**
	5	155	0.7500		8	287	0.8333
OPB-08	1	1868	0.6667		9	194	0.9167
	2	1156	0.5000	**OPF-O8**	1	2007	0.9167
	3	**794**	**1.0000**		2	1302	0.6667
	4	662	0.8333		3	**1047**	**1.0000**
	5	580	0.9167		4	876	0.5833
	6	476	0.9167		5	813	0.5833
	7	391	0.3333		6	**658**	**0.0833**
	8	335	0.3333		7	553	0.5833
	9	289	0.3333		8	433	0.9167
	10	238	0.8333		9	306	0.9167
	11	193	0.5833		10	265	0.9167
OPC-01	1	1172	0.3333		11	224	0.5000
	2	678	0.7500		12	153	0.5833
	3	550	0.5833		13	120	0.4167
	4	494	0.8333	**OPW-01**	1	1527	0.8333
	5	422	0.3333		2	1261	0.8333
	6	297	0.5000		3	1069	0.8333
	7	238	0.5000		4	832	0.9167
	8	201	0.2500		5	**673**	**1.0000**
OPD-02	1	3265	0.1667		6	**521**	**1.0000**
	2	1036	0.9167		7	384	0.3333
	3	794	0.2500		8	238	0.8333

4.5 Inter-genotype similarity indices (Sij)

The similarity indices (S_{ij}) for different genotype pairs with six different primers and their average were shown in Table 8. In the present study, inter-varietal/ inter-genotype means of the pair wise similarity indices (S_{ij}) ranged from 61.59% to 93.55% and average was 74.31%. The highest similarity indices (93.55%) was present between SVP-55 vs SVP-56 genotype pair. So, genetic distance was lower between that pair than rest of the genotype pairs. The second highest similarity indices (S_{ij}) was present between SVP-18 vs SVP-19 (86.73), followed by SVP-19 vs SVP-55 (85.5) and SVP-19 vs SVP-25 (85.04) genotype pairs. On the other hand, Asterix vs SIP-3 pair showed the lowest inter-variety similarity indices (61.59%) and genetic distance was higher between that pair than rest of the varietal pairs. The second lowest similarity indices (S_{ij}) was present between SIP-3 vs SVP-56 (63.75) followed by SIP-3 vs SVP-18 (63.98) genotype pair. All the 66 genotype pairs were not homogenous at different loci and different primers. Therefore, this study clearly indicated that, there was a high level of genetic similarity among different genotypes.

Many scientists reported similar type of observations on different potato varieties with RAPD primers. Kujal *et al.* (2005) reported pair-wise similarity value was of 52% to 86 % (average 65%) among 31 potato genotypes. Isenegger *et al.* (2001) studied 64 potato cultivars in Australia and found 67% to 90 % similarity among them. The results of above two studies were very close to present study.

On the other hand, Gauchan *et al.* (2012) reported 55.2% to 69% similarity value among 4 Nepali local potato cultivars studied with 10 RAPD primers, which was lower than the present study. This may happen due to cultivar variations. Again, Das *et al.* (2010) detected a wide range of similarity values (ranged from 29% to 93%) in 30 Indian potato cultivars with 13 RAPD primers.

Table 8. RAPD band sharing percentage inter-genotype similarity indices among the 12 potato genotypes across six primers

Varietal pairs	Primers						Average
	OPA-18	OPB-08	OPC-01	OPD-02	OPF-08	OPW-01	
Cardinal vs Diamant	88.89	72.73	0	72.73	73.68	100	68.01
Cardinal vs Asterix	57.14	66.67	100	76.92	90.91	92.31	80.66
Cardinal vs SIP-3	85.7	61.54	0	92.31	95.24	72.73	67.92
Cardinal vs SIP-5	100	61.54	80	75.71	90	83.33	81.76
Cardinal vs SVP-6	75	50	54.55	88.53	53.33	93.33	69.12
Cardinal vs SVP-18	89.89	60	40	92.31	84.21	93.33	76.62
Cardinal vs SVP-19	89.89	80	54.55	85.71	77.78	93.33	80.21
Cardinal vs SVP-25	89.89	50	57.14	80	70.59	93.33	73.50
Cardinal vs SVP-55	89.89	57.13	66.67	92.31	70	92.31	78.052
Cardinal vs SVP-56	89.89	61.54	54.55	72.73	80	100	76.46
Cardinal vs SVP-68	89.89	80	50	92.31	90.91	83.33	81.07
Diamant vs Asterix	75	66.67	0	66.67	84.21	92.31	64.14
Diamant vs SIP-3	75	87.5	0	83.33	66.67	72.73	64.21
Diamant vs SIP-5	88.89	87.5	0	76.92	66.67	83.33	67.22
Diamant vs SVP-6	88.89	66.67	0	90.91	50	93.33	64.97
Diamant vs SVP-18	100	46.15	0	83.33	62.5	93.33	64.22
Diamant vs SVP-19	100	76.92	0	76.92	66.67	93.33	68.97
Diamant vs SVP-25	100	66.67	0	71.43	71.43	93.33	67.14
Diamant vs SVP-55	100	70.59	0	83.33	94.12	92.31	73.39
Diamant vs SVP-56	100	62.5	0	60	94.12	100	69.44
Diamant vs SVP-68	100	76.92	0	83.33	73.64	83.33	69.53
Asterix vs SIP-3	66.67	71.43	0	85.71	85.71	60	**61.59**
Asterix vs SIP-5	57.14	71.43	60	80	85.71	72.7	71.16
Asterix vs SVP-6	85.71	76.92	54.55	76.93	53.33	85.71	72.19
Asterix vs SVP-18	75.7	54.55	57.14	85.71	73.68	85.71	72.08
Asterix vs SVP-19	75.7	90.91	54.55	80	66.67	85.71	75.59
Asterix vs SVP-25	75.7	46.15	57.14	87.5	58.82	85.71	68.50
Asterix vs SVP-55	75.7	66.67	66.67	85.71	90	100	80.79
Asterix vs SVP-56	75.7	57.14	75	66.67	90	92.31	76.14
Asterix vs SVP-68	75.7	72.73	50	85.71	95	72.73	75.31
SIP-3 vs SIP-5	85.71	100	0	93.33	80	66.67	70.95
SIP-3 vs SVP-6	85.71	82.55	0	92.31	57.14	66.67	64.06
SIP-3 vs SVP-18	75	53.33	0	100	88.89	66.67	63.98
SIP-3 vs SVP-19	75	80	0	93.33	82.35	66.67	66.23
SIP-3 vs SVP-25	75	82.35	0	87.5	75	66.67	64.42
SIP-3 vs SVP-55	75	84.21	0	100	73.68	60	65.48
SIP-3 vs SVP-56	75	77.78	0	83.33	73.68	72.73	63.75
SIP-3 vs SVP-68	75	80	0	100	85	88.89	71.48
SIP-5 vs SVP-6	75	82.35	93.33	85.71	42.87	76.92	76.03
SIP-5 vs SVP-18	88.89	53.33	54.55	93.33	66.67	76.92	72.28
SIP-5 vs SVP-19	88.89	80	93.33	87.5	58.82	76.92	80.91
SIP-5 vs SVP-25	88.89	82.35	72.33	94.12	50	76.92	77.44

Table 8. (Cont'd.)

Varietal pairs			Primers						Average
			OPA-18	OPB-08	OPC-01	OPD-02	OPF-08	OPW-01	
SIP-5	vs	SVP-55	88.89	84.21	92.31	93.33	73.68	72.73	84.19
SIP-5	vs	SVP-56	88.89	77.78	83.33	76.92	73.68	83.33	80.66
SIP-5	vs	SVP-68	88.89	80	25	93.33	76.19	60	70.57
SVP-6	vs	SVP-18	88.89	57.14	66.67	92.31	66.67	100	78.61
SVP-6	vs	SVP-19	88.89	71.43	100	85.71	54.55	100	83.43
SVP-6	vs	SVP-25	88.89	62.5	66.67	80	60	100	76.34
SVP-6	vs	SVP-55	88.89	88.89	85.71	92.31	46.15	85.71	81.28
SVP-6	vs	SVP-56	88.89	82.35	76.92	72.73	46.15	93.33	76.73
SVP-6	vs	SVP-68	88.89	71.43	22.22	92.31	40	76.92	65.30
SVP-18	vs	SVP-19	100	66.67	66.67	93.33	93.71	100	86.73
SVP-18	vs	SVP-25	100	57.14	50	87.5	85.71	100	80.06
SVP-18	vs	SVP-55	100	75	40	100	58.82	85.71	76.59
SVP-18	vs	SVP-56	100	80	57.14	83.33	58.82	93.33	78.77
SVP-18	vs	SVP-68	100	66.67	40	100	73.68	76.92	76.21
SVP-19	vs	SVP-25	100	57.14	66.67	94.12	92.31	100	85.04
SVP-19	vs	SVP-55	100	85.71	85.72	93.33	62.5	85.71	85.50
SVP-19	vs	SVP-56	100	66.67	76.92	66.67	62.5	93.33	77.68
SVP-19	vs	SVP-68	100	83.33	22.22	93.33	66.67	76.92	73.75
SVP-25	vs	SVP-55	100	77.78	80	87.5	66.67	85.71	82.94
SVP-25	vs	SVP-56	100	82.35	88.89	71.43	66.67	93.33	83.78
SVP-25	vs	SVP-68	100	71.43	60	87.5	98.82	76.92	82.45
SVP-55	**vs**	**SVP-56**	100	94.74	90.91	83.33	100	92.33	**93.55**
SVP-55	vs	SVP-68	100	75	28.57	100	63.16	72.73	73.24
SVP-56	vs	SVP-68	100	80	33.33	83.33	63.16	83.33	73.86

4.6 Nei's (1972) genetic identity and genetic distance

Pair-wise comparisons of Nei's (1972) genetic identity among 12 potato genotypes was calculated from the combined data of the six primers and the value ranged from 0.5370 to 0.9074. The highest Nei's genetic identity (0.9074) was observed in SVP-55 vs SVP-56 varietal pair; whereas the lowest genetic identity (0.5370) was estimated in SVP-68 vs SVP-6. The second largest genetic identity was 0.8184 and they were present in Cardinal vs SVP-68, SVP-68 vs SIP-3 and SVP-18 vs SVP-19 (Table 9). The differences between the highest and the lowest genetic identity indicates the presence of variability among 12 genotypes of potato.

Nei's (1972) genetic distance among 12 potato genotypes ranged from 0.0972 to 0.6217. The highest Nei's genetic distance (0.6217) was observed in SVP-6 vs SVP-68 genotype pair whereas the lowest genetic distance (0.0972) was estimated in SVP-55 vs SVP-56 genotype pair (Table 9).

Among the three popular cultivated varieties (Cardinal, Diamant and Asterix) Nei's (1972) genetic identity ranged from 0.6852 to 0.7778, where genetic distance ranged from 0.2513 to 0.3781. The highest Nei's genetic identity (0.7778) was observed in Cardinal vs Asterix varietal pair and the lowest genetic identity (0.6852) was estimated in Diamant vs Asterix varietal pair. On the other hand, the highest Nei's genetic distance (0.3781) was observed in Diamant vs Asterix varietal pair and the lowest genetic distance (0.2513) was estimated in Cardinal vs Asterix cultivar pair (Table 9). The result indicates that Cardinal and Asterix varieties are closely linked and Diamant and Asterix varieties are distantly linked among the three varieties.

Again, among Cardinal and other somaclonal variation genotypes Nei's (1972) genetic identity ranged from 0.5741 (at SVP-6) to 0.8148 (at SVP-68), while for Diamant it ranged from 0.5962 (at SVP-6) to 0.7593 (at SVP-68) and in case of Asterix it ranged from 0.5741 (at SVP-25) to 0.7407 (at SVP-55 and SVP-68). So, SVP-68 is very close to the three varieties among the somaclonal variations.

On the other hand, among Cardinal and other somaclonal variations Nei's (1972) genetic distance ranged from 0.2048 (at SVP-68) to 0.5550 (at SVP-6). Whereas for Diamant, it ranged from 0.2754 (at SVP-68) to 0.5232 (at SVP-6) and in case of Asterix it ranged from 0.3001 (at SVP-55 and SVP-68) to 0.5550 (at SVP-25). So, SVP-6 (to Cardinal and Diamant) and SVP-25 (to Asterix) is distantly linked accessions among the somaclonal variations.

Whereas, several scientists reported similar type of observation. Onamu *et al.* (2014) estimated a similarity coefficient of 0.55 to 0.89 among 15 potato cultivars (9 cultivars bred in Europe, N. America and Mexico, and 6 Mexican creole cultivars) which was very close to the present study. Yasmin *et al.* (2006) reported Nei's (1972) genetic identity among 6 potato cultivars ranged from 0.6530 to 0.8674 and genetic distance ranged from 0.154 to 0.558. They also reported that Nei's (1972) genetic identity and genetic distance between Cardinal and Diamant was 0.6530 and 0.558 respectively. Hoque *et al.* (2013) studied 12 potatoes with 8 RAPD primers in Bangladesh. They found that Nei's genetic identity was the highest (0.82050) in the varietal pair Cardinal and Diamant and it was the lowest (0.333) in Provinto and Granola. They detected Nei's genetic distance ranged from 0.137 to 0.970. On the other hand, Chakrabarti *et al.* (2001) detected 0.33 to 0.80 similarity value among 20 potato cultivars with 10 RAPD primers. Fu-cui *et al.* (2004) reported a high range (0.033 to 0.900) of genetic distance in 67 potato cultivars using 11 RAPD primers in China.

Table 9. Summary of Genetic identity (above diagonal) and Nei's (1972) genetic distance (below diagonal) values among 12 potato genotypes

Genotypes	Cardinal	Diamant	Asterix	SIP-3	SIP-5	**SVP-6**	SVP-18	SVP-19	SVP-25	**SVP-55**	**SVP-56**	**SVP-68**
Cardinal	****	0.7222	0.7778	0.7407	0.7037	0.5741	0.7593	0.7222	0.6481	0.7037	0.7222	0.8148
Diamant	0.3254	****	0.6852	0.7222	0.6111	0.5926	0.6296	0.6296	0.6667	0.7222	0.7037	0.7593
Asterix	0.2513	0.3781	****	0.6667	0.6667	0.6111	0.6481	0.6481	0.5741	0.7407	0.6852	0.7407
SIP-3	0.3001	0.3254	0.4055	****	0.7037	0.5741	0.6481	0.6111	0.6481	0.6296	0.6111	0.8148
SIP-5	0.3514	0.4925	0.4055	0.3514	****	0.6481	0.5741	0.6852	0.6481	0.7407	0.6852	0.6296
SVP-6	0.5550	0.5232	0.4925	0.5550	0.4336	****	0.7037	0.7778	0.6667	0.7222	0.6667	**0.5370**
SVP-18	0.2754	0.4626	0.4336	0.4336	0.5550	0.3514	****	0.8148	0.7407	0.6481	0.6667	0.7222
SVP-19	0.3254	0.4626	0.4336	0.4925	0.3781	0.2513	0.2048	****	0.7778	0.7222	0.6667	0.6481
SVP-25	0.4336	0.4055	0.5550	0.4336	0.4336	0.4055	0.3001	0.2513	****	0.7222	0.7407	0.6481
SVP-55	0.3514	0.3254	0.3001	0.4626	0.3001	0.3254	0.4336	0.3254	0.3254	****	**0.9074**	0.7037
SVP-56	0.3254	0.3514	0.3781	0.4925	0.3781	0.4055	0.4055	0.4055	0.3001	**0.0972**	****	0.7222
SVP-68	0.2048	0.2754	0.3001	0.2048	0.4626	**0.6217**	0.3254	0.4336	0.4336	0.3514	0.3254	****

4.7 UPGMA Dendrogram

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 12 genotypes of potato into two main clusters: A and B. The first major cluster (A) had 8 genotypes i.e. Cardinal, SVP-68, SIP-3, Diamant, Asterix, SIP-5, SVP-55 and SVP-56. The second major cluster (B) had 4 genotypes i.e. SVP-6, SVP-18, SVP-19 and SVP-25.

The first major cluster A was divided into two sub-cluster: AI and AII. Sub-cluster AI contained 5 genotypes i.e. Cardinal, SVP-68, SIP-3, Diamant and Asterix while Sub-cluster AII contained 3 genotypes i.e. SIP-5, SVP-55 and SVP-56. The sub-cluster AI was divided into 2 groups: AI GI and AI GII. The group A I G I contained 4 genotypes (Cardinal, SVP-68, SIP-3 and Diamant) which was further divided into 2 sub-groups: S1 and S2. The sub-group S1 had 3 genotypes (Cardinal, SVP-68 and SIP-3) and sub-group S2 had only one genotype i.e. Asterix. The sub-group 1 was divided into 2 classes: class 1 (Cardinal and SVP-68) and class 2 (SIP-3). The class 1 was further divided into 2 sub-classes containing Cardinal and SVP-68 separately. On the other hand, Sub-cluster AII was divided into 2 groups: Group AII GI (SIP-5) and AII GII (SVP-55 and SVP-56). The group AII GII was divided into two sub-group containing SVP-55 and SVP-56 separately.

Again, the second major cluster B was divided into two sub-cluster: BI and BII. Sub-cluster BI had only one genotype (SVP-6), and Sub-cluster BII had 3 genotypes (SVP-18, SVP-19 and SVP-25). Sub-cluster BII was divided into two groups: BII GI (SVP-18 and SVP-19) and BII GII (SVP-25). The group BII GI was divided into two sub-groups containing SVP-18 and SVP-19 separately.

The above results were shown in Figure 1. The results indicated that, different level of genetic identity and distance were present among the studied 12 potato genotypes.

Several scientists reported similar results to the present study. Hoque *et al.* (2013) studied an UPGMA dendrogram based on the Nei's genetic distances among 12 popular potato varieties in Bangladesh. These varieties were segregated into two main clusters. Then the clusters into sub-clusters, groups, sub-groups and classes. They reported that Cardinal, Diamant and Asterix belonged to same sub-cluster and their dendrogram was somewhat complex like the present study.

Yasmin *et al.* (2006) constructed UPGMA dendrogram based on the Nei's (1972) genetic distances, segregated 6 cultivars into two clusters and the clusters into sub-clusters and sub-clusters further into groups.

Brenna (2004) showed the genetic relationship among 12 potato varieties with a dendrogram. The dendrogram segregated the 12 varieties into 2 main clusters and then different sub-clusters, group, sub-groups and classes like the present study depending on the genetic similarity of 12 potato cultivars.

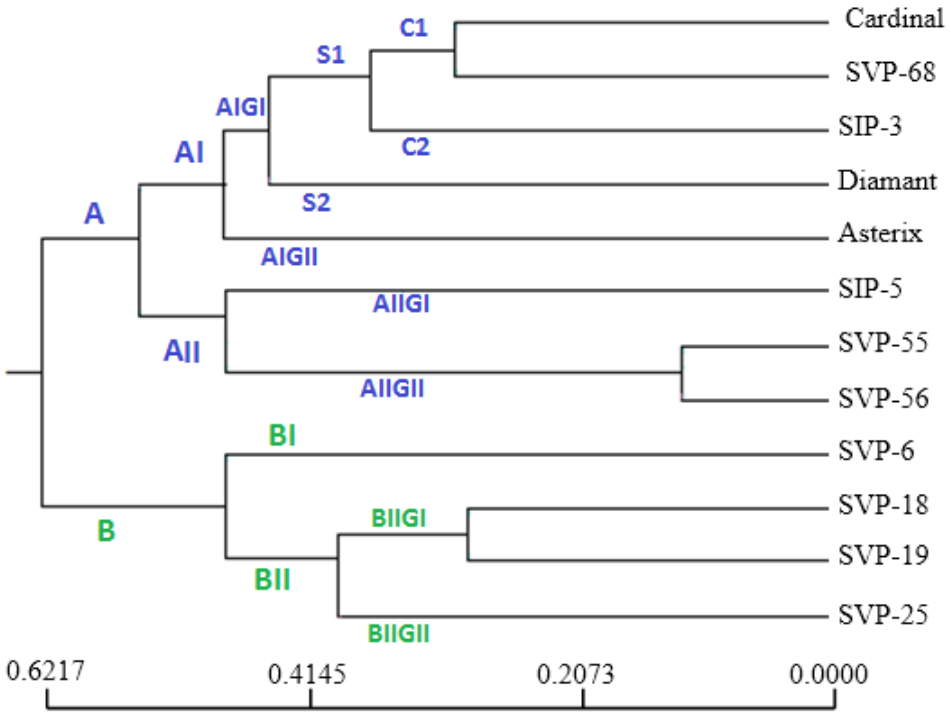
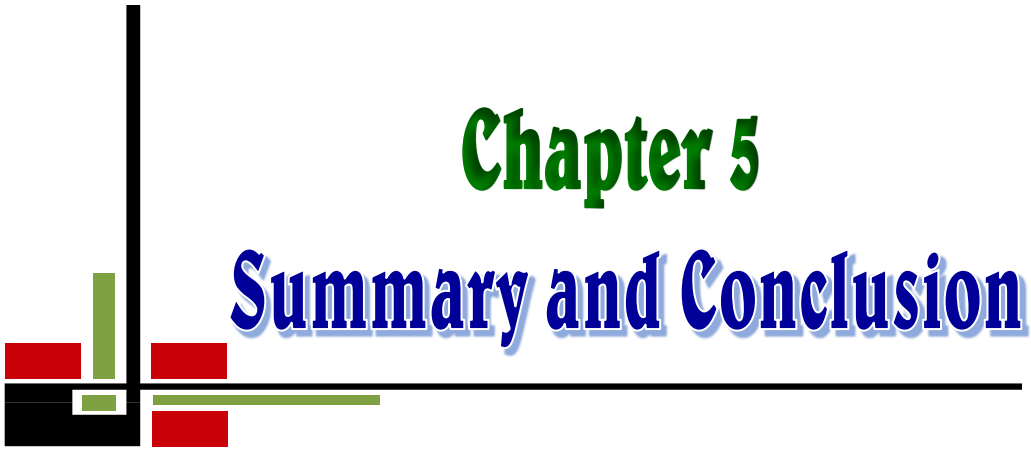


Figure 1. UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation among 12 Potato genotypes according to RAPD analysis



Chapter 5

Summary and Conclusion

CHAPTER V

SUMMARY AND CONCLUSION

Potato (*Solanum tuberosum* L.) is one of the most important food crop grown all over the world. Most of the varieties cultivated in Bangladesh are either imported from other countries or developed through conventional breeding procedures. Developing of new variety through somaclonal variation and their molecular characterization and diversity analysis is not common yet. The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka - 1207, during the period from November, 2013 to December, 2014 to study the DNA fingerprinting and genetic diversity of twelve potato genotypes through RAPD primers.

Out of the 12 materials three were popular potato varieties *viz*. Cardinal, Diamant and Asterix and nine (SIP-3, SIP-5, SVP-6, SVP-18, SVP-19, SVP-25, SVP-55, SVP-56 and SVP-68) were somaclonal variants potato genotypes created by the Department of Biotechnology, SAU. Six RAPD primers *viz.* OPA-18, OPB-08, OPC-01, OPD-02, OPF-08 and OPW-01were used for PCR amplification.

Selected six primers generated total 54 distinct and differential amplified bands (size ranged 88 to 3265 bp) i.e. average 9 bands per primer and 4.5 bands per potato genotypes. Out of them, 47 bands (87.04%) were polymorphic. Maximum (13) and minimum number (5) of bands were produced by primer OPF-08 and OPA-18 respectively. OPF-08 produced highest number (12) of polymorphic bands.

The gene frequency ranged from 0.083 to 1.00 and frequency of polymorphic loci ranged from 0 to 0.917. The highest gene frequency (1.00) i.e. the lowest frequency of polymorphic loci (0) was shown by the primer OPA-18 (at 400 and 240 bp), OPB-08 (at 740 bp), OPD-02 (at 34 bp), OPF-08 (at 1047 bp) and OPW-01 (at 673 and 521 bp). On the other hand the lowest gene frequency (0.083) i.e.

the highest frequency of polymorphic loci (0.917) was shown by the primer OPF-08 (at 658 bp).

The pair wise inter genotype similarity ranged from 61.59% to 93.55% with an average of 74.31%. The SVP-55 vs SVP-56 pair genotypes showed the highest similarity indices (93.55%) i.e. the lowest genetic distance. The lowest inter-genotype similarity indices (61.59%) i.e. the highest genetic distance was detected between Asterix vs SIP-3 genotype pair.

The Nei's (1972) genetic identity among 12 potato genotypes detected through six primers ranged from 0.5370 to 0.9074. The highest Nei's (1972) genetic identity (0.9074) was observed in SVP-55 vs SVP-56 and the lowest genetic identity (0.5370) was estimated in SVP-68 vs SVP-6 genotype pairs. On the other hand, the Nei's (1972) genetic distance among 12 potato genotypes ranged from 0.0972 to 0.6217. The highest Nei's genetic distance (0.6217) was observed in SVP-6 vs SVP-68 genotype pair, whereas the lowest genetic distance (0.0972) was estimated in SVP-55 vs SVP-56 cultivar pair.

Among the three popular cultivated varieties (Cardinal, Diamant and Asterix) Nei's (1972) genetic identity ranged from 0.6852 to 0.7778 and genetic distance ranged from 0.2513 to 0.3781. Cardinal vs Asterix varietal pair showed the highest Nei's (1972) genetic identity (0.7778) and the lowest genetic distance (0.2513). Diamant vs Asterix varietal pair showed the lowest genetic identity (0.6852) and the highest Nei's genetic distance (0.3781).

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithmetic Mean (UPGMA) segregated the 12 genotypes of potato into two main clusters. The first major cluster had 8 genotypes i.e. Cardinal, SVP-68, SIP-3, Diamant, Asterix, SIP-5, SVP-55 and SVP-56. The second major cluster had 4 genotypes i.e. SVP-6, SVP-18, SVP-19 and SVP-25.

This study revealed a sufficient amount of relationship and diversity among the studied 12 genotypes. It also indicated that, the RAPD method could be used as an effective tool for DNA fingerprinting and molecular diversity analysis of

various types of potato cultivars and newly created somaclonal variants. However, larger number of genotypes and higher number of primers would be necessary to construct an appropriate relationship and diversity but present type of study is widely acceptable in all concerns. The present study can be used as a guideline for future fingerprinting and genetic diversity study of potato varieties.

This study suggested some points for future consideration while starting DNA fingerprinting and diversity analysis in potato varieties and its somaclonal variants:

1. To obtain more precious result large number of RAPD primers are needed to be amplified.
2. Large number of varieties and their variant genotypes should be studied.
3. The genotypes and primers can be used in replication method.
4. Other molecular markers such as SSR, AFLP, SNP etc. should be used for diversity analysis in potato

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