MOLECULAR CHARACTERIZATION OF STRAWBERRY BY APPLYING DNA FINGERPRINTING TECHNIQUE USING SIMPLE SEQUENCE REPEATS (SSRs) MARKERS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MAHER DERNAIKA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

DECEMBER 2009

Approval of the thesis:

MOLECULAR CHARACTERIZATION OF STRAWBERRY BY APPLYING DNA FINGERPRINTING TECHNIQUE USING SIMPLE SEQUENCE REPEATS (SSRs) MARKERS

Submitted by **MAHER DERNAIKA** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İnci Eroğlu _____
Head of Department, **Biotechnology**

Prof. Dr. Fatih Yıldız _____
Supervisor, **Food Engineering Dept., METU**

Assist. Prof. Dr. Tülin Yanık _____
Co-Supervisor, **Biology Dept., METU**

Examining Committee Members:

Prof. Dr. Ufuk Gündüz _____
Biology Dept., METU

Prof. Dr. Fatih Yıldız _____
Food Engineering Dept., METU

Prof. Dr. Yavuz Beyatlı _____
Biology Dept., Gazi University

Assoc. Prof. Dr. Candan Gürakan _____
Food Engineering Dept., METU

Assist. Prof. Dr. Tülin Yanık _____
Biology Dept., METU

Date: December 2, 2009

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Maher Dernaika

Signature :

ABSTRACT

MOLECULAR CHARACTERIZATION OF STRAWBERRY BY APPLYING DNA FINGERPRINTING TECHNIQUE USING SIMPLE SEQUENCE REPEATS (SSRs) MARKERS

Dernaika, Maher
M.S., Department of Biotechnology
Supervisor: Prof. Dr. Fatih Yıldız
Co-Supervisor : Assist. Prof. Dr. Tülin Yanık

December 2009, 105 pages

In this study, strawberry fruit was taken as the studied model. An attempt was carried on trying to identify a unique DNA fingerprint in each of the selected different strawberry cultivars of *Fragaria x ananassa* Duch species available in Turkey. The basis of the study was to examine the fruit characteristics at the molecular level rather than at the morphological level. It is of great importance to differentiate and trace the origin of any variety by examining its DNA by using a very sophisticated molecular technique. In this case, DNA fingerprinting technique depending on the Simple Sequence Repeats (SSRs) markers which are also called Microsatellite markers were used. DNA fingerprinting technique reveals the specific DNA profile which is unique

as a fingerprint for a fruit specimen and this DNA profile is the same and constant throughout different parts of the fruit as well as its developmental stages. In this thesis work, nine primers flanking the SSR markers already available in the online databases were designed hoping to detect SSRs that could differentiate among the five selected cultivars of strawberry.

Keywords: DNA Fingerprinting, Microsatellites, Simple Sequence Repeat (SSR), DNA Molecular Markers, *Fragaria x ananassa* Duch.

ÖZ

BASİT DİZİ TEKRARI (SSRs) MAKÖRLERİ KULLANILARAK DNA PARMAKİZİ YÖNTEMİ İLE ÇİLEK ÇEŞİTLERİNİN MOLEKÜLER DÜZEYDE KARAKTERİZE EDİLMESİ

Dernaika, Maher
Yüksek Lisans, Biyoteknoloji Bölümü
Tez Danışmanı: Prof. Dr. Fatih Yıldız
Yardımcı Tez Danışmanı: Y. Doç. Dr. Tülin Yanık

Aralık 2009, 105 sayfa

Bu araştırmada Çilek meyvesi model olarak araştırılmıştır. Özel bir DNA parmakizi yöntemi ile Türkiye'de yetiştirilen seçilmiş çilek (*Fragaria x ananassa* Duch) çeşitleri, tanımlanmaya çalışılmıştır. Meyve karekteristiklerinin moleküler düzeyde belirlenmesi esas alınmıştır, morfolojik düzeyde değil. İleri düzeyde moleküler teknikler ve DNA araştırmaları ile cins farklılıklarının ortaya konması önemlidir. Araştırmada, DNA parmakizi tekniği, basit dizi tekrarı analizi (SSR), (mikrouydu markör analizi olarakta isinlendirilir) kullanılarak yapılmıştır. DNA parmakizi tekniği özel bir DNA profile vermektedir. Bu profil meyve örneğine özgün ve bitkilerde her organda aynı ve gelişme aşamalarındada değişmemektedir. Bu araştırma tezinde 9 primer tasarlanmıştır. Primerler mevcut sürekli yayınlanan databankalarında

bulunan, basit dizi tekrarı (SSR) noktalarının öncesi ve sonrasını belirleyen bir şekilde tasarlanmıştır. Bu ise seçilmiş çilek çeşitlerinin tanımlanmasında yardımcı olacağı düşünülmüştür.

Anahtar Kelimeler: DNA parmakizi, , Basit dizi tekrarı (SSR), Mikrouydu, DNA moleküler markörleri, *Fragaria x ananassa* Duch.

To My Dearest Mother…

ACKNOWLEDGEMENTS

First of all, I would like to express my unlimited thanks and gratitude to my supervisor Prof. Dr. Fatih Yıldlz who supported me greatly during my thesis study and for his indispensable guidance and unconditional patience. I owe him my sincere appreciation for being so kind and helpful as well as being so tender that I never felt myself as a foreigner away from my home country.

I would like also to thank all the jury members for accepting my humble invitation to the thesis jury and of course it is a great honor for me to present my study in their presence.

Very much thanks and gratitude full of kisses and hugs for my father and my mother who were with me all the time following up all my progress step by step, remotely supporting me by all means, and significantly encouraging me to reach my goal and accomplish my mission which I was sent for. To them I say, whatever I do, I can not pay back even a small portion from what you have done for me. I dedicate this study for you and I know that you are happier than me to see me finishing up my thesis. I am really proud of being a son of a wonderful parent. I also have to gratefully thank my brothers Mouzaffar and Moustafa and their families for being beside me all the way long supporting and encouraging me by all necessary means.

Wassim and Zeynep Rahim, two names that I will never ever forget in my life. I thank you from the deep of my heart for being beside me

whenever I needed you. My years in Turkey would not be as nice, safe, lovely, and spectacular as it is if they were not in it. They encouraged me and shared with me everything shareable until I became one of their family members. I wish you a descent life full of happiness and love with your little cutie baby Hana and the hopefully upcoming ones.

I also thank Dr. M. Emin Ergün and Dr. Burhan Erenoğlu from Atatürk Central Horticultural Research Institute Yalova/Turkey for their help and support in providing strawberry samples. I would like to thank Assoc. Prof. Dr. Candan Gürakan as well for the usage of her laboratory under the supervision of Houman Farhoud whose comments and suggestions were very much useful and appreciated. Also, I would like to thank Dr. Gökhan Duruksu who helped me a lot at the beginning of my study and his notes and remarks were more than important. I would like also to thank Fatma Coşkun and Derya Bahadırlı who were very kind and helpful in the laboratory during my study.

I owe much thanks to Prof. Dr. Mahinur Akkaya and Assist Prof. Dr. A.Elif Erson from whom I learnt vital issues considering my study as well as laboratory skills. In addition, I would like to thank Mohammed Alzamil and Wissam Abdallah for their encouragement and support throughout my study. Also, I profoundly thank Tülin Ateşoğlu and her beloved family for their kindness, love and caring through my years in Turkey. I also would like to thank all those who wished me luck and waited me eagerly to finish my master study. I wish I could mention all their names.

This study was supported by Research Fund Project of Middle East Technical University (BAP).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SSR: Simple Sequence Repeats

DNA: Deoxyribonucleic Acid

LOD: Logarithm of the Odds

m.u: map unit

MAS: Marker-Assisted Selection

PCR: Polymerase Chain Reaction

RFLP: Restriction Fragment Length Polymorphism

VNTR: Variable Number Tandem Repeats

RAPD: Randomly Amplified Polymorphic DNA

AFLP: Amplified Fragment Length Polymorphism

STS: Sequence Tagged Sites

SNP: Single Nucleotide Polymorphism

SCAR: Sequence-Characterized Amplified region

CAPS: Cleaved Amplified Polymorphic Sequence

SSCP: Single Strand Conformational Polymorphism

SSAP (or S-SAP): Sequence-Specific Amplification Polymorphism

ISSR: Inter Simple Sequence Repeats

bp: base pair

LTR: Long Terminal Repeat

GDR: Genome Database for Rosaceae

LB: Lysogeny Broth

DIG: Dioxigenine

YAC: Yeast Artificial Chromosome

BAC: Bacterial Artificial Chromosome

QTL: Quantitative Trait Loci

^{o}C: Degree Celcius

SDS: Sodium Dodecyl Sulfate

CTAB: Cetyl TrimethylAmmonium Bromide

V: Volt

UV: Ultra Violet

g: gram

ml: milliliter

µl: microliter

EDTA: Ethylene Dinitrilo Tetra Acetic acid

TE: Tris-EDTA

RNase: Ribonuclease

EST: Expressed Sequence Tags

Tm: melting Temperature

IDT: Integrated DNA Technologies

OD: Optical Density

M.M: Master Mix

min: minute

Kb: Kilobase

TBE: Tris-Borate-EDTA

EtBr: Ethidium Bromide

CHAPTER 1

INTRODUCTION

The aim of this study was the trial to differentiate among five different varieties of garden strawberry (*Fargaria x ananassa* Duch.) in Turkey by applying DNA fingerprinting technique depending on a very informative molecular marker which is simple sequence repeats (SSR).

Strawberry has been selected for such study due to many reasons. Strawberry is a unique species in the Rosaceae family because it is a perennial plant that grows very quickly, possesses a small genome, short reproductive cycle, and simplistic vegetative and reproducible propagation for genetic transformation. Moreover, it can grow in different types of weathers so that commercial production for fresh fruit market and processing industry has enlarged considerably enough to be ranked second in production among the berries species. Recently, genetic engineering and biotechnology applications are being employed in order to make a real improvement in not only strawberry qualities, but also to increase the breeding base and enhance the germplasm exploitation [1].

Biotechnology has been playing a very useful and indispensible role in many fields by now. In plants, the advent of molecular markers has greatly aided researchers to look at genetic applications from different angle and incredibly to assist the improvement of the conventional plant breeding programs. Besides, the usage of molecular markers has been

widely used in DNA fingerprinting and genetic diversity as well as in many applications of population genetics. From all the molecular markers available, SSR markers have been selected to differentiate among varieties of strawberry because SSR has become the marker of choice for fingerprinting purposes in most living organisms [2]. SSR markers are ideal in such studies because these markers are co-dominant, highly variable, very informative, and show high reproducibility of genotyping results [3].

CHAPTER 2

BACKGROUND

2.1 DNA Fingerprinting

As it is known, DNA is the sole genetic material in the living cells. The changes in the genetic characteristics are due to the sequential differences in the nucleotides of DNA. Even a tiny change in only one of the nucleotides may interrupt the sequential series of the arrangement and render it totally different. Even though the chemical structure of DNA is the same in all living organisms, the order of the nucleotides in DNA is the ultimate reason for the differences among them. By pointing out specific differences at certain sites in the genome among individuals, a unique DNA sequence called DNA fingerprint can be identified.

DNA fingerprinting is also famous as DNA typing or genetic fingerprinting. DNA fingerprinting is a method applied to identify individuals by their DNA structure [4]. DNA fingerprinting does not depend on morphological traits like in the usual fingerprinting in humans, but instead reveals the identity of an organism at the molecular level. This technique is about finding the genetic identity. This is done by studying the polymorphisms occurring at the molecular level in the base sequences of the genome. The basics of this genetic fingerprinting were discovered unexpectedly in 1984 by the geneticist Alec J. Jeffreys [5]. DNA fingerprinting was initially developed as a genetic mapping approach

and then many different applications like forensic casework and wildlife conservation were quickly found and introduced [6]. DNA fingerprinting is repeatedly used for genetic diversity studies as well as for relatedness among a wide range of organisms [7]. Moreover, DNA fingerprinting techniques show a huge potential as a tool for a broad spectrum in plant and fungal research areas, including genotype identification, population genetics, taxonomy, plant breeding, and diagnostics of plant, animal, and human pathogenic fungi [8].

DNA is a very long double-helix chemical strand contains specific essential regions called genes which are responsible for encoding functional proteins and in between there are bigger regions that do not encode proteins and called noncoding DNA or introns. By time, introns are more likely to accumulate changes. So, these regions are very much variable and are conventionally studied for distinguishing individuals.

Generally, DNA fingerprints are generally acquired by either of two strategies:

1. Classical hybridization-based fingerprinting which includes cutting of genomic DNA with a restriction enzyme and then electrophoretic separation of the obtained DNA fragments according to size; and finally the detection of polymorphic multilocus banding models by hybridization with a labeled probe.
2. PCR-based fingerprinting involves the *in vitro* amplification of specific DNA sequences with the usage of specifically or arbitrarily chosen short oligonucleotides called primers and a thermostable DNA polymerase. After this, the amplified fragments are separated

electrophoretically and then the detection of polymorphic bands is done by staining methods [8].

DNA fingerprinting depends profoundly on the existence of polymorphic sites in the genome. Polymorphisms are differences between individuals that occur in DNA sequences at the same locus in the chromosome. An individual may have only one sequence at a specific polymorphic locus in each chromosome, but if the population contains several to many different possible sequences at the same specific site, then the locus is considered "highly variable" within the population. DNA profiling finds out what polymorphisms a person possesses from the highly variable loci. Therefore, DNA profiling can be a powerful tool in distinguishing different individuals [4]. At the Molecular level, polymorphism can be classified into two categories: (1) sequence polymorphism (including nucleotide substitutions and insertions-deletions), and (2) number of tandemly repeated sequences in the repeated regions [9]. These informative polymorphic sites, that are conserved through time and influenced by the evolutionary processes, can be addressed as molecular markers.

2.2 Molecular Markers

Terminologically, there are three genetic markers: morphological markers, molecular markers (at DNA level), and biochemical markers (izozymes, proteins). A genetic marker is considered as ideal when possesses the following characteristics:

- Polymorphic.
- Multiallelic.
- Codominant: a heterozygous hybrid which at the same time expresses the traits of the homozygous parents; the heterozygotes can be differentiated from each of the homozygotes.
- Non-epistatic: the genotype can be identified from the phenotype directly, whatever the genotype at other loci may be. Codominance and non-epistasis can be described respectively as the absence of intra- and inter- locus interactions.
- Neutral: there are no phenotypic effects when there is an allelic replacement at the marker locus. Almost all molecular polymorphisms are neutral.
- Insensitive to environment: the genotype can be known directly from the phenotype whatever the environment is.

Morphological markers do not sufficiently meet these characteristics. They are not polymorphic enough and are generally dominant. Besides, they usually obstruct with other traits and can also be affected by the environment. But on the other hand, most biochemical and molecular markers have all these qualities. The major constraint of izozymes, which

are biochemical markers, is the small number of loci that can be detected. Markers at the DNA level are almost countless in number and are independent of the stage or organ examined, since DNA is the same in all tissues. Moreover, molecular markers are more advantageous because they can be directly used in molecular biology applications [9].

Plant breeders do their best to enhance and improve cultivars by crossing with other lines to acquire them new and beneficial traits. Normally, conventional breeding programs are very laborious and time consuming because it needs several crosses accompanied with long waiting time for new generations as well as the cautious selection of the desired phenotypes. Although recombinant DNA technologies and genetic engineering can be useful to overcome some limitations, they are still limited due to the shortage number of cloned genes and also due to the lack of standard transformation protocols in lots of crop species. In addition, genetic engineering procedures are very hard to be applied on polygenic traits. But with DNA molecular marker technology, there are some molecular breeding strategies that can be a solution for the many problems aroused by conventional breeding [10].

DNA molecular markers are used as an implement in order to study linkage analysis and to construct molecular linkage maps.

2.3 Linkage analysis

In genome, there are many genes scattered on different chromosomes. Some of the genes are inherited together and others are not. When two genes are inherited together, then they are linked. Linkage analysis is

the study of the linkage relation between genes [11]. Linkage can be occurred among genes or between a gene and a molecular marker. Genetic linkage analysis is a statistical method that is used to correlate the function of the genes to their location on chromosomes. The main idea is that markers which are found on the chromosome near genes have the tendency to bond together when passed on to offsprings. For example, if a disease is frequently passed to offsprings along with specific markers, then it can be concluded that the gene or the genes causing the disease are located close to these markers on the chromosome [12].

During meiosis, the homologous chromosomes are oriented in a way that they face each other and recombination process (crossing over) takes place as shown in the figure below.

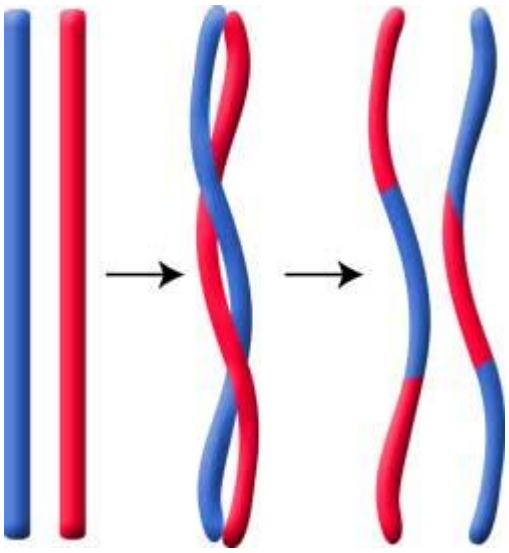

Figure 1 The crossing over of genetic material between two homologous chromosomes during meiosis [16]

Because of crossing over, alleles presented at the same chromosome may be separated and hence can be inherited by different daughter cells. When these two alleles are far away from each other, the probability of being separated is high. On the other hand, when they are close enough to each other, they are tending to be linked and inherited together [13]. Actually, there is a statistical estimation of the distance between any two studied loci called logarithm of the odds score (LOD score). If LOD score is 3 or more, then the two loci are close to each other on the chromosome and are likely to be linked and inherited together [11].The study of linkage analysis between genes and molecular markers is very useful to determine their relative positions on chromosomes [14]. For example, if we are searching for a disease gene in the genome, we apply linkage analysis. So, we try to find its location by finding an already known position of a molecular marker linked to that gene [15]. Genes which cause diseases are mapped by measuring recombination with respect to many different markers spread over the entire genome. In most of the cases, there will be many recombinations indicating that the disease gene and the marker are far apart from each other. However, some markers which are close enough do not recombine with the disease gene but stick to it and they are linked to each other. In best cases, two or more markers which flank the disease gene are identified and hence define a candidate region of the genome between 1 and 5 million bp in length. The gene responsible for the disease lies somewhere in this region confined by the identified markers [15].

2.4 Linkage mapping

Linkage maps demonstrate both the order and the relative positions of the genes on chromosomes. Linkage maps construction depends on linkage analysis and hence depends greatly on the crossing over event taking place during meiosis. The recombinant chromosomes, which are new combination of the origin chromosomes, let it possible for some traits to be inherited together and hence their genes are linked. So, by studying the traits through generations and recognizing the frequency of recombinants, we can map the relative position among genes [16]. When the number of recombinants occurring is studied, it is possible to determine the distance between the genes. This distance is called a genetic map unit (m.u.), or a centimorgan and is defined as the distance between genes for which one product of meiosis in 100 is recombinant. A recombinant frequency (RF) of 1 % is equivalent to 1 m.u. A linkage map is constructed by measuring the map distances between a number of traits presented on the same chromosome avoiding the significant gaps between the traits in order to be away from the inaccuracies that may occur because of the multiple recombination events [13]. By the same token, recombination frequency is the key process that can show us the relative positions of genes and/or molecular markers in linkage maps. By using linkage maps, researchers can locate different markers and define genes that are linked to already known molecular markers [13]. Also, linkage maps make it possible to understand evolution, synteny, and selection response [12]. In linkage mapping, mapping population should be acquired. A condensed map is only obtained when 10,000 markers are

identified on the chromosome and the distances between them are known. When a condensed map is obtained, it can be used in map-based cloning as well as in marker-assisted selection (MAS). A scheme of a linkage map is illustrated in figure 2.

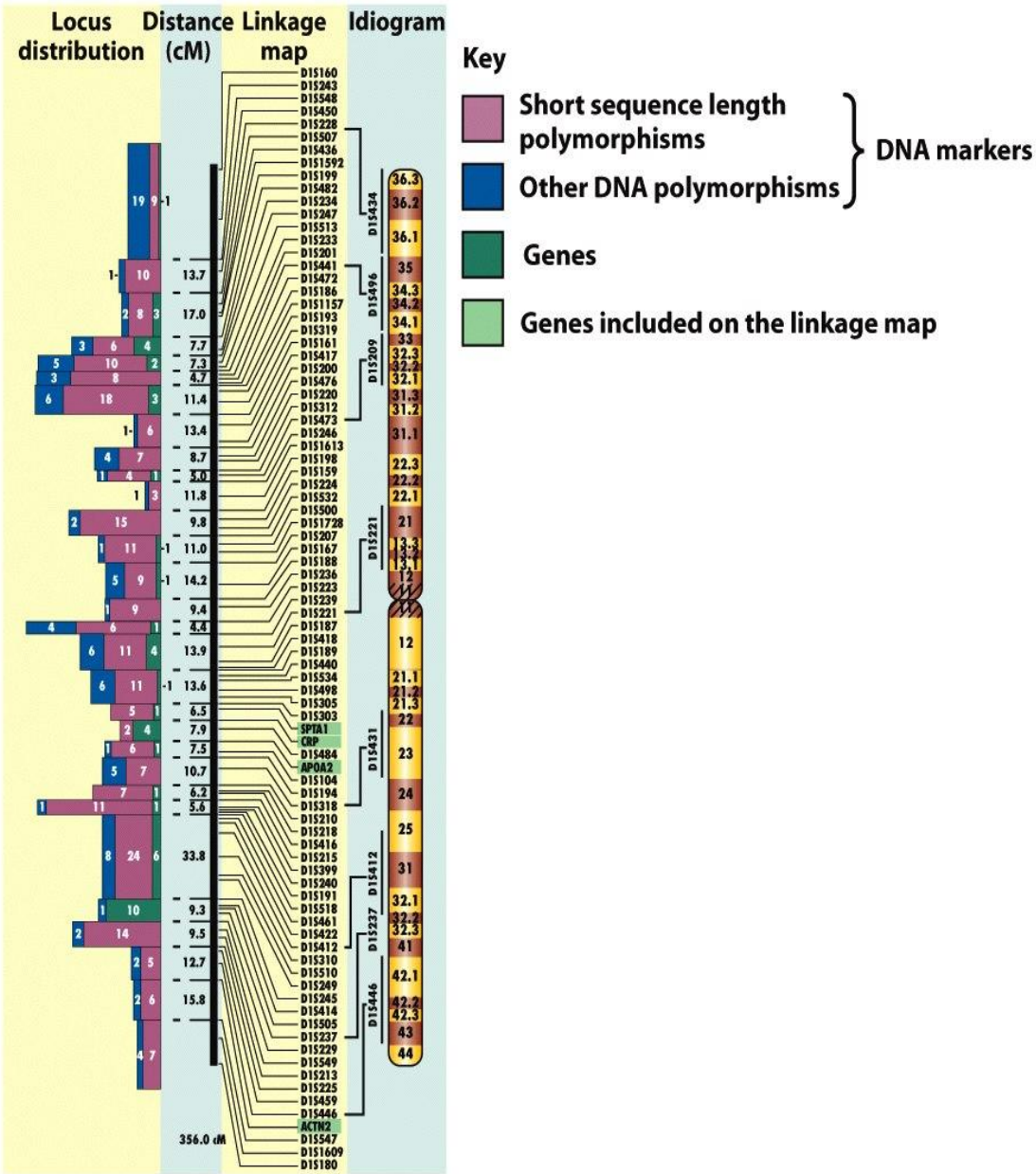

Figure 4-20
Introduction to Genetic Analysis, Ninth Edition
© 2008 W. H. Freeman and Company

Figure 2 Scheme of a detailed Linkage Map including genes and DNA markers and their distribution on the chromosome

2.5. DNA-Based Molecular Markers' Types

There are different types of molecular markers which are used to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In hybridization-based, DNA banding profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. PCR-based markers depends on the amplification of a particular DNA sequence or locus by a chosen oligonucleotide sequences called primers and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and then the banding patterns can be detected by different methods such as staining and autoradiography [18].

Hybridization-based markers are:

- RFLP: Restriction Fragment Length Polymorphism
- VNTR: Variable Number Tandem Repeats

PCR (Polymerase Chain Reaction)-based markers are:

- PCR-VNTR: Polymerase Chain Reaction-Variable Number Tandem Repeats
- RAPD: Randomly Amplified Polymorphic DNA
- AFLP: Amplified Fragment Length Polymorphism
- STS: Sequence Tagged Sites
- SNP: Single Nucleotide Polymorphism
- SCAR: Sequence-Characterized Amplified region
- CAPS or PCR-RFLP: Cleaved Amplified Polymorphic Sequence
- SSCP: Single Strand Conformational Profile (or Polymorphism)

- SSAP (or S-SAP): Sequence-Specific Amplification Polymorphism
- ISSR: Inter Simple Sequence Repeats
- SSR: Simple Sequence Repeats

2.5.1 Hybridization-based markers

2.5.1.1 Restriction Fragment Length Polymorphism (RFLP)

As the name implies, restriction enzymes are used to generate fragments of DNA. Restriction enzymes are enzymes that cleave DNA at a specific recognition site yielding DNA fragments differing in size [19]. RFLP is done by comparing the sizes of DNA fragments resulted from the cleavage of the DNA by the restriction enzymes. Actually, for two individuals, different DNA fragments can be resulted due to a change in one of the nucleotides that a restriction enzyme recognize that leads to an uncleaved fragment in one individual and a cleaved fragment in another. Firstly, isolation of a high quality DNA is achieved. Then, DNA is cut by a combination of restriction enzymes. After this, the digested DNA is fractionated by gel electrophoresis and then the fragments are transferred to a nitrocellulose membrane. Upon transformation, the DNA fragments are hybridized with radioactively labeled DNA probe sequences and then read by autoradiography [20].

It is worthy mentioning that RFLP needs a very high quality DNA to work. Besides, the development of the polymorphic probes is very expensive. Another drawback is that RFLP is relatively slow process. Due the usage of radioisotopes in this technique, only certified laboratories are allowed

to use it, but after the availability of the non-radioactive labeling systems, this technique is used more frequently. RFLP are often species-specific because it is a co-dominant marker [21].

2.5.1.2 Variable Number Tandem Repeats (VNTR)

In genome, there are sequences of repetitive DNA scattered along the DNA. These repetitive DNA may be up to more than 90% in some plant genomes. Repetitive DNA can be classified as interspersed or tandemly repeated. In interspersed repeats, the repeated DNA is located at different sites throughout the genome. While in tandem repeats, the repeated DNA is sequential consisting of two to several thousand basic motifs [8].

In VNTR, the tandemly repeated DNA is called minisatellites. Minisatellites are motifs that are 10 to 60 bp and show a lower degree of repetition at a given locus [8]. The generation of tandem repeats polymorphism is greatly believed to be done by unequal crossing over or by slippage during DNA polymerization process [22].

Actually, VNTR is a special case of RFLP. In VNTR, which is locus specific, none of the restriction sites that are cut by restriction enzymes is mutated and hence none of them is lost. In VNTR, the restriction sites are flanking the tandem repeated DNA sequences (minisatellites). So, due to the change of the number of repeats of the motifs, a change in the length of the tandem repeats is monitored. Hence, depending on the variation in tandem repeats, there will be multiple alleles that can be observed as different bands on gel electrophoresis [21].

As stated earlier, VNTR is a hybridization-based technique. So, like RFLP, VNTR is done by firstly isolating DNA from the organism of interest and then digested with suitable restriction enzymes. The restriction fragments are separated by gel electrophoresis and then blotted onto a membrane to be then hybridized with a labeled radioactive minisatellite probes. Finally, autoradiography is used for visualization [8].

It is worthy notifying here that VNTR technique can be PCR-based as well. In PCR-based VNTR, neither restriction enzymes nor hybridization with probes is done. Instead, the VNTR loci (minisatellites) are amplified by the usage of primers that corresponds to the both ends of the amplification region which is conserved. By the same token, the primers will be flanking the minisatellite region, and when the number of repeats of the minisatellite changes, the length of the amplified region changes which can be monitored on gel electrophoresis.

2.5.2 PCR-based markers

It is important before continuing any further to have a quick view at PCR. PCR is an indispensible method that can amplify any reasonable part of the DNA and have millions of copies of it in quite short time.

The polymerase chain reaction (PCR) is an *in vitro* method fantastically works on the amplification of DNA sequences by different automated cycles including steps of denaturation, annealing of primers and finally extension or synthesis of DNA fragments by using a thermocycler machine. PCR was invented and developed in the early 1980s by Kary Mullis, who was later awarded the Nobel Prize for Chemistry for the

invention in 1993. PCR permits the sensitive detection and analysis of very small quantities of nucleic acids. PCR has become a very famous and routine method in many diagnostic applications and has been very beneficial in the detection of infectious agents, mutations, translocations and gene amplifications, and also in molecular forensic applications including identity testing [23]. Moreover, PCR is widely used in molecular biology, microbiology, genetics, diagnostics, clinical laboratories, environmental science, hereditary studies, paternity testing, and many other applications [24]. The steps of PCR are shown in figure 3.

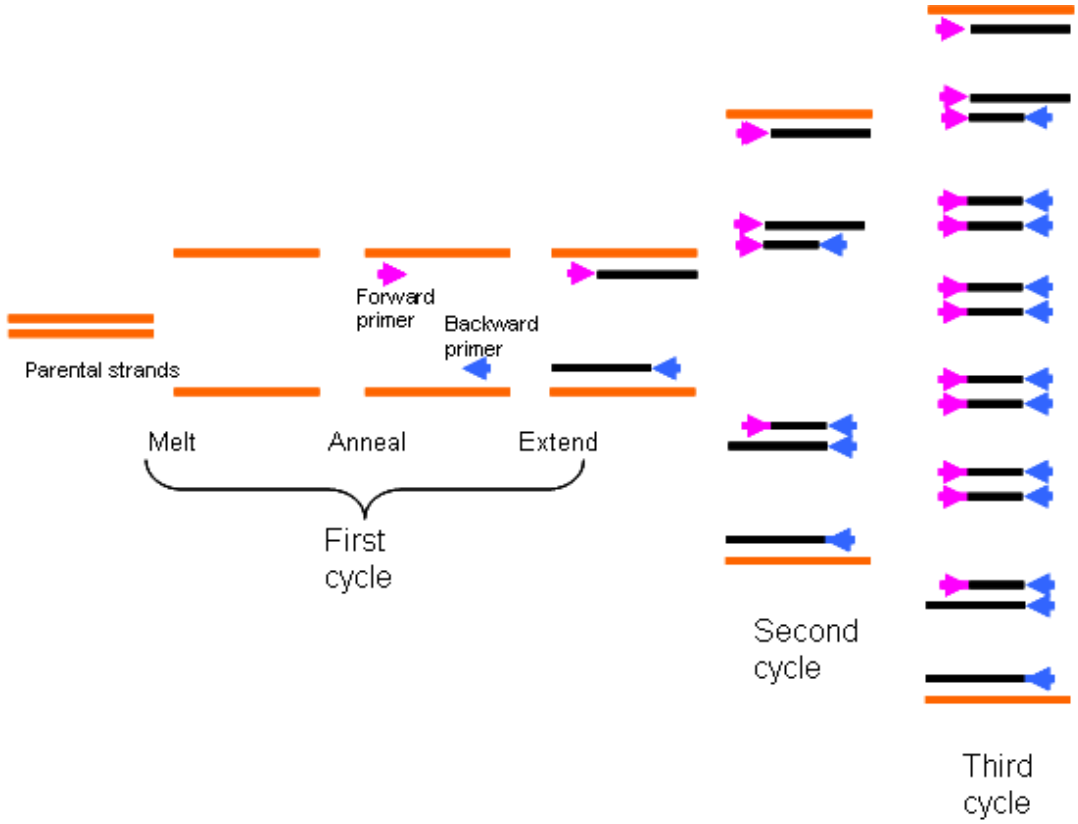

Figure 3 PCR technique starts with DNA and ends with many copies of the specific amplified fragment of DNA. For one cycle, the three main steps are melting DNA, annealing of primers, and extending DNA fragments by DNA polymerase. Generally, PCR is done for 30 cycles or so.

2.5.2.1 PCR-VNTR

PCR related VNTR was explained in the previous section *2.5.1.2*.

2.5.2.2 Randomly Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA is a technique that detects genomic DNA very quickly [25]. In RAPD, arbitrary, single, and short PCR primers are used in order to amplify DNA from total genomic DNA. Generally, the primers are 6 to 10 base pairs and hence the annealing temperature is low and around 37oC. The use of pure, high quality genomic DNA is critical for good RAPD analysis [26]. After PCR is done, different sizes of DNA fragments are obtained. The difference in DNA fragments between two samples is due to the changes that occur in DNA like insertions or deletions in the binding sites of primer, and in turn, polymorphism can be detected. Therefore, the absence or the presence of an amplification product let us differentiate between individuals [21]. RAPD has many advantages; they provide large numbers of markers and allow the detection of multi-locus genetic variation [27]. RAPD are fast, easy and cheap to perform and there is no need to know previous information about the genome under investigation [28]. Besides, it is not important to know the 5’ and the 3’ sequences of the designed primer. Moreover, there are already available commercial primers to use in RAPD technique [21].

However, some disadvantages of RAPD are studied and notified. RAPD are dominant markers so that it is usually can not distinguish

heterozygotes from their homozygous siblings [27]. Also, RAPD markers are anonymous markers and they are not very reproducible.

The usage of RAPD analysis in mapping and fingerprinting applications has been successfully done. In genetic mapping, the amplified products of RAPD that only pair with the segregating trait are informative. While in fingerprinting applications, RAPD is not informative when the difference (localized or dispersed) between two comparable genomes is limited to an extremely small genomic fraction [29].

2.5.2.3 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a DNA fingerprinting technique in which restriction fragments obtained by restriction enzymes can be detected and in this aspect it resembles the RFLP (restriction fragment length polymorphism) technique. However, there is a major difference which is the usage of PCR amplification instead of Southern hybridization for the detection of the restriction fragments [30]. This technique has three major steps: (1) cutting DNA with restriction enzymes and ligation of oligonucleotide adaptors, (2) amplification of certain restriction fragments by PCR, (3) studying gel of the produced fragments.

The detailed steps of this technique are as follows: first of all, isolated DNA is digested with a two restriction enzymes and then known adaptors are ligated to both ends of all the restriction fragments produced. Then, PCR is done by a specific primers correspond to the sequence of the adaptors and extended, toward the inside of the amplified fragment, at

the 3’ end by an arbitrary base. By doing this, only a subset of the restriction fragments is amplified. These fragments possess a base at the end that matches with the arbitrary base at the 3’ of the primers. After this, a second amplification is done but this time by designing primers that have two arbitrary bases at the 3’ end instead of one arbitrary base. Again, only fragments that have at the end complementary bases with the two arbitrary bases of the primers will be amplified. Actually, when only one arbitrary base is used, 1/16 of the total DNA fragments are amplified and when two arbitrary bases are used, 1/256 of the total DNA fragments are amplified and if three arbitrary bases are used, 1/4096 of the fragments are amplified. Finally, the amplified fragments are monitored on gel electrophoresis and up to 100 fragments can be detected. Moreover, interested bands can be cut out, cloned, and sequenced and then transformed into SCAR [9,28].

AFLP technique offers a very useful DNA fingerprinting technique when dealing with DNAs of any origin or complexity [30]. AFLP is very sensitive and requires lots of work as well as an expensive technique. In addition, the differentiation between homozygotes and heterozygotes needs band quantitation and also, the bands are very difficult to interpret because they are anonymous. In spite of all this, AFLP can efficiently amplify large numbers of fragments and both length and sequence polymorphisms can be detected reliably and reproducibly [21]. Furthermore, AFLP usage gives quick and reliable assays of 50 or more potentially polymorphic sites in a single experiment, and there is no need at all to have previous information of genome structure [31].

2.5.2.4 Sequence Tagged Sites (STS)

STS is a short DNA sequence which is approximately 200 to 500 base pairs. STS occurs one time in the genome and whose both location and sequence are identified. By running PCR with specific primers, STS can be easily detected [32]. STS is a marker that is useful for genetic and physical mapping of genes. Anyone who wants to amplify a STS marker can pick it up from available databases and run PCR after designing appropriate primers. STS are co-dominant markers that it is possible to differentiate between homozygoutes and heterozygotes. Besides, STS produces patterns that are simple and reproducible while examining gels [33]. STS DNA sequence may contain repetitive elements. Whenever the sequences at both ends flanking the repeat elements are unique and conserved, researches can uniquely identify this site of genome by using commonly tools available in laboratories [33]. So, when STS contain these genetic polymorphisms, they become very valuable markers at loci that let it possible to differentiate and distinguish different individuals. Even though STS required a previous knowledge of the location and the base sequence, they are used in shotgun sequencing and added a great help in sequence assembly [32].

2.5.2.5 Single Nucleotide Polymorphism (SNP)

In human genome, SNPs are the majority type of DNA sequence variation. SNP is a site on the DNA in which a single base-pair differes from one person to another. When SNP is detected in a small and unique portion of DNA, it provides a physical landmark and a genetic marker whose transmission from parent to child can be monitored [34]. SNP is considered as the most common DNA marker that shows a variation in the human genome [35]. To be considered as SNP, it must occur in at least 1% in the population. SNP occurs every 300 to 500 base pairs and can exist in both coding and non-coding regions of DNA. Alleles of SNPs that are close together tend to be inherited together. A set of associated SNP alleles in a region of a chromosome is called a haplotype [36]. Haplotype is a group of single nucleotide polymorphisms (SNPs) on a single chromatid that are associated with guidance. These associations accompanied by the identification of several alleles of a haplotype block, can clearly identify all other polymorphic sites in its region. Such studies are very important while inspecting the genetics of some common diseases [37]. From haplotypes, tag SNPs can be obtained. Tag SNPs are representative of SNPs in a certain region of the genome. Identification of genetic differentiation can be done without genotyping every SNP in a chromosomal region. Tag SNPs are very useful in whole-genome SNP association studies in which genotyping of hundreds and thousands of SNPs in the genome can be identified [38]. Figure 4 shows the relation between SNPs, halotypes, and tag SNPs.

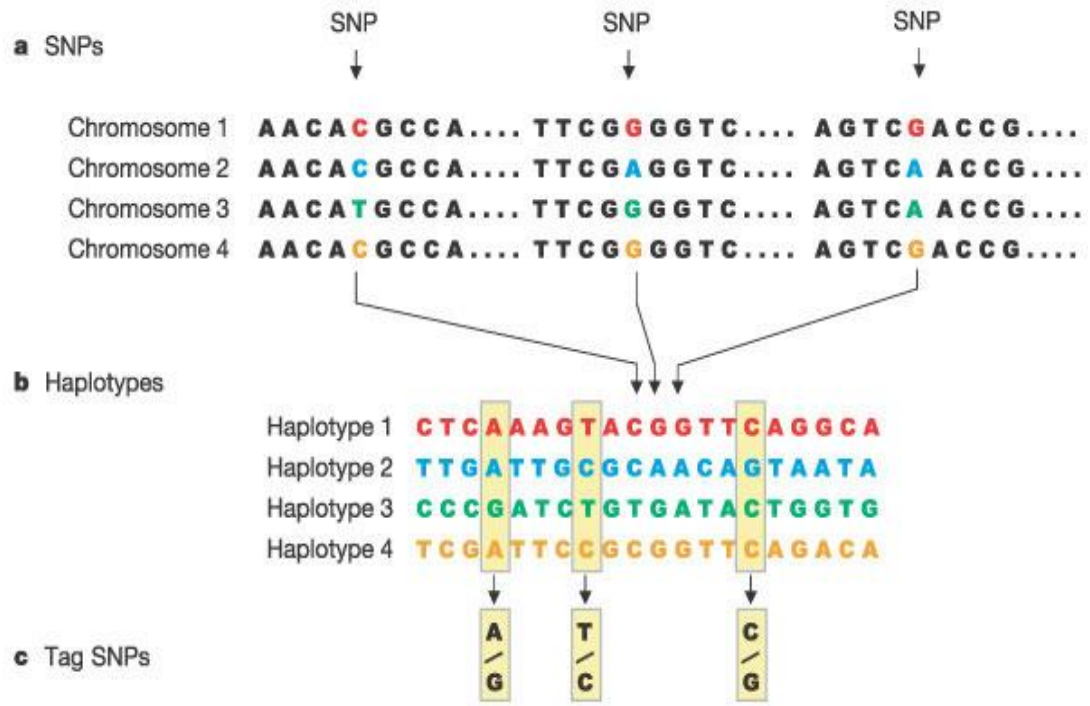

Figure 4 Unique SNPs in different chromosomes gathered together to form Haplotypes. By pointing out unique SNPs from different haplotypes, tag SNPs can be identified.

In the figure, it can be seen that there are four versions of the same chromosome region for four different people. Also, it can be noticed that there is a slight change among the four. Besides, it is obvious that each SNP has two alleles. In the second part of the figure, haplotypes are shown. In these haplotypes, there are 20 SNPs picked up from 6000 bases along DNA and brought close to each other including the three SNPs that are shown in the first part of the figure. In tag SNPs part, it is concluded that genotyping just the three tag SNPs from the 20 SNPs available is enough to identify the four haplotypes exclusively. For example, if a chromosome possesses the A–T–C pattern at these three

tag SNPs, it means that this chromosome matches the pattern determined for haplotype 1 [39].

Still for many crop plants there are not enough available validated SNP markers even though its crucial need in many studies regarding genetic variation, linkage mapping, population structure analysis, association genetics, map-based gene isolation, and plant breeding [40]. Finally, SNP has become a very promising and challenging field stimulating DNA technologists, population geneticists, and molecular genetics researchers that could bond them together than ever before [41].

2.5.2.6 Sequence-Characterized Amplified Region (SCAR)

SCARs markers correspond to one locus from the genetically defined loci that are produced by PCR with the use of specific oligonucleotide primers [42]. Actually, SCARs markers are derived from RAPD markers. When an informative RAPD marker is cloned and sequenced, it is not anonymous anymore. Then, specific PCR primers, usually 22 to 30 nucleotide long, are designed so that can amplify the already sequenced RAPD marker. Polymorphism is either maintained by the presence or the absence of the amplified bands or by the emergence of length polymorphisms which obtained by converting dominant RAPD loci into co-dominant SCAR markers [42]. The usage of long primers let SCARs markers to be more reproducible than RAPD markers. Besides, converting RAPDs into SCARs letting the dominant markers to be co-dominant markers and hence they are detectable in both homozygotes and heterozygotes [43].

SCAR is a useful technique for purity seed genetic testing as well as variety discrimination. Also, it seems that SCAR is applicable to species in which other techniques have failed. Particularly, SCAR could be very useful with species that were examined with RAPD and produced unreliable results [44].

2.5.2.7 Cleaved Amplified Polymorphic Sequence (CAPS)

Generally, CAPS are generated when a particular locus is amplified by specific primers or when an already amplified DNA fragments, that may be products of STS or SCAR, are digested with restriction enzymes and then the length polymorphism is studied by the gel electrophoresis analysis [45]. The same with RFLP, CAPS depends on the genetic differences between individuals according to the differences that may occur in the endonuclease restriction sites. These differences can be detected after digestion with restriction enzymes and studying the length of the DNA fragments [46].

The advantages of CAPS are:

- Most CAPS markers are co-dominant and locus-specific.
- Most CAPS genotypes are easily attained and clarified.
- CAPS markers can be simply shared among laboratories.
- There is no need to use radioactive isotopes in CAPS assays and hence it is agreeable to be performed in clinical settings analysis [47].

2.5.2.8 Single Strand Conformational Polymorphism (SSCP)

SSCP depends on the separation of single-stranded DNA electrophoretically. Even a small change in the sequences of the single-stranded DNA can result in a conformational change in their structure and hence a major difference in mobility through gel can be visualized [48]. When a single nucleotide is changed in a double stranded DNA, the variation cannot be distinguished by electrophoresis because the physical properties of the double strands are almost similar for both alleles. On the other hand, when DNA is denatured, the single stranded DNA undergoes a 3-dimensional folding and each strand acquired a unique conformational state based on its DNA sequence [49]. This change in the conformational state is enough to be detected as a shift in band migration. By doing so, the carriers of mutation can be easily detected because the mobility of the mutated and the normal strands is different. Besides, it is possible that some of the strands anneal together and form atypical bands called hetroduplex [50]. Single-stranded DNA is very flexible that is able to be arranged in a specific conformation due to the intramolecular interactions as well as base stacking depending solely on the sequence composition of DNA [51]. Figure 5 shows the detection of mutations by SSCP.

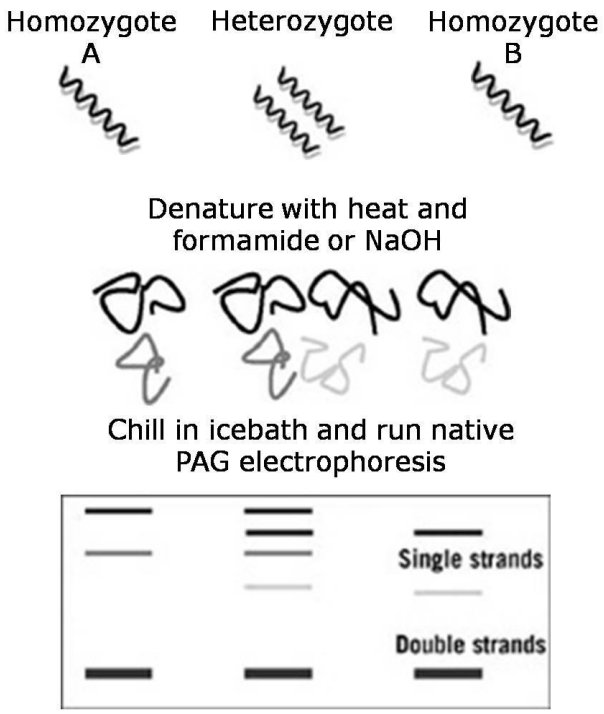

Figure 5 Mutation Detection with SSCP. When DNA is denatured, single stranded DNA are formed. The detection of mutation in the single strand DNA can be visualized by gel electrophoresis.

SSCP analysis includes several steps. Firstly, PCR amplification with primers flanking the mutated region [52]. The size of PCR fragments that can be studied are generally in the range of 175-250 bp. Optimization of the PCR reaction is important to decrease the unwanted products that may interfere with gel analysis [53]. Then, denaturation of the double stranded DNA by formamide and heat followed by chilling to avoid re-annealing of DNA strands. After that, separation of single-stranded DNA is done by running on non-denaturing acrylamide gels and from the DNA bands on the gel, comparison of the mobility is analyzed. Finally, a confirmation of mutation can be done by automated fluorescent DNA sequencing [52].

Actually, there is not much examples of SSCP done on plants for mapping or population genetics, but it is very useful and simple technique because it does not require digestion by restriction enzymes. It is also very useful for rapid screening of variants of amplification products when no information of substitutions is necessary [9].

2.5.2.9 Sequence-Specific Amplification Polymorphism (SSAP)

SSAP is a multiplex technique that utilizes the variation in the sequences flanking the sites of retrotransposons. It produces DNA amplified fragments that contain at one end retrotransposons long terminal repeat (LTR) and restriction site at the other end [54].

Retrotransposons are mobile gentic elements called transposable elements. Actually, transposable elements are divided into two groups depending on the transposition mechanism and the shape of propagation. The first group is retrotransposons, they are transposed by an RNA intermediate (can be converted to DNA by reverse transcription before reinsertion [55]). The second group is transposons which are transposed by excision and reintegration. The existence of these transposable elements along DNA make them likely useful as diagnostic tool that require plenty and reliable markers [56].

As mentioned earlier, SSAP produces DNA amplified fragments and hence the utility of PCR is crucial. First, genomic DNA is digested with restriction enzymes at the restriction sites and then an oligonucleotide adapter is added at the ends of the produced fragments. After that, by the usage of radioactively labeled LTR-specific primer and a primer

adaptor, SSAP bands are produced. It is worthy mentioning here that the specific primer amplifies only the outside region between the left LTR and the adjacent RE site. Such amplification can be obtained by inserting a selective base at the 3' end of the specific primer which is only compatible with the outside region of the left LTR, thus preventing the amplification of the transposon interior. See figure 6.

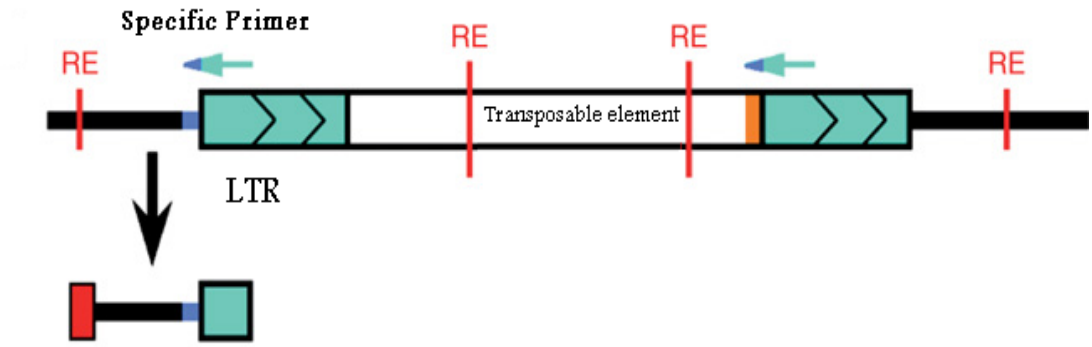

Figure 6 Digestion of DNA with restriction enzymes and the addition of oligonucleotide adapter at the end of the produced fragments. SSAP bands are produced by specific primers matching the LTR region and the adaptor added to the fragment.

After amplification by PCR, SSAP produced fragments that can be visualized as bands on sequencing gels or capillary electrophoresis so that a characteristic fingerprint of bands can be identified [57].

When SSAP is compared with traditional AFLP markers, SSAP shows higher polymorphism and displays better genome coverage due to the ubiquitous distribution, high copy number, and diverse genomic localization of retrotransposons in plant genomes. So far, S-SAP has been successfully implemented for genetic diversity and phylogeny analysis, and linkage mapping in many crops [58].

2.5.2.10 Simple Sequence Repeats (SSR)

Simple sequence repeats, which are also called microsatellites or short tandem repeats (STR), are tandem repeats of mono-, di-, tri-, tetra-, and penta- DNA nucleotide sequences that occur frequently along the genome. These simple sequences are repeated either few or several tens of times. As stated earlier in VNTR, these polymorphic repeats occurred due to unequal crossing over or due to slippage during DNA polymerization [22]. These simple DNA sequence repeats are generated through evolution and the sequences flanking the repeats are conserved through time. The co-dominant SSR markers are highly polymorphic, highly reproducible, abundantly present, and arbitrarily spread in the genome. In SSR, the differences in the number of the tandem repeats are the key in many genetics fields such as population genetics, genetic diversity, phylogenetic studies and others. Moreover, SSRs show high frequency of variation among different species, subspecies, and even among different varieties. So, SSR markers are very suitable to be applied in DNA fingerprinting and generate DNA fingerprints that can differentiate cultivars at the molecular level.

SSR technique produces amplified DNA fragments that vary in length among different samples. First of all, DNA isolation is done from different samples that are going to be differentiated by SSR markers. Then, amplification of the sequence repeats is commenced via PCR by using specific designed primers that are complementary to the conserved regions flanking the sequence repeats. Finally, gel electrophoresis is used to generate DNA bands that differ in length due to the difference in

numbers of the repeated sequences of the same locus in different samples and hence polymorphism is detected. See figure 7 below.

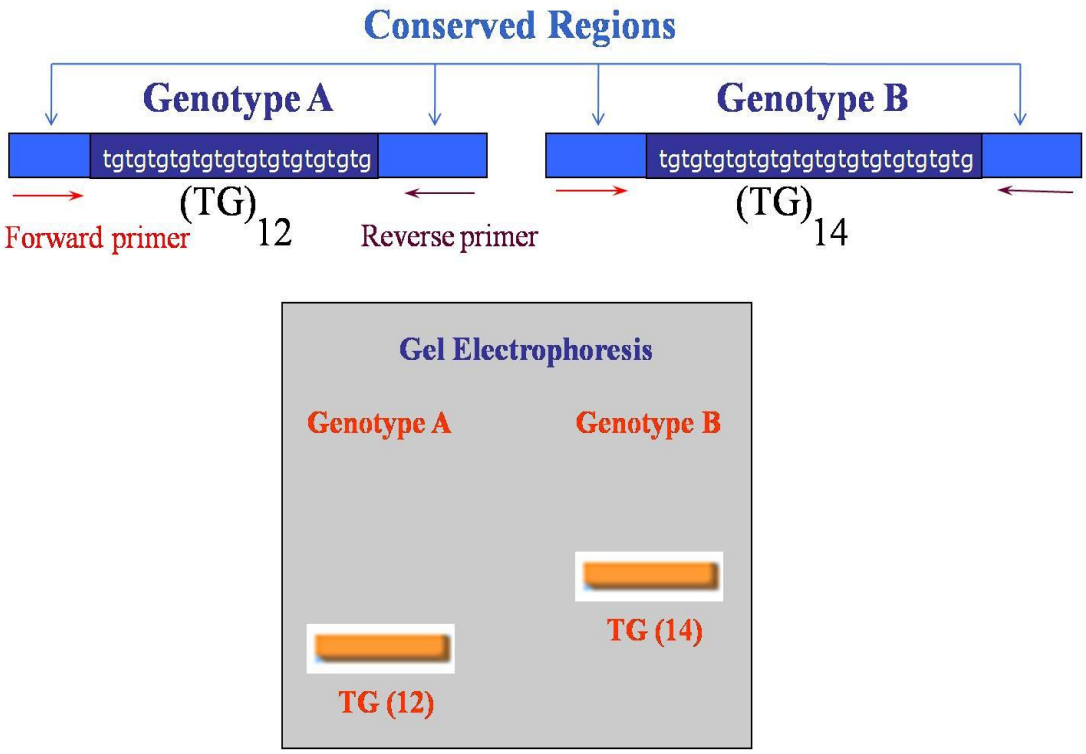

Figure 7 Two different SSR markers with different number of repeats can be detected by amplifying the SSR sites with primers matching the conserved region and the bands can be visualized on gel electrophoresis.

2.5.2.11 Inter Simple Sequence Repeats (ISSR)

ISSR is a technique based on the amplification of genomic fragments that are located between two SSR loci closely oriented to each other. This is accomplished by designing primers that are partly of an SSR sequence and partly of arbitrary 1-3 bases. Besides, the designed primers can be 5’ anchored or 3’ anchored with the arbitrary nucleotides [59,9]. As shown in figure 8 below, two amplified fragments can be obtained from two closely SSR when using 3’ and 5’ anchored primers.

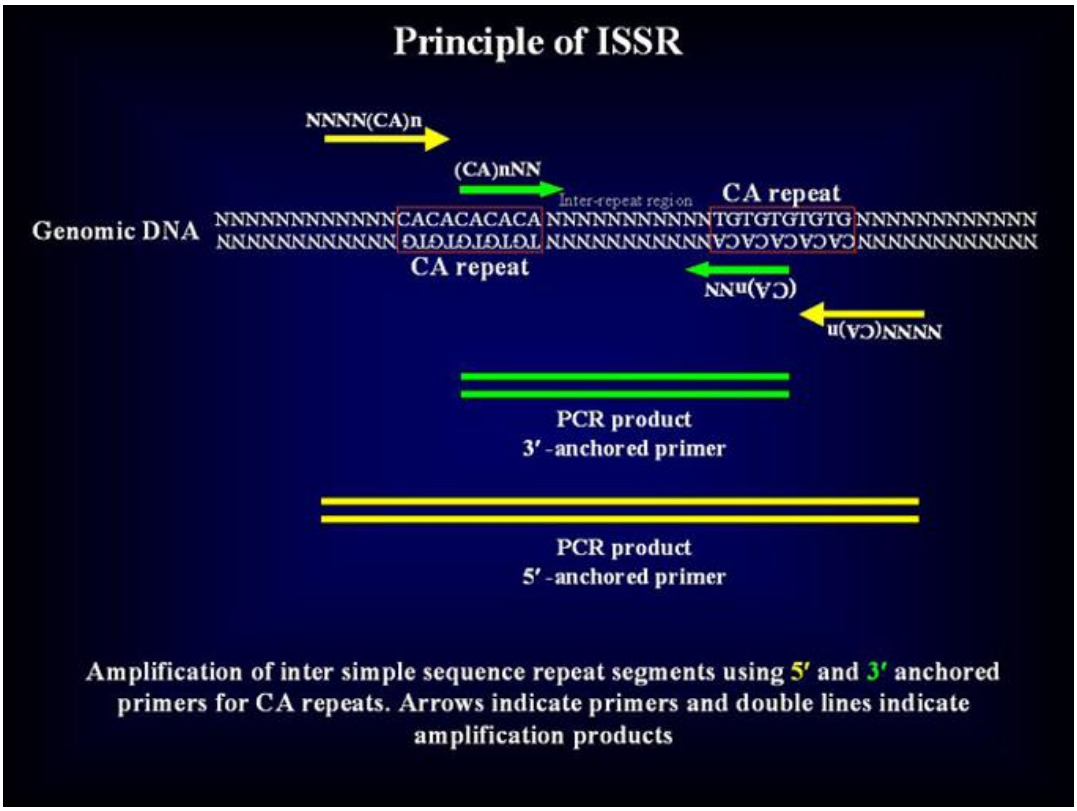

Figure 8 ISSR principle. Upon designing 5′ and 3′ anchored primers which include part of SSR sequence and 1 to 3 arbitrary bases, the site between two SSR markers can be amplified resulting in two products [61].

In ISSR method, there is no need for big quantity of DNA. ISSR are reproducible and highly polymorphic markers [60]. In addition, it is fast, non-costly, and requires no previous sequence information in similar to RAPD [59]. However, it is worthy to mention that ISSR are dominant markers and hence they are not as informative as SSR [60]. ISSR are actually sensitive that can differentiate closely related individuals as well as measure genetic diversity in germplasm [61].

2.6 Isolation of SSR Markers

Isolation and detection of SSR markers is a very laborious and tedious work. There are several techniques implemented for this purpose. By now, there are some established databases on the web in which isolated SSR markers are available for public. The available SSR markers databases are confined to certain families or specialized in certain species. For example, Genome Database for Rosaceae (GDR) is a big database that is specialized in Rosaceae family (http://www.bioinfo.wsu.edu/gdr/).

The isolation method of SSR markers includes several major steps as follows [21]:

- Genomic DNA isolation
- Fragmentation of DNA with restriction enzymes
- Construction of genomic DNA library
- Screening library with repeat DNA probes via colony hybridization
- Plasmid DNA isolation from clones containing putative markers
- Sequencing of the plasmid DNA
- Designing of PCR primers flanking the repeat region

There are many DNA isolation protocols available according to the organism of interest. Then, cutting DNA with restriction enzymes such as Sau3A-I, BamHI, HindIII is done preferably in several different tubes because sometimes too much DNA may be a reason for low digestion efficiency. After running the cut DNA on 1.5% gel electrophoresis, DNA fragments that range between 400 and 900 bases are isolated and

recovered on a membrane. After prospective DNA fragments are purified, they are ligated with cloning vectors such as pUC19 that are already digested with restriction enzymes. Then, the vectors with ligated DNA fragments are transformed into a competent cell and then plated onto Lysogeny broth (LB) petri dishes. The positive colonies are the ones that have the prospective DNA fragments insert. PCR tests can be done at this stage in order to estimate the size of the inserts. After the clones are pointed, they are transferred onto a membrane such as Hybond N+ nylon membranes. Then, hybridization of DNA fragments with labeled probes that consisted of different DNA repeats is done. The probes can be labeled with DIG (dioxigenine) oligonucleotide. Upon hybridization, screening for positive clones can be easily studied and identified. Then, plasmid DNA (vectors) are isolated from colonies, purified, and then sequenced. From the sequencing data, primers flanking the DNA repeats are designed and identified and then the amplification of the DNA repeat region is carried on and its size is shown. By this procedure the SSR markers are isolated and identified [21,62,63].

There are some other methods to isolate and identify SSR markers as well. For example, there is a method depends on the usage of AFLP markers. In this approach which can be applied for any organism, there is no need for the tedious library construction and usage of cloning, no need for screening of libraries, and no need for preservation of colonies. Instead, this technique consists of the combination of AFLP markers with an enrichment step which is done by using biotinylated target repeat oligonucleotide on the streptavidin coated magnetic beads [64].

2.7 Applications of molecular markers

The advent of molecular markers added a respectable aid and a high leap in the field of genetics. Different molecular markers show diverse optimum applications due to the characteristic differences among them. Molecular markers possess different techniques and produce different kinds of data. Even though one molecular marker is better than another in a certain subject, most of the time molecular markers produce accumulative and additive data that help a lot in elucidating some ambiguous patterns.

There are many outstanding applications that it is worthy mentioning them briefly. The usage of molecular markers in linkage analysis and in the construction of genetic maps let it possible to examine inheritance data for population, determine the relative distances among genes, and work on identifying certain genes. Another application is map-based or positional cloning, by which a gene with unknown product can be isolated from its position which is determined by linkage analysis. This is done by identifying two tightly linked markers to the gene of interest. Then the gene is identified by technique called chromosome landing and the usage of artificial chromosome vectors like yeast artificial chromosomes (YAC) and bacterial artificial chromosomes (BAC). Population genetics is one of the applications of molecular markers in which diversity, differentiation, and gene flow can be measured and studied. Also, quantitative genetics' applications were recharged by the advent of molecular markers. It is become possible to analyze genetic bases of morphological variations and to develop strategies for characterizing quantitative genes as in

quantitative trait loci (QTL). QTL is an area in DNA that is coupled with a phenotypic trait which its inheritance can be recognized due to the interactions between two or more genes and their environment. A very useful application is marker-assisted selection (MAS) which is a very useful tool for genetic improvement of plants. MAS is based on the identification and use of markers which are linked to the gene or genes controlling the trait of interest [9,65].

2.8 *Fragaria* (Strawberry)

Fragaria, commonly called strawberry, is a flowering plant and is a genus of the Rosaceae family. Strawberry is an accessory fruit that the red flesh is not developed from the ovaries (achenes) of the plant but from the bottom of the hypanthium which holds the ovaries confined in the pistil [66]. A hypanthium is a bowl-shaped part of a flower is composed of the bottoms of the sepals, petals, and stamens all stuck together [67]. So, the seeds embedded in the flesh are the actual fruits of the plant and the flesh itself is an enlarged modified receptacle tissue. With approximate same length, the leaves and the flowers of strawberry plant rise from nearly the ground. The flowering stems hold no leaves but flowers consisting of five white or pink petals, five sepals (two rings of five sepals form the green cap attached to the fruit), and numerous stamens. The leaves are divided into three oval toothed leaflets that are bright green from above and more pale with hairy makeup from below [68]. Below is a figure of different parts of strawberry plant.

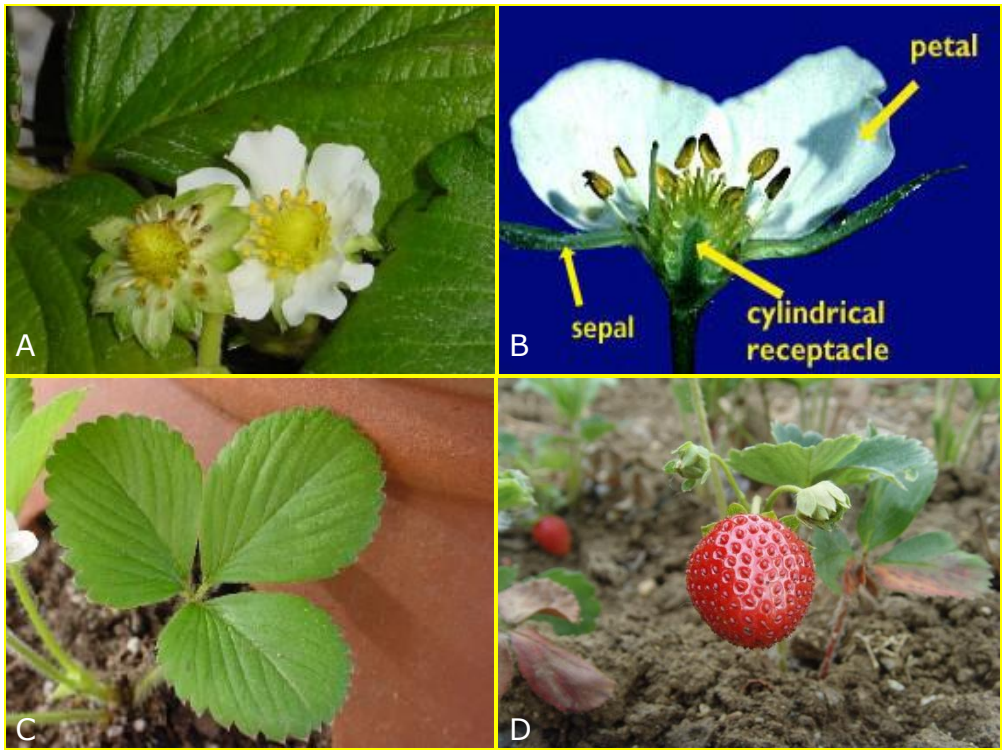

Figure 9 Different Parts of Strawberry Plant. A: Flower, B: Reproduction system, C: Leaves and D: Fruit.

Strawberries grow close to the ground and spread horizontally by propagating runners which are stolons having two nodes. The second node develops into a daughter strawberry plant [69].

Strawberries can be consumed either fresh or as an ingredient in different food preparations. It is identified that the cell wall contains pectic polysaccharides which is critical for cell to cell adhesion and provide mechanical strength [70]. Strawberries are very attractive for their physical appearance and for their physiological characteristics. Strawberries have bright colors, delicious taste, fine texture and fresh aroma. Also, strawberries have high economical and nutritional values such as essential minerals, organic acids, vitamins and antioxidant

properties [1]. Actually, strawberries contain much vitamin C and iron. Besides, strawberry fruit contains malic and citric acids, a volatile matter, sugar, mucilage, pectin, woody fibre and water [71]. A nice fact is that eating strawberry whitens the teeth. For some people eating strawberry may cause certain allergic reactions due to the little hairs on the fruit. This irritation can be avoided by rinsing strawberries by hot water before eating [72].

Generally, Fragaria consists of more than twenty species exhibiting different levels of polyploidy, having all a base chromosome number of seven [73]. Some species are diploid, having two sets of the seven chromosomes (14 chromosomes total). Others are tetraploid (four sets, 28 chromosomes total), hexaploid (six sets, 42 chromosomes total), octoploid (eight sets, 56 chromosomes total), or decaploid (ten sets, 70 chromosomes total) [74].

In this project, the focus is on the different and commercially important cultivars of the Fragaria x ananassa Duch species which is an octoploid and is commonly named garden strawberry. The Garden Strawberry was first bred in Brittany, France in 1740 by a cross between *Fragaria virginiana* from eastern North America and *Fragaria chiloensis* from Chile brought by Amédée-François Frézier, the former was noted for its flavor and the latter was noted for its large size [75]. *Fragaria x ananassa* is a very popular fruit crop and one of the most commonly cultivated around the globe. It is also a great source for macronutrients as well as a source for high levels of antioxidents [76,77]. *Fragaria x ananassa* is very easy to grow. It is perennial, winter hardy, and can survive in full sunshine whenever the soil is fertile and well drained. Healthy plants tends to

produce large amount of berries for three to four years, after which they should be replaced [78]. Strawberry cultivars can be divided into two groups; ever bearing or June bearing. Ever bearing are likely to produce throughout the growing season, whereas June bearing produce in spring and in the early summer. Besides, cultivars that are day neutral can produce fruit during the three months of planting in any season. As a fact, garden strawberry is the most cultivated fruit with the broadest distribution in the world. It can be grown in temperate and subtropical climates from Alaska to South America as well as in Europe, Asia, Africa, and Australia [79].

CHAPTER 3

LITERATURE REVIEW

Up to date, there have been many conducted works related to DNA fingerprinting in plants and especially by using simple sequence repeats (SSR) markers as the ultimate method. Typically, SSRs are greatly accepted as the markers of choice in many of the breeding programs because they are transferable, multi-allelic co-dominant markers, PCR-based, easily reproducible, randomly and widely scattered along the genome, and because their analysis may be automated [80].

Length polymorphism of SSR was detected in soybean. Forty three homozygous soybean genotypes were amplified at three loci and resulted in 6, 7, and 8 different alleles respectively. Besides, it is concluded that the longest repeat of CA was nine repeats and that CA/GT repeats are not polymorphic enough in soybean to be a an informative genetic marker. As for AT/TA repeats with a 15-repeats or more could be an alternative sequence repeats and an informative marker [81]. Based on the CA/GT repeats, it was concluded that these repeats occur one every 17.7 Kb in the genome of the alga *Chlamydomonas reinhardtii*. Upon amplification repeats region for four loci, it was shown that the four loci were highly polymorphic and they will be useful for identity testing, population studies, linkage analysis, and genome mapping in Chlamydomonas [82]. One study has succeeded in distinguishing twenty two mango cultivars by seven SSR markers with the use of SSR anchored

primers. After 40 primers usage, only seven primers produce reproducible and polymorphic bands. Then, a DNA fingerprinting table was constructed to distinguish among mango genotypes [83]. In another study, SSR polymorphism was investigated in 46 cultivars of olive tree (*Olea Europaea L.*). For five loci, sum of 26 alleles were spotted. 90% of all the cultivars had showed unique multi locus genotypes. Finally, it was confirmed that such SSR markers are very useful for cultivars identification [80]. SSR markers were used also to discriminate among durum wheat cultivars. Seven SSR markers were used to differentiate among 16 cultivars. The genotypes were distinguished by different alleles ranging from five to thirteen. At the end, it was shown that only three SSR markers were enough to differentiate among the sixteen durum wheat cultivars [84]. One of the studies worked on differentiating fifty cultivars of peach (*Prunus persica L. Batsch*) by using 26 SSR markers that have been isolated and sequenced by the same researchers. After carrying on 1300 PCR, they have succeeded to obtain expected products for all of the samples but two. All the SSR markers were polymorphic and produce different alleles per locus ranging from two to eight [85]. Genotyping of Anatolian doubled-haploid durum lines were done with SSR markers. In this study, ten highly polymorphic SSR markers were selected for differentiating and evaluating genetic relationships between and within the doubled-haploid lines. From the fifteen SSR markers started at the beginning, five of them produced monomorphic bands so that they were excluded [86]. Another study was conducted on 48 apricot (*Prunus armeniaca* L.) genotypes by using 37 SSR primers in order to screen relationships among them and characterize the different

genotypes. 31 of the SSR produced correct amplification and 20 of them produced polymorphic bands of all the 48 genotypes. From this study, 82 alleles were detected from the 20 loci. By combining the information obtained from the SSR markers, all the 48 genotypes were distinguished. It is emphasized that SSR markers are highly conserved and the available sequences of apricot will be useful for detection of homonymies and synonymies as well as to be used in plant breeding programs that can maximize genetic variability [87]. One more study differentiates among nineteen olive varieties by using fourteen SSR markers. All the markers show a high polymorphic level that ninety six alleles were detected. Only three SSR markers from the fourteen were chosen to distinguish all the studies varieties. The data obtained can be used for varietal analysis and creation of a database of all olive varieties in Slovenia affording more genetic information on the agronomic and quality characteristics of the olive varieties [88]. By approximately the same scenario, DNA fingerprinting of many cultivars from different species were studied and conducted by SSR markers and hence the aim of differentiation among cultivars were achieved. Such studies applied on olive varieties in Istria [89], Peanut (*Arachis hypogaea* L.) [90], grapevine (*Vitis vinifera* L.) [91], Tunisian pears (*Pyrus communis* L.) [92], tomato (*Solanum lycopersicum* L.) [93], Chinese cabbage (*Brassica rapa*) [94], Portuguese Olive Oil [95], Actinidia species [96], tomentosa cherry (*Prunus tomentosa* Thunb.) [97], barley (*Hordeum vulgare* L.) [98], Latvian and Swedish sweet cherry (*Prunus avium* L.) [99], Prunus rootstocks [100], Tunisian grapes (*Vitis vinifera*) [101], Chinese peach cultivars and landraces (*Prunus persica*) [102].

Up till now, no available studies of DNA fingerprinting have been conducted to differentiate among strawberry (*Fragaria x ananassa*) cultivars by SSR markers. One study has studied DNA fingerprinting of *Fragaria x ananassa* by RAPD markers. In this study, forty one cultivars which are grown in United States and Canada were examined by 10mers primers and 15 polymorphic fragments ranging in size between 450 and 1200 bp. In order to distinguish all the cultivars, ten of the markers obtained from only seven primers were needed [103]. One important study is done on *Fragaria Vesca* in which 21 SSR markers were developed and added to the 10 markers that have already been reported before. In this study, allelic diversity among four subspecies was reported. Besides, 26 SSR markers were able to distinguish 15 *F. vesca* accessions. When using the 31 primer pairs on three cultivars of *Fragaria x ananassa*, amplification of all of them were observed but five of them had big fragments which were unexpected. This study concluded that a cross species transferability of SSR loci can be occurred among Fragaria species [104].

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant Materials

In order to obtain pure wild type cultivars, strawberry fruits were collected from Atatürk Central Horticultural Research Institute – Yalova/Turkey. After collection of fruits and transport them to Ankara/Turkey, the samples were frozen at -20^{o}C until they were used in further processes. Five cultivars of the garden strawberry (*Fragaria x ananassa* Duch.) species were collected which are Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104.

4.2 DNA Isolation

It is very important to obtain a pure DNA which is free of any contaminations. The existence of contaminations such as proteins, polysaccharides, foreign DNA, and other unwanted materials greatly interferes with the obtained DNA and forbids its efficient usage in further applications. Therefore, it is very imperative to work as aseptically as possible and to choose DNA isolation protocol contains the necessary chemicals and reagents to remove any unwanted materials and has clear building steps to attain a pure DNA that can be applied in further processes such as PCR.

Several DNA isolation protocols have been tried for the extraction of DNA from the strawberry fruits. Some of them have failed and some of them have succeeded but to a certain extent and one has positively succeeded. DNA isolation protocols depending on sodium dodecyl sulfate (SDS) detergent or on Cetyl trimethylammonium bromide (CTAB) detergent were tried. Finally, a protocol that uses CTAB as a detergent was selected and it the protocol was modified slightly to obtain a higher yield and pure DNA. After isolation, the DNA solution was run in 0.8% agarose gel electrophoresis at 150V until the tracking dye reached one-third of the gel. The gel was stained with ethidium bromide and the DNA bands were shown by using a Bio-Rad UV Trans illuminator.

4.2.1 SDS-based DNA isolation (A modified protocol from current protocols in molecular biology, 1994)

In this protocol, 0.2g of fruit is crushed in liquid nitrogen until it became powdered. Then, the sample was transferred into a 1.5 ml eppendorf tube and 600µL Tris-EDTA (TE) buffer pH 8.0 containing 5% SDS was added and the solution was incubated at 65^{o}C for 1 hr. After cooling to room temperature, 700µl of chloroform:isoamylalcohol with a 24:1 volume to volume ratio was added and mixed by inverting the tubes. Then, a centrifugation step was performed by a microcentrifuge at 13000 rpm (13226x*g*) for 5 minutes at 4^{o}C. After centrifugation, the upper aqueous phase is transferred to a fresh tube and then two additional chloroform:isoamylalcohol extractions was done. To the upper phase, 1/10 volume of 3M sodium acetate (NaOAc) with pH 5.2 was added

followed by the addition of 2.5 volume of ice cold absolute ethanol to precipitate DNA. Then, centrifugation at 13000 rpm (13226x*g*) for 10 min was done to form a DNA pellet and next the supernatant was discarded. After that, an equal volume of 75% ethanol was added to wash DNA and centrifugation at 13000 rpm (13226x*g*) for 5 minutes was performed one more time. Then, ethanol was discarded and the little remaining ethanol was evaporated letting the tube at room temperature in fume hood to air dry for half an hour. In order to dissolve the precipitated DNA, 100 µL of TE buffer or sterilized distilled water was added. Finally, sample was treated with 50µg/ml RNase A at 37^{o}C for 30 min.

4.2.2 CTAB-based DNA isolation (The analysis of food samples for the presence of genetically modified organisms - extraction and purification of DNA, M.Somma)

First, 0.1g of fruit sample was crushed in liquid nitrogen and transferred into a 1.5 ml microcentrifuge tube and 300 µL of sterile distilled water was added and mixed. Then, 500 µL of CTAB buffer (Appendix B) was added and mixed. Before incubating the sample at 65^{o}C for 1 hour, 20 µL proteinase K (20mg/ml) was added. After incubation, 20 µL RNase A (10mg/ml) was added and mixed by inversion and then incubated at 65^{o}C for 10 minutes. Next, the sample was centrifuged for 10 minutes at 14000x*g* and the supernatant was transferred to a new microcentrifuge tube already containing 500 µL chloroform and was shaken for 30 seconds. The sample then was centrifuged for 5 minutes at 14000x*g* and the upper layer was transferred to a new tube. After this, two volumes of

CTAB precipitation solution was added and mixed by pipetting and let it incubated for 60 minutes at room temperature. After incubation, centrifugation was performed for 5 minutes at 14000x*g* and the supernatant was discarded. The obtained precipitate was dissolved in 350 µL NaCl (1.2 M) and 350 µL chloroform was added and the tube was shaken for 30 seconds. Then, an additional centrifugation step was done for 10 minutes at 14000x*g* to separate phases and the upper layer was transferred to a new tube. Upon the addition of two volumes ice cold ethanol, the sample was centrifuged for 15 min at 13000x*g* and the supernatant was discarded. Next, 500 µL 70% ethanol was added and the tube was shaken carefully and then last centrifugation step was done for 10 minutes at 14000x*g* and the supernatant was discarded. Finally, the pellet was air dried and re-dissolved in 100 µL sterile distilled water. The DNA was stored in refrigerator for further processes.

4.2.3 CTAB-based DNA isolation (short protocols in molecular biology)

After chilling a mortar and a pestle with liquid nitrogen, 0.2 g strawberry fruit was powdered by liquid nitrogen and transferred into a microcentrifuge tube. Then, warm CTAB buffer containing 2% 2-mercaptoethanol was added and the tube is inverted few times to mix the sample then incubated for 1 hour at 65^{o}C with occasional mixing. After incubation, 500 µL chloroform:isoamylalcohol (24:1 ratio) was added and mixed well by inversion. Then, the sample was taken for centrifugation for 5 minutes at 14000x*g*. After recovering the upper layer

into a new tube, 1/10 volume 65°C CTAB/NaCl solution was added and mixed well by inversion. Next, an extraction of the sample was done by chloroform:isoamyalcohol one more time and mixed then centrifuged for 5 minutes at 14000x*g* and again the upper aqueous layer was recovered. After that, one volume of CTAB precipitation solution was added and mixed well by inversion and then centrifugation of the sample at 2700x*g* for 5 minutes was done. Then, directly one volume of isopropanol was added and sample was centrifuged for 15 minutes at 7500x*g*. Finally, the pellet was washed with 70% ethanol and let it to air dry and then dissolved in 100 µL sterile distilled water. DNA was stored in refrigerator at 4°C. Next day, 1 µL RNase A (10mg/ml) was added and incubated for 15 minutes at 37°C.

4.2.4 CTAB-based DNA isolation (Miniprep protocols for isolation of plant DNA, revised by Kevin deHaan in 2005)

0.15 g of strawberry fruit was powdered in liquid nitrogen by already chilled mortar and pestle and was transferred into a 1.5 ml eppendorf tube. 700 µL CTAB buffer containing 0.4% 2-mecaptoethanol (2% 2-mercaptoethanol was also tried) was added to the sample and mixed by careful inversion. Then, the sample was incubated at 60°C for one hour and then cooled to room temperature for about ten minutes. After 700 µL chloroform:isoamylalcohol (24:1) was added and mixed well by inversion for 30 seconds, the sample was centrifuged at 14000x*g* for 5 minutes for the separation of the phases. After centrifugation, 650 µL of the upper aqueous phase was transferred into a new tube and again an extraction

step with chloroform:isoamylalcohol was done. To the newly 600 µL recovered upper layer, 700 µL of 99% cold isopropanol was added gently and then the samples was kept in freezer (-20^{o}C) for 10 minutes. After that, the two layers were mixed by inversion several times and the DNA fibers were precipitated. Then, the sample was centrifuged for 5 minutes at 14000x*g* to form the DNA pellet and the supernatant was poured off and 1 ml 70% cold ethanol was added. After keeping the samples in refrigerator for 10 minutes, an additional centrifugation step was done for 5 minutes a 14000x*g*. After that, supernatant was poured off and the remaining supernatant residues were removed by carefully pipetting them without dislodging the DNA pellet. Finally, the DNA pellet was let to air dry in a fume hood for one hour and then 100 µL of sterile distilled water was added and stored in refrigerator at 4^{o}C. Next day, 1 µL of RNase A (100mg/ml) was added and the sample was incubated at 37^{o}C for 30 minutes.

4.3 SSR Markers Sequences

The SSR marker repeat sequences of strawberry were provided from the Rosaceae family related GDR (Genome Database for Rosaceae) Database [105]. This database can be reached by following this link http://www.bioinfo.wsu.edu/gdr/. In this database, 437 strawberry SSR markers - which are universally recognized by unique accession numbers – were available in the EST (Expressed Sequence Tags) library that were generated from the fruits of strawberry. The SSRs from those ESTs were computationally derived by GDR group. By using a modified virgin

(CUGISSR) of a Perl script SSRIT, SSR analysis was done. In order to determine the location of SSR in the EST sequences depending on its relation with the putative coding region, CUGISSR uses the FLIP program which is a UNIX C program and that finds as well as translates the open reading frames in sequences [105]. Figure 10 shows SSR marker information taken from the GDR database website.

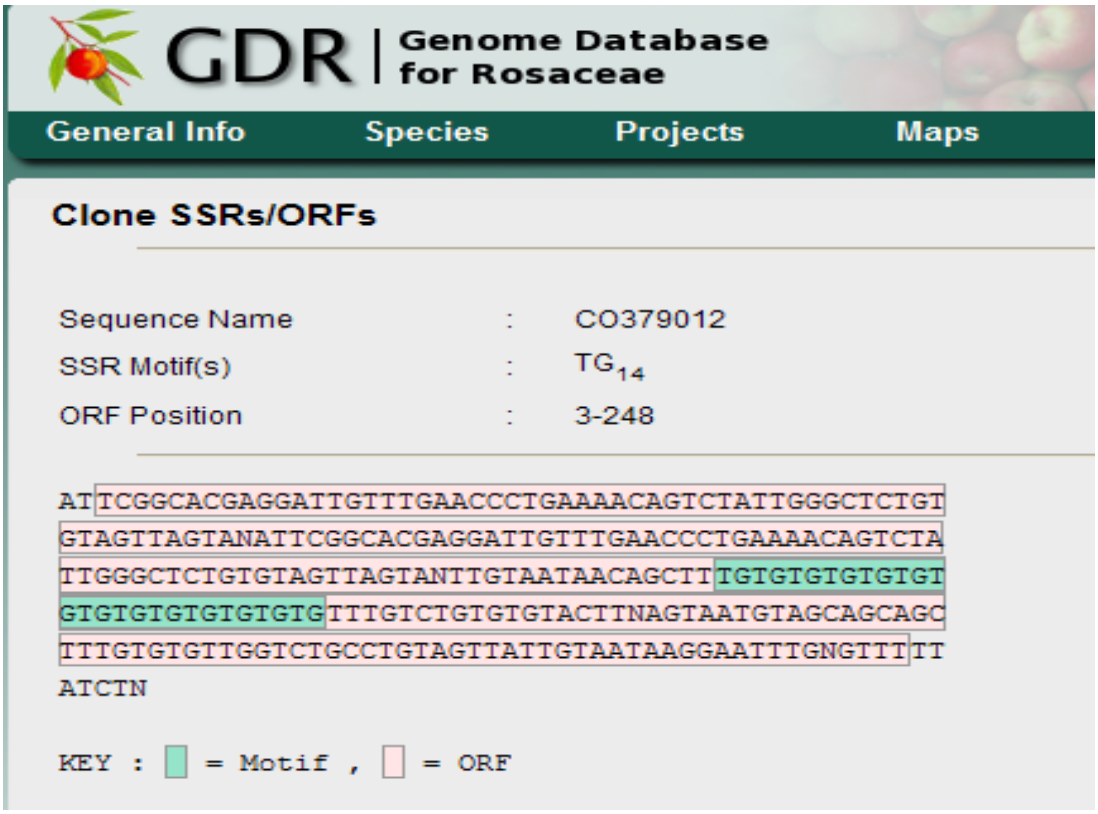

Figure 10 SSR Marker information taken from GDR website. A repeat sequence with an accession number CO379012 and which consists of 14 repeats of the dinucleotides TG (TG_{14}).

4.4 Primer Design

In order to amplify nine SSR marker region obtained from the GDR database, nine primers were designed in a way that flank the repeat motif region. Generally, the primers design is done by designing two primers, one is called the forward primer and the second one is called the reverse primer. There are some guidelines for designing primers so as to get a successful amplification of the interested region of DNA. These guidelines are:

- PCR primers are usually 15-30 nucleotides in length.
- The GC content of the primers should be 40 to 60%. The C and G nucleotides should be distributed uniformly throughout of the primer.
- The primer should be neither self-complementary in which the primer fold to itself and form a hairpin structure nor complementary to any other primer in the reaction to prevent primer-dimer formation.
- The 3’ end of the primer should be G or C in order to render the primer more stable and also to increase correct annealing at the site of addition of bases.
- The melting temperature Tm of the primer which is the temperature at which one-half of a double stranded DNA will dissociate and become a single strand DNA can be generally calculated by this formula Tm= 4 (G + C) + 2 (A + T). The melting temperature of the two primers should not differ by more than 5^{o}C [106,107].

For designing primers, both Primer3 website [108] and Integrated DNA Technologies (IDT) website [109] were used. In Primer3, primers were generated after introducing the DNA sequence containing the repeats of the SSR markers. Primer3 gives information about the primers including the length and GC content as well as the melting temperature of the given primers. Moreover, it gives the expected size of the amplified region by these primers. In addition to this indispensible information about primers, IDT gives even more essential information. By using the IDT Oligo Analyzer 3.1, many specific criteria of the primers were studied and shown. One of them is the hairpin formation of the primers at any temperature. If there is no hairpin formation at the melting temperature, the primers were further tested for self-dimer formation. Primers with 3' end base pair or/and more than three base pairs in the stem were rejected and the search for other primers were recommenced. When both the forward and the reverse primers in study pass the self-dimer test, hetero-dimer test was initiated to see if there is any unacceptable primer-dimer formation between the two primers. If there is no 3' end base pair and no more than three base pairs in the stem of the primers, then the primers were selected and ready to be synthesized by any related company.

After designing the primers flanking the repeats region, they were synthesized by Iontek Company Istanbul-Turkey.

4.4.1 Designed Primers

Primer3 and IDT websites were used to design and test primers. Nine primers were designed according to nine SSR markers of strawberry

taken from GDR database. Some of the different SSR markers contain the same number of repeats of the same sequences but with different loci in the genome and others have different number of repeats with different sequences. Table 1 shows the sequences of the designed primers as well as the type of the sequence repeats.

Table 1 SSRs and Designed Primers

Accession Number of SSR Marker	Motif (Repeat Sequences)	Designed primers
CO378681	TG_6	Forward:5′ CCCCTATTCGACAACCAATG 3′ Reverse:5′ GGCTACCAAAGAACACGAAC 3′
CO378665	TA_7	Forward:5′ GGAGAGTGTTGAGTGTTTAG 3′ Reverse:5′ TTAAATCTCCATCCAAACATAC 3′
CO378649	ACT_8	Forward:5′ TCGTCGAGTTCTACGCTTGCTG 3′ Reverse:5′ ACCGTGCAATCAAATCCCACTCTC3′
CO379012	TG_{14}	Forward:5′ GCACGAGGATTGTTTGAACC 3′ Reverse:5′ CTACAGGCAGACCAACACAC 3′
CO378708	ACT_7	Forward:5′ ACGCTTGCTGATGGAGAACTAC 3′ Reverse:5′ TCCTCAACCGTGCAATCAAATC 3′
CO378480	TG_6	Forward:5′ GTCCCCTATTCGACAACC 3′ Reverse:5′ TGGCTACCAAAGAACACG 3′
CO378521	TG_6	Forward:5′ ATTCGGCACGAGGGATTTG 3′ Reverse:5′ AAGGCCACAGATAATGACCAG 3′
CO378552	TG_5	Forward:5′ AATTCGGCACGAGGCTG 3′ Reverse:5′ GATGGAGAACATTACATGGCTACC3′
CO378635	TA_5	Forward:5′ATTCGGCACGAGGCTTCAC 3′ Reverse:5′CCTTTCCTGTTTTCACTAGCTCCATC 3′

4.5 Calculating Molarity and Diluting of Primers

Upon receiving primers, a sheet of information was supplied containing data about the primers like OD (optical density), Absorbance at 260 nm, Molecular weight, Picomol, and melting temperature and some more.

It is shown that 1 nucleotide approximately equal to 330 Dalton [110]. If molecular weight was not given, it could be calculated by multiplying 330 by the number of the primer nucleotides. Also, it is known that concentration of a primer is 37µg/ml when OD equals 1. Then, concentration determination could be done by the given OD of a primer multiplying by 37µg/ml and convert the answer to g/L. Finally, the molarity could be calculated by dividing the concentration in g/L by the molecular weight of the primer.

When primers were delivered, they were supplied as a precipitate in a micro tube. In order to make a primer solution at any needed concentration, a very useful equation was used as follows; µM = pmol/µl. Generally, a 100µM was firstly obtained from the new arrived primers. Then, to get any concentration needed, C1 V1 = C2 V2 equation was used easily where C stands for concentration and V stands for volume. By doing so, the dilution of the primers was completed and the diluted primers were stored at 4^{o}C with the criteria that they should be utilized during two-month period.

4.6 Polymerase Chain Reaction (PCR)

To start PCR, first the reaction components were brought to an already cleaned area with 70% ethanol. The reagents needed for PCR are listed below with their concentrations as given by the supplier:

1. Buffer solution: 10x Taq buffer with KCL and 10x Taq buffer with NH4SO4 (Fermentas)
2. dNTP Mix: 10 mM each (Fermentas)
3. $MgCl_2$: 25mM (Fermentas)
4. Taq DNA polymerase: 500U 5U/µl (Fermentas)
5. Primers (Iontek)
6. Isolated DNA: from strawberry fruits
7. Sterilized dH2O

It is worthy mentioning that the reagents were stored at -20^{o}C and they were brought on ice when their usage was needed for PCR.

In order to perform several parallel reactions, a master mix (MM) solution was prepared. Master mix solution contains sterilized distilled water, Taq buffer, dNTPs, primers, Taq DNA Polymerase, and MgCl2 in a single tube, which can then be transferred into individual PCR reaction tubes already containing DNA. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers. Below is a table showing the preparation of master mix and the final concentration of the reagents used in PCR.

Table 2 PCR Reagents and Master Mix Concentrations

PCR Reagents	Master Mix for 1 Reaction 50 µl (M.M)	Final Concentration in the Mix
10x Taq buffer with NH_4SO_4	5 µl	1x
dNTP Mix 10 mM	1 µl	0.2mM of each
$MgCl_2$ 25mM	4 µl	2mM
Taq DNA polymerase 500U 5U/µl	0.25 µl	1.25U/ µl
Primers: F and R 5µM each	5 µl each	0.5 µM
Sterilized dH_2O	27.75 µl	
Strawberry DNA	2 µl	Added separately before M.M

For each PCR tube, 48 µl of the Master Mix is added. So, depending on how many tubes we have, we multiply the above volumes by the number of the tubes in order to have a Master Mix containing solutions for every tube. In addition to the number of tubes necessary, one more tube is prepared which does not contain DNA in order to have a control tube and be sure later that there is no contamination in our samples.

To prepare PCR tubes, 2 µl DNA were added to PCR thin-walled 0.2ml tubes and then 48 µl from Master Mix were added to obtain a 50 µl as final volume in each PCR tube. Of course, preparing a control tube in which only Master Mix solution is added without DNA is very important.

4.6.1 PCR Steps

There are seven steps in performing PCR. PCR was done via thermal cycler (Techne). The steps of PCR and their functions are shown in the table below.

Table 3 PCR Steps and Functions

Steps of PCR	Temperature (°C)	Time	Function
Step 1	94	2 minutes	The first step of DNA denaturation
Step 2	94	30 seconds	The first step in the cycle (Denaturation)
Step 3	Between 52.5 and 60 (different for each primers)	30 seconds	The second step in the cycle (Annealing of primers)
Step 4	72	30 seconds	The third step in the cycle (Elongation and extension of primers, 72°C is optimum for Taq polymerase)
Step 5	Repeat of step2 to step4 for 34 times		Having large number of copies
Step 6	72	10 minutes	Fill-in the protruding ends of newly synthesized PCR products
Step 7	4	Forever	Storage

The detailed information of the PCR steps is explained as follows [106].

Step 1: Initial Denaturation Step

The complete denaturation of the DNA template at the begining of the PCR reaction is very important. The initial denaturation should be performed over an interval of 1-3min at 95°C if the GC content is 50% or less. If the initial denaturation is not more than 3min at 95°C, Taq DNA Polymerase can be added into the initial reaction mixture. If it is, then Taq DNA Polymerase should be added only after the initial denaturation to remain stable.

Step 2: Denaturation Step

Usually denaturation for 0.5-2min at 94-95°C is enough because the PCR product synthesized in the first amplification cycle is greatly shorter than the template DNA and is completely denatured under these conditions.

Step 3: Primers Annealing Step

Generally, the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2min is usually sufficient. However, if nonspecific PCR products are shown in addition to the expected product, the annealing temperature should be increased stepwise by 1-2°C.

Step 4: Extending Step

The extending step usually is performed at 70-75°C. The rate of DNA synthesis by Taq DNA Polymerase is highest at this temperature. It is

recommended to extend time for 1 min when the PCR fragments are up to 2 kb.

Step 5: Number of Cycles

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are enough.

Step 6: Final Extending Step

After the final cycle, the samples are usually incubated at 72°C for 5 to 15min to fill-in the protruding ends of newly synthesized PCR products. Besides, during this step, the terminal transferase activity of Taq DNA Polymerase adds extra A nucleotides to the 3'-ends of PCR products.

Step 7: Storage

Amplified DNA tubes are stored at 4^{o}C to be used for further processes.

4.7 Agarose Gel Electrophoresis

The protocol of agarose gel electrophoresis is done as follows [111]:

4.7.1 Preparation of the Gel

0.8% gels were prepared for whole Strawberry genomic DNA and 2.5% gels were used to examine the small amplified repeats region of SSR markers which are around 200 base pairs. Gel electrophoresis apparatus

(Thermo Maxicell Primo EC340) has a max gel tray of size 20x20 cm. So, gel was always preformed with different percentages (0.8 and 2.5) to a total volume of 200ml.

1. 1.6 g (gel for genomic DNA detection) or 5 g (gel for SSR fragments detection) of agarose powder were weighed and then dissolved in 200ml 0.5x TBE buffer in Erlenmeyer flask
2. The mixture was heated in microwave until it boils. If it continues to boil the flask may burst and the solution may be poured out. So, it was boiled a little for a few times. If the agarose was not dissolved completely, it was heated again until it boils a little (3-5s). During boiling a significant amount of water evaporates. So, the mixture was weighed before heating and when finished boiling the initial weight was restored in order to obtain the desired percentage gel.
3. After dissolving the gel, the solution was let free to cool a little until it reaches ~55^{o}C to prevent warping of the gel apparatus.
4. While the gel is cooling down, the tray was prepared in which the solution was poured. The gel casting tray was assembled and the comb was fitted into the provided slots. (it is ensured that there is enough space between the bottom of the comb and the plate (0.5–1.0 mm) to allow proper formation of the wells and avoid sample leakage.
5. After the gel solution is cooled down, Ethidium Bromide was added to the solution at a final concentration 0.5µg/ml. EtBr is a potent mutagen, so it was made sure that did not get any on fingers or on the body, and it was cared not to spread it around the lab.

Adding of EtBr was carried in laminar flow hood and by wearing gloves.

6. Then, the melted agarose was poured in, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the agarose are removed before the gel sets by using a pipette tip.
7. The gel was left free at room temperature in the hood about 30-40 min to solidify. It will look milky when it is set. When the agarose has set up, the comb was carefully removed from the gel by wiggling the comb slightly, so that it loosens its grip on the agarose, and then the comb was lifted vertically.
8. After solidification, the gel casting tray containing the set gel was placed in the electrophoresis tank and sufficient electrophoresis buffer of 0.5x TBE buffer was added to cover the gel to a depth of about 1 mm (or just until the tops of the wells are submerged). Also, there were no air pockets trapped within the wells.

4.7.2 Loading samples into the Gel

As said earlier in the DNA isolation section, the isolated DNA was dissolved in 100 µl sterile distilled water and stored at 4^{o}C. Also, the amplified SSR regions in the 50 µl PCR tubes were stored at 4^{o}C. In order to load DNA in the wells of the gels, a loading dye should be used to make DNA dense enough to set in wells successfully and act as an indicator showing how far DNA runs in the the gel. 6x orange loading dye (Fermentas) were used containing two dyes namely orange G and xylene cyanol FF which act as visual tracking during the migration of DNA, and

containing glycerol which make the DNA dense enough to settle DNA at the bottom of the well. Also, the loading dye contains EDTA which binds divalent metal ions and inhibits metal dependent nucleases.

To obtain best results, 15 µl of DNA was mixed with 3 µl of 6x orange loading dye thoroughly by pipetting gently up and down. By doing so, the mixture was ready to be loaded into the wells. Loading DNA into the wells was done as explained below.

1. A micropipette (Thermo Finnpipette) was used to transfer the contents of each tube into a well in the agarose gel.
2. Pipette tip was slightly located below the top of the well taking care not to hold the pipette too far in the well or else the sample would sometimes come out from the bottom.
3. Also, the pipette tip was not plugged against the side of the well because the sample would squirt out if there was enough pressure from the pipetteman.
4. Upon loading DNA, appropriate marker DNA O'RangeRuler DNA ready-to-use (Fermentas), were loaded as well flanking the sample lanes.
5. When all the samples have been loaded, the lid was carefully placed on the buffer chamber and connected to the electrodes of the power supply (Thermo EC250-90) and run the gel at a constant voltage (150V for genomic DNA and 90V for SSR amplified regions) so that the DNA will migrate towards the red anode (positive electrode).

6. The gel was run until the indicator dye has reached about two-thirds of the way down the gel.
7. Then, the power supply was turned off and after waiting for a couple of seconds, the leads were unplugged, and the lid was taken off the buffer chamber.
8. After removing the gel, supported by the gel tray, the drips of buffer were dried off by placing the tray on a paper towel and then the gel was placed in an ultra violet (UV) transilluminator cabinet (Bio-Rad) and the image of the migrated DNA in the gel was taken.

4.8 DNA Sequencing

DNA sequencing was done to three SSR markers of the five cultivars of strawberry. These three SSR markers are CO378681 which includes the TG_6 repeat, CO379012 which includes the TG_{14} repeat, and CO378708 which includes the ACT_7 repeat. DNA sequencing was done by RefGen Company at TeknoKent-Middle East Technical University Ankara, Turkey.

CHAPTER 5

RESULTS

5.1 DNA ISOLATION

As mentioned earlier, several DNA isolation protocols were tried but only one of them was adopted to be the best protocol for fruit DNA isolation. This protocol was CTAB-based and taken from Miniprep protocols for isolation of plant DNA. By applying this protocol, DNA was isolated successfully from five cultivars of garden strawberry (*Fragaria x ananassa*) namely; Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104. Figure 11 shows the bands of the isolated genomic DNA.

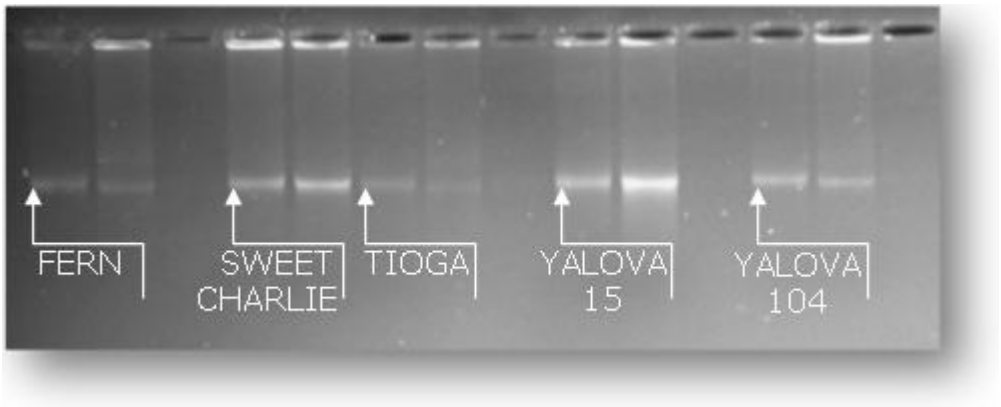

Figure 11 DNA bands of the isolated DNA from the five different cultivars of *Fragaria x ananassa* loaded onto 0.8% agarose gel. Every two wells of the gel indicated by an arrow correspond to one cultivar. The isolation protocol was done twice for each cultivar.

5.1.1 DNA Concentration

After isolating genomic DNA from strawberry fruits, DNA concentration was determined for each cultivar by NanoDrop Spectrophotometer. Table 4 shows the obtained concentrations.

Table 4 DNA concentrations of the five strawberry cultivars

Strawberry Cultivar	Abs 260nm	ng/µL	Abs 260/280
Fern	7.73	386.45	1.97
Sweet Charlie	17.18	859.24	2.09
Tioga	4.81	240.0	2.07
Yalova-15	11.98	598.0	1.86
Yalova-104	7.36	368.14	2.02

5.2 SSR amplification region

For each cultivar, PCR was performed using nine different primers flanking the sequence repeats that were designed before. After the PCR, the amplified fragments were examined in 2.5% agarose gel electrophoresis. The results which are shown in the following figures present all the cultivars with different primers. By the same token, the data are shown for different SSR markers loci for all the cultivars at the same time.

5.2.1 Amplification of CO378681 SSR marker from strawberry DNA

The first SSR marker possesses the sequence repeats TG_6. The designed primers successfully amplified the region containing the six repeats of the TG dinucleotide sequences. Figure 12 shows the amplified region of the marker which is about 220 base pairs and is consistent with the expected size.

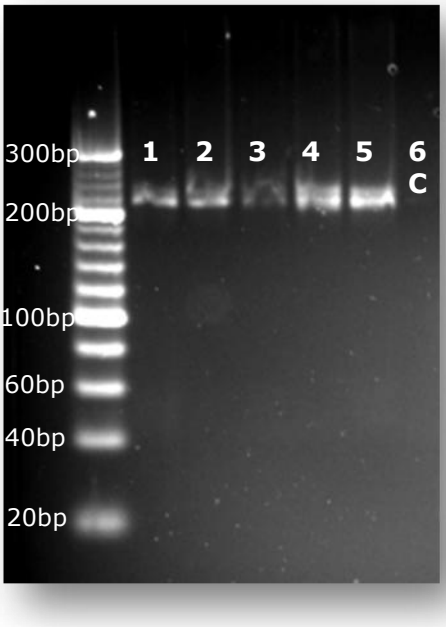

Figure 12 PCR products are loaded on 2.5% agarose gel after the amplification of the TG_6 SSR MARKER CO378681 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.2 Amplification of CO378665 SSR marker from strawberry DNA

The second SSR marker possesses the sequence repeats TA_7. The designed primers successfully amplified the region containing the seven repeats of the TA dinucleotide sequences. Figure 13 shows the amplified region of the marker which is about 90 base pairs and is slightly different than the expected size which is 101 base pairs.

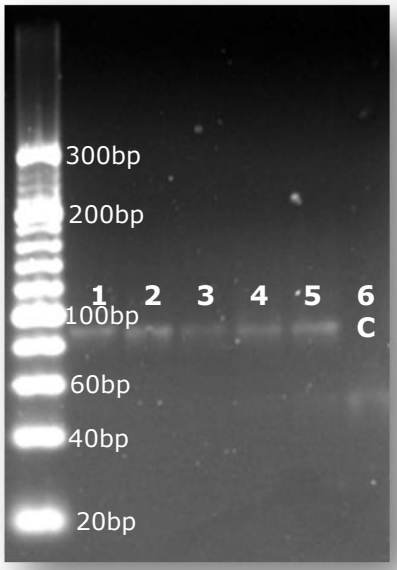

Figure 13 PCR products are loaded on 2.5% agarose gel after the amplification of the TA_7 SSR MARKER CO378665 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.3 Amplification of CO378649 SSR marker from strawberry DNA

The third SSR marker possesses the sequence repeats ACT_8. The designed primers amplified the region containing the eight repeats of the ACT trinucleotide sequences. Figure 14 shows the amplified region of the marker which is about 210 base pairs and is consistent with the expected size.

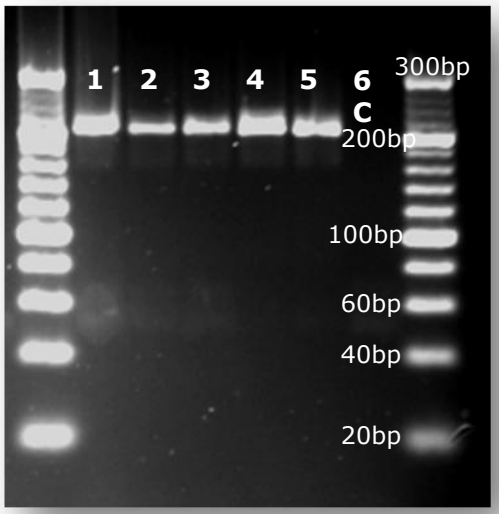

Figure 14 PCR products are loaded on 2.5% agarose gel after the amplification of the ACT_8 SSR MARKER CO378649 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.4 Amplification of CO379012 SSR marker from strawberry DNA

The fourth SSR marker possesses the sequence repeats TG_{14}. The designed primers amplified the region containing the fourteen repeats of the TG dinucleotide sequences. Figure 15 shows the amplified region of the marker which is about 157 base pairs and is consistent with the expected size.

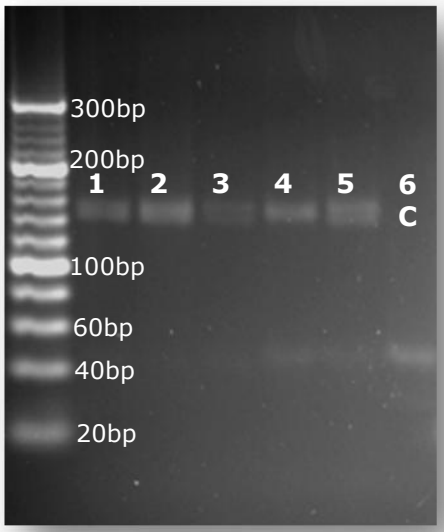

Figure 15 PCR products are loaded on 2.5% agarose gel after the amplification of the TG_{14} SSR MARKER CO379012 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.5 Amplification of CO378708 SSR marker from strawberry DNA

The fifth SSR marker possesses the sequence repeats ACT_7. The designed primers successfully amplified the region containing the seven repeats of the ACT trinucleotide sequences. Figure 16 shows the amplified region of the marker which is about 201 base pairs and is consistent with the expected size.

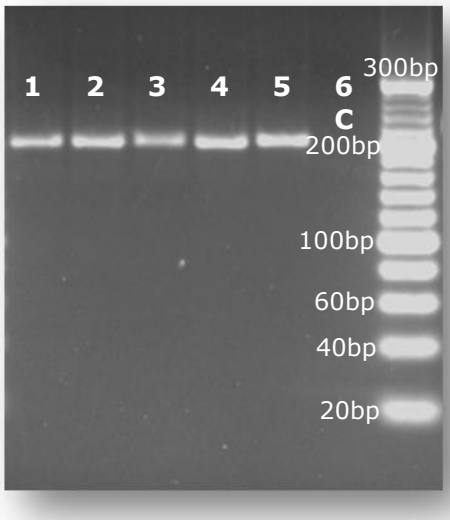

Figure 16 PCR products are loaded on 2.5% agarose gel after the amplification of the ACT_7 SSR MARKER CO378708 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.6 Amplification of CO378480 SSR marker from strawberry DNA

The sixth SSR marker possesses the sequence repeats TG_6. The designed primers successfully amplified the region containing the six repeats of the TG dinucleotide sequences. Figure 17 shows the amplified region of the marker which is about 235 base pairs and is slightly different than the expected size which is 222 base pairs.

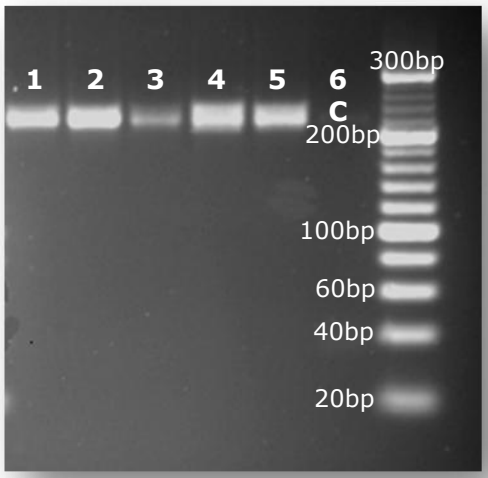

Figure 17 PCR products are loaded on 2.5% agarose gel after the amplification of the TG_6 SSR MARKER CO378480 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

5.2.7 Amplification of CO378521 SSR marker from strawberry DNA

The seventh SSR marker possesses the sequence repeats TG_6. The primers are designed to amplify the region containing the six repeats of the TG dinucleotide sequences. The expected size of the amplified region is 190 base pairs.

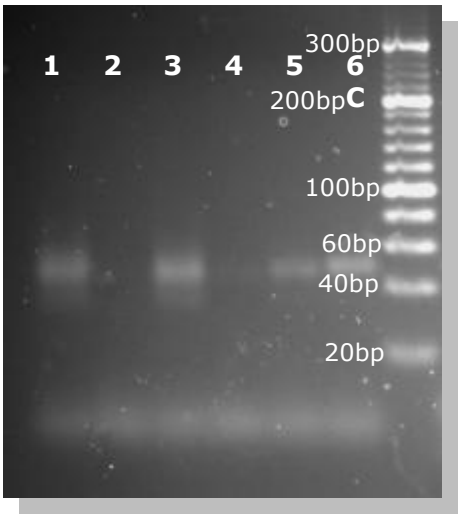

Figure 18 No PCR products are seen in the 2.5% agarose gel after the amplification of the TG_6 SSR MARKER CO378521 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

Figure 18 shows that the repeat region in all the five cultivars was not successfully amplified. The bands that are seen in the figure with an approximate size of 50 base pairs are not SSR marker amplified fragments.

5.2.8 Amplification of CO378552 SSR marker from strawberry DNA

The eighth SSR marker possesses the sequence repeats TG_5. The primers are designed to amplify the region containing the five repeats of the TG dinucleotide sequences. The expected size of the amplified region is 229 base pairs.

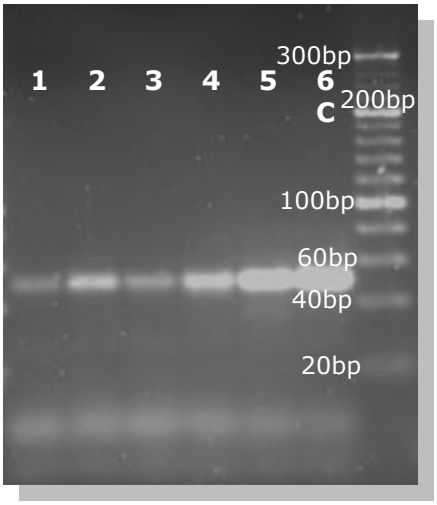

Figure 19 No PCR products are seen in the 2.5% agarose gel after the amplification of the TG_5 SSR MARKER CO378552 of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

Figure 19 shows that the repeat region in all the five cultivars was not successfully amplified. The bands that are seen in the figure with an approximate size of 50 base pairs are not SSR marker amplified fragments.

5.2.9 Amplification of CO378635 SSR marker from strawberry DNA

The ninth SSR marker possesses the sequence repeats TA_5. The primers are designed to amplify the region containing the five repeats of the TA dinucleotide sequences. The expected size of the amplified region is 166 base pairs.

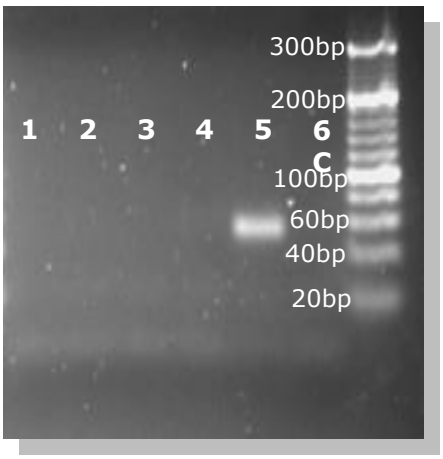

Figure 20 No PCR products are seen in the 2.5% agarose gel after the amplification of the TA_5 SSR MARKER CO378635 (1) of the five different cultivars of strawberry. 1,2,3,4,5, and 6 corresponds to the five cultivars Fern, Sweet Charlie, Tioga, Yalova-15, and Yalova-104, control (No DNA) respectively.

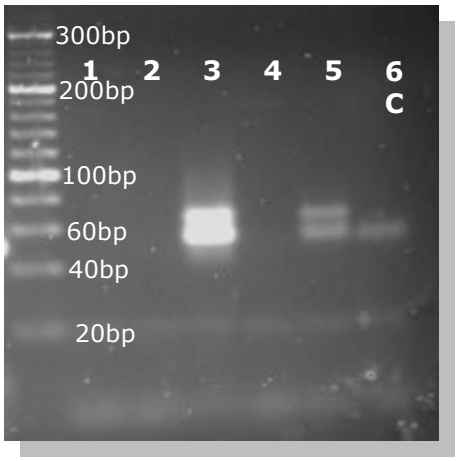

Figure 21 No PCR products are seen in the 2.5% agarose gel after the amplification of the TA_5 SSR MARKER CO378635 (2) of the five different cultivars of strawberry.

Figure 20 and 21 show that the repeat region in all the five cultivars was not successfully amplified. The bands that are seen in the two figures with an approximate size of 50 base pairs are not SSR marker amplified fragments.

5.3 DNA Sequencing

5.3.1 DNA Sequencing of CO378681 SSR marker

Table 5 shows the result of the DNA sequencing of CO378681 SSR marker of the five strawberry cultivars which includes the TG repeats.

Table 5 DNA sequencing of CO378681 SSR Marker with TG repeats

Strawberry Cultivar	DNA Sequence	Number of TG repeats
Fern	CCTCTTAGTATTACCTAGTGATAGGAGGAAGATGA GTCTCGTCATAAGGATTTGTGAGAATCAATAACGTA CTCTGTTTATTATTTGTGATGTAGTTTGATCCTGTCT GTGAGTAATTGATCGAGTAATAGATGTACTCCTC**TG TGTGTGTG**TCGTGCATGTTGTTGTTGTTTGTTAGC CACCATGGTTGG	5
Sweet Charlie	CGGCTTATTATTACCTAGTGATAGGAGAAGCGGAG TCTCGTCATAAAGGATTTGTGAGAATCAATAACGTA CTCTGTTTATTATTTGTGATGTAGTTTGATCGAGTCT GTGAGTAATTGATCGAGTAATAGATGTACTCTAC**TG TGTGTGTG**TCATTCATGTGTTTGTTGTTTGGTAGCC ACCCCTGTCTCCGTCGGGGGATATCAATTATAAATT CCCCTCACACACCTCTGG	5
Tioga	CGCTTATTATTACCTAGTGATAGGAGGAGAAGGAG TCTCGTCATAAGGATTTGTGAGAATCAATAACGTAC TCTGTTTATTATTTTGTGGTGTTAGTTTGATCCTGTC TGCGAGTAATCGATCGAGTAACATACGCTGTCCGC **TGTGTGTGTG**TCATGCTCGTGTGCGTTGTTTGGCA GCCACAATGGT	5
Yalova-15	TAGTCTTATTATTACCTAGTGATAGGAGAAGAAAGG AGTCTCCTCATAAAGGATTTGTGAGAATCAATAACG TGCTCTGTTTATTATTTTTTTTTGTTAGTTTGATCCT GTCTGCGAGTAATTGAATGAGTAACATACGCTGTCT GG**TGTGTGTGTG**TCATGTTCGTGTTCGTTGTATGC CATCCACATCGGTTG	5
Yalova-104	GAGTCTTATTATTACCTAGTGATAGGAGAAGACGGA GTCTCGTCATAAAGGATTTGTGAGAATCAATAACGT TCTCTGTTTATTATTTTTGTGTAGTTTGATCCTGTCT GTGAGTAATTGATTGAGTAACATACGCACTCTGG**T GTGTGTGTG**TCATTCTCGTGTTCGTTCTATGCCATC CACATTGGTTG	5

5.3.2 DNA Sequencing of CO379012 SSR marker

Table 6 shows the result of the DNA sequencing of CO379012 SSR marker of the five strawberry cultivars which includes the TG repeats.

Table 6 DNA sequencing of CO379012 SSR Marker with TG repeats

Strawberry Cultivar	DNA Sequence	Number of TG repeats
Fern	CCGGAGTCTTTGGGCTCTGTGTAGTTACTATTGTAT AACAGCTT**TGTGTGTGTGTGTGTGTGTGTGTGTG TG**TTTGTGTGGCAGGGTATGTATGTTTCTCTTTCTG TGTGTGTAGAAAAGTATATAGA	14
Sweet Charlie	CCGAAAGTTAATAGGGCTCTGTGTAGTTACTATTGT ATACAGCTT**TGTGTGTGTGTGTGTGTGTGTGTGT G**TAGTTGTGTGGTAGTGAACATATGTTTTTGTTTT GTGTGGGGAGGAGAGTATAT	13
Tioga	CTAAAGTCATTGGGCTCTGTGTAGTTAGTATTGTAT ACAGCTT**TGTGTGTGTGTGTGTGTGTGTGTGTGT G**TTTGTCTGTGTGTGTCAGTATGTTTGTTGATTTGT CTGTGTGGGTCTGCCTGTAGAAGGGGGGGCACGG CACCCAATTGGCCGCATAGTGAATCGTATTACGCG CGCTCCTGGCCGTCGTTTTAATCCC	14
Yalova-15	CGCAGTCTATTGGGCTCTGTGTAGTTACTATTGTAT ACAGCTT**TGTGTGTGTGTGTGTGTGTGTGTGTGT G**TCTGTGTGTTTTGAAATGTCTCCTTTTTTTTTTGTG TGGCTCGGCCAGTAAATAGAATCTGCTTATCTCTCC CACAGATCCCTGCACCATCAGTATTACTGGATATAC	14
Yalova-104	CCGCCAAATTCTATTGGGCTCTGTGTAGTTACTATT GTATACAGCTT**TGTGTGTGTGTGTGTGTGTGTGT GTG**TACTTGTCTGTGTGTGTCACCTCTGTTTGTGTG CTGCCTGTGGAGGTCAGCCTGCATATAGACCGCCC CCCCCCCCCCCCCCCAACCCAATTAGCTCATATTGTT TTTCCCCCC	13

5.3.3 DNA Sequencing of CO378708 SSR marker

Table 7 shows the result of the DNA sequencing of CO378708 SSR marker of the five strawberry cultivars which includes the ACT repeats.

Table 7 DNA sequencing of CO378708 SSR Marker with ACT repeats

Strawberry Cultivar	DNA Sequence	Number of ACT repeats
Fern	TCCCATACTGGGGGGGAGGGGAAAAGTAGGGTGC TTCCCGGTAAAAAACTTGGGGGGAGAGAATAAAAC GTTTCTGGCGCCCTGGGCTCGGATGGGCTGG CCTTACTGCACCAAGAAGTTGGGGGGGGGAAGGA AACCTACCAGTGCCCTGCCCCTTTCTTGTGTTCTTC	0
Sweet Charlie	TTGGGATGTAATGCATGGT**ACTACTACTACTACTACTACT**AGGTGAAAGGTGAAAGTGACGACTGAGAAA GAAGAATAAGGAAGAGAAGGAGGATTGTACATTCT TTTTTAAGAGATTTAAAAGTTACTGTATGTAAAAGAT TGAGAGTGGGATTTGATTGGCACGGTTGAGGAA	7
Tioga	TAGGATGTTTGCTGGT**ACTACTACTACTACTACTACTACT**ATGGTGAGTGGTGGTGACGACTGCTGAAAA AGAATAATGAAGAAAAGGAGGATGGTTGTTTCTTCT TTTTTAAAATATTTAGTTATTGCTGGTTGTAAATTAT TAGTGGTGTTATTTTGTTGCGTTGATGAAGA	8
Yalova-15	TTGGTAGTGTGCTGGT**ACTACTACTACTACTACTACT**AGGTGAAAGGTGAAAGTGACGACTGAGAAAGA AGAATAAGGAAGAGAAGGAGGATTGTACATTCTTTT TTAAGAGATTTAAAAGTTACTGTATGTAAAAGATTG AGAGTGGGATTTGATTGCACGGTTGAGGAAAACTT GTAGCCCCGCAGCACACCGAAAAAAAAAAAAATCT TTCTTTTTTCCTCTCATCGGGGGGGGGGGAATTCAA	7
Yalova-104	GTTTGGTGATATGCTGGT**ACTACTACTACTACTACTACTACT**AGGTGAAAGGTGAAAGTGACGACTGAG AAAGAAGAATAAGGAAGAGAAGGAGGATTGTACAT TCTTTTTTAAGAGATTTAAAAGTTACTGTATGTAAAA GATTGAGAGTGGGATTTGATTGGCACGGTTGAGGA AAAAAACTGGCCTAAAACGAACAAAGGGACACAAA	8

CHAPTER 6

DISCUSSION

Generally, DNA fingerprinting in plants depending on the usage of Simple Sequence Repeats SSR molecular markers is a very useful and informative method. This technique is widely used and internationally accepted as a strong process to differentiate among different cultivars. Even though this technique was used successfully resulting in positive PCR products in agarose gel electrophoresis, differentiation among the five cultivars of garden strawberry (*Fragaria x ananassa*) was not accomplished because the results obtained from the gel were not informative. However, when DNA sequencing has done to certain SSR markers, a slight difference in numbers of the sequence repeats was seen and hence a differentiation among some of the cultivars can be studied. In SSR studies, one may find informative SSR markers that show different alleles at the same locus, and one may find non-informative SSR markers that have same allele sizes at the same locus. In this work, DNA fingerprinting using SSR markers method was conducted and applied successfully and when only the agarose gel electrophoresis results were taken into account, it could be concluded that the SSR markers which were examined in the garden strawberry can not differentiate among the five selected cultivars because in all the cultivars each SSR marker shows only homozygote alleles that are equal

in size. However, when DNA sequencing results were taken into consideration, some results showed a consistency with the agarose gel electrophoresis results but others showed a difference. These differences in the results is because agarose gel electrophoresis technique does not have the ability to differentiate among DNA that have a slight change in their sizes but DNA sequencing can monitor a tiny change even a one base difference.

By scanning the results, it is obviously seen that DNA isolation from the fruits of different cultivars of the garden strawberry was done successfully. Figure 10 shows the isolated DNA of all the cultivars in a way that each two wells correspond to one cultivar. Each band of the two was carried by the same DNA isolation protocol but with different samples of the same cultivar. By doing so, it is clear that the performed protocol was a successful one without the doubt that any contamination or errors have been interfered. In DNA isolation step, four protocols were tried. In SDS protocol, DNA could not be isolated at all. This protocol was performed for several times and in each time a slight modification was applied but again DNA was not isolated when examining it by agarose gel electrophoresis (data not shown). When CTAB was used as a detergent, three protocols were carried out. When used the protocol derived from "The analysis of food samples for the presence of genetically modified organisms - extraction and purification of DNA", successful DNA isolation was not observed even though some modification steps were introduced. For instance, in this protocol 2-mercaptoethanol was used instead of proteinase K and in another trial both of them were used in the same protocol, but still no DNA was isolated. The remaining two CTAB

protocols were successfully enough to isolate DNA from strawberry fruits, but the one derived from "Miniprep protocols for isolation of plant DNA" has shown to obtain high DNA yield especially when we made a slight modification by changing the concentration of 2-mercaptoethanol from 0.4% to 2%.

Concerning primer design, it is clear from the results that most of the already designed primers have successfully amplified the expected regions. From figures 12 to 17, the DNA bands of the amplified SSR molecular markers regions are observed. Confidently, it is assured that these bands are for the SSR marker regions because they are in the range of the expected size, they have the same base pairs size for each marker meaning that all possess the same allele, and they show no DNA bands at all the control wells which contain every reagent of PCR except the DNA template. This last point emphasizes that there is neither DNA contamination nor false DNA bands generation in these results. In the seventh, eighth, and ninth markers, it can be seen that no amplification bands are observed. So, the amplification of these SSR regions was not successfully done. There could be several reasons for such negative results. It is worthy mentioning by the way that the same PCR reagents and concentrations were used in all the PCR reactions done. So, the problem may be in the primers that were designed for these SSRs keeping in mind that the same designing procedure steps were used for the other successfully designed primers as well. The most probable reason is that at these three SSR loci, the already identified and sequenced SSR markers from *Fragaria vesca* are not transferable to *Fragaria x ananassa* like the other markers from which we obtained

positive results; hence, the regions flanking the SSR repeats in the these three loci are different in ananassa and in turn the designed primers would not anneal and produce an amplification fragments. The observed short bands in these figures however are due to the presence of primers that have not annealed to DNA. Besides, these observed bands are very small with respect to the expected sizes of the SSR fragments. Even if it is said that these short bands could be alternative alleles for the expected ones, it can not be right because the bands are short enough that even if all the repeats are deleted, still the bands have to be bigger than the obtained ones. Another imperative point is that the same band sizes were obtained in the control wells of the gel, and if figures 13, 14, and 15 are checked thoroughly, which are positive results, it can be found that there are bands similar to those obtained from the seventh, eighth, and ninth markers. These bands are between the 40bp and 60bp bands of the DNA marker and their existence is due to the presence of primers that have not annealed to DNA. So, obtaining bands at this level is for sure not considered as DNA amplified fragments especially for the SSR repeat region fragments. In the ninth marker and from figures 20 and 21, one may have doubt that the obtained bands are amplified fragments of SSR region due to the existence of one band in the first figure corresponding to Yalova-104 cultivar but not for others and then bands are obtained from Tioga cultivar as well in the second picture. As stated before, these are not DNA amplified bands because they are too small to be an SSR different allele from the expected one and the same size fragments were obtained in the control well that does not contain DNA. The reason that these bands were obtained for certain cultivars is

probably because of the different primers association in different cultivars.

As for the positive results and data obtained from the successful amplification of the six SSR repeat regions, it can be said that some of the SSR markers which were provided from the GDR database are shown to be non-informative SSRs with respect to the five cultivars of *Fragaria x ananassa* available in Turkey and that these markers could be informative in other cultivars of *Fragaria x ananassa* in different countries or even informative in other cultivars of other Fragaria species like *Fragaria vesca* for example. Nevertheless, two SSR markers namely CO379012 and CO378708 with the TG and ACT repeats respectively showed the differences in the numbers of repeats among the cultivars. As seen from table 6 regarding CO379012 SSR marker , Fern, Tioga, and Yalova-15 strawberry cultivars have the TG sequence repeated for 14 times, but Sweet Charlie and Yalova-104 cultivars have the TG sequence repeated for 13 times. Moreover, CO378708 SSR marker shown in table 7 showed that sweet Charlie and Yalova-15 have the ACT sequence repeated for 7 times, but Tioga and Yalova-104 cultivars have the ACT sequence repeated for 8 times. When gathering these two SSR markers together we can differentiate among certain cultivars. For example, if an unknown cultivar sample from these five cultivars is to be identified, a check with the two SSR markers will be done. If a 13 repeats of TG sequence is obtained from the first marker, then the unknown sample could be one of the two cultivars Sweet charlie or Yalova-104. After that, the second SSR marker with the ACT repeats is checked. If the ACT

repeats are 7, then the unknown sample is Sweet Charlie. If the ACT repeats are 8, then the unknown sample is Yalova-104 cultivar.

In many similar studies which followed DNA fingerprinting method by using SSR markers, researchers obtained non-informative SSR markers in addition to the informative ones. When informative SSR markers were found, they were able to differentiate among the studied cultivars. For example, in [84] 16 durum wheat cultivars were differentiated by using three SSR markers from the total seven markers studied. When a closer look at the results is given, some of the markers can be found that have the same allele sizes for six, seven and even nine cultivars. So, when the cultivars differentiation is aimed among small number of cultivars, the finding of informative SSRs become harder and the need for trying more SSR markers becomes crucial.

This work would open the door to continue working with cultivars of strawberry in Turkey following the same method and of course trying more different SSR markers available at databases in order to finally be able to discriminate among all the cultivars. Cultivar differentiation studies are very important and useful in many ways. The cultivars would be identified and possess unique fingerprints that could be beneficial in many further agricultural processes. Besides its importance in agriculture, it adds essential touch in horticulture as well as plant breeding purposes. In addition, cultivars differentiation is indispensable in plant proprietary rights protection [112]. By now there are many organizations that protect different plant varieties by providing patent-like rights for developers, breeders, and owners of new varieties for the reproduction and distribution of their cultivars. For instance, there is an

organization called The Plant Variety Protection Act (PVPA) which beside all the mentioned points, they ensures that developers would benefit from their cultivars and assures that they got recover of their research costs [113]. Moreover, in order to prevent unauthorized growers from producing more fruits, there are legal steps are followed. When suspect fruits are seen in orchards or in markets, an investigation is commenced. Samples of the fruits or the suspected plants are taken to laboratory and the fingerprints of the samples will determine if they are authorized or unauthorized samples. From the fingerprints, different varieties could be distinguished even though they may look the same and identical at the morphological level [114].

Up till now, many molecular markers have been discovered and identified. Even though many studies are done on these topics and contributed greatly into the realm of science, still lots of processes and applications are awaiting to be applied in order to flourish its essence widely. Cultivar identification have been performed for many years by now but not extended enough to catch up with the exponential rising and growing of markers' discovery. Nowadays, due to the increasing advances of technology especially in plant genomics and DNA studies, a necessity for contribution and application of molecular markers is indispensable in order to have better plans and developed new applications in the future.

REFERENCES

1. Yonghua Qin, Jaime A. Teixeira da Silva, Lingxiao Zhang, Shanglong Zhang. Transgenic strawberry: State of the art for improved traits. Biotechnology Advances 26: 219–232. 2008
2. J.I. Hormaza. Molecular characterization and similarity relationships among apricot (Prunus armeniaca L.) genotypes using simple sequence repeats. Theor Appl Genet 104:321-328. 2002
3. D. Poljuha, B. Sladonja, E. Setic, A. Milotic, D. Bandelj, J. Jakse, B. Javornik. DNA fingerprinting of olive varieties in Istria (Croatia) by microsatellite markers. Scientia Horticulturae 115: 223–230. 2008
4. Reference Answers, http://www.answers.com/topic/genetic-fingerprinting, last visited on December 2009.
5. Bio Solutions, http://biosolutions.6te.net/articles_files/dna_fin.html, last visited on December 2009.
6. Chambers GK. DNA fingerprinting: a versatile technology. Australas Biotechnol. Mar-Apr;4(2):99-102. 1994
7. Weising K, Ramser J, Kaemmer D, Kahl G. Multilocus DNA fingerprinting and genetic relatedness in plants: a case study with banana and tomato. EXS.;69:45-59. 1994
8. Weising Kurt, Nybom Hilde, Wolff Kristen, and Meyer Wieland. DNA fingerprinting in plant and fungi. CRS Press. 1995

9. Dominique de Vienne. Molecular Markers in Plants Genetics and Biotechnology. Inc. Enfield. 2003.
10. Lalitha Sunil Kumar. DNA markers in plant improvement: An overview. Biotechnology Advances 17: 143–182. 1999
11. Medicine Net, http://www.medterms.com/script/main/art.asp?articlekey=4166, last visited on December 2009.
12. Maayan's Homepage, http://bioinfo.cs.technion.ac.il/superlink/, last visited on December 2009.
13. Wikipedia, http://en.wikipedia.org/wiki/Genetic_linkage, last visited on December 2009.
14. David Edwards. Linkage analysis using loglinear models. Computational Statistics & Data Analysis Volume 13, Issue 3, Pages 281-290. April 1992
15. The Human Genome, http://genome.wellcome.ac.uk/doc_WTD020778.html, last visited on December 2009.
16. Genome News Network, http://www.genomenewsnetwork.org/resources/whats_a_genome/Chp3_2.shtml, last visited on December 2009.
17. Barley World, http://barleyworld.org/css430_09/lecture%205-09/notes5_09.htm, last visited on December 2009.
18. Swati P. Joshi, Prabhakar K. Ranjekar and Vidya S. Gupta. Molecular markers in plant genome analysis. Plant Molecular Biology Group, Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India. 1999.

19. Nathans D, Smith H. Restriction endonucleases in the analysis and restructuring of DNA molecules. Annu Rev Biochem 44:273- 293, 1975.

20. Botstein D, White RL, Skolnick M Davies RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32: 314-331. 1980

21. Mahinur Akkaya. Plant Molecular Markers and Applications in Genome. METU. Biotechnology Course. 2007.

22. Ronald L.Phillips and Indra K. Vasil. DNA-Based Markers in Plants.1994

23. Mark F. Evans. The polymerase chain reaction and pathology practice. Diagnostic Histopathology Volume 15, Issue 7, Pages 344-356. July 2009.

24. Horizon Scientific Press, http://www.horizonpress.com/pcr/, last visited on December 2009.

25. Azfer, A. Bashamboo, N. Ahmed and S. Ali. Random amplification of polymorphic DNA with conserved sequences reveals genome specific monomorphic amplicons: Implications in clad identification. J.Biosci 24, pp. 101–107. 1999.

26. P. Chattopadhyay. RAPD markers system as a useful tool for rapid identification of the origin of lizard contaminants in food. Food and Chemical Toxicology 41: 1719–1725. 2003.

27. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey S. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531-6535. 1990

28. Weising K, Nybom H, Wolff K, Meyer W. DNA fingerprinting in plants and fungi. Boca Raton: CRC Press. 1995.

29. University of Oslo http://www.bio.uio.no/fellesavdelinger/DNA-KAFFE/Kaffe_Resources/RAPD.html, last visited on December 2009.

30. Vos P, Hogers R, Bleeker M, Reijans M, van der Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, Vol. 23, No. 21. 1995.

31. F. Geuna, M. Toschi, and D. Bassi. The use of AFLP markers for cultivar identification in apricot. Plant Breedind 122. 526-531 2003.

32. Wikipedia, http://en.wikipedia.org/wiki/Sequence-tagged_site, last visited on December 2009.

33. National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechSTS.shtml, last visited on December 2009.

34. Pui-Yan Kwok and Zhijie Gu. Single nucleotide polymorphism libraries: why and how are we building them? Molecular Medicine Today Volume 5, Issue 12, Pages 538-543, 1 December 1999.

35. Hsueh-Wei Chang, Cheng-Hong Yang, Chang-Hsuan Ho, Cheng-HaoWen, Li-Yeh Chuang. Generating SNP barcode to evaluate SNP–SNP interaction of disease by particle swarm optimization. Computational Biology and Chemistry 33:114–119. 2009.

36. Annenberg Media, http://www.learner.org/courses/biology/textbook/genom/genom_8.html, last visited on December 2009.
37. Wikipedia, http://en.wikipedia.org/wiki/Haplotype, last visited on December 2009.
38. Wikipedia, http://en.wikipedia.org/wiki/Tag_SNP, last visited on December 2009.
39. The International HapMap Project. Nature 426, 789-796. 18 December 2003.
40. Martin W Ganal, Thomas Altmann, and Marion S Röder. SNP identification in crop plants. Current Opinion in Plant Biology Volume 12, Issue 2, Pages 211-217, April 2009.
41. Anthony J. Brookes. The essence of SNPs. Gene 234:177–186. 1999.
42. I. Paran and R. W. Michelmore. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85: 985-993. 1993.
43. Chang Bioscience, http://www.changbioscience.com/primo/pcr/eSequencesCharacteriz.htm, last visited on December 2009.
44. Menezes, C.C., Vantoai, T.T., Mcdonald, M.B., Sediyama, E.T. Sequence characterized amplified region (scar) technique used for variety discrimination in vinca (catharanthus rosues l.). Revista Brasileira de Sementes. 24:299-305. 2003

45. Konieczny A, Ausubel FM. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4(2):403-10. 1993.

46. Wikipedia, http://en.wikipedia.org/wiki/Cleaved_Amplified_Polymorphic_Sequence, last visited on December 2009.

47. National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechCAPS.shtml, last visited on December 2009.

48. Biology @ Davidson, http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Parker/method.html, last visited on December 2009.

49. Wikipedia, http://en.wikipedia.org/wiki/Single_strand_conformation_polymorphism, last visited on December 2009.

50. Wine ÓJ. J. Genes and Behavior. 2000.

51. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. Proc Natl Acad Sci USA; 86:2766-70. 1989.

52. Single-Stranded Conformation Polymorphism (SSCP) on Applied Biosystems Capillary Electrophoresis Systems. Applied Biosystems. Publication 116AP01-02. 2006

53. Fong Ah, and Judelson. Single-Strand Conformation Polymorphism (SSCP) Analysis. Oct 2000.

54. Tian Tang, Jianzi Huang, Yang Zhong, Suhua Shi. High-throughput S-SAP by fluorescent multiplex PCR and capillary electrophoresis in plants. Journal of Biotechnology 114: 59–68. 2004.

55. R. Waugh, K. McLean, A. J. Flavell, S. R. Pearce, A. Kumar, B. B. T. Thomas, and W. Powell. Genetic distribution of Bare–1-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). Mol Gen Genet 253:687-694. 1997

56. Alexander V. Vershinin, Theodore R. Allnutt, Maggie R. Knox, Mike J. Ambrose, and T. H. Noel Ellis. Transposable Elements Reveal the Impact of Introgression, Rather than Transposition, in Pisum Diversity, Evolution, and Domestication. Mol. Biol. Evol. 20(12):2067–2075. 2003

57. Naeem H Syed and Andrew J Flavell. Sequence-specific amplification polymorphisms (SSAPs): a multi-locus approach for analyzing transposon insertions. Nature Protocols 1, 2746 – 2752. 2007.

58. L. Porceddu, A., Albertini, E., Barcaccia, G., Marconi, G., Bertoli, F.B. and Veronesi, F.Mol. Development of S-SAP markers based on an LTR-like sequence from Medicago sativa. Genet. Genom. 267, pp. 107–114. 2002.

59. Mehmet Ali Sudupak. Inter and intra-species Inter Simple Sequence Repeat (ISSR) variations in the genus Cicer. Euphytica 135: 229-238, 2004

60. A Silkworm Microsatellite Database, http://sunserver.cdfd.org.in:9999/PHP/SILKSAT/index.php?f=protocol_issr, last visited on December 2009.

61. Dhanikachalam Velu, Kangayam M. Ponnuvel, Murugiah Muthulakshmi, Randhir K. Sinha, Syed M.H. Qadri. J. Analysis of genetic relationship in mutant silkworm strains of Bombyx mori using inter simple sequence repeat (ISSR) markers. Genet. Genomics 35: 291−297. 2008

62. Arnaud ESTOUP and Julie TURGEON, Personal communication. Protocols available by WEB at the address http://www.inapg.inra.fr/dsa/microsat/microsat.htm, last visited on December 2009.

63. R. Zamora-Bustillos, R. Rodriguez-Canul, and F. J. Garcia De Leon. Isolation and characterization of eight polymorphic microsatellite markers from pink conch (Strombus gigas). Molecular Ecology Notes 7, 597–599. 2007

64. E. E. Hakki and M. S. Akkaya. Microsatellite isolation using amplified fragment length polymorphism markers: no cloning, no screening. Molecular Ecology 9, 2149–2154. 2000

65. Wikipedia, http://en.wikipedia.org/wiki/QTL, http://en.wikipedia.org/wiki/Marker_assisted_selection, last visited on December 2009.

66. Wikipedia, http://en.wikipedia.org/wiki/Fragaria, last visited on December 2009.

67. Gourt, http://articles.gourt.com/en/hypanthium, last visited on December 2009.

68. Botanical Online, http://www.botanical-online.com/fotos6.htm, last visited on December 2009.

69. Purdue University, http://www.hort.purdue.edu/hort/courses/HORT414/Strawberrylecture.html, last visited on December 2009.

70. Ilse Fraeye, Griet Knockaert, Sandy Van Buggenhout, Thomas Duvetter, Marc Hendrickx, Ann Van Loey. Enzyme infusion and thermal processing of strawberries: Pectin conversions related to firmness evolution. Food Chemistry 114:1371–1379. 2009

71. Botanical, http://www.botanical.com/botanical/mgmh/s/strawb95.html, last visited on December 2009.

72. The Fruit Pages, http://www.thefruitpages.com/grapes.shtml, last visited on December 2009.

73. A.M. Hadonou, D.J. Sargent, F. Wilson, C.M. James, and D.W. Simpson. Development of microsatellite markers in Fragaria, their use in genetic diversity analysis, and their potential for genetic linkage mapping. Genome 47: 429–438. 2004.

74. Gourt, http://articles.gourt.com/en/strawberry, last visited on December 2009.

75. Wikipedia, http://en.wikipedia.org/wiki/Garden_strawberry, last visited on December 2009.

76. Ki Woo Kim, Jeong Joon Ahn, Joon-Ho Lee. Micromorphology of epicuticular wax structures of the garden strawberry leaves by electron microscopy: Syntopism and polymorphism. Micron 40: 327–334. 2009.

77. Fabrizio Carbone , Fabienne Mourgues, Franco Biasioli, Flavia Gasperi, Tilmann D. Mark, Carlo Rosati, Gaetano Perrotta. Development of molecular and biochemical tools to investigate fruit quality traits in strawberry elite genotypes. Mol Breeding 18:127–142. 2006.

78. The Garden Helper, http://www.thegardenhelper.com/strawberries.html, last visited on December 2009.

79. Flori Data, http://www.floridata.com/ref/F/frag_xan.cfm, last visited on December 2009.

80. P. Rallo, G. Dorado, A. Martín. Development of simple sequence repeats (SSRs) in olive tree (Olea europaea L.). Theor Appl Genet 101:984–989. 2000.

81. Mahinur S. Akkaya, Arvind A. Bhagwat, and Perry B. Cregan. Length Polymorphisms of Simple Sequence Repeat DNA in Soybean. The Genetics Society of America. 1992.

82. Tae-Jin Kang and Marvin W. Fawley. Variable (CA/GT) n simple sequence repeat DNA in the alga Chlamydomonas. Plant Molecular Biology 35: 943–948, 1997.

83. Wichan Eiadthong, Keizo Yonemoria, Akira Sugiura, Naoki Utsunomiya, Suranant Subhadrabandhu. Identification of mango cultivars of Thailand and evaluation of their genetic variation using the amplified fragments by simple sequence repeat-(SSR-) anchored primers. Scientia Horticulturae 82: 57-66. 1999.

84. N. Dograr, S. Akin-Yalin, and M. S. Akkaya. Discriminating durum wheat cultivars using highly polymorphic simple sequence repeat DNA markers. Plant Breeding 119, 360-362. 2000.

85. Raffaele Testolin, Teresa Marrazzo, Guido Cipriani, Roberta Quarta, Ignazio Verde, Maria Teresa Dettori, Marco Pancaldi, and Silviero Sansavini. Microsatellite DNA in peach (Prunus persica L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. Genome 43: 512–520. 2000.

86. Erdogan E. Hakki, Cigdem Savaskan, and Mahinur S. Akkaya. Genotyping of Anatolian doubled-haploid durum lines with SSR markers. Euphytica 122: 257–262, 2001.

87. J.I. Hormaza. Molecular characterization and similarity relationships among apricot (Prunus armeniaca L.) genotypes using simple sequence repeats. Theor Appl Genet 104:321–328, 2002.

88. Dunja Bandelj, Jernej Jakse, and Branka Javornik. DNA Fingerprinting of Olive Varieties by Microsatellite Markers. Food Technol. Biotechnol. 40 (3) 185–190, 2002.

89. D. Poljuha, B. Sladonja, E. Setic, A. Milotic, D. Bandelj, J. Jakse, B. Javornik. DNA fingerprinting of olive varieties in Istria (Croatia) by microsatellite markers. Scientia Horticulturae 115: 223–230. 2008.

90. Ronghua Tang, Guoqing Gao, Liangqiong He, Zhuqiang Han, Shihua Shan, Ruichun Zhong, Cuiqiu Zhou, Jing Jiang, Yangrui Li, Weijian Zhuang. Genetic Diversity in Cultivated Groundnut Based on SSR Markers. Journal of Genetics and Genomics, 34(5): 449-459, 2007.

91. Huseyin Karatas¸ Dilek Degirmenci, Riccardo Velasco, Silvia Vezzulli, Cagrı Bodur, Y. Sabit Agaoglu. Microsatellite fingerprinting of homonymous grapevine (Vitis vinifera L.) varieties in neighboring regions of South-East Turkey. Scientia Horticulturae 114: 164–169, 2007.

92. W. Brini, M. Mars, J.I. Hormaza. Genetic diversity in local Tunisian pears (Pyrus communis L.) studied with SSR markers. Scientia Horticulturae 115: 337–341. 2008.

93. Solomon Benor, Mengyu Zhang, Zhoufei Wang, Hongsheng Zhang. J. Genet. Assessment of genetic variation in tomato (Solanum lycopersicum L.) inbred lines using SSR molecular markers. Genomics 35: 373−379. 2008.

94. CUI Xiu-min, DONG Yu-xiu, HOU Xi-lin, CHENG-Yan, ZHANG Jing-yi, and JIN Min-feng. Development and Characterization of Microsatellite Markers in Brassica rapa ssp. chinensis and Transferability Among Related Species. Agricultural Sciences in China, 7(1): 19-31. 2008.

95. P. Martins-Lopes, S. Gomes, E. Santos, and H. Guedes-Pinto. J. Agric. DNA Markers for Portuguese Olive Oil Fingerprinting. Food Chem. 56, 11786–11791. 2008.

96. A.E. Korkovelos, A.G. Mavromatis, W.G. Huang, M. Hagidimitriou, A. Giakoundis, C.K. Goulas. Effectiveness of SSR molecular markers in evaluating the phylogenetic relationships among eight Actinidia species. Scientia Horticulturae 116:305–310. 2008.

97. Qijing Zhang, Guijun Yan, Hongyan Dai, Xinzhong Zhang, Chunmin Li, Zhihong Zhang. Characterization of Tomentosa cherry (Prunus

tomentosa Thunb.) genotypes using SSR markers and morphological traits. Scientia Horticulturae 118: 39–47. 2008.

98. Aihua Wang, Zhiyong Yu, Yi Ding. C. R. Genetic diversity analysis of wild close relatives of barley from Tibet and the Middle East by ISSR and SSR markers. Biologies 332: 393-403, 2009.

99. Gunars Lacis, Isaak Rashal, Silvija Ruisa, Viktor Trajkovski, Amy F. Iezzoni. Assessment of genetic diversity of Latvian and Swedish sweet cherry (Prunus avium L.) genetic resources collections by using SSR (microsatellite) markers. Scientia Horticulturae 121: 451–457. 2009

100. Mariem Bouhadida, Ana Maria Casas, Maria Jose Gonzalo, Pere Arus, Maria Angeles Moreno, Yolanda Gogorcena. Molecular characterization and genetic diversity of Prunus rootstocks. Scientia Horticulturae 120:237–245. 2009.

101. N. Zoghlami, L. Riahi, V. Laucou, T. Lacombe, A. Mliki, A. Ghorbel, P. This. Origin and genetic diversity of Tunisian grapes as revealed by microsatellite markers. Scientia Horticulturae 120: 479–486. 2009.

102. Zhongping Cheng, Hongwen Huang. SSR fingerprinting Chinese peach cultivars and landraces (*Prunus persica*) and analysis of their genetic relationships. Scientia Horticulturae 120:188–193. 2009.

103. Chemda Degani, Lisa J. Rowland, Amnon Levi, Jerzy A. Hortynski, and Gene J. Galletta. DNA fingerprinting of strawberry (Fragaria × ananassa) cultivars using randomly amplified polymorphic DNA (RAPD) markers. Euphytica 102: 247-253, 1998.

104. A.M. Hadonou, D.J. Sargent, F. Wilson, C.M. James, and D.W. Simpson. Development of microsatellite markers in Fragaria, their use in genetic diversity analysis, and their potential for genetic linkage mapping. Genome 47: 429–438, 2004.

105. Sook Jung, Margaret Staton, Taein Lee, Anna Blenda, Randall Svancara, Albert Abbott, and Dorrie Main. GDR (Genome Database for Rosaceae): integrated web-database for Rosaceae genomics and genetics data. Nucleic Acids Research, 36(Database issue): D1034–D1040. January; 2008.

106. Fermentas Life Sciences, http://www.fermentas.com/techinfo/pcr/dnaamplprotocol.htm, last visited on August 2009.

107. Masato Mitsuhashi. Technical Report: Part 2. Basic Requirements for Designing Optimal PCR Primers. Journal of Clinical Laboratory Analysis 10:285-293 (1996).

108. Steve Rozen and Helen J. Skaletsky. In: Krawetz S, Misener S (eds) Primer3 on the WWW for general users and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386, 2000.

109. Integrated DNA Technologies, http://eu.idtdna.com/SciTools/SciTools.aspx?cat=DesignAnalyze, last visited on December 2009.

110. Sambrook et al. Molecular Cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1659 p. 1989.

111. Fred M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J.G. Seidman, John A. Smith, Kevin Struhl. Current Protocols in Molecular Biology. John Wiley & Sons, Copyright 2005.

112. Kurt Weising, Hilde Nybom, Kirsten Wolff, and Günter Kahl. DNA Fingerprinting in Plants: Principles, Methods, and Applications, Second Edition. CRC Press 2005.

113. B. Erker and M.A. Brick. The Plant Variety Protection Act. http://www.ext.colostate.edu/pubs/crops/00301.html, last visited on December 2009.

114. Michael Striem. Fresh News from Sun World, DNA Fingerprinting Simplifies Identification of Proprietary Plants, http://www.sun-world.com/Sun-WorldFruit/Newsletter/News06, last visited on March 2006.

APPENDIX A

LIST OF REAGENTS

REAGENTS	**BRAND**
Agarose Powder	Prona
Boric Acid	Merck
BromoPhenol Blue	Merck
Chloroform	Merck
Cetyl trimethylammonium bromide (CTAB)	Merck
dNTP Mixture 10mM each	Fermentas
Ethylene Dinitrilo Tetra Acetic acid (EDTA) Titriplex III disodium salt dehydrate	Merck
Ethanol	
Ethidium Bromide	Gene Choice
HyroChloric Acid (HCL)	Merck
Isoamyl Alcohol	Merck
Isopropanol	Delta

Magnesium chloride 25mM $MgCl_2$	Fermentas
2-MercaptoEthanol	Merck
O'RangeRuler 20 bp DNA Ladder, ready-to-use	Fermentas
6X Orange DNA Loading Dye	Fermentas
Primers	İontek
Proteinase K	Fermentas
Ribonuclease A (RNase A)	Fermentas
Sodium Acetate (NaOAc)	
Sodium Chloride	Carlo Erba
Sodium Pellets	Merck
Sodium Dodecyl Sulfate	Merck
Sucrose	
Taq DNA Polymerase	Fermentas
Taq Buffer	Fermentas
Tris (hydroxymethyl) Aminomethane (TRIS)	Merck

APPENDIX B

PREPARATION OF SOLUTIONS

Chloroform:Isoamyl alcohol 24:1 (50ml)

Mix 48ml of chloroform with 2 ml isoamyl alcohol and store at RT.

(Do not autoclave)

2x CTAB Buffer 200ml pH 8:

CTAB	4.0 g (2 %)
100 mM Tris pH 8	20 ml of 1.0 M solution or 3.15g
20 mM EDTA	8 ml of 0.5 M solution or 1.5g
1.4 M NaCl	16.4 g NaCl
2-mercaptoethanol (optional)	400 µl (0.2 %)

Beta mercaptoethanol Added just before use; 20 µl per 10 ml solution

(Autoclave)

CTAB Precipitation solution 200ml:

5g/l CTAB	1g
0.04M NaCl	0.5g

Add 100ml of deionized water

Adjust pH to 8 with 1 M NaOH

Fill up to 200ml and autoclave

Store buffer at 4^{o}C for max. 6 months

EDTA (ethylene dinitrilo tetra acetic acid), 0.5 M, pH 8.0 (100ml):

EDTA Titriplex III disodium salt dehydrate, MW: 372.24g/mol

Weigh 18.61g of powder EDTA and mix them with 80ml distilled water. By mixing the solution with a magnetic stirrer in a beaker and using a pH meter, adjust the pH to 8.0 with 10M NaOH or by adding ~2.2g NaOH pellets. EDTA only dissolves when the pH reaches 8 or higher. (Autoclave)

70% Ethanol (100ml):

Mix 70ml of absolute Ethanol with 30ml sterile dH2O.

(Do not autoclave)

10x Loading Dye (50ml):

Sucrose	2.5g
Bromophenol Blue	0.5g
1M Tris pH 8.0	0.5ml
0.5M EDTA pH 8.0	1.0ml
dH2O	48.5ml

NaOH, 10 M

Dissolve 20g of NaOH pellets in 25ml dH2O and add dH2O to 50ml.

The preparation of 10 M NaOH involves a highly exothermic reaction, which can cause breakage of glass containers. Prepare this solution with extreme care in plastic beakers. To 25 ml of dH2O, slowly add 20g of NaOH pellets, stirring continuously. When the pellets have dissolved

completely, adjust the volume to 50ml with dH2O. Store the solution in a plastic container at room temperature. Sterilization is not necessary.

NaOH, 1 M

Dissolve 4g of NaOH pellets in 50ml dH2O and complete the volume to 100ml dH2O.

NaOAc 3M, (sodium acetate) pH 5.2 (100ml):

Sodium Acetate-3 H20 MW=136.08

Mix 40.8g of NaOAc with 80ml dH2O. By magnetic stirring, adjust pH to 5.2 with glacial acetic acid. Bring volume to 100ml with dH2O

(Autoclave)

Tris 1M, pH 8.0 (100ml):

Tris (hydroxymethyl) aminomethane MW: 121.14g/mol

Weigh 12.1 g of powder Tris and mix it with 80ml dH2O. By mixing with magnetic stirrer, adjust the pH to 8.0 by adding concentrated HCl. Add the volume to 100ml by dH2O.

Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used.

(Autoclave)

1x TE (Tris/EDTA) buffer, pH 8.0, 10mM Tris-HCl, 1mM EDTA (100ml):

1st method: 500ml

Add 0.6057g of Tris and 0.18612g of EDTA in 400ml dH2O. By mixing in magnetic stirrer adjust pH by adding HCl until pH 8.0 is reached. Complete to 500ml with dH2O.

2nd method: (best) 100ml

Mix 1 ml of 1M Tris-HCl, pH 8.0 (10mM) with 200µL (0.2ml) of 0.5M EDTA, pH 8.0 (1mM), and add 98.8 ml of dH2O

(Autoclave)

TE buffer containing 5% SDS (100ml):

Weigh 5g of powder SDS and mix it with TE buffer to a final volume 100ml. check pH and adjust if necessary with 1M NaOH.

If SDS crystallizes from solution, it can be redissolved with gentle stirring at 40°C.

(Autoclave)

TBE (Tris/borate/EDTA) electrophoresis buffer 5x stock solution, 1L:

54 g of Tris base

27.5 g of boric acid

20 ml of 0.5 M EDTA (pH 8.0)

Add up the volume to 1 liter.

5x stock solution is more stable than 10x because the solutes do not precipitate during storage. (Autoclave)