DETERMINATION OF GENETIC DIVERSITY IN SOME TUNISIAN CITRUS VARIETIES USING MOLECULAR MARKERS

Master's Thesis

Amna GABSİ

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Master's Thesis

Department of Advanced Technologies

Programme in Biotechnology

Supervisor: Prof. Dr. Emel SÖZEN

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FINAL APPROVAL FOR THESIS

This thesis titled DETERMINATION OF GENETIC DIVERSITY IN SOME TUNISIAN CITRUS VARIETIES USING MOLECULAR MARKERS has been prepared and submitted by Amna GABSİ in partial fulfillment of the requirements in "Eskişehir Technical University Directive on Graduate Education and Examination" for the Degree of Master's in Advanced Technologies Department has been examined and approved on 08/07/2022.

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ABSTRACT

DETERMINATION OF GENETIC DIVERSITY IN SOME TUNISIAN CITRUS VARIETIES USING MOLECULAR MARKERS

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Eskişehir Technical University, Institute of Graduate Programs, July 2022

Supervisor: Prof. Dr. Emel SÖZEN

Citrus is one of the most important fruit plants consumed all over the world. Tunisia is well known for its *Citrus* fruits production which have a big importance in agricultural and medicinal sector. In this study, genetic variation among 36 *Citrus* genotypes of which 8 were developed in Tunisia were determined using two molecular markers: ISSR and SCOT. The 10 ISSR primers generated a total of 270 clear and reproducible bands (ranged between 250 and 3888 bp), of which 268 were polymorphic (99.2%). On the other hand, 10 SCOT primers amplified a total of 240 reproducible bands (ranged between (150 and 3357 bp), of which 228 were polymorphic (95%). Average polymorphism information content was calculated as 0.249 for ISSR, and 0.27 for SCOT primers. The highest pairwise genetic similarity obtained from ISSR analysis was between Powell and Novalina (0.545) and lowest pairwise genetic similarity was between Aforer and Panacher (0.102). Whereas SCOT analysis indicated that the genetically most close varieties were Orange Lane Late and Novalina (0.615), and least close varieties were Eureka and Tomatera (0.169). Additionally, UPGMA dendrograms revealed the different levels of sub clustering that separated varieties according to the respective *Citrus* group and allowed for the discrimination of all the genotypes. The results of this study showed that genetic diversity and relationships among *Citrus* varieties could be successfully determined by ISSR and SCOT markers for breeding programs.

Keywords: Citrus, Genetic variation, Molecular markers, SCOT, ISSR

ÖZET

BAZI TUNUS CITRUS VARYETELERİNDE GENETİK ÇEŞİTLİLİĞİN MOLEKÜLER MARKERLER KULLANILARAK BELİRLENMESİ

Amna GABSİ

İleri Teknolojiler Anabilim Dalı

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Danışman: Prof. Dr. Emel SÖZEN

Citrus, tüm dünyada tüketilen en önemli meyve bitkilerinden biridir. Tunus, tarım ve tıp sektöründe büyük öneme sahip olan narenciye üretimi ile tanınmaktadır. Bu çalışmada, 8'si Tunus'ta geliştirilen 36 *Citrus* genotipi arasındaki genetik varyasyon, iki moleküler belirteç ISSR ve SCOT kullanılarak belirlenmiştir. 10 ISSR primeri, 268'i polimorfik (%99.2) olmak üzere büyüklükleri 250 ile 3888 bç arasında değişen toplam 270 net ve tekrarlanabilir bant oluşturmuştur. Diğer taraftan, 10 SCOT primeri, 228'i polimorfik (%95) olmak üzere büyüklükleri 150 ile 3357 bç arasında değişen toplam 240 tekrarlanabilir bnat amplifiye etmiştir. Ortalama polimorfizm bilgi içeriği ISSR için 0.249, SCOT primerleri için 0.27 olarak hesaplanmıştır. ISSR analizinden elde edilen en yüksek genetik benzerlik değeri Powell ve Novalina arasında (0.545), en düşük genetik benzerlik değeri ise Aforer ve Panacher (0.102) arasında tespit edilmiştir. SCOT analizi ise genetik olarak en yakın çeşitlerin Orange Lane Late ve Novalina (0.615), en uzak çeşitlerin ise Eureka ve Tomatera (0.169) olduğunu göstermiştir. Buna ek olarak, ISSR ve SCOT verilerinden elde edilen UPGMA dendrogramları, çeşitleri farklı alt kümeleme seviyelerinde ilgili *Citrus* grubuna göre ayırmış ve tüm genotiplerin ayırt edilmesine olanak sağlamıştır. Bu çalışmanın sonuçları, ıslah programları için ISSR ve SCOT markörleri ile *Citrus* çeşitleri arasındaki genetik çeşitliliğin ve akrabalığın başarılı bir şekilde belirlenebileceğini göstermiştir.

Anahtar Sözcükler: Citrus, Genetik varyasyon, Moleküler belirteçler, SCOT, ISSR.

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Amna GABSİ

08/07/2022

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Eskişehir Technical University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

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GLOSSARY OF SYMBOLS AND ABBREVIATIONS

AFLP	:	Amplified Fragment Length Polymorphism
CTAB	:	Cetyltrimethylammonium bromide
°C	:	Degrees Celsius
DNA	:	Deoxyribonucleic acid
DNTP	:	Disoxy Adinine tri-phosphate
EDTA	:	Ethylene diamine tetra acetate
ISSR	:	Inter Simple Sequence Repeats
mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
min	:	Minute
ml	:	Millilitre
NaCl	:	Sodium chloride
ng	:	Nanogram
nm	:	Nanometre
PCR	:	Polymerase Chain Reaction
PIC	:	Polymorphism information content
RAPD	:	Random amplified polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	Round per minute
s	:	Second
SCOT	:	Start Codon Targeted Polymorphism
SSR	:	Simple-sequence repeats
T	:	Temperture
TBE	:	Tris-borate-EDTA
µm	:	Micromolar
µg	:	Microgram
µl	:	Microlitre

1. INTRODUCTION

Citrus trees have been among the most significant fruit-bearing plants consumed in the world and are native to Southeast Asia. There are two different classifications for the genus *Citrus* L.: in 1977 Tanaka categorized it into 162 species, while Swingle and Reece subdivided it into only 16 species in 1967 (Xiang and Roose, 1988). *Citrus maxima* (Burm.) Merr. (pummelo), *Citrus reticulata* Blanco (mandarin), and *Citrus medica* L. have just been included in a new categorization established by Mabberley in 1997. While *Citrus* is diploîd, there were some naturally present or synthetically created polyploids (*Fortunella hindsii*, Tahiti lime) (Xiang and Roose, 1988).

Tunisia is a Mediterranean nation known for its citrus fruit production, which plays a significant role in the nation's agricultural and healthcare sectors. Fresh fruit is provided by Tunisia for both domestic and foreign markets for 6 months out of the year. The *Citrus* cultivar named the maltese oranges is the most exported. *Citrus* plants are characterized by a high degree of variety and diversity within and between most cultivars, which offers a significant opportunity for selection. The breeding of such priceless fruit plants relies on selecting the best genotypes. The development of molecular technology enhances the common perception regarding the challenges and dispensations of genetic variation within species. In this situation, the creation of molecular methods is required for the identification of specific cultivars in order to provide better management and plant exploitation. Following the selection of features that are adaptable to various conditions, genetic diversity analysis is required to create improved varieties and conserve *Citrus* resources (Mahjbi et al.,2015; Zanganeh and Sheidai, 2022).

The aims of the present study are to investigate the potential of two different molecular markers, SCOT and ISSR , to reveal molecular polymorphism, distinguish and explorate the genetic diverseness of 36 different economically and medicinally important *Citrus* varieties grown in Tunisia.

1.1. The Genus *Citrus*

More than 140 countries worldwide currently farm *Citrus* in general. One of the most prized cultivated fruits in this area, *Citrus* is one of the main features of the Mediterranean chorography. *Citrus*, however, did not originate in the Mediterranean Basin but rather in Southeast Asia's "*Citrus* belt," which is roughly between 40° N and 40° S latitude (Zhong and Nicolosi, 2020).

One of the most widely produced fruits on earth, *Citrus* contributes for over 20 percent of all fruit output; in 2012, *Citrus* production was predicted to be 131.3 million tonnes (8.8 kha). In the production of *Citrus*, sweet oranges make up 52.6% of the total, followed by mandarins (21.1%), lemons and limes (11.2%), pomelo and grapefruit (6.2 %) (http-1). A large variety of *Citrus* is categorized based on the fruit's shape, quality, embryo type, inflorescence, growth, as well as the habit of the trees and adaptability. Over a long history of cultivation and widespread distribution, sexual congeniality among *Citrus* species has been causing a considerable diversity, which over time has resulted to a complex system of taxonomy and nomenclature (Omura and Shimada, 2016).

1.2. The *Citrus* Taxonomy

Subdivision: *Angiospermae*

Class: *Dicotyledoneae*

Order: *Geraniales*

Family: *Rutaceae*

Subfamily: *Aurantioideae*

Citrus is a usual term and a genus of flowering plant in the Rutaceae family (Ani and Abel, 2018). Taxonomy and phylogeny of *Citrus* have been based primarily on geographic and morphological data. Two distinct classification ways are accepted for *Citrus* taxonomy: The Swingle system (1943) and the Tanaka system (1977), which include 16 and 162 species respectively (Zhong and Nicolosi, 2020).

Phylogenetic studies by Scora (1975) and Barrett and Rhodes (1976) suggested that *Citrus* diversity is based around three ancestral species:, mandarin (*C. reticulata* Blanco), grapefruit (*C. maxima* (Burm)) and citron trees (*C. medica L.*). Recent molecular studies have concluded the involvement of a fourth additional ancestral taxon, (*C. micrantha* Wester) (Froelicher et al., 2011).

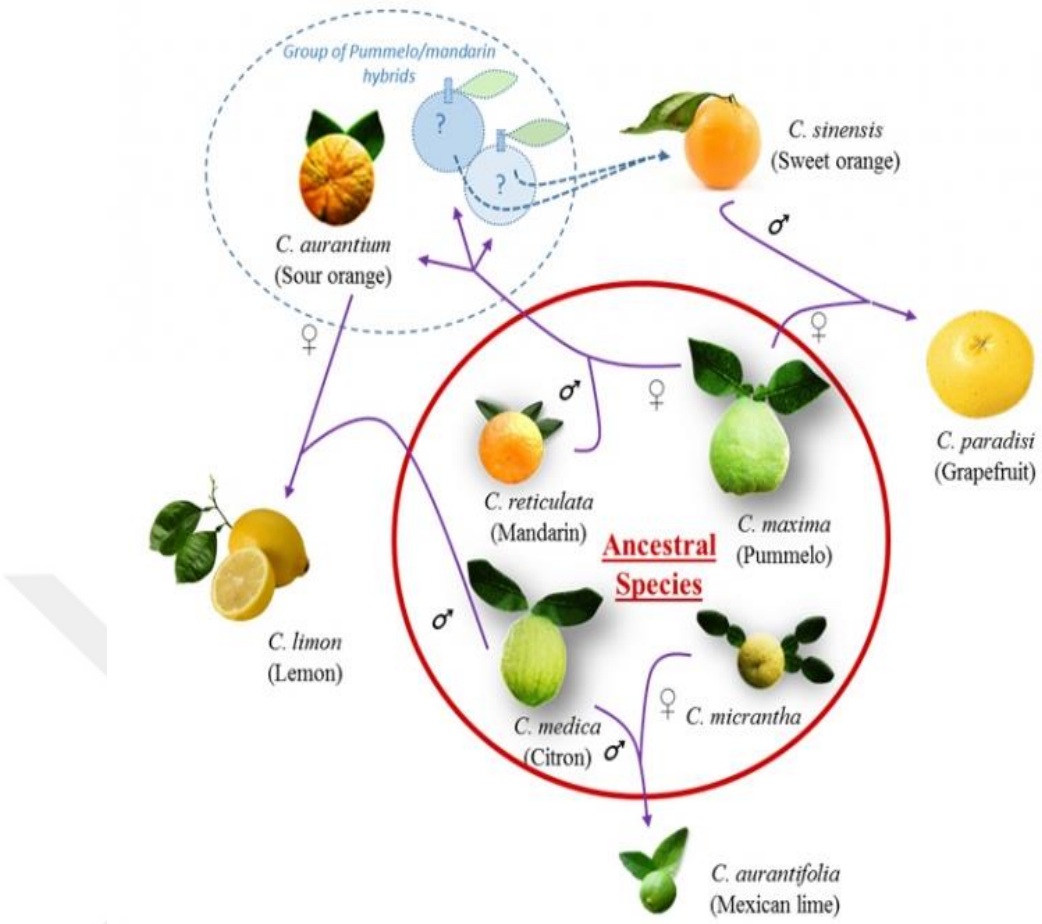

Figure 1.1. *Phylogenetic origins of major secondary Citrus species with the maternal and paternal ancestors (dotted lines are hypothetical cross) (Luro et al, 2017).*

- The common orange tree (*Citrus sinensis*) and the sour orange tree (*Citrus aurantium*) are considered to be hybrids between grapefruit trees and mandarin trees (Figure 1.1).
- The Mexican lime (*Citrus aurantifolia*) probably results from a direct hybridization between a citron (*Citrus medica*) tree and *C. micrantha.*
- Lemons (*Citrus limon*) derive from a hybridization between a citron tree and a bitter orange tree.
- The pomelo (*Citrus paradisi*) would be a hybrid between a *C.maxima* and *Citrus sinensis* (Luro et al, 2017).

In these cultivable species, the diversity of phenotypic traits was mostly a result of fixed somatic mutations, as well as apomixis, which is present in *Citrus* species, or grafted for clonal propagation. Another significant topic of debate concerning *Citrus* and its related groups' sexual congeniality is the continuing reformulation of *Citrus* taxonomy

and phylogeny. Many trials based on molecular techniques have been conducted to classify the genus *Citrus* and related genera based on their genomic data. Nuclear genomic markers used in phylogenetic analysis include simple repeats, isozymes, intersimple repeats, restriction fragment length polymorphisms, etc. In order to evaluate maternal origins, polymorphisms in the chloroplast and mitochondrial genomes have also been studied (Horibata and Kato, 2020).

1.3. The Genus *Citrus* Morphological Characteristics

Citrus trees are modest, 3–10 m tall, green shrubs or trees. Initially flat and angular, young branches eventually develop cylindrical with single (rarely paired) spines in the axils. *Citrus* trees have unifoliate leaves with distinct winged petioles that are typically joined at the base of the blade (Fig.1.2. U). *Citrus* flowers are fragrant, borne individually or in small clusters in the leaf axils, and have 4 to 8 petals, although typically only 5. These petals are white (Fig. 1.2 M) or pink (Fig. 1.2 N) on the outside, overlapping, and thick. The calyx is subglabrous, cup-shaped, and has three to five lobes (Ollitrault et al., 2020). The fruit's skin is often green throughout its early development and turns yellow or orange when it is ripe. *Citrus* trees produce berries, which are soft, indehiscent fruits with many seeds with their only hard element (Fig. 1.2). They are specifically hesperidia, in which the fruit's fleshy portions are segmented and covered in a separate skin (Fig 1.2 A and B). The flattened or oblong seeds (Fig. 1.2.C and D) have smooth or ridged seed coats, are attached close to the nucleus (Fig. 1.2.A), and hold one to multiple embryos (Fig. 1.2.E-J). The spindle-shaped, stalked pulp vesicles that make up the segments contain a very watery large-celled tissue (Fig.1.2. A and B) (Ollitrault et al., 2020).

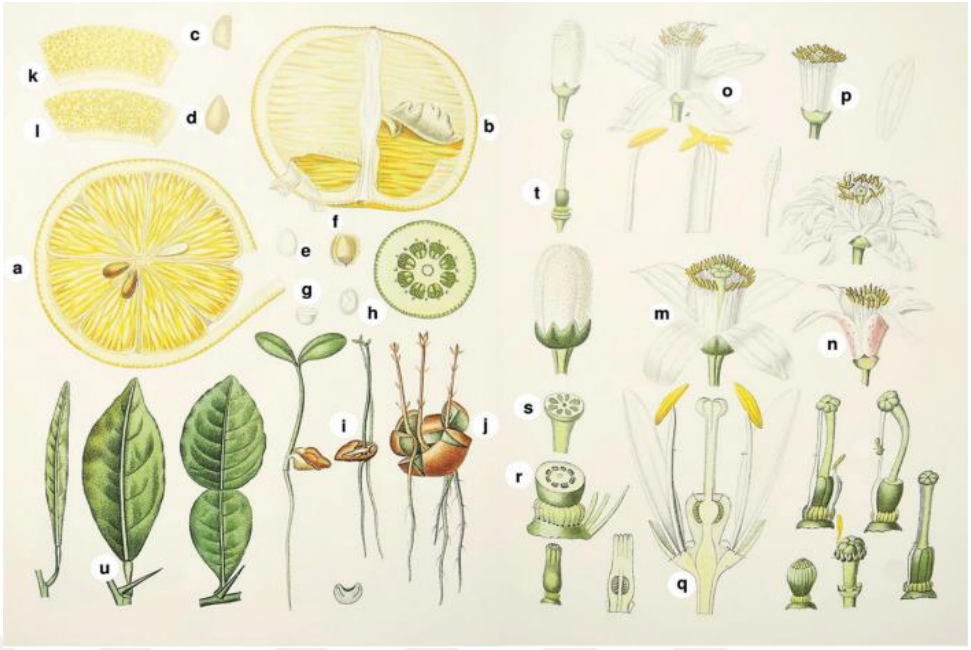

Figure 1.2. *Citrus fruit in cross-section., B: a citrus fruit's longitudinal segment; C: citrus seed that is semi-deltoid; D: obovoid citrus seed; E: a citrus seed sliced longitudinally; F: citrus seed covered in seed coats, G: cross-section through a citrus seed; H: citrus seed with many embryos; I and J: polyembryonic citrus seedling; K and L: piece of orange peel with oil glands outside; M: open citrus flower; N: open lemon flower; O: open orange flower; P: citrus flower stamens; Q: a citrus bloom seen from a longitudinal slice; R and S: a cross-section of a citrus fruit's ovary; T: pistil (ovary, style and stigma) of a citrus flower; U: citrus leaves with one foliation. (Risso and Poiteau, 1818).*

1.4. The Genus *Citrus* Genetic Characteristics And Database

The fundamental chromosomal number in all *Citrus* varieties and similar genera is n =9 (Krug, 1943). The majority of *Citrus* species are diploid, and the average genome size is between 0.73 and 0.82 picograms of DNA per diploid genome, or 714 Mb and 802 Mb in the case of *C. sinensis* L. (Arumuganathan and Earle, 1991). The largest *Citrus* species, *C. medica L.*, has a genome size of 398 Mb/haploid, while the smallest species, *C. reticulata* Blanco, had a genome size of 360 Mb (Ollitrault et al., 2020).

Citrus has had 21,976 different independent sequences documented up to this point in the DNA data bank of Japan (DDBJ). The Spanish *Citrus* Genomics Consortium also created 25 cDNA libraries with 12,672 cDNA probes that cover a variety of tissues at various stages of development and under a variety of environmental stress (Forment et al, 2005).

1.5. The Repartition of Citrus in The Worldwide and Their Ecological Conditions

Citrus are appreciated for their nutritional and medicinal value, and are generally distributed in the coastal areas of several countries as well as in the Mediterranean region. In recent years, world *Citrus* production has grown considerably to reach 82 million tonnes in 2009-2010 (United States Department of Agriculture/Foreign Agricultural Service, 2010).The optimum ecological conditions for *Citrus* plants to grow and produce fruits are at altitudes between 40° north (Corsica, Japan) and 40° south (New Zealand), from the hot/humid climates of the equator to the subtropical climates of the Mediterranean Basin (Jardak et al., 2020).

Based on genomic, phylogenetic and biogeographical analyses, scientists proved that the main origin of *Citrus* plants are the tropical and subtropical regions of Southeast Asia, northeast India, southwest China, Myanmar, the Indochinese Peninsula and the Malaysian Archipelago (Jardak et al., 2020).

Citrus domestication assumably started with the identification and asexual propagation of selected, possibly hybrid or mixed individuals, rather than continual selection from a breeding population either for annual crops. *Citrus* is also the result of vast and complex domestication operation, which, together with the sexual compatibility between *Citrus* and related genera and the frequency of bud mutations, make the taxonomy and phylogeny of *Citrus* fruits very complicated. Human selection, leading to the development of most varieties known to us, came much later (Ani and Abel, 2018).

1.6. *Citrus* Production and Their Importance in Tunisia

Citrus fruits accounted for 24,000 ha in Tunisia in 2018, ranking second to just the olive tree in terms of agricol and economic value (Zouaghi et al. 2019). The primary *Citrus* varieties planted in Tunisia are "Maltaise demi sanguine" (30%), following by navel oranges (24%) Clementines (16%), and lemon trees (15%) (Figure 1.3). Tangerines, Late Valencia, and other less notable varieties make up approximately 15%. *Citrus aurantium*, a variety of sour orange, seems to be the most prominent rootstock because of its higher able to adapt to environment conditions.

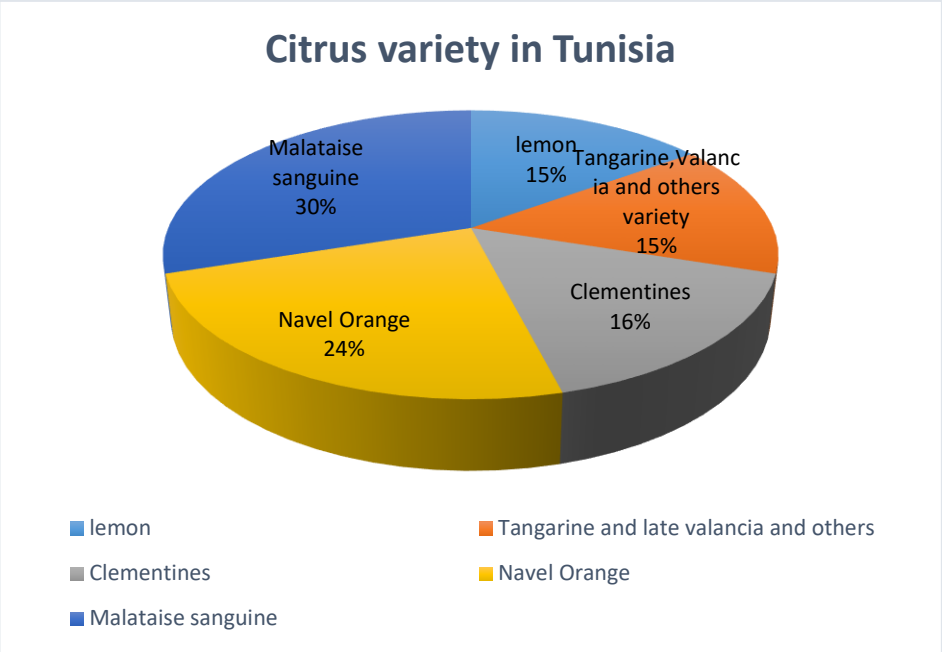

Figure 1.3. *Citrus variety grown in Tunisia*

Citrus fruits are found mainly in the Cap Bon region of Tunisia, which covers 11,000 hectares. While the districts of Jendouba, Beja, Bizerte, Ariana, Ben Arous, and Manouba each contribute 15% to the production of citrus (Farhat et al., 2016).

By providing new plantation sections, *Citrus* agriculture has increased their cultivation area from 13,500 ha in 1990 to 27,000 ha in 2018, producing an average of 346,000 tons, or about 5% of all fruit plantations (Jardak et al., 2020).

Maltese alone accounts for 84 % of Tunisia's total exports of *Citrus* fruits, which are primarily sold to France (who ranked first in selling Maltese at 82 %), Algeria (which came in second at (13 %), Libya (4 %), and other nations in Europe and the Middle East. Tunisia exports *Citrus* fruits at a rate of six percent of the nation's overall production (Farhat et al., 2016).

In addition to the significance of *Citrus* in Tunisia's agricultural economy, Maltese by-products have been identified as a good source of oleic acid, which is known for its therapeutic properties and ability to prevent cardiovascular disease (Dhifi and Mnif, 2012).

The export of Neroli essence which is used in the manufacture of high-end perfumes brings the country 5 million Tunisian dinars in foreign currency each year. Neroli essence refers to oil extracted from orange blossom, especially sour orange. It is rare and precious

oil because it takes 1,000 kg of flowers to obtain only one kilo of neroli. Nevertheless, Tunisia exports 700 kilograms of neroli to the French city of Grasse, recognized as the world perfumes capital since the 18th century (https-2).

1.7. Plant Breeding And Importance of Genetic Variations

Plant breeding is a process of co-evolution between humans and edible plants. People learned to plant seeds in the ground at a specific time of year. This therefore led to the start of plant cultivation and the first production of plants (Muellner, 2019).

Civilization could not exist without agriculture, and agriculture could not sustain the civilized world without modern crop varieties. From this point of view, it is clear that plant breeding is one of the main foundations of civilization. The key to plant breeding is the selection of better types among the variants, in terms of yield and quality of edible parts; ease of cultivation, tolerance to environmental stresses; harvesting and processing; and pest resistance (Toro and Caballero, 2005).

The difficulty in plant breeding is in simultaneously enhancing all the desirable traits. Employing vegetative propagation, plant breeders focus all of their efforts to causing polymorphism in a single cycle from which they conduct clonal selection. As a result, selection concentrates on either the spontaneous mutations found in the orchard, the genotypes obtained by hybridization, induced mutagenesis, or even after applying various biotechnological techniques (somatic hybridization, transformation) (Ollitrault et al., 2012).

The total amount of genetic traits in a species' gene pool is known as genetic diversity. Although the determining gene trait is always unique, diversity is caused by variations in the gene's allele coding sequences. Breeding programs' main resource is genetic polymorphism. In order to maintain long-term genetic diversity and the capacity to adapt to shifting environmental conditions and demands over time, breeding research should be conducted on a broad genetic base (Dirik, 1994). Studies show that genetic diversity creates a genetic barrier against various biotic and abiotic stresses (Hughes and Stachowicz, 2004). Hajjar et al in 2008 showed that the increase in genetic diversity is critical for the development of cultivars resistant to plant diseases and pests, and therefore for the species improvement. Nonetheless, it has also been demonstrated by genetic diversity and genetic mapping studies, that DNA markers are a valuable method in plant breeding (Pradeep et al., 2002).

Plant breeding using molecular markers has played an important role in modern plant breeding over the past two decades (Moose and Mumm, 2008). Many useful markers have been developed and applied to improve parental selection in breeding programs (Grover and Sharma, 2016).

Several genetic techniques such as selection or genetic engineering can constitute an alternative to the natural transfer of genes (Ulukan, 2007).

1.8. Markers For Plant Breeding

Use of morphological (phenotypic), biochemical, and molecular (DNA) markers allows for the identification of genetic diversity. Environmental factors typically have an impact on morphological characteristics; they show dependence on environmental conditions throughout cultivation; and, usually, the detection of these traits requires a mature plant (Munankarmi et al., 2022). On the other hand, DNA-based molecular markers are many, accurate, superior, and offer unbiased genetic diversity attribution and identification of a specific sequence in the DNA pool; yet, each molecular marker seems to have its own benefits and drawbacks (Semaga et al., 2006).

1.8.1. Morphological markers

Morphological markers can be used without special tools and are simple to use. Visual distinctions can be made between characteristics including seed structure, growing conditions, bloom color, and other significant agronomic properties using morphological markers. Since the dawn of humanity, people have effectively studied usage variation in plant breeding using a variety of morphological indicators. However, a number of morphological markers have drawbacks, including their scarcity, sensitivity to environmental variables, and dependence on plant growth stages (Nadeem et al., 2018; Karaköy et al., 2014).

1.8.2. Biochemical markers

This markers can be considered as naturally excited markers, such as secondary metabolites, seed storage proteins and isozymes, they are multimolecular forms of enzymes encoded by severals genes but having the same acts. Biochemical markers have been successfully applicable in the finding of genetic diversity, gene flow, population structure and subdivision (Nadeem et al, 2018; Mondini et al, 2009).

Different modifications to DNA coding regions or post-translational protein modifications cause variations in these indicators. Biochemical markers are influenced by population structure. They are more rare and less common than molecular markers, less expensive, detect less polymorphism, and are affected by diverse extraction techniques, plant tissues, and developmental phases (Nadeem et al., 2018).

1.8.3. Cytological markers

This markers belongs to variations of the number, banding patterns, size, shape, order, or position of chromosomes.These divergences highlight differences in repartation of euchromatin and heterochromatin in chromosomes. As a matter of fact, it can be used in the classification of normal and mutated chromosomes and these markers can also be used in the preparation of physical mapping (Nadeem et al., 2018; Jiang et al., 2013).

1.8.4. Molecular markers

This markers are nucleotide sequences resulting from base by point mutation such as insertion, deletion, duplication and translocation are the foundations of these polymorphisms; but, they do not necessarily affect gene activity and can be studied through the polymorphism exciting among the nucleotide sequences of several individuals. Various types of DNA molecular markers have been successfully progress and applied in genetics and breeding activities in different agricultural harvest. The use of each one or the other mechanisms will rely on both the population being studied and the phenotype and genotype analyzed in the study. Moreover, often, in a research etude, the researcher does not limit himself to use a single analysis of molecular marker, but on the contrary, carries out a combination of several of them (Heikrujam et al., 2015).

An ideal DNA marker should be co-dominant, highly reproducible, evenly dispensed over the genome, and able to reveal the highest level of polymorphism (Mondini et al, 2009). Molecular markers are classified into 3 groups based on:

(i) Process of action of genes (dominant or co-dominant markers).

(ii) Detection procedure ((PCR) hybridization or polymerase chain reaction).

(iii) Method of transmission (fathernal and maternal inheritance of organelles, biparental,or maternal nuclear inheritance) (Semaga et al., 2006).

1.8.4.1. Molecular markers based on hybridization

RFLP (Restriction Fragment Length Polymorphism)

RFLP was the only hybridization-based marker system and the first molecular marker method. RFLPs are transmitted natural Mendelian traits. They have their DNA rearrangements due to evolutionary processes, unequal crossover, mutations within fragments, and point mutations (called InDels) within the restriction enzyme recognition site (Schlötterer and Tautz, 1992).

The initial step in the RFLP process is the isolation of pure DNA before DNA is cut at a specific locus. Restrictions enzymes obtained from bacteria are combined with the DNA, resulting in a massive number of fragments of various lengths (Upta et al., 2021). When these fragments are separated, they are run through a polyacrylamide gel electrophoresis (PAGE), which creates a series of bands. Each band reflects a fragment of varying lengths, and hybridization with a tagged probe allows for the visualization of these bands. These probes, which are typically species-specific and range in size from 0.5 to 3.0 kb, can be found in cDNA or genomic banks.

The benefits of RFLP markers

(i) RFLP is used to create genomic maps.

(ii) In linkage analysis and selection, RFLPs are codominant and reliable markers.

(iii) Can be simply determined by a homozygous or heterozygous individual.

Disadvantages of RFLP markers

(i) The large amount of DNA required for restriction digestion and Southern blotting.

(ii) Expensive, time-consuming and dangerous (Idrees et al., 2014).

1.8.4.2. Molecular markers based on polymerase chain reaction (PCR)

In 1983, the PCR method was discovered by Cary Mullis. This is a scientific technique in molecular biology which could amplify a few copies of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence, without the application of any living organisms (Joshi and Deshpande, 2010). The basis of the technique is the enzyme named "*Taq* polymerase" isolated from the bacterium (*Thermus aquaticus*) that is resistant to high temperatures (Nadeem et al., 2018). There are 3 major steps in the PCR technique:

- **Denaturation step :** DNA is denatured at high temperatures (from 90 - 97°C).

• **Annealing step:** The annealing step happens at a lower temperature, 50-60°C. Primers anneal to the DNA template strands to prime extension.

• **Extension step :** The extension of the primers by *Taq* polymerase occurs at approx 72°C for 2-5 minutes, extension starts at the end of the annealed primers to create a complementary copy strand of DNA (Figure 1.4) (Nadeem et al., 2018).

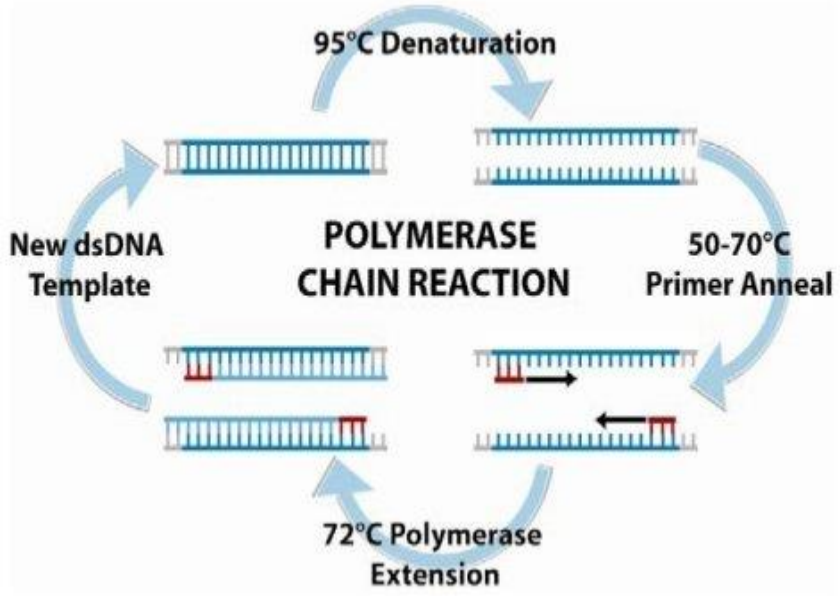

Figure 1.4. *PCR Steps (Lui and Batt, 2009)*

RAPD (Random amplified polymorphic DNA)

One of the most popular molecular marker approaches is PCR-based random amplified polymorphic DNA (RAPD) amplifications. These markers are modest, straightforward, short oligonucleotide primers, typically 10 bp in length, of arbitrary sequence to build profiles of bands. Using RAPD markers, the objective is to collect fragments of various sizes (Williams et al., 1990). RAPDs are unaffected by environmental factors and can be used at any stage of plant development (Sadhu et al., 2020). These primers hold with complementary sequences in the genome, and PCR amplification occurs when regions between opposing primer sites are at amplifiable distances. The basic steps of RAPD include:

(i) The highly pure DNA, isolation

(ii) Adding a unique arbitrary primer,

(iii) (PCR) Polymerase chain reaction,

(iv) The PCR products are typically determineted on 1.5-2.0% agarose gels and tinted with ethidium bromide (EtBr) (Idrees et al., 2014).

It is quickly being used by the research community in diffrent areas of crop refenment. This technique has been too used successfully in phylogenetic studies (Yadav et al., 2016), genome mapping (Valader et al., 2018) and evolutionary biology in a wide range of other crop species (Gupta and Bhagyawant, 2021).

Amplified Fragment Length Polymorphism (*AFLP*)

The AFLP technique can be considered a mixture of both RAPD and RFLP techniques, which combines RFLP's the power with the flexibility of PCR-based technology by ligating primer recognition sequences (adapters) to restricted DNA (Lynch and Walsh, 1998).

In this technique, the DNA sequence is not necessarily known to be amplified beforehand (Mueller and Wofenbarger, 1999). AFLP tags can be produced for DNA from any organism without upfront investment in primer development and sequence analysis. Good quality of DNA can be used for digestion, but the DNA should be without restriction enzymes and free of PCR inhibitors. One of the great advantages of AFLP markers is that they are easily multiplex able, which permits them to augment their performance considerably (Jones et al., 1997).

Furthermore, it is extremely reproducible, reliable and does not require any DNA sequence information from the studied organism. Nevertheless, it is deep in information due to its capacity to analyse a vast number of polymorphic loci simultaneously with only single combination of primers on a gel compared to RFLPs and microsatellites (Russell et al., 1997). Their main drawback is that when a fragment with low sequence homology is presented between samples, the number of common AFLPs will be very low and the technique is no longer useful. AFLP fragments are visualized either by AgNO3 staining on denaturing polyacrylamide gels with autoradiography, on agarose gel, or on automatic DNA sequences (Jones et al., 1997).

Microsatellites (*SSR*)

Microsatellites, also called short tandem repeats (STRs), single repeats (SSR**s**), or variable number tandem repeats (VNTR), are repeated single sequences of 1 to 8 base pairs up to 100 times (Adal et al., 2015).

These elements are found in non-coding and coding regions in prokaryotic and eukaryotic genomes studied to date, as well as in mitochondrial and chloroplast DNA (Provan et al., 2001). The DNA sequences around the microsatellites are marked as primers and amplified by the PCR method (Yorgancilar et al., 2015).

For the analysis of the PCR reactions, the markings of the primers of the microsatellites can be done by a fluorophore, by a radioactive element or devoid of marking. Detection systems will differ depending on whether one option or another is used, ranging from self-reading laser detection systems to simple agarose gels. The main advantages of this type of marker are their large number (Adal et al., 2015) and their co-dominant inheritance, which, unlike dominant markers, provides complete genetic information. This is why they are probably the most widely used molecular markers in laboratories in the world (Garrido et al., 2018).

The disadvantages of SSR markers are the requirement for a known sequence to be amplified (Weising et al., 2005). The development of new microsatellites is very expensive and time-consuming (Coates and Byrne, 2005). SSR markers are frequently used in genetic mapping and diversity determination (Gülşen and Mutlu., 2005).

Inter Simple Sequence Repeat (ISSR)

ISSRs have been used for a wide range of organisms and was developed by Zietkiewicz et al.(1994). ISSR results in the amplification of DNA segments located between two identical microsatellite repeat regions but of opposite orientation (Figure 1.5). The latter can be undefined or in most of the cases, defined at the 3 'or 5' end having 1 to 4 corrupted bases, which are extended in surrounding sequences. Primers generally having a size of 15 to 30 bases are used in this technique; they can be di-, tri- and tetra- or penta-nucleotide repeats. The temperature used in ISSR PCR is around 45-60°C; the length of the amplified sequences is 200 to 2000 bp (Singh et al., 2014). Compared to RAPD, ISSR s are also simple and easy to understand and no prior knowledge of DNA sequences is required (Idrees et al., 2014). Moreover, ISSR markers are fast, highly reproducible, and do not require the use of radioactivity, they are also highly polymorphic and are used in genetic diversity, gene tagging, and phylogeny studies, evolutionary biology and disease mapping genome (Reddy et al., 2002).

ISSR PCR is a technique that just overcomes the problems of previous methods such as high cost of AFLP, poor reproducibility of RAPD and flanking sequences to develop species-specific primers for SSR polymorphism (Idrees et al., 2014).

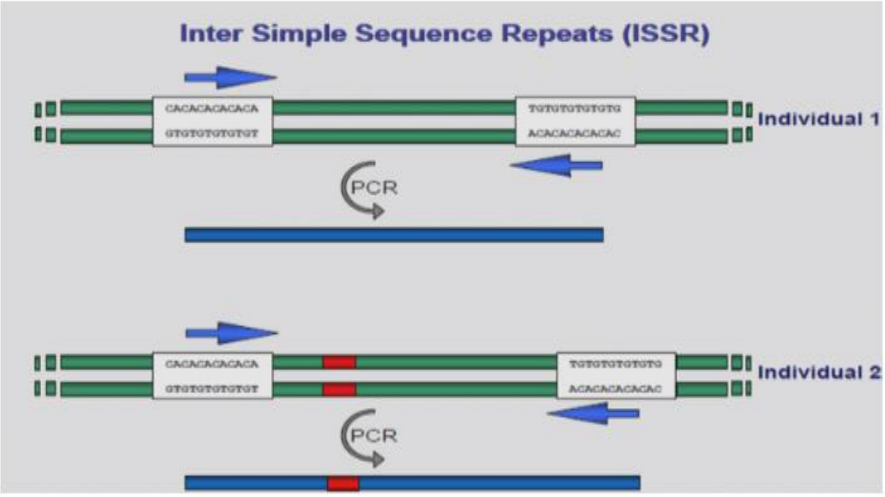

Figure 1.5. *Principles of the amplification of DNA with a single oligo-nucleotide primer in ISSR marker*

Start Codon Targeted (SCoT)

In principle, SCOT is similar to RAPD and ISSR because the same unique primer is used as the forward and reverse primer (Collard and Mackill, 2009). SCOT reactions amplify single segments of DNA 15-19 metres in length (Figure 1.6). It is a promising gene-targeted marker that targets the conserved region flanking the translation initiation codon (ATG) of plant genes (Singh et al., 2017).

SCoT markers have valuable characteristics like reproducibility, simplicity, novel and gene-targeted, the SCoT marker has been used in a variety of applications such as genetic diversity analysis (Singh et al., 2017), population structure, the genetic relationship between individuals of one or different species (Rajesh et al., 2015), QTL identification (Yan et al., 2016), differential gene expression and stress-related gene screening (Luo et al., 2014), offspring fingerprinting assessment with their parents, analysis of male and female genotypes (Heikrujam et al., 2015).

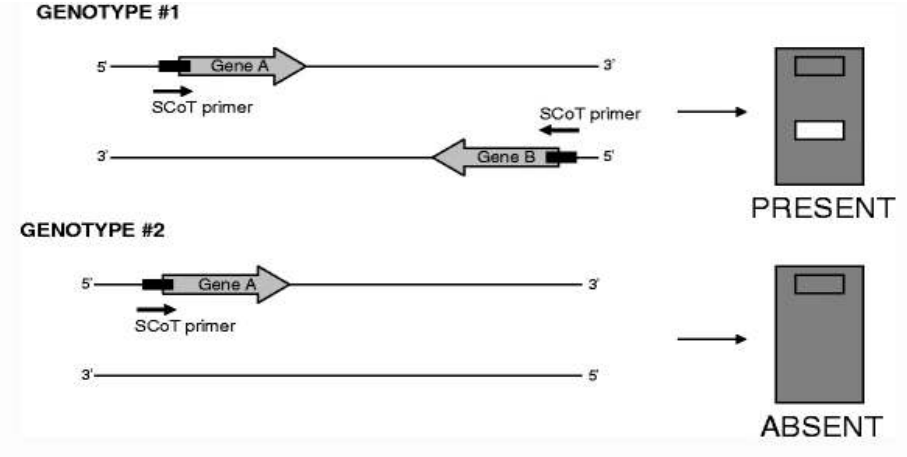

Figure 1.6. *Principles of the amplification of DNA with SCoT primer in plant (Collard et al., 2009)*

1.9. Previous Studies On *Citrus*

Considering the genetic diversity and interrelationships between *Citrus* species, several studies were performed to deciphere specific markers.

Barkley et al (2006) used twenty-four SSR markers to detect molecular polymorphisms among 370 mostly sexually derived *Citrus* accessions from the collection of *Citrus* germplasm. 275 bands was detected, and the average of polymorphism information content was 0.625.

Malik et al, (2012) in India 22 cultivars of *Citrus sinensis* (L.) (sweet orange) analyzed on the basis of morphological markers and RAPDs. 99 bands were generated with 20 RAPD primers, 51 bands were polymorphic (51.83%). A pairwise similarity value among cultivars ranged from 0.48 to 1.00 (mean 0.77).The level of polymorphism was high and a genetic similarities was observed within *C. sinensis* suggested that the cultivars have a low level of genetic diversity despite high morphological variability. This study indicated the presence of low genetic diversity, which could be explicated by the fact that much of the observed phenotypic variations may be due to certain somatic mutations.

Polat et al (2012) studied the genetic relationships and diversity among 51 accessions of sour orange and their relatives using SSR and SRAP molecular markers. 21 SRAP primer were tested and produced 167 polymorphic amorces, the PIC value was 0.47. Although the 17 SSR primers produced 30 polymorphic fragments, with 0.39 as PIC value. The combination between SSR and SRAP data produced a similarity range

from 0.71 to 1.00 among the accessions. They determined that genetic diversity in sour orange accessions is low, and some accessions were very identical .

Later, Khiavi et al. (2016) showed significant variability between 29 *Citrus* genotypes at both morphological and molecular levels. Through the morphological characters and ISSR markers comparison, 32 qualitative characteristics revealed a similarity ranging from 0.19 to 0.88. 8 ISSR primers amplified 102 fragments with an average of polymorphism rate 66.67%. PIC analysis showed ranges from 0.42 to 0.49. The results show that primer sets can distinguish genetic differences between the genotypes with good precision. The study discover that molecular and morphological markers could be successfully used to distinguish genetic diversity in *Citrus*.

On top of that, a study was performed to verify the parentage genetic diversity the and structure analysis between 20 Egyptian *Citrus* rootstocks and the affiliated species (El Zayat et al., 2021). By using molecular markers, 14 LTR-IRAP and 26 SSR in addition to 20 LTR-REMAP markers.The results of genetic structure analysis support the monophyletic nature of the *Citrus* species, also provide identification and disposition of species and their hybrids like lemon, lime, citron, grapefruit, sour orange , sweet orange, pomelo, mandarin, and fortunella and resulted in their placement in individual or superposition groups.

Moreover, given the extremely low production and productivity of acid lime, a study in Nepal aimed at optimizing ISSR-PCR reaction and cycling conditions for PCR amplification and genetic diversity assessment of acid lime cultivars from eastern agro-ecological zone, Nepal. In this study, 4 different cycling conditions were assessed for the determination of the optimum range for ISSR-PCR profiling. The results from this study were successfully used for ISSR-PCR based genetic diversity assessment of Nepalese acid lime genotypes to find out the elite cultivars of Eastern Nepal (Munankarmi et al.,2022)

In Tunisia several studies was undertaken on *Citrus* varieties due to their economic and therapeutique importance. In order to study the genetic diversity on Tunisian *Citrus* species and contribute on citrus breeding information, several studies were conducted, and different molecular markers have been used.

Mezghani et al. (2012) in this study, on green *Citrus* aphid *Aphis spiraecola* using random amplified polymorphic DNA (RAPD) markers, this analysis conducted on 141 individuals with 5 primers revealed only 50 polymorphic RAPD markers, indicating a

low genetic diversity that might result from the lack of sexual phase for used species in Tunisia.

In Debbabi et al, study (2014), AFLP (Amplified Fragment Length Polymorphism) markers were utilized on *Citrus sinensis* tunisian variety. Using this technic, a total of 330 of polymorphic markers were revealed with 3 AFLP primer combinations. A high level of polymorphism was observed wich the (PIC) ranged from 0.16 to 0.22 with an average of 0.18 per primer pair.

Similarly, two other molecular markers, RAPD and SSR were used in order to evaluate the genetic diversity of 19 Tunisian sweet oranges genotype (Lamine et al., 2018). The values of PIC obtained in SSR analysis was between 0,38 to 1,00 among the genotypes studied, while the same values ranged from 0.33 to 0.84 in RAPD analysis. However, 3 primers combination showed higher genetic diversity than one primer and two primer combination with an average coefficient of 0.60.

Hajjar et al (2008) used 20 nuclear SSR markers and four indel mitochondrial markers to determining the genetic diversity of Tunisian *Citrus* rootstocks (*C. aurantium*, sour orange*; C. sinensis*, swee*t orange; C. limon*, lemon; and *C. aurantifolia*, lime). The differentiation of this varietal groups was distinct. Each group displayed a relatively high allelic diversity, a strong heterozygosity and a differentiation between this varietal induces this genetic diversity of Tunisian *Citrus* rootstocks, this phenotypic diversity within these groups results from multiple introductions, somatic mutations and rare sexual recombination events.

Another study by Ben-romdhan et al. (2016) aimed to experience a set of 15 informative nuclear SSR molecular markers on 8 Tunisian *Citrus* species. The results of this study clearly indicated that the use of nSSR molecular markers are highly polymorphic belonging to 8 *Citrus* species. Twelve SCoT markers were used to establish phylogenetic relationship and to reveal polymorphism among 7 species from *Citrus* L. genus. The polymorphism information content (PIC) of 0.884 illustrated the efficiency of the tested SCoT primers in highlighting polymorphism, which suggests the effectiveness of the SCoT markers for assessing genetic diversity, characterization as well as identification of the species of *Citrus*.

Lamine et al., (2018) analyzed 57 volatile chemicals with different qualities and quantities in the leaves of 4 *Citrus* species using gas chromatography coupled with mass spectrometry (GC-MS) with these compounds they were able to identify the taxonomy

and phylogeny of *Citrus* genus and proved its complementarity with the previous existing taxonomy.

In addition, Chamandoosti (2020) carried out a study based on *in vitro* culture of different vegetale tissues. Basal media and plant growth regulators concentrations of *Citrus latifolia* were optimized in this study and thus confirmed that direct and indirect organogenesis is possible.

The same year, Jardak et al (2020) used epicotyl segments of popular Tunisian varieties (*Citrus sinensis)* for regeneration and genetic transformation procedure. The results of this study successfully showed that epicotyl segments were good explants for *in vitro* regeneration.

As it's known, field trials performed during plant breeding work are time-consuming, expensive, and labor-intensive practices. Therefore, it is very important to determine the genetic diversity and the affinity levels of plant varieties with the help of molecular marker technologies, especially for *Citrus* fruits which have a very important value in Tunisia simultaneously with the world.

Here, we studied genetic diversity of 36 *Citrus* varieties, 7 of which were Tunisian origin, belonging different main *Citrus* groups (lemon, orange, bigarde, mandarin, kumkuat and pomelo), using the molecular markers ISSR and SCOT.

2. MATERIAL AND METHODS

2.1. Material

In this study we used 36 *Citrus* varities grown in Tunisia (Table 2.1). Clean, healthy and fresh green leaves from adult plants were collected in September from the Mabrouka company which has the first highly sophisticated *in vitro* laboratory on an industrial scale in Tunisia. All samples were labelled and stored in plastik zip bags containing silica gel until DNA isolation.

Table 2.1. *The characteristics of the studied Citrus varieties (https-3)*

Aforer	Moroco	Is a very well-liked late variety that is high-quality for export. It has a few seeds and a thin, smooth skin that is simple to peel. is medium in size Less seeds, greater fruit coloring, and ease of peeling like a clementine are its main advantages of murcott. The flesh is orange-colored, juicy, and seedy when cross-pollinated, but low-seeded when not, with a rich, sweet flavor..	
Bigarde	Tunisia and North Africa	Because its fruits resemble regular oranges but are significantly more bitter, a fruit tree sometimes known as the bitter orange. The rind is typically more thicker, surface-rough, and difficult to peel. The bitter orange is extremely juicy and seedy.	
Citronier beldi(limette)	Tunisia	It is a very juicy yellow fruit excellent in cooking. That mediterranean limette is acidless, hence even more insipidly sweet; chalazal spot is cream-colored instead of purple.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Clementina cassar	Tunisia	Seedless fruit, contains seeds when the orchard includes mandarins or tangerines. Fruit sizes can range from medium to medium-small. Deep orange to reddish-orange in color, surface smooth and shiny yet slightly pebbly due to abundant oil glands. Red orange flesh that really is soft and melt-in-your-mouth, juicy, sweet in flavor, subacid, and aromatic.	
Eureka	California	The fruit has a good flavor, a smooth, medium-thin rind, a lot of juice, a lot of acidity, few seeds, and a high juice content. picking is made simpler because the trees are less thorny than those of many other lemon varieties. Eureka productivity starting in late winter and continuing through spring till early summer.	
Hernandina	Spain	It is a late-maturing type that is only modestly productive and can resist the chilly winter temperatures. The fruit's pulp, which ripens before the fruit's color and is also highly juicy, sweet, and it might include some seeds.	
Kaffin	Maroco	Although they color up better and develop a little earlier than regular clementines, kaffin fruit do not retain well on the tree.The fruit is juicier than many other types, and it has a good flavor and taste.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Kumkuat	China	In the ground or in pots, it grows quite easily. More tolerant of the cold than a lemon tree.The peel is also edible and not bitter. Has elliptical or oval in shape. These fruits weigh 10–11 g, 15–16 % juice content, 15–17 % brix, 4–5 %acid and 50–55 mg ascorbic acid/100 ml.	
Leunari	Sicilia	Beautiful lemon tree, with four distinct seasons, elongated fruits, and a sharp neck in the peduncular zone.Juicy pulp, quite acid, also slightly pointed leaves. Resistance to cold -5°C (temperatures must rise above zero during the day)	
Lime douce	Comes from the Mediterranean basin.	It is frequently employed as a root stock, primarily for orange trees. The tunisian sweet lime may unintentionally lose its leaf in the winter, but it will begin growing anew in the spring. Is the Mediterranean basin's delicious lemon.	
Ma3(Nour)	Moroco	It is a productive type that can withstand the chilly winter temperatures. The fruit is of good color, although the rind quality is frequently coarse. The interior is rich, flavorful, and incredibly tender.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Maltese balerin	Tunisia	A late orange variety characterized by seedless fruit with a juicy, slightly bloody colored pulp, resembles maltaise sanguine in all other respects but barlerin is a late maturing selection.	
Maltese demi sanguin	Tunisia	A delicious orange with no acid and pink-tinted flesh from lycopene. The spherical fruit is medium in size, seedy, and has a smooth, medium-thick orange peel. It is also quite juicy. The tree bears fruit between February and early March and develops to a small to medium size with a round appearance.	
Mariana		Production method: in vitro culture Tolerant to drought, asphyxia, salinity, chlorosis and very resistant to nematodes.	
Marisol	Spaine	A robust variety of citrus tree that can be prickly when young. Although the fruit is larger than clemenules, the tree tends to lose its fruit after a very heavy rain. The interior is juicy and delicate. It's common to interpret the advent of marisol clementines in stores as the start of autumn.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Mapo	Italy	It grows in regions with a warm and subtropical climate. Tree of medium vigor, fruit with thin skin, the flesh is yellow-orange when ripe, pleasantly acid taste. Maturity of the fruit december/january.	
Meski ansli	Tunisia	Belonging to the white orange family Productive just in february	
Meski malti	tunisia	Belonging to the white orange family Productive just in january and fabruary	
Moro	Sicilia	Its juice is sweet and brilliant. Due to the high pigment content of anthocyanin, this blood orange has the most intense color. The tone is an intense deep purple that is on the verge of turning black. It misses the violet to blue tints that some other deep blood oranges have. There is no such thing as a "typical" moro because the fruit's size and form vary so greatly from region to region and country to country. Small fruit production is a tendency of several moro varieties.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

New Hall	California	Issued by spontaneous mutation on w. Navel and propagated by P. Hackneu in Peru. It is an early, productive variety with remarkable taste qualities.	
Nova	Florida	Is a hybrid between an orange and a clementine mandarin. Medium-sized fruit often has a subglobose shape and an easily peeled, slightly pebbled, thin reddish-orange rind. The dark orange, juicy flesh has a delicate texture and a sweet, rich flavor. Although isolated Nova fruits are seedless but less common, they are still generally seedy.	
Nules	Spaine	It is a seasonal variety, very productive and profitable, seedless fruit, juicy fruit with a pleasant taste.	
Oranger double fine	Variety resulting from a mutation of double fine.	The fruit is moderate to small, practically seedless, and more or less heavily flushed with rose-colored specks when it is mature. Medium-thick, stiff, leathery, very smooth, fine-textured, strongly adhering, and occasionally challenging to peel, is the rinse.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Oranger lane late	Australia	Discovered by spontaneous mutation by w. Navel in 1950. It is a late variety .	
Oranger novalina	California And Propagated In Spain In 1933.	It is a productive variety with smooth skin and good coloring when fully ripe, very similar to w. navel.	
Orograndi	the Region of Murcia Spain	A beautiful mandarin with a sweet, delicious flavor. It has a significant amount of juice that is of the highest caliber, and it has a very vivid orange hue. long time frame.	
Panacher	California	Is a variegated variety of Eureka that first appeared in a Burbank, California, private garden in 1931. A mature fruit's flesh and juice are pink. The fruit's peel and leaves are both colored. An attractive ornamental plant with the added benefit of edible fruit is the variegated lemon.	
Pomplomose Star Ruby	Texas	It is a fruitful variety that starts producing right away (after the second year), but it is less vigorous than other kinds, grows slowly, and the tree and fruit continue to be smaller than other grapefruit types. Strongly colored meat and juice are present, as well as slightly more sugar and acid.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Powel	In Mediterranean	It has a perfect balance between acids and sugars and is seedless, maturity from april to may.	
Rubis	Tropical Africa	The fruit has an intense orange color . It has a good juice content and its pulp is juicy and tender, the skin is easy to peel, characteristic of all clementines.	
Sakasli	Discovered in Tunisia	The quality of blood maltese is better than other varieties ripening at the same time. An early maturing, low acid variety which is reported to have more decay and ships less well.	
Sanguinelli	Spain	It is a semi-late blood variety with a deep purplish red hue in both its pulp and epidermis that results from a spontaneous mutation of double fine. The medium-sized, thornless, and fruitful trees have the ability to produce the most intensely colored blood oranges, however they don't have as dark an interior as tarroco or moro.	
Taroco	Sicilia	It is a bloody variant, distinct from the Italian sanguinelli kind, with good-quality fruits that have a tendency to drop. A medium-sized, moderately prolific tree, tarocco.	

Table 2.1. (Continue) *The characteristics of the studied Citrus varieties (https-3)*

Tomatera		Clementine with a spherical and flattened shape, it releases an incredibly sweet juice with a low acidity rate .	
Valancia	Florida	Was thought to be a seedling from a paperrind-type sweet orange.	
Wagshintone Novel	Brazil	It is a variety of sugar naveltres, large caliber and long shape and very productive.	

2.2. Method

The genetic diversity between *Citrus* varieties were achieved by PCR technique using two different markers : ISSR and SCoT. The sequence of our study is as follows : DNA Isolation, measure of DNA purity and quantities, PCR amplification, agarose gel electrophoresis of PCR samples, gel visualisation, band analysis and finally statistical analysis.

2.2.1. DNA extraction

Genomic DNA from *Citrus* leaves was isolated by CTAB (Cetyl trimethylammonium bromide) method (Doyle and Doyle, 1990). The lysis buffer used in this method was prepared as indicated in (Table 2.2).

Table 2.2. *The components of CTAB extraction buffer*

Components	Quantity
NaCl	4.095 g
Tris 1M (pH=8)	5 ml
CTAB (Cetyl trimethylammonium bromide)	1 g
EDTA (Ethylenediaminetetraacetic acid) (0.5 M)	2 ml
PVP (Polyvynilpyrolidone)	0.5 g
Distilled water	is made up to 50 ml and autoclaved

After sterilization, 200 µl of β-mercaptoethanol was added to the buffer. The steps of the 2XCTAB method were given below:

- CTAB (Hexadecyltrimthylammonium bromide) buffer was preheated at 65°C.
- 100 mg of plant tissue was ground in liquid nitrogen and transferred to 2 ml eppendorf tube. 1000 µl of CTAB buffer was added on it and slowly pipetted until it becomes homogeneous.
- The mixing tube was incubated at 65°C for 30 minutes and the tubes were turned upside down at regular intervals.
- Equal volume of chloroform: isoamyl alcohol was added into the tube and inverted for 10 minutes.
- After the mixture was centrifuged at 10000 rpm for 10 minutes, the upper liquid part was transferred to a new 1.5 ml tube.
- Isopropanol was added 2/3 of the transferred amount from the previous step and kept at -20°C for 2 hours after several inversions.
- At the end of the incubation, the tube was centrifuged at 10,000 rpm for 10 minutes.
- The DNA pellet was washed with 300 µl of 70% ethanol twice and stored in a fume hood or outdoors until pellet dried.
- Dried DNA pellet was dissolved in sterile deionized water and kept in frezeer.

2.2.2. Measurement of DNA quantity and purity

Before PCR analysis, it is important and necessary to know the quality and concentration of DNA obtained from plant samples. For this reason, first 5 µl of each isolated DNA samples were loaded on a 0.8% agarose gel along with 1kb DNA ladder (Fermentas) and run at 100V for 30 min. After the run, the gel was photographed using

the UVIpro gel documentation system (Uvitec, Cambridge, UK). DNA isolation was repeated for the samples that appeared in the form of very faint or light paths on the agarose gel.

Purity and quantity determinations of the samples were accomplished with Nanodrop Spectrophotometer (ND-1000, Wilmington, USA) at 260 and 280 nm wavelengths. According to the DNA amounts calculated from the OD results, DNA samples were diluted with distilled water as 5 ng/µl for PCR studies.

2.2.3. PCR analysis

2.2.3.1. ISSR analysis

In this study, 10 ISSR primers previously available in our laboratory were used. PCR reactions were established with DNAs obtained from plant samples, negative control and relevant primers. Information on the ISSR primers used in this study was given in Table 2.3.

Table 2.3. *ISSR Primer sequences and annealing temperature*

Primer	T (C)°	Sequences
ISSR-4	50	ACACACACACACACACC
ISSR-6	50	GAGAGAGAGAGAGAGAC
ISSR-810	50	GAGAGAGAGAGAGAGAA
ISSR-851	55	GTGTGTGTGTGTGTGTYG
ISSR-847	52	CACACACACACACACARC
ISSR-309	48	GAGAGAGAGAGAGAGAA
ISSR-807	52	AGAGAGAGAGAGAGAGT
İSSR-855	53	ACACACACACACACACYT
ISSR-834	53	AGAGAGAGAGAGAGAGYT
ISSR-859	55	TGTGTGTGTGTGTGTGRC
ISSR-310	48	AGAGAGAGAGAGAGAGT

PCR reactions were prepared with the amounts indicated in Table 2.4 in a volume of 25 µl. PCR cycling conditions are given in (Table 2.5).

Table 2.4. *ISSR PCR Components*

Components	Volume
dH_2O	13.8 µl
10X Taq Buffer	2.5 µl (1X)
25mM $MgCl_2$	1. µl (2 µM)
2,5mM dNTP	2.5 µl (2.5 µM)
2,5mM Primer	2 µl (2.5 µM)
DNA	3 µl
Taq Polimeraz	0.2 µl
Total	25 µl

Table 2.5. *ISSR PCR cycle conditions*

	Temperature (°C)	Duration	Number of cycle
Predenaturation	94	4 min	1
Denaturation	94	45 s	45
Annealing	50-53	45 s	
Elongation	72	90 s	
Final elongation	72	7 min	1

ISSR PCR reactions were prepared in a total volume of 25 µl containing 10 ng/µl of DNA, 2.5 µl of 5 × PCR Buffer, 1 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs, 2 µl of 2 mM Primer and 0.2 µl *Taq* DNA polymerase (either Thermo or Biolabs). A negative control (without DNA) was included in each reaction for control of DNA contamination.

PCR reactions were performed with an Applied Biosystems Veriti gradient thermal cycler. PCR cycles started with a 4 min predenaturation at 94°C then 45 cycles of 45s at 94 °C, 45 s between 50-53 °C and 1.5 min at 72 °C. At the end of those cycles there was an extension for 7 min at 72 °C. PCR products were stored refrigerated at +4°C until run on gel.

2.2.3.2. SCOT analysis

In SCOT analysis 10 SCOT primers previously available in our laboratory were used. Information about these SCOT primers was given in (Table 2.6).

Table 2.6. *SCOT primers sequences and annealing temperature*

Primer	T(C°)	Sequences
SCoT-3	56	CAACAATGGCTACCACCG
SCoT -12	56	ACGACATGGCGAACCAACG
SCoT -15	52	ACGACATGGCGACCGCGA
SCoT -16	52	ACCATGGGCTACCACCGAC
SCoT -17	52	ACCATGGCTACCACCGAG
SCoT -18	54	ACCATGGCTACCACCGCC
SCoT -21	53	ACGACATGGCGACCCACA
SCoT -28	54	CCATGGCTACCACCGCCA
SCoT -32	52	CCATGGCTACCACCGCAC
SCoT -34	50	ACCATGGCTACCACCGCA

PCR reactions were prepared with the amounts indicated in (Table 2.7) in a volume of 25 µl. PCR cycling conditions were given in (Table 2.8).

Table 2.7. *SCOT PCR Components*

Components	Volume
dH_2O	8.3 µl
10X Taq Buffer	2.5 µl (1X)
25mM $MgCl_2$	2 µl (2 µM)
2,5mM dNTP	2 µl (2.5 µM)
2,5mM Primer	5 µl (2.5 µM)
DNA	5 µl
Taq Polimeraz	0.2 µl
Total	25 µl

Table 2.8. *SCOT PCR cycle conditions*

	Temperature (°C)	Duration	Number of cycle
Predenaturation	94	3 min	1
Denaturation	94	1 min	
Annealing	50-53	1min	35
Elongation	72	2 min	
Final elongation	72	5 min	1

SCOT PCR reactions (25 µl) contained 50 ng/µl of DNA, 2.5 µl of 5 × PCR Buffer, 2 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs, 5 µl of 2 mM Primer and 0.2 µl Taq DNA polymerase (Thermo or Biolabs). A negative control (without DNA) was included in each reaction for control of DNA contamination.

PCR reactions were performed with an Applied Biosystems Veriti gradient thermal cycler. PCR cycles started with a 3 min predenaturation at 94°C then 35 cycles of 1 min

at 94 °C, 1 min between 50-53 °C (according to the primer's annealing temp.) and 2 min at 72 °C. At the end of those cycles there was an extension for 5 min at 72 °C. PCR products were stored refrigerated at +4°C until run on gel.

2.2.4. Agarose gel electrophoresis of PCR products

PCR products were separated on a 1.5% agarose gel containing Redsafe (Intron Bio) by horizontal gel electrophoresis (Thermo, Midicell Promo). PCR products were loaded as 6 µl PCR sample and 2 µl of 6X Loading Dye (Thermo) to each well. In order to determine the band sizes two different DNA ladders were used depending on the availability in the lab: 100 bp Plus DNA ladder (Thermo) and 100 bp DNA ladder (ABM) (Figures 2.1, 2.2). Approximately 3 µl of DNA ladder was loaded into the first and the last wells. The gel then was run at 100 V for 40-50 min in a gel tank (Thermo, Midicell Primo). Gels were visualized and photographed with UV documentation system (Uvitec, Cambridge, UK).

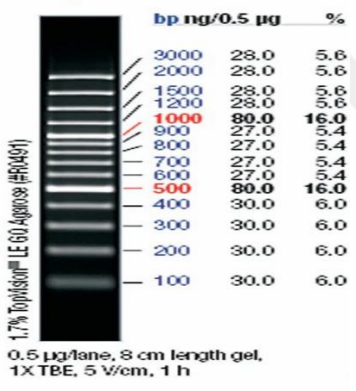

Figure 2.1. *Thermo GeneRuler 100 bp plus*

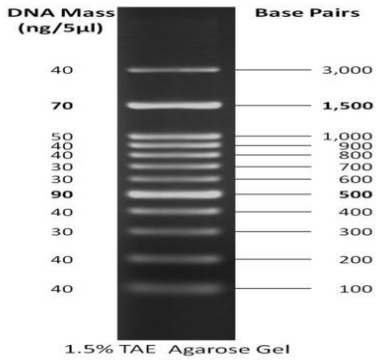

Figure 2.2. *ABM Opti-DNA markers 100bp plus*

2.2.5. Data analysis results

The bands were analyzed with the Total Lab CLIQS 1D program by using gel photograph of each primer. A binary matrix was prepared by counting the bands either present (1) or absent (0). The "polymorphism information content" (PIC) of the primers used in the study was calculated with the help of the following formula:

PIC = $1- \sum (p_{ij})^2$ (Smith et al., 1997). In this formula, p_i and p_j are indicated the frequency of alleles i,and j, respectively. This calculation was used to find PIC value of each band amplified with a specific primer and then average value was calculated.

The genetic similarity values between *Citrus* varieties were calculated by Jaccard coefficient using the DendroUPGMA program. Dendrograms for both ISSR and SCOT markers were constructed using the MEGA software version 11.

3. RESULTS

3.1. Measurement Of DNA Quantity And Purity Following DNA Isolation

In this study, DNA was isolated from 36 different *Citrus* varieties by the CTAB method. The quantity and purity measurements of the isolated DNA samples were carried out by spectrophotometry at wavelengths of 260 and 280 nm. The results were shown in Table 3.1.

Table 3.1. *The quantity and purity measurements of DNA samples*

Sample Name	DNA purity (260/280 nm)	DNA quantity (ng/µl)
Aforer	2.01	263.4
Bigarde	1.99	107.2
Citronier beldi	1.90	67.1
Clementina cassar	1.94	94.5
Eureka	1.91	135.5
Hernandina	1.94	101.1
Kaffin	1.93	191
Kumkuat	1.52	29
Leunari	2.06	159.4
Lime douce	2.0	111
Ma3(nour)	1.69	169.8
Maltese balerin	1.75	137.2
Maltese demi sanguin	1.90	148.2
Mariana	1.97	127.2
Marisol	2.0	204.1
Mapo	1.96	176.4
Meski ansli	1.1	136.8
Meski malti	1.9	20.5
Moro	1.89	65
New hall	1.5	36.6
Nova	1.85	137.1
Nules	1.92	192.7
Oranger double fine	1.48	119.8
Oranger lane late	1.12	76.1
Oranger novalina	1.61	147.5
Orograndi	1.98	219.5
Panacher	1.98	90.8
Pomplomose star ruby	2.07	118.2
Powel	1.99	99.7
Rubes	2.04	278.4
Sanguinelli	1.53	137.3

Table 3.1. (Continue) *The quantity and purity measurements of DNA samples*

Taroco	2.09	103
Tomatera	1.83	99
Valancia	2	113
Washintone novel	1.94	211

3.2. PCR Analysis

PCR analyses were performed using 10 ISSR and 10 SCOT primers with DNA samples of 36 different *Citrus* varieties.

3.2.1. ISSR PCR analysis

For ISSR analyses we have previously screened 15 ISSR primers. Only 10 of them produced reproducible banding pattern, others either did not amplified bands or the bands very faint. For this reason we excluded these primers and continue our study with 10 ISSR primers giving bright and reliable bands.

PCR results obtained from 10 different ISSR primers were loaded in agarose gels and run along with 100 bp plus DNA ladder. The gels were photographed digitally and given in figures 3.1-3.10.

Gel images of PCR reactions represent banding patterns of 36 *Citrus* varieties: 1/Aforer-2 /Bigarde-3/Citronier Beldi-4/Clementina Cassar-5/Eureka-6/Hernandina-7/ Kafin-8/ Kumkuat-9/Leunari-10/Lim Douce-11/Ma3(Nour)-12/Maltese Balerin-13/Maltese Demi Sanguin-14/Mariana-15/Marisol-16/Mapo-17/Meski Ansli-18/Meski Malti-19/Moro-20/New Hall-21/Nova-22/Nules-23/Oranger Double Fine-24/Oranger Lane Late-25/Oranger Novalina-26/Orograndi-27/Panacher-28/Pomplomose Star Ruby-29/Powel-30/Rubes-31/Sakasli-32/Sanguinelli-33/Taroco-34/Tomatera-35/Valancia-36/Washintone Novel , which were loaded in the same order on all agarose gels

ISSR-4: Figure 3.1 shows that ISSR-4 primer amplified 36 bands in total, the largest band was 2532 bp and the smallest band was 443 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

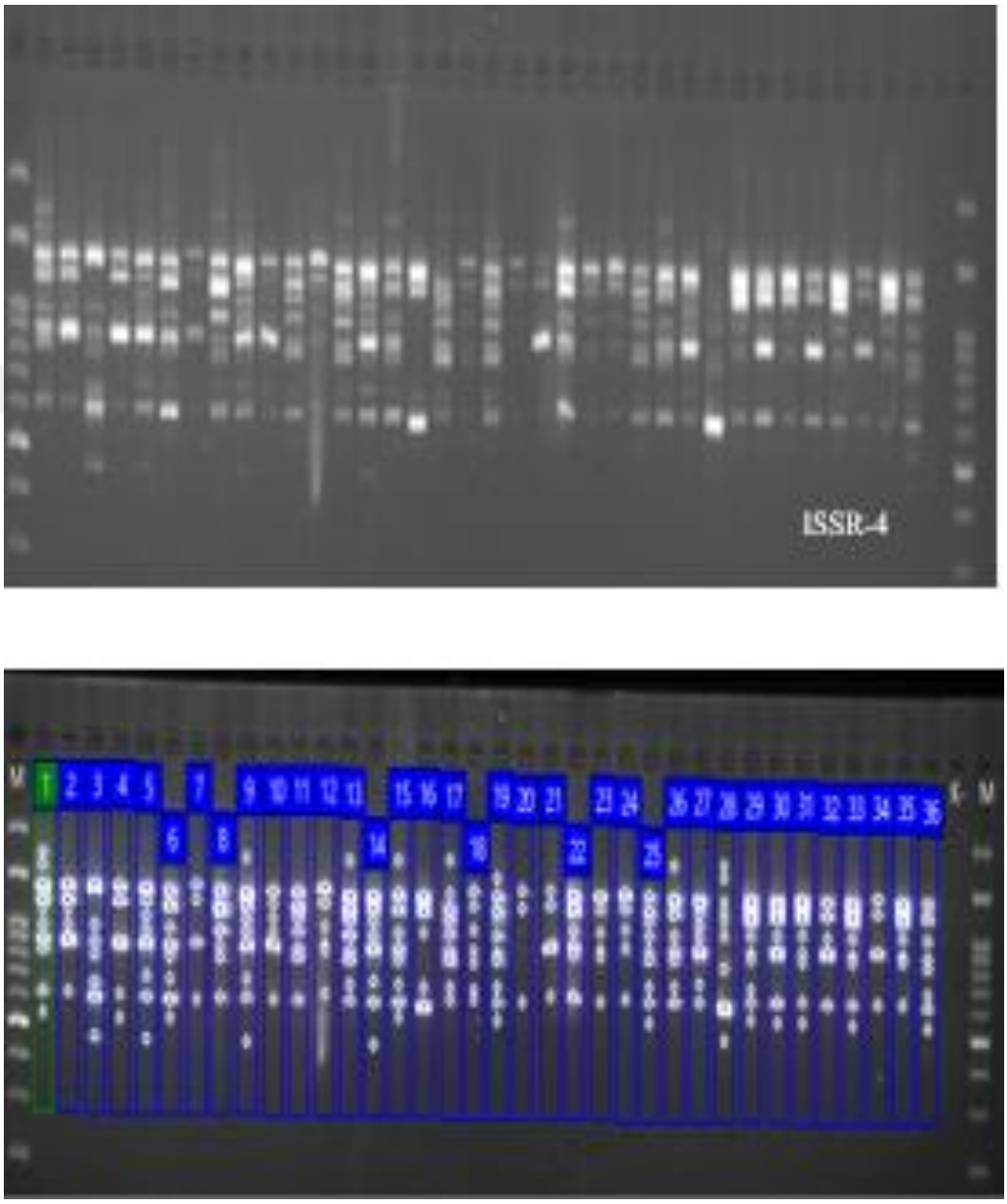

Figure 3.1. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-4 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

ISSR-6: Figure 3.2 shows that ISSR-6 primer amplified 23 bands in total, the largest band was about 3888 bp and the smallest band was 431 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

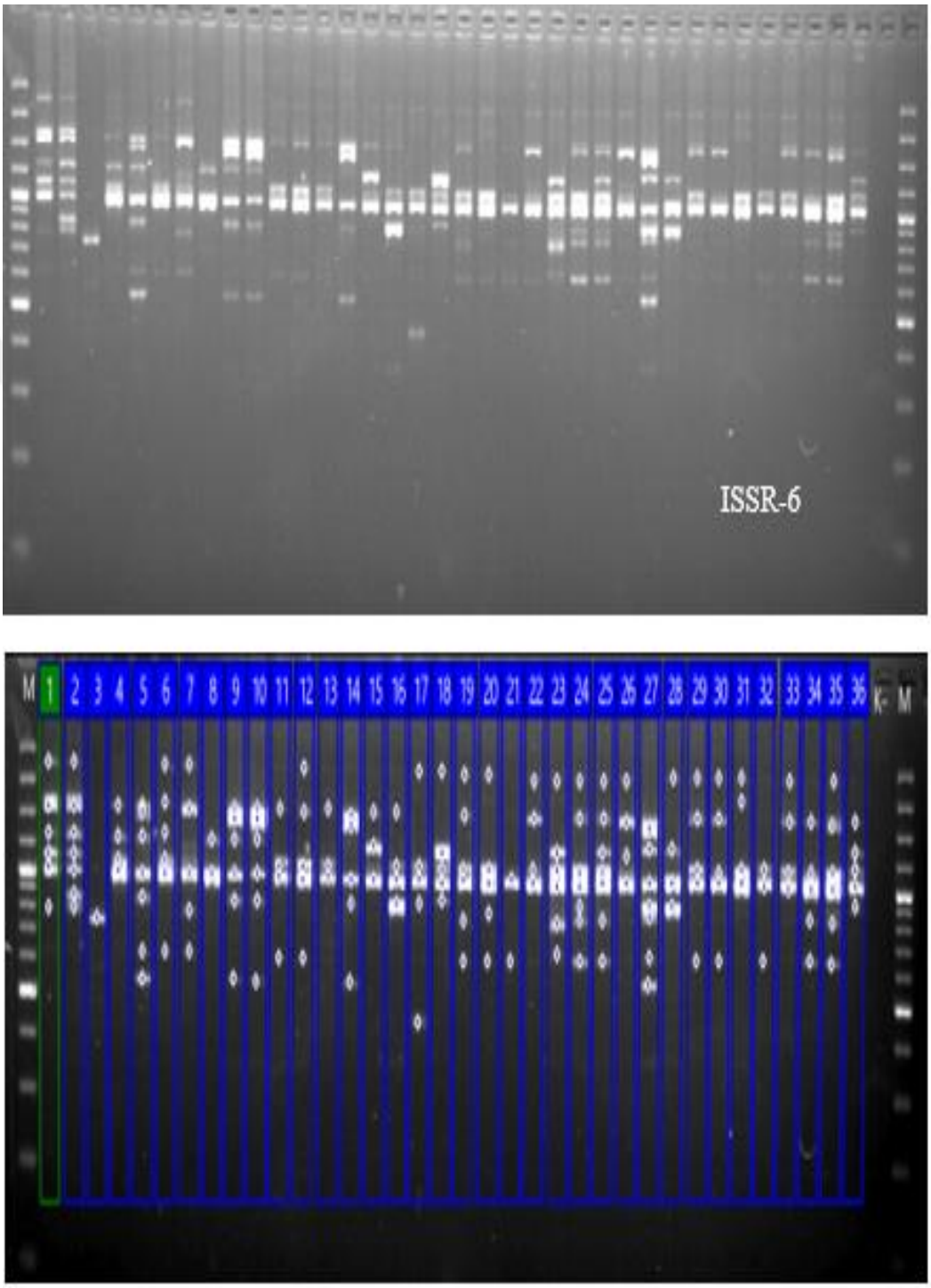

Figure 3.2. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-6 primer (A). Bands detected with Totallab Cliqs program (B). M:100 bp plus marker; K-: - control*

ISSR-309: Figure 3.3 shows that ISSR-309 primer amplified 31 bands in total , the largest band was about 1667 bp and the smallest band was 375 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

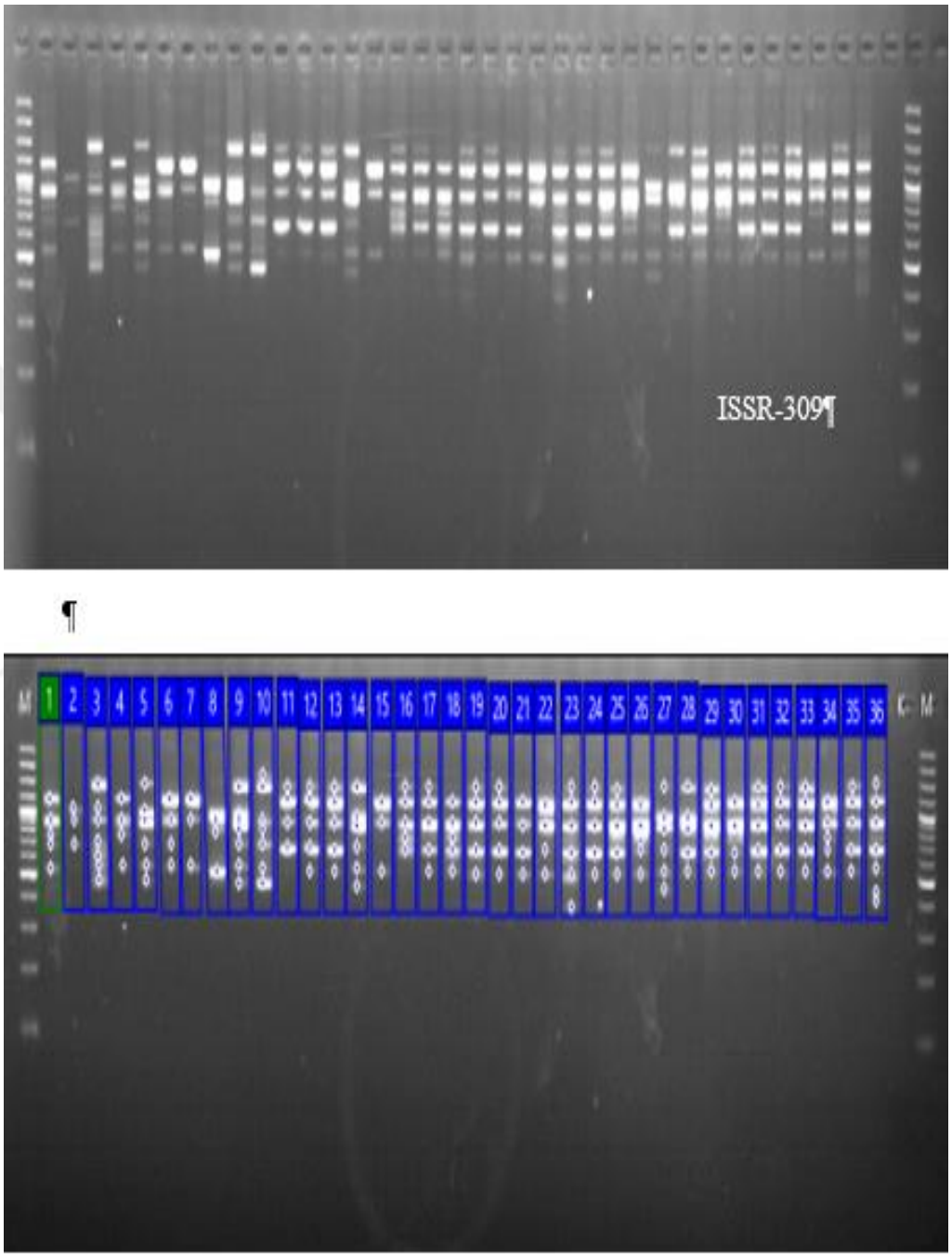

Figure 3.3. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-309 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: control.*

ISSR-310: Figure 3.4 shows that ISSR-310 primer amplified 34 bands in total, the largest band was about 2454 bp and the smallest band was 254 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

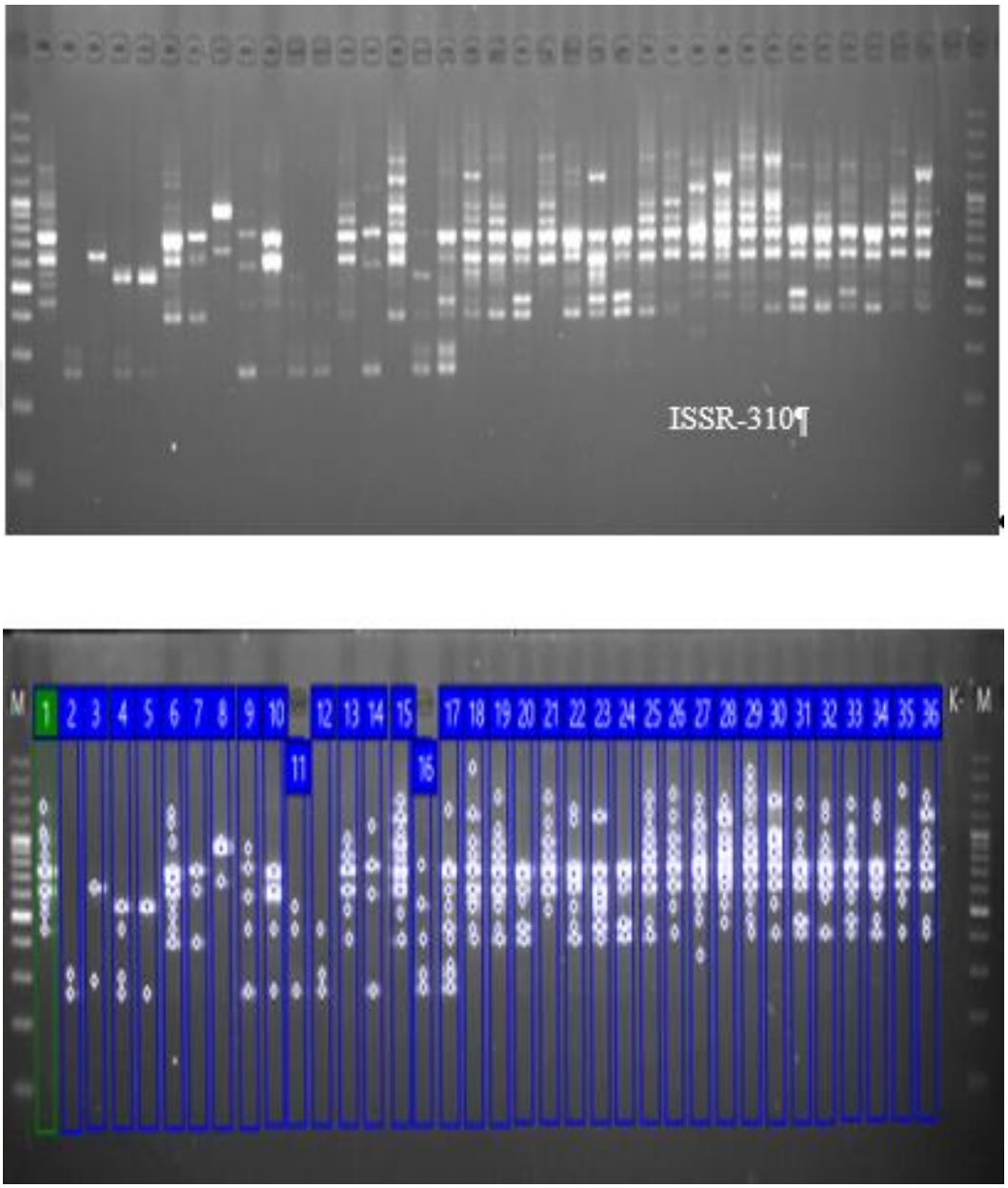

Figure 3.4. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-310 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

ISSR-807: Figure 3.5 shows that ISSR-807 primer amplified 21 bands in total, one was monomorphic, the others were polymorphic. The largest band was 2817 bp and the smallest band was 412 bp. The polymorphism rate of this primer was 95.2%.

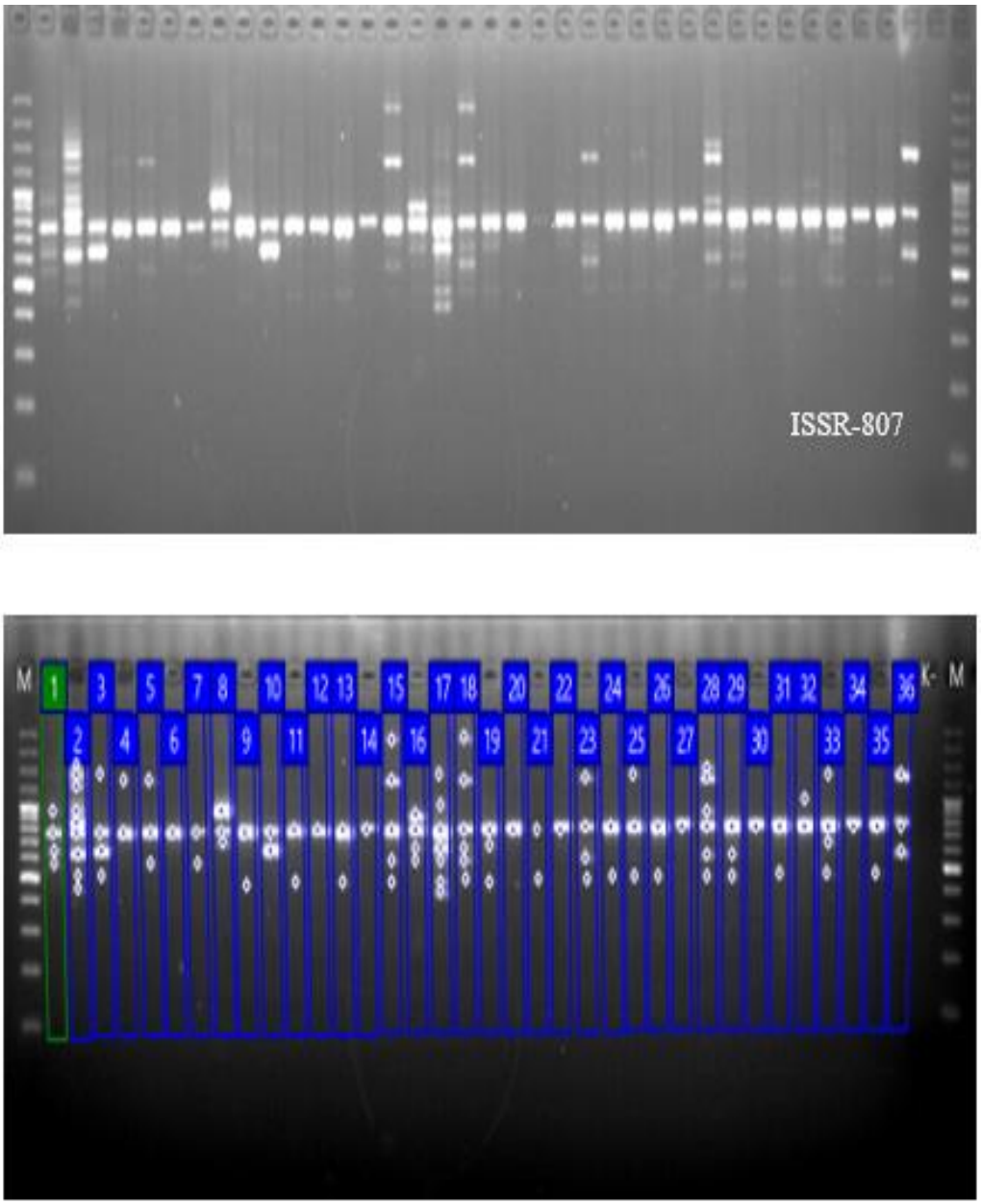

Figure 3.5. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-807 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

ISSR-810: Figure 3.6 shows that ISSR-810 primer amplified 22 bands in total, one was monomorphic, the others were polymorphic which gave 95.4% polymorphism. The largest band was 2858 bp and the smallest band was 452 bp.

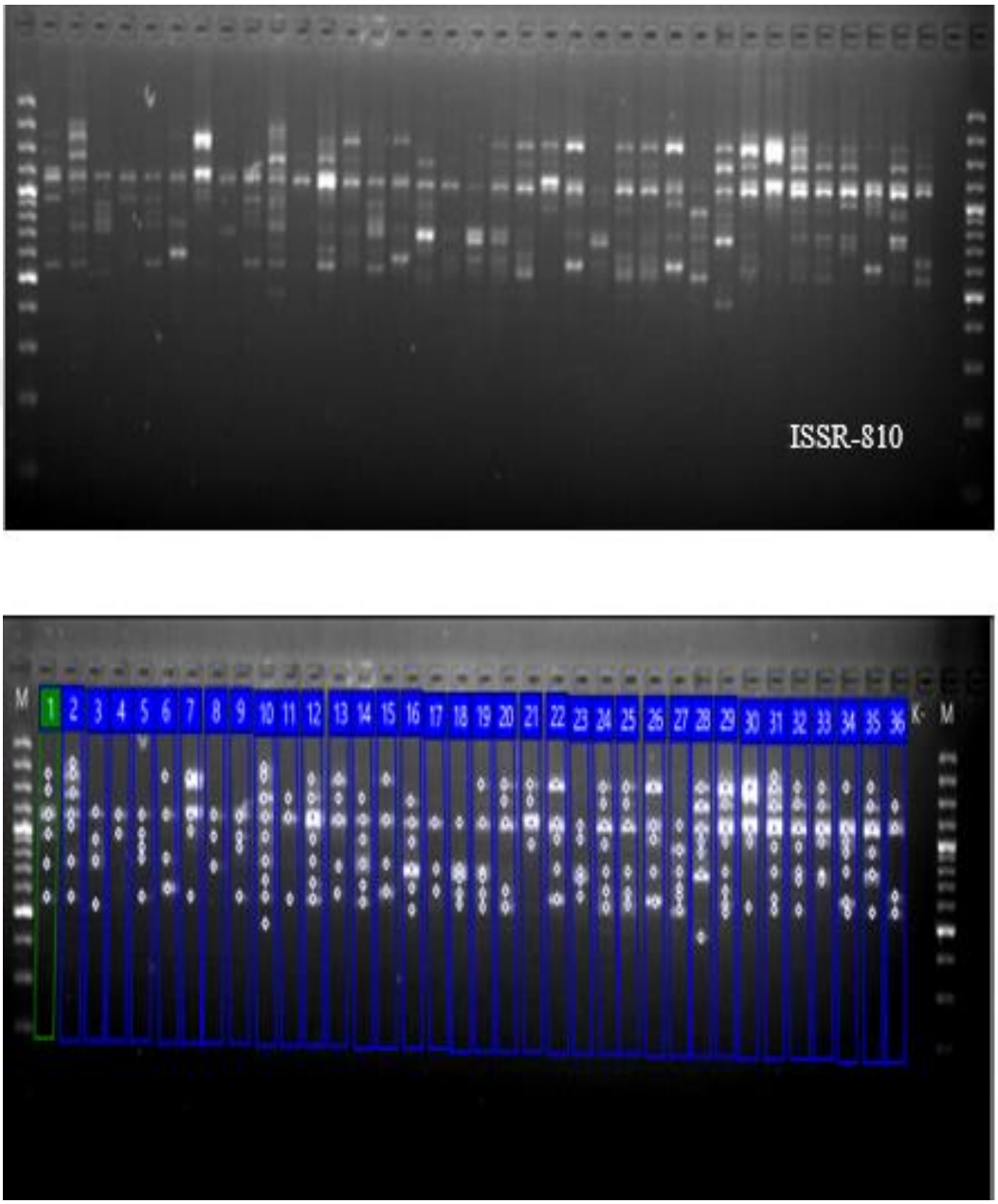

Figure 3.6. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-810 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

ISSR-843: Figure 3.7 shows that ISSR-843 primer amplified 39 bands in total, the largest band was about 2349 bp and the smallest band was 300 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

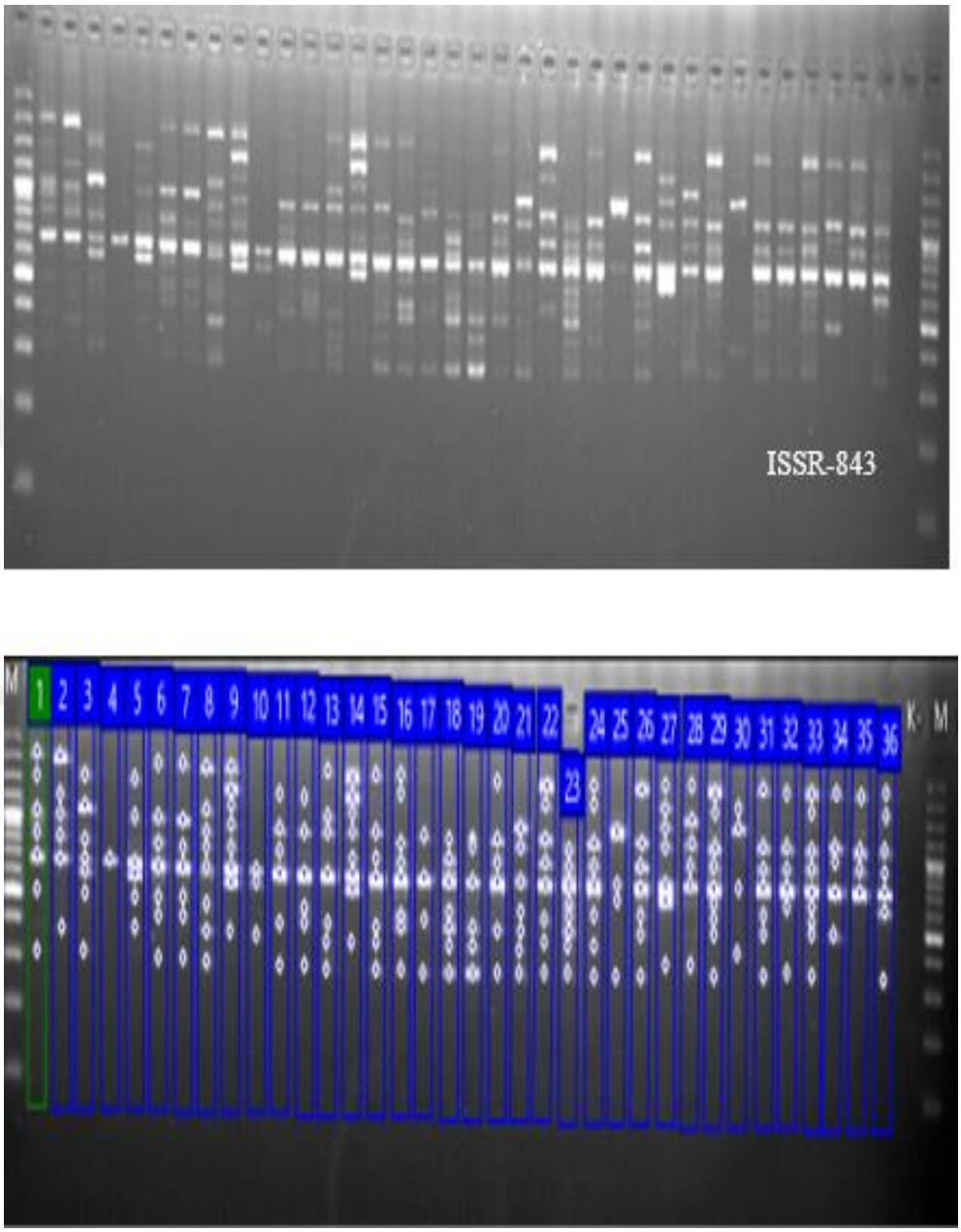

Figure 3.7. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-843 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

ISSR-847: Figure 3.8 shows that ISSR-847 primer amplified 20 bands in total, the largest band was about 1915 bp and the smallest band was 512 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

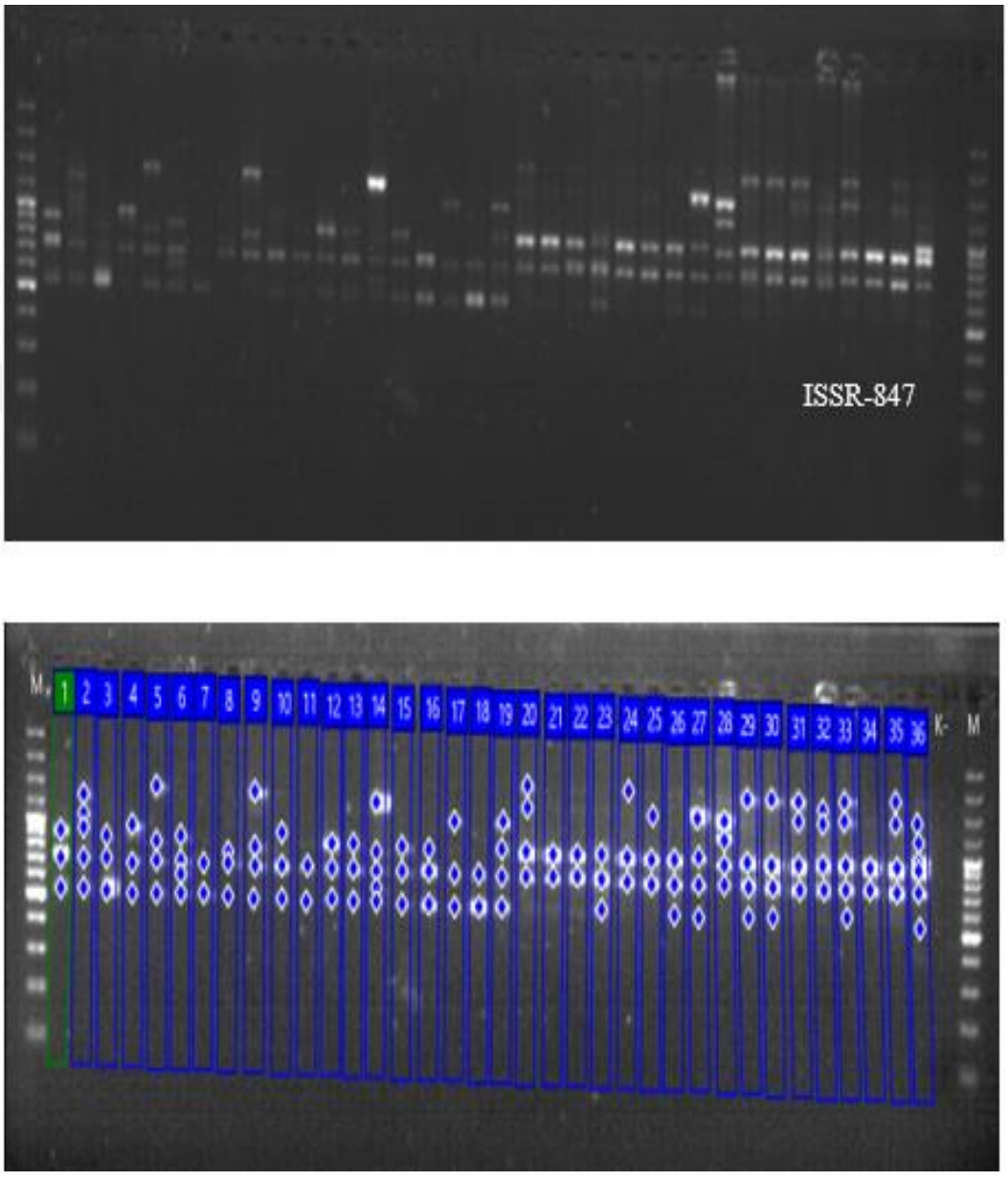

Figure 3.8. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-847 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

ISSR-851: Figure 3.9 shows that ISSR-851 primer amplified 25 bands in total, the largest band was about 1819 bp and the smallest band was 390 bp, all bands were polymorphic which gave 100% polymorphism for ISSR-851 primer.

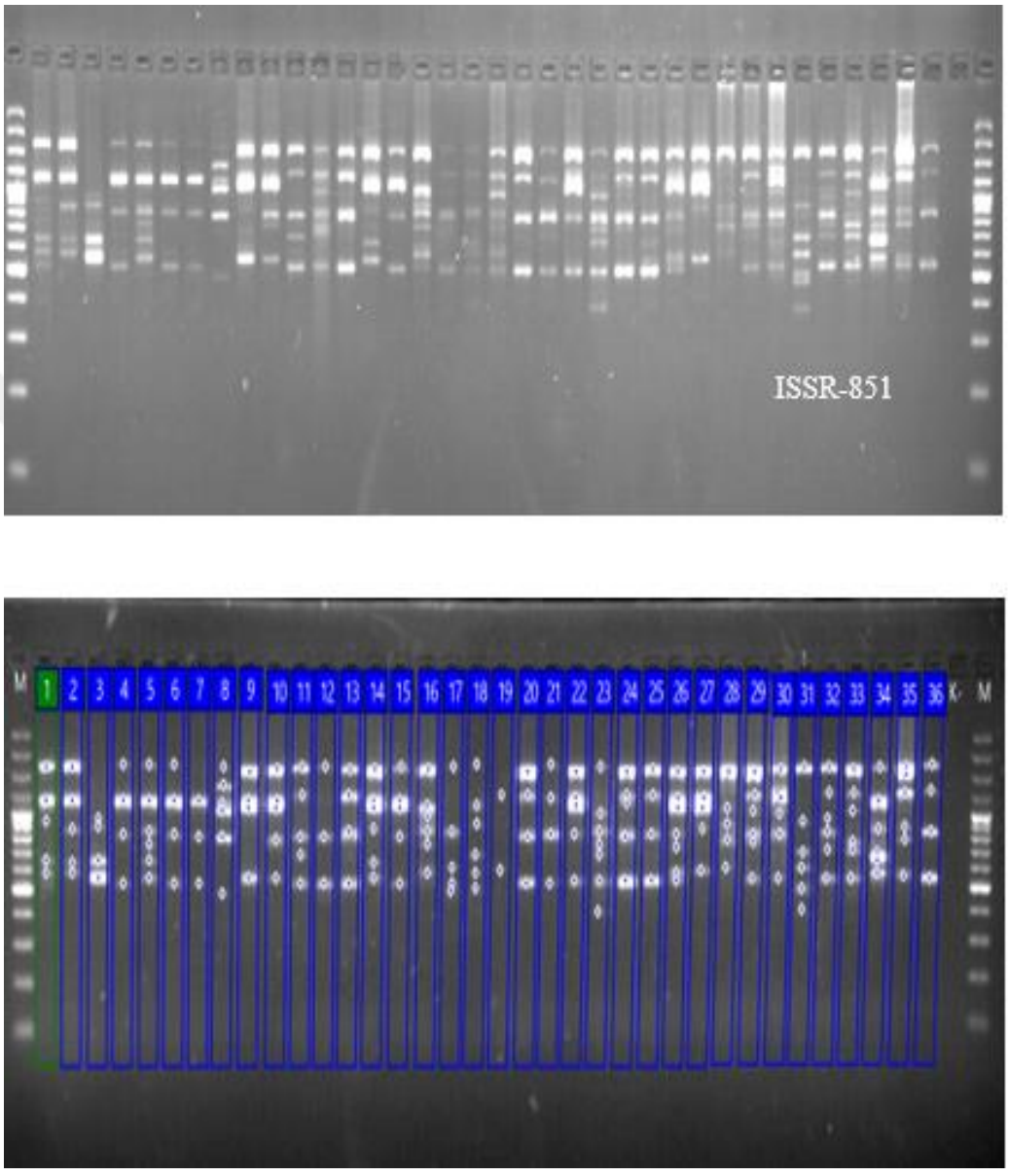

Figure 3.9. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-851 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

ISSR-855: Figure 3.10 shows that ISSR-855 primer amplified 19 bands in total, the largest band was about 2509 bp and the smallest band was 371 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

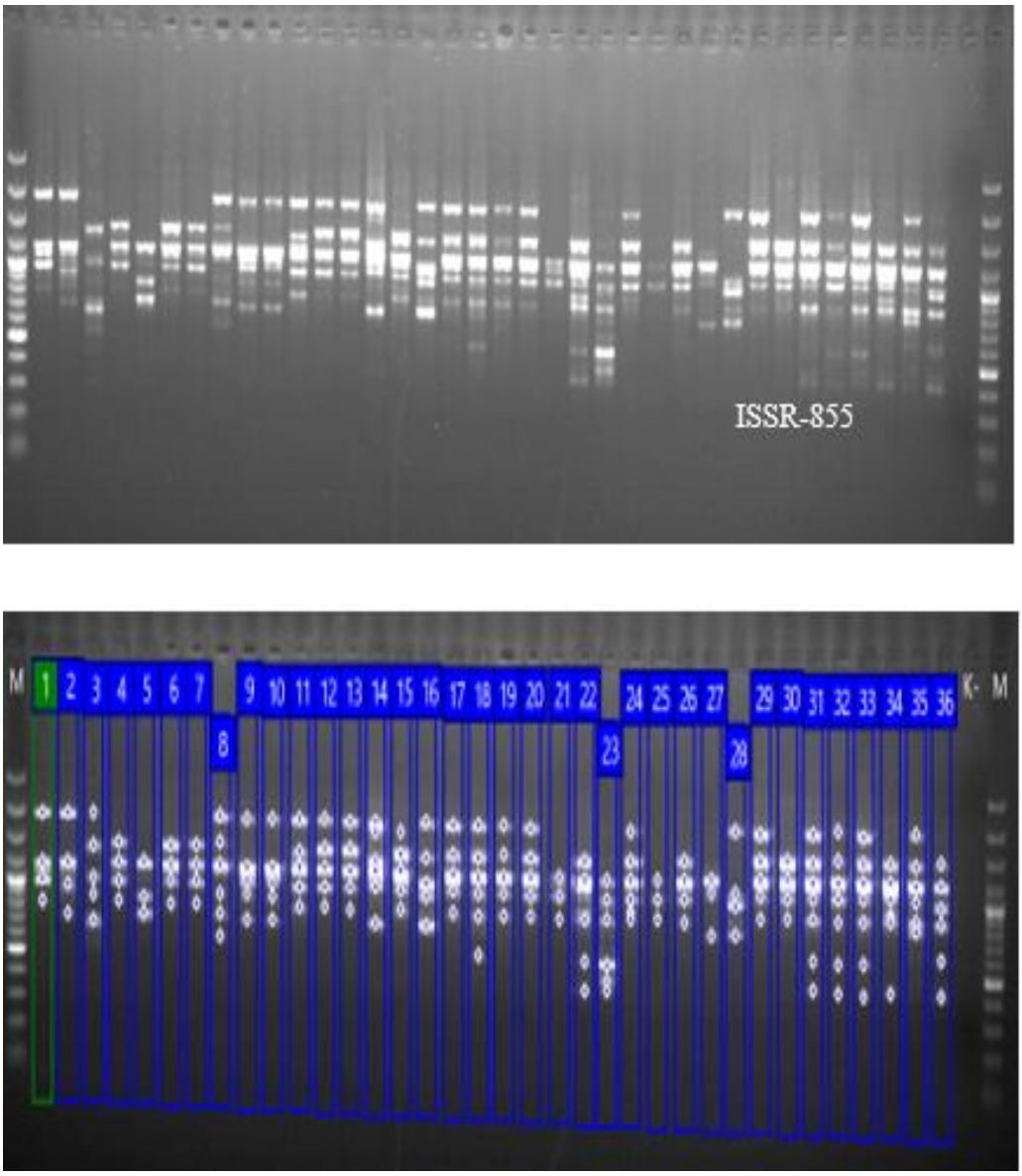

Figure 3.10. *PCR results showing band patterns between 36 Citrus varieties (1-36) with ISSR-855 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

3.2.1. Assessment of ISSR analysis

Bands obtained using 10 ISSR primers were scored as present (1) or absent (0) using Phoretix 1D Pro software and a binary matrix was produced. The analysis of ISSR patterns shows that all the 10 primers amplified reproducible and easily interpretable banding pattern. A total of 270 bands was generated from the 36 *Citrus* varieties studied with an average of 27 bands per primer. Among these bands 268 were polymorphic (99.2%). The size of the amplified bands ranged from 254 bp (ISSR-310) to 2858 bp (ISSR -810). The number of bands per primer ranged from 19 for the ISSR -855 primer to 39 for ISSR-843 primer

The polymorphism information content (PIC) ranged from 0.150 (ISSR-807) to 0.297 (ISSR-855) with an average of 0.249 (Table 3.2).

Table 3.2. *Characteristics of bands resulted from 10 ISSR primers*

Primer	TA (C°)	Band size (bp)	TB	MB	PIC	PPB
ISSR-4	50	(443-2532)	36	0	0.269	100%
ISSR-6	50	(431-3888)	23	0	0.288	100%
ISSR-309	48	(375-1667)	31	0	0.215	100%
ISSR-310	48	(254-2454)	34	0	0.276	100%
ISSR-807	52	(412-2817)	21	1	0.150	95.2%
ISSR-810	50	(452-2858)	22	1	0.250	95.4%
ISSR-843	53	(300-2349)	39	0	0.242	100%
ISSR-847	52	(512-1915)	20	0	0.235	100%
ISSR-851	55	(390-1819)	25	0	0.271	100%
ISSR-855	53	(371-2509)	19	0	0.297	100%

TA: *Annealing Temperture,* **TB***: Total number of bands;* **MB**: *Monomorphic bands;* **PIC:** *Polymorphism Information content;* **PPB**: *Percentage of polymorphic bands*

Genetic similarity matrix (Table 3.3) between *Citrus* varieties was calculated based on Jaccard's similarity coefficient. The genetically closest varieties were found to be as Powell and Novalina, with a genetic similarity coefficient of 0.545. The cultivars with the lowest genetic similarity were found to be 0.102 between varieties Aforer and Panacher.

Table 3.3. *Genetic similarity matrix between Citrus varieties based on Jaccard's similarity coefficient*.

	EUREKA	LEUNARI	LIMEDOUCE	CITRONIERBELDI	PANACHER	AFORER	HERNANDINA	KAFFIN	MA3	NOVA	MARISOL	CLEMENTINACASSAR	MAPO	NULES	OROGRANDI	RUBIS	TOMATERA	VALANCIA	ORANGERNOVALINA	ORANGERLANELATE	WASHINTONENOVEL	NEWHALL	POWEL	MALTESEBALERIN	MALTESEDEMISANGUIN	SANGUINELLI	TAROCO	MORO	ORANGERD	SAKASLI	MESKIANSLI	MESKIMALTI	BIGRADIER	KUMKUAT	POMPLOM	MARIANA
EUREKA	1	0.4	0.365	0.274	0.178	0.195	0.195	0.236	0.286	0.165	0.178	0.217	0.179	0.174	0.191	0.169	0.146	0.157	0.169	0.209	0.125	0.159	0.158	0.219	0.188	0.198	0.188	0.161	0.22	0.149	0.151	0.155	0.198	0.173	0.109	0.377
LEUNARI		1	0.387	0.215	0.198	0.216	0.216	0.195	0.274	0.131	0.198	0.208	0.229	0.209	0.185	0.205	0.193	0.152	0.151	0.202	0.144	0.195	0.165	0.15	0.153	0.165	0.136	0.121	0.129	0.133	0.134	0.15	0.205	0.241	0.106	0.416
LIMEDOUCE			1	0.211	0.167	0.157	0.212	0.205	0.254	0.138	0.207	0.221	0.181	0.149	0.167	0.157	0.161	0.172	0.17	0.17	0.151	0.16	0.172	0.239	0.221	0.186	0.189	0.189	0.146	0.202	0.128	0.168	0.229	0.16	0.156	0.364
CITRONIERBELDI				1	0.09	0.188	0.173	0.143	0.209	0.122	0.155	0.136	0.184	0.15	0.141	0.128	0.12	0.133	0.118	0.131	0.112	0.147	0.113	0.127	0.147	0.175	0.193	0.151	0.159	0.165	0.181	0.17	0.146	0.194	0.132	0.183
PANACHER					1	0.102	0.125	0.094	0.106	0.157	0.183	0.128	0.144	0.193	0.196	0.174	0.191	0.244	0.227	0.213	0.155	0.179	0.26	0.134	0.151	0.23	0.18	0.231	0.211	0.217	0.144	0.16	0.115	0.1	0.318	0.222
AFORER						1	0.342	0.338	0.278	0.288	0.317	0.303	0.485	0.355	0.333	0.253	0.268	0.207	0.191	0.218	0.196	0.244	0.24	0.152	0.169	0.154	0.221	0.17	0.177	0.183	0.253	0.226	0.235	0.244	0.213	0.161
HERNANDINA							1	0.517	0.373	0.343	0.543	0.458	0.347	0.373	0.385	0.269	0.238	0.18	0.247	0.247	0.158	0.276	0.253	0.197	0.276	0.18	0.196	0.222	0.177	0.158	0.198	0.267	0.154	0.26	0.163	0.137
KAFFIN								1	0.426	0.386	0.476	0.42	0.365	0.354	0.329	0.333	0.29	0.2	0.264	0.319	0.173	0.367	0.253	0.226	0.224	0.154	0.174	0.188	0.21	0.159	0.237	0.207	0.2	0.188	0.1	0.163
MA3									1	0.356	0.382	0.469	0.426	0.328	0.324	0.288	0.216	0.213	0.243	0.26	0.185	0.317	0.25	0.283	0.277	0.152	0.172	0.231	0.193	0.185	0.25	0.235	0.167	0.203	0.111	0.253
NOVA										1	0.371	0.345	0.328	0.379	0.391	0.381	0.333	0.257	0.403	0.343	0.225	0.349	0.305	0.234	0.308	0.192	0.182	0.273	0.217	0.21	0.212	0.244	0.134	0.197	0.159	0.116
MARISOL											1	0.354	0.373	0.364	0.41	0.365	0.293	0.202	0.286	0.271	0.217	0.286	0.274	0.177	0.32	0.176	0.204	0.273	0.237	0.191	0.261	0.303	0.163	0.237	0.184	0.183
CLEMENTINACASSAR												1	0.397	0.317	0.313	0.295	0.254	0.232	0.194	0.211	0.2	0.242	0.222	0.224	0.167	0.149	0.171	0.169	0.177	0.139	0.236	0.221	0.181	0.222	0.106	0.143
MAPO													1	0.38	0.338	0.253	0.237	0.235	0.217	0.263	0.18	0.333	0.253	0.246	0.296	0.163	0.194	0.265	0.2	0.167	0.3	0.239	0.176	0.26	0.16	0.198
NULES														1	0.522	0.352	0.384	0.308	0.355	0.411	0.259	0.362	0.349	0.173	0.221	0.259	0.27	0.216	0.31	0.23	0.262	0.233	0.146	0.175	0.156	0.154
OROGRANDI															1	0.403	0.359	0.274	0.403	0.317	0.244	0.286	0.424	0.208	0.286	0.274	0.283	0.273	0.337	0.258	0.22	0.275	0.176	0.151	0.184	0.158
RUBIS																1	0.366	0.324	0.32	0.356	0.256	0.304	0.4	0.217	0.25	0.273	0.313	0.241	0.277	0.272	0.214	0.216	0.167	0.216	0.138	0.148
TOMATERA																	1	0.287	0.268	0.284	0.271	0.284	0.286	0.236	0.203	0.287	0.253	0.213	0.291	0.286	0.202	0.258	0.198	0.173	0.12	0.191
VALANCIA																		1	0.4	0.419	0.434	0.333	0.405	0.304	0.28	0.387	0.337	0.282	0.302	0.362	0.2	0.215	0.169	0.171	0.189	0.163
ORANGERNOVALINA																			1	0.536	0.41	0.47	0.545	0.264	0.311	0.312	0.275	0.375	0.329	0.31	0.239	0.253	0.154	0.128	0.2	0.174
ORANGERLANELATE																				1	0.358	0.59	0.469	0.3	0.311	0.296	0.275	0.325	0.395	0.31	0.267	0.267	0.167	0.155	0.2	0.161
WASHINTONENOVEL																					1	0.329	0.352	0.203	0.202	0.267	0.304	0.213	0.245	0.239	0.215	0.192	0.135	0.148	0.157	0.143
NEWHALL																						1	0.447	0.281	0.294	0.215	0.274	0.365	0.268	0.247	0.316	0.265	0.171	0.158	0.193	0.193
POWEL																							1	0.268	0.294	0.311	0.402	0.367	0.34	0.337	0.258	0.27	0.135	0.122	0.233	0.175
MALTESEBALERIN																								1	0.464	0.324	0.329	0.397	0.289	0.357	0.221	0.269	0.154	0.139	0.138	0.192
MALTESEDEMISANGUIN																									1	0.371	0.321	0.464	0.351	0.347	0.266	0.312	0.157	0.173	0.129	0.165
SANGUINELLI																										1	0.513	0.329	0.366	0.514	0.213	0.256	0.143	0.143	0.189	0.138
TAROCO																											1	0.379	0.352	0.519	0.227	0.216	0.162	0.176	0.228	0.146
MORO																												1	0.345	0.357	0.299	0.341	0.135	0.135	0.204	0.155
ORANGERDOUBLEFINE																													1	0.36	0.275	0.33	0.131	0.118	0.186	0.139
SAKASLI																														1	0.242	0.269	0.172	0.11	0.229	0.12
MESKIANSLI																															1	0.393	0.174	0.163	0.17	0.156
MESKIMALTI																																1	0.119	0.193	0.208	0.149
BIGRADIER																																	1	0.171	0.153	0.189
KUMKUAT																																		1	0.154	0.151
POMPLOMOSESTARRUBY																																			1	0.115
MARIANA																																				1

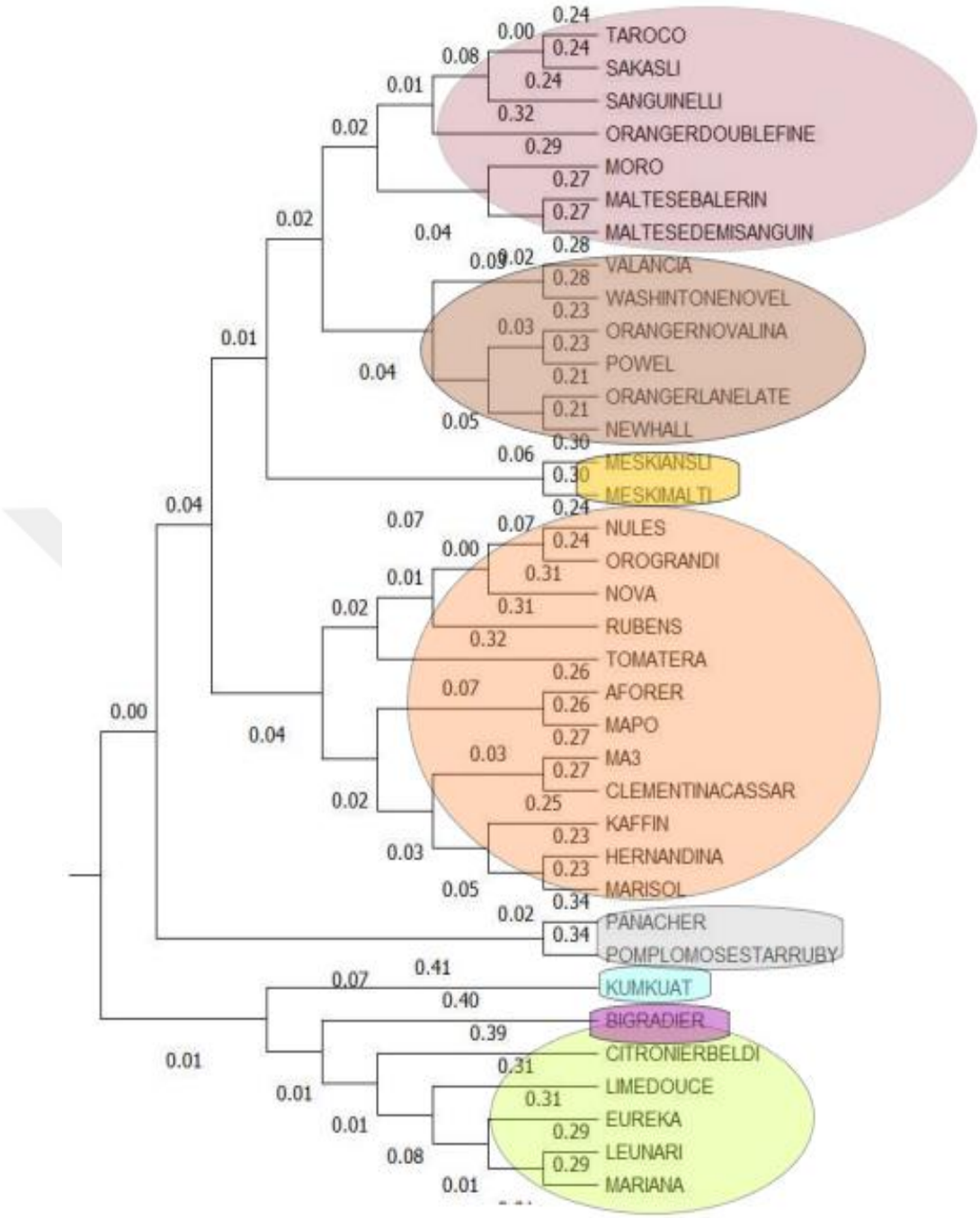

Figure 3.11. *Genetic similarity dendrogram of 36 Citrus varieties using ISSR markers*

The 1-0 binary matrix was transferred to the Dendro:UPGMA program, where the genetic similarity values were calculated. Then, the results obtained were transferred to the MEGA program in Newick format and the genetic tree was formed.

According to the dendrogram, 2 main clusters were formed. The first cluster included the varieties Lemons, Bigarde, Kumkuat; the second main cluster composed of 2 subgroups; the first subgroup contained Panacher and Star Ruby and second subgroup included orange and mandarine varieties. This second large group also further divided into subgroups that contained blood orange, sweet orange and white orange varieties, respectively.

3.2.2. SCOT PCR analysis

For SCOT analyses we have previously screened 13 SCOT primers. Only 10 of them produced reproducible banding pattern, others either did not amplified bands or the bands very faint. For this reason we excluded these primers and continue our study with 10 SCOT primers giving bright and reliable bands.

PCR results obtained from 10 different SCOT primers were loaded in agarose gels and run along with 100 bp plus DNA ladder. PCR results obtained from 10 different SCoT primers were given in figures 3.12-3.21.

Gel images of PCR reactions represent banding patterns of *Citrus* varieties: 1/Aforer-2 /Bigarde-3/Citronier Beldi-4/Clementina Cassar-5/Eureka-6/Hernandina-7/ Kafin-8/ Kumkuat-9/Leunarise-10/Lim Douce-11/Ma3(Nour)-12/Maltese Balerin-13/Maltese Demi Sanguin-14/Mariana-15/Marisol-16/Mapo-17/Meski Ansli-18/Meski Malti-19/Moro-20/New Hall-21/Nova-22/Nules-23/Oranger Double Fine-24/Oranger Lane Late-25/Oranger Novalina-26/Orograndi-27/Panacher-28/PomplomoseStarRuby-29/Powel-30/Rubes-31/Sakasli 32/Sanguinelli-33/Taroco-34/Tomatera-35/Valancia-36/Washintone Novel

SCOT-3: Figure 3.12 shows that SCoT-3 primer amplified 22 bands in total, the largest band was about 3375 bp and the smallest band was 421 bp, one band was monomorphic, the others were polymorphic which gave 95.4% polymorphism.

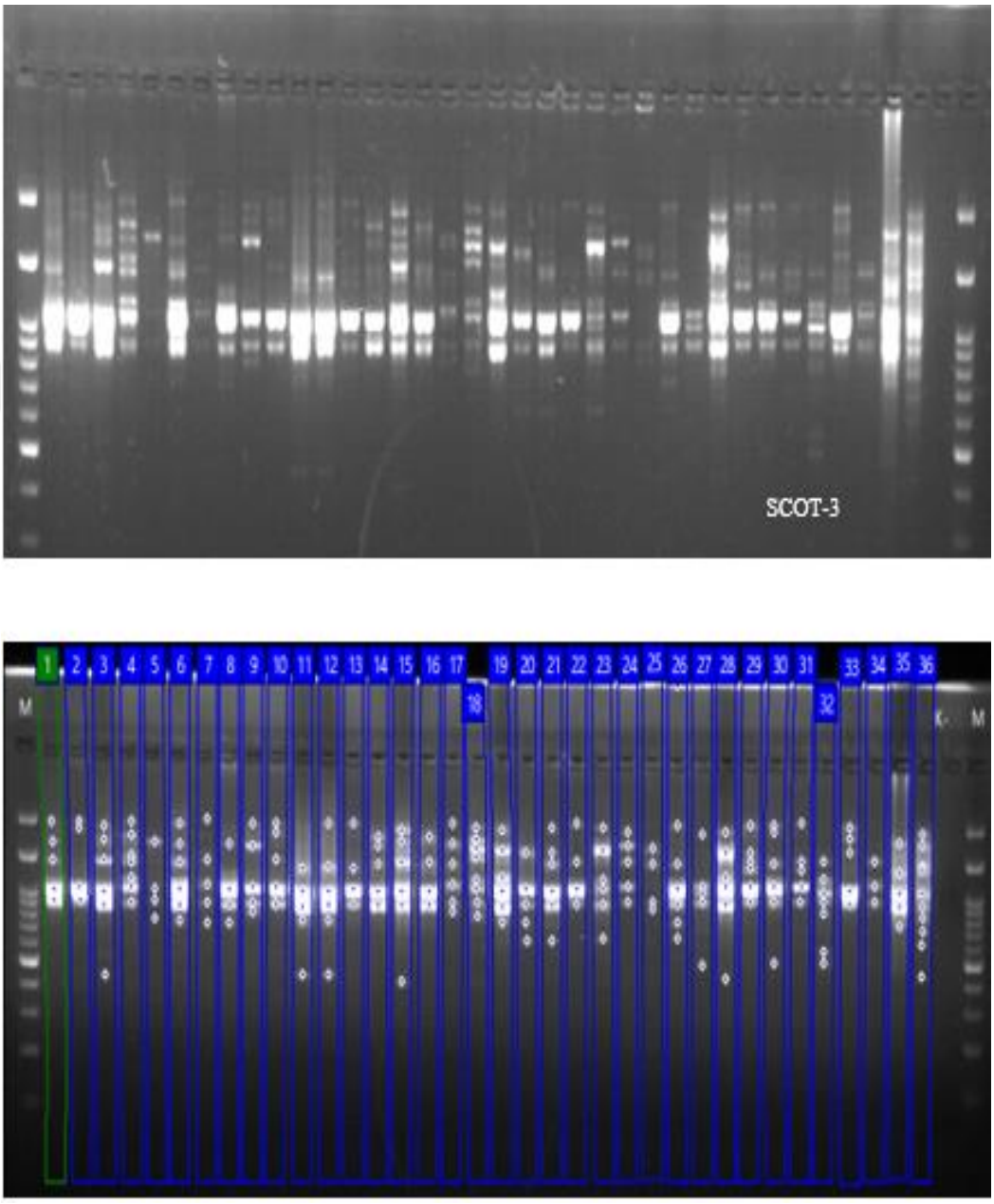

Figure 3.12. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-3 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: -control.*

SCOT-12: Figure 3.13 shows that SCOT-12 primer amplified 48 bands in total, the largest band was about 2272 bp and the smallest band was 371 bp, one band was monomorphic, the others were polymorphic. The polymorphism rate of this primer was calculated as 97.9%.

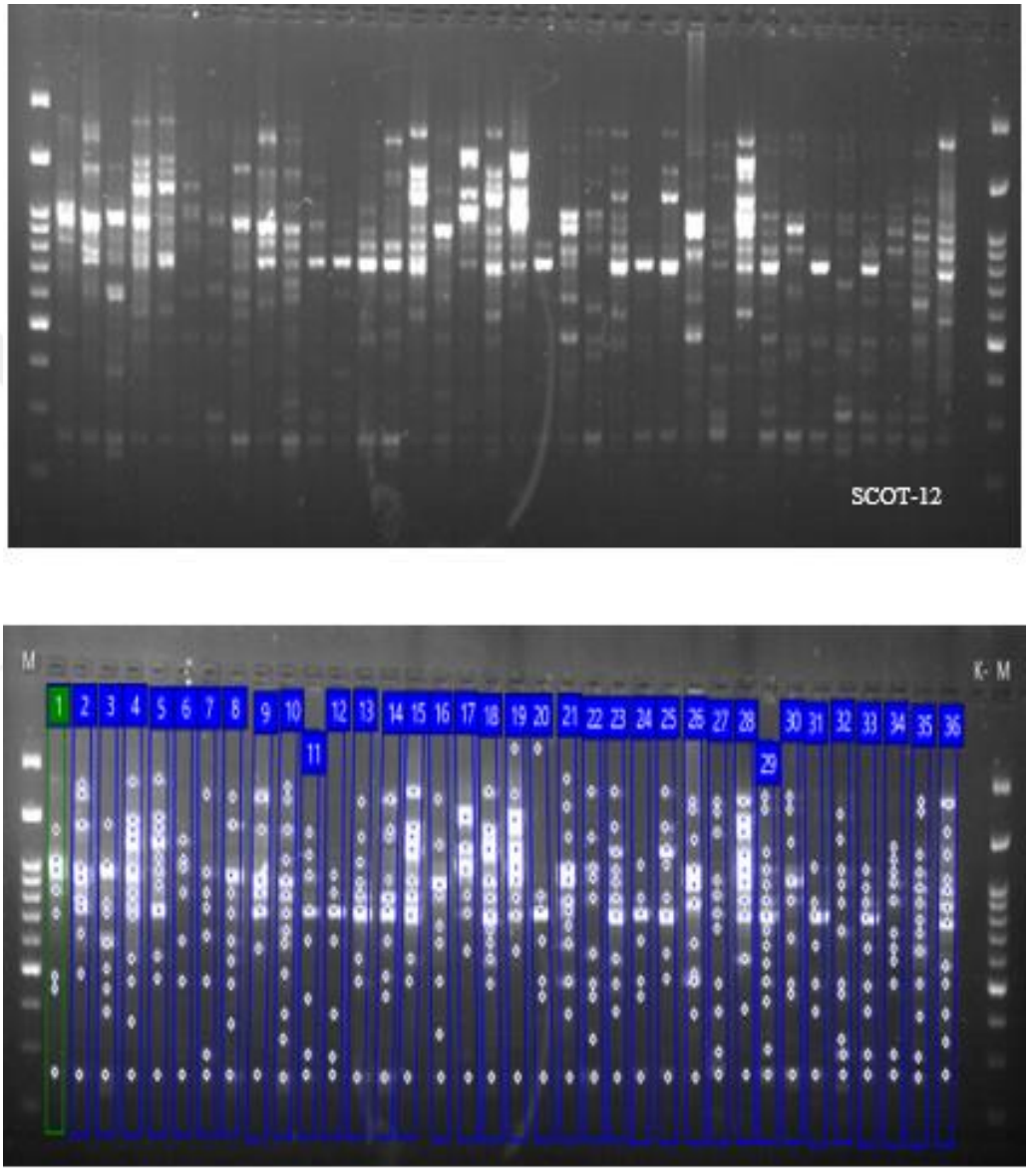

Figure 3.13. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT12 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

SCOT-15: Figure 3.14 shows that SCOT-15 primer amplified 22 bands in total, the largest band was about 2135 bp and the smallest band was 306 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

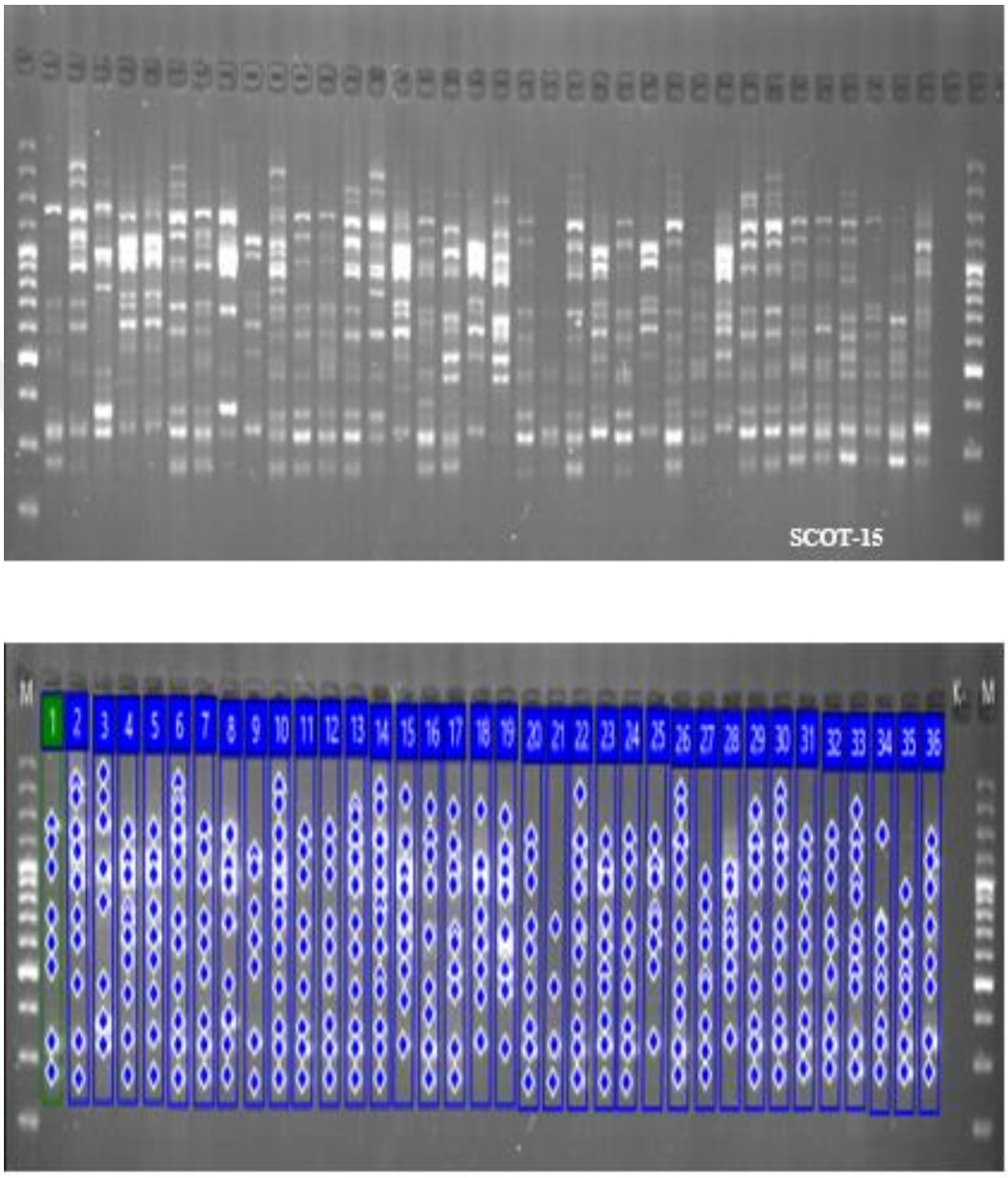

Figure 3.14. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-15 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

SCOT-16: Figure 3.15 shows that SCOT-16 primer amplified 28 bands in total, the largest band was about 3222 bp and the smallest band was 210 bp, one bands was monomorphic the others were polymorphic which gave 96.4 % polymorphism.

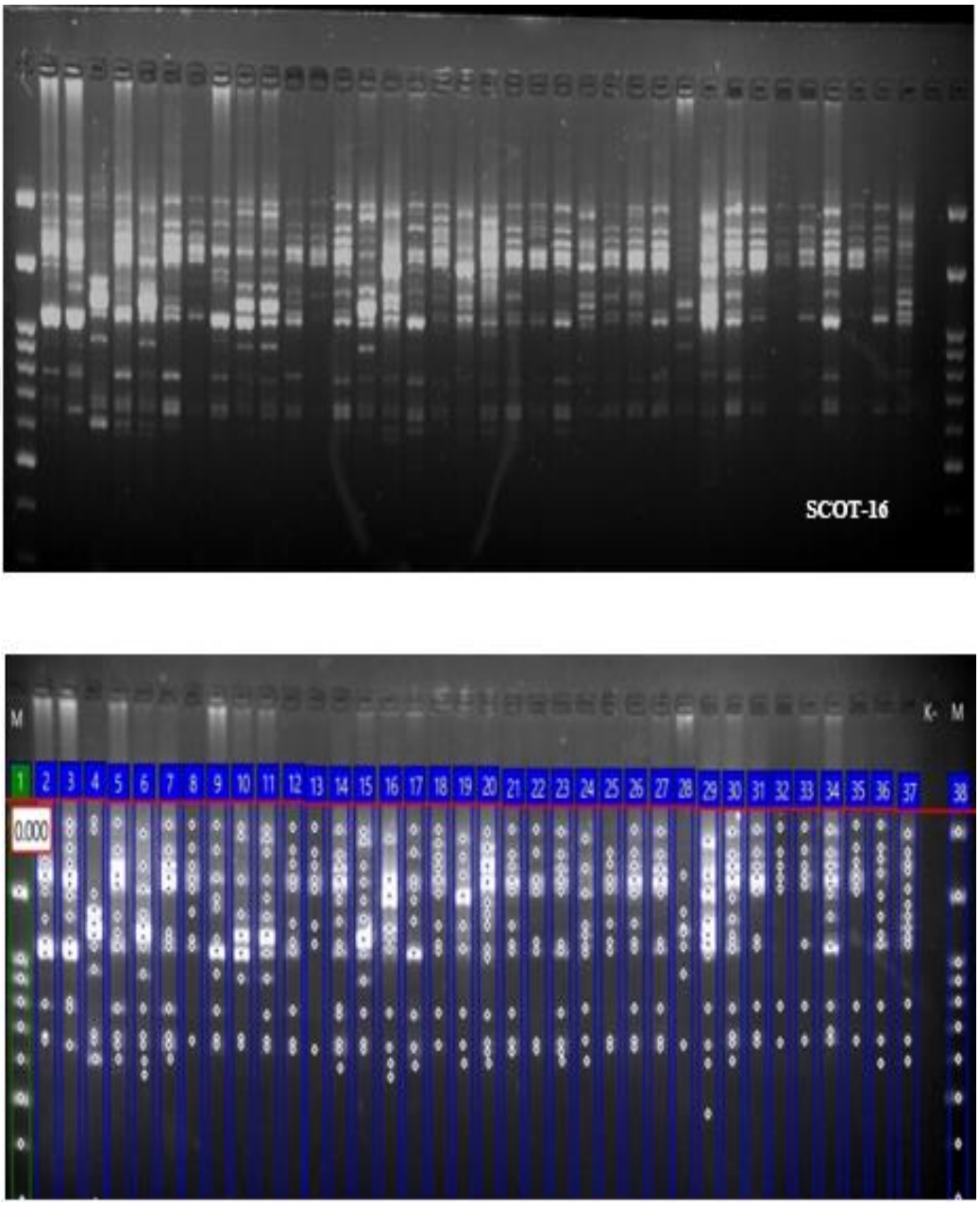

Figure 3.15. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT16 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

SCOT-17: Figure 3.16 shows that SCOT-17 primer amplified 30 bands in total, the largest band was about 1900 bp and the smallest band was 150 bp, there were 2 bands monomorphic, the others were polymorphic which gave 93.3% polymorphism.

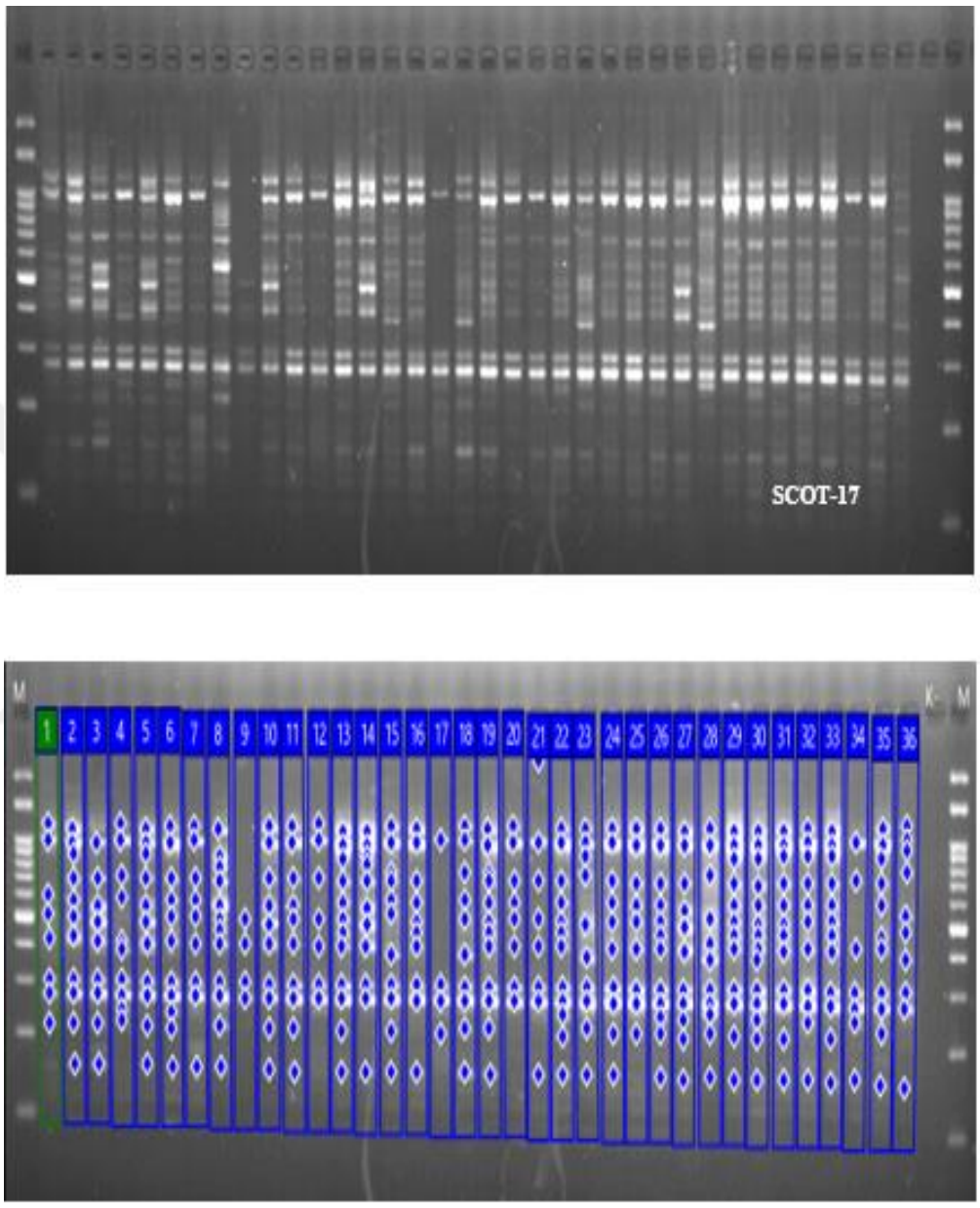

Figure 3.16. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-17 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

SCOT-18: Figure 3.17 shows that SCOT-18 primer amplified 18 bands in total , the largest band was about 3110 bp and the smallest band was 315 bp, in these bands 3 bands were monomorphic, the others were polymorphic which gave 83.3% polymorphism for this primer.

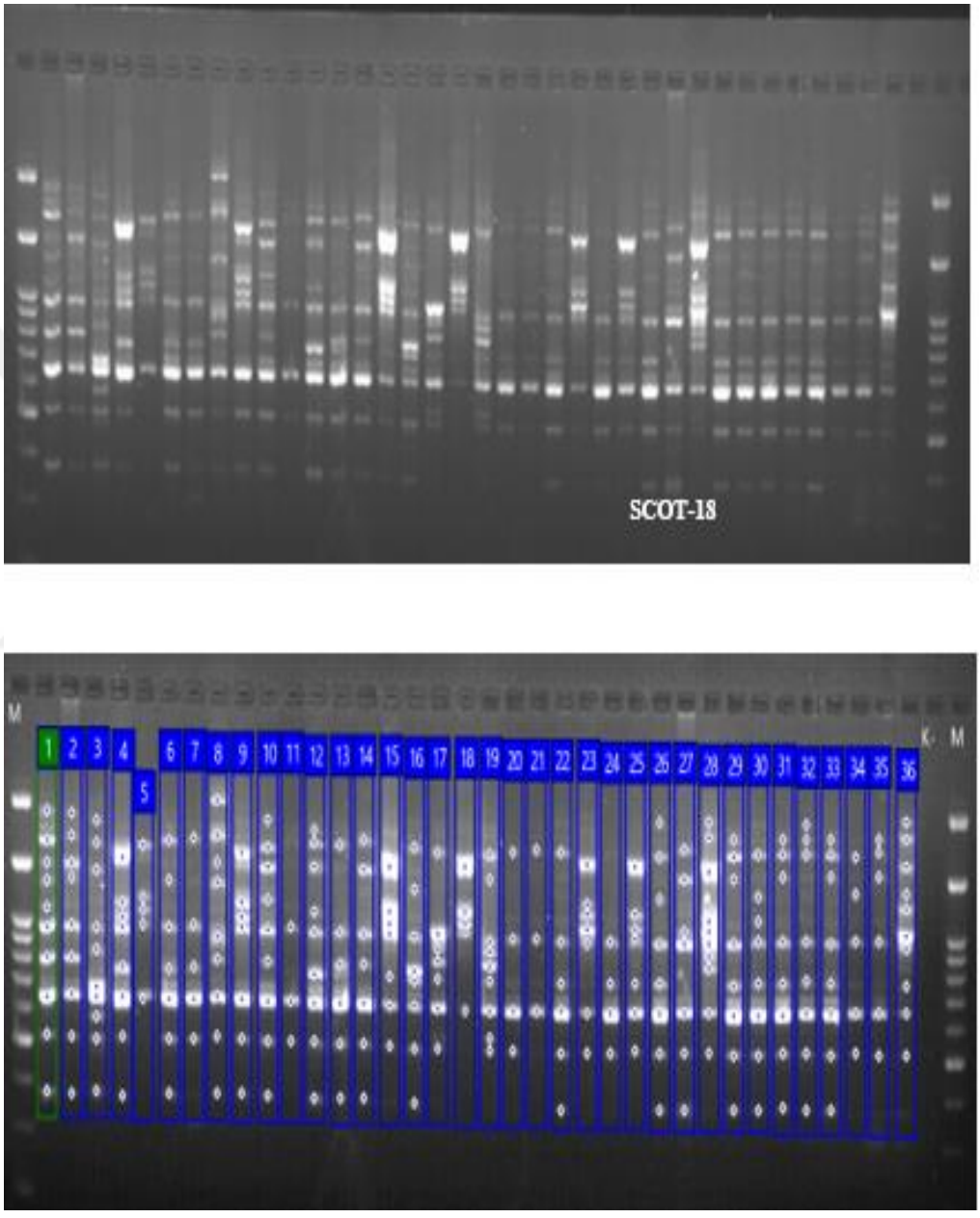

Figure 3.17. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-18 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

SCOT-21: Figure 3.18 shows that SCOT-21 primer amplified 20 bands in total, the largest band was about 2486 bp and the smallest band was 260 bp, in these bands just one monomorphic, the others were polymorphic which gave 95% polymorphism.

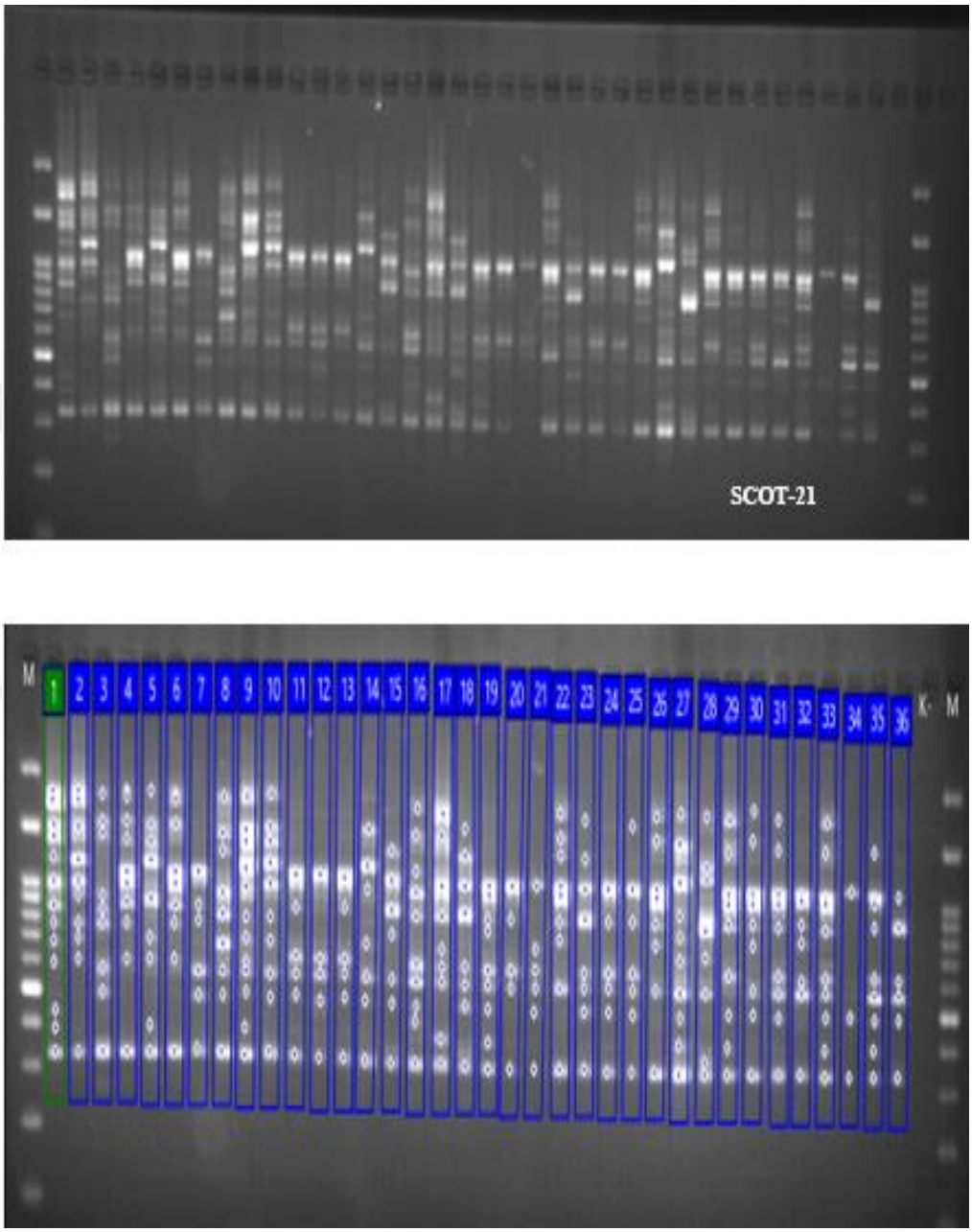

Figure 3.18. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-21 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

SCOT-28: Figure 3.19 shows that SCOT-28 primer amplified 14 bands in total, the largest band was about 2500 bp and the smallest band was 345 bp, in these bands there is 3 bands monomorphic, the others were polymorphic which gave 78.5% polymorphism.

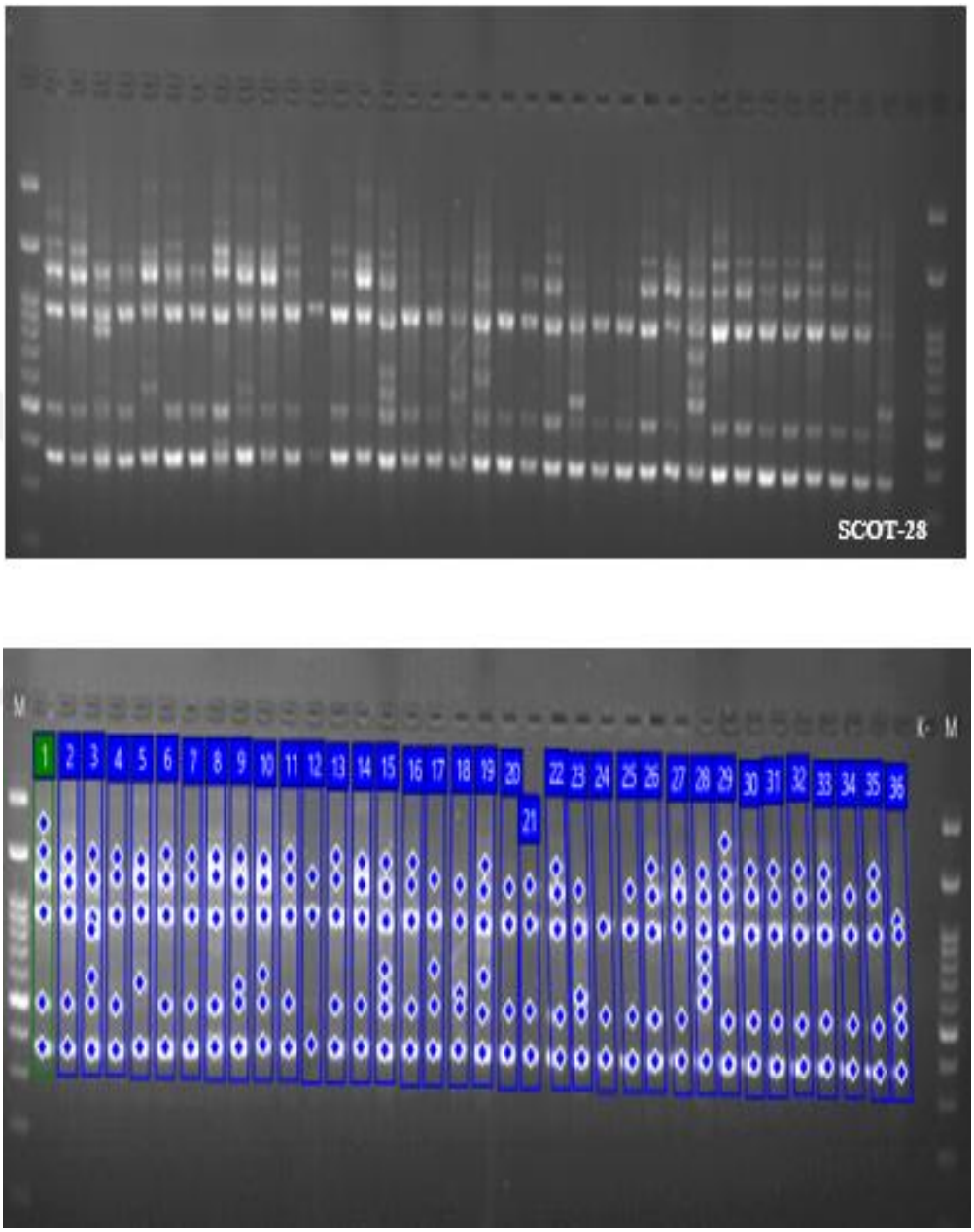

Figure 3.19. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-28 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control.*

SCOT-32: Figure 3.20 shows that SCOT-32 primer amplified 23 bands in total, the largest band was about 3130 bp and the smallest band was 174 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

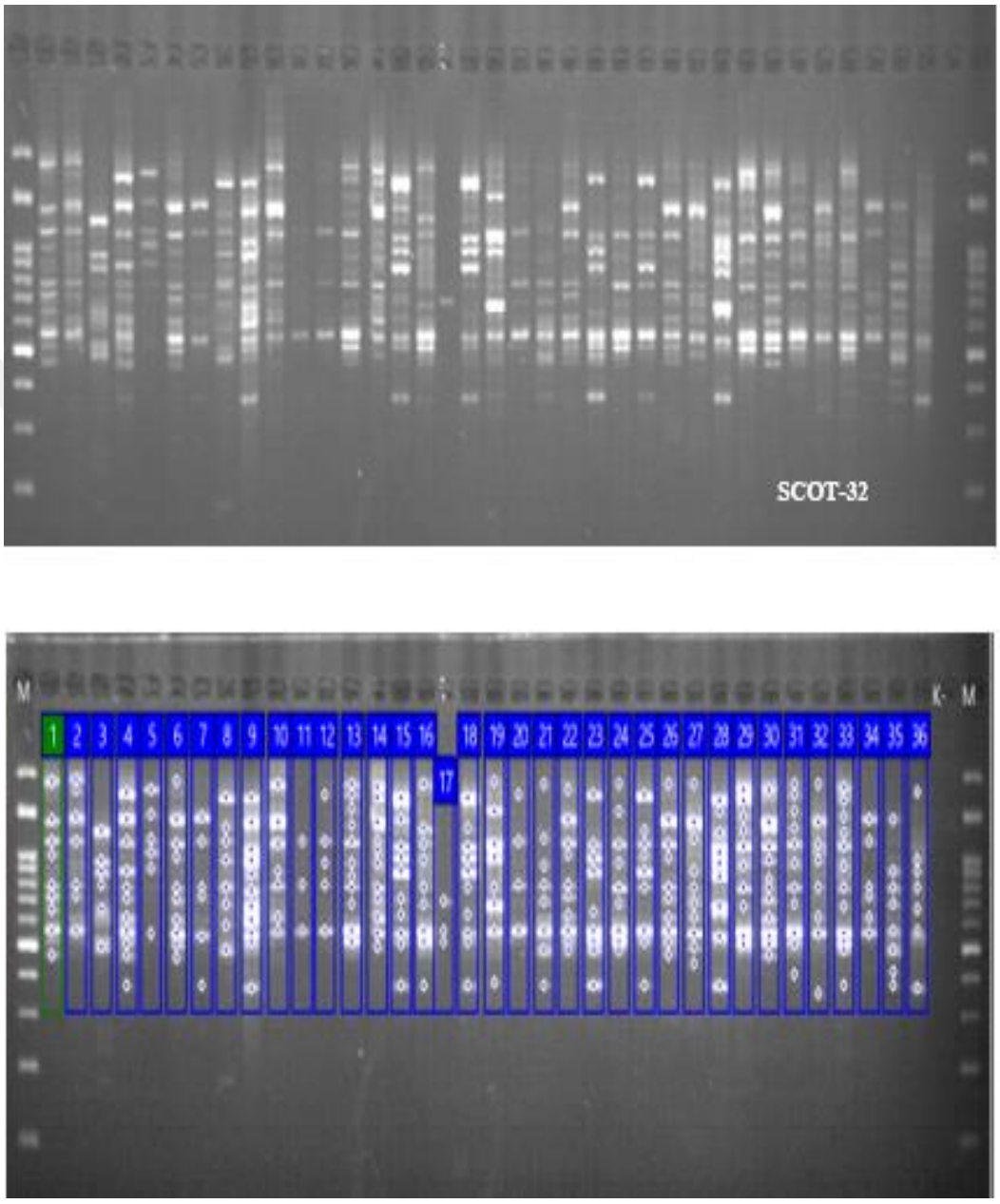

Figure 3.20. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-32 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: - control*

SCoT-34: Figure 3.21 shows that SCoT-34 primer amplified 26 bands in total, the largest band was about 3130 bp and the smallest band was 600 bp, all bands were polymorphic which gave 100% polymorphism for this primer.

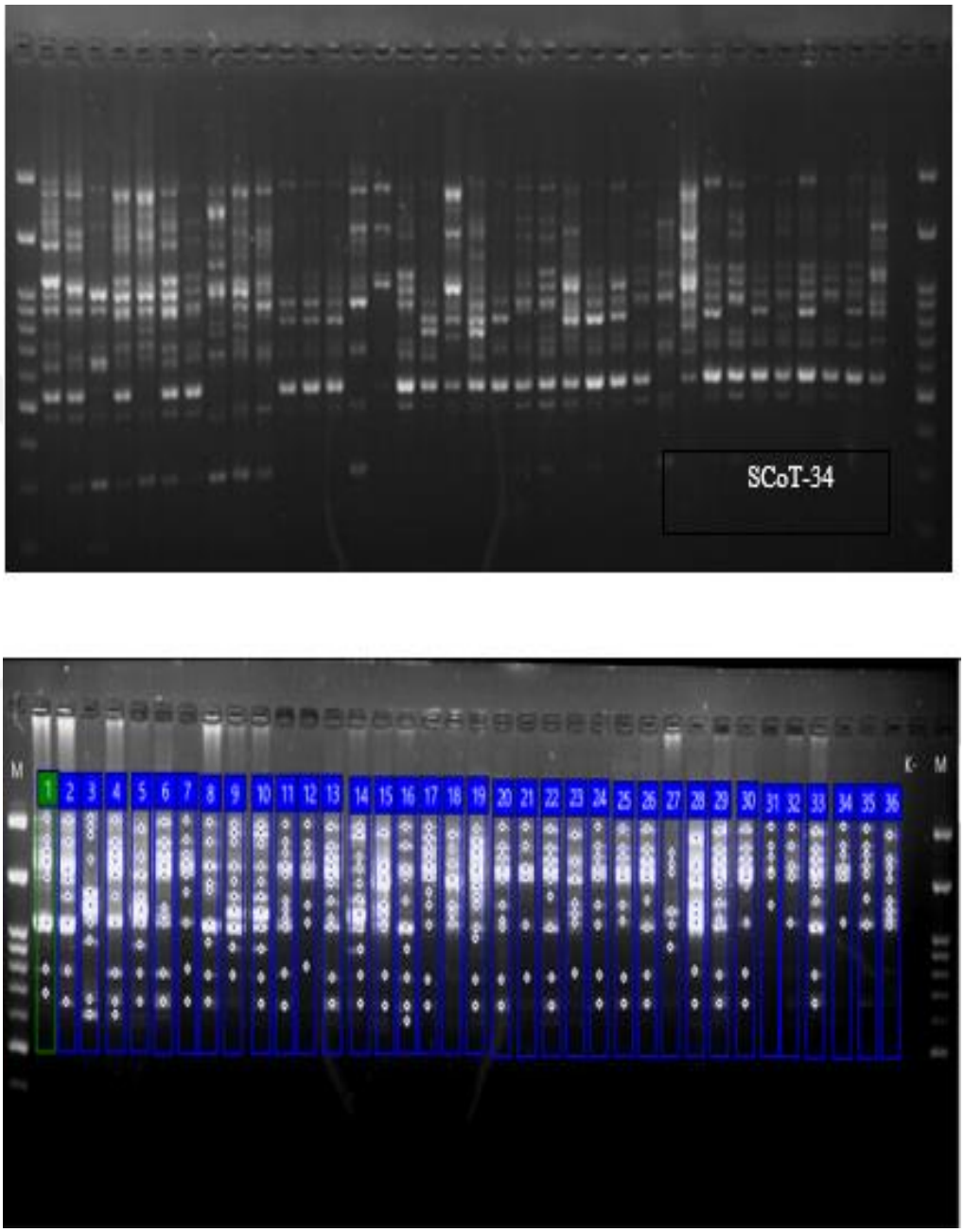

Figure 3.21. *PCR results showing band patterns between 36 Citrus varieties (1-36) with SCOT-34 primer (A). Bands detected with Totallab Cliqs program (B). M: 100 bp plus marker; K-: -control.*

3.2.3. Assessment of SCoT analysis

Bands obtained using 10 SCoT primers were scored as present (1) or absent (0) using Phoretix 1D Pro software and a binary matrix was produced. The analysis of SCOT patterns shows that all of the 10 primers used produced reproducible and easily interpretable banding patterns. A total of 240 bands was generated from the 36 *Citrus* varieties with an average of 24 bands per primer. Among these bands 228 were polymorphic (95%) The size of the amplified bands ranged from 150 bp (SCOT-17) and 3222 bp (SCOT-16). The number of bands per primer ranged from 14 for the SCOT-28 to 48 for SCOT-12 primer. It was found that SCOT-15, SCOT-32 and SCOT-34 primers provided 100% of polymorphic bands. While SCOT-28 primer provided 78.5% of polymorphic bands (Table 3.4). The polymorphism information content (PIC) ranged from 0.150 (SCOT-28) to 0.362 (SCOT-32) with an average of 0.27 which showed a high content of polymorphism and the primers used were very discriminative.

Table 3.4. *Characteristics of bands resulted from 10 SCOT primers*

Primer	TA (C°)	Band size (bp)	TB	MB	PIC	PPB
ISSR-4	50	(443-2532)	36	0	0.269	100%
ISSR-6	50	(431-3888)	23	0	0.288	100%
ISSR-309	48	(375-1667)	31	0	0.215	100%
ISSR-310	48	(254-2454)	34	0	0.276	100%
ISSR-807	52	(412-2817)	21	1	0.150	95.2%
ISSR-810	50	(452-2858)	22	1	0.250	95.4%
ISSR-843	53	(300-2349)	39	0	0.242	100%
ISSR-847	52	(512-1915)	20	0	0.235	100%
ISSR-851	55	(390-1819)	25	0	0.271	100%
ISSR-855	53	(371-2509)	19	0	0.297	100%

TA: *Annealing Temperature,* **TB**: *Total number of bands;* **MB**: *Monomorphic bands;* **PIC**: *Polymorphism Information content;* **PPB**: *Percentage of polymorphic bands*

Genetic similarity matrix between *Citrus* varieties was calculated from SCOT data based on Jaccard's similarity coefficient (Table 3.5).

Table 3.5. *Genetic similarity matrix between Citrus varieties based on Jaccard's similarity coefficient.*

	EUREKA	LEINARI	CITRONIER	LIMEDOUC	AFORER	MA43	NULES	HERNANDINOVA	KAFFIN	CROGRANI	MARISOL	CLEMENTITOMATER	RUBENS	MARPO	WASHINTOVALANCIA	PONVEL	ORANGERN	ORANGERL	NEWHALL	MALTESED	MALTESBA	MORO	TAROCCO	SANGUINE	ORANGERSCSAKSU	MESKANSI	MESKMAL	PONPLOM	PANAGHER	BIGRADER	MARIANA	KUMKUAT
EUREKA	1	0.325	0.394	0.376	0.336	0.333	0.296	0.352	0.235	0.278	0.31	0.302	0.359	0.169	0.225	0.231	0.203	0.221	0.261	0.273	0.221	0.235	0.271	0.248	0.267	0.318	0.25	0.317	0.25	0.271	0.266	0.242	0.266	0.333	0.363	0.26
LEINARI		1	0.4	0.413	0.286	0.278	0.278	0.312	0.248	0.281	0.273	0.325	0.315	0.202	0.244	0.314	0.269	0.262	0.291	0.319	0.244	0.27	0.325	0.272	0.31	0.31	0.262	0.353	0.294	0.284	0.269	0.244	0.258	0.283	0.388	0.325
CITRONIER			1	0.541	0.317	0.374	0.364	0.344	0.248	0.248	0.312	0.294	0.267	0.191	0.282	0.347	0.231	0.233	0.301	0.275	0.31	0.282	0.338	0.25	0.299	0.28	0.252	0.276	0.305	0.296	0.279	0.199	0.289	0.336	0.474	0.359
LIMEDOUC				1	0.326	0.39	0.391	0.414	0.285	0.325	0.35	0.324	0.315	0.213	0.331	0.386	0.238	0.266	0.338	0.288	0.31	0.295	0.385	0.286	0.338	0.329	0.267	0.279	0.326	0.267	0.254	0.233	0.331	0.451	0.492	0.364
AFORER					1	0.495	0.397	0.483	0.393	0.469	0.467	0.413	0.432	0.239	0.397	0.325	0.254	0.285	0.382	0.32	0.345	0.355	0.407	0.386	0.341	0.439	0.361	0.341	0.434	0.266	0.242	0.188	0.281	0.44	0.354	0.305
MA43						1	0.54	0.51	0.457	0.544	0.505	0.486	0.402	0.25	0.388	0.407	0.362	0.382	0.456	0.475	0.316	0.576	0.545	0.508	0.423	0.473	0.413	0.456	0.485	0.327	0.33	0.219	0.284	0.382	0.412	0.369
NULES							1	0.555	0.423	0.471	0.549	0.466	0.413	0.252	0.483	0.482	0.383	0.35	0.462	0.404	0.435	0.457	0.466	0.407	0.398	0.467	0.438	0.367	0.45	0.322	0.315	0.206	0.304	0.443	0.389	0.339
HERNANDI								1	0.36	0.329	0.521	0.442	0.508	0.215	0.447	0.432	0.286	0.31	0.408	0.37	0.398	0.385	0.454	0.384	0.378	0.467	0.378	0.347	0.426	0.293	0.267	0.193	0.267	0.47	0.391	0.374
MOVA									1	0.446	0.476	0.402	0.339	0.253	0.338	0.404	0.289	0.352	0.356	0.333	0.406	0.438	0.389	0.404	0.31	0.405	0.356	0.338	0.382	0.271	0.312	0.23	0.3	0.377	0.27	0.359
KAFFIN										1	0.468	0.49	0.442	0.229	0.416	0.425	0.319	0.385	0.388	0.381	0.429	0.478	0.409	0.412	0.342	0.438	0.364	0.307	0.417	0.294	0.265	0.192	0.223	0.398	0.333	0.36
CROGRANI											1	0.5	0.445	0.269	0.556	0.442	0.388	0.454	0.345	0.478	0.514	0.505	0.513	0.459	0.377	0.538	0.461	0.39	0.514	0.283	0.208	0.212	0.349	0.442	0.348	0.311
MARISOL												1	0.408	0.217	0.416	0.388	0.381	0.366	0.4	0.446	0.454	0.389	0.445	0.413	0.381	0.471	0.358	0.456	0.442	0.317	0.387	0.333	0.279	0.377	0.394	0.366
CLEMENTIT													1	0.203	0.423	0.364	0.291	0.333	0.397	0.417	0.375	0.328	0.353	0.35	0.328	0.431	0.368	0.338	0.368	0.307	0.331	0.277	0.263	0.364	0.321	0.294
TOMATERP														1	0.302	0.193	0.244	0.303	0.279	0.225	0.26	0.265	0.248	0.293	0.223	0.277	0.317	0.217	0.269	0.211	0.241	0.207	0.35	0.235	0.228	0.174
RUBENS															1	0.395	0.356	0.395	0.533	0.44	0.494	0.394	0.451	0.347	0.346	0.464	0.456	0.379	0.436	0.244	0.238	0.212	0.36	0.395	0.338	0.311
MARPO																1	0.318	0.312	0.424	0.374	0.404	0.377	0.496	0.316	0.382	0.381	0.347	0.296	0.407	0.294	0.288	0.227	0.268	0.39	0.352	0.39
WASHINTO																	1	0.504	0.494	0.491	0.472	0.407	0.349	0.381	0.313	0.385	0.331	0.4	0.375	0.26	0.285	0.269	0.285	0.223	0.297	0.206
VALANCIA																		1	0.575	0.436	0.457	0.46	0.377	0.389	0.339	0.5	0.395	0.373	0.445	0.273	0.32	0.254	0.342	0.281	0.311	0.281
PONVEL																			1	0.55	0.532	0.495	0.48	0.439	0.373	0.528	0.384	0.419	0.518	0.254	0.296	0.264	0.326	0.359	0.365	0.319
ORANGERN																				1	0.615	0.556	0.446	0.426	0.367	0.448	0.366	0.5	0.471	0.333	0.304	0.265	0.26	0.285	0.285	0.244
ORANGERL																					1	0.607	0.467	0.433	0.336	0.43	0.345	0.36	0.451	0.312	0.271	0.246	0.293	0.308	0.328	0.275
NEWHALL																						1	0.456	0.408	0.369	0.444	0.369	0.412	0.424	0.308	0.3	0.22	0.254	0.339	0.325	0.281
MALTESED																							1	0.535	0.54	0.561	0.509	0.482	0.614	0.306	0.32	0.255	0.31	0.308	0.427	0.355
MALTESBA																								1	0.418	0.553	0.495	0.451	0.559	0.273	0.289	0.242	0.278	0.293	0.303	0.261
MORO																									1	0.525	0.46	0.412	0.514	0.333	0.315	0.269	0.265	0.339	0.326	0.308
TAROCCO																										1	0.565	0.496	0.69	0.312	0.357	0.281	0.346	0.45	0.376	0.359
SANGUINE																											1	0.44	0.556	0.284	0.322	0.216	0.357	0.347	0.282	0.272
ORANGERC																												1	0.481	0.289	0.383	0.307	0.314	0.296	0.305	0.286
SAKASU																													1	0.274	0.357	0.254	0.322	0.383	0.355	0.347
MESKANSI																														1	0.525	0.274	0.198	0.252	0.283	0.185
MESKMAL																															1	0.341	0.264	0.258	0.278	0.193
PONPLOM																																1	0.289	0.209	0.229	0.175
PANAGHER																																	1	0.342	0.259	0.258
BIGRADER																																		1	0.384	0.344
MARIANA																																			1	0.395
KUMKUAT																																				1

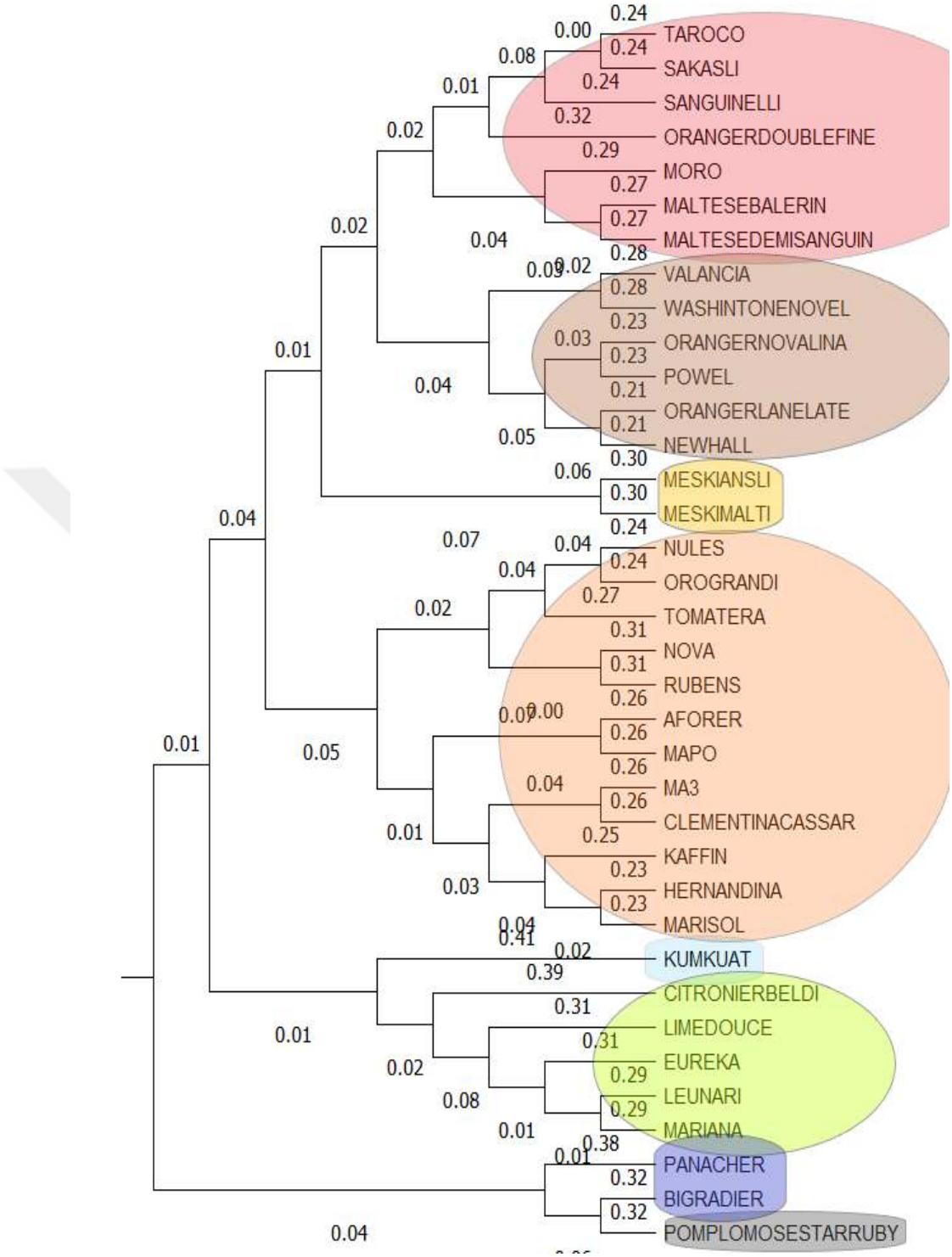

Figure 3.22. *Genetic similarity dendrogram of 36 Citrus varieties using SCOT markers*

The genetically closest varieties were found to be as Orange Lane Late and Novalina, with a genetic similarity value of 0.615. The varieties with the lowest genetic similarity value were found to be 0.169 between Eureka and Tomatera.

The 1-0 binary matrix was transferred to the Dendro:UPGMA program, where the genetic similarity values were calculated by using Jaccard coeficient. Then, the results obtained were transferred to the MEGA program in Newick format and UPGMA dendrogram was generated.

According to the dendrogram, 2 main clusters formed. The first cluster included the varieties Panacher, Bigarde, Pomelo Star Ruby. The second main group was mainly composed of 2 subclusters; the first included lemon and kumkuat and a second large subgroup contained mandarine varieties which was further divided into three subgroups that contained blood orange, sweet orange and white orange varieties, respectively.

4. DISCUSSION AND CONCLUSION

4.1. ISSR And SCOT Amplification

In this study, dominant molecular markers ISSR and SCOT were used to determine the genetic variation between 36 *Citrus* varieties. Among these markers some of them have not been studied before on these *Citrus* varieties to determine their genetic polymorphism levels.

The 10 ISSR primers used in this study gaves a total of 270 bands. The least prominent band (19 bands) was obtained from primer ISSR-855, most of the bands (38 bands) were obtained from the ISSR-843 primer, 27 was the average of bands per primer. 268 of the 270 bands formed were polymorphic, so the observed polymorphism rate was 99.2%. Primer polymorphism rates varied between 95.2% and 100%. The primer with the lowest polymorphism rate was ISSR -807, which gave 21 bands one of which was monomorphic and with 95.2% polymorphism. The primers with the 100% polymorphism rate were ISSR -4, ISSR-6, ISSR-309, ISSR-310, ISSR-843, ISSR-847, ISSR-851 and ISSR-855, which gave 17 bands with 94.12% polymorphism. Our results were closely similar to study of Shasavar et al. (2007) in which they used 6 primers on 33 *Citrus* varieties and collected 234 amplified bands with an average of 39 bands per primer. Also very close with the result obtained by Marak et al. (2010) who used 6 ISSR primers within 10 species of *Citrus* which resulted 233 bands, all of them were polymorphic. In contrast, our obtained band numbers were lower than what were obtained by ISSR markers on 46 *Citrus* cultivars by Fang et al. (1998). They reported that 10 ISSR primers generated 642 polymorphic fragments, this diffrence may explicated from using different cultivars and different ISSR primers. Sankar et al. (2001) used 50 ISSR primers within the same group of *Citrus grandis*. The primers ISSR-855, ISSR-851, ISSR-847, ISSR-843, ISSR-810 and ISSR-807 amplified 11, 6, 7, 10, 3, 4 bands respectively. Compared to our results these numbers are low, since with the same primers and *Citrus* varieties belonging to differents species of *Citrus*, the obtained band number was 19, 25, 20, 39, 22 and 21, respectively. These results confirms the theory that the diversity augment from species to other and decreased among the same species, that's why in this study we concentrate to analyse different species for augment genetic knowledge so that breeding programs for Tunisian *Citrus* can be more successful.

The number of bands obtained from SCOT marker in this study was 240 and the polymorphism rate was about 95%. These numbers were higher than that of Mahjbi et al.

(2015), who studied 54 Tunisian *Citrus* cultivars with SCOT markers and obtained 132 bands of which 124 were polymorphic (93.9 %) and an average of bands per primer was 12.4. Also our SCOT results were higher than those obtained by Juibary et al. (2021) which were 64 bands from 25 *Citrus* cultivars. Therefore it can be said that our chosen SCOT markers are more informative than those utilized by Mahjbi et al (2015) and Juibary et al (2021). Coming to the performance of SCOT primer, we observed that polymorphism rates per primer varied between 78.5% and 100%.

The size of SCOT amplicons in our study ranging up to 3222 bp was higher than that of obtained by Han et al. (2014) who found band sizes ranging from 150 to 2100 bp for *Citrus* species but lower than the result of Mahjbi et al. (2015) in which band sizes ranged up to 3800 bp. On the other hand, the size of amplified ISSR fragments in our study ranged between 254 and 3888 bp which was higher than that of reported by Fang et al. (1998) and Shasavar et al. (2006), in these studies band sizes were ranging from 80 to 3000 bp and from 100 to 3000 bp, respectively.

The average polymorphism rate of 10 SCOT primers used in our study was 95% which was close to Guo et al. (2012) which was around 93.1%. Moreover when the ISSR and SCOT primers used in this study were compared, the ISSR primers showed higher rate of polymorphism with 99% which was very close to the results obtained by Chibame et al (2010) with ISSR primers showing 100% polymorphism. These results make ISSR markers the leading marker for polymorphism studies in *Citrus* varieties.

4.2. The Polymorphism Information Content (PIC)

The polymorphism information content (PIC) value is used to evaluate polymorphism in a widely used marker locus in genetics. This number shows which marker alleles are passed down from the parents to the next generation. When table 3.4 are examined, it can be seen that the PIC values of ISSR primers varied between 0.150 and 0.297, with an average value of 0.249. The PIC value for dominant markers (like ISSR, RAPD, SCOT, and AFLP) has a maximum value of 0.5 while the range for codominant markers is from 0.5 to 1.0 (Chesnokov and Artemyeva, 2015). The primer with the lowest PIC value (0.150) was ISSR -807 and the primer with the highest PIC value (0.297) was ISSR -855. Coming to SCOT marker PIC values ranged from 0.150 (SCOT-28) to 0.362 (SCOT-32) with an average of 0.27 (Table 3.6). These results indicated that among the ISSR and SCOT primers used in the study we can obtain the

most polymorphism information from the ISSR -855 and SCOT-32 primers, and the least polymorphism information from the ISSR-807 and SCOT-28 primers, in a population consisting of 36 *Citrus* varieties. The values of both ISSR and SCOT markers in our analysis were below 0.5 as expected for a dominant marker. However, the value of ISSR primers were different in the study of Munankarmi et al (2018) who reported the average value as 0.89. Also, Mahjbi et al. (2016) showed that the () values of SCOT primers ranging from 0.616 (SCOT-19) to 0.944 (SCOT-13) with an average of 0.884. Since ISSR and SCOT markers are dominant and maximum PIC value for a dominant marker was stated to be as 0.5, probably PIC values in the study of Mahjbi et al.(2016) and Munankarmi et al (2018) were miscalculated.

Studies by Uzun et al. (2010) employing 12 ISSR markers on *Citrus* grapefruit and pomelo, indicated that these primers amplified less polymorphic bands than that of our study. Considering grapefruit is a hybrid created by crossing pomelos and oranges, the lowset of polymorphic bands comparing to our results can be explained by pamelo and grapefruit closely related groups but in our study we compared *Citrus* varieties belonging to various species that may cause the level of diversity more higher.

In addition, these results are comparable to Sülü et al. (2020) study with different types of markers SSCP, ISSR , and SSR on the mandarine and lemon varieties, the values of (PIC) ranged from 0.61-0.99, 0.31-0.96, and 0.33-0.96, respectively. ISSR marker was more effective to determine the genetic differences between the *Citrus* varieties compared to SSCP and SSR markers. The PIC values of both ISSR and SCOT markers in our study were higher and imply that these two types of markers are more informative in *Citrus*.

4.3. The Genetic Similarity of *Citrus* Varieties

The Jaccard similarity values obtained by ISSR analyses between studied *Citrus* varieties ranged from 0.102 to 0.545. The low genetic similarity was estimated between the ‘Aforer’ and ‘Panacher’. This low genetic similarity is confirmed with morphologic characters for example panacher has sour flavor, its fruit has yellow to green color and is big in size whereas Aforer is a mandarine with a sweet flavor, its fruit has an orange color and is moderate in size.

The highest similarity was recorded between 2 sweet orange varieties in the same cluster. This result reflects the high genetic divergence between ‘Orange novalina’ and ‘Powell’. Compared with the similarity coefficient obtained by Debbabi et al (2014) using

AFLP markers on Tunisian *Citrus* cutivars the coefficient was ranged from 0.15 to 0.96 with an average of 0.76, which the lowest genetic similarity value of 0.15 has been scored between both ‘Meski sifi’ and ‘Malti ahmar’ and ‘Meski sifi’ and ‘Meski ansli’. ‘Lsen asfour’ and ‘Moro’ were the most similar (0.96) varieties. This high coefficient value can explicated by studying very close species of *Citrus*, but in our study we used different varieties from lemon to mandarine which proved why our similarity coefficient were lowset then Debbabi et al. (2014).

The UPGMA dendrogram based on ISSR analysis (Fig 3.11) generated two major clusters. The first one included five sub-clusters (blood oranges, sweet oranges, white oranges, mandarins, pomelo), this distribution of cultivated *Citrus* genotypes confirmed the hybrid relationship between these groups, in which sweet orange should be a direct inter-specific hybrid between a pomelo and mandarin. Furthermore, according to Barkley et al. (2006), mandarin accounted for the majority of the genetic makeup of sweet oranges while pomelo accounted for a much smaller part. The local variety "Meski" is genetically quite close to the sweet orange tree and the mandarin tree, according to the UPGMA dendrogram. The theory that the meski is a cross between a mandarin and a sweet orange was supported by this outcome. The second major cluster includes three species (kumkuat, bigarde, lemons) which was confirmed by Ramadugu et al. (2013) suggesting that sour oranges ‘bigarde’ were found to be of hybrid origin, with pomelo and mandarin parentage, this hypothesis proved by the UPGMA dendrogram which clarify the neighbor between ‘star ruby’, bigarde and mandarins group. Similar to our groupings, dendrogram obtained by Youssef et al (2014) within *Citrus* species in Egypt contained two mains clusters at 0.18 level of similarity and 6 groups of *Citrus* (lemons, mandarins, sweet oranges, sour oranges, grapefruit and pomelo) close to our findings.

These results indicated that ISSR markers are very useful tool for identifying polymorphism and differentiating *Citrus* varieties. It was observed that mandarin and orange species demonstrate genetic convergence as well as differences with lemons compared to other orange and mandarin species. In addition, the bigarde, pomelo, and lemon species are very closely related genetically. This species' shared appearance and shared major characteristics, such as a bitter to acid flavor, supported their genetic closeness.

The coefficient of genetic similarity of Jaccard obtained by SCOT analysis ranged from 0.169 to 0.615. The genetically closest varieties were ‘Orange lane late’ and

‘Novalina’, with a genetic similarity coefficient of 0.615. The cultivars with the lowest genetic similarity were found to be between ‘Eureka’ and ‘Tomatera’ (0.169). This low genetic similarity is also confirmed with morphologic characters. As a matter of fact, ’Eureka’ is a Limon with high acidity, its fruit has a yellow color , and small in size, in contrast ‘Tomatera’ is a mandarine with sweet flavor, its fruit has an orange color and is moderate in size. Also, the UPGMA dendrogram (Fig 3.22) generated two major clusters which were similar to the dendrogram obtained from ISSR markers in this study. The first cluster included six sub-clusters (blood oranges, sweet oranges , white blood oranges, mandarins, lemons and kumkuat). The second major cluster includes three species (panacher, pomelo and bigarde), compared with the previous dendrogram these sub-groups repartition were clearly different with lemon groups, kumkuat and panacher which can be explicated by the use of different primers.

To conclude, the SCOT and ISSR molecular markers have proved their usefulness in the characterization of genetic diversity of *Citrus* varieties. Our results can be used in breeding studies for the development of *Citrus* varieties grown in Tunisia, the genetic diversity and genetic relatedness levels of parent cultivars will be determined and these data can be used to obtain new cultivars.

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2018 : Diş cerrahisinde asistanlik (Tunus, 1 ay)

2017 : Temmuz: Anatomi ve patolojik sitoloji laboratuvarı stajı (Tunus, 1 ay)

2017 : Biyoteknoloji araştırma laboratuvarında lisans sonu çalışmas stajı (3 ay)

2016 : Temmuz: Anatomi ve patolojik sitoloji laboratuvarında staj (Tunus, 1 ay)

2015 : Biyoloji laboratuvarında staj hastanıda (Tunus, 1 ay)

EĞİTİM BİLGİLERİ

2021-2022 : Yüksek lisans tezinin hazırlanması ve sınavı (Türkiye)

2020-2021 : Yüksek lisans ders dönemi biyoteknoloji (Türkiye)

2019-2020 : Yüksek lisans ders dönemi biyoteknoloji (Türkiye)

2018-2019 : Dil çalışması (Türkçe; C1 seviyesi)

Lisans : Monastir Yüksek Biyoteknoloji Enstitüsü

2016-2017 : Lisans 3 "Tıbbi biyoteknoloji" (B)

2014-2016 : Lisans 1 ve 2 "Genel biyoteknoloji" (AB)

2013-2014: Bakalorya "Deneysel Bilimler" (Djerba AB)

LABORATUVAR BECERİLERİ

Biyokimya ve Hücre ve moleküler biyoloji teknikleri : PCR, elektroforez, kromatografi, nano drop, DNA ekstraksiyonu, protein ekstraksiyonu, ELISA, mikroskopi, hücre kültürü, mikrotomi

DİLLER:

Arapça: Anadil

Türkçe: Akıcı konuşma ve yazma (C1)

Fransızca: Ikinci dil

İngilizce: Akıcı konuşma, yazma ve akademik (B1)

BILGISAYAR BILIMI

Microsoft program, Total Lab CLIQS 1D, DendroUPGMA program, MEGA software version

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