Bethlehem University

Faculty of Science

Biotechnology Master Program

Molecular Phylogeny of Palestinian Date Palm Cultivars by Microsatellite Simple Sequence Repeats and Inter Simple Sequence Repeats Markers

By

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In partial fulfillment of the requirements for the Degree Master of Science

November 2014

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) is considered one of the major fruit crops in Palestine and one of the oldest cultivated plants. Date crops contribute to the economical, industrial, and religious concepts in the Middle East and North Africa. Since studies on the genetic diversity and relatedness of date palm germplasm are limited in Palestine, the aim of this study is to analyze the genetic relatedness among twelve cultivars collected from the Jericho City area in Palestine using Simple Sequence Repeat (SSR) and Inter- Simple Sequence Repeat (ISSR). These cultivars are Zahidi, Hayani, Hejazi, Khadrawy, Barhy, Majool, Dejlet Noor (Deek Noor), Amery, Ibrahemi, and three cultivars known as Baladi.

DNA was extracted and purified from the young leaves by applying a phenol-chloroform extract followed by DNeasy DNA extraction kit. Microsatellites (Simple Sequence Repeats; SSR) and Inter Simple Sequence Repeats (ISSR) were amplified by PCR using 3 SSR and 9 ISSR primers, respectively. The size of the amplicons of the SSR and ISSR products ranged between 180-240bp and 140 - 3100bp, respectively. A matrix representing the banding profiles was used to establish a phylogenetic tree of the twelve cultivars using the Fingerprint Analysis with Missing Data (FAMD) software and the Neighbor Joining method.

The phylogram obtained by SSR and ISSR encompasses three clusters. The first cluster contained one cultivar Ameri. The second cluster included 5 cultivars, which was further divided into two sub-clusters; the first sub-cluster contained one cultivar; Baladi 3, and the second sub-cluster encompasses four cultivars, Ibrahimi, Khadrawi, Dejlat Noor and Hayani. The third cluster was divided into two sub-

clusters; the first one contains Majool and Berhy, while the other one holds Balady 2, Zuhadi, Hijazi, and Baladi 1. The fidelity of the phylogram was assured by the bootstrapping and Principal Coordinate of Analysis (PCoA) methods.

Despite the well-known morphological differences among some cultivars, they were closely related in this phylogram. Examples include Ibrahemy and Khadrawi, Dejlat Noor and Hayani, as well as Hijazi and Baladi 1. The phylogram differentiates among three cultivars that are known as Baladi. These three cultivars were named as Baladi1-3 to facilitate their use in this study. To the best of my knowledge, this is the first study done in Palestine with emphasis on the genetic relatedness among date palms cultivars using SSR and ISSR.

تحديد الطرز الوراثي لأهم أصناف النخيل في فلسطين باستخدام التسلسل البسيط المتكرر والمناطق التي تقع بين هذه التسلسلات

هلا علي محمد الشولي

الملخص

النخيل (*Phoenix dactylifera* L) هو واحد من محاصيل الفاكهة الرئيسية في فلسطين ويعد واحدا من أقدم النباتات المزروعة. في منطقة الشرق الأوسط وشمال أفريقيا، كما لها أهمية اقتصادية تساهم في قطاع الصناعات الزراعية، كما أنها جزء من المفاهيم الدينية لشعوب هذه المناطق. وبالرغم من أهمية النخيل الا أن الدراسات حول التنوع الجيني وصلات القرابة بين أصناف النخيل محدودة في فلسطين. تهدف هذه الدراسة إلى تحديد الطرز الجينية والعلاقات التطورية بين 12صنفا تم جمعها من منطقة أريحا في فلسطين وذلك بدراسة المادة الوراثية لهذه الاصناف باستخدام طرق ووسائل تكنولوجية حديثة مثل: (Simple Sequence Repeats; SSR) و (Inter Simple Sequence Repeats; ISSR). وقد شملت الدراسة هذة الاصناف: زهيدي، الحياني، حجازي، خضراوي، برحي، مجول، دجلة نور (نور الديك)، العامري، ابراهيمي وثلاثة أصناف المعروفة باسم البلدي.

تم استخراج DNA من الأوراق الجديدة باستخدام مادة الفينول و الكلوروفوم وكذلك باستخدام احد الطرق المتوفرة تجاريا وهي DNA DNeasy Extraction Kit. تم مضاعفة المادة الوراثية باستخدام PCR لثلاث مناطق من SSR وبالنسبة ISSR فقد تم استخدام 9 بادئات (primers)، تراوح حجم المناطق المضاعفة من SSR 180-240 نيوكلتيد ومن ISSR 140 - 3100 نيوكلتيد. واستخدمت هذه النتائج لعمل مصفوفة لإنشاء شجرة النشوء والتطور للأصناف المدروسة باستخدام برنامج FAMD.

توزعت الأصناف قيد الدراسة في شجرة القرابة على ثلاث مجموعات: المجموعة الأولى تحتوي على صنف واحد هو العامري. وتضمنت المجموعة الثانية 5 أصناف. وقد تم تقسيم هذه المجموعة إلى مجموعتين فرعيتين؛ احتوت المجموعة الفرعية الأولى صنف واحد هو البلدي 3. أما المجموعة الفرعية الثانية فتشمل أربعة أصناف، الإبراهيمي، خضراوي، دجلة النور والحياني. تم تقسيم المجموعة الرئيسية الثالثة إلى مجموعتين فرعيتين؛ احتوت الأولى على أصناف مجول وبرحي، في حين احتوت الثانية على أصناف بلدي 2، زهيدي، حجازي، والبلدي 1. وتم التأكد من مصداقية شجرة القرابة باستخدام طريقتين احصائيتين هماBootstrapping وPCoA .

على الرغم من الاختلافات الشكلية المعروفة بين بعض أصناف النخيل المستخدمة في هذه الدراسة الا أن النتائج تظهر أنها ترتبط ارتباطا وثيقا في شجرة القرابة. ومن الأمثلة على ذلك صنفي الابراهيمي والخضراوي، كذلك دجلة النور والحياني، وأيضا صنفي حجازي والبلدي 1. ومن الجدير ذكره أن شجرة القرابة تميز بين الثلاثة أصناف التي تعرف باسم البلدي لدى المزارعين. وقد تم تسمية هذه الأصناف الثلاثة ببلدي 1-3 لتسهيل التمييز بينها في هذه الدراسة. التي تعد الأولى في فلسطين من حيث دراسة، القرابة الوراثية بين بعض أهم أصناف النخيل باستخدام تقنية SSR وISSR.

DECLARATION

I declare that the Master Thesis entitled " Molecular Phylogeny of Palestinian Date Palm Cultivars By Microsatellite Simple Sequence Repeats and Inter Simple Sequence Repeat Markers" is my own original work, and hereby certify that unless stated otherwise, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Dedication

My thanks are due to everyone supported and assisted me to do this thesis

I want to extend my endless thankfulness to my husband, kids, and family

Acknowledgment

I am grateful to my supervisor **Dr. Omar Dar Issa**, Director of the UNESCO Biotechnology Center at Bethlehem University, for advising me to select this topic to be my thesis and providing me with valuable guidance and support during my study which made it a great experience and pleasure for me.

Special thanks to **Dr. Naim Iraki** for his advice.

I owe a huge gratitude to my sincere friends in the Biotechnology master program, especially **Mariam Al-Ajrami**, who supported me. Furthermore, I would like to express my special thanks to my colleagues at the UNESCO Biotechnology Center.

We acknowledge the Agriculture Department of Jericho and the Jordan Valley of the Palestinian Ministry of Agriculture, especially for **Mahran Brahma Abu Serag** for supplying the palm date samples used in the present work.

May Allah give them long, prosperous and happy life (Ameen).

List of abbreviations:

AFLP	Amplified Fragment Length Polymorphism
FAMD	Fingerprint Analysis with Missing Data
ISSR	Inter Simple Sequence Repeat
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RELP	Restriction Fragment Length polymorphism
rpm	Round per minute
SNP	Single Nucleotide polymorphisms
SSR	Simple Sequence Repeat

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CHAPTER 1

1. Introduction

1.1. Background

Date palms (*Phoenix dactylifera* L), also called the "Tree of Life", have a long history of cultivation and utilization in the Middle East and North Africa for at least 5,000 years (Chao and Krueger, 2007). Date crops play a central role in the economy and social life in these regions in addition to industrial products. This tree, with its unique characteristics, has the ability to survive in dry and semi-arid regions. Hence, the important characteristics of the date palm are its tolerance to environmental stress, limited input requirement, long productive life, long lifespan (some trees live for hundreds of years), and high production of fruits which can be preserved for many years (Marsafari and Mehrabi, 2013). The geographic distribution of palm covers the regions between 10°N and 39°N in the Northern Earth and 5°S to 51°S in southern Earth (Al Khalifah, 2013).

The Date Fruit has been used as source of food for the last 5,000 years and has played an important role in world religions such as Christianity and Islam. It is considered to be traditional food for pilgrims to Makka and a major breakfast element during the holy month of Ramadan. The date palm is reported to have an important nutrition value which consist of 72–88% of sugar (carbohydrates) (Abdrabbo, 2013), minerals (i.e., iron, potassium, calcium, chlorine, copper, magnesium, sulfur, and phosphorus), amino acids, and vitamins (Al-Shahib and Marshall 2003). Moreover, antioxidant and anti-mutagenic activities are also found in date fruit (Vayalil, 2002).

1.2 Economic Importance of the Date Palm in Palestine

The date palm has great economic importance especially in the Middle East. Furthermore, its fruit value goes beyond the traditional consumption in the Arab World. Palm cultivation was known in Palestine for thousands of years and figured prominently between Palestinian farmers. As a tree of great economic value, along with great religious status, the date palm can live for hundreds of years and palm cultivation spread in the region of Jericho, the Jordan Valley, the Gaza Strip and, most particularly, in Deir al-Balah and Khan Younis. It is also known that the name of the historic City of Jericho means the "City of Palms" referencing the presence of many palm tree farms (Qutub, 1997).

Palm farmers in Palestine achieved qualitative leap with their cultivation of palm trees in terms of quality and quantity of the product during the period 2006 - 2012. During this time, Palestinian farmers were able to cultivate 59,000 palm seedlings, the equivalent of 4249 dunams of trees.

In mid-2012, an estimated 6071 dunams were cultivated with Palestinian palm trees in the Jordan Valley while the previous year, the estimated numbers of palm trees, according to figures from the Ministry of Agriculture, until the end of June 2011 was about 85,000 thousand trees. According to expectations, the amount of production of pure Palestinian dates will double, about 5,000 tons, by 2015.

The annual consumption of dates is 4,000 tons (Ministry of Agriculture). This means that 85% of the Palestinian date production goes to the domestic market and only 15% of date production is exported (http://www.wafainfo.ps/atemplate.aspx?id=8701).

The date palm industry produces a variety of dates such as: fresh and dried dates, whole dates and pitted (stoned) dates, date paste, date syrup and date wine. Date by-products which include cull dates, immature dates, date pedicels, date seeds, date press-cake and date molasses, are used as fodders to feed domesticated livestock (Chao and Krueger, 2007).

1.3 Geographical Distribution of the Date Palm

There are more than 3,000 date varieties in the world. According to the Food and Agriculture Organization of the United Nations (FAO) Statistics in 2011, the global production of dates reported in table 1 (FAOSTAT, 2011) stated that Israel reported a total of 37,008 tons of dates while The Arab World produced 73% of the total world production (Al Khalifa, 2013).

Table 1.1: Arab World production of dates in tons.

Country	**Production in ton**
Egypt	1,373,570
Saudi Arabia	1,122,822
Iran	1,016,608
Algeria	724,894,000
Iraq	619,182
Sudan	432,100
Oman	268,011
United Arab Emirates	239,164
Tunisia	180,000
Libya	165,948

Agriculture plays a significant role in the Palestinian civilization with date palms being cultivated since ancient time. Jericho in the West Bank and Deir Al-Balah in Gaza strip are the two major cities that cultivate palm trees. In the Palestinian territories there is an increase in date production resulting in 4,688 metric tons of cultivated dates in 2011 (FAO, 2013). There are many species of palm trees cultivated in Palestine with some being more than 50 years old.

Among the difficulties facing the date palm culture in Jericho and Gaza are: (i) The limited amount of water available for agriculture. (ii) Problems in fertilization and pollination. (iii) The lack of good propagation systems. (iv) The lack of overall planning for the agricultural section. (v) Obstacles from occupation.

1.4 Date Palm Taxonomy and Morphology

The date palm, *Phoenix dactylifera L.*, is a long lived dioecious species, diploid (2n=36) and belongs to the genus *Phoenix* (Haider et al., 2012), a member of the *Phoeniceae monocotoledonous* family *Palmae* (Zhao et al., 2013). Phoenix, which means purple or red in the Greek language and it refers to the color of the fruit and *dactylifera*, which means "finger", referring to the fruit appearance or shape (Chao and Krueger, 2007). The palm family contains 183 genera and 2500 species that are divided into five subfamilies: *Nypoideae, Calamoideae, Ceroxyloideae, Coryphoideae* and *Arecoideae* (Dransfield et al., 2005).

According to variety and growth conditions, date fruit varies in shape, size, weight and color (dark brown – reddish- yellowish brown). They are oblong in shape or may be almost round. The length and width may vary from 18-110 mm to 8-32 mm and the average weight per fruit is between 2 to 60 grams (Zaid, 2002).

The palm tree grows up to 10 m high. Its trunk (stem or stipe) is vertical, cylindrical and columnar of the same girth all the way up. The leaves of a palm tree are 3 to 6 m long and have a normal life span between three to seven years. The greatest width of the leaf midrib attains 0.5 m, but elsewhere it is only half this size and rapidly narrows from the base upwards. The leaf midrib or petiole is relatively triangular in cross section with two lateral angles and one dorsal. It is bare of spines for a short distance but full of spines on both sides thereafter. The intermediate zones have spine-like leaflets. (Qutub, 1997; AlKhalefah, 2013). Furthermore, roots play a critical role in the water absorption, transport and water storage in palms.

Date palms trees are gonochoric which means they have separate male and female plants. The plant is characterized by its ending in a lump of leaves with each leaf having an axillary bud which can produce offshoots and give rise to inflorescences in the mature phase (Litz, 2005). As is well known, the palm is propagated sexually through a vegetative method by offshoots; an adult date palm in its lifetime produces 5-15 offshoots (Al Kaabi et al., 2003). Plants are produced through seeds are not practiced in traditional cultivation because most of the trees from seeds have different genotypes from the mother and the female to male ratio is 50:50.

1.5 Diseases

Date palm trees cultivation requires attention by advanced science, not only in the development methods of propagation and profitable species, but also in disease control. This serious problem which could kill millions of trees. The cause of palm tree death often occur by fungi like Bayoud disease, fruit rot, graphiola leaf spot and diplodia disease. In addition, black scorch, brown leaf spot, belaat disease, brittle leaves disease, al wijam disease and lethal yellowing also result in tree mortality (Djerbi et al., 2003; Qutub, 1997).

A major problem facing farmers in Jericho is pest of date palm, the Red Palm Weevil, *Rhynchophorus ferruginous* (Blumberg, 2008). It is also called the hidden enemy, the Red Palm Weevil is invasive and spends all its life stages inside the trunk of the tree. It remains undetected until the signs of the disease begin to manifest. However, the manifestation of the disease is the beginning of the destruction and by then, it is too late to save the dying tree. The Weevil feeds on the apex stem and infects trees that do not exceed twenty years old (Al Omari, 2012). Unfortunately, the Palestinian agricultural sector does not have a clear strategy on how to combat these problems.

1.6 Date Palm Cultivars in Palestine

Thousands of date palm cultivars exist in different parts of the world and they are very difficult to distinguish by the plants morphology. Nevertheless, they could be identified by the character of the fruit, which are produced only after 4-5 years (El Kichaoui1 et al., 2013). The fruits of the Phoenix genus are drupes of variable sizes, depending on the species, with a single grooved seed.

The most common date palm cultivars in the West Bank includes, but not limited to, Zahidi, Hayani, Hejazi, Khadrawy, Barhy, Majool, Dejlet Noor (Deek Noor), Amery, Ibrahemi, and Baladi. Baladi cultivar is a problem because there are many date palm cultivars named Baladi; hence, in this study for instance, we had to collect more samples from many trees with the same name. These cultivars are classified by agronomists and farmers, which mainly based on fruit characteristics which might be misleading in some cases.

Studies on the genetic diversity of date palm germplasm have been limited in Palestine. One of these studies was conducted in the Gaza strip by using random amplification of polymorphic DNA (RAPD) to study the genetic diversity among the six cultivars (El Kichaoui, 2013). Therefore, the major aim of the current study is to establish a phylogenic tree for those cultivars.

1.7 Diversity and Genetic Markers

With the number of the palm tree varieties distributed in the world being approximately 5,000, Date palms have high genetic diversity. There are about 450 varieties that are found only in Saudi Arabia (Al Kalifah, 2013). The most widely grown varieties are those globally characterized morphologically and their markers depend on phenotypic traits of leaves, spines and fruits, sensitivity to environmental factors which may change due to interaction with environmental conditions (Elshibli and Korpelainen, 2009). Furthermore, there are several morphological markers that are considered phenotypic traits or characters. The most common morphological or phenotypic character used for the date palm is the morphology of leaves, spines and fruits. Such morphological features are sensitive to environmental factors and can be observed only in mature trees. Another marker is the biochemical marker, which includes allelic variants of enzymes, the so called isozymes (Zehdi et al., 2004).

Molecular techniques may be a more reliable approach for cultivar discrimination. DNA-based markers have been used in the assessment of genetic diversity in plant species for different purposes (Arabnezhad, 2011). It provides information regarding the large numbers of polymorphisms for genetic analyses, relationship between cultivars and analyzes relatedness between or within different populations (species and individuals). Some of these markers can be used to identify genes controlling fruit quality traits, disease resistance genes and also used to create genetic maps (Trojanowska and Bolibok, 2004). There are many types of DNA

markers that have been used in genotyping of date palm, each with its own advantages and disadvantages. On one hand, Molecular markers are more accurate and can identify date palm cultivars and quantify their genetic diversity and phylogenic relationships (Cho et al., 2000). While DNA-based Molecular markers have been used to study the genetic variation of date palm cultivars (Elmeer, 2011). These markers are selective as they are usually located in non-coding regions of the DNA. There is another type of markers called expressed sequence tags (ESTs) that are developed from expressed regions in the genome (Zhao et al., 2013).

1.7.1 Dominant and Co-dominant Markers

DNA markers are useful in detecting differences between cultivars of the same or different species. These markers are called polymorphic; markers that do not distinguish between genotypes are called monomorphic. Polymorphic markers may also be described as co-dominant or dominant based on whether or not the marker can detect the differences between homozygotes and heterozygotes. Co-dominant markers indicate differences in size while dominant markers are either present or absent (Collard et al., 2005).

1.7.2 Different types of DNA markers:

Several molecular markers have been used for measuring date palm genetic diversity e.g. co-dominant include: Amplified Fragment Length Polymorphism (AFLP) (Vos, 1995), Simple Sequence Repeats (SSRs) (Hamza et al., 2012), and Single-Nucleotide Polymorphisms (SNPs). The dominant markers include Randomly Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Inter Simple Sequence Repeat markers (ISSR). Microsatellite markers are expected to provide a valuable and highly informative resource for genetic mapping and diversity analysis in date palm (Elmeer et al., 2011).

The advantages of DNA markers are: i) they measure the diversity directly at the DNA level and hence, characters are not influenced by the environment and they are independent of the physiological stage of the plant; ii) they have ability to obtain large amounts of data in a short time; iii) they can be used as a non-destructive test of polymorphism; iv) they provide the possibility of obtaining data on non-living material. Needless to say, they also have disadvantages as well. Most of these marker

protocols are time consuming and expensive and for some of them the amount of polymorphism is low and the application is complicated (Soliman et al., 2003).

1.7.3 RAPD and RLFP

Randomly Amplified Polymorphic DNA (RAPD was commonly used for estimation of the genetic diversity in plant species, which detects DNA polymorphisms amplified by primers. This technique has been applied to identify date palm on the variety level. However, the technique is laborious and therefore not well suited for studies involving a large number of samples. Hence, a common problem experienced with RAPD analysis is its poor reproducibility. It is therefore essential to optimize the PCR to obtain reproducible and interpretable results (Williams et al., 1990; Rhouma-Chatti et al., 2011).

Restriction fragment length polymorphism (RFLP) is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The problem in using this method was in the needs to use different kits adapted to the size of the genome being analyzed. Developing locus-specific markers from individual fragments can be difficult (Ajmone-Marsan et al., 1997; Vos et al., 1995).

1.7.4 Microsatellite Markers

Microsatellites have been employed widely because they can be readily amplified by PCR and the large amount of allelic variation at each locus. These markers are copious, distributed throughout the genome and are highly polymorphic compared with other genetic markers. Microsatellite markers were used to investigate the genetic diversity and relationship among date palm cultivars (Billotte et al. 2004).

1.7.4 a. Simple Sequences Repeat Marker (SSR)

Simple sequence repeat (SSR) markers have been used in plant genetic diversity. They are highly polymorphic and highly reproducible. SSR contains 1 to 6 nucleotide units repeated in tandem and randomly spread in eukaryotic genomes. SSRs are very polymorphic due to variation in the number of repeat units and they can be found in both coding and non-coding regions (Akkak et al., 2009). The repeat length at specific SSR loci is easily assayed by PCR using specific primers for conserved regions flanking the repeat array (Abass, 2013).

There are two strategies used to create SSR markers: searching for sequences containing microsatellites in the available data bases, and constructing the marker and screening the genomic library with complementary probes to microsatellite sequences (Glenn and Schable, 2005). SSRs have several advantages, for example: microsatellites allow the identification of many alleles at a single locus, little DNA is required, they are distributed all over the genome, and the analysis could be semi-automated and performed without the need of radioactivity. But their use is still limited because of the long and laborious steps required to develop them (Billotte et al., 2004; Akkak et al., 2009).

1.7.4 b. Inter Simple Sequence Repeat Markers (ISSR)

ISSR-PCR is a genotyping technique based on amplification of DNA segments in the regions between microsatellites. It has been used for many purposes such as genetic fingerprinting cultivar identification and phylogenetic analysis. They seem to be suitable for phylogenetic studies, the evaluation of genetic diversity and cultivar identification. The major advantages of this method is its low cost and it is not time-consuming step of genomic library construction (Zietkiewicz et al., 1994; Askari et al., 2011).

Chapter 2

2.1 General objectives:

To establish a phylogenetic tree and to study the genetic relatedness among the most common date palm varieties in Palestine using the molecular markers; SSR and ISSR.

2.2 Specific objectives:

- To optimize and apply a reliable protocol for DNA extraction form palm leaves.

- To discriminate between date palm cultivars based on SSR and ISSR markers.

- To examine the efficiency of the 3 SSR primers and 9 ISSR primers for studying genotyping and identification of the cultivars.

- To compare between the two techniques.

Chapter 3

Methodology

3.1 Samples collection

Young leaves were collected from date palm trees. Eleven samples, each from a different tree, were collected from each cultivar (Zahidi, Hayani, Hejazi, Khadrawy, Barhy, Majool, Dejlet Noor (Deek Noor), Amery, Ibrahemi, and Baladi). The Balady samples selected randomly from trees harboring small dry fruits. The origin of tested date palms is from the Station of the Ministry of Agriculture and other farms in Jericho as shown in Table 3.1.

Each sample was collected was based on the knowledge of an the agricultural engineer , and each sample was cleaned by removing the dried leaves and taken in clean bags with labels, and stored in a refrigerator (4°C) till analyses.

Table 3.1: List of the names of the date palm cultivars used in this study.

No.	**Cultivar name**	**Origin of Cultivar**
1	Zahedi	Jericho Station
2	Hayani	Farmer (Jericho)
3	Hejazi	Jericho Station
4	Khadrawy	Farmer (Jericho)
5	Berhy	Jericho Station
6	Majool	Jericho Station
7	Dejlet Noor	Jericho Station
8	Amery	Farmer (Jericho)
9	Ibrahemi	Farmer (Jericho)
10	Baladi 1	Jericho Station

11	Baladi 2	Jericho Station
12	Balady3	Jericho Station

3.2 DNA extraction

Total genomic DNA was extracted from 100 mg of young fresh leaves of each cultivar. The leaves were first washed carefully with distilled water and using blade to remove the waxy layer and then cut the leaf samples in a small pieces and were grounded to a fine powder in liquid nitrogen by using pestle and mortar.

The powder was put in a tube and mixed with an equal volume of Phenol:Chloroform (24:1), then it was shaken for 0.5-1 hour. The mixture was then centrifuged at 10K rpm for 10 min. The top layer (aqueous) was removed to a new labeled tube and the DNA was precipitated using Sodium acetate and 100% Ethanol. After that, the tubes were vortexed and centrifuged and the precipitate was washed with 70% Ethanol and air-dried. The DNA was further purified by following the steps of the (QIAGEN) for DNeasy Plant Mini Kit. In a second trial, the DNA was extracted using the DNeasy Kit without the phenol-chloroform steps. After that, the quantity of the extracted DNA was determined by running on 1% agarose gel stained by Ethidium Bromide and visualized under UV light as well as using a Nanodrop Spectrophotometer. Finally, the DNA samples were stored at -20˚C till use.

3.3 PCR amplification of SSR and ISSR marker

A set of four SSR primers and nine ISSR primers were tested to amplify the extracted DNA. These primers are listed in below (table 2 and 3) with their characteristics. These primers were selected based on previous studies conducted by Elmeer, 2011 and Zehdi, 2002.

For PCR amplifications, a 20 µl reaction mixture was used and it contained between 20 and 30 ng of total genomic DNA (1 µl), 30 pg of primer (0.5 µl), these components were added to AccuPower® PCR PreMix tube (Bioneer Corporation – Hylabs). Amplifications were performed in 9600 Perkin Elmer Thermal Cycler as follows: One step of 95˚C for 3 min followed by 35 cycles of three steps:

Denaturation at 94˚C for 30 sec, annealing depending on primer for 30 sec, extension at 72˚C for 1 min, and a final extension step for 7 min at 72˚C.

To reduce the possibility of cross contamination in the amplification reactions, a master reaction mixture was routinely prepared and a negative control was used. Amplification products were separated by electrophoresis on 1.6% agarose gels stained with Ethidium Bromide in 1X TBE buffer at 100 mA for 1-1.5hr. Gels were photographed using a BioDoc-It imaging system, UVP. Additionally, SSR and ISSR analyses were repeated twice for all samples and only clear bands produced in both replicates were scored and their sizes were estimated by using a 100bp DNA ladder.

Table 3.2: List of SSR primers and there theoretical annealing temperatures.

Primer name	**Primer sequence 5'□3'**	**Annealing temp. (Tm)**	**Expected size**
DP157	F: TGG ACA ATG ACA CCC CTT TT R: GCC CAC ACA ACA ACC TCT CT	56°C 60°C	180-244
DP160	F: AAG AGC GAC AAT CAT GAC CA R: GGA AAT TGA AGG GCA TCT TG	56°C 56°C	108-136
DP168	F: GCA GCA AAG CCC TTA GGC R: GGT GTT ATG TGC AGC CAA TG	58°C 58°C	163-175

Table 3.3: List of ISSR primers used in this study and there annealing temperatures.

	Sequence	**Annealing temp. (Tm)**
1	(AGG)6	60°C
2	(ACTG)4	48°C

3	(GACAC)4	64°C
4	(AG)10G	64°C
5	(AG)10C	64°C
6	(AG)10T	62°C
7	(CT)10A	62°C
8	(CT)10G	64°C
9	(CT)10T	62°C

CHAPTER 4

Results

4.1 Yield of DNA Extraction

Isolation of DNA highly pure is important to PCR amplification, and date palm leaves are hard and fibrous to extract DNA from it. In addition, the plants contain polyphenolic compounds and polysaccharides which imposes difficulties for PCR experiments. Our trials to extract and purify the genomic DNA, using the DNeasy Plant Mini Kit without the involvement of a Phenol-chloroform step, yielded very small impure amounts of DNA. Therefore, such DNA was not used for the subsequent PCR amplifications.

Nevertheless, we successfully isolated DNA from date palm plant by using an additional step (Phenol-chloroform step). The A260/ A280 values were in the range of 1.7-2. White DNA pellets formed and were quickly soluble in TE buffer. The results of the agarose gel test and PCR or RAPD analysis indicated that polysaccharides had been efficiently removed and that the DNA quality had been improved. Also, the readings showed that our method was efficient in removing polysaccharides, polyphenols and RNA. The DNA obtained was free of any contaminating proteins, polysaccharides, or colored pigments

4.2 Molecular characterization & PCR amplification

In this study we applied the Microsatellites markers and the results indicated a high level of polymorphism for eleven cultivars by using SSR and ISSR marker. The following primers were used in SSR-PCR: DP157, DP160 and DP169 while the primers: DP157, DP160, DP168, (AGG)6, (ACTG)4, (GACAC)4, (AG)10G, (AG)10C, (AG)10T, (CT)10A, (CT)10G and (CT)10T were used for the ISSR-PCR. Only the clear bands of PCR products were measured as polymorphic markers, The DP157 primer product showed no bands (result not shown), the primer DP160 indicated only one allele in cultivars except for the last one which showed no band (fig 4.1). The primer DP169 showed high heterozygosity and identified 1-3 alleles in between cultivars (fig 4.2). ISSR markers produced a different level of polymorphism among the different cultivars and some of the results are shown in the below figures (fig 4.3). The number of polymorphic bands per primer varied between 5-26 and the analysis based on the intensity size from 140 bp to 3100bp (table 4.1). A minimum of 5 and maximum of 26 DNA fragment were obtained using (GACAC)4

and (AG)10C respectively. All other primers generated an intermediate number of the bands with an average 16.6. The primers that showed weak were discarded.

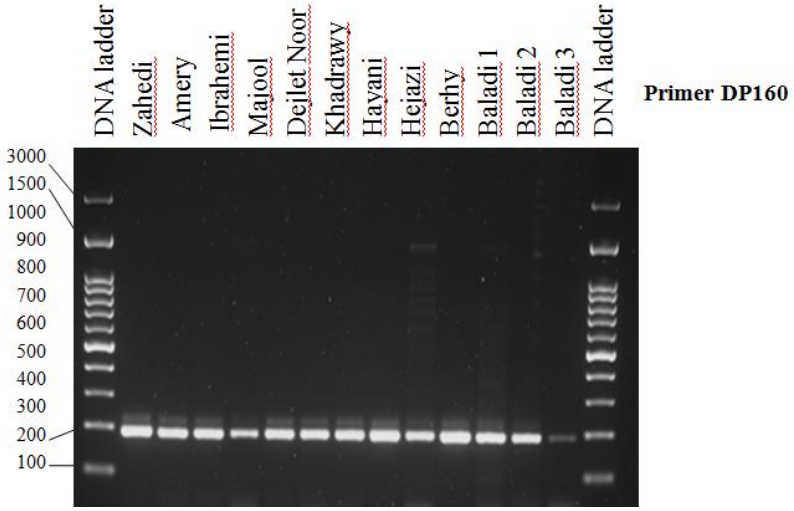

Figure 4.1: SSR-PCR band profiles generated by primer DP160

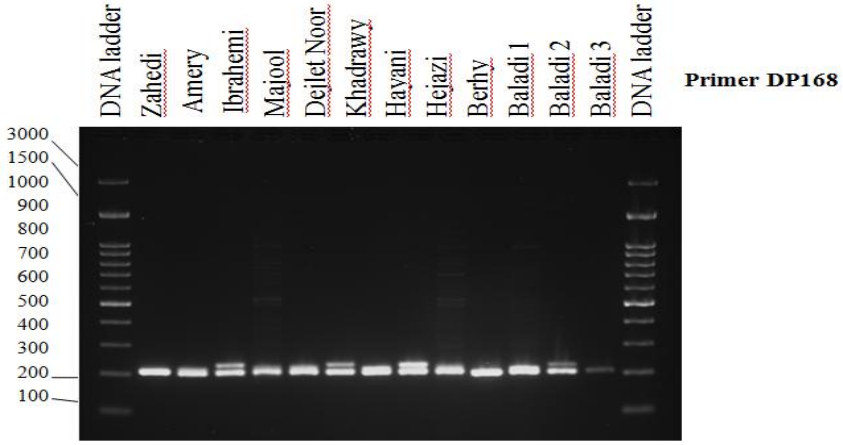

Figure 4.2: SSR-PCR band profiles generated by primer DP169

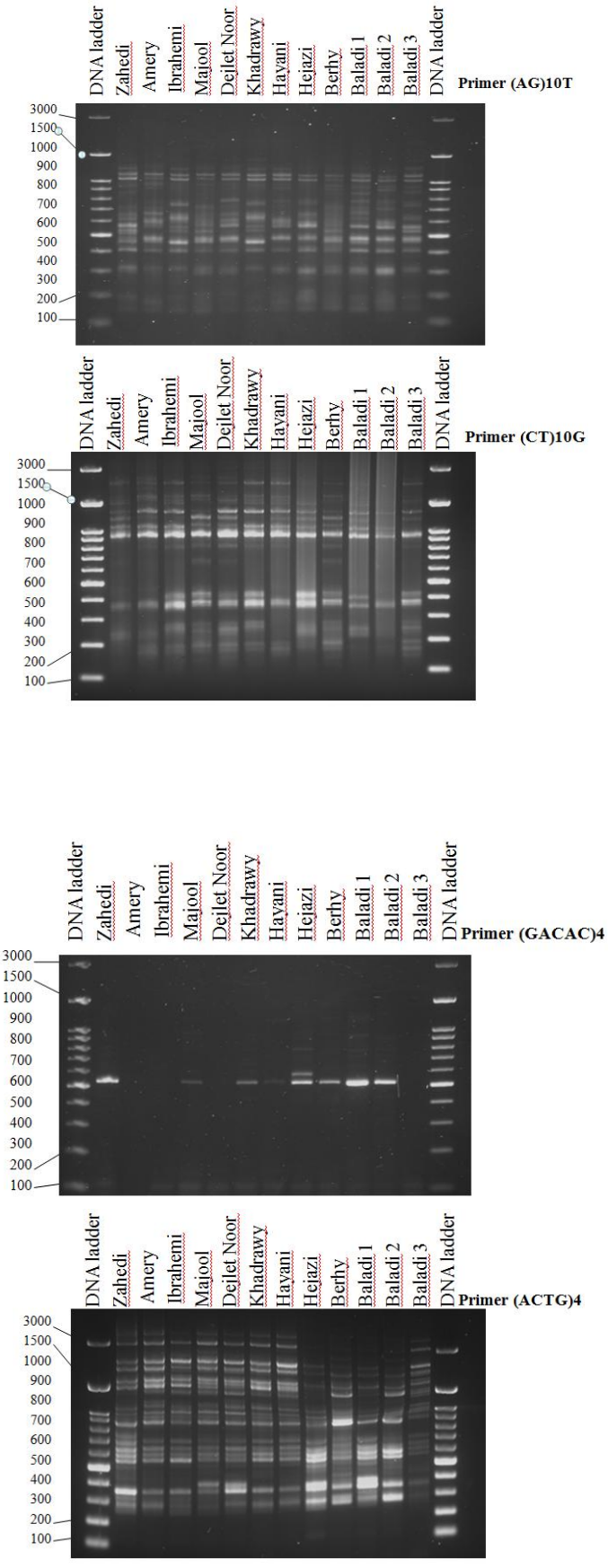

Figure 4.3: PCR products of ISSR generated by primers (AG)10T, (CT)10G, (GACAC)4 and (ACTG)4

4.3 Analysis of SSR & ISSR profiles

Each polymorphic band was treated as a separate character and scored as present (1) or absent (0) to generate binary matrix. Furthermore, each primer was analyzed and calculated the Total Number of Band, the Number of Polymorphic Bands and Number of Exclusive Band shown in Table 4.1. SSR and ISSR data were then computed using the Fingerprint Analysis with Missing Data (FAMD) software to generate a relationship among twelve cultivars (Schluter, 2006), and to establish Neighbor-joining tree for 12 cultivars .

Table 4.1: Characteristics of ISSR banding profiles produced in varieties of date palms cultivated in Palestine.

Primer	Total No. of bands (TNB)	No. of scorable bands	No. of poly. bands	% of poly. Bands (P)	NEB	Size (bp)of fragment
(ACTG)4	30	25	17	68	2	240-3100
(AG)10C	28	26	22	84	0	200-2700
(AG)10G	26	24	18	75	1	150-2000
(AG)10T	24	19	16	84	0	140-2100
(AGG)6	10	9	8	88	1	350-2400
(CT)10A	15	14	14	100	1	400-3000
(CT)10G	23	22	19	86	0	160-2300
(CT)10T	7	6	5	83	1	600-1300
(GACAC)4	10	5	5	100	2	500-920
DP160	1	1	1	100	0	180
DP168	3	3	2	66	0	190-240

The phylogenetic diagram (Phylogram) shows similarity and genetic relatedness among the date palm cultivars and the analysis indicated three cluster groups as shown in (Fig 4.4). Cluster 1 encompasses only one cultivar, Ameri, while cluster 2 contains five cultivars Balady 3, Ibrahemy, Khadrawi, Deek Alnur and Hayani. The third Cluster is the large one which contains sex cultivars Majool, Berhy, Balady2, Zuhadi, Hijazi, Balady 1. In addition, the analysis of data by Principal Coordinate of Analysis (PCoA) was used to confirm the phylogram and to show the pattern of relationships contained in the matrix by placing the cultivars on three axes. The plotted pattern below supports the genetic diversity and its existence among cultivars which makes it easy to distinguish them from one another (fig 4.5).

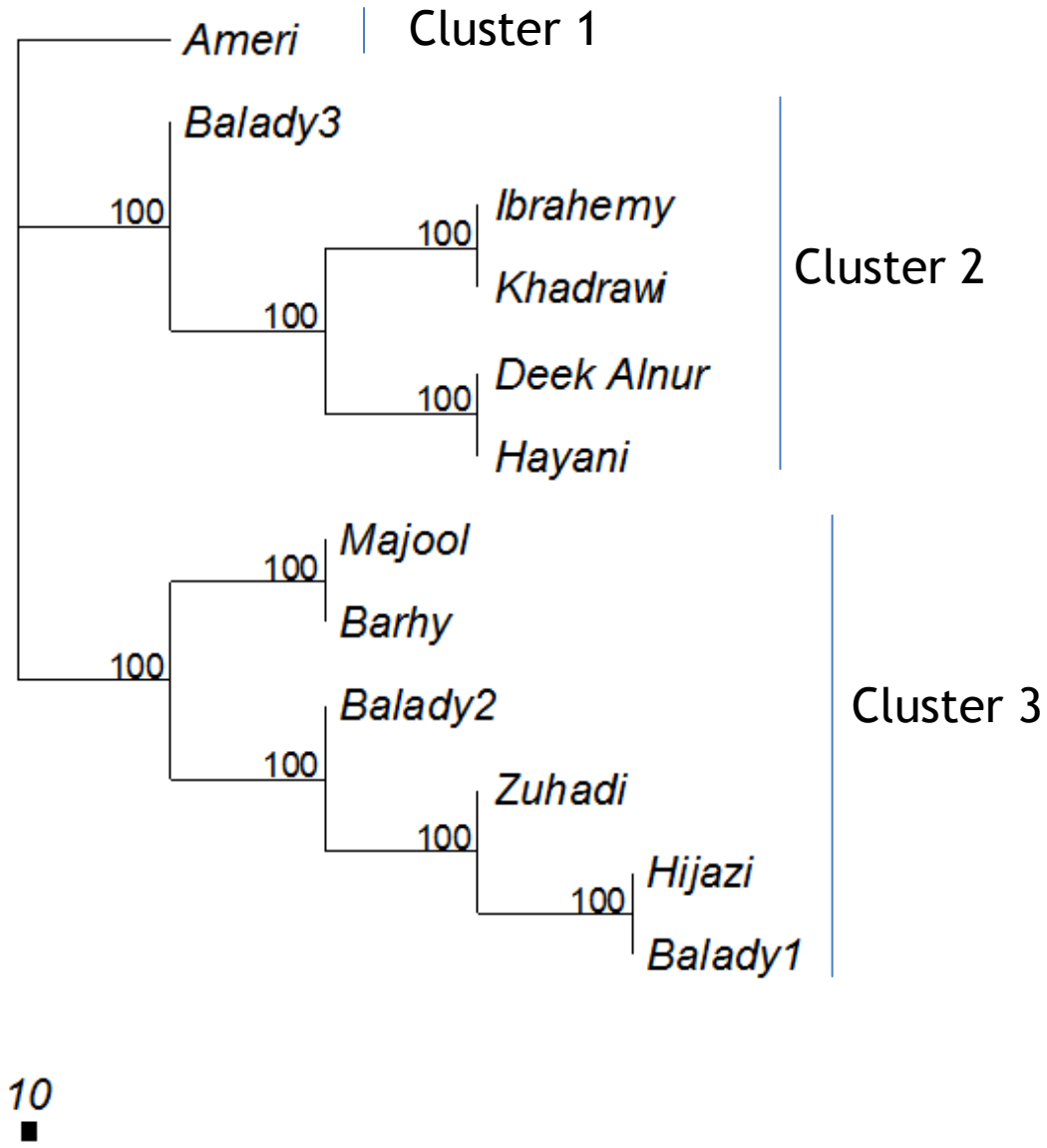

Figure 4.4: Phylogenetic tree showing the relatedness among 12 date palm cultivars used in this study.

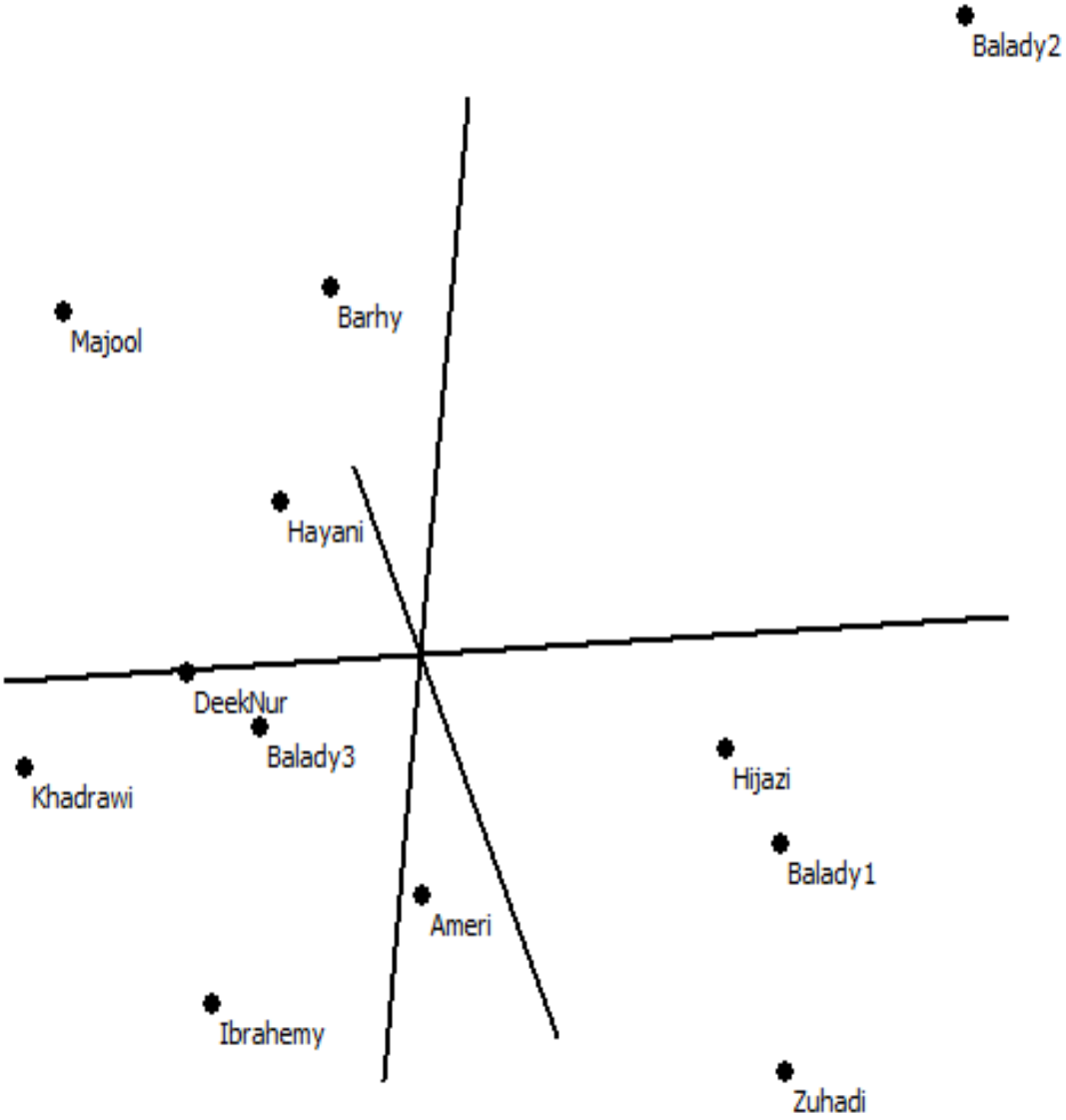

Figure 4.5: Principal Coordinate of Analysis (PCoA) of 12 date palm cultivars based on the results of the SSR-PCR and ISSR-PCR markers used in this study

CHAPTER 5

Discussion

One of the most important issues that appeared before the PCR reaction is the DNA extraction. This poses a problem in date palm plant, because the presence of polyphenols. They are a powerful oxidizing agents and tends to bind with extracted DNA. The presence of polyphenols reduces the yield and the purity of the DNA, which makes it useless for most studies. Polysaccharides are known to inhibit PCR reactions. They alter the results in many reactions and, therefore, lead to wrong interpretations (Porebski *et al.*, 1997; Hemphill *et al.*, 2006). Polyphenol contamination of DNA makes it resistant to restricting the enzymes and this interaction is irreversible between phenolic compounds and proteins (including nucleic acids) (Cheng *et al.*, 2003). To obtain good readings, the leaves were frozen before they were used and grounded. Liquid nitrogen was also added. The leaves were also mixed with phenol-chloroform before using the kit to get more yield of DNA. The quantity and quality was also tested for DNA prepared.

In this study we have chosen two techniques, SSR and ISSR technology. This was essential in order to add to the number of molecular markers that are suitable in the characterization of a date palm. Studies on the diversity of date palm germplasm have been limited in Palestine. One of these studies was done in Gaza strip by using random amplification of polymorphic DNA (RAPD). This method was used to study the genetic diversity among the six cultivars (El Kichaoui, 2013). However, Microsatellites are now the leading and the most applied method for most of molecular genetic studies. Microsatellites are also excellent markers for high-throughput analyses, and fluorescent techniques.

One of the main challenges of this research is selecting the markers for their specific purposes. The ideal genetic marker depends on highly polymorphic results, show co-dominant inheritance and be evenly distributed throughout the genome. In addition, marker sequences should be easy to access, and analysis, but no marker type could meet all requirements. However, based on our study, we found out that we can

choose from the different molecular marker systems and select the one that best suits our research needs. So many factors should be considered when choosing the molecular markers such as quantity and quality of available DNA.

Marker system availability in the this study of microsatellite analysis was used to investigate the genetic diversity in 12 date palm samples collected from trees in Jericho farms in Palestine. Recently, microsatellite markers were intensively used to investigate the genetic diversity and relationship among date palm cultivars. Simple sequence repeat (SSR) molecular markers are co-dominant, highly polymorphic and highly reproducible (Akkak et al., 2009). Three markers were selected randomly from a list that was highly recommended in a Qatar study shows (++) status of amplification (Elmeer et al., 2011). The DP157 primer product showed no polymorphism. The primer DP160 amplified monomorphic banding patterns at 180 bp except the last cultivar (Baladi 3) which indicated a very weak band compared to others (fig 4.1). The primer DP169 showed heterozygosity and the number of alleles per locus detected was1-3 alleles among cultivars. For example, Zahedi, Ibrahimi, Khadrawy, Hejazi and Baladi 2 cultivars showed one band, while on the other hand, Amery, Majool, Dejelt Noor, Hayani, Berhy Baladi 1 and Baladi3 cultivars produced two bands.(fig 4.2). However, the number of alleles per locus detected in this study were lower than those scored by (Elmeer et al., 2011). The number of alleles varied between 4 in primer DP168 and 12 in primer DP157 (Elmeer et al., 2011) . The two primers used in this study successfully produced clear amplified SSR band sizes ranging from 180 bp to 240 bp similar to the results reported by (Ahmad And Al-Qaradawi 2009) and (Elmeer et al., 2011). These result ranged from 100-300 bp.

Inter-simple sequence repeats (ISSR) marker are dominant molecular marker and considered to be a powerful technique for the analysis of genetic diversity of germplasm. It shows a high mean value for the number of effective alleles and high yield of amplified DNA fragments (Hamza et al., 2012). Because the level of polymorphism among the cultivars tested is necessary for the reliability of a molecular marker technique; ISSR techniques has high reproducibility among date palms and more informative than others (Marsafari and Mehrabi, 2013). A total of 9 primers were selected from previous study on Tunisian date palm cultivars. (Zehdi et al. 2002). The 9 primers used for ISSR produced reproducible and a measurable patterns. The amplification profiles were screened for the presence of polymorphisms

among the genotypes analyzed. Primer (AG)10C yielded the highest number of fragments which produced 26 bands, while the lowest number of fragments was observed for primer (GACAC)4, which produced 5 fragments. Of the 9 primers used, two had all bands polymorphic (100%) (CT)10A and (GACAC)4. The remaining primers showed polymorphic bands that ranged from 68% ((ACTG)4) to 88% ((AGG)6) with a total average of 59.8% per primer. In profiles generated for all primers, the sizes of the fragments ranged from 140- to 3100-bp (table 4.1).

The phylogram (Fig. 3) is in agreement with the (PCoA) analysis. Three main clusters were observed. The first cluster contained one cultivar, Ameri. The second cluster included 5 cultivars. This cluster was divided into two sub-clusters; the first sub-cluster contained one cultivars Baladi 3, and the second sub-cluster was further divided into two groups; one containing two cultivars, Ibrahimi and Khadrawi and the second containing Dejlat Noor and Hayani. The third cluster was divided into two sub-clusters as well. The first one contained Majool and Berhy and the other one is Balady 2 with sub-cluster of Zuhadi and another group of Higazi and Baladi 1.

Depending on dendrogram construction, there are four groups most closely related cultivars studied: (Ibrahemy and Khadrawi), Ibrahimy has oblong fruit and medium size and it has purple to black color with broadly wrinkled skin and dried flesh. Khadrawi has obovoid-oblong fruit shape and pale green color in beser stage turning yellow with reddish brown with fissured skin. The second group is (Dejlat Noor and Hayani). Dejlat Noor has linear-oblong Fruit and light yellow color in beser turns to reddish brown, with wrinkled skin. On the other hand; Hayani has oblong fruit shape and dark red color and the skin separates easily. The third group (Majool and Berhy). Majool is a very robust, ellipsoid-oblong fruit and it has orange-red color of beser stage. It turns to dark brown or black with loose broadly wrinkled skin, while Berhy has broadly ellipsoid or globose with flat base fruit. It has a lemon yellow color in beser stage and turns golden brown color with wrinkled skin. The fourth group is (Hijazi and Baladi 1). Hijazi has ovate fruit shape with big size, in beser stage yellow in color and turns to yellow-green with low fibers content, but Baladi has small fruit with wrinkled skin and brown color. Its flesh is very dry and has a low moisture content (Al-Khalifah et al., 2013) (El Kichaoui et al., 2013). According to morphological characteristics, fruit shape and taste, Balady cultivars should be very similar to one another, but the results showed that they are genetically

different. Baladi 3 is in a second cluster and Baladi 2 in a third cluster with Balabi 1 but in another sub-cluster.

Table 5.1: A summary of the morphological characteristics of ten date palm cultivars from Jericho.

Zuhadi	Fruit shape: ovate Fruit color: golden brown Fruit size: small Fruit skin: mild wrinkled	
Amri	Fruit shape: oblong Fruit color: reddish-yellow Fruit size: madium Fruit skin:mild wrinkled	
Ibrahemy	Fruit shape: oblong Fruit color: purple to black Fruit size: long Fruit skin: wrinkled	
Majool	Fruit shape: ellipsoid-oblong Fruit color: dark brown Fruit size: medium Fruit skin: wrinkled	
Dejlat Noor	Fruit shape: linear-oblong Fruit color: reddish brown Fruit size: long Fruit skin: wrinkled	
Khadrawi	Fruit shape: oboviod-oblong Fruit color: reddish brown Fruit size: small Fruit skin: mild wrinkled	
Hayani	Fruit shape: oblong Fruit color: dark red Fruit size: medium Fruit skin: smooth	

Hijazi	Fruit shape: ovate Fruit color: red Fruit size: big Fruit skin: smooth	
Berhy	Fruit shape: broadly ellipsoid Fruit color: lemon yellow- golden Fruit size: big Fruit skin: smooth	

The Palestinian cultivars were differentiated by using morphological characteristics of the fruits, color and form of the fruit. In fact, most of the date palm cultivars grown in Palestine can be distinguished at the production stage using morphologic and taste characteristics. A study done in Gaza Strip using 42 RAPD primers to study the genetic diversity among six cultivars (Hayani, Bentaisha, Halawy, Zahedi, Berhi and Amery), showed that two clusters where the first one contained Amri and sub-cluster: Bentaisha and Hayani and the second cluster contained Zahedi and sub-cluster: Halawy and Berhi. In our study, Ameri was in its own cluster. However, in the Gaza study, it was grouped with Hayani. On the other hand, our study yielded the same result for Zuhadi and Berhy that they are in the same cluster but in deferent sub-cluster.

Microsatellites proved useful and effective for phylogenetic studies and cultivar identification among different Palestinian date palm. The same is true for studies in Egypt (Adawy et al. 2002,), Tunisia (Zehdi et al. 2004, Hamza et al. 2012), Sudan (Elshibli and Korpelainen, 2008), Oman (Al-Ruqaishi et al. 2008), Iraq (Khierallah et al. 2011), Qatar (Ahmed and Al-Qaradawi 2009, Elmeer et al. 2011), Syria (Haider et al. 2012) and Libya (Racchi et al. 2013). Recently, date palm genome was de novo sequenced and annotated (Al-Dous et al. 2011). The analysis of the SSR & ISSR genotypes allow evaluating the genetic differences based on distances among the cultivars tree. The results are an evidence that the genetic diversity exists and it distinguish them easily.

The selected primers generated an appropriate amplification pattern with clear, consistent, and reproducible bands. The efficiency of microsatellite markers in discriminating all the cultivars examined confirms their usefulness for identification.

Since each variety was identified by a unique profile, it is possible to generate an individual profile useful in the documentation of date palm. This would help in the management of plant accessions and in the breading stages. Furthermore, farmers are faced with a problem of distinguishing cultivars that clonally propagated by offshoots. Since the identity determination is only possible after the first flowering and fruiting stages at the age of about 5-7 years, early identification of cultivars would prove valuable.

DNA markers are more suitable and ubiquitous to most applications (comparative morphology, anatomy, epidemiology and phylogeny). They are highly polymorphic in nature, frequently occurring in genomes, highly reproducible and easy to access.

CHAPTER 6

Conclusion and Recommendations

Molecular markers are efficient tools for cultivar identification and estimation of relatedness through DNA analysis. In this investigation, two types of molecular makers SSR and ISSR were used on the genetic polymorphism among Palestinian date palm cultivars (Zahidi, Hayani, Hejazi, Khadrawy, Barhy, Majool, Dejlet Noor (Deek Noor), Amery, Ibrahemi, and Baladi 1 2 3).

The results of this study showed clear and high yields of amplified DNA fragments. SSR and ISSR method appears to be a powerful technique for the analysis of genetic diversity of date palm germplasm. Many primers are now known for future genetic diversity analysis of date palm and for the identification of available varieties. Three primers in SSR and 9 primers in ISSR showed a high number of bands and had been discriminated among the 12 cultivars. ISSR markers are molecular markers but ISSR markers showed a higher value for the number of effective bands.

The cultivars studied were genetically different using morphological characteristics of the fruits (color and form of the fruit). In fact, most of the date palm cultivars grown in Palestine can be distinguished at the production stage using morphologic and taste characteristics. The SSR and ISSR techniques are useful to differentiate the cultivars that cannot be discriminated by the morphology. Nevertheless, the study the genetic diversity of Baladi genotypes is important. The Neighbor-Joining cluster phylogram based on the microsatellite markers grouped the 12 date palm cultivars into three main clusters: In cluster 1 out of one cultivar only Ameri. Cluster 2 contained five cultivars Balady 3, Ibrahemy , Khadrawi, Deek Alnur and Hayani. Cluster 3 is the larger one containing six cultivars Majool, Berhy, Balady2, Zuhadi, Hijazi, Balady 1. It was identified that the lowest distance was between (Ibrahemy and Khadrawi), (Dejlat Noor and Hayani), (Majool and Berhy) and (Hijazi and Baladi 1). Balady cultivars should be very similar, yet the results showed that they are genetically different. Baladi 3 is in a second cluster and Baladi 2 in a third cluster with Balabi 1 but in another sub-cluster.

World-wide date palm genome needs further investigations and also the identification of proper markers that may assist in identifying the economically and

agronomical important cultivars. Genetic differentiation may cover specific traits that contribute to adaptive mechanisms in phenotype. I believe that further investigations to identify specific traits for drought adaptation as well as possible drought responsive genes are needed.

Genotypic and morphological diversity seems to be a general characteristic feature of date palm germplasm as revealed in many research including different production areas. This diversity represents requirements which should be well recognized and maintained for evolutionary responses in a constantly changing environment and for breeding purposes to improve the agronomical and commercial characters of date palm. In Palestine, some absent date palm cultivars need more study. Cultivar nomenclature and classification is still based on fruit characteristics including morphological and physical traits. We suggest to use combination of different methods for the analysis of date palm cultivars, Due to the large amount of diversity that exists among germplasm, it is difficult to screen and document all of them. Many of markers can be applied as a group according to specific adjectives, different markers systems and their combinations may have considerable value when screening wide date palm collections for the characterization of germplasm.

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