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Genotypic characterization of eleven barley (*Hordeum vulgare* L.) cultivars grown in Palestine using RAPD, ISSR and SSR genetic markers.

By

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Abstract

The use of molecular markers for the detection of DNA polymorphism is one of the most significant developments in the field of molecular biology. In this study, three types of molecular markers: RAPD, ISSR, and SSR (microsatellites) were used to determine the genetic relatedness among the eleven barley cultivars grown in Palestine. These cultivars includes: Baladi, Nabawi, Rihane, ICARDA 6, 16, 20, 27, 29, 30, 33, and 34. Five primers of each molecular marker were used. Amplification products of the three methods represent 102 loci of the barley genome in addition to the wheat out group. Ninety three out of the total loci were polymorphic, which correspond to 91%. Moreover, 22 exclusive markers (loci) were identified for several barley cultivars of which 8 markers belong to the wheat out group. Such private loci could be used for cultivar identification. In general, an average of 59.4 loci per cultivar was recorded.

The phylogenetic trees show that RAPD and SSR markers are highly polymorphic. Less polymorphism was revealed by the ISSR method. The polymorphism obtained by the three methods was sumed up and used to establish a phylogenetic tree for the eleven barley cultivars. UPGMA trees show two major clusters. The first one encompasses cultivars Rihane and ICARDA16, whereas the second cluster is further subdivided into two subclusters. The first subcluster contains cultivars ICARDA 6, 27, 33, 30, 20 and Baladi while the second subcluster is included of ICARDA29, 34 and Nabawi cultivars. The tree also shows that cultivars Baladi and ICARDA 33 are very closely related. Similar clustering patterns were obtained by the Principal Coordinate Analysis (PCA) which supports the accuracy of the phylogenic tree.

The results of this study indicate the presence of high genetic diversity among the tested barley cultivars which makes them of a special value pertaining to any breeding program aiming at increasing their productivity and the development of tolerance to biotic and abiotic stresses. To the best of our knowledge, this is the first such study for the barley (*Hordeum vulgare L*) grown in Palestine.

تحديد الطرز الوراثية لاصناف شعير تزرع في فلسطين باستخدام ثلاث طرق جزيئية: التضاعف العشوائي المميز للاصناف (RAPD)، ما بين المتسلسلات البسيطة المتكررة (ISSR)، والمتسلسلات البسيطة المتكررة (SSR).

ماهر ياسين محمـــــد الردايدة

ملخص

استخدام العلامات الجزيئية (molecular markers) في الكشف عن التنوع الجيني في DNA كانت اهم التطورات التي حدثت في معرفة الطرز الجينية للاصناف المختلفة من النباتات.

في هذه الدراسة، تم استخدام ثلاث طرق جزيئية: التضاعف العشوائي المميز للاصناف (RAPD)، ما بين المتسلسلات البسيطة المتكررة (ISSR)، المتسلسلات البسيطة المتكررة (SSR)، وذلك من اجل تحديد العلاقة الجينية بين ١١ نوع من اصناف الشعير المختلفة التي تزرع في فلسطين. هذه الاصناف هي: البلدي، النبوي، الريحان، الايكاردا ٦،١٦،٢٠،٢٧،٢٩،٣٠ و ٣٣ .

تم استخدام خمسة primer لكل نوع من العلامات الجزيئية ، نواتج المضاعفة في الطرق الثلاث تمثل ١٠٢ موقع من مجموع المادة الوراثية للشعير ومجموعة القمح الخاضعة للدراسة. وجدت اختلافات وراثية في ٩٣ موقع وهي تشكل ٩١%، اضافة لذلك لقد تم تشخيص 22 موقعاً مميزاً (منها ٨ للقمح). ظهرت كعلامة مميزة للصنف بعينه ولم تظهر في

باقي الأصناف مما يمكن استخدامها للتعريف بذلك الصنف. وبشكل عام فان معدل وجود مواقع فيها اختلافات هو ٥٩.٤ موقع لكل صنف.

وعن طريق استخدام طريقة Neighbor-joining (NJ)، تبين وجود اختلاف جيني كبير بين العلامات الجزيئية RAPD , و ISSR ، تنوع جيني قليل ظهر في ISSR . التنوع الجيني الذي تم الحصول عليه من الطرق الثلاث جمع وتم بناء شجرة واحدة لاصناف الشعير الاحد عشر.

باستخدام طريقة Unweighted Pair Group Method with Arithmetic Mean (UPGMA) تم انشاء شجرة العلاقات التطورية بين الأصناف حيث احتوت مجموعتين رئيسيتين: المجموعة الاولى مكونة من الريحان والايكاردا ١٦، بينما المجموعة الثانية انقسمت الى مجموعتين فرعيتين: المجموعة الفرعية الاولى ضمت الايكاردا ٦، ٢٧، ٣٣، ٣٠، ٢٠، والبلدي. اما المجموعة الفرعية الثانية فقد احتوت على الايكاردا ٢٩، ٣٤، والنبوي. كما اظهرت الشجرة ان الصنفين البلدي والايكاردا ٣٣ بينهما تماثل كبير. نفس النتائج تم الحصول عليها عند استخدام طريقة Principal Coordinate Analysis (PCA) مما يدعم دقة الشجرة التي تم بناؤها باستخدام طريقة (Neighbor-joining (NJ.

تشير نتائج هذه الدراسة الى وجد اختلافات وتنوع جيني كبير من اصناف الشعيرالمزروعة في فلسطين والخاضعة لهذه الدراسة، مما قد يساعد في اي مشروع يهدف الى تحسين المحصول ومقاومة هذه الاصناف للظروف البيئية الضارة الحية (مثل الامراض والحشرات) وغير الحية (مثل درجات الحرارة والجفاف). ولكن حسب علمنا، تعتبر هذه اول دراسة تحاول تحديد الطرز الوراثية لاصناف مختلفة من الشعير في فلسطين.

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DECLARATION

I declare that the Master Thesis entitled "Genotyping of the Palestinian barley (*Hordeum vulgare L.*) cultivars using RAPD, SSR and ISSR genetic markers " is my own original work, and hereby certify that unless stated, 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 acknowledgement is made in the text.

Name and signature: Maher Yasin Mohammed Al-Radaydeh

Date: ……....

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Signature: Maher Yasin Mohammed Al-Radaydeh

Date: August 1, 2016

Dedication

To

My parents, wife and children with love and respect

for their patience, support and encouragement

Acknowledgement

I would like to express my deepest respect and my great thanks to my supervisor Dr. Omar DarIssa, the director of the UNESCO Biotechnology Education and Research Center at Bethlehem University, for his guidance, and encouragements at all stages of my work.

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List of Abbreviation:

ISSR	Inter-Simple Sequence Repeats
SSR	Simple Sequence Repeats
RAPD	Random Amplified Polymorphic DNA
RFLP	restriction fragment length polymorphisms
AMP-PCR	Anchored Microsatellite Primer
PCR	Polymerase Chain Reaction
NJ	Neighbor-joining
UPGMA	Unweighted Pair Group Method using Arithmetic average
ICARDA	International Center for Agricultural Research in the Dry Areas.
DNA	Deoxyribonucleic acid
UV	Ultra Violet
PCA	Principal Coordinate Analysis
FAO	Food Agriculture Organization of the United Nations
NARC	National Agricultural Research Center

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CHAPTER 1

1. Introduction

1.1 Barley (*Hordeum vulgare L.*); origin, characteristics and uses

Barley (*Hordeum vulgare* L.) belongs to the subfamily Triticaceae of the family Poaceae. There are two subspecies of barley one is *vulgare* and the second is *spontaneum*. Barley is one of the earliest known crops and of great importance for the mankind worldwide (Dakir *et al*., 2002). It is one of several crops that originated in the Fertile Crescent including historical Palestine (Badr *et al*., 2000). The Fertile Crescent includes parts of Jordan, Lebanon, Palestine, Syria, South-eastern Turkey, Iraq and Western Iran. Around 7,000 BC, cultivated barley was domesticated from a wild relative species called *Hordeum spontaneum* which is still common in the area till today (Azhaguvel and Komatsuda, 2007). The world production of barley ranks fourth among cereals crop, after corn, rice, and wheat (FAO STAT, 2014). According to the FAO estimation (FAO 2016), the total production of barley worldwide in 2016 was 147.5 million metric tons, harvested from 47.9 million hectares. Russia, Germany, France were the leading producers of barely. Table 1

Barley is a diploid (2n) with 14 chromosomes, largely self-fertilizing species with large genome 5,1 Mb (Dolezel *et al*.,1998; Doležel and Lucretti, 1995; Mayer, K. F. *et al*., 2012). Barley is a widely adaptable crop. It has a short growing season is more tolerant of drought,

low soil fertility and soil salinity than other major cereals (Maniruzzaman *et al*., 2014; Baum *et al*., 2004; Nevo, 2012; Zohary *et al*., 2012).

Barely spikes are dense and up to 10 cm long. The barley spike is composed of triplets (one central and two lateral spikelets) . In wild barley only the central spikelet is fertile, while the other two are reduced (not fertile) producing cultivars known as two-row barleys. Six-row spike barleys are produced when mutation happened in single gene called *vrs1* that is recessive to the dominant allele responsible for the two-rowed spike (*Vrs1*) as recent studies showed (Komatsuda *et al*.2006).

About 75% of the world production of barley is used for animal feed, 20% is malted for use in alcoholic and non-alcoholic beverages, and 5% as part in several food products, such as bread barley, (Blake *et al*., 2011). Barely grains are rich of vitamins, such as Vitamin B6 and K, as well as amino acids such as Tryptophan and Histidine (Arcade *et al*., 2000). Moreover, barley grains are particularly high in soluble dietary fiber, which significantly reduces the risk of serious human diseases including type II diabetes, cardiovascular disease and colorectal cancers that affect millions of people worldwide (Collins, H. M., 2010).

Table1: Ranking of countries in term of barely production in 2013 according to the Food and Agriculture Organization database. (FAO 2013)

Rank	**Country/Region**	**Barley production (tons)**
1	Russia	15,388,704
2	Germany	10,343,600
3	France	10,315,900
4	Canada	10,237,100
5	Spain	10,057,600
6	Turkey	7,900,000

1.2 *Hordeum vulgare* in Palestine

In Palestine, about 5100 dunums are cultivated with barley according to the Palestinian Central Bureau of Statistics of 2012. This makes barley in the second place among cereals crop after wheat. Barley is a winter crop, which is grown in temperate regions under diverse cultural conditions covering a wide geographical range of Palestine. Since it is more tolerant than wheat to drought conditions, barley cultivation is successful in areas with an average annual rainfall of 250 - 300 mm. Rain-fed barley yield about125 kg per dunum while irrigated barley produces between 300-500 kg / dunum depending on the cultivar and cultivation conditions.

There are more than ten types of barley species cultivated in Palestine including ICARDA cutivars, Baladi. Rihan, and Nabawi. These cultivars possibly variations in their genetic makeup because of the apparent differences in their productivity, morphological features, and crop quality. Cultivars of the ICARDA group are the result of breeding programs carried out by ICARDA (Stephensen *et al*., 2008). Unfortunately, there are no adequate information about the breeding parents and conditions in the literature.

Germplasm collections in the form of grains of the cultivated barley species are available in several gene banks in Palestine including that of the National Research Center of the Palestinian Ministry of Agriculture. Although, the most prevailing phenotypic variations among these cultivars have been known to the local researchers and farmers, still the genetic diversity has not been evaluated adequately. Such evaluation is important for the conservation and proper use of plant genetic resources. For example the possible existence of high degree of genetic diversity within the Palestinian landraces would assist in barley breeding programs through the use of such diversity as a potential source for genes to develop commercial

varieties (Soleri and Smith, 1995). Till now, such genetic variability among the Palestinian barley landraces is maintained at the level of small scale farming systems. Unfortunately, most of the research studies on revealing the diversity of barley germplasms have focused on morphological and physiological traits (Massood and Chaudhry, 1987).

1.3 Genome organization of *H. vulgare*

Cultivated barley (*Hordeum vulgare ssp spontaneum*), derived from its wild progenitor *H. spontaneum*, is among the world's earliest domesticated and most important crop plants. It is diploid (2n =14) consisting of 5.1 gigabases(Dolezel *et al*.,1998). Barely chromosomes are relatively large ranging from 6 – 13.7 µm. Whole genome sequencing of barley is complicated because of its large genome, about 12 times the size of the rice genome, and includes over 80% of repetitive DNA (Schulte *et al*., 2009; Wicker *et al*., 2009). The seven barley chromosomes are named from 1H to 7H. They are evolutionary homologous to wheat chromosomes and other Triticeae linkage groups (Klaus *et al*., 2009). Chromosome 1H is considerably smaller than chromosomes 2H to 7H and contains approximately 4,600 to 5,800 genes while the whole barley genome encompasses 38,000 to 48,000 genes. Recent research showed that much of genes for abiotic stress tolerance in barley are located on chromosomes 2H, 5H and 7H (Wehner *et al*., 2015).

1.4 Molecular markers

Dominant and co-dominant molecular markers provide valuable and powerful tools to detect and characterize the genetic diversity, genotype identification, and genetic mapping within and between the barley species and in the assessment of genetic variation and genetic relationships

at the cultivar level. Molecular markers are used to track loci and genome regions in crop plants, and therefore many disease resistance genes, tolerances to abiotic stresses and quality traits have been identified (Lörz and Wenzel, 2004).

In this study, three different genetic markers (Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR or microsatellite), Inter-Simple Sequence Repeat (ISSR), were applied to reveal the genetic diversity among eleven barley cultivars grown in Palestine. To the best of my knowledge, this is the first study for revealing the genetic relatedness among the Palestinian barley cultivars using three different genetic markers.

1.4.1 Random amplified polymorphic DNA (RAPD)

RAPD-PCR has become widely used in genetic diversity/polymorphism, cultivar identification, genome mapping, population and evolutionary genetics, and plant and animal breeding. It is based on the amplification of DNA fragments using short (10 nucleotides) random primers. In this technique a previous knowledge of the template sequence (of the organisms' genome) is not required (Williams *et al*., 1993). In RAPD analysis, a single, short oligonucleotide primer is able to anneal and prime at multiple locations throughout the genome, which in turn can produce a spectrum of amplification products that are characteristics of the template DNA. Moreover, the produced amplified fragment depends on the sequence of the primer and the target genome as well as on the thermal cycling conditions. The lengths of the amplified fragments are within the range of 100 bp to 2kb. This technique is simple, rapid, multi loci (covers large parts of the genome), and valid to numerous genomes from simple prokaryotes to the most complicated eukaryotes. It requires only small amounts of DNA,

(about 10 ng per reaction) (Senthil, K., and Gurusubramanian, G., 2011), low costs compared to other marker technologies, also it gives a results in a few hours. However, the reproducibility of the RAPD markers is not consistent and requires a lot of efforts for optimization. Also, nearly all RAPD markers are dominant where most RAPD fragments result from the amplification of one locus and so it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (one copy) or homozygous (two copies).

1.4.2 Inter-simple sequence repeats (ISSR)

Inter-simple sequence repeat (ISSR) is a PCR based method for the genotyping of organisms. The technique is based on the amplification of genomic regions between microsatellite loci along a DNA template using primers designed to anneal to the microsatellite itself (Cano *et al*.,2005). Such primers are usually 16-25 bp long where a single primer is used in a PCR reaction. The amplification may result in multiple fragments with different sizes (Zietkiewicz *et al*., 1994). To avoid smearing of the fragments in gel electrophoresis, a modified version of the method known as Anchored Microsatellite Primer AMP-PCR has been developed. An extra random non-microsatellite nucleotide is added either to 3` or 5` end of the primer. Therefore, ISSR-PCR uses primer composed of di, tri- or tetranucleotide repeat units (Wu *et al*., 2005), one or two anchoring nucleotides at either the 3' or 5' end (primer consists of a repeated motif with one or several non-motif nucleotides at the 5'-end, e.g. 5'–$GA(AC)_8$–3' or at the 3'-end, e.g. 5'–$(AC)_8AG$–3'). This is important to target the end of a microsatellite region and to

prevent primer dimerization (Reddy *et al*. 2002). PAGE (polyacrylamide gel electrophoresis) or agarose gel are used to separate the amplification products.

This technique has been used in many applications such as, cultivar identification to show the genetic variability and relationships among species, phylogenetic analysis, genetic fingerprinting (Wang, *et al.,* 2009), assessment of hybridization in many plant and animal species (Gupta *et al*.,2008), genetic mapping, clone and stain identification(Essadki *et al*., 2006; Monte-Corvo *et al*., 2001), evolution and molecular ecology of closely related species (Bornet & Branchard 2001; Reddy *et al*. 2002), to identify genetic trait loci (Arcade *et al*., 2000).

This method is a simple, non-time consuming, and inexpensive that requires small amount of DNA samples. Also, ISSR is reproducible, highly specific and highly polymorphic between two organisms (Esselman *et al*., 1999; Wang *et al*. 2012; Shafiei-Astani *et al*., 2015). ISSR markers are easy to use, and methodologically less demanding compared to other dominant markers, making it an ideal genetic marker for organisms whose genetic information is lacking (Ng, W. L., and Tan, S. G., 2015).

ISSR-PCR technique permits to screen quickly a wide part of the genome without prior DNA sequence knowledge. ISSR bands are considered as dominant markers but have high reproducibility when compared to RAPD markers (Fang and Roose, 1997; Nagaoka and Ogihara, 1997).

1.4.3 Simple Sequence Repeats (SSR)

Microsatellite markers, or simple sequence repeats (SSR) (Tautz, 1989), also known as, short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs), have been widely used for marker assisted breeding and variety identification due to their high level of polymorphism and ease of use. SSRs have recently become important genetic markers in a wide range of crop species including barley and wheat. The method has been usesd in genetic diversity studies (Senior *et al*., 1998), cultivar discrimination (Sarri *et al*., 2006), and to determine genetic relationships between varieties (Sefc *et al*., 1997). SSR is the smallest class of simple repetitive DNA sequences in high organism's genome. The microsatellite repeats can be di-nucleotide, tri-nucleotide, tetra- nucleotide, or penta- nucleotide and gives the specific polymorphism between species (Zietkiewicz *et al*., 1994). The repetitive satellite DNA can be separated from total genomic DNA via density-gradient centrifugation. SSR polymorphism between two varieties is due to the difference in the length of the repeat between the two conserved sequences.

The unique sequences flanking such repetitive motifs are used to design forward and reverse primers for detection of length polymorphism via PCR (Litt and Luty, 1989; Weber and May, 1989). SSR detects the allelic variation by way of repeat numbers within a locus and one pair of SSR primer deal with one locus (Weising *et al*., 1992). They appear to be ubiquitous in higher organisms, although the frequency of microsatellites varies between species.

They are abundant, dispersed throughout the genome, and show higher levels of polymorphism than other genetic markers. SSR markers are co-dominant, as two alleles may be identified at each locus, frequently and evenly distributed throughout the genome, and mutations among

them do not affect the survival of the individual. They are highly polymorphic (multi-allelic) (Belaj *et al*., 2003), and highly reproducible compared with other techniques (José, 2011). This makes them an excellent molecular marker system for many types of genetic analyses, including linkage mapping, germplasm surveys, and phylogenetic studies (Liu *et al*., 1996).

CHAPTER 2

Objectives

2.1 overall objectives:

To reveal the genetic relatedness among eleven barley cultivars grown in Palestine

2.2 Specific objectives

1. To establish a phylogenetic tree for 11 cultivars of barley grown in Palestine using RAPD, ISSR, and SSR molecular markers and to assess the accuracy of the tree by the Principal Coordinate Analysis.
2. To compare the efficacy of the three genetic markers in revealing the polymorphism among the Palestinian barley cultivars.
3. Allocation of markers/loci for genotype (cultivar) identification.

CHAPTER 3

Materials and Methods

3.1 Sample collections and culture conditions

Grains of eleven cultivars of barley, usually cultivated in different regions in West Bank, Palestine, were provided by the National Research Center in Jenin, Palestine. These cultivars are Baladi, Nabawi, Rihane, and ICARDA 6, 16, 20, 27, 29, 30, 33, and 34. Seed germination was conducted on wet cotton covered by Wattman paper. The germinated seedlings were transferred to plastic pots containing autoclaved vermiculite and incubated at 23-26◦C under 16 hour photoperiod for one month. Leaf samples were collected from each cultivar for genomic DNA extraction and purification.

3.2 DNA Extraction

For each cultivar, about 100 mg of a young leaf sample was ground in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted and purified from the ground leaves using DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. The DNA was eluted with 50µl of TE buffer and stored at -80°C till used. DNA was extracted from a wheat leaf to serve as an out group in the subsequent experiments. The quality of the extracted DNA

was assessed by gel- electrophoresis in 1% agarose gels, and the concentration of DNA (Table 2) was determined using the nanodrop spectrophotometer (NanoDrop ND-1000 spectrophotometer).

Table 2: Quantity and purity of the DNA extracted from different barley cultivars using the Qiagen DNA extraction kit.

Sample No	**Barley Cultivar**	**DNA Con. *(ng/ µl)***	**DNA Purity (260/280)nm**
1	Rihane	113	1.85
2	ICARDA (16)	52.8	1.82
3	ICARDA (6)	110	1.88
4	ICARDA (27)	115	1.87
5	ICARDA (29)	77.2	1.84
6	ICARDA (33)	94.1	1.84
7	ICARDA (34)	103	1.89
8	Nabawi	70.2	1.91
9	Baladi	85.7	1.82
10	ICARDA (30)	62.3	1.86
11	ICARDA (20)	62.3	1.94

3.3 PCR amplification of RAPD

Five RAPD markers were used (Table 3). The PCR reactions were carried out in 20 µl reaction mix containing 50 ng of template DNA, 0.4 µM of each primer, and ultra-pure distilled water (Biological Industries). Then, all components were added to AccuPower® PCR PreMix tube (Bioneer Corporation - Hylabs) containing optimum amounts of $MgCl_2$, deoxy nucleotide mixture, Taq DNA Polymerase and its buffer. Additional $MgCl_2$ was added to achieve a concentration of 3 mM.

PCR amplification was performed in a cycler (MJ research) as follows: an initial denaturation step for 3 min at 94°C followed by 35 cycles of 45 seconds at 94°C, 1 min annealing at 37°C, 90 seconds of extension at 72°C followed by a final extension step of 7 minutes at 72°C. PCR amplification were assessed by gel electrophoresis, using 1.4% agarose gel in 1X TBE buffer at 120 mA for 1.5 hour, then PCR product visualized by UV gel documentation system (BioDoc-It imaging system, UVP).

3.4 PCR amplification of ISSR

For the ISSR-PCR, five primers were used (Table 3). The conditions of the PCR reactions and the thermal cycling were similar to those of the RAPD-PCR except that the primers are different and the annealing temperature was set to 50°C. Amplification products were resolved by electrophoresis on 1.6% agarose gels stained with Ethidium Bromide in 1X TBE buffer at 100 mA for 90 minutes.

3.5 Microsatellite (SSR-PCR)

PCR amplifications for five SSR markers (Table 4) were carried out in 20 µl reaction mix containing 50 ng of template DNA, 0.4 µM of each primer (forward and reverse), and ultra-pure distilled water (Biological Industries). Then, all components were added to *AccuPower*® PCR PreMix tube (Bioneer Corporation - Hylabs) containing optimum amounts of $MgCl_2$, deoxy nucleotide mixture, Taq DNA Polymerase and its buffer.

PCR amplification was performed with the following thermal cycles: 3 min at 94°C followed by 35 cycles of 45 seconds at 94°C, 1 min at 55°C , 90 seconds at 72°C followed by a final step of 7 min at 72°C. The annealing temperatures for SSR and ISSR primers were calculated for each primer pair according to the Amplifx software, version 1.5.4.

PCR amplification were assessed by gel electrophoresis, using 2% agarose gel in 1X TBE buffer at 150 mA for 1.5 hour, then PCR product visualized by UV gel documentation system (BioDoc- It imaging system, UVP).

3.6 Gel documentation and scoring of bands

Gels were photographed using a BioDoc-It imaging system, UVP. Only clear bands were scored and their sizes were estimated using a 100 bp DNA ladder (Invitrogen).

Table 3: List of the RAPD and ISSR primers used in this study. The primers were designed according to Eshghi *et al*., 2012.

Primer Name	Marker type	Sequence 5 → 3
S32	RAPD	TCGGCGATAG
S39	RAPD	CAAACGTCGG
S134	RAPD	TGCTGCAGGT
OPC06	RAPD	GAACGGACTC
OPC08	RAPD	TGGACCGGTG
ISSR6	ISSR	TCTCTCTCTCTCTCTCA
ISSR7	ISSR	ACACACACACACACACT
ISSR11	ISSR	TATATATATATATATART
ISSR14	ISSR	GTGTGTGTGTGTGTGTYC
ISSR15	ISSR	ACCACCACCACCACCACC

R = Purine, Y = Pyrimidine

Table 4: List of the SSR primers used in this study.The primers were designed according to Maniruzzaman *et al*., 2014.

Primer Name	Marker type	Sequence 5 → 3	Chr No.	motif
Bmag0211	SSR	ForATICATCGATCTTGTATTAGTCC Rev ACATCATGTCGATCAAAGC	1H	(CT) 16
Bmac0134	SSR	For CCAACTGAGTCGATCTCG Rev CTTCGTTGCTTCTCTACCTT	2H	(AC)28
Bmac0181	SSR	For ATAGATCACCAAGTGAACCAC Rev GGTTATCACTGAGGCAAATAC	4H	(AC)20
Bmag0040	SSR	For AGCCCGATCAGATTTACG Rev TTCTCCCTTTGGTCCTTG	6H	(AC)20
Bmag0131	SSR	For TTICAGAAACGGAGTITTG RevCCTCCACACAAAAAA TCC	3H	(AG)16 G(AG) 15

3.7 Phylogenetic analysis

The banding profiles of the dominant RAPD and ISSR markers and the co-dominant marker SSR were based on their presence or absence (1 or 0) where only clearly identified bands were considered as potential polymorphic markers. A similarity matrix was calculated by the Nei method (Nei, 1972) which states that GD=1-dxy/ (dx+dy-dxy),where GD = Genetic distance between two genotypes, dxy = total number of shared loci (bands) in two genotypes, dx= total number of loci (bands) in genotype x and dy= total numbers of loci (bands) in genotype y. A rooted phenogram was obtained by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA method) using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 2.10e (Rohlf, 2002). A local wheat cultivar was used as an outgroup. Genetic relationships among the cultivars were further assessed by means of the Principal Coordinate Analysis (PCA) using Jaccard's coefficient. Data matrix and statistics were calculated using the pfamd (Fingerprint analysis with missing data) software version 1.31 (Schlüter & Harris, 2006). Moreover, A phylogram was constructed using the Neighbor Joining NJ method based on the sum of the data collected by the three different genetic markers.

CHAPTER 4

Results and discussion:

This is the first study that reveals the genetic relatedness among the Palestinian cultivars of barley in Palestine using three different genetic markers. Amplification products of the three methods represent 102 loci of the barley genome in addition to the wheat outgroup. Figure 1, shows the amplification products of part of the primers used in this study.

Collectively, the 93 out of the total loci were polymorphic which correspond to 91% (Table 5). This means that the primers used in this study were powerful in revealing such a high percentage of difference among the cultivars. Moreover, there are 22 different bands (loci) that appeared only in individual cultivars (Table 5). Such private bands could be considered as genotype identity that would help in the identification of specific cultivars when the available morphological data is unsufficient to differentiate among them. Only 9 of the 102 loci provide no polymorphisms. Table 5 shows also that out of the102 loci produced by the three markers, an average of 59.4 loci were present per cultivar.

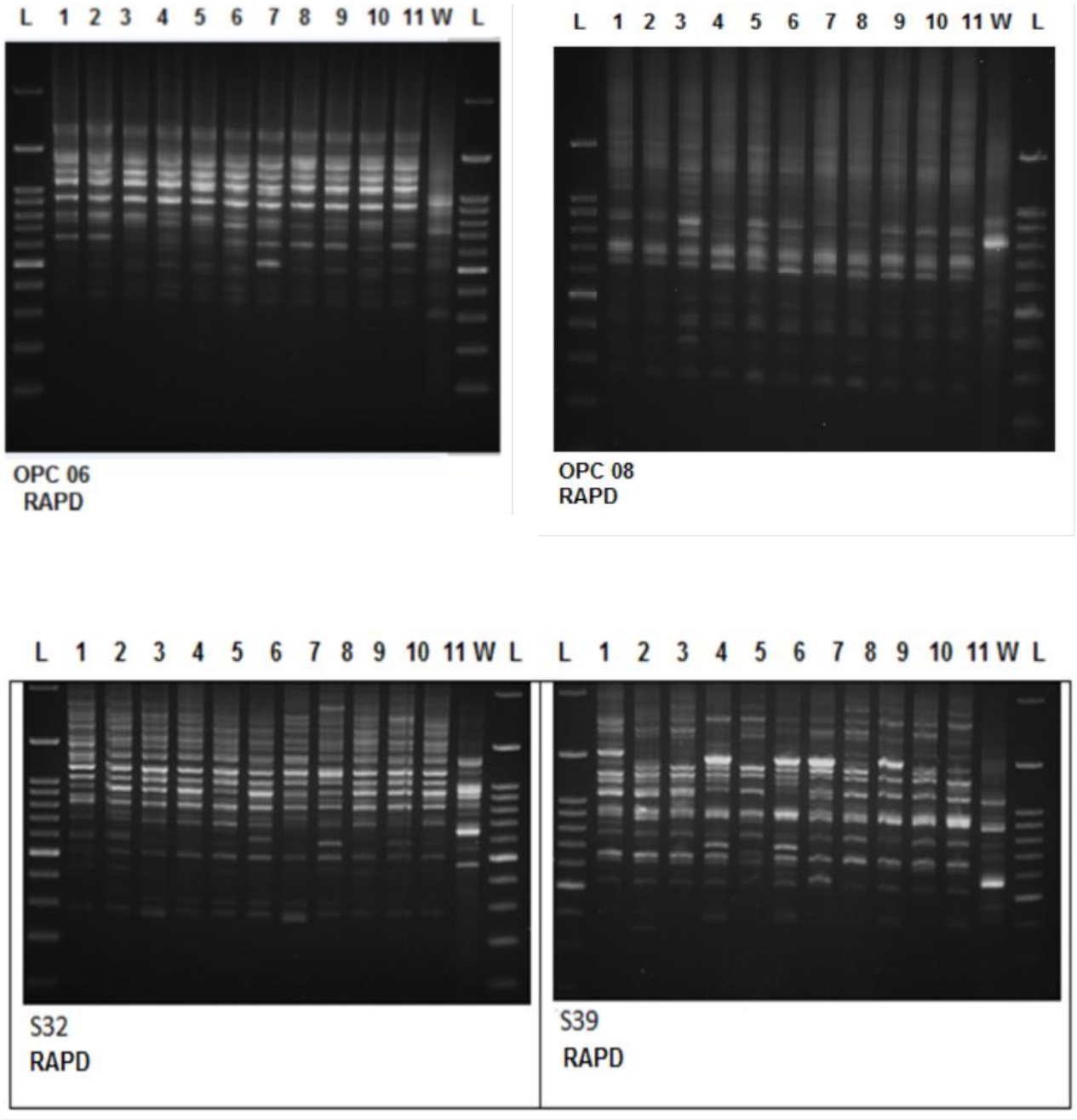


Figure 1 (A): PCR amplification products of RAPD primers (OPC 06, OPC 08, S32, and S39.

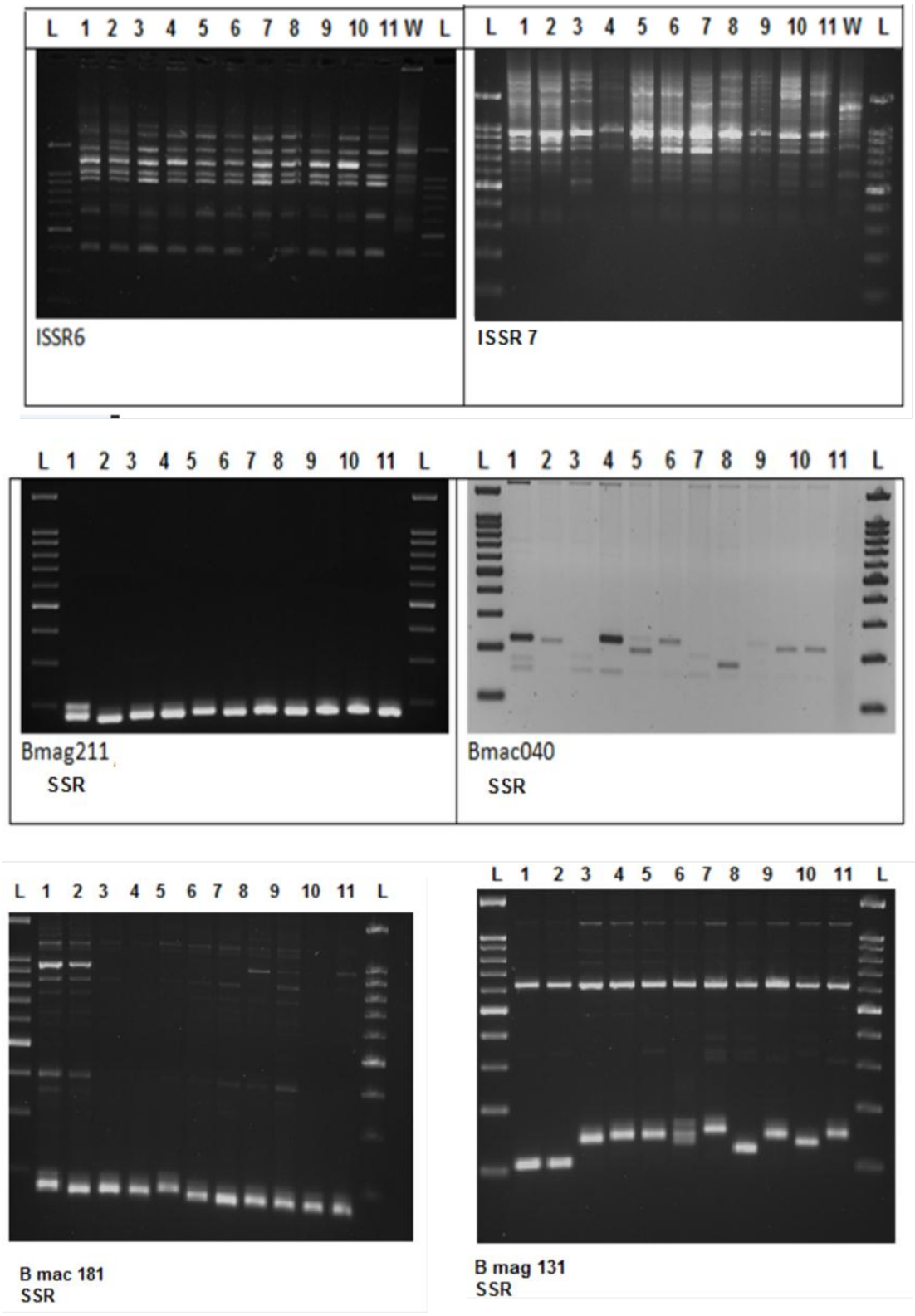


Figure 1(B): PCR amplification products of ISSR (ISSR6 and ISSR 7) and SSR (Bmag211, Bmac040, Bmag 131, and Bmac181).

A comparasion between the different molecular markers, the results in Table 5 show that the highest polymorphism was obtained by the SSR markers (100%) while the percent polymorphic bands/loci for RAPD and ISSR were 91% and 82%, respectively. It worth mentioning that the number of successful markers were only 2 (ISSR6 and 7) for ISSR method since the amplicons produced by the other 3 primers were unscorable due to smearing. More optimization is needed for these 3 primers. In addition, the percent of the private loci, those where the band was scored for one cultivar, were the highest for the SSR markers (50%), while for RAPD and ISSR the percent was only 14.9% and 17.6%, respectively (Table 5). In total, 22 private bands were obtained of which 8 belong to the wheat out group while the rest 14 belong to 6 of the 11 barley cutivars (Table 6). These results indicate that the SSR markers are the most powerfull in revealing polymorphism among the different barely cultivars and in providing genotype identities. RAPD and ISSR are comparable in their efficiencies to discriminate among the various cultivars. However, the high reproducibility of ISSR markers would be an extra reason to consider it in such studies by researchers.

Table 5: Data Matrix Statistics

Marker type	Sum	RAPD	ISSR	SSR
Number of successful markers*	12	5	2	5
Individuals	12	12	12	11**
Loci	102	67	17	18
Number/% of polymorphic bands found	93 (91%)	61 (91%)	14 (82%)	18(100%)
Number of fixed bands found	9	6	3	0
Mean band presences per individual	59.416	42.33	11.75	5.81
Number/percent of private loci	22 (21.5%)#	10 (14.9%)	3 (17.6%)	9 (50%)

* Number of the primers/primer pairs that lead to scorable amplification products.

**No amplification products where produced for wheat using SSR markers and so was excluded.

Percent of loci where a locus is present only in one cultivar, therefore could be used for genotype identification.

Table 6: Cultivars where a private band was allocated. For each cultivar, the marker that produced the private band and their numbers are shown.

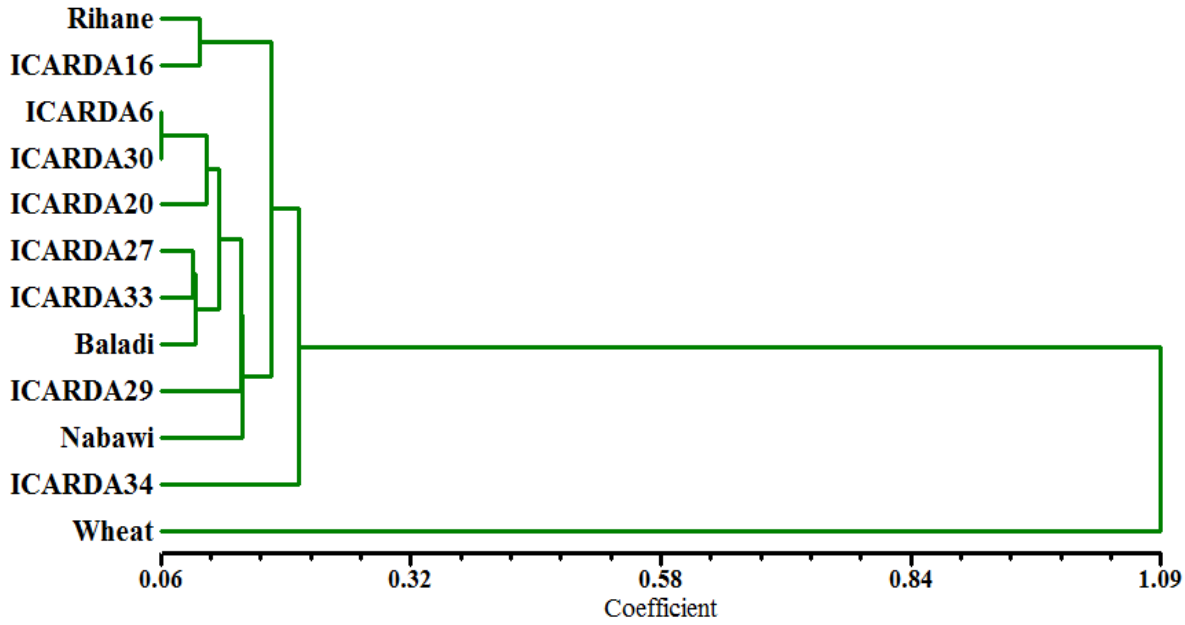
Cultivar	Markers	Number of private bands/loci
Rihane	S 39	1
	B mag 211	1
	Bmac 134	1
ICARDA 34	S 134	1
	OPC 06	1
	B mag131	1
ICARDA 30	B mag 131	1
ICARDA 16	B mag 211	1
	ISSR 6	2
	B mag 211	1
ICARDA 29	Bmag181	1
	Bmac 134	1
Nabawi	B mag131	1
Wheat	S 32	1
	S 134	2
	ISSR 7	1
	OPC 06	3
	OPC 08	1

The genetic distance among the barley cultivars as calculated by Jaccard coefficients are shown in figure 2. These data constitute a basis on which the phylogenetic trees were constructed. Smaller values in figure 2 correspond with closer genetic relatedness between the two cultivars that share that value.

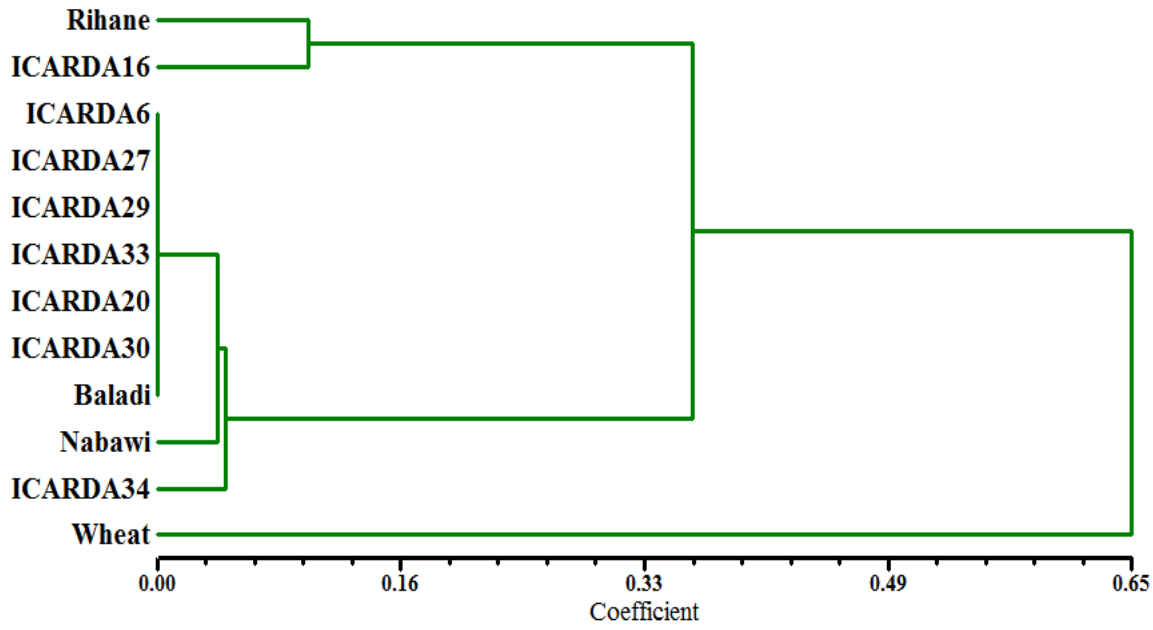
Rihane ICARDA16 ICARDA6 ICARDA27 ICARDA29 ICARDA33 ICARDA34 Nabawi Baladi ICARDA30 ICARDA20 Wheat
0.0000000000
0.2463768116 0.0000000000
0.3513513514 0.3472222222 0.0000000000
0.3846153846 0.3815789474 0.1492537313 0.0000000000
0.4303797468 0.3648648649 0.2318840580 0.2500000000 0.0000000000
0.3157894737 0.3108108108 0.2000000000 0.1428571429 0.2465753425 0.0000000000
0.4074074074 0.4444444444 0.3066666667 0.2987012987 0.3670886076 0.2727272727 0.0000000000
0.3378378378 0.3783783784 0.2463768116 0.2142857143 0.3150684932 0.2602739726 0.2702702703 0.0000000000
0.3243243243 0.3424657534 0.2058823529 0.1470588235 0.2535211268 0.1176470588 0.2800000000 0.2428571429 0.0000000000
0.3466666667 0.3424657534 0.1515151515 0.1739130435 0.2285714286 0.1971830986 0.3026315789 0.2428571429 0.1764705882 0.0000000000
0.3815789474 0.3783783784 0.1940298507 0.1617647059 0.2428571429 0.1857142857 0.2933333333 0.2571428571 0.1363636364 0.1363636364 0.0000000000
0.8441558442 0.8219178082 0.7746478873 0.8026315789 0.7972972973 0.8076923077 0.7948717949 0.8266666667 0.7972972973 0.7808219178 0.8108108108 0.0000000000

Figure 2: Maximum similarities among the barley cultivars as calculated by the Maximum Jaccard coefficients.

The phylogenetic trees in figure 3 show that RAPD and SSR markers are highly polymorphic. Less polymorphism was revealed by the ISSR method. This is mainly due to the fact that clear and well-resolved amplification products were successfully obtained by two out of the five tested primers. In other words, less information was used to construct the tree using the ISSR markers (Figure 3B) than the trees of the RAPD (Figure 3A) and the SSR method (Figure 4A) where the five primers were successful in amplifying clear and distinct bands.

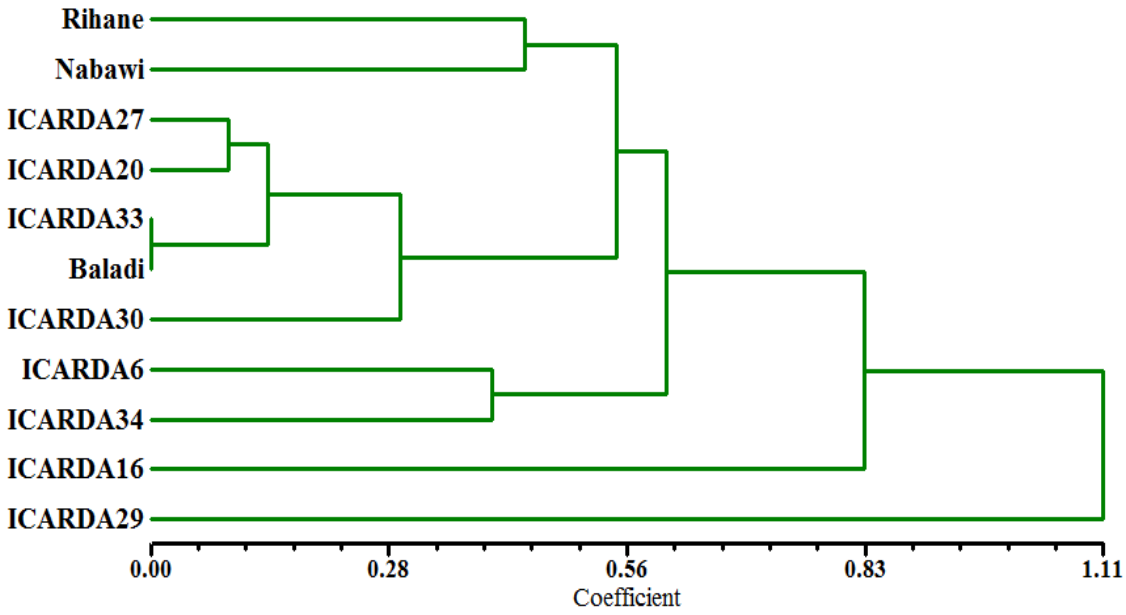


A: RAPD

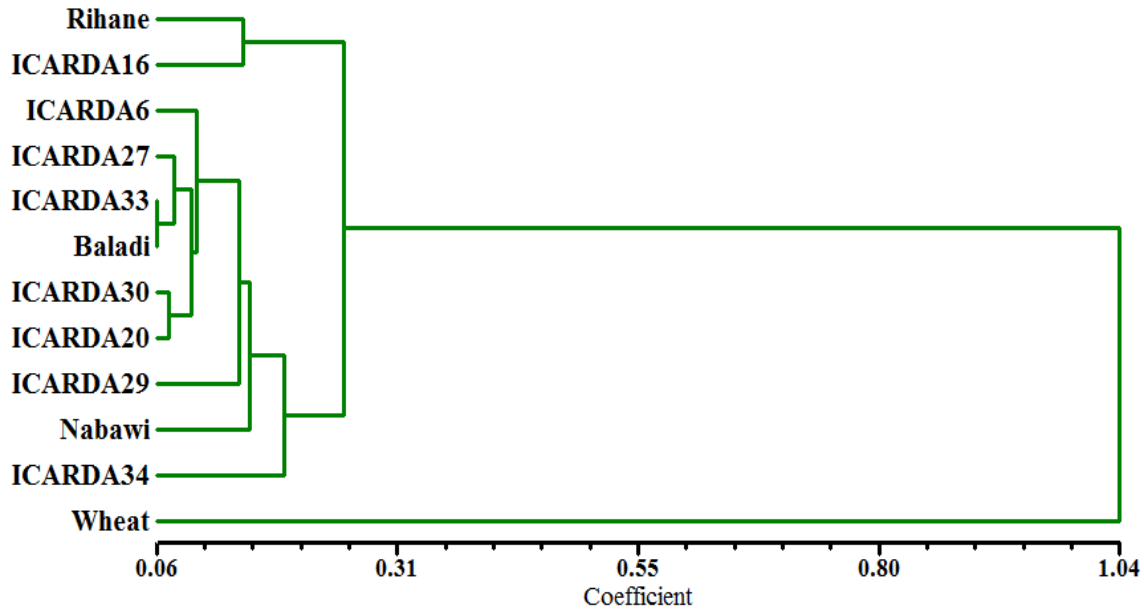


B: ISSR

Figure 3: Phenograms for the dominant markers (A) RAPD and (ISSR). The UPGMA phenograms were constructed using Nie72 factor and were rooted to the out group wheat. The coefficient in the scale bar represents genetic distance.



A: SSR



B: Sum of the three methods.

Figure 4: Phenograms for (A) SSR and (B) for Sum of the three methods. The phenograms were constructed as described in figure 3.

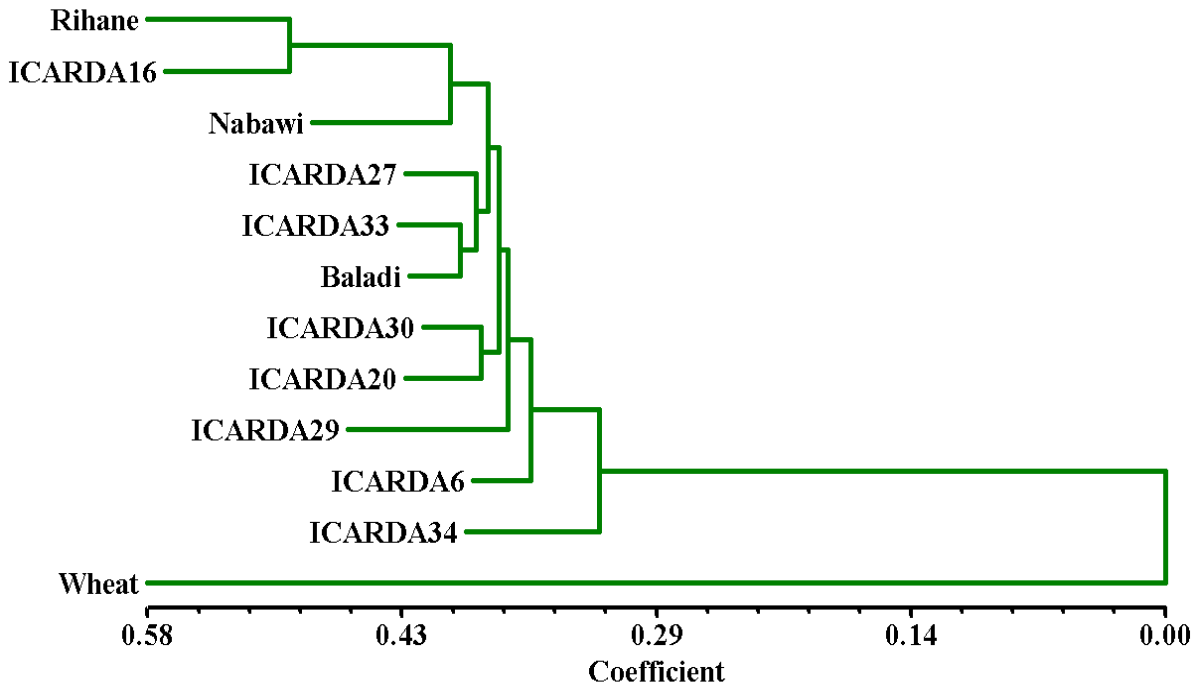


Figure 5: Phylogenetic trees revealing the genetic relatedness and evolutionary history among the barley cultivars. The tree was constructed using the NJ method according to the collective data obtained by the three different markers using wheat as an out group.

The polymorphism obtained by the three methods was summed up and used to establish a phenogenetic tree for the eleven barley cultivars (Figure 4). The genotypes clustered into two major groups. The first group constitutes cultivars Rihane and ICARDA16 which were shown to be closely related by most of the trees constructed in this study (Figure 3A, B). The second group encompasses two subclusters, where cultivars ICARDA 6, 27, 33, 30, 20 and Baladi are the first subcluster while ICARDA29, 34 and Nabawi make the second subcluster. The tree also shows that cultivars Baladi and ICARDA 33 are identical which means that the formers might constitute one of the parents in the breeding program that resulted in the later cultivar. The accuracy of the UPGMA tree in figure 4B was statistically supported by the principal

coordinated analysis conducted on the collected data (Figure 6). Similar clustering patterns were obtained in the three dimensional analysis. The phylogram (Figure 5) which was constructed using the sum of data obtained by the three genetic markers (RAPD, ISSR, and SSR) show similar clustering patterns to those obtained by the phenogram (Figure 4B). However, the phylogram provide additional information which is the evolutionary history of the barley cultivars as demonstrated by the differences in the lengths of the branches. For example, cultivars Baladi and ICARDA33 are closely related to each other as shown by both type of trees. However, in the phylogram, these two cultivars are further resolved so that the shorter branch length of the Baladi cultivar (Figure 5) indicates that less evolutionary changes has been accumulated in this cultivar in comparison to his relative ICARDA33. This result supports the hypothesis that Baladi might be indeed a parent in the breeding program leading to the development of ICARDA33.

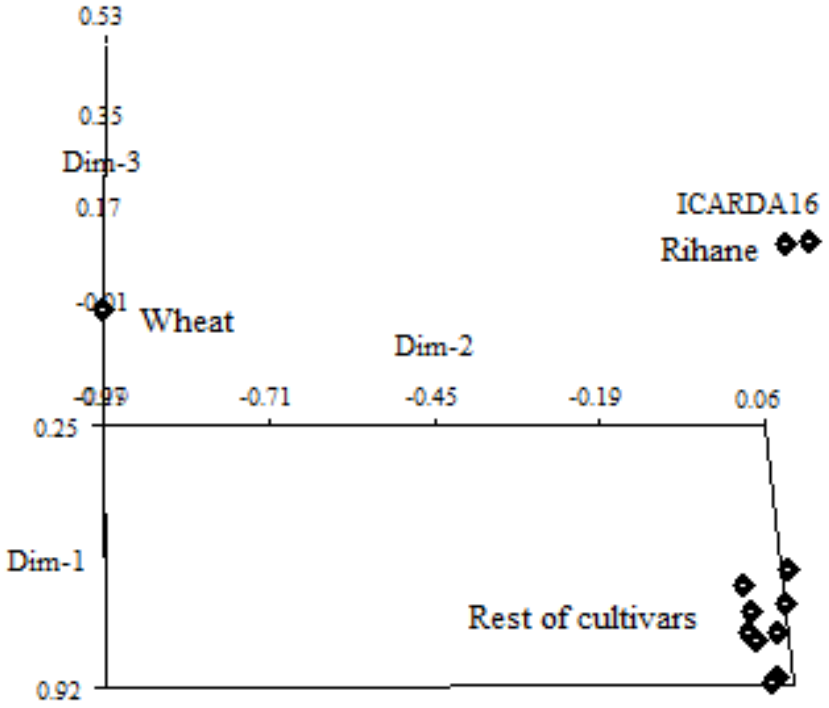


Figure 6: Principal Coordinate Analysis (PCA).

This three dimensional analysis was conducted for the sum of the data collected by the three genotyping methods. The results support the accuracy of the UPGMA tree in figure 2D.

CHAPTER 5

Conclusion and Recommendations

This is the first study in Palestine to reveal the genetic relatedness among the locally cultivated barley cultivars using modern molecular markers namely, RAPD, ISSR, and SSR. Moreover, the powerfulness of the three genetic markers was compared. It was found that SSR markers reveal more polymorphism among the cultivars than the other two methods. Such polymorphism is the basis for constructing phylogenetic trees and discrimination among closely related cultivars. In addition, the SSR markers were also more powerful in allocating private bands which could be used in the future by breeders and researchers as genotypic identities for the studied cultivars. The markers RAPD and ISSR produced comparable polymorphism despite the fact that only two ISSR markers were successfully used in this study in comparison to five RAPD markers. The fact that ISSR markers are much more reproducible than the RAPD ones, togethe with the possibility that more polymorphism might be obtained when five ISSR markers are employed, make the latter markers a better choice for revealing genetic relatedness among barely cultivars. Practically, from our experience it seems that PCR optimization is more laborious for ISSR than the case for RAPD markers.

Moreover, this study provides preliminary indications about the origin of the ICARDA cultivars grown in Palestine and their genetic relatedness to the other local ones included in this study. This is important, particularly due to the lack of sufficient information about the

origin of these cultivars which are believed to be the product of a breeding programs conduced by ICARDA.

Finally, these results would be of value in any breeding program aiming at the development of barely cultivars resistant to biotic and abiotic stresses and of higher productivity. The 14 genotypic identities (without wheat) produced by the employed markers might be used in marker-assisted breeding to map stress-tolerance related genes.

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