Hebron University

College of Graduate Studies

Genetic diversity of Palestinian grapevine (*Vitis vinifera* L.) cultivars using different DNA-based markers.

By:

Arwa M. Naser Mujahed

Supervisor:

Dr. Rezq Basheer-Salimia

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Natural Resources and it's Sustainable Management, College of Graduate Studies.

Hebron University, Palestine.

2013

بسم الله الرحمن الرحيم

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This thesis was successfully defended and approved on September 08, 2013

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Dedication

I dedicate this thesis to my husband "Ahmed Al-Sharif" for his endless love, support and encouragement and to my wonderful children Akram and Asal.

A special feeling of gratitude to my loving parents, whose words of encouragement and push for tenacity ring in my ears. My sisters and brothers have never left my side and are very special. I also dedicate this thesis to my best friends who have supported me throughout the process.

This work was done specially to assist my lovely homeland **"Palestine"**...

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List of Contents

Contents

List of Tables

List of Figures

Abstract

Identification of the Palestinian grapevine cultivars is still obscure. Development of a common database based on molecular analysis would seem to be priority to determine the true number of the existing cultivars and their relationships. The main goals of this study were to determine the number of genetically different grapevine cultivars that were actually collected in Palestine using DNA-based RAPD and ISSR techniques; to infer possible cases of synonymy and homonymy; and to evaluate the genetic relationships of the characterized cultivars.

A total of 36 grapevine cultivars were surveyed throughout Bethlehem and Hebron regions of West-Bank, Palestine. Genomic DNA was extracted from fresh leaves of single adult trees using the DNeasy Plant Mini Kit (Qiagen Inc.). 25 RAPD primers (Sigma-Aldrich) and 20 ISSR primers were used for the amplification of the DNA banding patterns followed by PCR reactions. From these primers, 21 RAPD primers and 18 ISSR primers were produced clear and informative bands and therefore were used in this study, however, the remaining primers which produced indistinct and ambiguous bands were excluded from the analysis.

RAPD primers produce 186 amplified loci in which 124 were polymorphic with average polymorphism 68.1%, whereas, ISSR primers produce 57 amplified loci, in which 55 were polymorphic with average polymorphism 88%. Primers OPG-13 (from RAPD group) and S-31 (from ISSR group) presented the maximum number of amplified bands and thereby, were considered as the most powerful primers.

Concerning the relatedness of the grapevine cultivars, RAPD primers showed an average genetic distance of 0.07 (93% similarity) between Jandali-taweel-mofarad and Jandali-kurawi-mlzlz cultivars, whereas, the maximum genetic distances of 0.50 exhibited between Romi-aswad-habe-tawele and Jandali cultivars. Regarding the ISSR primers, the distance range was 0.05 to 0.76 between (Jandali-tawel-mofrod and Jandali.Kurawi.Mlzlz) and (Shami and Marawi.Hamadani.Adi) respectively.

Dendrogram constructed by UPGMA based on RAPD, ISSR and combinations of RAPD and ISSR banding patters revealed that high genetic relatedness were exist between many examined grapevine cultivars such as (Shami.Mtrtsh.Mlwn and Shami.Aswad), (Jandali.Tawel.Mafrod and Jandali.Kurawi.Mlzlz), (Baluti.Abiad and Zaini.Haba.Tawela), (Shami and Betuni) and (Darawishi and mtartash). Therefore, we might assume that each pair is genetically one cultivar but with different names. Moreover, several cultivars were

commonly named as the same name; however, our analysis showed that these cultivars are genetically different such as Jandali, Daboki, Marawi and Romi with their synonyms.

On the other hand, there were several distinctive cultivars such as Romi.Aswad.Haba.Tawela, Marawi.Hamadani.Adi and Dabuki.Aswad.Baladi that might be used as promising cultivars toward future breeding programs in Palestine.

Based on the obtained synonyms and homonyms the total number of the examined cultivars could be reduced into 20 instead of 36 grapevine genotypes.

Keywords: *Vitis venifera*, Palestine, DNA-based markers, RAPD, ISSR.

Introduction

Grapevine (*Vitis vinifera* L.) is a crop of major economic importance worldwide used for the production of table, wine grapes, raisins, and juices (Jaladet *et al*., 2009). According to the Food and Agriculture Organization, more than 7.6 million hectares of the world are dedicated to grapes (FAOSTAT, 2008) and the area devoted to vineyards is increasing dramatically by about 2-3% per year.

Approximately 71% of the world grape production is used for wine, 27% as fresh fruit, and only 2% as dried fruit. A portion of grape production goes to producing grape juice to be re-constituted for fruits canned "with no added sugar" and "100% natural".

The history of grapevine cultivation parallels the history of civilization along the Mediterranean basin. From the earliest events of grape and wine production, dating back to before 6000 BC in Transcaucasia region (Mcgovern and Rudolph, 1996) to the present there has been an important trade of grapevines and their products, mainly wine and raisins, promoted by different cultures like Phoenicians, Greeks, Romans, and Muslims (Snoussi *et al*., 2004).Viticulture and viniculture of grape were highly important in ancient time in the near east regions that include Palestine. According to archaeological finds and ancient scripts such as Bible and others, these industries were highly popular and widely practiced (Klein *et al*., 2006).

In Palestine, grapevines are considered the second important fruit crop after olive in terms of both areas covered as well as economic returns. Due to the unique geographical and ecological environment for growing high quality table grapes, its growing and production are still restricted to the southern part of West-Bank especially Hebron and Bethlehem areas (Sultan, 2005). According to the recent Palestinian statistics, the total agricultural area is about 170.000 ha, includes 105.000 ha planted with fruit trees, of which 7.600 ha are grown to grapevines cultivation (PCBS, 2010).

Like other crops, Palestinian grape is suffering from different problems including: pest infections, drought, disappearance of some inherited cultivars, and miss-named (mis-identified cultivars or called by different names in different areas) cultivars. Due to urbanization, many of our cultivars have been subjected either to genetic deterioration and/or to disappearance leading thereby to lose a great pool of grape genetic materials that might include some interesting traits such as drought and insect resistance. Therefore, determination of genetic variability and proper cultivar identification in grapevine would be of major importance in

improvement programs and in germplasm characterization and conservation to control genetic erosion.

In general, characterization of fruit cultivars could be done at phenotypical and/or, bio-chemical, and/or molecular levels.

Over the phenotypic as well as biochemical levels, DNA-based markers provided a wealth of polymorphisms, enabling the identification of cultivars and the construction of saturated genetic maps in many higher plants (Grando and Frisinghelli, 1998), Morphological characters have traditionally provided signatures of varietal genotype and purity. However, molecular characters that more quickly and accurately reveal genetic differences without the obscurance of environment provide significant advantages in genetic analysis, germplasm characterization, and improvement programs (Hussein, *et al*., 2005)

Molecular markers have been implemented in several countries for cultivar identification, recognition of synonyms, and to establish genetic diversity and relatedness (Francesca, *et al*., 2010).

Various types of molecular markers have been some described in the literature, which are listed in alphabetical order as follows: allele specific associated primers (ASAP ; Gu *et al*., 1995), allele specific oligo (ASO; Beckmann, 1988), allele specific polymerase chain reaction (AS-PCR; Landegren *et al*., 1988) amplified fragment length polymorphism (AFLP; Vos, *et al*.,1995), anchored microsatellite primed PCR (AMP-PCR; Zietkiewicz, *et al*., 1994), anchored simple sequence repeats (ASSR; Wang *et al*., 1998), arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990), cleaved amplified polymorphic sequence (CAPS; Akopyanz, *et al*., 1992, Konieczny and Ausubel, 1993), degenerate oligonucleotide primed PCR (DOP-PCR; Telenius, *et al*., 1992), diversity arrays technology (DArT; Jaccoud, *et al*., 2001), DNA amplification fingerprinting (DAF; Caetano-Anolles, *et al*., 1991), expressed sequence tags (EST; Adams, *et al*., 1991), inter-simple sequence repeat (ISSR; Zietkiewicz, *et al*., 1994), inverse PCR (IPCR; Triglia, *et al*., 1988), inverse sequence-tagged repeats (ISTR; Rohde, 1996), microsatellite primed PCR (MP-PCR; Meyer, *et al*., 1993), multiplexed allele-specific diagnostic assay (MASDA; Shuber, *et al*., 1997), random amplified microsatellite polymorphisms (RAMP; Wu, *et al*., 1994), random amplified microsatellites (RAM; Hantula, *et al*., 1996), random amplified polymorphic DNA (RAPD; Williams, *et al*., 1990), restriction fragment length polymorphism (RFLP; Botstein, *et al*., 1980), selective amplification of microsatellite polymorphic loci (SAMPL; Morgante and

Voge, 1994), sequence characterized amplified regions (SCAR; Paran and Michelmore, 1993), sequence specific amplification polymorphisms (S-SAP; Waugh, *et al.*, 1997),sequence tagged microsatelite site (STMS; Beckmann and Soller, 1990), sequence tagged site (STS; Olsen, *et al.*, 1989), short tandem repeats (STR; Hamada, *et al.*,1982), simple sequence length polymorphism (SSLP; Dietrich, *et al.*, 1992), simple sequence repeats (SSR; Akkaya, *et al.*, 1992), single nucleotide polymorphism (SNP; Jordan and Humphries, 1994), single primer amplification reactions (SPAR; Gupta, *et al.*, 1994), single stranded conformational polymorphism (SSCP; Orita, *et al.*, 1989), site-selected insertion PCR (SSI; Koes, *et al.*,1995), strand displacement amplification (SDA; Walker, *et al.*, 1992), and variable number tandem repeat (VNTR; Nakamura, *et al.*, 1987).

In Vitis, numerous molecular studies have been conducted toward characterization of grape species in different countries (Pollefeys and Bousquet, 2003). However, almost no studies have been found in the literature on Palestinian grapes neither at phenotypical nor at molecular levels.

The main goals of the present research were to determine the number of genetically different grapevine cultivars that were actually collected in Palestine using DNA-based RAPD and ISSR techniques; to infer possible cases of synonymy and homonymy; and to evaluate the genetic relationships of the characterized cultivars

Study Objectives

The aims of this study are:

(a) To determine the number of genetically different grapevine cultivars that were actually collected in Palestine using DNA-based RAPD and ISSR techniques,

(b) To infer possible cases of synonymy and homonymy,

(c) To evaluate the genetic relationships of the characterized cultivars.

Chapter One
Literature Review

Chapter One: Literature Review

1.1. Botanical classification of grapes:

The grapevine (*Vitis vinifera*) belongs to the family Vitaceae, which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental–temperate climatic conditions ((Ren *et al.*, 2000).

Over time, mainly *Vitis* species are acquired significant economic interest; however, some other species (for example the North American *V. rupestris*, *V. riparia* or *V. berlandieri*), are used as breeding rootstock due to their resistance against grapevine pathogens, such as *Phylloxera*, *Oidium* and mildews (Basheer-Salimia and Hamdan, 2009) .

Two forms of Vitis constitutes a great majority of cultivated cultivars exists: *Vitis vinifera* L. subsp. *vinifera* (or *sativa*), and the *Vitis vinifera* L. subsp. *sylvestris* "mainly wild form" (Zohary, 1995, Sefc *et al.*, 2001, This *et al.*, 2004).

Grapes also may classify according to the fruit color as red, black and white (pale-green in color). Each kind has its own particular characteristics for table grape and wine making.

1.2. Origin and domestication of grapes:

The cultivation and domestication of grapevine appears to have occurred in a geographical area between the Black Sea and Iran (McGovern and Rudolph, 1996, Zohary, 1995). From this area, cultivated forms would have been spread by humans in the Near East, Middle East and Central Europe. As a result, these areas may have constituted secondary domestication centers (Grassi, *et al.*, 2003, Arroyo-Garcia, *et al.*, 2006). Continuously, grape cultivation seems to spread gradually from the eastern Mediterranean areas westwards. For example, the Egyptians were using grapes from approximately 3000 BC, and pictures showing vines growing on structures date back to around 1500 BC (Singer, *et al.*, 1954). In Greece and Crete, the beginnings of viticulture would have started during the fifth millennium BC (Valamoti, *et al.*, 2007). In Italy, the most ancient testimonies of grapevine cultivation date back to the ninth century BC (Di Vora and Castelletti, 1995). In Spain and in the Maghreb, the Phoenician influence during the first part of the last millennium BC appears to have played a significant part in the establishment and development of viticulture and viniculture. In France, it was thought that the emergence of viticulture was concomitant with the foundation of Marseille (600 BC) by the Greek Phocaeans (Rivera Nunez and Walker, 1989).

For the modern grapevine, analysis of genetic diversity in the wild (Grassi, *et al.*, 2003, Snoussi, *et al.*, 2004) as well as in the cultivated compartment (Bowers, *et al.*, 1999, Sefc, *et al.*, 2000) allows us to propose hypotheses based on historical biogeography of domestication and dispersal routes, in relation to human migrations and exchanges (This, *et al.*, 2004, Vouillamoz and Grando, 2006) although we cannot date these events.

1.3. Plant description:

Grapevine is mostly woody perennial, tree-climbing vines. The grapevine starts as an under-story plant, growing rapidly and upward, clinging to any other supporting system. In fact, viticulturist can manipulate it in many ways and change the manner in which it trained.

It has a shallow root system and a main weak trunk (the portion of the vine from the ground to about the fruiting wire), and up-ward branches (called canes or cordons) that support the shoots, leaves and fruit.

Canes, which were the previous year's shoots and a non-count cane, which are shoots that arising from latent buds. Along the cane are nodes, separated by internodes. At this point in the season, the nodes are where the following season's shoots will arise. Positioned at alternate sides of the cane are compound buds, so called because they contain three (the primary, secondary and tertiary) pre-formed shoots. Each of these will have six to nine leaf primordia and, in some cases, flower cluster primordia already formed.

The compound bud is designed for overwintering, and is protected by tough bud scales and woolly fibers. Primary buds are less winter hardy than secondary or tertiary buds. If the primary bud is killed by cold temperatures in winter, the secondary bud will grow; if the secondary bud is killed the tertiary bud can grow. The largest clusters are found on the primary buds; in comparison, the secondary bud has inferior clusters and results in a lower yield. Tertiary buds generally do not have clusters, but following a severe winter or early-season frost damage, at least the vine is able to grow to normal size from those.

Arising from the node positions are leaf petioles, tendrils, flower clusters and, as already mentioned, they house the axillary and compound buds. At the distal (furthest from origin, as opposed to basal, closest to the origin) end of the petiole is the leaf blade. Once the plant bears, it produces fruits, which are true berries, containing the seed within.

1.4. Grape reproduction (breeding):

Three processes have had a significant impact on the development of cultivated grapevines: sexual reproduction, vegetative propagation and somatic mutations. New genotypes are produced by sexual reproduction, by either crossing or self-fertilization. Because individual grapevine plants have highly heterozygous genotypes, any progeny produced from seed is a novel combination of parental alleles, resulting in phenotypic variation and segregation of traits in a progeny population (Franks, *et al.*, 2002).

1.5. Economical importance:

Grapevine (*Vitis vinifera* L.) is a crop of major economic importance used for the production of table and wine grapes, raisins, and juices (Bohm and Zyprian, 1998). Recent statistics (FAOSTAT, 2008) showed that this crop covers about 7.6 million hectares and produced about 616,309 million tones, in which 71% of the world grape production is used for wine, 27% as fresh fruit, and only 2% as dried fruit.

Many of the world's countries produce at least some grapes. However, the top ten producers (Italy, France, USA, Spain, China, Turkey, Iran, Argentina, Chile and Australia) are responsible for more than 70% of world production, and it is notable that Italy is the top producing country.

In Palestine, grape is covered about 7178 ha (PCBS, 2007). In addition, grapevine ranks the second among fruit crops grown in Palestine in terms of planted area and economic returns.

Generally, grape is used for a myriad of products. Indeed, most grapes are grown for the production of wine however; the remaining is used as fresh fruit, preserves, juice and raisins. Recently, the trend for growing grapes for the fresh market is increased dramatically worldwide.

Nutritionally, grapes contain poly-phenolic antioxidants, vitamins, and minerals. Since, grapes may promote heart health, help prevent cancer, support immunity and strengthen bones. Moreover, grapes contain ellagic acid substance that blocks the production of enzyme that cancer cell needs to grow (Palma and Tylor, 1999).

1.6. Environmental conditions:

As with many organisms, the environment has a major impact on how the grapevine grows. Since the vine is a perennial plant, it exists under a set of varying conditions and, in a vineyard

setting, the plants experience variation through both space and passage of time. Growth and development of the vine (and thus composition of the fruit) is modified by environment.

1.6.1. Climatic requirements:

Climatic factors including temperature, water availability, and light are the main limiting factors of grape distribution and production. In many areas, winter low temperatures define which grapes can be grown. The limits can be modified by lake influences, slope and many cultural practices. In other areas, the climate is too mild for easy commercial production of grapes, as their growth habit in these conditions encourages uneven cropping.

Naturally occurring dormancy is occurred by low temperatures among the growing season. Once the leaves have fallen, the vine is in a state of eco-dormancy, which is controlled by the environment (e.g. cold temperatures) as well as in a state of endo-dormancy (through physiological factors within the plant). Therefore, buds will not develop immediately even if environmental conditions improve. While vines do not have a chilling requirement (i.e. needing a certain amount of time below 10°C in order to emerge from endo-dormancy) per se, cold temperatures do facilitate the process, decreasing the length of time needed for the plant to start growing again when environmental conditions improve (Schnabel and Wample, 1987).

In terms of a requirement for light there is little needed for a grapevine to grow in the wild. In low light situations, the vine grows upward and, once it reaches bright light it fruits, with the focus being the bringing of whatever seeds are in the fruit to maturation and then rendering the fruit enticing enough for something to take it away.

In case of water requirement of the vine depends on its situation. A vine can survive on very little water, but it will not be able to sustain a crop in doing so. Addition of fruit into the equation means a greater canopy area is needed to ripen it, which requires a supply of water to allow the stomata to be open and photosynthesis to occur. Many grape-growing regions of the world rely on natural rainfall to supply water to their vines, but many are also reliant on water brought to the vines. If there is no mechanism to supply vines with water, the amount and quality of the crop is dependent on rainfall and soil water-holding capacity. If the viticulturist has a dry ripening season, then water application becomes a (potentially useful) management tool. A balance must be struck between applying enough water to maintain vine photosynthesis and fruit development without applying too much, resulting in overly vigorous vines that require excessive management.

1.6.2. Soil requirements:

Grapevines can grow in a wide-range of soil types. Generally, all *Vitis venifera* are grafted on numerous American rootstocks that thereby, overcome the problem properties. Indeed, with the use of rootstocks a single cultivar, if grafted, can have roots that are completely different, and even then, there can be particular rootstock/cultivar combinations that are best suited to certain sites. With this in mind, when evaluating soils it is best to confirm that the most deleterious characteristics are not present. These include drought, excessively low or high nutrient levels or pH and insufficient organic matter.

In addition, it could easily grow and produce crop hydroponically or in sand culture. If soil serves its primary purposes anchorage and reservoir for water and an appropriate range of nutrients, there is little direct effect of soil on grapevine growth and even fruit composition, though there is debate on this issue (Bodin and Morlat, 2006, Coipe, *et al.*, 2006, Huggett, 2006, Van Leeuwen and Seguin, 2006).

However, the interaction of physical characteristics of soil and the origin of its parent material can have quite important effects on the magnitude of grapevine growth. For example, the chalk soils found in the Champagne region of France have a desirable combination of high water-holding capacity yet good drainage, porosity and permeability that makes them well suited to grapevine growth and grape production (Huggett, 2006).

The proportion of gravel in soil can have an effect on the root to shoot ratio, with high-percentage gravel soils reducing shoot growth relative to root growth (Trought, *et al.*, 1999).

1.7. Grapevine diversity:

Ampelographic characters are very often not sufficient to identify a given variety. The use of alternative methods based on DNA markers has proven a valid tool for characterization and detection of synonymies among grapevines (Grover, *et al.*, 2001).

Determination of genetic diversity in a gene pool is the key to any crop improvement, indeed, the analysis of genetic diversity and relatedness among different individuals, species or populations is the central topic in genetics (Smykal, 2006). For estimation of genetic diversity and establishment of varietals identity, various types of marker systems are being used by plant breeders (Choudhury, *et al.*, 2007).

The last two decades have seen an exponential increase in the number of plant sequences in databases and the explosion of investigations on the molecular functions and

physiological roles of these genes (Burstin, *et al.*, 2007). The functions of thousands of genes have been identified (Ostergaard and Yanovsky, 2004). These tools can now be used to address the question of phenotypic plasticity "which genes control plant functioning in which environments" and to provide some clues about which forces shaped natural variation and the strategies that should be used to breed more adapted cultivars (Paran and Zamir, 2003, Reymond, *et al.*, 2003, Koornneef, *et al.*, 2004, Mitchell-Olds and Schmitt, 2006).

Genomic structure of grapes (*Vitis venifera* L.) is considered as attractive model for research since it is diploid plant and has a small genome size of 475–500 Mb relative to other plants. Moreover, it is approximately four times the size of Arabidopsis and one-sixth the size of the corn genome (Thomas, *et al.*, 1993, Lodhi and Reisch, 1995), consisting of 19 chromosomes. The genotypes of grape varieties are highly heterozygous and nearly all modern cultivated varieties (cultivars) are hermaphroditic, self-fertile and out-cross easily. Over the decade, there has been a rapid increase in genomic resources available for grapevine research.

In fact, sexual and asexual multiplication and mutations have had a major role in the expansion and diversification of grapevine in which, the number of different varieties held in germplasm collections around the world is exceeding 10,000 (Alleweldt, and Dettweiler, 1994).

Characterization of these varieties (cultivars) could be achieved either by morphological (traditional) method and/or by molecular makers (AFLP, RAPD, RFLP, ISSR, SSR, etc) which is the most recent approach.

Biochemical markers like isozyme were also used for the recognition of the grape fruits. In contrast to the environment (Pelsy and Merdinoglu, 2002), DNA markers have the maximum efficiency for reforming tasks related to plants.

In vitis, recent improvements using different tools such as RFLP (Bourquin, *et al.*, 1993), RAPD (Ye, *et al.*, 1998), AFLP (Cervera, *et al.*, 2000), ISSR (Sabir, *et al.*, 2009), and SSR (Bowers and Meredith, 1996, Sefc, *et al.*, 2001) have provided valuable information on biodiversity of its material worldwide.

Screening the literature, rare studies exists about the Palestinian grapevine cultivars except those reported by Sultan (2005) who stated that around 50 table-grapevine cultivars are exist in Palestine including early, medium, and late cultivars in which the majority are white once. Unfortunately, his assumption based on his experiences rather than on scientific analysis.

1.7.1. Phenotypical and morphological characterization:

Morphological markers have been used for many years for identification and characterization of plant genotypes and therefore, it considered as a traditional methods for varietal identification (Ohmi, *et al.*, 1993). In grapes, several reports demonstrated the usefulness of these markers in documenting variability among genotypes (Sabir, *et al.*, 2009).

However, morphological characters can often yield ambiguous results due to high plasticity for many traits, as well as phenotypic modifications caused by environmental differences. For example, plants ability to tolerate multiple stresses through morphological adjustments is a major feature that determines species survival and colonization, and hence the ecological breadth of the variety (Bazzaz, *et al.*, 1979). Further, trichomes in plant are considered as morphological trait that can impose resistance to herbivore insects (Medeiros, 2009). As a result, ampelographic characters are very often not sufficient to identify a given variety.

1.7.2. Molecular Characterization:

The limitations of phenotype-based genetic markers led to the development of more general and now wide- spread use of DNA-based markers, which proved to be powerful tools to estimate genetic diversity of species, as well as genotype identity. In fact, molecular markers offer numerous advantages over conventional morphological based approaches, since they are stable and detectable in all tissues regardless of growth, differentiation, development, or defense status of the cell. In addition, DNA markers are not confounded by the environment, pleiotropic, and epistatic effects (Kumar, *et al.*, 2009).

A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA; (b) a specific piece of DNA with a known position on the genome, or (c) a gene whose phenotypic expression is usually easily discerned, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, chromosomes, or locus (King and Stansfield, 1990). Since the markers and the genes they mark are close together on the same chromosome, they tend to stay together as each generation of plants is produced. As scientists learn where markers occur on a chromosome, and how close they are to specific genes, they can create a genetic linkage map .

In grapes, assessment of genetic relatedness and diversity has been investigated by using RFLP, AFLP, SSR, ISSR, and RAPD methods (Bowers, *et al.*, 1999).

1.7.2.1. RFLP (Restriction Fragment Length Polymorphism):

RFLP is the most widely used hybridization-based molecular marker. RFLP markers were first used in 1975 to identify DNA sequence polymorphisms for genetic mapping of a temperature-sensitive mutation of adeno-virus serotypes (Grodzicker, *et al.*, 1975). It was then used for human genome mapping (Botstein, *et al.*, 1980), and later adopted for plant genomes (Helentjaris, *et al.*, 1986, Weber and Helentjaris, 1989).

The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. Although two individuals of the same species have almost identical genomes, they will always differ at a few nucleotides due to one or more of the following causes: point mutation, insertion/deletion, translocation, inversion and duplication.

There are, however, several limitations for RFLP analysis:

I. It requires the presence of high quantity and high quality of DNA (e.g., Potter and Jones, 1991, Roy, *et al.*, 1992).

II. It depends on the development of specific probe libraries for the species.

III. The technique is not amenable for automation.

IV. The level of polymorphism is low, and few loci are detected per assay.

V. It is time consuming, laborious, and expensive (Yu, *et al.*, 1993).

VI. It usually requires radioactively labeled probes.

RFLP is applied to the genomic DNA of grape cultivars by using heterozygous probes derived from phage M13 or the human probe 33.6 (Striem, *et al.*, 1990)

1.7.2.2. AFLP (Amplified Fragment Length Polymorphism):

AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA (Lynch and Walsh, 1998). The key feature of AFLP is its capacity for "genome representation": the simultaneous screening of representative DNA regions distributed randomly throughout the genome. AFLP markers can be generated for DNA of any organism without initial investment in primer/probe development and sequence analysis.

In grapes AFLP marker has been used in grape identification and discrimination by many authors like Cervera, *et al*., 1998, Scott, *et al*., 2000, Fossati, *et al*., 2001, and Fanizza, *et al*., 2003.

The limitations of AFLP comparing with other markers include:

a) It requires more number of steps to produce the result.

b) It requires template DNA free of inhibitor compounds that interferes with the restriction enzyme.

c) The technique requires the use of polyacrylamide gel in combination with $AgNO_3$ staining, radioactivity, or fluorescent methods of detection, which will be more expensive and laborious than agarose gels.

d) It involves additional cost to purchase both restriction and ligation enzymes as well as adapters.

e) Like RAPD, most AFLP loci are dominant, which does not differentiate dominant homozygotes from heterozygotes. This reduces the accuracy of AFLP markers in population genetic analysis, genetic mapping, and marker-assisted selection.

1.7.2.3. SSR (Simple Sequence Repeats):

Microsatellites consist of tandem repeats of mono-, di-, tri- or tetra-nucleotide patterns (Burstin, *et al*, 2001). They are also referred to as simple sequence repeats (SSRs). These sequences provide an interesting source of markers, as they are frequent in eukaryote genomes and are well distributed throughout the genome. The sequencing of the flanking regions of a microsatellite allows one to design specific primers to amplify the fragment containing this microsatellite using PCR. The length polymorphism of the amplified fragment is then visualized following agarose or acrylamide gel electrophoresis. These markers are highly polymorphic, and their length polymorphism results from a variable number of tandem repeats (VNTR), probably stemming from replication slippage and/or unequal recombination. High levels of polymorphism have already been observed with this kind of marker (Saghai-Maroof, *et al*., 1994, Innan, *et al*., 1997, Burstin, *et al*., 2001).

Microsatellites are now widely used in plants for genetic mapping, genetic diversity assessment, population genetics and marker-assisted selection (Gupta and Varshney, 2000) and

for investigating genetic diversity among cultivars. SSR maps now exist for a large range of plant species including grape (Adam- Blondon, *et al*., 2004).

1.7.2.4. RAPD (Randomly Amplified Polymorphic-DNA):

In 1991, Welsh and McClelland developed a new PCR-based genetic assay namely Randomly Amplified Polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. Generally, RAPD based PCR amplification process consists of 30 or 40 repeated cycles. This is done on automated thermocycler (Newton and Graham., 1997).

RAPD marker has been used on large scale in population genetics analysis of biological variations and in study the relativity among the species on different levels. RAPD has attracted interest for identification of plant material. It has been used for different crop plants, such as potato (Mori, *et al*., 1993), broccoli and cauliflower (Hu and Quiros, 1991), cocoa (Wilde, *et al*., 1992), apple (Koller, *et al*., 1993) and apricot (Gogorcena and Parfitt, 1994). In addition, studies have also been carried out for identification of grapevine cultivars (Collins and Symons, 1993, Buscher, *et al*., 1993).

The RAPD technique is fast and easy, since it does not require knowledge of the sequences of the markers and can produce abundant polymorphic fragments. RAPD analysis is one of the techniques that has been used successfully to reveal genetic variations (Kocsis, *et al*., 2005). Notwithstanding the limitations, RAPD markers have proven to be a highly effective and efficient method for the genetic analysis (Gogorcena, *et al*., 1993, Buscher, *et al*., 1993, Ye, *et al*., 1998, Ulanovsky, *et al*., 2002). Large numbers of data sets can be generated because different RAPD primers are commercially available (Fanizza, *et al*., 2000).

1.7.2.5. ISSR (Inter Simple Sequence Repeat):

The Inter Simple Sequence Repeat (ISSR) technique, composed of a microsatellite sequence between two SSR priming sites oriented on opposite DNA strands, was approved as a simple, quick and reliable tool used in various grape materials for certain purposes (Zietkıewicz, *et al*., 1994, Moreno, *et al*., 1998, Dhanorkar, *et al*., 2005, Sabir, *et al*., 2009).

ISSRs have been used in assessing genetic relationships among various accessions of different species (Bornet and Branchard, 2001, Fang, *et al*., 1998, Lanham and Brennan, 1999, McGregor, *et al*., 2000).

ISSRs have been also successfully employed to identify grapevine cultivars. The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes. The microsatellite repeats used as primers for ISSRs can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers used can be either unanchored (Meyer, *et al.*, 1993, Gupta, *et al.*, 1994, Wu, *et al.*, 1994) or more usually anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz, *et al.*, 1994).

ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher stringency. The annealing temperature depends on the GC content of the primer used and ranges from 45 to 65^{o}C.

The amplified products are usually 150–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi, *et al.*, 2000). The primers are not proprietary and can be synthesized by anyone. The technique is simple, quick, and the use of radioactivity is not essential .

ISSR markers usually show high polymorphism (Kojima, *et al.*, 1998) although the level of polymorphism has been shown to vary with the detection method used.

Chapter Two
Materials
and
Methods

Chapter Two: Materials and Methods

2.1. Plant materials

Healthy grape leaves were collected from the middle-region of the newly growing shoots from 36 assumed cultivars (>50 years) throughout Hebron and Bethlehem districts (Table 1, Figure 1).

Table 1: List of assumed grapevine cultivars and their site collection.

No.	Cultivar name	Collecting area
1	Shukhi	Al-Arub, Hebron
2	Malikat.Libnan	Al-Arub, Hebron
3	Betuni	Beit Enun, Hebron
4	Dabuki	Beit Enun, Hebron
5	Jandali	Beit Enun, Hebron
6	Miskt.El.Eskandaria	Beit Enun, Hebron
7	Mtartash.Fhaisi	Beit Enun, Hebron
8	Halawani	Beit Enun, Hebron
9	Zaini	Beit Enun, Hebron
10	Shami	Beit Enun, Hebron
11	Bairuti	Beit Enun, Hebron
12	Mtartash	Halhul, Hebron
13	Fhaisi	Al-Arub, Hebron
14	Salti.Khdari	Al-Arub, Hebron
15	Hamadani	Al-Arub, Hebron
16	Baluti	Al-Arub, Hebron
17	Darawishi	Al-Arub, Hebron
18	Marawi.Hamadani.Adi	Al-Bak´a, Hebron
19	Shami.Mtartash.Mlawan	Al-Bak´a, Hebron
20	Halawani.Baladi.Valantika	Al-Bak´a, Hebron
21	Hamadani.Ma´tar.(Faransi)	Al-Bak´a, Hebron
22	Shami.Aswd	Al-Bak´a, Hebron
23	Roomi.Aswad	Al-Bak´a, Hebron
24	Marawi.Haba.Tawela	Al-Khader, Bethlehem

25	Zaini.Baladi.Dabuki.Baladi.Dabuki.Haba.Tawela	Al-Khader, Bethlehem
26	Jandali.Tawel.Mafrod	Al-Khader, Bethlehem
27	Jandali.Kurawi.Mlzlz	Al-Khader, Bethlehem
28	Aswad.Baladi	Al-Khader, Bethlehem
29	Zaini.Haba.Tawela	Yatta, Hebron
30	Marawi.Haba.Tawela.Shabeh.EL.Beruti	Assun, Bethlehem
31	Baluti.Abiad	Assun, Bethlehem
32	Dabuki.Aswad.Baladi	Assun, Bethlehem
33	Jandali.Shabh.Dabuki	Assun, Bethlehem
34	Halawani.Haba.Kabera	Assun, Bethlehem
35	Hamadani.Kadem	Assun, Bethlehem
36	Roomi.Aswad.Haba.Tawela	Assun, Bethlehem

Figure 1: Grapevine collection sites (map prepared by Land Research Center, Halhoul, Hebron, 2012).

2.2. DNA extraction, purification, and quantification

2.2.1. DNA extraction and purification

Two medium – healthy, young leaves of each assumed cultivar were ground with pestle and mortar using liquid nitrogen to fine powder. A weight of 100 mg of leaf powder of each cultivar was transferred into a 1.5 mL eppendrof tube and mixed with, 400 µL of buffer AP1 and 4 µL of RNase A stock solution (100 mg/mL) were added to the tube, and the mixture was vigorously vortexed, incubated at 65 °C, and mixed 2-3 times during incubation by inverting. A quantity of 130 µL of buffer AP2 was added to the lysate, which was mixed, incubated on ice for 5 minutes, and then centrifuged at 20,000 x g. The supernatant was applied to the QIA-shredder Mini Spin Column and it was carefully centrifuged at 20,000 x g for 2 minutes so as not to disturb the pellet. The flow-through fraction (liquid) was transferred to a new 2 mL eppendrof tube without disturbing the cell-debris pellet and a quantity of 1.5 volumes of buffer AP3/E was added to the cleared lysate and mixed by pipetting. A quantity of 650 µL of the mixture was applied to the DNeasy Mini Spin Column placed in a 2 mL collection tube which was centrifuged at 6,000 x g while its flow-through was discarded. The rest of the mixture was applied as aforementioned. Subsequently, the DNeasy Mini Spin Column was placed in a new 2 mL collection tube, and 500 µL of buffer AW was added to it. The tube was centrifuged at 6,000 x g for 1 min, and the flow-through was discarded, reusing the collection tube in the next step. A total volume of 500 µL of the same buffer was used once more with centrifuging at 20,000 x g for 2 min. The DNeasy Mini Spin Column was transferred to a 1.5 mL eppendrof tube and a quantity of 30 µL of buffer AE was added twice with a separation time of at least 5 min between them. Before storing at 20 °C, the tube was centrifuged at full speed for 1 min.

2.2.2. Estimation of DNA quantification

DNA quality and quantity was tested on 0.8% agarose gel electrophoresis using Lambda DNA as a standard. Other measurements also done for DNA concentration and purity using spectrophotometer. Final concentration of DNA was adjusted to 50 ng/µl.

2.2.3. Randomly Amplified Polymorphic DNA (RAPD) / PCR reaction mixture and program

A total of twenty five RAPD primers "10 mer" (Sigma-Aldrich, Table.2.) were used for the amplification of random DNA banding patterns according to Karataş H. and Agaoglu Y.S. (2010).

PCR reactions were carried out in a 25 µl volume mixture containing: 5 µl of a total DNA (30 ng), 4 µl primer (5 µM), 2 µl dNTPs (200 mM) (Fermentas), 2.5 µl Taq buffer (10X), 2 µl magnesium chloride (25 mM) and 1.5 U of Taq DNA polymerase (Hy Labs). Consequently, DNA was amplified by PCR on a Peltier Thermal Cycler-200 (MJ Research. Inc, Watertown, MA) and the PCR program was: 1 cycle, 94 °C (3 min); 35 cycles, 94 °C (1 min), 53 °C (1 min), 72 °C (1;30 min) 1 cycle, 72 °C(5 min), and then cooling down to 4 °C.

Table 2: List of the used RAPD primers

No.	**Primer name**	**Primer sequence (5`→3`)**
1	OPG-02	GGCACTGAGG
2	OPG-03	GAGCCCTCCA
3	OPG-06	GTGCCTAACC
4	OPG-08	TCACGTCCAC
5	OPG-11	TGCCCGTCGT
6	OPG-12	CAGCTCACGA
7	OPG-15	ACTGGGACTC
8	OPG-17	ACGACCGACA
9	OPG-18	GGCTCATGTG
10	OPN-05	ACTGAACGCC
11	OPN-11	TCGCCGCAA
12	OPN-13	AGCGTCACTC
13	OPN-16	AAGCGACCTG
14	OPN-20	GGTGCTCCGT
15	OPO-05	CCCAGTCACT
16	OPT-20	ACACACGCTG

17	OPW-08	GACTGCCTCT
18	OPR-12	ACAGGTGCGT
19	OPG-13	CTCTCCGCCA
20	OPG-05	CTGAGACGGA
21	OPS-05	TTTGGGGCCT
22	OPW-13	CACAGCGACA
23	OPX-01	CTGGGCACGA
24	OPE-17	CTACTGCCGT
25	OPD-14	CTTCCCCAAG

2.2.4. RAPD-Gel processing

Amplified products (25 µl) were mixed with 5 µl of orange gel loading buffer and analyzed by electrophoresis in 2% agarose gels (Hy Labs) in 1X TAE buffer at 4 volt/cm for 4h as well as detected by staining with ethidium bromide (Sigma). A 100 bp DNA ladder was used as standard marker (Fermentas). Consequently, amplicons were visualized and photographed black and white on Polaroid type film with UV trans-illuminator (ImageMaster® VDS).

DNA bands were scored (1) for presence and (0) for absence for each primer- genotype combination. Only reliable and clear bands were scored for the estimation of genetic similarity.

2.2.5. Inter Simple Sequence Repeats (ISSR) / PCR reaction mixture and program

Twenty ISSR primers (Table 3) were used for the amplification of random ISSR banding patterns according to Sabir *et al.*, (2009). PCR reactions were carried out in a 25 µl volume mixture containing: 5 µl of a total DNA (50 ng), 4 µl primer (5 µM), 2 µl dNTPs (200 mM) (Fermentas), 2.5 µl Taq buffer (10X), 2 µl magnesium chloride (25 mM) and 1.5 U of Taq DNA polymerase (Hy Labs). Consequently, DNA was amplified by PCR on a Peltier Thermal Cycler-200 (MJ Research. Inc, Watertown, MA) and the PCR program was: 1 cycle,

94 °C (3 min); 40 cycles, 94 °C (1 min), 54 °C (1 min), 72 (2 min) 1 cycle, 72 °C(7 min), and then cooling down to 4 °C.

Table 3: List of the used ISSR primers.

No.	Primer name	Primer sequence (5`→3`)
1	#4	5`GAG AGA GAG AGA GAG AYG3`
2	#811	GAG AGA GAG AGA GAG AC
3	S-17	GAG AGA GAG AGA GAG AT
4	S-19	GAG AGA GAG AGA GAG AA
5	#9	ACA CAC ACA CAC ACA CYG
6	S-13	ACA CAC ACA CAC ACA CYC
7	S-14	AGA GAG AGA GAG AGA GT
8	S-16	AGA GAG AGA GAG AGA GG
9	S-27	BDB CAC ACA CAC ACA CA
10	S-31	AGA GAG AGA GAG AGA GVC
11	UBC-855	ACA CAC ACA CAC ACA CYA
12	#890	VHV GTG TGT GTG TGT GT
13	#841	GAG AGA GAG AGA GAG AYC
14	S-30	HVH TGT GTG TGT GTG TG
15	#840	GAG AGA GAG AGA GAG AYT
16	#836	AGA GAG AGA GAG AGA GYA
17	#826	ACA CAC ACA CAC ACA CC
18.	#825	ACA CAC ACA CAC ACA CT
19	#820	GTG TGT GTG TGT GTG TC

20	#818	CAC ACA CAC ACA CAC AG
Where Y: (C,T); B: (C,G,T); D: (A,G,T); H: (A,T,C) V: (A,C,G); and R: (A,G).		

2.2.6. ISSR-Gel processing

Amplified products (25 µl) were mixed with 5 µl of orange gel loading buffer and analyzed by electrophoresis in 3% agarose gels (Hy Labs) in 1X TAE buffer at 4 volt/cm for 4h as well as detected by staining with ethidium bromide (Sigma). A 100 bp DNA ladder was used as standard marker (Fermentas). Consequently, amplicons were visualized and photographed black and white on Polaroid type film with UV trans-illuminator (ImageMaster®VDS).

DNA bands were scored (1) for presence and (0) for absence for each primer-genotype combination. Only reliable and clear bands were scored for the estimation of genetic similarity.

2.2.7. RAPD and ISSR Data analysis

Data matrix was utilized to generate genetic similarity data among genotypes using. Jacquard's similarity coefficient formula as the following:

$$S_{ij,Jaccard} = \frac{n_{11}}{n_{11} + n_{01} + n_{10}}$$

Where *nxy* is the number of characters that have state *x* in individual *i* and state *y* in individual *j*. Un-weighted pair group method using arithmetic averages (UPGMA) (Schluter and Harris, 2006) phenogram was then calculated from the Jaccard's similarity using fingerprint analysis with missing data (FAMD) software version 1.108 beta. Tree view software (Win32) version 1.6.6 was used to visualize the resulted trees. All trees presented in this study were mid-pointed.

Chapter Three
Results

Chapter Three: Results

3.1. RAPD results

3.1.1. Genetic variations and relatedness among grapevine accessions based on RAPD

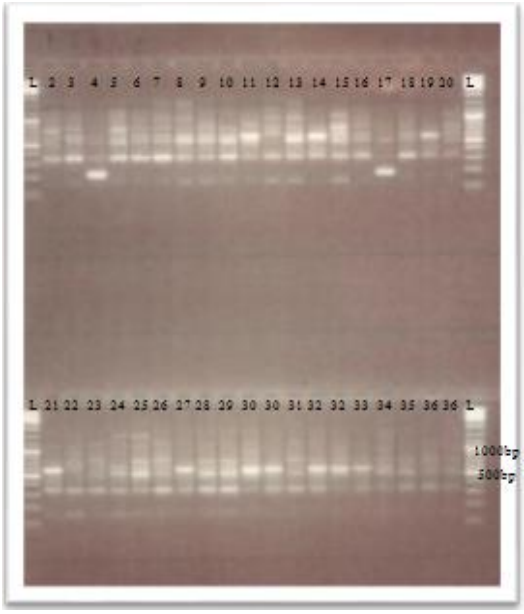
Among the 25 tested primers used to investigate the pattern of genetic variation among 36 accessions of grape grown at the southern region of West-Bank, Palestine; 21 primers produced reasonable amplification products with high intensity and pattern stability (Table 4). However, the remaining four primers (OPN-11, OPN-20, OPG-17, OPN-13) exhibited ambiguous, light, and non-clear complex amplification products, and therefore was excluded from our analysis.

A total of 186 DNA fragments (loci) separated by electrophoresis on agarose gels, were detected (Table 4), ranging in size from 150 to 1400 bp. Of these fragments, 124 (62.5%) were polymorphic and 62 (37.5%) were monomorphic. Our results also revealed an average of 7.7 loci per primer (Tables 4). A minimum of 2 and a maximum of 15 DNA fragments were obtained using (OPG-8, OPG-15 and OPR-12) and (OPG-13) primers, respectively (Table 4). Therefore, the later primer (OPG-13) is considered as the most powerful primer (Table 4, Figure 2). The maximum percentage of polymorphic markers was 100.0 with OPG-11 primer.

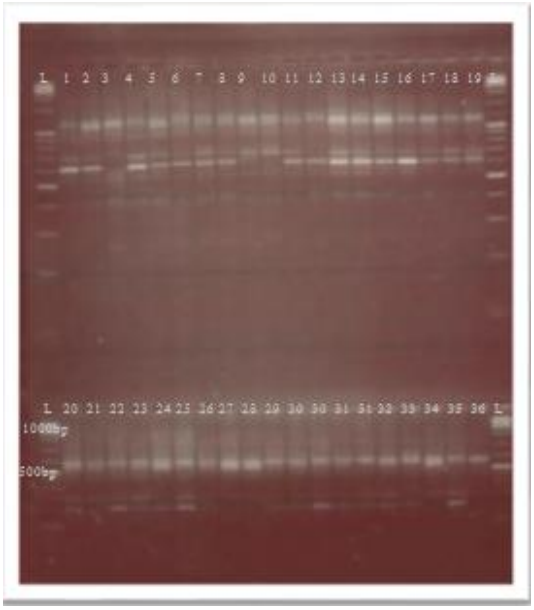
Table 4: Analysis of the polymorphism obtained with RAPD markers

				Approximate band size (bp)			
primers name	**Total No. RAPD bands**	**Mono-morphic band**	**Poly-morphic bands**	**Min**	**Max**	**Poly-morphic (%)**	**Primer case**
OPG-3	8	3	5	280	1000	62.5%	Included
OPO-5	4	2	2	300	600	50%	Included
OPE-17	11	1	10	150	1200	91%	Included

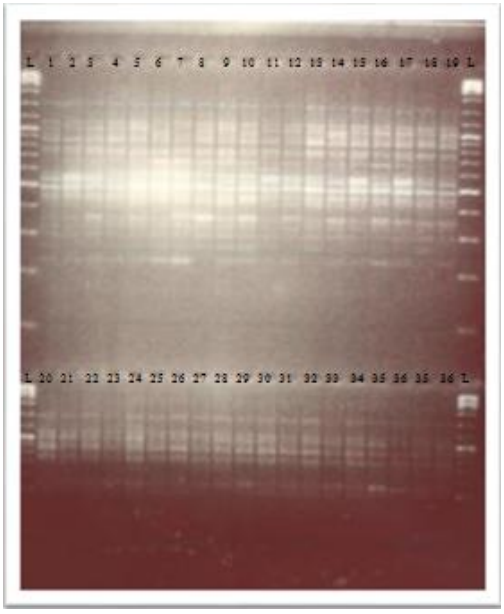
OPG-12	10	1	9	150	900	90%	Included
OPN-11	9	6	3	300	1050	33.3%	Excluded
OPG-13	15	5	10	100	1200	67%	Included
OPN-16	11	4	7	300	1200	63.6%	Included
OPX-1	11	4	7	200	1150	63.6%	Included
OPG-5	4	1	3	300	900	75%	Included
OPS-5	11	2	9	250	1400	82%	Included
OPG-2	8	4	4	380	1200	50%	Included
OPW-13	12	6	6	210	1050	50%	Included
OPD-14	9	1	8	250	1100	89%	Included
OPG-6	9	1	8	250	1000	89%	Included
OPG-11	9	0	9	150	1200	100%	Included
OPG-18	9	2	7	300	1200	78%	Included
OPN-20	6	4	2	250	1000	33.3%	Excluded
OPG-8	2	1	1	350	1300	50%	Included
OPT-20	5	2	3	200	800	60%	Included
OPG-15	2	1	1	400	800	50%	Included
OPG-17	4	3	1	500	1200	25%	Excluded
OPN-5	5	2	3	500	1300	60%	Included
OPW-8	5	2	3	350	800	60%	Included
OPR-12	2	1	1	500	1000	50%	Included
OPN-13	5	3	2	350	1000	40%	Excluded
Total	**162**	**46**	**116**			**68.1 %**	



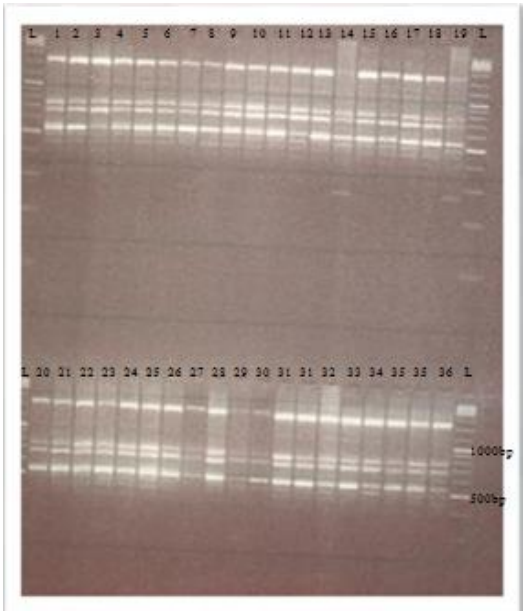
OPE-17



OPD-14



OPG-13



OPG-17

Figure 2: Examples of RAPD banding patterns generated in Palestinian grapevine cultivars using OPE-17, OPD-14, OPG-13 and OPG-17 primers (100 bp ladder).

3.1.2. Genetic distances

The data matrix size analyzed incuded 6696 entries, 4443 of which were for present loci (1) and 2253 for absent loci (0). Accordingly, the Jaccard coefficient was calculated and

presented in table 5. The genetic distance matrix showed an average distance range from 0.07 to 0.50 with a mean of 0.29. The maximum genetic distance value of 0.50 (50%) was exhibited between Romi.Aswad.Habe.Tawele and Jandali genotypes, whereas the lowest genetic distance of 0.07 (93% similarity) was exhibited between Jandali.Tawel.Mofarad and Jandali-Kurawi.Mlzlz genotypes.

3.1.3. UPGMA analysis

UPGMA dendrogram clustered the grape genotypes into two major clusters (Figure 3). The cluster I, consists of 18 genotypes which are divided into three main sub-clusters namely Ia, Ib, and Ic in which all are related to an isolated genotype (Id, Romi.Aswad.Habe.Tawela) as a distinctive cultivar.

The sub-cluster (Ia) included two small groups (Jandali.Kurawi.Mlzlz and Jandali .Tawel.Mafrod, which are related to Jandali.Shabh.Dabuki), as well as (Hamadani.Kadem and Marawi.Shabh.Beruti, related to Dabuki.Aswad.Baladi), in which all three genotypes are related to Halawani.Habe.Kabera.

The sub-cluster (Ib) included highly related Darawishi and Mtartash genotypes. Sub-cluster (Ic) is composed of genotypes (Baluti.Abiad and Zaini.Habe.Tawela) and (Aswad.Baladi and Zaini.Baladi.Dabuki.Baladi), in which all related to Sulti.Khdari genotype. The minor group is composed of Marawi.Habe.Tawela and Hamadani, related to Fhaisi.

The cluster II also composed of 18 genotypes, which grouped as IIa, IIb, IIc, and IId. Group IIa consists of 11 genotypes accordingly to their relationship as the following: (Shami.Aswad, Shami.Mtartash.Mlwan, Hamadani.Ma´tr.Faranci, Marawi.Hamadani.Adi, and Romi.Aswad). In addition to, Dabuki and Zaini (which are closely related), Shami , Betuni , Baluti, and Bairuti.

Group IIb is composed of (Miskat.El.Eskandaria and Jandali which are highly related), as well as (Malikat.Libnan, Shukhi and Halawani). Group IIc and grope IId which are composed of only one genotype each (Halawani.Baladi.Valantiki and Mtartash.Fhaisi), respectively. In addition, both later genotypes (IIc and IId) are related to the other 16 grape genotypes.

Table 5: Jaccard´s distance index generated for the 36 local Palestinian grapevines' RAPD data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.Mar.Hamadani.Adi	0.00																																			
2.Shami.Mr.Mwn	0.15	0.00																																		
3.Halawani.Bald.Valntiki	0.29	0.29	0.00																																	
4.Hamadani.Matr.Faranci	0.13	0.15	0.20	0.00																																
5.Shami.Aswad	0.16	0.08	0.25	0.11	0.00																															
6.Romi.Aswad	0.24	0.23	0.29	0.21	0.16	0.00																														
7.Shukhi	0.35	0.30	0.32	0.27	0.27	0.24	0.00																													
8.Malkt.Libnan	0.35	0.31	0.31	0.27	0.27	0.26	0.17	0.00																												
9.Jandali	0.29	0.32	0.28	0.26	0.26	0.24	0.29	0.27	0.00																											
10.Miskat.Eskandaria	0.32	0.31	0.31	0.25	0.26	0.26	0.29	0.23	0.22	0.00																										
11.Mtartash.Fhaisi	0.31	0.23	0.36	0.29	0.26	0.29	0.32	0.31	0.35	0.31	0.00																									
12.Halawani	0.32	0.32	0.29	0.29	0.30	0.25	0.24	0.27	0.27	0.25	0.27	0.00																								
13.Zaini	0.24	0.25	0.30	0.24	0.27	0.25	0.30	0.30	0.31	0.27	0.27	0.25	0.00																							
14.Betuni	0.21	0.23	0.30	0.21	0.22	0.28	0.29	0.29	0.28	0.26	0.26	0.22	0.20	0.00																						
15.Shami	0.23	0.16	0.31	0.23	0.19	0.25	0.30	0.32	0.30	0.32	0.26	0.27	0.22	0.14	0.00																					
16.Bairuti	0.26	0.25	0.25	0.20	0.21	0.28	0.31	0.31	0.32	0.27	0.28	0.28	0.29	0.19	0.20	0.00																				
17.Dabuki	0.24	0.26	0.29	0.22	0.27	0.25	0.33	0.32	0.34	0.29	0.31	0.27	0.18	0.24	0.22	0.23	0.00																			
18.Baluti	0.26	0.26	0.32	0.26	0.27	0.25	0.29	0.28	0.29	0.25	0.31	0.20	0.27	0.15	0.19	0.23	0.20	0.00																		
19.Salti.Khdari	0.38	0.38	0.43	0.37	0.38	0.41	0.37	0.37	0.46	0.40	0.39	0.39	0.35	0.37	0.38	0.41	0.36	0.39	0.00																	
20.Fhaisi	0.37	0.33	0.42	0.35	0.32	0.40	0.37	0.35	0.44	0.39	0.32	0.40	0.37	0.35	0.34	0.35	0.36	0.36	0.19	0.00																
21.Hamadani	0.35	0.38	0.47	0.36	0.39	0.43	0.41	0.39	0.46	0.42	0.42	0.41	0.40	0.35	0.39	0.37	0.37	0.36	0.18	0.20	0.00															
22.Darawishi	0.41	0.38	0.40	0.35	0.36	0.38	0.35	0.38	0.40	0.41	0.40	0.37	0.40	0.39	0.40	0.37	0.41	0.38	0.25	0.26	0.27	0.00														
23.Mtartash	0.36	0.36	0.40	0.34	0.32	0.35	0.40	0.40	0.41	0.39	0.39	0.36	0.37	0.31	0.35	0.35	0.39	0.36	0.27	0.23	0.26	0.14	0.00													
24.Marawi.Haba.Twela	0.25	0.28	0.39	0.25	0.28	0.35	0.38	0.37	0.38	0.36	0.41	0.39	0.34	0.32	0.34	0.33	0.33	0.35	0.22	0.20	0.18	0.24	0.21	0.00												
25.Zaini.Bal.Dabk.Bal	0.42	0.41	0.43	0.38	0.41	0.42	0.41	0.37	0.45	0.37	0.37	0.40	0.39	0.36	0.42	0.37	0.40	0.39	0.19	0.25	0.22	0.22	0.26	0.22	0.00											
26.Jandali.Twel.Mafrod	0.42	0.42	0.42	0.37	0.39	0.42	0.39	0.35	0.36	0.37	0.38	0.39	0.43	0.37	0.43	0.37	0.44	0.38	0.25	0.22	0.23	0.24	0.24	0.22	0.20	0.00										
27.Jandali.Kuravi.Mizlz	0.39	0.41	0.40	0.35	0.38	0.39	0.39	0.35	0.39	0.37	0.37	0.40	0.43	0.38	0.44	0.37	0.43	0.38	0.25	0.24	0.23	0.22	0.22	0.21	0.18	0.07	0.00									
28.Aswad.Baladi	0.39	0.39	0.43	0.34	0.38	0.40	0.37	0.36	0.41	0.36	0.38	0.36	0.38	0.34	0.41	0.38	0.37	0.34	0.18	0.26	0.21	0.16	0.24	0.21	0.14	0.20	0.16	0.00								
29.Zaini.Haba.Twela	0.39	0.39	0.40	0.35	0.39	0.38	0.38	0.37	0.46	0.39	0.37	0.37	0.33	0.35	0.39	0.39	0.33	0.38	0.20	0.29	0.28	0.24	0.28	0.26	0.15	0.28	0.22	0.12	0.00							
30.Marawi.Shabh.Beruti	0.38	0.42	0.46	0.38	0.43	0.42	0.43	0.42	0.47	0.43	0.44	0.40	0.39	0.38	0.41	0.41	0.37	0.39	0.29	0.33	0.21	0.28	0.31	0.26	0.26	0.28	0.22	0.19	0.14	0.00						
31.Baluti.Abiad	0.40	0.39	0.42	0.37	0.38	0.40	0.40	0.35	0.47	0.39	0.40	0.41	0.37	0.36	0.38	0.38	0.36	0.38	0.17	0.25	0.24	0.27	0.29	0.25	0.17	0.25	0.21	0.15	0.08	0.17	0.00					
32.Dabuki.aswad.baladi	0.40	0.40	0.42	0.34	0.39	0.41	0.43	0.40	0.44	0.39	0.39	0.40	0.35	0.37	0.41	0.39	0.39	0.39	0.28	0.28	0.29	0.29	0.30	0.25	0.25	0.28	0.23	0.17	0.17	0.19	0.18	0.00				
33.Halawani.H.Kabera	0.39	0.39	0.36	0.34	0.37	0.39	0.42	0.41	0.46	0.43	0.40	0.42	0.43	0.39	0.42	0.35	0.41	0.43	0.30	0.31	0.26	0.26	0.27	0.27	0.23	0.27	0.22	0.22	0.22	0.22	0.23	0.22	0.00			
34.Jandali.Shabh.Dabuki	0.45	0.45	0.43	0.41	0.43	0.45	0.41	0.42	0.42	0.45	0.45	0.46	0.47	0.42	0.45	0.37	0.46	0.43	0.32	0.33	0.24	0.26	0.31	0.29	0.28	0.20	0.15	0.24	0.26	0.20	0.23	0.29	0.21	0.00		
35.Hamadani.Kadem	0.37	0.40	0.46	0.39	0.41	0.42	0.42	0.41	0.46	0.43	0.43	0.41	0.41	0.37	0.41	0.40	0.38	0.35	0.26	0.28	0.15	0.25	0.28	0.23	0.23	0.25	0.19	0.15	0.21	0.08	0.18	0.20	0.20	0.18	0.00	
36.Romi.Aswad.H.Twela	0.48	0.46	0.43	0.45	0.45	0.47	0.47	0.45	0.50	0.46	0.44	0.46	0.48	0.44	0.45	0.39	0.43	0.44	0.36	0.36	0.36	0.34	0.36	0.38	0.32	0.35	0.32	0.31	0.30	0.32	0.27	0.36	0.26	0.28	0.29	0.00

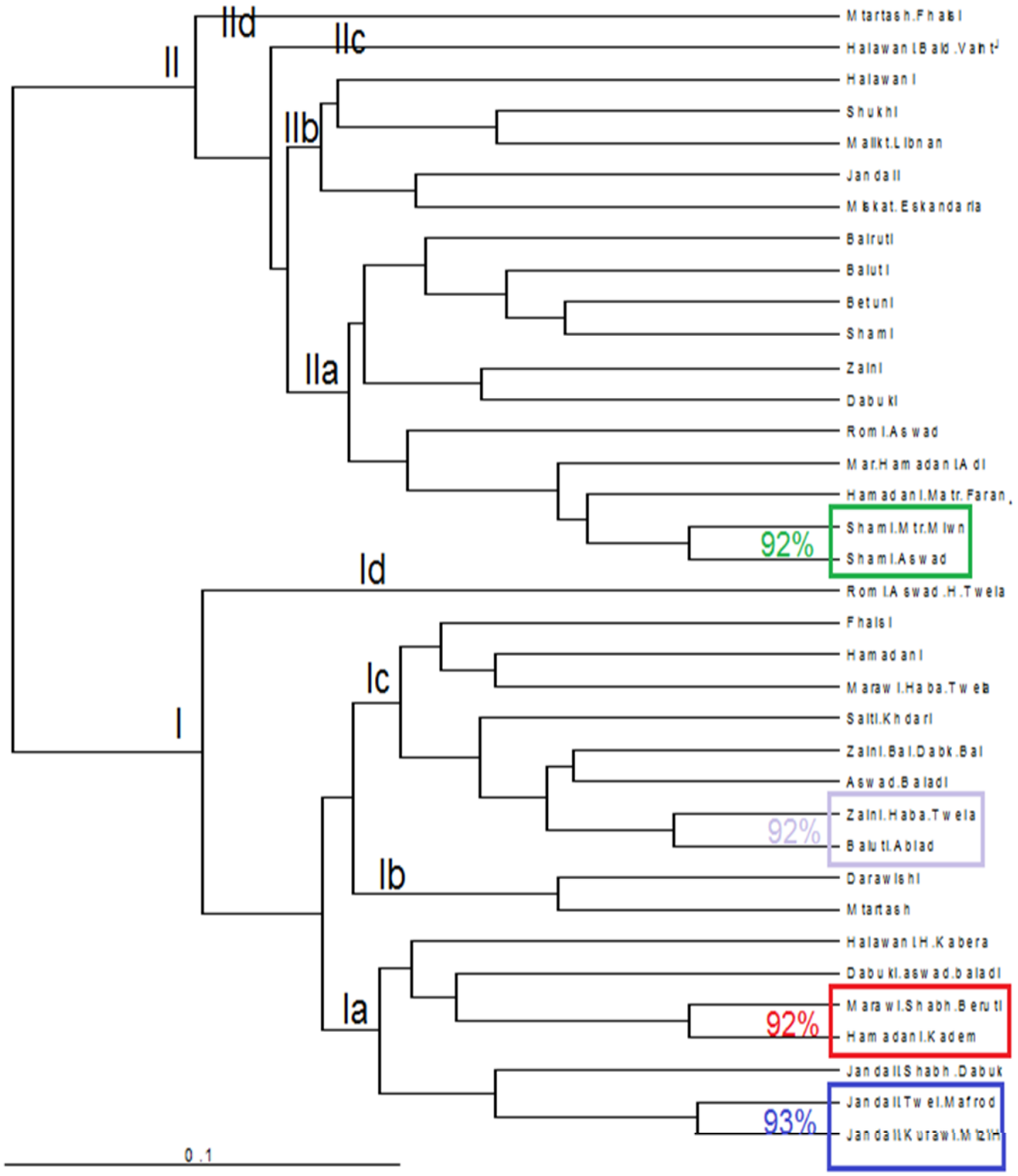


Figure 3: Dendrogram of 36 local Palestinian Grapes constructed by UPGMA based on RAPD banding patterns

3.2. ISSR results

3.2.1. Genetic variations and relatedness among grapevine accessions based on ISSR

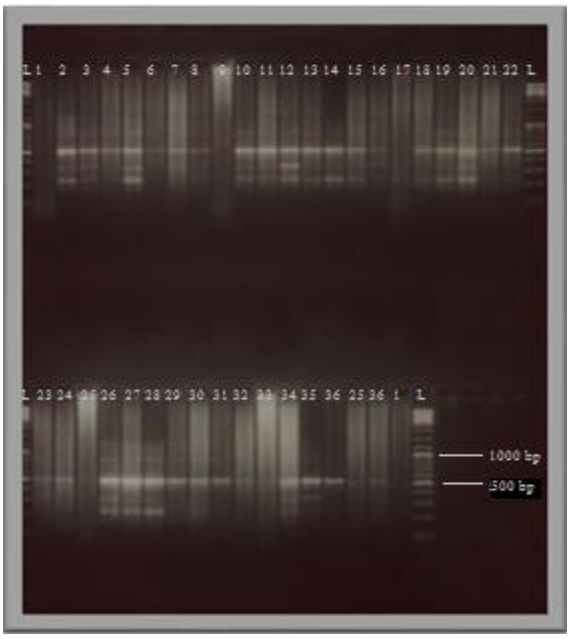
Among the 20 tested primers used to investigate the pattern of genetic variation between 36 accessions of grape grown at the southern region of West-Bank, Palestine; 17 primers produced reasonable amplification products with high intensity and pattern stability (Table 6), whereas, only 2 primers (S-27 and #841) exhibited ambiguous, light, and non-clear complex amplification products and primer #840 produce no amplification, and therefore were excluded from our analysis.

A total of 57 DNA fragments (loci) separated by electrophoresis on agarose gels, were detected (Table 5), ranging in size from 150 to 900 bp (Figure 4). Of these fragments, 55 (88%) were polymerphic and 2 (3.5%) were monomorphic. Our results also revealed an average of 3.1 loci per primer (Tables 5). A minimum of 1 and a maximum of 10 DNA fragments were obtained using (S-17, #820 and #841) and (S-31) primers, respectively (Table 6). Therefore, the later primer (S-31) is considered as the most powerful primer among the tested once (Figure 4). The maximum percentage of polymorphic markers was 100% with 17 primers (Table 5), however, the minimum percentage was 80% with only one primer (S-31).

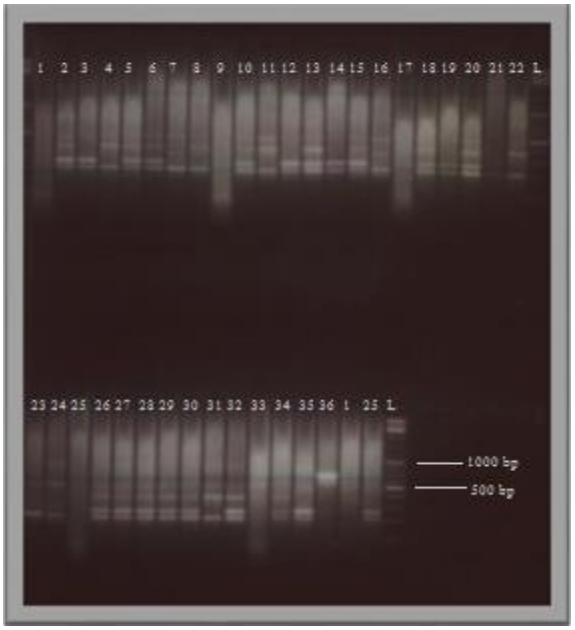
Table 6: Analysis of the polymorphism obtained with ISSR markers

		Approximate band size (bp).					
primers name	**Total No. ISSR bands**	**Min**	**Max**	**Mono-morphic band**	**Poly-morphic bands**	**Poly-morphic (%)**	**Primer case**
# 9	6	200	800	0	6	100%	Included
S-13	2	300	400	0	2	100%	Included
# 4	5	150	400	0	5	100%	Included
S-17	1	400	400	0	1	100%	Included
S-19	3	250	600	0	3	100%	Included
S-14	3	250	500	0	3	100%	Included
S-16	5	150	400	0	5	100%	Included
S-27	4	250	450	4	0	0%	Excluded
S-31	10	180	800	2	8	80%	Included
# 818	5	250	700	0	5	100%	Included
# 820	1	300	300	0	1	100%	Included
# 825	2	300	400	0	2	100%	Included
# 826	2	400	450	0	2	100%	Included
#836	2	600	700	0	2	100%	Included
S-30	2	300	400	0	2	100%	Included
#841	1	350	350	1	0	0%	Excluded
#890	2	300	400	0	2	100%	Included

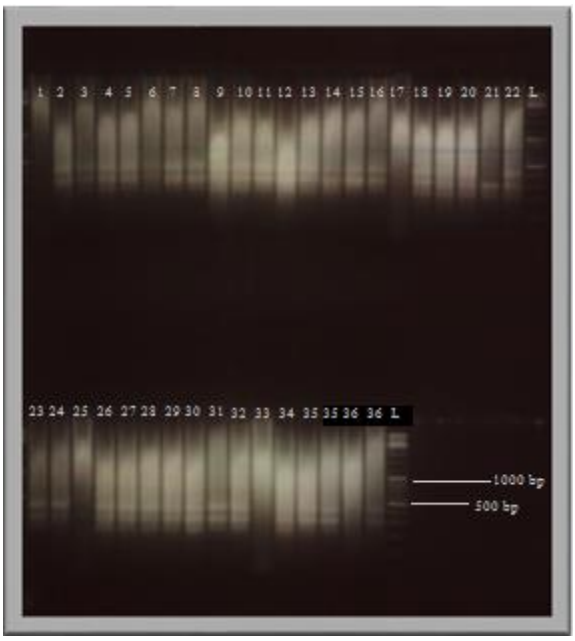
UBC-855	1	300	300	0	1	100%	Included
# 811	5	280	900	0	5	100%	Included
Total	**62**			**7**	**55**	**88%**	



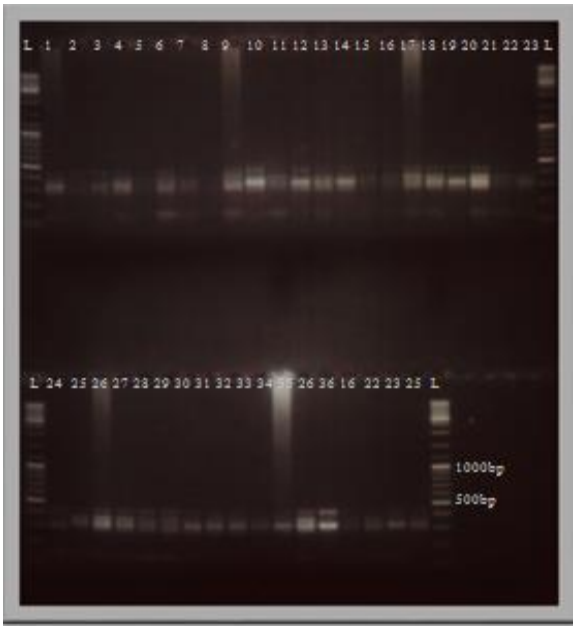
S-14



#9



S-13



S-16

Figure 4: Examples of ISSR banding patterns generated in Palestinian Grapevine cultivars using S-14, #9, S-13, and S-16 primers (100 bp ladder).

3.2.2. Genetic distances

The data matrix size analyzed included 2232 entries, 1288 of which were for present loci (1) and 944 for absent loci (0). Accordingly, the Jaccard coefficient was calculated and presented in table 7. The genetic distance matrix showed an average distance range from 0.05 to 0.76 with a mean of 0.405. The maximum genetic distance value of 0.76 (24% similarity) was exhibited between (Shami and Marawi.Hamadani.Adi) as well as (Bairuti and Marawi.Hamadani.Adi) genotypes, whereas the lowest genetic distance of 0.05 (95% similarity) was exhibited between (Jandali.Tawel.Mofarad and Jandali.Kurawi.Mlzlz) along with (Shami.Aswad and Shami.mtartash.mlwn) genotypes.

3.2.3. UPGMA analysis

UPGMA dendrogram clustered the grape genotypes into eight major clusters in addition to an isolated genotype Marawi.Hamadani.Adi as a distinctive one (Figure 5). Cluster "1", consists of only two genotypes: Marawi.Habe.Tawela and Jandali. Cluster "2" contains Darawishi and Shukhi genotypes which both are related to Mtartash. Cluster "3" is further divided into two highly related small groups (Jandali.Kurawi.Mlzlz, Jandali.Tawel.Mafrod, and Zaini.Baladi.Dabuki.Baladi) and (Sulti.Khdari, Zaini, Zaini.Habe.Tawela, Hamadani, Aswad.Baladi, Baluti.Abiad and Marawi.Shabh.Bairuti), in which all also are connected into Mtartash.Fhaisi cultivar. Cluster "4" composed of (Baluti and Dabuki) related to Halawani.

Cluster "5" is sub-divided into small groups including ("Bairuti and Shami" related to Betuni), (highly related Shami.Aswad and Shami.Mtartash.Mlwn), (Miskat.El.Eskandaria and Halawani.Baladi.Valantiki), (Romi.Aswad and Hamadani.Ma´tar.Faranci), and another two related individual genotypes Malikt.Libnan and Fhaisi.

Cluster "6" consists of Jandali.Shabh.Dabuki and Halawani.Habe.Kabera. Cluster “7” composed of only one genotype Romi.Aswad.Habe.Tawela. Finally, cluster "8" consists of Hamadani.Kadem and Dabuki.Aswad.Baladi.

Table 7: Jaccard´s distance index generated for the 36 local Palestinian grapevines' ISSR data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.Mar.Hamadani.Adi	0.00																																			
2.Shami.Mtr.Mwn	0.67	0.00																																		
3.Halawani.Bald.Valntiki	0.64	0.16	0.00																																	
4.Hamadani.Matr.Faranci	0.58	0.20	0.21	0.00																																
5.Shami.Aswad	0.68	0.05	0.16	0.15	0.00																															
6.Romi.Aswad	0.62	0.30	0.31	0.17	0.30	0.00																														
7.Shukhi	0.68	0.27	0.24	0.23	0.28	0.21	0.00																													
8.Malkt.Libnan	0.73	0.20	0.26	0.24	0.21	0.26	0.20	0.00																												
9.Jandali	0.51	0.49	0.43	0.32	0.46	0.38	0.44	0.54	0.00																											
10.Miskat.Eskandaria	0.62	0.20	0.11	0.15	0.16	0.26	0.20	0.29	0.38	0.00																										
11.Mtartash.Fhaisi	0.64	0.32	0.33	0.24	0.29	0.37	0.35	0.29	0.49	0.29	0.00																									
12.Halawani	0.64	0.28	0.29	0.32	0.29	0.37	0.27	0.33	0.49	0.24	0.28	0.00																								
13.Zaini	0.65	0.37	0.38	0.26	0.34	0.31	0.33	0.34	0.45	0.30	0.22	0.30	0.00																							
14.Betuni	0.68	0.31	0.33	0.27	0.28	0.33	0.30	0.28	0.48	0.28	0.31	0.23	0.18	0.00																						
15.Shami	0.76	0.29	0.34	0.33	0.26	0.38	0.35	0.21	0.59	0.33	0.33	0.33	0.23	0.16	0.00																					
16.Bairuti	0.76	0.33	0.38	0.40	0.33	0.41	0.32	0.21	0.59	0.37	0.33	0.36	0.31	0.24	0.14	0.00																				
17.Dabuki	0.59	0.33	0.30	0.24	0.29	0.34	0.32	0.37	0.42	0.21	0.29	0.20	0.23	0.24	0.33	0.40	0.00																			
18.Baluti	0.60	0.36	0.33	0.32	0.33	0.33	0.39	0.40	0.32	0.28	0.43	0.28	0.30	0.23	0.36	0.40	0.19	0.00																		
19.Salti.Khdari	0.63	0.29	0.26	0.21	0.26	0.27	0.28	0.33	0.41	0.17	0.29	0.25	0.16	0.24	0.26	0.37	0.17	0.24	0.00																	
20.Fhaisi	0.64	0.33	0.22	0.25	0.30	0.27	0.24	0.26	0.39	0.21	0.29	0.29	0.31	0.29	0.30	0.30	0.30	0.29	0.26	0.00																
21.Hamadani	0.63	0.36	0.30	0.21	0.33	0.27	0.24	0.26	0.41	0.26	0.25	0.33	0.20	0.28	0.26	0.30	0.26	0.33	0.14	0.22	0.00															
22.Darawishi	0.71	0.38	0.36	0.31	0.39	0.29	0.14	0.28	0.48	0.32	0.38	0.35	0.29	0.30	0.32	0.28	0.36	0.42	0.32	0.29	0.20	0.00														
23.Mtartash	0.65	0.47	0.42	0.33	0.45	0.31	0.25	0.38	0.43	0.38	0.40	0.44	0.35	0.36	0.38	0.34	0.41	0.41	0.34	0.31	0.23	0.21	0.00													
24.Marawi.Haba.Twela	0.53	0.47	0.37	0.31	0.44	0.33	0.34	0.40	0.31	0.32	0.43	0.43	0.40	0.42	0.50	0.50	0.36	0.35	0.32	0.37	0.28	0.38	0.37	0.00												
25.Zaini.Bal.Dabk.Bal	0.63	0.30	0.31	0.22	0.27	0.24	0.26	0.30	0.42	0.23	0.26	0.30	0.24	0.26	0.31	0.34	0.27	0.30	0.20	0.31	0.16	0.33	0.31	0.33	0.00											
26.Jandali.Twel.Mafrod	0.65	0.38	0.40	0.31	0.36	0.33	0.30	0.39	0.36	0.32	0.35	0.35	0.33	0.34	0.39	0.39	0.36	0.31	0.28	0.36	0.24	0.38	0.33	0.38	0.09	0.00										
27.Jandali.Kurawi.Mizlz	0.65	0.38	0.40	0.31	0.36	0.33	0.34	0.43	0.36	0.32	0.35	0.31	0.29	0.30	0.35	0.39	0.36	0.31	0.24	0.33	0.24	0.38	0.33	0.42	0.14	0.05	0.00									
28.Aswad.Baladi	0.63	0.40	0.38	0.25	0.37	0.30	0.35	0.37	0.41	0.33	0.33	0.33	0.20	0.28	0.30	0.37	0.30	0.33	0.22	0.30	0.14	0.28	0.34	0.36	0.20	0.24	0.20	0.00								
29.Zaini.Haba.Twela	0.63	0.40	0.38	0.25	0.37	0.30	0.32	0.33	0.38	0.33	0.29	0.33	0.23	0.32	0.33	0.37	0.30	0.33	0.22	0.30	0.10	0.28	0.30	0.32	0.16	0.16	0.16	0.10	0.00							
30.Marawi.Shabh.Beruti	0.65	0.33	0.35	0.22	0.34	0.24	0.26	0.27	0.45	0.30	0.33	0.33	0.21	0.26	0.23	0.27	0.30	0.37	0.20	0.27	0.11	0.18	0.24	0.36	0.24	0.33	0.29	0.16	0.20	0.00						
31.Baluti.Abiad	0.67	0.27	0.32	0.23	0.27	0.24	0.33	0.27	0.47	0.35	0.34	0.34	0.29	0.30	0.28	0.31	0.31	0.34	0.24	0.32	0.20	0.30	0.32	0.41	0.25	0.33	0.30	0.20	0.20	0.13	0.00					
32.Dabuki.aswad.baladi	0.66	0.61	0.53	0.54	0.59	0.51	0.56	0.59	0.50	0.51	0.67	0.58	0.57	0.53	0.60	0.64	0.51	0.42	0.50	0.53	0.50	0.56	0.59	0.37	0.53	0.56	0.56	0.50	0.50	0.53	0.55	0.00				
33.Halawani.H.Kabera	0.71	0.36	0.34	0.36	0.33	0.38	0.39	0.44	0.46	0.33	0.40	0.40	0.41	0.39	0.44	0.40	0.41	0.37	0.37	0.38	0.40	0.43	0.38	0.48	0.27	0.28	0.28	0.40	0.40	0.41	0.31	0.59	0.00			
34.Jandali.Shabh.Dabuki	0.70	0.42	0.37	0.39	0.40	0.40	0.38	0.40	0.45	0.36	0.42	0.42	0.40	0.41	0.36	0.32	0.40	0.39	0.36	0.28	0.32	0.38	0.40	0.43	0.36	0.34	0.30	0.32	0.32	0.36	0.33	0.58	0.36	0.00		
35.Hamadani.Kadem	0.67	0.59	0.50	0.43	0.56	0.53	0.50	0.60	0.47	0.44	0.59	0.62	0.55	0.60	0.64	0.67	0.53	0.61	0.48	0.54	0.48	0.50	0.53	0.48	0.55	0.57	0.57	0.51	0.51	0.51	0.56	0.48	0.56	0.59	0.00	
36.Romi.Aswad.H.Twela	0.67	0.51	0.46	0.37	0.49	0.42	0.43	0.52	0.47	0.41	0.48	0.44	0.41	0.47	0.51	0.54	0.41	0.45	0.37	0.43	0.33	0.36	0.42	0.36	0.38	0.40	0.36	0.25	0.29	0.38	0.35	0.48	0.38	0.40	0.45	0.00

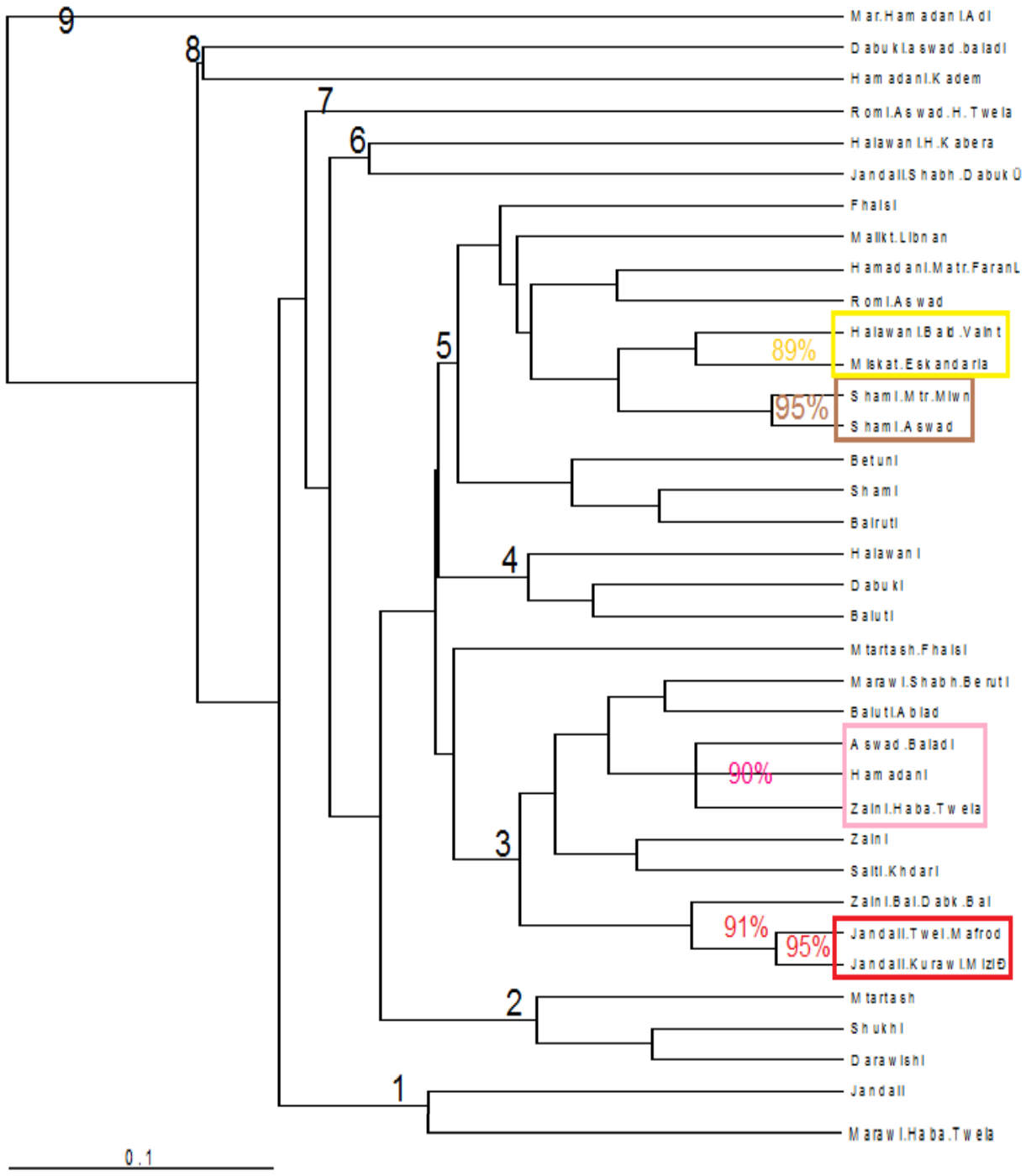


Figure 5: Dendrogram of 36 local Palestinian Grapes constructed by UPGMA based on ISSR banding patterns

3.3. Combinations of RAPD and ISSR results

3.2.2. Genetic distances based on combinations between both markers (RAPD+ISSR)

Jaccard´s coefficient was calculated and presented in table 8. The genetic distance matrix showed an average distance range from 0.06 to 0.54 with a mean of 0.57. The maximum genetic distance value of 0.54 (46% similarity) was exhibited between Romi.Aswad.Habe.Tawela and Marawi.Hamadani.Adi genotypes, whereas the lowest genetic distance of 0.06 (94% similarity) was exhibited between Jandali.Tawel.Mofarad and Jandali.Kurawi.Mlzlz genotypes.

3.2.2. UPGMA analysis based on combinations between both markers (RAPD+ISSR)

UPGMA dendrogram clustered the grape genotypes into two major clusters (Figure 6). The cluster I consists of 18 genotypes which are sub-divided into four main sub-clusters namely Ia, Ib, Ic and Id. The sub-cluster (Ia) included (Hamadani.Kadem and Dabuki.Aswad.Baladi).

The sub-cluster (Ib) (Jandali.Shabh.Dabuki and Halawani.Habe.Kabera). Sub-cluster (Ic) is composed of 13 genotypes as the following: (Mtartash and Darawishi), (highly related Jandali.Kurawi.Mlzlz and Jandali.Twel.Mafrod, in which both associated with Zaini.Baladi.Dabuki.Baladi), (Zaini.Habe.Tawela and Aswad.Baladi, in which both are related, Marawi.Shabh.Bairuti as well as Baluti.Abiad), and (Hamadani, Sulti.Khdari, Fhaisi, and Marawi.Habe.Tawela). The sub-cluster (Id) contains only Romi.Aswad.Habe.Tawela genotype.

The cluster II also composed of 18 genotypes which sub-divided into IIa, IIb, in addition to an isolated sub-cluster IIc (Marawi.Hamadani.Adi). Sub-cluster IIa consists of 8 genotypes accordingly to their relationship as the following: (Shami and Betuni related to Bairuti), (Dabuki and Zaini), (Halawani and Baluti), and Mtartash.Fhaisi.

Sub-cluster IIb composed of (Malikat.Libnan related to Shukhi), (highly related Shami.aswad and Shami.Mtrtsh.Mlwn), and (Hamadani.Ma´tr.Faranci), (Romi.Aswad), (Miskat.El.Eskandaria), (Halawani.Baladi.Valantiki), and Jandali genotypes.

Table 8: Jaccard´s distance index generated for the 36 local Palestinian grapevines "RAPD+ ISSR" data combination

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1.Mar.Hamadani.Adi	0.00																																			
2.Shami.Mtr.Mwn	0.31	0.00																																		
3.Halawani.Bald.Valntiki	0.39	0.26	0.00																																	
4.Hamadani.Matr.Faranci	0.26	0.16	0.20	0.00																																
5.Shami.Aswad	0.32	0.07	0.23	0.12	0.00																															
6.Romi.Aswad	0.35	0.25	0.29	0.20	0.20	0.00																														
7.Shukhi	0.45	0.30	0.30	0.26	0.27	0.23	0.00																													
8.Malkt.Libnan	0.46	0.28	0.30	0.27	0.26	0.26	0.18	0.00																												
9.Jandali	0.34	0.36	0.31	0.27	0.31	0.28	0.33	0.34	0.00																											
10.Miskat.Eskandaria	0.41	0.28	0.26	0.22	0.24	0.26	0.27	0.25	0.26	0.00																										
11.Mtartash.Fhaisi	0.41	0.26	0.35	0.28	0.27	0.31	0.33	0.30	0.39	0.31	0.00																									
12.Halawani	0.41	0.31	0.29	0.29	0.30	0.29	0.25	0.29	0.33	0.24	0.28	0.00																								
13.Zaini	0.37	0.29	0.33	0.24	0.29	0.27	0.31	0.31	0.35	0.28	0.26	0.26	0.00																							
14.Betuni	0.36	0.25	0.31	0.23	0.24	0.30	0.29	0.29	0.34	0.27	0.27	0.22	0.19	0.00																						
15.Shami	0.40	0.20	0.31	0.25	0.21	0.29	0.32	0.29	0.38	0.33	0.28	0.29	0.23	0.15	0.00																					
16.Bairuti	0.43	0.27	0.29	0.26	0.25	0.32	0.31	0.28	0.40	0.30	0.30	0.30	0.29	0.21	0.18	0.00																				
17.Dabuki	0.34	0.28	0.29	0.23	0.28	0.28	0.33	0.34	0.36	0.27	0.30	0.25	0.20	0.24	0.25	0.28	0.00																			
18.Baluti	0.35	0.29	0.33	0.27	0.29	0.28	0.32	0.31	0.30	0.26	0.35	0.22	0.28	0.17	0.24	0.28	0.20	0.00																		
19.Salti.Khdari	0.46	0.36	0.38	0.32	0.35	0.37	0.34	0.36	0.45	0.34	0.36	0.35	0.29	0.33	0.35	0.39	0.30	0.35	0.00																	
20.Fhaisi	0.45	0.33	0.37	0.33	0.32	0.36	0.34	0.32	0.43	0.35	0.31	0.37	0.35	0.34	0.33	0.34	0.34	0.34	0.21	0.00																
21.Hamadani	0.44	0.38	0.42	0.32	0.38	0.39	0.36	0.35	0.45	0.37	0.37	0.39	0.34	0.33	0.35	0.35	0.34	0.35	0.17	0.21	0.00															
22.Darawishi	0.50	0.38	0.39	0.34	0.37	0.35	0.29	0.35	0.42	0.39	0.40	0.36	0.37	0.37	0.38	0.34	0.39	0.40	0.27	0.26	0.25	0.00														
23.Mtartash	0.44	0.39	0.41	0.34	0.36	0.34	0.36	0.40	0.41	0.39	0.40	0.38	0.36	0.32	0.36	0.35	0.39	0.38	0.29	0.26	0.25	0.16	0.00													
24.Marawi.Haba.Twela	0.32	0.33	0.38	0.26	0.32	0.35	0.37	0.38	0.37	0.35	0.41	0.40	0.35	0.35	0.38	0.38	0.34	0.35	0.25	0.25	0.20	0.28	0.25	0.00												
25.Zaini.Bal.Dabk.Bal	0.49	0.38	0.39	0.33	0.37	0.37	0.36	0.35	0.44	0.33	0.34	0.37	0.34	0.33	0.39	0.36	0.36	0.36	0.19	0.27	0.20	0.26	0.27	0.25	0.00											
26.Jandali.Twel.Mafrod	0.49	0.41	0.42	0.35	0.38	0.39	0.37	0.36	0.36	0.35	0.37	0.38	0.40	0.36	0.42	0.37	0.41	0.36	0.26	0.26	0.23	0.29	0.27	0.27	0.16	0.00										
27.Jandali.Kurawi.Mzlz	0.47	0.40	0.40	0.34	0.37	0.37	0.38	0.38	0.38	0.36	0.36	0.37	0.39	0.35	0.42	0.38	0.41	0.36	0.25	0.26	0.23	0.28	0.26	0.27	0.16	0.06	0.00									
28.Aswad.Baladi	0.46	0.39	0.42	0.31	0.38	0.37	0.37	0.36	0.41	0.35	0.36	0.35	0.32	0.32	0.38	0.38	0.35	0.34	0.19	0.27	0.19	0.21	0.28	0.26	0.16	0.22	0.18	0.00								
29.Zaini.Haba.Twela	0.47	0.39	0.39	0.32	0.38	0.35	0.36	0.36	0.44	0.37	0.35	0.35	0.30	0.34	0.37	0.38	0.32	0.37	0.20	0.29	0.23	0.26	0.28	0.28	0.16	0.24	0.20	0.11	0.00							
30.Marawi.Shabh.Beruti	0.47	0.40	0.43	0.33	0.41	0.37	0.38	0.37	0.47	0.39	0.41	0.38	0.34	0.34	0.36	0.36	0.35	0.38	0.26	0.31	0.18	0.25	0.29	0.29	0.26	0.30	0.25	0.18	0.16	0.00						
31.Baluti.Abiad	0.48	0.35	0.39	0.33	0.35	0.35	0.38	0.32	0.47	0.38	0.38	0.39	0.34	0.34	0.35	0.36	0.34	0.37	0.19	0.27	0.23	0.28	0.30	0.30	0.20	0.28	0.24	0.17	0.13	0.16	0.00					
32.Dabuki.aswad.baladi	0.46	0.45	0.44	0.39	0.44	0.44	0.46	0.45	0.45	0.42	0.48	0.45	0.41	0.41	0.46	0.46	0.42	0.40	0.34	0.35	0.35	0.37	0.38	0.28	0.34	0.37	0.33	0.28	0.27	0.31	0.30	0.00				
33.Halawani.H.Kabera	0.49	0.38	0.36	0.35	0.36	0.39	0.41	0.42	0.46	0.40	0.40	0.42	0.42	0.39	0.42	0.37	0.41	0.41	0.32	0.33	0.31	0.31	0.30	0.33	0.24	0.27	0.24	0.29	0.28	0.29	0.26	0.34	0.00			
34.Jandali.Shabh.Dabuki	0.53	0.44	0.41	0.40	0.42	0.44	0.40	0.41	0.43	0.42	0.44	0.45	0.45	0.42	0.42	0.36	0.44	0.42	0.33	0.31	0.27	0.30	0.34	0.33	0.31	0.24	0.20	0.27	0.28	0.26	0.27	0.37	0.26	0.00		
35.Hamadani.Kadem	0.45	0.45	0.47	0.40	0.45	0.45	0.44	0.46	0.46	0.43	0.48	0.47	0.45	0.44	0.48	0.48	0.42	0.42	0.33	0.36	0.26	0.33	0.36	0.30	0.34	0.35	0.31	0.27	0.31	0.23	0.31	0.27	0.32	0.31	0.00	
36.Romi.Aswad.H.Twela	0.54	0.47	0.44	0.43	0.46	0.45	0.46	0.47	0.50	0.45	0.45	0.46	0.46	0.45	0.47	0.44	0.43	0.44	0.36	0.38	0.35	0.34	0.38	0.37	0.34	0.36	0.33	0.29	0.30	0.34	0.30	0.39	0.29	0.32	0.34	0.00

Figure 6: Dendrogram of 36 local Palestinian Grapes constructed by UPGMA based on RAPD+ISSR banding patterns combination

Chapter Four
Discussion

Chapter Four: Discussion

Classical ampelography and other morphometric methods are useful for cultivar identification but are not enough dependable for grape genotypes identification (Galet, 1979, Swanepoel and De Villiers, 1987), since they are affected by environment. In fact, the same cultivar may have different names and varied cultivars may have the same name.

During the last decades, DNA molecular markers have been used intensively to characterize a wide range of plant species (Herrera, *et al.*, 2002), including grapevines across the world, however almost nothing were done in Palestine.

In the present study, the level of polymorphism among 36 grapevine (*Vitis venifera* L.) accessions commonly grown in Palestine were estimated using two PCR-based marker techniques including RAPDs and ISSRs.

4.1. Genetic variations and relatedness among grapevine accessions based on RAPDs

The presence of the different patterns generated by RAPD primers shows variance between the grapevine accessions from the genetic point of view using twenty-five primers, which selected from the literature based on their high ability to produce polymorphic RAPD markers. Similar primer number even less were used by Karatas and Agaoglu, (2010). However, 4 primers (OPN-11, OPN-20, OPG-17, OPN-13) were excluded from the analysis since they produce weak, unclear, and unreadable patterns, in addition, to their low polymorphisms (Less than 50%). Other researchers reported that some primers seem to be more efficient than others in producing stable and reproducible DNA fingerprints (This, *et al.*, 2004).

Primer selection is essential for discrimination analysis. Obviously, the more bands scored and plants studied, the higher the statistical significance of the calculation will be. About 100 bands should be enough to obtain statistically significant results (Kocsis, *et al.*, 2005). In our study, among 162 bands obtained from 21 primers; 116 were polymorphic which presents high level of DNA polymorphisms (68.1%). In fact, the polymorphism presented at our study is comparably similar even higher to those exhibited by Karatas and Agaoglu, (2010) who obtained 112 polymorphic bands with 65.49%. Continuously, maximum number of polymorphic band obtained in our study is 15 comparing to only 7 polymorphic band created by the same authors. This implies high efficiency of our examined primers.

The values of the genetic distances ranged from 0.07 for the most closely related cultivars (Jandali.Tawel.Mafrod and Jandali.Kurawi.Mlzlz) to 0.50 for the most distantly related cultivars (Romi.Aswad.Habe.Tawela and Jandali). Therefore, we might assumed that (Jandali.Tawel-Mafrod and Jandali.Kurawi.Mlzlz) are identical accessions with different names. The same trend goes also with (Marawi.Shabh.Bairuti and Hamadani.Kadem), (Zaini.Habe.Tawela and Baluti.Abiad) and (Shami.Aswad and Shami.Mtartash.Mlwn) by 0.08 distance. Our assumption is in agreement with Francesca and others (2010) who reported that the genetic distance between the identical accessions was equal to 0. For the remaining genotypes, the genetic distance matrix showed a high level of divergence at the DNA level. Several authors also mentioned the existence of homonyms and synonyms in grapevine varieties that have been cultivated for centuries (Borrego, *et al.*, 2002). Actually, this confusion is mostly based on environmental and agronomical characteristics. In fact, ecological similarities and parallelism within the population composed of varieties adapted to a certain geographical region, is an important factor, which should be taken into consideration (Ulanovsky, *et al.*, 2002, Agaoglu, *et al.*, 2006).

On the other hand, different authors agree with the existing difficulties to detect intra-varietal polymorphism in grapevines (Gogorcena, *et al.*, 1993, Sefc *et al.*, 2000). According to Ulanovsky and others (2002), the genetic intra-varietal variability has been attributed into two main factors: (a) a probable polyclonal origin of the varieties and (b) an accumulation of somatic mutations over the centuries increases variability.

The size of amplified fragments varied from 150 bp to 1400 bp. This interval was narrower than the results obtained by Pollefeys and Bousquet (2003) and Dalbo and others (2000), who reported fragments between (400 bp and 2000 bp) and (200 bp and 2500 bp), for different grape cultivars in America and Canada, respectively. Indeed, the narrower amplified fragments exhibited by our cultivars might related to the smaller and restricted cultivated areas (Hebron and Bethlehem regions, about 2000 Km^2) in which our grapevines are grown comparing with the very large studied areas of America and Canada.

The average of 7.7 amplicons (loci) per primer presented in our study (Table 4) was sufficient to produce useful fingerprints for many fruit crop genotypes such as (Khadari, *et al.*, 1995, Galderisi, *et al.*, 1999 and Basheer-Salimia, *et al.*, 2012). Therefore, we may confidently assume that the RAPD technique can solve one of the major problems associated with varietal identification in Palestinian grapes.

Based on the average genetic relatedness among the 36 examined Palestinian grape assumed genotypes, RAPD UPGMA dendrogram analysis divided the cultivars into two major clusters in which each cluster is made up of eighteen cultivars. The most closed cultivars were Jandali.Kurawi.Mlzlz and Jandali.Tawel.Mfrod (93%), Hamadani.Kadem and Marawi.Shabh.Bairuti (92%), Baluti.Abiad and Zaini.Habe.Tawela (92%), and Shami.Aswad and Shami,Mtartash.mlwn (92%), however, the most distant once were Romi.Rswad.Habe.Tawela and Jandali cultivars (50%). Interestingly, Romi.Aswad.Habe.Tawela as well as Mtartash.Fhaisi cultivars respectively were separated and identified as distant genotypes.

These results might emerges that our region has a very rich and varied clonal grape genetic structure.

4.2. Genetic variations and relatedness among grapevine accessions based on ISSR

In this study we evaluate 20 ISSR primers (dinucleotide repeats, commonly used for grape identification) to identify and discriminate the Palestinian grapes (Wang, *et al.*, 1994, Moreno, *et al.*, 1998). Many authors stated that, few ISSR primers (5 primers) were needed to generate diagnostic and reproducible fingerprint profiles and therefore distinguishing between the varieties (Moreno, *et al.*, 1998).

Of these primers screened initially on 36 grapevine cultivars, 18 primers yielded an altered interval of polymorphism from 1 to 8. However, the other two primers (S-27 and #841) were excluded from the analysis since they produced no polymorphism. Reddy, *et al.*, (2002) and Herrera, *et al.*, (2002) reported that some primers were more efficient in recognizing a complementary site in the plant genome. Comparing with Herrera and others (2002), our results revealed higher polymorphic bands (55 bands) using 18 primers relating to 40 bands using 11 primers (which are parallel to our examined primers). Interestingly, primer #820 that showed only one band in Merlot cultivar cultivated in Chile also revealed the same number of bands (1 band) with our Bairuti genotype so these genotypes maybe related, however, further investigation is needed to support this assumption.

Among the evaluated 20 primers; 6 were GA repeats (the maximum), 5 were AC repeats, 4 were AG repeats, 3 were GT repeats, and 2 were CA repeats. Remarkably, 3 motifs (AG) of nucleotide repeats were realized, with 100% polymorphism (Table 6).

Consequently, the primers revealed significant differences in resolving polymorphism, in which the primers containing (GT)n repeats and (CA)n repeats were the most polymorphic. The same is true for the average number of bands per primer.

Thomas and others, (1993) found the repeats GA and GT as the most highly represented in the Vitis genome. Further, Dhanorkar and others, (2005) recorded the repeats AC as more common among ISSR primers selected by using Indian grapes in a more recent study.

Contradictory to the fact that AT motifs in the plant kingdom have generally been approved as the most plentiful repeat (Casasoli, *et al.*, 2001), here, in grapes our results revealed 4 AG motifs among the 20 tested primers which confirm the results of Dhanorkar, *et al.*, (2005) who also registered 6 AG motifs in Indian grapes. Consequently, the relative abundance of nucleotide repeats in the grapevine genome indicates differences between different studies conducted on *Vitis* species.

The size of amplified fragments varied from 150 bp to 900 bp. This interval was narrower than the results obtained by Dhanorkar, and others, (2005) and Sabir and others, (2009), who reported fragments between (300 bp and 1500 bp) and (300 bp and 2500 bp), for different grape cultivars in India and Turkey, respectively. Indeed, the narrower amplified fragments exhibited by our cultivars might related to the smaller and restricted cultivated areas (Hebron and Bethlehem regions, about 2000 Km^2) in which our grapevines are grown comparing with the very large studied areas of India and Turkey.

The percentage of polymorphism revealed in the present analysis is much higher than those reported by Moreno, *et al.*, (1998), Dhanorkar, *et al.*, (2005), Sabir, *et al.*, (2009). This could be attributed to either the pre-selection of our primers for their abilities to generate clear and polymorphic-band-patterns and/or the intra-varitial differences as it is presented in our study rather than the inter-varietal differences among the examined cultivars.

The values of the genetic distances ranged from 0.05 for the most closely related cultivars (Jandali.Tawel.Mofarad and Jandali.Kurawi.Mlzlz) and (Shami.Mtaratsh.Mlwn and Shami.Aswad) to 0.76 for the most distantly related cultivars (Marawi.Hamadani.Adi and Shami) and (Marawi.Hamadani.Adi and Bairuti). These results confirm that these cultivars could be the same cultivar but with different names (synonyms). Vignani, (1996) set a precedent that closely related individuals could be considered under a common name.

UPGMA dendrogram (Figure 5) clustered the grape genotypes into eight major clusters including 2, 3, 11, 3, 11, 2, 1 and 2 genotypes, respectively. In addition to that, cluster 9 (Marawi.Hamadani.Adi) was isolated as a distinctive genotype. Interestingly, some examined cultivars showed high similarities with each other such as (Jandali.Tawel.mafrod and Jandali.Kurawi.Mlzlz, by 95%; Shami.Aswad and Shami.Mtartash.Mlwn, by also 95%), which might assumed that these cultivars are highly correlated and therefore might be the same cultivars with different names. The most distant cultivars were between (Marawi.Hamadani.Adi and Shami, by 24%) and (Marawi.Hamadini.Adi and Bairuti, by 24%). Therefore, these cultivars could be useful and interesting grape genotypes for any future breeding program in Palestine. In addition, since ISSR represents an efficient tool for estimating the genetic variability and the genetic relationships among our examined grapevine genotypes therefore, ISSR markers could be a useful technique for grapevine genotype identification's and characterizations.

4.3. Genetic variations and relatedness among grapevine accessions based on combinations between both markers (RAPD+ISSR)

Evaluation of phylogenetic relationships is an essential element of plant characterization and conservation, needed in order to establish effective breeding programs. RAPD and ISSR-PCR have been used for the fast and large-scale identification of grapevine varieties and clones (Pollefeys and Bousquet, 2003, Jaladet, *et al.*, 2008, Sabir, *et al.*, 2009, Karatas and Agaoglu, 2010). Both protocols require a preliminary screening of a modest number of primers in order to define which are the most appropriate for any application. Here, combinations of RAPD and ISSR markers has been performed to evaluate the genetic relationships among the Palestinian grapevine cultivars.

The dendrograms constructed by the two separated approaches showed general similarities among the examined grapevine cultivars as the following: (Shami.Mtartash.Mlwn and Shami.Aswad), (Jandali.Tawel.Mafrod and Jandali.Kurawi.Mlzlz), (Baluti.Abiad and Zaini.Haba.Tawela), (Shami and Betuni) and (Darawishi and Mtartash). Interestingly, combinations of both markers (RAPD and ISSR) are also revealed the same similarities and relatedness. These results means that each pair is probably the same cultivar with two different names (synonym). Furthermore, the distant cultivars exhibited by the combinations including Romi.Aswad.Habe.Tawela, Marawi.Hamadani.Adi and Dabuki.Aswad.Baladi, would be an interesting cultivars that could support grapevine-breeding programs in Palestine.

Since the combinations of RAPD and ISSRs almost presented similar results to each individual marker with minor differences, therefore we confidently assume that each marker could be sufficient for identification and discrimination between the Palestinian grapevine cultivars. This of course decreases the costs, time and efforts of cultivar selection.

Chapter Five Conclusion and Recommendations

Chapter Five: Conclusions and Recommendations

1. Molecular approaches including RAPD and ISSR are quick, reliable, efficient and useful tools for grapevine identification, and they are an essential preliminary step toward any breeding programs.

2. OPE-17, OPD-14, OPG-6 and OPG-11 were efficient and powerful RAPD primers for grapevine discrimination, whereas, #9, #4, S-16, S-31, #818, and #811 primers were the most powerful and highly effective ISSR primers.

3. Many cultivars were presented with different names such as (Shami.Mtartash.Mlwn and Shami.Aswad), (Jandali.Tawel.Mafrod and Jandali.Kurawi.Mlzlz), (Baluti.Abiad and Zaini.Habe.Tawela), (Shami and Betuni) and (Darawishi and Mtartash), however, each pair is genetically one cultivar but with different names.

4. Several cultivars were commonly named as the same name; however, our analysis showed that these cultivars are genetically different such as Jandali, Dabuki, Marawi and Romi with their synonyms.

5. There were several distinctive cultivars such as Romi.Aswad.Habe.Tawela, Marawi.Hamadani.Adi and Dabuki.Aswad.Baladi that might be used as promising cultivars toward future breeding programs in Palestine.

6. Since the combinations of RAPD and ISSRs almost presented similar results to each individual marker, therefore each marker could be sufficient for identification and discrimination between the Palestinian grapevine cultivars.

7. Our region has a very rich and varied clonal grape genetic structure.

8. Urgent action by the Ministry of Agriculture and the National Agricultural Research Center must be taken toward conservation the existing and threating grapevine cultivars.

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Abstract in Arabic

الملخص

التنوع الوراثي لأصناف العنب الفلسطينية باستخدام بادئات تعتمد على"DNA"

إن تحديد عدد أصناف العنب الفلسطينية لا تزال غير معروفة وعليه فإن وضع قاعدة بيانات حقيقية تهدف الى تحديد التباينات الوراثية بين هذه الأصناف باستخدام البصمة الوراثية يعتبر أولوية ملحة. تهدف هذه الدراسة الى تحديد عدد أصناف العنب الفلسطينية المختلفة جينيا وتقييم التنوع الوراثي وتحديد التباينات الوراثية بينها باستخدام بادئات RAPD وISSR.

شملت الدراسة على 36 صنف عنب تم جمعها من مناطق مختلفة من محافظتي بيت لحم والخليل. حيث تم استخلاص الحمض النووي الرايبوزي منقوص الاوكسجين (DNA) لهذه الأصناف من أوراق العنب الطازجة باستخدام DNeasy كيت (QIAGEN-Company) . هذا وتم استخدام 25 بادئة RAPD و20 بادئة ISSR. من هذه البادئات، 21 بادئة RAPD وبادئة 18 ISSR، أنتجوا حزما (Bands) واضحة وغنية بالمعلومات، وبالتالي تم استخدامها في هذه الدراسة، في حين تم استبعاد البادئات المتبقية والتي أنتجت نطاقات غير واضحة في التحليل.

أظهرت نتائج ال RAPD، 186 حزمة منها 124 حزمة متعددة الأشكال، حيث بلغ متوسط تعدد الاشكال 68.1 ٪، في حين أعطت نتائج ال ISSR، 57 حزمة منها 55 حزمة متعددة الأشكال مع متوسط تعدد الأشكال 88٪. وقد انتج البادئ OPG 13- (من مجموعة RAPD) والبادئ S- 31 (من مجموعة ISSR) الحد الأقصى لعدد الحزم و بالتالي فهي تعتبر البادئات الأقوى.

بالنسبة لمستوى القرابة الوراثية بين أصناف العنب ، فقد أظهرت نتائج بادئات ال RAPD أن درجة التقارب بين الأصناف تراوحت من0.07 (93 % تشابه) بين (جندلي.طويل.مفرود و جندلي. كروي.ملزلز) الـــى 0.50 (95% تشابه) بين (رومي اسود حبة طويلة و صنف جندلي). أما فيما يتعلق بنتائج بادئات ال ISSR ، فقد تراوحت درجة التقارب بين الأصناف بين 0.05 بين (جندلي. طويل.مفرود و جندلي كروي ملزلز) الـــى 0.76 بين (صنف الشامي و صنف مراوي.حمداني.عادي).

وقد أظهرت نتائج تحديد درجة القرابة الوراثية باستخدام التحليل العنقودي (UPGMA cluster) لكل من بادئات الـRAPD وبادئات ال ISSR و كذلك اتحاد بادئات RAPD+ISSR ، أظهرت درجة تقارب وراثية عالية بين العديد من أصناف العنب التي شملتها الدراسة مثل (شامي.اسود و شامي.مطرطش.ملون), (جندلي.كروي.ملزلز و جندلي.طويل مفرود), (زيني.حبة.طويلة و بلوطي ابيض), (شامي و بيتوني) و (دراويشي و مطرطش). وعليه فإننا نتوقع أن كل زوج من هذه الأصناف من الناحية الوراثية هو صنف واحد ولكن بأسماء مختلفة. من ناحية أخرى، فان هناك أصناف متعددة تعرف محلياً بنفس الاسـم، ولكن أظهرت النتائج أن هذه الأصناف تختلف وراثيا مثل أصناف الجندلي، والدابوقي، والمراوي والرومي مع مرادفاتها.

أيضاً بينت النتائج أن هناك عدة أصناف مميزة جينيا مثل صنف الرومي.اسود.حبة.طويلة ، مراوي.حمداني.عادي و دابوقي.اسود.بلدي والتي يمكن ان تكون أصناف واعدة نحو برامج التكاثر المستقبلية للعنب في فلسطين .

استنادا إلى المترادفات والمتشابهات من أسماء أصناف العنب التي تم الحصول عليها فقد تم تخفيض العدد الإجمالي للأصناف المدروسة الى 20 صنفا مختلفا وراثياً بدلاً من 36 صنفا.