Deanship of Graduate Studies

Al-Quds University

Molecular Characterization of Palestinian Local Zucchini (*Cucurbita pepo* L.)

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M.Sc. Thesis

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Molecular Characterization of Palestinian Local Zucchini

(*Cucurbita pepo* L.)

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Dedication

To whom was the best help for me on this journey, to the permanent bond and shoulder, to who planted the seed of love of learning and knowledge in us, to my dear father Osama.

To the one who fights the universe in our path, to who does not forget me in her praying to my dear mom Khawla

To my husband Mohammed, who has been a constant source of support and encouragement during the challenges of graduate college and life.

To my child Laith, you are my inspiration to achieve greatness, you have made me stonger, better and more fulfilled than I could have ever imagined.

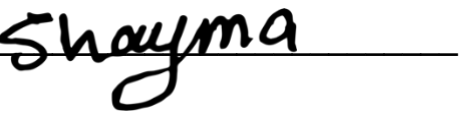
To those who are a piece of the heart, my brothers zeidan, Luay, Mustafa and Omar.

To all my supportive friends during this journey

Shayma' Al-Jubeh

Declaration

I certify that the thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not be submitted for a higher degree to any other university or institution.

Signed: 

Shayma' Osama Al-Jubeh

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Shayma' Aljuba May, 2023

Abstract

Cucurbita pepo L. is one of the most economically important crops in the Cucurbitaceae, and it gains this value from the culinary uses of immature fruit that's referred as "summer squash". In Palestine, the local zucchini which is one of the varieties of summer squash is very popular for the consumers and there is a high market demand on it. Palestinian zucchini is widely grown in the areas of Hebron, Bethlehem, and Ramallah and it is characterized by long growing season, and its leaves are dark green, in addition to its green fruits with a strong aroma, flavor and taste.

This study was performed to assess the genetic diversity of the local Palestinian zucchini using SSR markers. Thirty three zucchini samples were collected in which twenty-six of them were local accessions obtained from the Union of Agricultural Work Committees (UAWC). The other seven accessions were commercial accessions gathered from a local agricultural equipment store. In addition to the zucchini samples, six accessions from other species of the Cucurbitacaea family were studied, including gourd, snake melon, and muskmelon. All of the samples were grown in an open field in order to make note of some morphological traits and to make visual documentation.

The accessions were assessed using 50 SSR markers; only nine primers were used in the analysis as they have produced polymorphic bands. Out of the nine, primer UAWC 39 produced a unique band for the gourd accessions while producing no bands for the muskmelon or snake melon accessions. Therefore, this primer can be a potential primer to differentiate between zucchini and gourd. On the other hand, primer UAWC 13 produced a unique band for the muskmelon and snake melon accessions, while gourd and zucchini accessions produced identical bands. Accordingly, this primer can be used to differentiate zucchini and gourd accessions from snake melon and muskmelon accessions.

The nine SSR primers used in the analyses produced total 27 polymorphic SSR loci.

According to these results, the genetic distance matrix was calculated according to the Jacquard's similarity coefficient and the distance between the squash accessions ranged between (0.00 – 0.68). The lowest genetic distance was observed between two commercial accessions; therefore it was assumed that these two accessions are identical with different commercial names.

Furthermore, a neighbor joining tree was constructed according to the genetic distance matrix that was calculated using Jacquard similarity coefficient formula. NJ tree clustered the accessions to four major groups and it has shown how the accessions of gourd, muskmelon, and snake melon are away from squash accessions. In addition to that, it has also shown how there is a genetic variation among the local accessions of zucchini. This genetic diversity among local zucchini accessions is a source of genetic material that can be used for classical breeding or new biotechnological techniques to produce new varieties that are better suited to the changing environment if the need arises. Therefore we recommend that these local accessions continue to be maintained, are further characterized, and are made available to scientists and farmers for crop improvement.

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AFLPs: Amplified Fragment Length Polymorphism.

ARIJ: Applied Research Institute –Jerusalem.

DNA: Deoxyribonucleic acid.

ESTs: Expressed Sequence Tags.

FAMD: Fingerprint Analysis with Missing Data.

HA: Hectare.

ICARDA: International Center for Agricultural Research in the Dry Area.

ISSR: Inter Simple Sequence Repeat.

Kg: Kilo gram.

µL: Microliter.

mL: Milliliter.

mm: millimeter.

MOA: Ministry of Agriculture.

NARC: National Center for Agricultural Research.

NJ: Neighbour Joining.

PAGE: Polyacrylamide gel electrophoresis.

PARC: Palestinian Agricultural Relief Committees.

PCO: principal coordinate analysis.

PCR: Polymerase chain reaction.

RAPD: Random Amplified Polymorphic.

DNA RFLP: Restriction fragment length polymorphisms.

RH: Relative humidity.

SCAR: Sequence characterized amplified region.

SNP: Single nucleotide polymorphisms.

SRAP: Sequence-related amplified polymorphism.

SSR: Simple sequence repeats.

Subsp: Subspecies.

TAE: Tris / acetate/ EDTA.

UAWC: Union of Agricultural Work Committees.

UPGMA: Unweighted pair group method with arithmetic mean.

USA: United States of America.

UV: Ultraviolet.

Chapter One: Introduction

1.1 General introduction

Cucurbita pepo L. is a species with variable fruit features (Duchesne, 1786). This species is a member of the Cucurbitaceae family, which includes 825 species and 118 genera and is a significant plant family that provides edible products and useful fibers to humans (Bisognin, 2002).

Like other Cucurbits, *Cucurbita pepo* has 20 pairs of chromosomes (Mercado & Lira, 1994). Around the turn of the 16th century, transoceanic travelers helped this species spread from its native Central America and Mexico to other continents (Whitaker, 1947).

Cucurbita pepo is considered one of the most economically important species of the Cucurbitaceae, and it gains this importance from the culinary uses of the immature fruit that is referred to as “summer squash" (Formisano et al., 2011). In 2017, the area harvested with pumpkin, squash, and gourd around the world was 2,078,450 ha with an annual production of 27,449,481 tones (http://www.fao.org/faostat/en).

In Palestine, local zucchini, which is one of the varieties of summer squash, is very popular among consumers and there is a high demand for it in the markets. Local zucchini is considered one of the main local summer crops produced by rain-fed agriculture that does not require fertilizers or chemical pesticides, and only needs fertile land rich in organic matter, making it fully suitable for the Palestinian climate and does not require high production costs.

Palestinian local zucchini is widely grown in Hebron, Bethlehem, and Ramallah. It is characterized by its long growing season, and its dark green leaves, in addition to its green fruits with a strong aroma, flavor, and taste (UAWC, 2018). According to Palestinian agricultural statistics in 2017, the total area planted with zucchini was 23,762 dunum with 60,858 tons produced annually (MOA, 2017).

Figure 1.1: The fruit of Palestinian local zucchini

Zucchini is a tap rooted plant, and the stem is hairy, rough, and hollow. The elliptical dark green leaves are heart shaped and may consist of 3 lobes. The leaf surface is hairy with stiff bristles, while the edges of the leaves are irregularly wavy. The yellow flowers are independent of each other on these monoecious plants, and the fruit is green, regularly striped, and the base of the fruit is usually wider than the top.

Figure 1.2: A Palestinian zucchini plant

Palestinian farmers prefer to grow zucchini in one go due to the spread of many viruses that inflict heavy losses on zucchini harvests. Also, there is another phenomenon in zucchini that limits production and reduces the quality of the fruits, which is the phenomenon of bleaching (the appearance of white spots on the leaves). This phenomenon is directly related to the fall of the fruits, and the green color of the fruits turns to pale yellow. Farmers try to overcome the problem of viruses and bleaching by growing zucchini under white gauze (Harb & Iseed, 2021).

The seed bank in the Union of Agricultural Work Committees works to cultivate and propagate local zucchini in addition to many other local crops with the goal of producing and improving the local seeds and supplying them to farmers (UAWC,2018). Because they are the source of local plant genes, which are crucial for maintaining the potential of local plants, local seeds are a significant national treasure that must be protected. Utilizing these genes has the potential to significantly contribute to the management of agricultural pests and the reduction of pesticide use (Shtayeh, 2005).

In order to expand a crop's genetic basis, recognize cultivars, and select parental varieties for breeding programs, it is crucial to have a better understanding of the molecular diversity of the crop (Ferriol, et al,; 2001). In order to establish a sound breeding strategy for the production and identification of superior cultivars for use in nurseries, it is important to consider the genetic variation among nutritionally valuable food accessions varying in nutritional traits, which can often correlate to enhancing human health (Meru et al., 2019)

This study will identify genetic diversity of Palestinian local zucchini. This purpose will be accomplished with the use of molecular genetic markers, which have been shown to be more efficient in most circumstances due to their ability to overcome the limits of older approaches. These markers are utilized extensively for cultivar genotyping and provide a vital tool for precise genotyping (Azofeifa, 2006).

1.2 Study objective

This study was done to identify the genetic diversity in the Palestinian local zucchini using SSR molecular marker.

Chapter Two: Literature Review

2.1 Cucurbitaceae Family

All members of the Cucurbitaceae family are collectively referred to as "cucurbits" and they are among the most significant vegetable crops grown worldwide. The majority of these species are grassy, tendril-bearing, and sensitive to freeze so they are planted in temperate and tropical environments annually. Squash and pumpkin (*Cucurbita pepo* L.) are the most common cucurbit crops, along with melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus* L.) (Weng & Sun, 2012).

Cucurbits are a major part of the daily diet of humans since they are low in fat and there is a high amount of water in the fruit, making them a favorite choice among those who are interested in proper nutrition.

Peeled Cucurbita seeds are referred to as oily seeds since they have 50% oil and 35% protein. Although most species of oil are unsaturated and usually edible, a few species have a combination of trienoic fatty acids that makes them inedible but improves their commercial usefulness as drying oils. On the other hand, Cucurbit seed proteins appear to be edible and their nutritional value is raised by the addition of certain amino acids (Mansour et al. 1993).For example, watermelon seeds are a common snack because they are low in moisture and rich in carbs, lipids, and protein. On 140,000 hectares, China produces more than 200,000 tons of developed watermelon varieties with large seeds per year (Zhang 1996).

2.2 *Cucurbita pepo* L

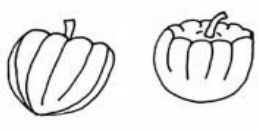
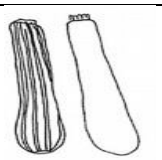
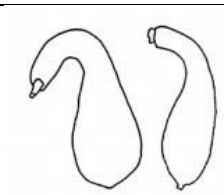
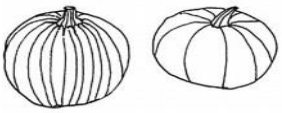
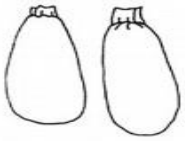
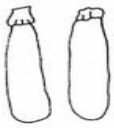
The most genetic diversity within the Cucurbita genus is found in *Cucurbita pepo* , according to recent research utilizing SSR markers to examine changes within and across species (Gong et al. 2013). Taking into consideration the morphological characteristics of the fruits, the shape varies from spherical to very long or flat, and their weight can reach 20 kg or more. They can also have longitudinal ribs, wrinkles or ridges. The color of the fruit varies widely as well, they can be green, orange, or yellow, and their shades range from dark to almost white and from bright to pale. Their patterns also vary widely, including longitudinal lines, mottling, and multicolor patterns. As a result, a single fruit may have different colors on its surface. Taking into consideration the interior part of the fruit, the color of the interior differ from light green to bright yellow or orange (Paris et al. 2012) and seeds are between 8.8 to 23.3 mm long, and the width varies from 5.0 to 12.5 mm(Paris & Nerson, 2003), but still beige in color.

Figure 2.3: *Cucurbita pepo* mature fruits that show a wide range of variation in fruit size, shape, and color. (Paris et al. 2012)

Genetic links between cultivars of a crop species are of great importance in order to have a scientifically accepted infraspecific grouping of cultivars. However, the degree to which these classifications are valuable relies on how quickly farmers, distributors, merchants, consumers, and breeders can all recognize them. Additionally, groups should have a global reach rather than be completely based on the market types prevalent in a single country or area (Paris, 2008).

Paris (1986) divided edible-fruited C. pepo into eight cultivar groups, including the Zucchini Group, the Cocozelle Group, the Vegetable Marrow Group, the Straightneck Group,the Crookneck Group, and Scallop Group, based mostly on fruit shape. Additionally, the Pumpkin Group and the Acorn Group are two cultivar groupings of C. pepo that are often produced for their mature fruits (Table 1). The Warted Gourd Group, the Oviform, Smooth-Rinded Gourd Group and the Round, Smooth-Rinded Gourd Group, are the three types that are not produced for food but rather for decoration. There are now thought to be 11 cultivar groupings in the *Cucurbita pepo* genus (Paris, 2000).

Table 2.1: Infraspecific classification of cultivated *Cucurbita pepo* (Paris, 2015)

Cultivar-group	Fruit shape	
Acorn	Turbinate (topped), wrinkled, wide at the peduncle end, curved at the stylar end.	
Cocozelle	Long to very long with cylindrical shape	
Crookneck	Elongated fruit, with a thin curved neck	
Pumpkin	Round: globular, oval, short oblong	
Scallop	Flattened, with scalloped margins	
Straightneck	Cylindrical in shape, having a constriction or short neck towards the stem end	
Vegetable Marrow	Short, tapering cylindrical, thin at the peduncle end, broad at the stylar end,	
Zucchini	Uniformly cylindrical	

Isozyme polymorphism studies indicated a basic difference among *Cucurbita pepo* that goes beyond cultivar groupings (Decker,1985; Andres,1987). This division is botanically expressed by three subspecific categorizations:

1. *Subsp. texana:* includes Oviform, SmoothRinded Gourd Groups, Acorn, Scallop, Straightneck and Crookneck
2. *Subsp. pepo*: includes Round, Smooth-Rinded Gourd, and Warted Gourd Groups, Pumpkin, Zucchini, Vegetable Marrow, and Cocozelle
3. *Subsp. fraterna* : Although wild plants have been spotted in northeastern Mexico (Andres, 1987), no cultivars that are closely related to them have been found (Paris et al., 2003)

2.2.1 Origin and Domestication:

Zucchini which is one variety of summer squash is a cultivated plant of *Cucurbita pepo* L. species, which in the genus *Cucurbita*, that belong to Cucurbitaceae family, the subfamily *Cucurbitoideae* and the tribe *Cucurbiteae* (Jeffrey, 1990).

Archeological, linguistic, and historical sources agree with the idea that *Cucurbita* L. genus is native to Mexico and America and were spread in the 16th century to other continents by transoceanic passengers (Whitaker, 1947).

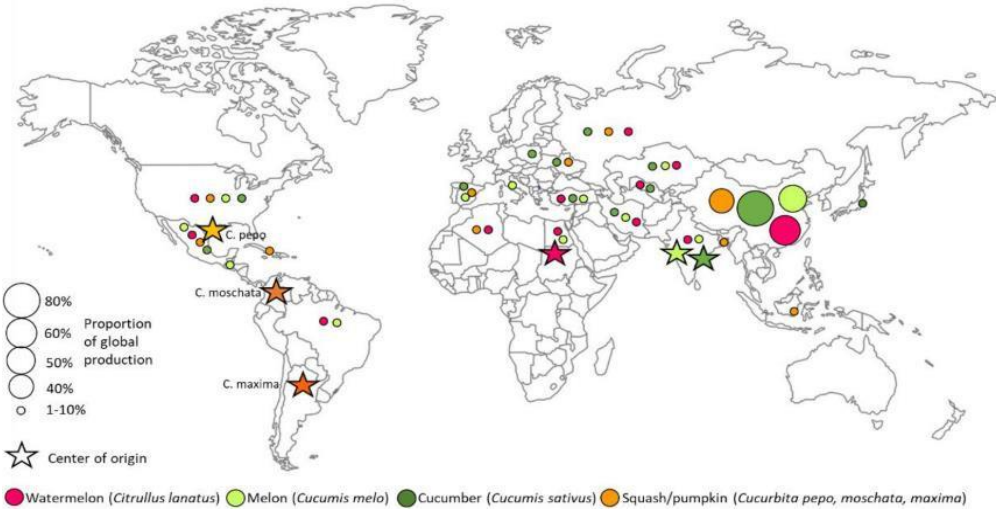


Figure 2.4: Origins of domestication and regions of production and of major cucurbit crops. (Grumet et al., 2021)

Southern Mexico's archaeological evidence of *Cucurbita pepo* domestication goes back 10,000 years (Smith, 1997). Domesticates were discovered more than 4,000 years ago in northern Mexico and the United States. Domestication in Mexico and U.S is considered to have occurred at least twice (Decker, 1988).

The origin of the word "Squash" is " Asq" that is a Native American word which mean the immature fruit, and the plural form is asquash (Trumbull, 1876). Therefor, "Squash" is applied on the fruit of Cucurbita that is non round, long, edible when immature (Paris, 2016).

2.2.2 Botanical description

Zucchini are the edible immature fruits of *Cucurbita pepo* L., and it's a short-season crop with a high value (Paris, 1996). Zucchini grows in a temperate environment where the temperatures range from 24°C to 29°C within the day, and nighttime temperatures range from 16°C to 24°C to give the best development. Although the plant is drought-resistant, it needs an adequate amount of water to sustain optimal development and yield.

Cucurbita pepo L. is an annual plant with creeping stems in some varieties and climbing stems in other varieties that can reach up to 15 m long. This plant has a branched - shallow root growing from strong taproot. The stems are scabrous while the leaves are lobed (Lu & Jeffrey 2011).

Summer squash plants are called monoecious plants, as they have both staminate and pistillate flowers. Perfect environmental conditions lead to synchronizing the anthesis of the two different flowers. Although staminate flowers are differentiated first, pistillate flowers can develop more quickly. The flowers are obvious and large, with an orange-yellow color from the corolla, consisting of 5 or 6 petals fused at the base, while the sepals are short and green. Both flowers bloom in the morning, shrink before midday, and are no longer open again (Paris, 2008).

Sex expression of summer squash flowers is influenced by several factors, one of them is

temperature, in which cool temperatures promote development of pistillate flowers leading to reduce the ratio of male flowers and affecting pollination process. Another important factor is light levels, studies show that female flowers production is enhanced by high light levels and shades can reduce its number. It can also be affected by the nutritional status of the plant, for example, the formation of pistillate blooms may be affected by the presence of an excessive nitrogen in the soil (Loy, 2004; Wien, 1997). Hormones can also affect Sex expression, whether it was produced by the plant or synthetic ones applied to it (Kumar, 2016).

As the male and female flowers are separated, mechanical pollination is essential because the pollen is clustered, sticky, and thick and cannot be distributed by wind. The honeybee is the most common pollinator as it is attracted to the nectar produced (Free, 1970).

2.2.3 Fruit development

After fruit setting, cucurbit fruit grows rapidly for a while until its growth rate slows down. Following pollination, cellular expansion rather than an increase in the cell count is mostly responsible for the growth in fruit size. Depending on whether the fruit was picked while it was young, like summer squash and cucumbers, or when it was ripe, the cucurbits are classified into two primary types. Summer squash may be ready for harvesting by size as soon as 3 days after pollination, depending on the needs of the market (Kumar, 2016).

2.2.4 Harvesting and storage

Summer squash is harvested young, with attractively bright, delicate and delicious fruits 2-5 days beyond anthesis. Fruits that are older are losing their shine gradually, becoming tough and invalid. Therefore, harvest is carried out every 2-3 days, usually for several weeks, during the entire harvest period. Fruits are heavily scraped, bruised, or otherwise injured during harvesting; therefore packaging must be done carefully. Summer squash is especially subject to drainage. Therefore, in the event of inhibiting water loss, 85–95% RH and a

constant circulation of Air should be kept immediately to avoid mold. On another hand, temperature must be kept above 5 °C to avoid chilling injuries (Paris & Tadmor, 2017).

2.2.5 Economic importance

Cucurbita pepo is an economically important crop as it is grown in a large area of the world and it is consumed in a large quantity. In 2017, the area harvested with Pumpkin, squash and gourd around the world was 2078450 ha and it had an annual production of 27449481 tones (http://www.fao.org/faostat/en). Because of their economic importance, people in the Middle East have tended to use greenhouses as well as open-field to grow these cultivars (Rouphael & Colla, 2005).

Summer squash is a plant of economic importance and is grown all over the world for its oil and medical benefits (Fu et al., 2006). The potential of use for different summer squash types have been reported as pharmaceutical effects comprising antidiabetic, antihypertensive, antitumor, antibacterial, immuno modulative, antibacterial, antihypercholesterol, antiparasitary antibody, antalgic and anti-inflammatory effects (Fu et al., 2006; Esuoso et al., 1998). As a source of manganese and vitamin C, it also gives us a great mix of conventional antioxidant nutrients, Studies recently confirmed that antioxidants maintain their function after steaming or freezing in summer squash (Kumar *et al.,* 2016).

Summer squash seeds have been employed to treat various disorders, especially worm diseases (Lewis et al., 1997) as they are employed in Eritrea, Sudan, and Ethiopia to cure tapeworm. Extracts from summer squash seeds, especially in Europe, were used for many years in traditional medicine as a treatment of miction due to benign hyperplasia of the prostate system (Younis et al., 2000).

2.3 Local Seeds in Palestine

Local seeds are an important national wealth that must be preserved, because they are the source of local plant genes, which are essential for maintaining the potential of local plants.

Utilizing these genes has the potential to significantly help manage agricultural pests and reduce the use of pesticides (Shtayeh, 2005).

The use of plant sources, such as local varieties and their wild genes, are considered basic components in the development of appropriate genetic material. They also constitute a safety valve to maintain food security and the sustainability of agriculture and plant production, through their ability to tolerate and continue to produce in the face of changes in climate and biological and non-biological conditions (Hremat 2001).

Palestinian local seeds are the most important agricultural inputs, because of the presence of many species, varieties, and local breeds; this is due to the diversity of land conditions and climate in the region (Alrajabi, 2013). Local seeds enable farmers to collect and store seeds for the next season, unlike commercial seeds that also require the farmer to purchase fertilizers and chemical pesticides necessary for plant growth, which leads to continuous erosion of soil fertility.

Most of the Palestinian local seeds are under a high risk of disappearing. For many years, Israeli and foreign chemical and hybrid seed companies have overflowed the market with hybrid and improved seeds, which caused Palestinian local seeds to disappear. This results in an imbalance in the environment, an increase in costs, and a dependence on Israeli and other foreign seed and chemical companies, ensuring the continuity of control over our food while depriving us of real food security (Kurzm, 2010).

The commercial interest behind flooding the Palestinian market with commercial hybrid seeds and their fertilizers and chemical pesticides directly threatens local seeds. Taking into consideration the fact that local seeds are suitable for our arid or semi-arid climate conditions and do not need much water, unlike hybrid commercial seeds, the Palestinian farmers must return to producing local seeds annually with the aim of reusing them in the next season, especially during the cultivation of grains and vegetables. Considering that until a few years

ago the Palestinian farmer relied on their self-produced local seeds, which are more affordable and disease-resistant due to their characteristics, but now farmers tend to ignore the fact that they use a lot of water, chemical fertilizers and pesticides that are bad for the soil, and need a variety of other services in the belief that commercial improved seeds and seedlings produce more and make farming easier (Kurzm, 2010).

Some of the local agricultural institutions and associations have worked to improve many species and varieties, especially rain-fed ones. One of these institutions is the Union of Agricultural Work Committees (UAWC), which established a local seed bank in 2010 as an extension of the Union's work in improving and reproducing local seeds. This step came to protect the local varieties from extinction or loss as a national and heritage goal, in addition to the elucidating the importance of saving and documenting these seeds, reviving the local seeds and transferring traditional knowledge about them, and benefiting from the characteristics of these local seeds.

Over the past ten years, the Union of Agricultural Work Committees has worked to develop and improve the local seed bank. As a result, it has succeeded in collecting and preserving more than 50 local varieties, which are included in more than 550 accessions from 14 plant families. They also worked on preserving local vegetable varieties in their early stages, as more than 20 new varieties were preserved, all of which are medicinal and rare plant varieties, such as black carrots, white cucumbers, yellow cauliflower, local tomatoes, local lettuce, and several aromatic and medicinal varieties.

The process of preserving the seeds takes place under organized scientific management. Several models have been developed since the bank's inception to organize its work and focus on tracking any sample that enters the bank until it reaches the storage unit. In addition to collecting results found in the laboratory and drying unit through clear and sequential steps until the sample reaches the storage unit with high quality in terms of a high purity rate

and a tested moisture percentage. Focus was placed on purity along several axes, including purifying and separating varieties based on the morphological description and geographical consumer demand.

The work of the seed bank did not stop at preserving local seeds, but rather went beyond this goal to reproducing these seeds in safe ways using innovative and modern techniques. As the units have been greatly developed to move from the regular production stage to the environmentally smart intensive production stage that is compatible with the needs of each variety. Among these techniques are propagation inside plastic houses, using pollinators, cultivation in cages, cultivation on roofs, and the establishment of permanent propagation units for medicinal plants. In addition, one of the most important objectives of the seed bank is to conduct descriptive studies of local plants at the morphological and molecular levels.

In addition to the Union of Agricultural Work Committees, other institutions work to preserve local seeds (alhamad,2018), such as:

The Palestinian Agricultural Relief Committees (PARC): These committees work on the issue of producing local seeds, as they trained a large number of farmers to choose desirable seeds and inform them how to store these seeds, and train a technical cadre of agricultural engineers to identify and collect local seeds in local agricultural areas. It also established a central bank for local seeds in 2011 as well as small local banks in several farmer communities, where more than 150 varieties of local crop plants that are suitable and adapted to weather conditions and soil conditions were collected.

The Applied Research Institute Jerusalem (ARIJ): The ARIJ Foundation has worked since its inception to focus on the issue of local seeds. The institute launched a program to collect seeds of local varieties of economic field crops in Palestine in 1996, during which the institution collected 14 samples of local varieties such as wheat, lentils, chickpeas, and barley.

The Ministry of Agriculture (MoA): The Ministry of Agriculture is the main incubator for all institutions working in the agricultural sector. It has placed a high value on preserving biodiversity in general and local seeds in particular through its branches, which are primarily represented in the National Center for Agricultural Research (NARC), which is considered one of the Ministry of Agriculture's most important departments that has worked in collaboration with many local and international institutions on the issue of selecting and preserving municipal seeds and distributing them among farmers throughout the country.

The International Center for Agricultural Research in the Dry Areas (ICARDA): The ICARDA Foundation worked on the issue of local seeds in cooperation with the National Center for Agricultural Research, where its expertise was utilized in establishing a unit for preserving genetic assets at the National Center for Agricultural Research, and collecting about 2000 local and wild plants and preserving them in the asset unit, in addition to purifying and reproducing the seeds of field crops such as wheat, barley, chickpeas, and lentils, and the seeds of some local vegetables such as tomatoes, onions, pumpkins, and watermelons.

2.4 Genetic Diversity

A basic source of biodiversity is genetic diversity, which can be identified as any metric quantifying the magnitude of genetic variability within a population. For more than 80 years, evolutionary scientists have focused primarily on the study of genetic variation (Wright 1920; Fisher 1930), which is fundamental to crop improvement. Plant breeders employ a variety of marker methods for estimating genetic diversity and establishing varietal identity (Choudhury, 2007).

Historically, morphological features, especially those agro morphological traits of importance to consumers, have been used to study crop genetic variation. Although expensive technology is not necessary for morphological characterization, extensive land

areas are frequently needed for these studies, raising the expense. Furthermore, such traits are frequently subject to phenotypic plasticity. Later, biochemical methods like isozyme and protein electrophoresis were used to reduce the influence of environmental influences (Hunter and Merkert, 1957). Although this approach is quick since it only needs tiny amounts of biological material, the resolution of variety is constrained because there are only a certain number of enzymes accessible for biochemical analysis, which are the main material in these methods, as it responsible for the separation of proteins into distinct banding patterns. Afterward Since 1990, numerous molecular methods, including RAPD, AFLP, and SSR, have been employed to evaluate genetic diversity. Such molecular markers provide improved sampling of the plant genome while minimizing environmental effect, improving the accuracy of genetic variation assessments. For the evaluation of genetic diversity, more than 30 different types of molecular markers are presently accessible. These markers have been widely employed to assess the genetic variation of agricultural plants and have been crucial in defining the genetic diversity of crops (Mondini et al. 2009).

2.5 Molecular markers

The fact that molecular markers are persistent and detectable in all tissues independent of cell proliferation, differentiation, development, or defensive state gives them significant benefits over conventional morphological characterization. Furthermore, DNA markers are not mistaken for environmental pleiotropic and epigenetic impacts (Kumar, et al., 2009).

Over the past 20 years, a variety of genetic markers have been accessible, each with unique advantages and disadvantages. Within these markers there are different categories, as they can be broadly classified according to their generation of their development as the following:

- The first generation, including Restriction fragment length polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPDs), and their modification.
- The second-generation, including Simple sequence repeats (SSRs), Amplified

Fragment Length Polymorphism (AFLPs), and their modified forms.

- The third generation, including single nucleotide polymorphisms (SNPs), expressed sequence tags (ESTs) and many more.

These markers have another classification according to their method of analysis as the following:

1) Sequence-based marker techniques, such SNPs technique.
2) PCR-based techniques: several methods including SSR, AFLP and RAPD methods fall under this category.
3) Non PCR-based techniques: such as RFLPs that relies on hybridization

A particular molecular marker can be involved in particular study only if at least accomplished few of the desirable properties which can be mentioned as: (Agarwal et al., 2008) (Hatzopoulos et al., 2002)

a) Efficiently and evenly distributed over the genome.
b) Occurs frequently in the genomes.
c) Co-dominant.
d) Polymorphic.
e) A quick, easy, and affordable procedure.
f) No prior knowledge of the sample's genome is necessary.
g) Stable over generations.
h) Small amount of DNA samples requires.

Various DNA molecular markers in the genetics and breeding practices of various agricultural crops have successfully been established and implemented. In this section we'll offer brief details about some of the molecular markers.

2.5.1 Restriction Fragment Length Polymorphism (RFLP)

The most used hybridization-based molecular marker is restriction fragment length polymorphism. The first time RFLP markers were utilized was in 1975 to trace the genetics of an adenovirus serotype with a temperature-sensitive mutation (Grodzicker, et al., 1975). Later, it was accepted for plant genomes after being utilized for human genome mapping (Botstein et al., 1980) (Helentjaris, et al., 1986, Weber and Helentjaris, 1989).

The first step of RFLP technique is isolating a pure DNA, then restriction enzymes are added. These enzymes cut DNA in certain places (so-called sites of recognition), producing many fragments of various lengths. In the past, fragments were separated using polyacrylamide or agarose gel electrophoresis, which produced a sequence of bands with varying lengths that represented the DNA cut fragments. The bands were then transferred to a membrane and observed by hybridization to particular probes (Liu & Cordes, 2004).

The base pair deletions, transpositions, translocations, inversions, and mutations that cause an increase or decrease in recognition sites and result in fragments of different lengths and polymorphism are the major causes of the variability that results in the RFLP pattern. The restriction enzymes won't cut the fragment if the recognition site differs by a single base pair. However, if both bands are visible, it is thought to be heterozygous for the marker and this happens when mutation occurs on one chromosome but not the other (Madhumati et al., 2014). The co-dominant nature of RFLP markers and the absence of requirement for prior sequence knowledge are their main advantages for this technique (Madhumati et al., 2014). However, several limitations have been reported for this technique:

- Low level of polymorphism (Liu & Cordes, 2004).
- Radioactively tagged probes are needed.
- Specialized probe libraries for the species are required.
- It requires a lot of effort, time, and money (Yu, et al., 1993).

- Great amount and good quality of DNA must exist for it (Potter & Jones, 1991) (Roy, et al., 1992).

2.5.2 Randomly Amplified Polymorphic DNA (RAPD)

This method is a PCR-based method that was created by Williams et al. in 1990 (Williams et al, 1990). By utilizing a random, single, and short primer, PCR is used to amplify genomic DNA. Amplification occurs when two hybridization sites are comparable to one another and move in the opposite direction (Nadeem et al., 2018). The length and size of the primer and the target genome determine the amplified fragments produced by PCR, which are then separated on an agarose gel and stained with ethidium bromide. By examining the presence or absence of various bands in the electrophoresis, it is possible to detect polymorphism brought on by rearrangements or mutations, either at or between the primer-binding sites (Jiang, 2013).

This technique has some factors affecting its reproducibility such as: DNA quantity and quality, magnesium chloride concentration, PCR buffer, Taq polymerase and annealing temperature (Wolff, Schoen, & Peters-Van Rijn, 1993). Primers also can play an important role, where the chosen primer should have a minimum GC level of 40% since any primer with a GC content below 40% cannot withstand the high annealing temperature (72 o C) (Williams et al., 1990).

RAPD technique had a high level of polymorphism and it has several features that make it simple and easy to be done such as (Jiang, 2013):

- The method is rapid, easy, and effective.
- Since random primers are widely accessible, no sequence data are required for primer synthesis.
- Due to the use of PCR, only modest quantities of DNA are needed.
- The relevant RAPD products can be cloned, sequenced, and then utilized to create

or modify additional PCR-based markers.

Due to their sensitivity to the reaction conditions, RAPDs' principal issues include limited reproducibility and the requirement for highly standardized experimental techniques. In addition to that, since short random primers are employed, care must be taken to prevent DNA samples from becoming contaminated. Other limitations include the lack of locus specificity of RAPD markers, the inability to interpret band profiles in terms of loci and alleles (marker dominance), and the possibility that similar-sized fragments may not be homologous. (Williams et al., 1990)

2.5.3 Amplified Fragment Length Polymorphism (AFLP)

The limitations of the RAPD and RFLP approaches have been overcome by improving AFLP markers (Vos et al., 1995). AFLP technique combines the flexibility of PCR-based technology with the power of RFLP with, where DNA was digested and PCR is then performed (Lynch & Walsh, 1998). The AFLP markers are economical and pre-sequence information is not needed. Additionally, this procedure may be utilized with both excellent quality and partially damaged DNA; nevertheless, the DNA used in this process should not include any PCR inhibitors or restriction enzymes (Blears et al.,1998).

This technique has three main steps (Vos et al., 1995):

I. DNA restriction and oligonucleotide adapter ligation: The DNA is cut using two restraint enzymes, and the resultant fragments are then joined with oligonucleotides that will act as primer binding sites for PCR amplification.
II. Selective amplification of sets of restriction fragments: The ligated fragments are amplified using a selective primers.
III. Gel analysis of the amplified fragments.

With just little amounts of DNA samples (0.05–0.5 mg) from any origin or complexity, the AFLP technique can detect small sequence variations, and its results are highly reliable and

reproducible. The key feature of AFLP is its ability to detect many polymorphic bands in a single lane. This ability, together with the simultaneous analysis of all the bands on a gel, makes AFLP an incredibly effective method (Blears et al., 1998). However, according to Madhumati et al., (Madhumati et al., 2014) this technique has some limitations such as:

- Since most AFLP loci are dominant, dominant homozygotes and heterozygotes cannot be distinguished from each other, and that in turn lower its accuracy.
- It takes additional steps to achieve the desired result.
- The method requires the use of polyacrylamide gels, which are more expensive and time-consuming than agarose gels.
- It costs extra to buy adapters as well as restriction and ligation enzymes.
- The restriction enzyme needs template DNA free of inhibitory substances to function properly.

2.5.4 Simple Sequence Repeats (SSRs)

SSR markers are multi-allele, co-dominant, highly polymorphic and informative genetic markers, and they are the suitable marker for many applications. According to that, a considerable amount of effort has been invested into developing them in many species during the past ten years, and they have been frequently used marker for plants genotyping (Mason, 2015).

The majority of eukaryotic species' genomes contain tandemly repeating mononucleotide (T), dinucleotide (TA), trinucleotide (GAA), tetranucleotide (CCAT), pentanucleotide (GTTGC), and hexanucleotide (ATTACG) units. These DNA segments are known as simple sequence repeats (SSRs) or microsatellites (Burstin, et al, 2001). These markers are numerous and equally dispersed across the whole genome in all chromosomal regions. They have been identified in non-gene sequences, introns, and gene-coding regions(Liu & Cordes, 2004). The mitochondria (Rajendrakumar et al., 2007)and chloroplast, also contain them

(Provan et al., 2001).

This method is a PCR-based method that uses primers to amplify the desirable microsatellite region. These primers could be either unlabeled primers pairs or one of the pairs could be radiolabeled or fluorolabeled. The performance and effectiveness of genotyping procedures have been greatly enhanced by the use of fluorescent, tagged microsatellite primers and laser detection. However, this choice is more expensive than using unlabeled primers due to the high cost of the fluorescent label (Agarwal et al., 2008).

Using acrylamide or agarose gel electrophoresis, the length polymorphism of the amplified fragment from unlabeled primers is visualized. Due to the presence of varied amounts of repeats in microsatellite regions, these markers are extremely polymorphic. Additionally, mismatches, recombination of double-strand DNA, the transfer of mobile elements (retrotransposons), and slippage of single-strand DNA can all contribute to the occurrence of SSRs (Kalia et al., 2011).

SSR maps are currently available for a wide variety of plant species, including Rose (Yan et al. 2005), cherry (Dirlewanger et al. 2004), cassava (Mba et al. 2001), melon (Ritschel et al. 2004), soybean (Song et al. 2004), kiwi (Fraser et al. 2004), and maize (Sharopova et al. 2002).

2.5.5 Inter Simple Sequence Repeat (ISSR)

The basic idea behind this technology, which was created by Zietkiewicz et al., is the amplification of DNA segments that are situated between two identical but oppositely orientated microsatellite repeat areas, at a distance that allows for amplification (Zietkiewicz et al., 1994). Despite rare reports of co-dominant segregation, ISSRs primarily segregate as dominant markers (Tsumura et al., 1996).

This technique is a PCR based technique that is simple, quick, easy to understand, doesn't require prior knowledge of DNA sequences, doesn't require the use of radioactivity, and

typically exhibits high polymorphism (Madhumati et al., 2014). In a single primer PCR reaction, this method uses microsatellites as primers. These primers are usually long primers (15–30 bases) and can be di-, tri-, tetra-, or penta-nucleotide. The method allows for the efficient utilization of high annealing temperatures, which typically range from 45 to 65 ºC and depend on the GC content of the primer. The final products are typically 200 to 2000 bp long and are visible on a polyacrylamide or agarose gel (Madhumati et al., 2014). On the other hand, ISSRs' principal drawbacks are that they are usually dominant markers.

2.6 Previous studies

Previous studies utilized some molecular markers in the assessment of Cucurbita genetic relatedness.

In order to determine whether the aqueous extracts of the fruits with different phenotypic characteristics have similar hypoglycemic effects, Hernández, M., et al. (2018) conducted a comparative investigation of the genetic diversity of C. ficifolia leaves gathered in some parts of Mexico by AFLP. The bands produced by the nine different primer combinations showed that there is little genetic variability in the C. ficifolia samples from different parts of Mexico. Despite the great degree of similarity discovered, two clades were produced in the dendrogram that was created, and these clades were categorized by fruit features, regardless of where the growing crop was and how it affected blood sugar levels.

Commercial cultivars and Spanish landraces were among the 69 accessions that Ferriol et al., (2003) examined for genetic diversity. These accessions represent the significant type of diversification that has occurred in Europe since this species' introduction from America.With the aid of both SRAP and AFLP markers, the accessions were separated into two subspecies using principal coordinates analysis and cluster analysis using the UPGMA method. However, between the two marker systems, there were differences in the gene diversity and genetic identity scores among morphotypes and subspecies. In comparison to

AFLP markers, the information provided by SRAP markers was more consistent with the morphological variability and the evolutionary history of the morphotypes.

Kaźmińska,K. et al. (2016) used a set of 23 SSR markers obtained from *Cucurbita pepo* and C. moschata to analyze the genetic diversity within a collection of 85 C. maxima accessions.Using neighbor joining analysis, two major clusters were easily distinguished according to their locations, while Principal coordinate analysis grouped the accesions according to cultivar group and region of origin.

Chapter Three: Material and methods

3.1 Plant material

Thirty-three zucchini accessions were used in this study; twenty-six of them were local accessions collected from the Union of Agricultural Work Committees (UAWC), and the other seven zucchini accessions were commercial accessions gathered from a local agricultural equipment store. In addition to the zucchini samples, six cultivars from other species of the Cucurbits family were studied, including gourd, snake melon, and muskmelon (Table 2). The plants were grown in a greenhouse, then their third and fourth leaves were taken for DNA extraction, after that they were transferred to an open field in order to complete their growth and to take morphological data related to fruit size, shape and colors and make visual documentation. When the plants were left in the greenhouse, the fruit development was not complete because bees were required for the pollination process, which did not occur.

Figure 3.5: The plants used in this study grown in a green house

Table 3.2: List of the 39 accessions and their collection sites

Accession number	UB-Code	Location\notes
OTU 1	57	Halhol
OTU 2	172	Halhol
OTU 3	133	Dora
OTU 4	233	Dora
OTU 5	318	Dora
OTU 6	323	Dora
OTU 7	85	Beit Aula
OTU 8	173	Kharas
OTU 9	281	Kharas
OTU 10	198	Al-Aroub
OTU 11	200	Dora
OTU 12	248	Hebron city
OTU 13	254	Jenin
OTU 14	268	Tarqomia
OTU 15	312	Dahria
OTU 16	91	Nablus - 02
OTU 17	94	Jenin - M1
OTU 18	16	Halhol 2008
OTU 19	94	Dora2010
OTU 20	202	Dora2011
OTU 21	398	Aroub
OTU 22	452	Dora
OTU 23	412	Dora
OTU 24	213	Dora
OTU 25	257	Dora
OTU 26	348	Wad fooken
OTU 27	408	Gourd
OTU 28	404	Muskmelon
OTU 29	423	Snake melon
OTU 30	512	Gourd
OTU 31	509	Muskmelon
OTU 32	375	Snake melon
OTU 33	C1	commercial
OTU 34	C2	Commercial
OTU 35	C4	Commercial
OTU 36	C5	Commercial
OTU 37	C6	commercial
OTU 38	C7	Commercial
OTU 39	C8	commercial

3.2 DNA extraction

Each plant's DNA was extracted using the "DNeasy Plant Mini Kit/Qiagen" procedure. Fresh, young plant leaves weighing 100 mg were first crushed using a pestle and mortar before being added to a 1.5 mL Eppendorf tube and combined with 400 µL of AP1 buffer and 4 µL of RNase solution. Then the mixture was vortexed before incubating in the water bath at 65°C. After 10 min, the tube was taking out of the water bath and 130 µL of AP2 buffer were added and mixed with the lysate, then incubated on ice for 5 minutes.

After that, the lysate was centrifuged for 5 min at 20,000 x g and supernatant was transferred to the QIAshredder Mini Spin Column and centrifuged at that same speed for an additional 2 minutes. The flow- through was transferred into a new tube and mixed by pipetting with 1.5 volume of AW1 buffer, then 650 µl of the mixture was transferred into a DNeasy Mini spin column and centrifuged at 6000 x g for 1 min, the flow-through was then discarded and the spin column was placed into a new 2ml collection tube and 500 µL of AW2 buffer was added to it and centrifuged at that same speed for an additional 1 minute. The flow-through was discarded after the centrifuge and another 500 µL of AW2 buffer was added and centrifuged at 20,000 x g for 2 min. The spin column was then carefully removed from the collection tube and transferred to a new 2 ml tube. 100 µL of AE buffer was then added, and the mixture was incubated at room temperature for 5 minutes before being centrifuged at 6000 x g for 1 minute. The final step was then repeated by adding another 100 L of AE buffer, and the mixture was then incubated and centrifuged.

3.2.1 DNA quantification

On 0.8% agarose gel electrophoresis, the quantity and quality of DNA were evaluated.

Then DNA purity and concentration was determined using Nano drop. DNA was modified to have a final concentration of 30ng/µl.

3.3 SSR (Simple Sequence Repeats) primer sequence design

Fifty SSR primers of C.pepo (table 3) were used according to Gong et al., (2008) that were designed in their study with the following parameters:

- GC content between 20% and 80%, with 50% being ideal.
- An annealing temperature of 60C^{o} is ideal.
- The size of PCR products ranges between 80–300 bp.
- The optimal length of the primer is 20 nucleotides.
- A minimum of five tri- or seven di-nucleotide repetitions.

Table 3.3: The sequences of fifty SSR primers used to detect genetic variation among zucchini accessions.

	F.Primer	R.Primer
UAWC 1	AGGAGTGGTGGGCTAATACG	TGAAATTGAGGGAGGGAGAG
UAWC 2	GGTGACGGCAAGAAAAGCTA	GCTGACCCTCTCTCCCTCTC
UAWC 3	GCAGAGGAGAAGTGGGTTTG	CTTTATCCGACCAAGCGTTC
UAWC 4	AAGGGTTCGTCGGAGAAACT	CAACGACTCCCAGCACCTAT
UAWC 5	AACAATCCTCGCCTCAAATC	ATGAAAATGGGAAGCCAGAG
UAWC 6	TTGAACATGATTCTCCACAA	TTTTTGGGTAAGCACAAAGT
UAWC 7	CTTCGTCGACACCAATTCC	GAAGACGAAGATGACGTGGA
UAWC 8	GCCGGAACCAGACTTCTC	CCCTCCCTTCCCATTAAAC
UAWC 9	ACCCTAAGCCAGATATTCATGC	GGGCTTCATGCATCTAGTTTG
UAWC 10	CACCGACGACTCCATCATC	CTTCTTGTCCCCAAAATCACA
UAWC 11	CCCCTAATCGCTCTCTCTCG	CTCCAATGGGAATGGCAAC
UAWC 12	GATCGAATTGGGGCTTCTT	CCTCCGTACATCCCCAGA
UAWC 13	TCAACCAAGTGCCAATCTCA	ACTGATCCACCGACTGATACG
UAWC 14	CACGAAGATTTGATGGCCTTA	GGATTGGGATGGTGAAGATG
UAWC 15	GGGCTACTTCCCCTAAAGATG	ACGATGCGTATTCACACTCAA
UAWC 16	ACGACATGAGGGAAGATTCG	TTCAATGCCATTCGCCTAC
UAWC 17	ATTCCGACTTGGGGATACCT	TGTCCTATCGCTTCGGAGAT
UAWC 18	ATTCATGTCATACTACCGACTTC	CGGATTTATAAGATGGCAAG
UAWC 19	ATACTTGCTCCCCAAGTTTA	AAATAAAAAGACAACGTAATGGT
UAWC 20	AGAAGGAGGAGAGCGGAAAG	ATAAGATCCCAACCCCAACC
UAWC 21	AATGCGTTTGAACAAAGCTG	GGCTGTTAAAATTTCCTCGATT
UAWC 22	CCGTAGAGATGTCAGAGACAAGG	AGGGATGCTCATCACACCTC
UAWC 23	TTTATTCCGTCGTCGCTTAC	CCCGCTGTTTTTCACTTG
UAWC 24	TCTGGTCTTGGGGTGGTTCT	AGAAACAAAGTGGCGGGTGT
UAWC 25	GGGTGTGTTGAGGATTGGTT	ATCACATTTTCTCCCCACCA

UAWC 26	TCACTTTACAACCAGAAGCTGA	CACTTTGCTGCTCATCCAC
UAWC 27	AGAGCTGCGGGAAAAGGT	TTCTCTCCCATTCTGCCACT
UAWC 28	ACCTCCTTACTACCAAGCTCCT	TTGTCACATCCCTGAGTTTG
UAWC 29	CCACACACCAATCGTTGAAG	CGCAGAATCTCGAAACACAA
UAWC 30	AAACGGTTGAGGAGGATGAC	AGGTCATTTGGCGTGATAACT
UAWC 31	TAGGCTGGATTTGCTGGTGA	TCCATGTCATCCCAGTTGCT
UAWC 32	GCATTTCACTCCCGATTCTC	GTCTCGGAAATCCAATCCAC
UAWC 33	GCACTTGAATCTTCGTCAAC	CGAGAAAGAATTAACGAGCA
UAWC 34	GTGCCCGTCAGTCGGAAT	TGTCGACGAAGATAGCAATAGCA
UAWC 35	CGTTGGCCATGGAAGTCT	CCCTATATGAGACCGCGAGA
UAWC 36	ACCTACCGTCACACCCACAT	CCACCTGAAAACAGGGCTAA
UAWC 37	CATGAAACGATATGAGTAGG	TGAGGTATTTTAGGAATAAAGA
UAWC 38	CCCTCATTAGGGAGTCTACTG	ACTATGCCAGCCGTTATTC
UAWC 39	GGCGAAAAGGAAGAACGAAT	TTTTTCTCCCCCTTCCACAT
UAWC 40	CACGAAGATTTGATGGCCTTA	GGATTGGGATGGTGAAGATG
UAWC 41	TCGGAGAAACTCGACACTCC	TCCCAGCACCATCAGGATAC
UAWC 42	AAAGGTTTCCACATCCTTG	GAAAAGGAAAAAGTGTTCAAAG
UAWC 43	CCCTAGACCCATCATAGTCG	ACATTTGGTTACTTCCCCATT
UAWC 44	GAAGTGCCGTGGCTGAGAG	TTCAGCTTCCCGGATTATGG
UAWC 45	ACTGCTCAATAAGGCAAGGA	AAACAAGAGTGCACAAACAGG
UAWC 46	CACGACTCCCAGAATCAATC	GATTGGAAACTCGCTCCATA
UAWC 47	GTGGAAGTTACTGCGATTGG	GCAAAGAATGTCCTCAGCAG
UAWC 48	AGAGCTGCGGGAAAAGGT	TTCTCTCCCATTCTGCCACT
UAWC 49	TGCTCTCTTTGAATTCAGCAT	AGGGATCTTCCATTTCCAAT
UAWC 50	AAACATGGCGATGTCCCATTA	GGGAGACGCCATTGAAGAAG

3.4 SSR (Simple Sequence Repeats) PCR reaction mixture and program

PCR reactions were carried using GoTaq® Green Master Mix in 25µl volumes containing: 12.5µl Green master mix, 1.5 µl (30 ng) of templet DNA, 1.0 µl Reverse primer (0.4µM), 1.0 µl Forward primer (0.4µM), and 9.0 µl of Nuclease-Free Water. PCR was performed as follows: denaturation at 95°C for 2 min, followed by seven cycles of 45 s at 94°C, 45 s at 68°C (with each cycle the annealing temp decreases 2°C), and of 60 s at 72°C. Products were subsequently amplified for 30 cycles at 94°C for 45 s, 54°C for 45 s, and 72°C for 60 s, with a final extension at 72°C for 5 min (Gong et al., 2008).

3.5 Evaluation of PCR amplicons and generation of binary data matrix

Using 1X TAE buffer and a 2.0% agarose gel with ethidium bromide, PCR products were electrophoretically separated. Under UV light, the amplified products were visualized and photographed.

For each primer-genotype combination, DNA bands were scored (1) for presence and (0) for absence. Only reliable and clear bands were scored in order to estimate genetic similarity.

3.6 SSR data analysis

Using the Jacquard similarity coefficient formula, a data matrix was used to produce genetic similarity data between genotypes.

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

Where n_{xy} is the number of characters that have state x in individual i and state y in individual j. The distance matrix was then calculated using the fingerprint analysis with missing data (FAMD) software, followed by the construction of a neighbor joining tree. The resulting tree was visualized using the Dendroscop program.

Furthermore, the squared Euclidean distances matrix between all pairs of individuals using FAMD software was used for the study of principal coordinates (PCO), which was then projected in three dimensions using FAMD software. In addition to that, Shannon index was calculated using the same software to detect the diversity within the zucchini accessions.

Chapter Four: Results and Discussion

This study was performed to assess the genetic diversity of local Palestinian zucchini using SSR markers. In order to accomplish this, thirty-three zucchini samples were collected; twenty-six of them were local accessions collected from the Union of Agricultural Work Committees (UAWC), and the other seven zucchini accessions are commercial accessions gathered from a local agricultural equipment store. In addition to the zucchini samples, six cultivars from other species of the Cucurbits family were studied, including gourd, snake melon, and muskmelon. The plants were grown in a greenhouse, then their third and fourth leaves were taken for DNA extraction, after that they were transferred to an open field in order to complete their growth and to take morphological data related to fruit size, shape and colors and make visual documentation.

4.1 phenotypes of the zucchini accessions

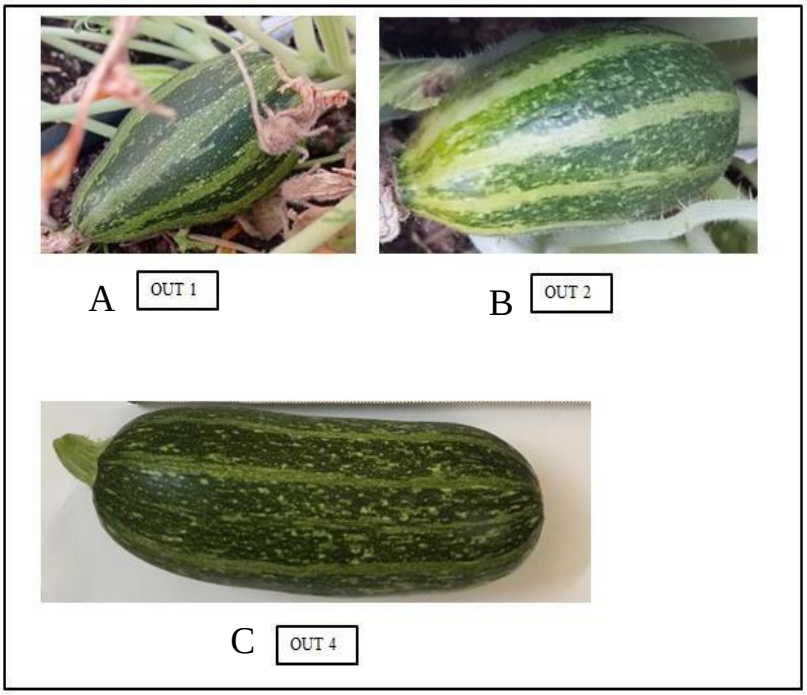
In order to have some morphological data related to fruit size, shape, and color, the plants were transferred to an open field to complete their growth because when they were left in the greenhouse the fruit development did not complete because bees were required for the pollination process, which did not occur. Unfortunately, not all of the samples have grown well as many problems have appeared and the plants were replanted more than once. This agrees with Azofeifa (2006) who reported that depending on morphological and agronomic characters have a tremendous amount of restrictions, since their expression may be subjected to many environmental factors.

One of the main problems is the viruses that affect the zucchini plants and the bleaching phenomenon. This phenomenon is directly related to the fall of the fruits, and the green color of the fruits turns pale yellow. According to the UAWC seed bank records, Farmers try to overcome the problem of viruses and bleaching by growing zucchini under white gauze (UAWC, 2018)

4.1.1 Morphological characterization of zucchini fruit

Figure 6 shows visual documentation of the young summer fruits taken from various local samples. Samples showed variation for several morphological parameters. First, there was a clear difference in parameters related to color. As for major color, samples OTU 1,2,4,6, 7, 8, 9, 12, 13, 14, and 15 have the deep green major color expected for local accessions, while samples OTU 5, 16, and 17 have a markedly lighter pale green major color.

The presence of pale green fruits among the local accessions was an unexpected result, as all local accessions are supposedly distinguished by their deep green color according to UAWC seed bank records (UAWC, 2018). The fact that samples OTU 5, 16, and 17 produced fruit of a pale light green color indicates that they may in fact be a hybrid zucchini accessions.



OUT 5

OUT 6

OUT 7

OUT 8

OUT 9

OUT 12

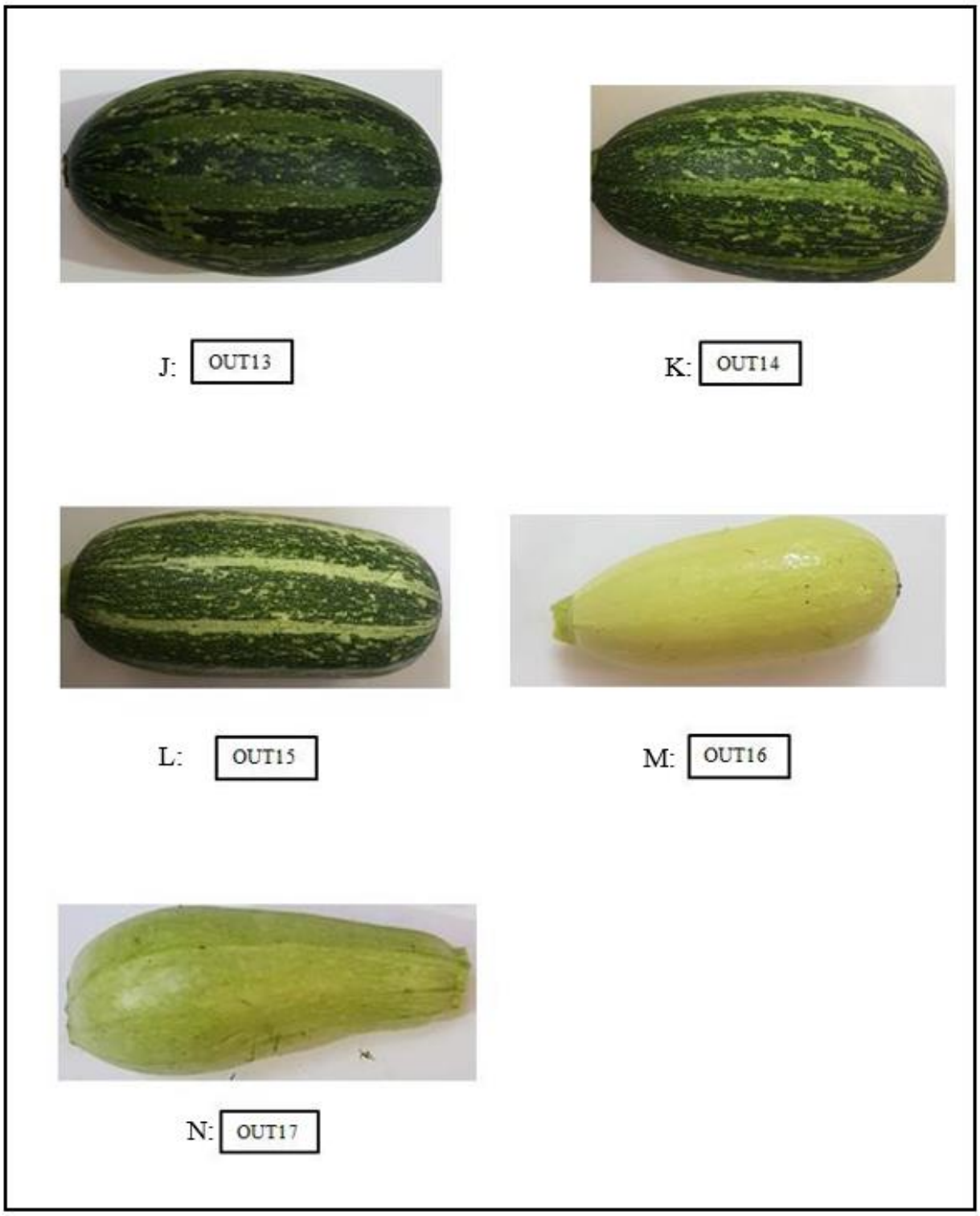


Figure 4.6: Fruit shape for some local Palestinian squash accessions

All of the samples displaying the dark green major color also had at least some degree of lighter linear stripes. These stripes varied in their width, as some of the samples had wide stripes like OTU 1 while sample OTU 4 for example, had thin yet well-defined stripes. On the other hand, other samples had stripes that were not as well defined, perhaps due to the non-uniformity of the deep green major color, creating a blemished appearance of the major color that merged into the stripes.

There was also considerable diversity in the shapes of fruits with regards to width and degree of tapering at the stem. Samples OTU 1 and 2 were very wide and oval and slightly tapered at the stem end. Samples OTU 4, 5, 6, 12, 15, and 16 were more elongated and of relatively uniform width along the length of the fruit. Samples OTU 13 and 14 had an almost perfectly oval shape with no tapering at the stem. Samples OTU 7, 8, 9, and 16 had an elongated shape with slight tapering at the stem, while sample OTU 17 had an attractive elongated shape with a neck region.

Overall, the observation of the morphology of the samples shows that there is a morphological diversity among the accessions. Also, the marked difference in the color of samples OTU 5, 16, and 17 suggests that these accessions were misidentified as local accessions while they may be hybrid accessions. This is considered an important result as it necessitates a re-check of the existing entries in the seed bank. In addition more conformational steps, including molecular tests, need to be done before any approval of new entries.

In addition to the morphological characteristics of the fruit, figure 7 shows the dark green heart shaped leaves with irregularly wavy edges. It was also noted how the stem is hairy, rough, and hollow as shown in Figure 8.

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Figure 4.7: Zucchini plant stem and leaves

Figure 4.8: Leaves of zucchini plant

4.1.2 Measurements of the zucchini accessions fruit

In addition to the previous morphological characteristics, various measurements such as the length and weight of the zucchini fruit, and the weight of 100 seeds of the zucchini were taken for some accessions as shown in Table 4. The average weight for the fruit was 160 g with an average lenght of 10.8 cm, while the average weight of 100 seeds was 10.2. This result is in agreement with Paris (2008) who reported that the fruits of zucchini are harvested when they are shiny and have the preferred size for the fruit which usually ranges from 100 g to 200 g.

As seen from Table 4, sample OTU 16 had a higher weight of 100 seeds than the rest of the samples. As it was suggested earlier, based on morphological characteristics, sample OTU 16 may be a hybrid accession that was misidentified by the seed bank as a local accession, which could explain the marked increase in weight of 100 seeds for OTU 16. To test for a significant difference in the weight of 100 seeds between the local and commercial accessions, the weight of 100 seeds was collected for five different commercial samples. The average weight of 100 seeds for these samples was 14 g, while the average weight of 100 seeds for the samples in Table 4 was 10.2 g, which was found by the t-test to be a significant difference (P values = 0.0011). This result proves that there is a significant difference in the weight of 100 seeds between the local and commercial zucchini accessions. Accordingly, additional studies should be done to take into consideration the weight of the seeds as a preliminary and rapid method to differentiate between local zucchini seeds and commercial zucchini seeds.

Table 4.4: Measurements for the zucchini fruit

Accession	Height (cm)	Weight (g)	100\U seed weight (g)
OTU 1	10.5	160	11.7
OTU3	11	200	8.26
OTU 4	10	118	9.34
OTU6	11	168	9.92
OTU7	11	135	9.54
OTU8	11.8	172	7.00
OTU 9	11.5	145	12.00
OTU12	11.5	140	9.55
OTU13	11	184	9.19
OTU14	8.5	99	11.6
OTU15	10.5	151	10
OTU16	12	250	15

4.2 SSR amplification data

The assessment of diversity among local and commercial zucchini accessions based on morphological characteristics showed remarkable diversity. This morphological diversity alludes to substantial underlying genetic diversity. Increased knowledge of this molecular diversity and the development and assessment of molecular markers may be of great value for farmers and for use in breeding programs. This is because the reliance on morphological markers has several drawbacks. For one, plants must be observed at various life cycle stages in order to study morphological markers, which require space and time and other resources. In addition, morphological characteristics are strongly affected by environmental conditions (Azofeifa, 2006). On the other hand, genetic markers can be studied from DNA samples taken from seedlings at the earliest stages of growth, which greatly reduces the time and space needed to carry out the assessment. Not only have molecular markers been shown to produce characterizations consistent with morphological markers in *Cucurbita pepo* . L (Nacar et al.

2012), it has been suggested that molecular markers may provide more accurate detection of differences (Rellstab et al. 2016). A comparative assessment of various molecular markers has shown SSR markers to be superior in terms of discriminatory power (Smýkal et al. 2008).

Therefore, this study used SSR primers to assess the genetic diversity among local and commercial zucchini accessions.

In this study 50 SSR Primers were used to investigate the pattern of genetic variation among 33 accessions of Palestinian zucchini in addition to another six accessions of other crops from the cucurbit family, consisting of gourd, snake melon, and muskmelon. Among these 50 SSR primers, only nine primers produced results with polymorphic bands that can be used in our analysis. This result is in accordance with Ntuli et al., (2015) result who reported that out of 55 SSR primers tested; only 10 primers were selected for their reproducibility and high polymorphism. The nine SSR primers used in this study produced a total of 27 polymorphic SSR loci. The size of the amplicons ranged from 90 bp for the primer UAWC 32 and the largest amplicon was 425 bp for the primer UAWC 24, which was considered the most powerful primer.

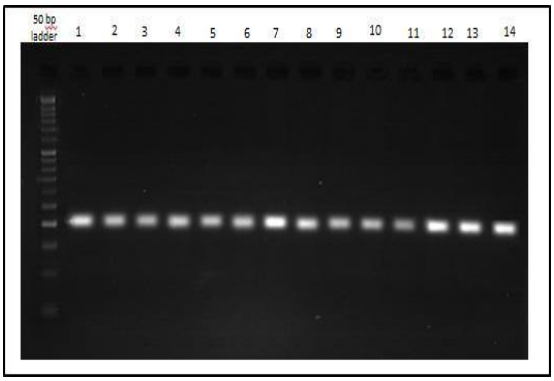


Figure 4.1: Amplicons produced using primer UAWC 22 that was excluded from the analysis because of monomorphic bands.

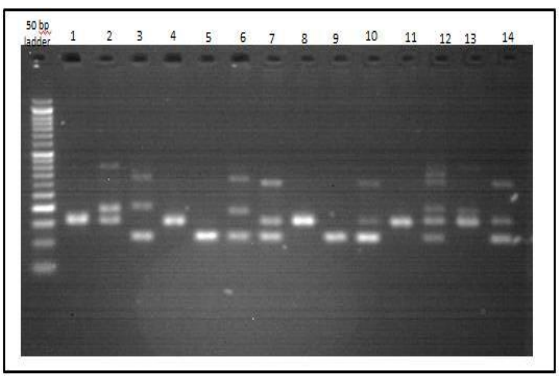


Figure 4.2:Amplicons produced using primer UAWC 24

Out of the nine primers used in our analysis, primer UAWC 39 produced a unique band for the gourd accessions while producing no bands for the muskmelon or snake melon accessions (Figure 11). Therefore, this primer can be used to differentiate between zucchini and gourd.

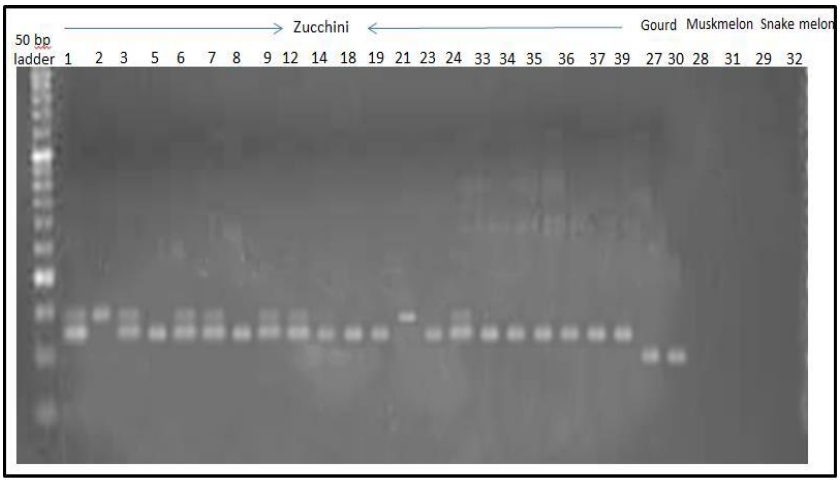


Figure 4.3; Agarose gel showing amplification products using primer UAWC39, showing a unique band for gourd. Lanes 27 and 30 are gourd samples, lanes 28 and 31 are muskmelon samples, lanes 29 and 31 are snake melon, and the remaining lanes are for zucchini samples

On the other hand, primer UAWC 13 produced a unique band for the muskmelon and snake melon accessions, while gourd and zucchini accessions produced identical bands (Figure 12). Accordingly, this primer can be used to differentiate zucchini and gourd accessions from snake melon and muskmelon accessions.

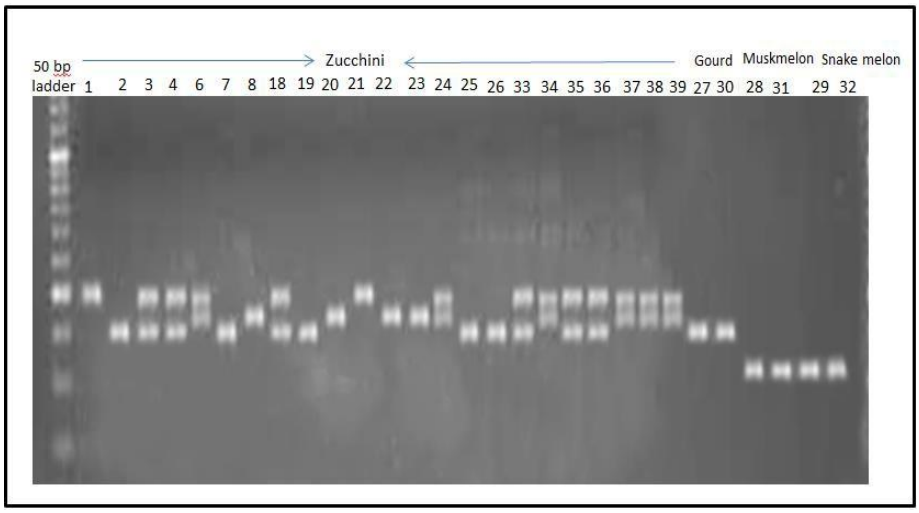


Figure 4.4: Agarose gel showing amplification products using primer UAWC 13, showing unique bands for muskmelon and snake melon. Lanes 27 and 30 are the gourd samples, lanes 28 and 31 are muskmelon samples, lanes 29 and 32 are snake melon, and the remaining lanes are for zucchini

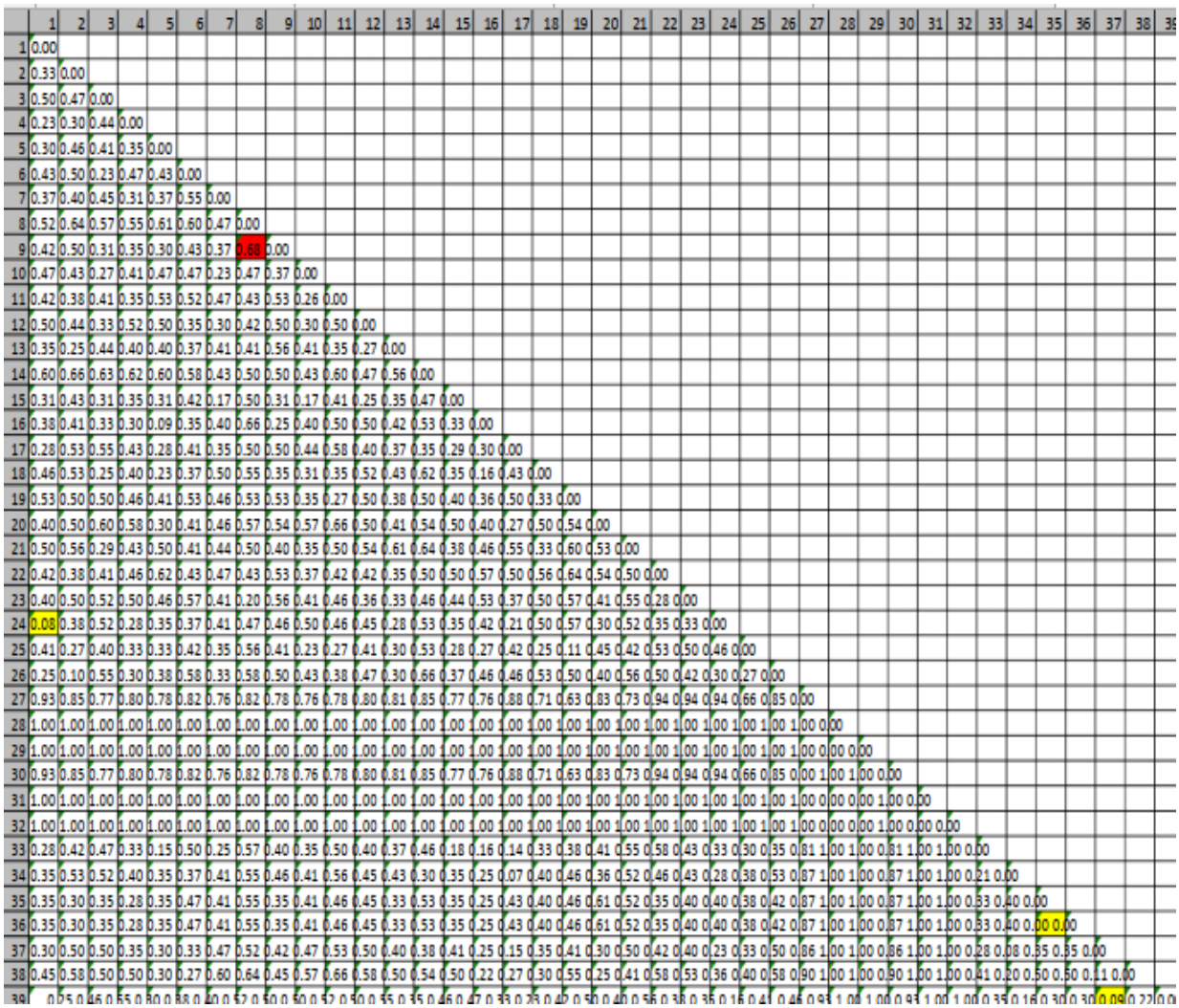
4.3 SSR data analysis

Based on the data collected using the polymorphic SSR primers, FAMD software was used to generate a genetic distance matrix according to Jacquard's similarity coefficient, and according to this matrix a neighbor joining tree was constructed. Squared Euclidean distances matrix between all pairs of individuals was also calculated using FAMD for the study of principal coordinates (PCO). In addition, genetic diversity was calculated by Shannon index (I) that was 3.9 which indicate that there is a high diversity within the samples. This high score is consistent with Barzegar et al,. (2013), who interpreted this score as due to the selection of polymorphic loci previously reported in other studies

4.3.1 Genetic distance

The genetic distance matrix was calculated according to Jacquard's similarity coefficient, which included 1053 entries and is shown in Table 4.

Table 4.1: Genetic distance matrix calculated according to Jacquard's similarity coefficient



The genetic distance matrix shows an average distance ranging from 0.00 to 1.00. The maximum genetic distance value was 1.00, which was between the zucchini accessions and the muskmelon and snake melon accessions. The genetic distance between the zucchini and gourd accessions ranged between 0.7 - 0.9, which was expected as zucchini and gourd are from the same species. The lowest genetic distance of 0.00 was within the accessions of muskmelon, snake melon and gourd, due to the fact that even if some primers produced bands for these accessions, the bands happened to not be polymorphic. This result is in agreement with Gong *et al* (2008) who suggested that SSRs are not easily transferable

between genera within the *Cucurbitaceae*.

In addition to these accessions, the lowest genetic distance of 0.00 was also observed between OTU 35 and OTU 36. This was expected, as both of these accessions are commercial accessions. Moreover, upon inquiry it was discovered that some commercial zucchini accessions are distributed under more than one trade name while being the same accession. Therefore, it is reasonable to assume that OTU 35 and OTU 35 are identical accessions with different commercial names. Our assumption is consistent with Francesca *et al.* (2010) who reported that identical accessions has a genetic distance that equal to 0.

On another hand, excluding the gourd, muskmelon, and snake melon accessions, the genetic distance range was (0.07–0.68), which was close to Inan et al. (2012) as the genetic distance range was (0.04 -0.87), whereas it was much more than Paris et al., (2002) results as the genetic distance range was (0.52 - 0.96). According to barzegar et al., (2013) this result is expected, as most of the accessions used were local, whereas in Paris et al. (2002) study, accessions were mainly improved commercial cultivars.

The lowest genetic distance of 0.07 was exhibited between OTU 34 and OTU 17. This result was unexpected, as OTU 34 is a commercial accession while OTU 17 is a local accession However, when morphological characterization was taken into consideration, it was noted that accession OTU 17 has the same fruit shape and color of the commercial accessions (Figure 13). This morphological similarity taken together with the low genetic distance between OTU 17 and the commercial accessions leads to believe that OTU 17 is a commercial accession that was misidentified at the seed bank as a local accession.

Figure 4.5: The fruit taken from accession OTU 17

4.3.2 Principal Coordinates Analysis (PCO)

The 3-D plot of the principal coordinate analysis (PCO) of the squared Euclidean distances between all 39 accessions is shown in Figure 14.

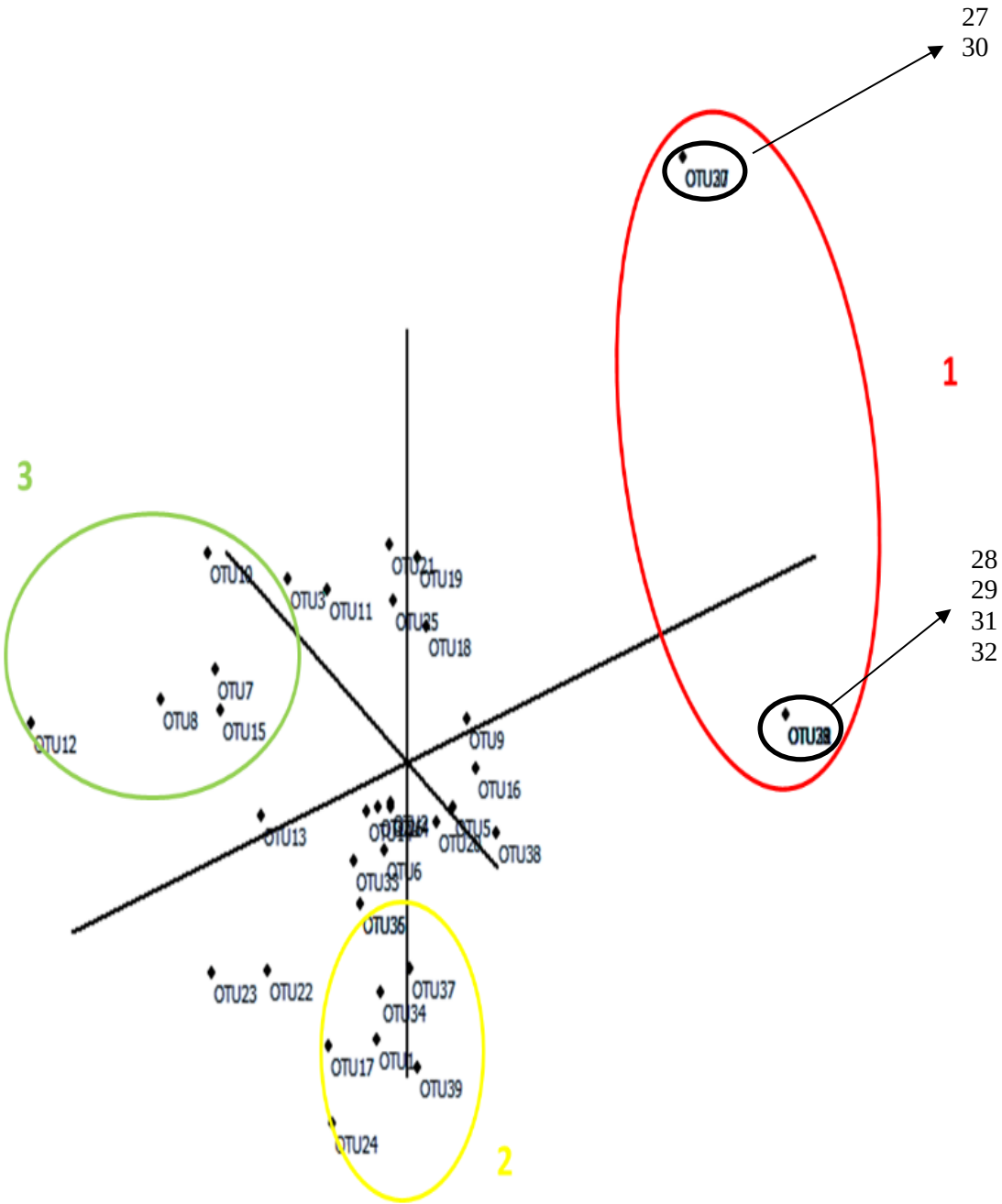


Figure 4.6: 3-D plot of the principal coordinate analysis for 39 accessions according to the squared Euclidean distances.

Figure 14 clearly shows how accessions OTU 27, 28,29,30,31 and 32, which are the gourd, muskmelon, and snake melon accessions, clustered in group 1 far away from the zucchini accessions. The upper coordinate in group 1 represents OTU 27 and 30, which are both gourd accessions and the remaining muskmelon and snake melon accessions clustered in the lower coordinate. The commercial accessions clustered close together in group 2 (shown in yellow), but there were also other local accessions located within the same group. The fact that some local accessions clustered closer to the commercial accessions suggests that some level of hybridization has happened and a number of local accessions may not be entirely pure. This may be due to farming practices, as farmers are known to plant different zucchini accessions in an open field at the same time, and also in close proximity so conditions for cross pollination exist. The figure also shows how accessions OTU 7, 8, 10, 12 and 15 (group 3 shown in green), which are local accessions, formed a distinct cluster.

4.3.3 Cluster Analysis

An unrooted Neighbor-Joining (NJ) phylogenetic tree was constructed according to the Jacquard's similarity coefficient as shown in Figure 16. The figure shows how the accessions are divided into 4 major clusters. Cluster I consists of accessions OTU 27, 28, 29, 30, 31, 32, which are the gourd, snake melon, and muskmelon accessions, and as expected these appear at a distance away from the zucchini accessions.

Cluster II consists of accessions OTU 25, 19, 11, 10, 15, 7, 12, 23, 8, 22, 14, which are all local accessions. Cluster III consist of accessions OTU 38, 37, 39, 34, 17, 20, 24, 1, 16, 5, 33, 36, 35, 4, 26, 2, and 13. Most of the accessions in this cluster are the commercial accessions, but it does contain a number of accessions that were identified as local accessions by the seed bank. It was notice in this cluster how accessions OTU 17 and 34 are closer to each other, which was expected as they were found to have a low genetic distance of 0.07 by the genetic distance analysis, and how accession OTU 33, which is a commercial accession,

is closer to accessions OTU 5 and 16, which are registered as local accessions. This is further corroboration for the explanation suggested earlier. By taking into account the morphological characteristics as well as distance and clustering analysis, it appears that accessions OTU 5, and 16 are in fact commercial accessions.

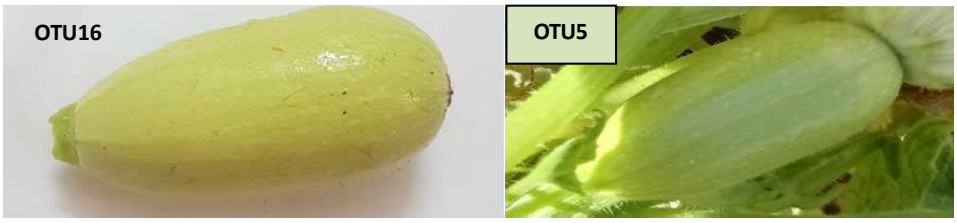


Figure 4.7: Fruit shape of accessions 5 and 16

It was also notice in the cluster analysis how accessions OTU 35 and 36 are presented in the cluster analysis as the same accessions. This was expected, as in the genetic distance analysis they had the lowest genetic distance of 0.00 between them.

Cluster IV consist of accessions OTU 21, 18, 6, 3, and 9, which are local accessions from assorted locations.

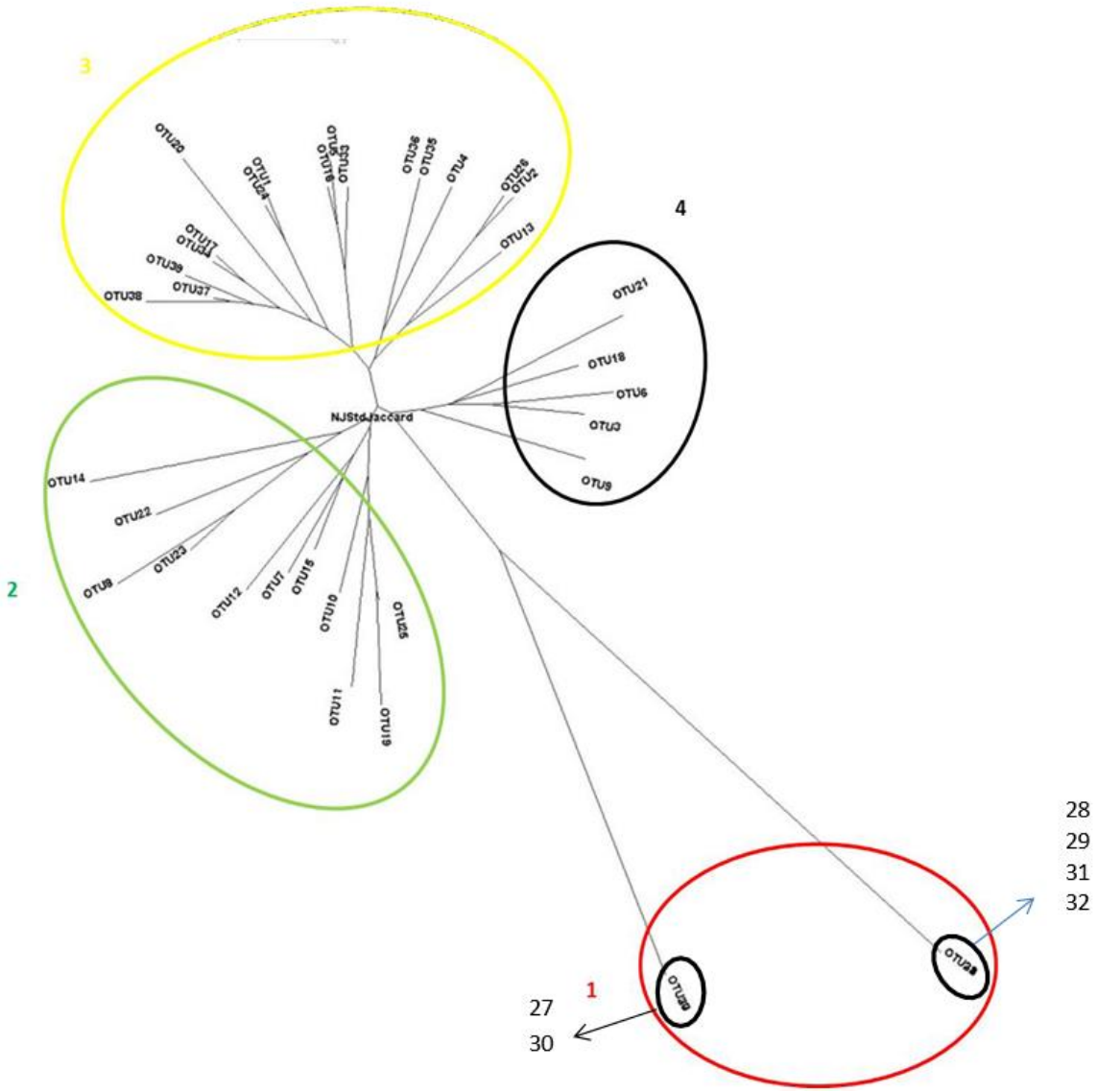


Figure 4.8: Mid-pointed NJ tree of all accessions according to the Jacquard's similarity coefficient.

Chapter Five: Conclusion and Recommendations:

5.1 Conclusion

This study was performed to assess the genetic diversity of local Palestinian zucchini accessions using SSR markers. In order to accomplish this, thirty-three zucchini samples were collected; twenty-six of them were local accessions collected from the Union of Agricultural Work Committees (UAWC), and the remaining seven zucchini accessions were commercial accessions gathered from a local agricultural equipment store. In addition to the zucchini samples, six accessions from other species of the Cucurbits family were studied, including gourd, snake melon, and muskmelon. All of the samples were grown in an open field in order to take morphological data related to fruit size, shape and colors and to make visual documentation.

The observation of the morphology of the samples shows that there is a morphological diversity among the accessions. Also, the marked difference in color of samples OTU 5, 16, and 17 compared to the other local samples suggests that these accessions were misidentified as local accessions and they may in fact be hybrid accessions. This is considered an important result as it necessitates a re-check of the existing entries in the seed bank.

The study of morphological and agronomic characters to assess diversity entails a tremendous amount of limitation, since their expression may be subject to environmental or phenotypic factors. On the other hand, approaches for identifying and characterizing organisms that rely on the use of molecular genetic markers have generally turned out to be

more effective since they get beyond the drawbacks of conventional techniques. These markers are employed globally for these applications because they offer a useful tool for establishing precise genotyping of crops (Azofeifa, 2006).

Accordingly, SSR markers were employed in this study to assess the genetic diversity of Palestinian zucchini. These markers were used in this investigation because SSR markers are abundant, co-dominant, highly informative and polymorphic (Marquez-Lema et al., 2010; Nybom, 2004).

Fifty SSR primers were used in this study. Out of these primers, only nine primers produced reasonable results with polymorphic bands, and these nine primers were used in the analysis. The monomorphism of the other SSR markers concurs with Ntuli et al. (2015), who reported monomorphism in some SSR markers in his study.

One of the nine primers that produced polymorphic bands, primer UAWC 39 produced a unique band for the gourd accessions while producing no bands for the muskmelon or snake melon accessions. Therefore, this primer can be a potential primer to differentiate between zucchini and gourd. In addition, primer UAWC 13 produced a unique band for both snake melon and muskmelon accessions.

Based on the data collected using the polymorphic SSR primers, FAMD software was used to generate a genetic distance matrix according to Jacquard's similarity coefficient, and according to this matrix a neighbor joining tree was constructed. Squared Euclidean distances matrix between all pairs of individuals was also calculated using FAMD for the study of principal coordinates (PCO). In addition, genetic diversity was measured by Shannon index (I) which was 3.9. This high score is consistent with Barzegar et al,. (2013), who interpreted this score as due to the selection of polymorphic loci previously reported in other studies

The genetic distance matrix showed average distances ranging from 0.00 to 1.00. The lowest

genetic distance of 0.00 was found between the accessions of muskmelon, snake melon, and gourd, as even if some primers produced bands for these accessions, the bands were not polymorphic between these accessions. In addition to these accessions, a genetic distance value of 0.00 was observed between two commercial zucchini accessions, indicating that they are both identical. Additionally, low genetic distance was observed between two zucchini accessions, one of them being a commercial accession while the other was identified as a local accession by the seed bank. When morphological notes were taken in consideration in addition to genetic distance data, it was concluded that this accession is a commercial zucchini accession that was misidentified by the seed bank as a local accession.

The neighbor-joining (NJ) tree was constructed based on the data generated from the genetic distance matrix. NJ tree clustered the accessions into four major groups, showing how the accessions of gourd, muskmelon, and snake melon are away from squash accessions. In addition to that, it has also shown how there is genetic variation among the local accessions of zucchini, as they clustered in three different groups while the commercial accessions were all in the same group. This is consistent with Formisano et al., (2011) study, in which the variation among landraces belonging to the Zucchini group was larger than that of the commercial cultivars.

NJ tree has also shown that some level of hybridization has happened and a number of local accessions may not be entirely pure as some of the local accessions were within the same group of the commercial accessions.

A focal discovery made in this study is the considerable genetic variation found between local zucchini accessions. This finding is reassuring considering the risks associated with the narrow genetic variation found in commercial accessions. The huge coverage of monocultures that happened with most crops following the green revolution has resulted in food crises in staple crops such as potato (Bruneel et al., 2020) and corn (Condon et al.,

2018), and could result in similar problems for crops like zucchini if care is not taken to preserve natural genetic diversity in seed and germplasm banks. The cultivation of traditional local varieties and landraces of crops has been put forth as a source of resilience to counter agricultural shocks (Hellin et al., 2014). In fact, the importance of genetic diversity conservation is evident in international agreements that have been formed such as the UN Convention on Biological Diversity and International Treaty on Plant Genetic Resources. This is particularly important in the face of accelerating climate change, which in addition to altered temperature and rainfall patterns, can allow for increased range of plant pathogens and insect vectors. The genetic diversity of local zucchini preserved in the seed bank are a source of genetic material that can be used for classical breeding or new biotechnological techniques to produce new varieties that are better suited to the changing environment if the need arises.

5.2 Recommendation

Referring to this work, the following recommendations for future work would be outlined:

- More studies need to be done on the morphological characterization of Palestinian zucchini
- Further studies need to be done using the primers (UAWC 13, UWAC 39) with more accessions from the gourd, snake melon, and muskmelon, as they could be differentiated primers.
- The seed bank need to put into place a new protocol to verify the identity of accessions before accepting any new accessions, and re-check existing accessions.
- The local zucchini accessions need to be continue maintained, further characterized and be available for scientists and farmers for crop improvement.

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Abstract in Arabic

التوصيف الوراثي لنبات الكوسا البلدي في فلسطين

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الملخص:

تم اجراء هذا البحث من اجل دراسة التنوع الوراثي لنبات الكوسا البلدي في فلسطين. حيث يعتبر الكوسا أحد اهم انواع القرعيات في فلسطين ذات الطلب العالي في الأسواق المحلية كونها محببة لدى المستهلكين. يتميز الكوسا البلدي بموسم نمو طويل و ثمار خضراء داكنة اللون ذات رائحة و نكهة قوية ومميزة، وتزرع بشكل كبير في مناطق الخليل و بيت لحم ورام الله.

شملت الدراسة ثلاثة وثلاثون عينة من الكوسا، حيث كانت ستة وعشرون منها عبارة عن كوسا بلدي تم جمعها من بنك البذور التابع للجان اتحاد العمل الزراعي والسبع عينات الأخرى كانت عينات تجارية من السوق المحلي. بالاضافة لأصناف الكوسا فقد تم دراسة ستة عينات من أصناف اخرى من عائلة القرعيات وهي القرع والشمام والبطيخ. ومن أجل اتمام الدراسة تم استخدام الواسم الجزيئي SSR marker.

من أجل اتمام الدراسة تم استخدام الواسم الجزيئي " SSR marker" حيث تم استخدام ٥٠ برايمر ولكن فقط تسعة منها تم استخدامها في التحليل لأنها انتجت نتائج مرضية من أجل اتمام هدف الدراسة. واحدة من النتائج المهمة التي ظهرت باستخدام هذه البرايمرات كانت باستخدام برايمر (UAWC39) حيث أظهر علامات خاصة ومميزة لنباتات القرع بينما لم يظهر أي علامات للبطيخ أو الشمام وبذلك من الممكن استخدامه للتفريق بين الكوسا و القرع. من ناحية أخرى، انتج البرايمر (UAWC13) علامات خاصة للشمام والبطيخ بينما تشارك القرع بعلامات خاصه مع الكوسا.

بناء على النتائج التي ظهرت باستخدام الواسم الجزيئي تم استخدام برنامج FAMD من أجل دراسة التنوع الوراثي للعينات. بالبداية تم حساب المسافة الوراثية بين العينات باستخدام مؤشر جاكارد حيث تراوحت المسافة بين عينات الكوسا بين (0.00 – 0.68). لوحظت أدنى مسافة وراثية بين مدخلين تجاريين ؛ لذلك تم افتراض أن هذين المُدخلين متطابقان مع أسماء تجارية مختلفة.

بالاضافة لذلك، تم انشاء شجرة قرابة neighbor joining وفقا لمصفوفة المسافة الوراثية. قد قسمت الشجرة العينات لأربع مجموعات رئيسية و أظهرت أن مدخلات القرع و البطيخ والشمام في مجموعة خاصة بعيدة عن الكوسا و بذلك فقد تقسمت عينات الكوسا لثلاث مجموعات رئيسية وهذا يدل على وجود تنوع وراثي بين هذه العينات. كما أظهرت ان بعض عينات الكوسا البلدي قد تكون هجينة لكونها على مسافة قريبة جدا من العينات التجارية وفي نفس المجموعة التي تضم العينات التجارية.

هذا التنوع الوراثي بين مدخلات الكوسة البلدية هو مصدر للمواد الوراثية لتي يمكن استخدامها لإنتاج أصناف جديدة أكثر ملاءمة للبيئة إذا دعت الحاجة. لذلك نوصي بمواصلة الحفاظ على هذه المُدخَلات البلدية وتوصيفها بشكل أكب وإتاحتها للعلماء والمزارعين لتحسين المحاصيل.