ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

Morphological characterizations, genetic diversity and population structure of date palms (*Phoenix dactylifera* L.) in Ethiopia

PhD dissertation

By Workia Ahmed Tegegne

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Addis Ababa, Ethiopia

Morphological characterizations, genetic diversity and population structure of date palms (*Phoenix dactylifera* L.) in Ethiopia

Workia Ahmed Tegene

A Thesis Submitted to Institute of Biotechnology, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology (Agricultural Biotechnology)

Suppervisors:

Tileye Feyissa (PhD, Professor)

Kassahun Tesfaye (PhD, Professor)

Sumaira Farrakh (PhD, Associate Professor)

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Approved by Examining Board:

Name	**Date**	**Signiture**
Prof Tileye Feyissa (Advisor)		
Prof Kassahun Tesfaye (Co-advisor)		
Dr. Sumaira Farrakh (Co-advisor)		
Dr. Tsegaye Getahun (Internal examiner)		
Dr. Yemisirach Melkie (External examiner)		

Declaration

I, Workia Ahmed Tegegne, hereby declare that this dissertation and its entirety is my bona fide work and I have submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University. This dissertation or its part has not been submitted to other University anywhere for the award of any degree. All sources of materials used for the dissertation have been duly acknowledged.

PhD candidate:

Workia Ahmed Signature ______________Date_____________

Major advisor:

Tiliye Feyissa (Prof) Signature ______________Date_____________

Co-advisors:

Kassahun Tesfaye (PhD) Signature ______________Date_____________

Sumaira Farrakh (PhD) Signature ______________Date_____________

September, 2024

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List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CTAB	Cetyl Trimethyl Ammonium Bromide
DAPC	Discriminate Analysis of Principal components
He	expected hetrozygosity
Ho	observed hetrozygosity
I	Shannon information index
ISSR	Inter - Simple Sequences Repeats
LD	linkage disequiliberium
MAF	major allele frequency
Na	observed number of alleles
Ne	effective number of alleles
NJ	neighbour-joining
NG	number of genotype per locus
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PIC	Polymorphic information content
RAPD	Random Amplified Polymorphic DNA
SNPs	Single Nucleotide Polymorphisms
SSR	Simple Sequence Repeats
TWAS	The World Academy of Sciences

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Abstract

Date palm tree is aperennial plant that belongs to Arecaceae family and it significantly contributes to agricultural sustainability and socio-economic values for many countries including Ethiopia. Scientific evidences from molecular and morphological genetic data of date palms are the baseline for establishment of gene banks, conservation and to develop breeding programs. In Ethiopia, there is lack of genetic information about date palm trees. Therefore, the objective of this dissertation is to evauate the genetic diversity and population structure of date palms in Ethiopia. In this study, 45 morphological traits for 11 individual date palms, 10 Inter Simple Sequence Repeat (ISSR) markers for 113 date palm genotypes, 10 Simple Sequence Repeat (SSR) markers for 124 date palm genotypes and 4 Single Nucleotide Polymorphism (SNPs) markers for 15 date palm genortypes were used. The morphological diversity analysis was based on principal component analysis using the total 45 traits showed 37% variations exist among 11 individuals. The morphological data analysis using 25 vegetative traits alone as well as the data from 20 reproductive traits alone 29% and 32% of variations was observed respectively. Fourty three morphological traits exhibited significant differences at p <

0.05 in analysis of variance and 23 traits showed significant variances at $p < 0.05$ in homogeneity of variance analysis among cultivars. Dendrograms indicated clear genetic relationships of cultivars. From ISSR analysis, a total of 241 DNA fragments were generated by all primers and each primer showed 100% of polymorphism. The highest (37) and the lowest (10) number of bands were produced by (AGG)6 and (AG)10C primers, respectively. AMOVA result showed 49% and 51% within and among population diversity, respectively, and also, the first three PCoA accounted for 10.55%, 9.17% and 7.68% variations. The genotyes were also clustered according to their geographical location. From SSR analysis: a total of 112 of alleles were detected, Neighbour-joining clustering analysis based on dissimilarity coefficient values the date palm popuatons grouped into five major categories. Population structure analysis at the highest K value grouped the genotypes into three groups. Principal coordinate analysis explained a total variation of 17.33%. The discriminant analyses of principal components also separated date palm genotypes into eight clusters. From SNPs data, genetic variations were shown in phylogenetic tree, nucleotide content and pairwise distance analysis of date palm genotypes. Generally, evaluation of genetic diversity and phylogenetic structure of date palms using molecular and morphological markers is very important. The overall findings of this study are helpfull to design genetic improvement, conservation and germplasm introduction programs of date palms in Ethiopia and elsewhere.

Keywords: AMOVA, date palm, genetic diversity, ISSR, SNPs and SSR markers, morphological traits, polymorphism, population structure

Chapter 1

1. Introduction

1.1. General Introduction

Date palm tree (*Phoenix dactylifera* L.) is a diploid plant with 2n = 36 chromosome number, a perennial monocotyledonous plant belonging to the Arecaceae family (Mirbahar *et al*., 2014; Flower *et al*., 2019). It is one of the oldest known fruit-bearing trees that have long history as early as 4000 B.C. 'Phoenix' is a very old name that was used to call for this plant by Theophrastus, indicating that the tree was 1st introduced to the Greeks by the Phoenicians (Orwa *et al*., 2009; Al-Alawi *et al*., 2017). The scientific name of date palm tree is called *phoenix dactylifera* L.; the word dactylifera originates from a Greek word 'dactylos' and a Latin word 'fero'; 'dactylos' which means the elongated shape of the fruits which is resembling the "fingers" of the hand and 'fero' which means "I bear". So, the name of the species *dactylifera* means "finger-bearing" which refers to the fruit bunches produced by a palm tree (Orwa *et al*., 2009; Al-Alawi *et al*., 2017).

Date palm is distributed into arid and semi-arid parts of the world. Since ancient time, the palm trees could be beneficial for different purposes and it has got a spiritual and religious meaning; for example, 'Palm Sunday' a feast celebrated by Christians and Jews is rooted in the blessing and strategic placement of palm branches while, in ancient Greece, the tree also symbolized Apollo who was born under a date palm on the island of Delos (Popenoe, 1924). Additionally, the dates and date palms are mentioned in the religious text of Judaism, Christianity and Islam. About the importance of date palm over 20 times mentioned in the Holy Quran such as, in Surah

Maryam, at the point when Mary brought forth the Christ Jesus (Peace Be upon Him) under a palm tree, she heard a voice advising her: “Shake the trunk of the palm tree towards thee: it will drop new, ready dates upon thee. Eat, then, and drink, and let thine eye be cheered!” (Qur’an 19: 25-26) and the health benefit of ‘Ajwa’ dates variety has been documented in hadith: “If somebody takes seven Ajwa dates in the morning, neither magic nor poison will hurt him that day” (El-Far *et al*., 2016).

Due to the old history and the wide spreading and exchanging of dates across the globe, the exact origin of date palm is still unknown but, it most probably originated from the ancient Mesopotamia area currently known as southern Iraq or western India and later expanded throughout North Africa during Roman civilization (Chao and Krueger, 2007; Gros-Balthazard and Flowers, 2021). Many archaebotanical remains and the documented history of phoeniciculture in ancient texts in the gulf region of the Tigris and Euphrates River valleys are important clues for origin of domesticated date palm in these places (Gros-Balthazard and Flowers, 2021).

Date palms have a great socio-economic impact and prominent contribution in agricultural sustainability in many arid and semiarid parts of the world due to its food, industrial, commercial, medicinal and ornamental values (Johnson, 2012; Al-Khalifah and Shanavaskhan, 2012; El-Far *et al*., 2016). Date fruits are nutritionally rich in carbohydrate containing 70% of sugar like glucose, sucrose and fructose; and also packed essential minerals and vitamins (Aljaloud *et al*., 2020). In food industry, dates have been used to process pickles, chutney, jam, jelly, date-in-syrup, date butter, candy, date bars and confectionary products (Ghnimi *et al.*, 2016). Due to the exceptional character of this palm with its long-term productivity, it is also called “tree of life” and “Bread of the Desert” (Simon, 1978) and the sacred tree (Mariana, 2007).

Research studies have shown that the date fruits extract is a good sources of compounds like ferulic acid, ϱ-coumaric acid, ϱ-hydroxybenzoic acid, certain oxidative enzymes, steroids, flavonoids and contained small amounts of vitamins C, B1 (thiamine), B2 (riboflavin) and nicotinic acid, which are used for antioxidants, antibacterial and anticancer activities (Assirey, 2015; Meer *et al*., 2017). Different parts of date palm is used for different purposes (Fatima *et al*., 2016); trunks to construct houses, hives, bridges and as packing material (Al-Jabri, 2014); terminal buds and young leaves can be cooked as vegetables while rachises are used for paper making (Khiari *et al*., 2011; El Hadrami and Al Khayri, 2012).

Date palm has been introduced to Ethiopia from Middle East countries approximately 200 years ago by Arabian traders (Aregawi Lemlem *et al*., 2018). Cultivation of date palm began in Afar region mainly at Afambo and Asayta and other places nearby Awash River and then spread to Errer Gota and Dredawa areas (Hussen, 2010; Ben-Salah, 2015). Currently, dates production from these areas is mainly used for local consumption and income sources through trading to nearby towns. In these areas, different unknown varieties of date fruits are produced which have different shapes and colours at the last of its fruiting stage. Around 10 years ago, 14 *in vitro* date palm varieties have also been introduced to Ethiopia, and cultivated in three places: Humodoyta site (Afambo), Asayta and Melka Werer agricultural research centre (Melka Werer) for adaptation and improvement programme.

The date palms are perennial and dioecious plants. The natural characteristics and breeding system of this crop can be suitable to enhance the diversification among date palm populations. Therefore, evaluation of genetic diversity and phylogenetic relationships using morphological and molecular markers in between and among date palm populations at regional, national and global level is the most important groundwork for breeding, genetic improvement and conservation programme. However, to date, there were no research studies to determine the genetic diversity, population structure and geneflow of date palms in Ethiopia.

Different types of molecular markers have been reported on the assessment of genetic diversity and phylogentic relationships of date palm cultivars in many countries such as Egypt (El-Assar *et al*., 2005; Abd-Alla, 2010), Tunisia (Bodian *et al*., 2012; Zehdi *et al*., 2004), Morocco (Bodian *et al*., 2012), Nigeria (Yusuf *et al*., 2015), Pakistan (Mirbahar *et al*., 2014) and Syria (Haider *et al*., 2012) and using morphological markers also reported in Tunisia (Hamza *et al*., 2011; Kadri *et al*., 2019; Karim *et al*., 2021; Ouarda *et al*., 2012), Algeria (Simozrag *et al*., 2016; Bedjaoui and Benbouza, 2018; Abdelkrim *et al*., 2020), Iraq (Khierallah and Azhar, 2016), Egypt (Eissa *et al*., 2009; Ibrahim *et al*., 2014; El - Sharabasy and Rizk, 2005), Sudan (Elsafy *et al*., 2015), Pakistan (Haider *et al*., 2015; Faqir *et al*., 2018), Iran (Khankahdani and Bagheri, 2019), Saudi Arabia (Al-Khalifah *et al*., 2012), Morocco (Elhoumaizi *et al*., 2002) and Nigeria (Odewale *et al*., 2013).

1.2. Objective of the study

1.2.1. General objective of the study

The general objective of this research is to study the genetic diversity and population structure of Ethiopian date palm genotypes using molecular and morphological markers.

1.2.2. Specific objectives

- ✓ To evaluate the genetic diversity and population structure of date palm genotypes using microsstelite markers
- ✓ To assess the genetic divaersity and phylogenetic structure of date palm populatins using inter simple sequences repeats (ISSR) markers
- ✓ To evaluate the phylogenetic relationship of date palm cultivars using morphological traits
- ✓ To identify the genetic relationships of Ethiopian date palms cultivars using SNPs markers.

1.3. Significance and limitations of the study

Identification of genetic diversity and assessment of population structure of any plants at regional, national and international levels is the primary groundwork for conservation, genetic improvement and for sustainable utilization of plant genetic resources. Therefore, the main significance of this study is to provide basic information on molecular genetic diversity of date palm genotypes using SSR, ISSR and SNPs that are collected from different geographical location of Ethiopia. Moreover, morphological diversity evidences from *in vitro* date palm cultivars that are planted and adapted at Melka Werer agricultural research center were obtained. Besides, the information obtained in this study would be initiative for breeders and researchers for developing new cultivars that having good agronomic traits and better adaptation to the region.

During study of this research there was some limitations related to sample collection. In Kereuda, Berga and Mego sites we have collected fewer samples than others. This is due to there was no offshoots on oldest mother plants and also it is also difficult to collect young leaves on the top of the tree. Therefore, this factor may be affecting the result of the study.

Chapter 2

Literature review

2.1. Botany and ecology of date palm

Date palm is an evergreen dioecious perennial monocotyledonous tree that belongs to Arecaceae/palm family. There are over 2600 species of palm trees that are classified into more than 200 genera. Like other monocoyledon date palm has no tap root and root hairs; roots are adventitious and fibrous emerging from a region of the root initiation zone. Secondary roots appear on the primary root and these produce additional tertiary and quaternary lateral roots which have no much difference in diameter and length with the primary and secondary roots (Broschat, 2013). Trunk is un-branched cylindrical single stem reaching up to 30 m high with equal diameter and covered by bases of fallen leaves (Figure 2.1). The top part also carries fan-shaped or feather-like fronds (Broschat, 2013). Leaves of the date palms are glabrous, grayish-green fronds having 3 to 6 meters in length and 120 to 240 numbers of pinnae per frond depending on the cultivars. Leaflets are distichous, 16 - 45 cm long and nearly 2 cm broad, sharply pointed, the lowest converted in to spines in form, pinnate, induplicate (V-shaped) and erect, with basal leaf spines. The petioles of fronds also covered by spines (thorns); these spines vary in length, thickness, their number and groups of spines attached at a point (can be single, two, or three) (Chao and Krueger, 2007; Marwat, *et al.*, 2012).

In date palm, cluster of flowers are called inflorescences. The inflorescences of female and male trees are not similar, but both are bounded by a hard fibrous cover during early stage of flower development to protect the immature flowers from heat and sunlight.

Female inflorescences are born on 90 - 120 cm long rachis and arranged in spirals. The rachis of male inflorescence is much smaller, 12 - 25 cm long. Both female and male flowers usually have three sepals and three petals (Chao and Krueger, 2007). Male inflorescence is packed tightly at the end of the rachis; when a flower open, it has six stamens enclosed by waxy scale-like petals and sepals and each stamen contained two little yellowish pollen sacs. While, the female inflorescence is less densely packed than male's inflorescence and on flowers opening, they show more yellowish or yellow green colours (Chao and Krueger, 2007; Marwat, *et al.*, 2012; Al-Alawi *et al.*, 2017).

A fruit is single-seeded of a cylindrical, rounded or ovoid shape contained an edible fleshy part which is yellowish-brown to reddish-brown and having different size (2.5 - 5) × (1 - 1.5) cm depending on the variety. Anatomically, components of a single fruit are a pericarp, mesocarp, endocarp and one seed (also called kernel, pit, or pyrene) (Figure 2.2b). Date fruits have five consecutive developmental stages; these are Hanabauk, Kimri, Khalal (or Bisr), Rutab, and Tamras shown (Figure 2.2a) and at the final of three stages the fruit is edible due to decreasing bitterness, increasing sweetness, and improving tenderness, and succulence (Ghnimi *et al.*, 2016). Fruits carried by spikelets and each spikelet attached to top part of peduncle to form a bunch and the number of spikelets and bunches vary based on the cultivar type and environmental conditions (El Hadrami and Al Khayri, 2012) when ripe, range from bright red to bright yellow in colour, depending on variety. Seeds of date palm is classified as stony type and the adaxial side of seeds characterized by longitudinally grooved with varying depth and width; the dorsal sides of seeds have convex shape and also contained micropyle (Marwat, *et al.*, 2012; Ghnimi et al., 2016).

Ecologically, the date palms can be grown in different types of soil from which deep sandy loam is the most suitable and desirable one due to its water holding capacity with good drainage. It can also adapt and tolerate saline and alkaline soil environment but the growth and fruiting rates are diminished. Date palm tree can grow in arid regions which are located between 15 ^{o}N to 35 ^{o}N with an average temperature ranging from 12.7 to 27.5°C. Indeed, date palm have shown ecological plasticity and high adaptation to arid conditions where the annual precipitation rarely exceed 250 mm combined with hot summers up to 50°C and sustaining short periods of frost at cold winter reaching to -10°C. The date palm produces fruits in areas having long hot summer and mild temperature during flowering (February to April) and fruit ripening (May to August) should be 25 to 29°C (Chao and Krueger, 2007).

2.2. Use and socio-economic importance of date palm

Among plants, date palm is one of the most important plant for human that having food, medicinal, industrial, commercial, ornamental, cultural, architectural and ecological values. There are no discarding date palm parts as wastes and each of plant part as well as its residues have direct and indirect benefits by using the row materials or products of a palm tree, such as date fruits are the main staple food for local people and contain carbohydrates up to 71.2–81.4% dry weight and low concentrations of ash, protein and lipid (1.68–3.94%, 1.72–4.73% and 0.12–0.72%, respectively) (Assirey, 2015; Ghnimi *et al.*, 2016). In addition, the date fruit contains amino acids and vitamins that are essential for our health like aspartic acid, proline, alanine, glycine, valine, leucine, low concentrations of threonine, serine, isoleucine, tyrosine, arginine, phenylalanine, lysine, methionine, histidine, nicotinic acid and small amounts of vitamins C, B1 thiamine, B2 riboflavin (Assirey, 2015).

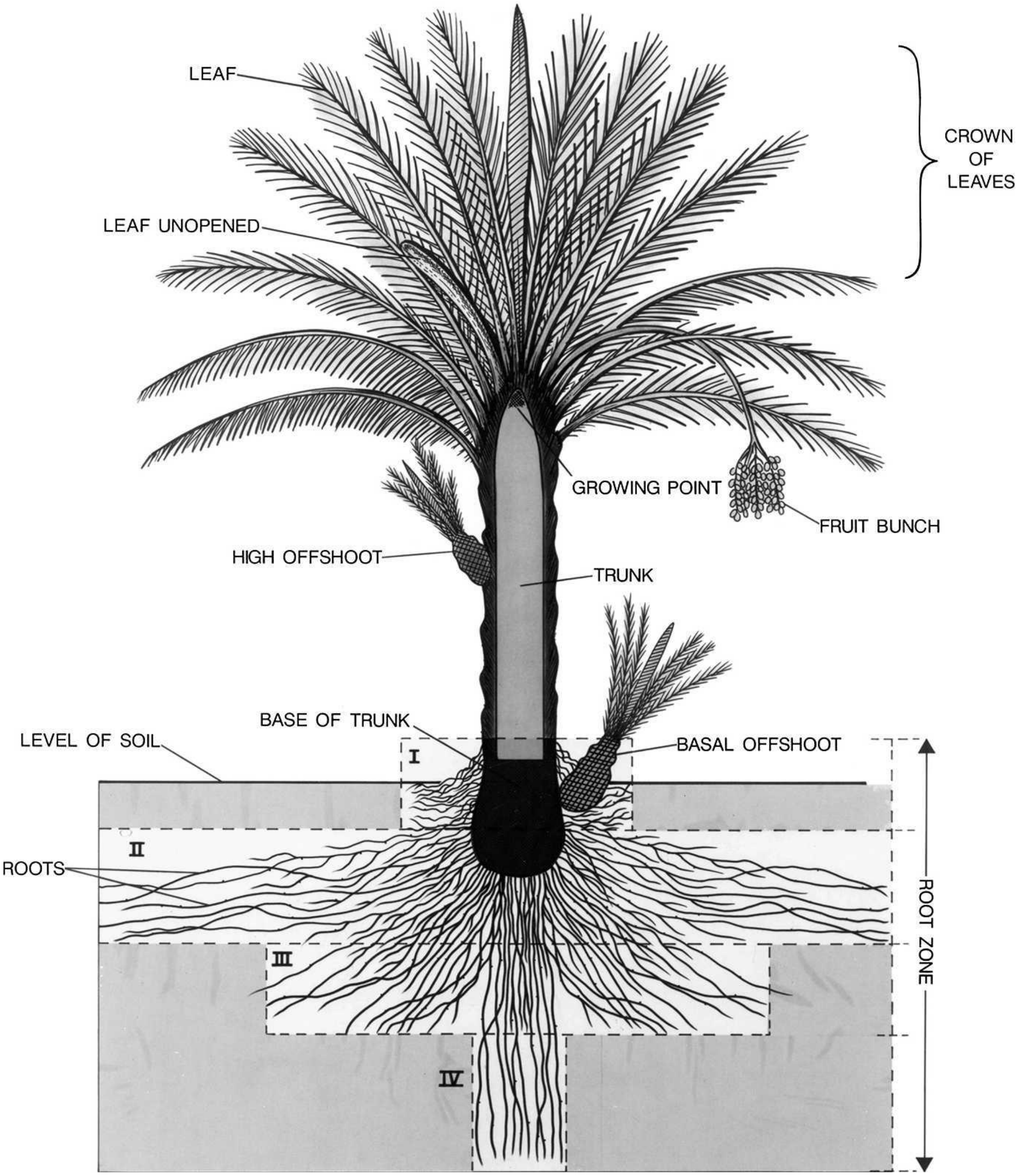


Figure 2.1 Morphological feature of date palm tree (Chao and Krueger, 2007)

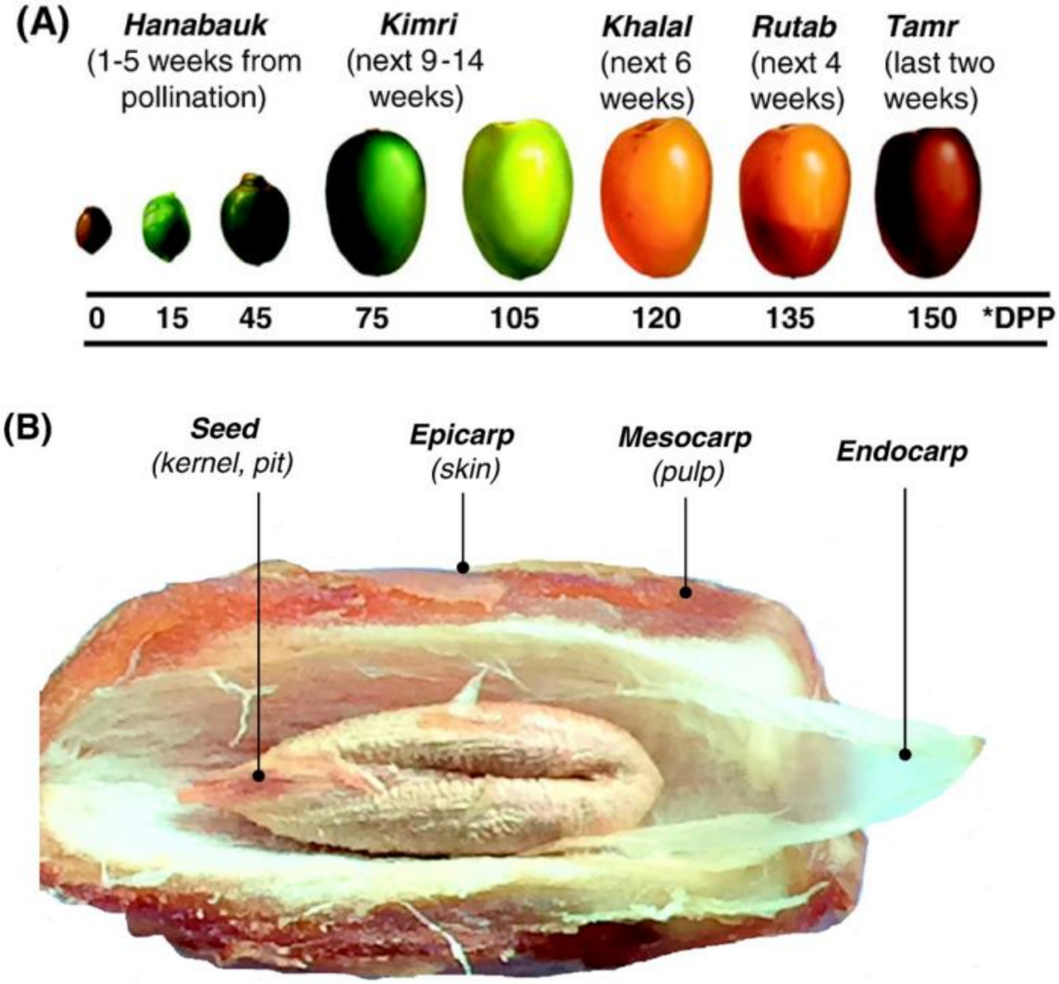


Figure 2.2 Dates developmental stage and its anatomy **A**) Different fruiting stages of date palm according to DPP* (days post-pollination) showing Khalal, Rutab and Tamr, the three edible stages of the fruit **B**) The anatomy of the date fruit at Tamr stage showing the epicarp, mesocarp, endocarp and seed (Ghnimi *et al*., 2016).

Several research studies revealed date fruits have medicinal properties that have beneficial effect on human health and diseases prevention, such as strong antioxidant, anticancer and antiviral activity, gastroprotective effects, antimutagenic properties, and hepatoprotective effects (Mohamed and Al-Okbi, 2004; Alhaider *et al*., 2017). The dates extract used as pre-treatments to reduced necrosis and edema, and restored the cardiomyocytes architecture and preserved cardiac muscle fiber morphology (Alhaider *et* *al*., 2017). From dates many value added products could be made in industry such as pickles, chutney, jam, jelly, date-in-syrup, date butter, candy, date bars (Ghnimi *et al.*, 2016). The trunk of palms are used to construct houses, hives, other types of furniture (Figure 2.3d,e), packing material for local transportation of fruits and vegetables, light foot bridges, tables, benches, flowerpots, small artworks, and shoe soles (Johnson, 2012; Al-Jabri, 2014; Fatima *et.al*., 2016).

The whole leaves of date palm are convenient to build fences and roof; the mid-ribs and leaflets of fronds are used to make window screens, mats for sitting, fish traps, chicken coops, carrying sacks, baskets, staple dishes, hand fans, food mats and covers, ropes, and brooms and brushes (Shabib *et al*., 2017; Darwish and Eldeeb, 2023) (Figure 2.3a, b). Like lettuce the terminal buds eaten as a salad while rachises are used for paper making (Barreveld, 1993; Khiari *et al*., 2011; El- Hadrami and Al Khayri, 2012). The fruit stalks could be used for decorations in houses (Kumar *et al*., 2014**)** and the fruit stalk also used for fuel and to make rope and; the fibre, for cordage and packing material (Barreveld, 1993). Seeds of date palm immerse in water to become stock feed for camels, sheep, goats and horses, or crushed dried seed added to chicken feed and seeds also roasted and powdered used as an extender to coffee or flour (Hadrami and Al-Khayri, 2012). Sap (juice) can be processed traditionally from date palm and used as a beverage either fresh or fermented (Barreveld, 1993; Marwat, *et al.*, 2012) (Figure 2.3c).

Date palm fiber produced from trunk and leaves can be transformed into several applications such as in textile, sports item, baggage, automobiles, cabinets, mats and also used for making machine parts like transmission cloth, air-bag tying cords, conveyor belt cord and some cloths for industry uses (Chao and Krueger, 2007; Ghori *et al*., 2017).

Date palm residues could be an important and sustainable source for clean and green energy to meet the rising demand taking place in date palm producing countries (Tahir *et al*., 2021).

Trees of date palm offer socio-economic benefits for local peoples of these date palm producing countries. In addition to being a staple food, it also serves as income generation by selling the fruit as well as its products, and also date palm cultivation and production can create job opportunities for local people during harvesting and, processing and packing of date palm products in industries (Johnson, 2012; Fatima *et al.*, 2016). The long life span and drought resistance nature of the date palms could have great ecological and environmental eminent by forming "oases" (green areas in the dessert) in dessert. These oases provide many ecosystem services that are used as habitats for diverse wildlife including small plants, animals and microorganisms (Grenade, 2013) and enhancing settlements of human by forming comfortable microclimate which is more suitable for sustaining life and reducing the damage caused by sandstorms and wind erosion (Shabib *et al*., 2017; Bisht *et al*., 2020).

2.3. Genetic diversity and genetic markers on date palms

Obtaining sufficient information on the extent and pattern of genetic diversity, population differentiation occurring over geographical ranges, and understanding of the ecological and genetic relationships among individuals and populations are essential for establishing guidelines on utilization and conservation of the genetic resources of a species (Elshibli, 2009; Zehdi-Azouzi *et al*., 2015). Characterisation and analysis of the available genetic diversity therefore constitute an indispensable step with regard to the development of breeding and conservation strategies.

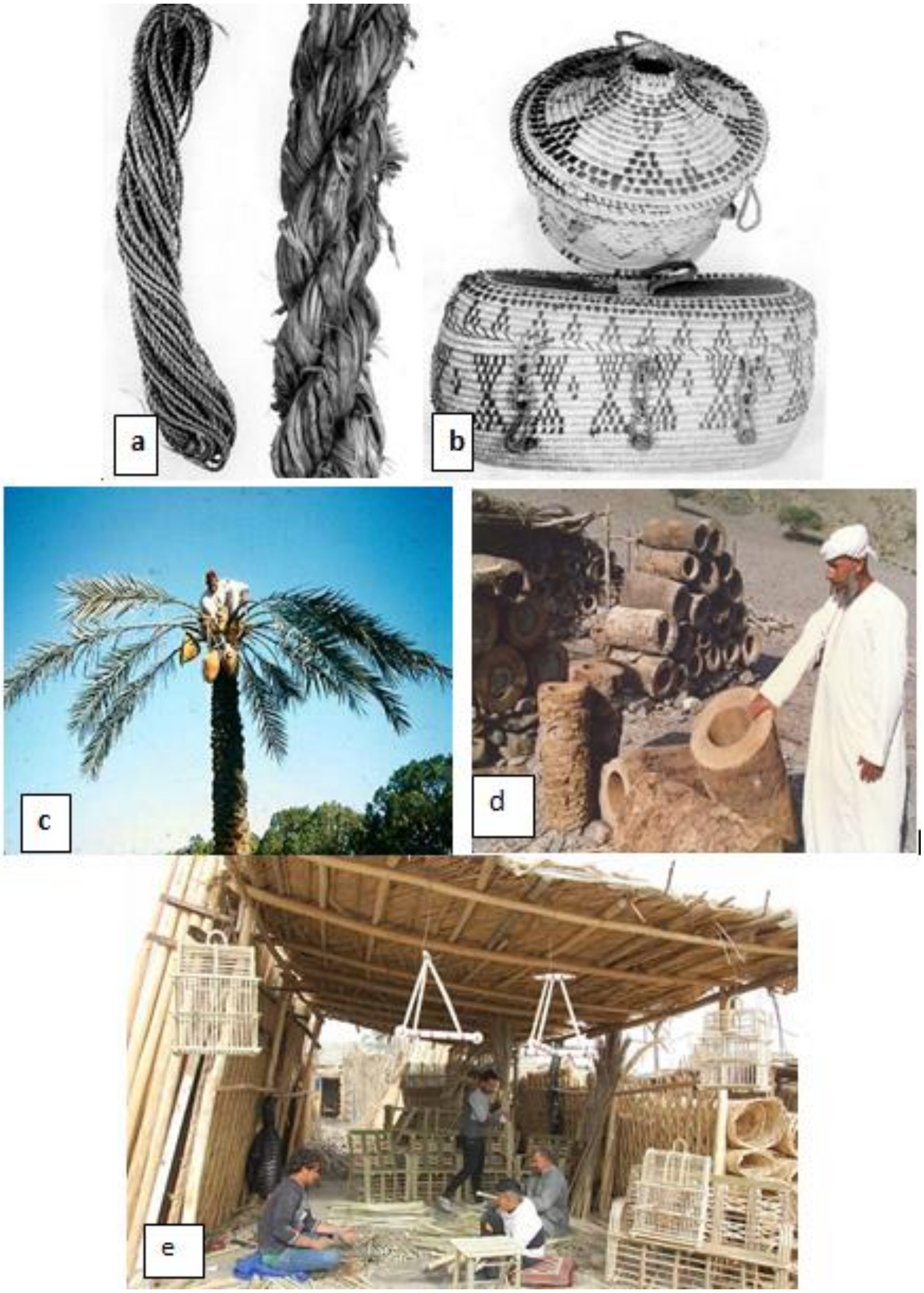


Figure 2.3 Uses of parts of date palm tree a) heavy rope made from shredded date palm fruit stalks b) Containers for domestic use made out of fibre of the leaflets wrapped around cores derived from fibre cut off the fruit stalk **(**Barreveld, 1993**)** c) harvesting sap from a date palm tree d) hives made from date palm trunk e) traditional crafts for making home furniture using date palm parts

There are more than 2000 date palm varieties that are grown around the globe which have difference in colour, flavour, shape, size and ripening time (Elmeer *et al*., 2017; Yusuf *et al*., 2015). The most common characteristics that are used to identify variation among date palm varieties are using morphology of leaves, spines and fruit, particularly for introduced date palm cultivars (Al-Khalifah *et al*., 2012; Ahmed and Al-Qaradawi, 2009).

Assessment of diversity and phylogenetic relation of date palm cultivars using morphological markers have been reported in Tunisia (Hamza *et al*., 2011; Ouarda*et al*., 2012; Kadri *et al*., 2019; Karim *et al*., 2021), Algeria (Simozrag *et al*., 2016; Bedjaoui and Benbouza, 2018; Abdelkrim *et al*., 2020), Iraq (Khierallah and Azhar, 2016), Egypt (El-Sharabasy and Rizk, 2005; Eissa *et al*., 2009; Ibrahim *et al*., 2014), Sudan (Elsafy *et al*., 2015), Pakistan (Haider *et al*, 2015; Faqir *et al*., 2018), Iran (Khankahdani and Bagheri, 2019), Saudi Arabia (Al-Khalifah *et al*., 2012), Morocco (Elhoumaizi *et al*., 2002), Nigeria (Odewale *et al*., 2013) and Ethiopia (Workia Ahmed *et al*., 2023). However, morphological traits are often unreliable or imprecise indicators of plant genotype because they are influenced by environmental conditions and vary with the developmental stages of plants (Mirbahar *et al*., 2014; Elmeer *et al*., 2017).

The identification and evaluation of genetic diversity among cultivars on the bases of morphological markers is not possible until the onset of fruiting, which takes three to five years (Elmeer and Mattat, 2015; Yusuf *et al*., 2015; Khalifa *et al*., 2016**)**. Furthermore, characterising varieties requires a large set of phenotypic data that is difficult to access statistically and shows variations because of environmental factor effects (Haider *et al*., 2012; Zhao *et al*., 2013; Elmeer *et al*., 2017). Furthermore, biochemical approaches, including isoenzyme analysis, have been used to characterize date palms but such biochemical markers are limiting due to low levels of polymorphism (Al-Jibouri and Adham, 1990) and discrimination among closely related cultivars and clones based on morphometric descriptors is often difficult (Ahmed and Al-Qaradawi, 2009; Haider *et al*., 2012). Consequently, DNA based techniques have been developed and proved effective to assess genetic diversity, because they provide a nearly unlimited potential of markers to uncover differences at the molecular level and overcome the disadvantages of morphological markers and biochemical approach using isoenzymes (zehdi *et al*., 2004; Haider *et al*., 2012; Racchi *et al*., 2014).

Several DNA marker systems have been reported on the genetic diversity within and among date palm cultivars in many middle east countries such as SSR (Zhao *et al*., 2013), ISSR (Elsheikh *et al*., 2014), ISSR and SRAP (Khalifa *et al*., 2016), SSR (Racchi *et al*., 2014) in Libya; ISSR, SSR, RAPD (Srivashtav *et al*., 2004) in India; ISSR & RAPD (Marsafari and Mehrabi, 2013), RAPD (Modareskiain *et al*., 2015), SSR and RAPD (Marsafari and Mehrabiin, 2013) in Iran; RAPD (Mirbahar *et al*., 2014), in Pakistan; RAPD, ISSR (Khierallah *et al*., 2014) in Iraq; SSR (zehdi *et al*., 2004) in Tunisia; ISSR and RAPD (Elmeer *et al*., 2017), SSR (Ahmed and Al-Qaradawi, 2009; Elmeer and Mattat, 2015) in Qatar; RAPD (Emoghene *et al*, 2015), SSR (Yusuf *et al*., 2015) in Nigeria; RAPD and morphological marker (Al-Khalifah *et al*., 2012) in Saudi Arabia; RAPD and ISSR (Haider *et al*., 2012) in Syria; RAPD (Sedra *et al*., 1998), SSR (Bodian *et al*., 2014) in Morocco; SSR (Moussouni *et al*., 2017) in Algeria; SSR and ISSR in Ethiopia (Workia Ahmed *et al*., 2021; Takele *et al*., 2021). Indeed, many research studies on date palm genetic diversity using different molecular markers were reported in detail (Rahman *et al*., 2022).

2.4. Breeding and genomics status of date palm

Crop improvement in date palm is very slow using conventional methods. This is because breeding of date palm is hindered by the long generation time and high heterogeneity in nature (El Hadrami and El Hadrami, 2009; Al-Khayri, 2012). Therefore, plant tissue culture can be used to reduce the time required to achieve crop improvement in terms of fruit size and quality and height of trees. In Kuwait, breeding of date palm cultivars have successfully implemented through convectional breeding techniches by pollinating tall date palm by dwarf date palm (T x D) and followed by plant tissue culture techniques to produce hybrid date palms (Sudhersan *et al.,* 2009). In that program, female date palm cultivars ‘Barhi’, ‘Madjhool’, and ‘Sultana’ were pollinated with male pollen of dwarf date palm (*Phoenix pusilla*). Fertilization followed by fruit set was achieved in all three female date palm cultivars. However, seed development was arrested followed by embryo abortion. Immature hybrid embryos from the three cultivars were successfully isolated from the sterilized immature fruits and cultures were established *in vitro*. Tall X Dwarf hybrid plantlets of date palms were acclimatized and preceded planted in the field. This is believed to be the first successful trial on T x D hybrid production in date palms (Sudhersan *et al*., 2009).

Although other attempts at date fruit production of new genotypes with better resistance to bayoud *(Fusariumor)* or good fruit quality have been conducted in USA, Algeria and Morocco, so far these research did not lead to the expected results due to the difficulties encountered in working with date palm, requiring a long time for regenerating F1 and F2 progenies and for back-crossing them (up to 30 years) (El Hadrami and El Hadrami, 2009; Rivas *et al*., 2012). Further, few studies have been published on MAS employment in date palm breeding and improvement. Khierallah (2015) reported the use of AFLP markers to study two F1 and F4 backcrossed populations to tentatively map traits related to the fruit and seed weights, seed volume and the total soluble solids (TSS), as well as other measured morphological characters. In that study, AFLP markers on parental clones and tested 35 primer combinations were used to detect polymorphic markers for further construction of a linkage map**.** However, from socio economics point of view of date palm, the research works on breeding and improvement is inadequate. Thus, further date palm breeding programs using biotechnological approachs will be necessary in ordert o enhance the quality of dates, yields and to produce disease resistance cultivars. Therefore, chloroplast, mitochondrial and nuclear genomes sequencing and making genetic maps of date palm cultivars can simplify breeders work.

The date palm contains 36 chromosomes (2n=36) and there are more than 2,000 date palm varieties grow around the globe; (Yusuf *et al*., 2015; Elmeer *et al*., 2017). Previously, in some cultivars of date palm the nuclear DNA, mitochondria DNA and chloroplast DNA sequences achievements have been made. In comparison with the high number of date palm cultivars present in the world molecular genetics research investment made on some date palm cultivars and the overall molecular toolbox for the date palm is still limited and not efficient relatively with other fruit crops.

The first draft nuclear genome for a Khalas variety female date palm was assembled based on data generated from the Illumina GAII sequencing platform by a research team in Qatar (Al-Dous *et al*., 2011), which results in an assembly of 56000 scaffolds representing 380 Mb in size, annotating 25000 genes of the genome. Subsequently, at the same time they sequenced other eight cultivars, including females of the Deglet Noor and Medjool varieties and their backcrossed males, identified >3.5 million polymorphic sites, including >10,000 genic copy number variations that segregated with gender and were useful in discriminating between male and female (Al-Dous *et al*., 2011). Although in Saudi Arabia a second draft of the date palm genome was published in 2013 (Al-Mssallem *et al*., 2013) for the same cultivar and assembled around 88000 scaffolds, which represent around 605 Mb in size and annotating 41660 genes. Additionally, they also constructed transcriptomic profiles for fruit development based on pyrosequencing data (Al-Mssallem *et al*., 2013).

The transcriptomic data indicated that its stress resistance and sugar metabolism-related genes tend to be enriched in the chromosomal regions where the density of single-nucleotide polymorphisms is relatively low and the date palm's unique sugar metabolism that underlies fruit development and ripening. Yang *et al.* (2010) revealed the complete chloroplast (cp) genome sequence of date palm cultivar Khalas using the next-generation sequencing method - pyrosequencing (Roche GS FLX). They also described details in genome assembly, annotation, and comparative analysis, as well as information on sequence variations and trancriptomics for the date palm cp genome.

Based on next-generation sequence platforms using pyrosequencing (Roche GS FLX) and ligation-based sequencing (Life Technologies SOLiD), the mitochondrial (mt) genome of date palm (*Phoenix dactylifera* L.) into a circular molecule of 715,001bp in length was assembled (Fang *et al*., 2012). Based on the data from three date palm cultivars (Khalas, Fahal, and Sukry), they also investigated RNA editing sites and SNPs within the species (Fang *et al*., 2012). Alfaifi *et al*. (2015) reported expressed sequence tags (ESTs) generated from a normalized cDNA library of the date palm cv. Sukary to uncover transcriptomic profiles. The functional annotation showed that the majority of the ESTs were associated with binding (44%), catalytic (40%), transporter (5%) and structural molecular activities (5%). Further, Alfaifi *et al*. (2015) reported that the analysis showed that some ESTs were categorized as stress/defense and fruit development related genes. Moreover, Mathew *et al.* (2013) have generated genome-wide genotyping data for 13,000 - 65,000 SNPs in a diverse set of date palm fruit and leaf samples, which identified genomic regions with high densities of geographically segregating SNPs and also observed higher levels of allele fixation on the recently described X-chromosome than on the autosomes.

According to Yaish *et al.* (2017), RNA-seq libraries have been generated from leaves and roots of NaCl-treated and control seedlings of Khalas date palms to understand the molecular bases of salt tolerance or adaptation in this important crop plant. Consequently, the study revealed that genes and pathways that are associated with responses to elevated NaCl levels and thus may play important roles in salt tolerance providing a foundation for functional characterization of salt stress-responsive genes in the date palm. The Mathew *et al.* (2014) constructed the first genetic map for date palm and identified the putative date palm sex chromosome based on a modified genotyping-by-sequencing approach (GBS). Thus, the resulted in 4000 markers were placed on the map using nearly 1200 framework markers spanning a total of 1293cM. Also they integrated the genetic map, derived from the Khalas cultivar, with the draft genome and placed up to

19% of the draft genome sequence scaffolds onto linkage groups for the first time. Besides, a medium density genetic map of 111date palm was constructed using 53 individuals from BC1 and 30 individuals from F_1 populations (Al-Mamari, 2013). The BC1 map consisted of 28 SSR and 242 SNP distributed into 29 linkage groups with total genetic length of 1.486.7cM, while the F1 map consisted of 21 SSR and 570 SNP distributed into 30 linkage groups with total genetic length of 2,385.6cM. A total of 25 combined linkage groups were possible by combining both BC1 and F1 maps through common markers. Hazzouri *et al*. (2015) also presented a comprehensive catalogue of approximately seven million SNPs in date palms based on whole genome re-sequencing of a collection of 62 cultivars, and then population structure analysis indicated a major genetic divide between North Africa and the Middle East/South Asian date palms, with evidence of admixture in cultivars from Egypt and Sudan. Rahman *et al*. (2022) reviewed in detail about the transcriptomic studies, trait-specific markers studies, whole-genome re-sequencing of date palms and displayed the advances in genome sequencing of the date palm in diagram (Figure 2.4). Recently, whole mitochondrial and chloroplast genome of 171 Tunisian date palm cultivars were also sequenced to determine their diversity and evolutionary relationships (Hamza *et al*., 2023).

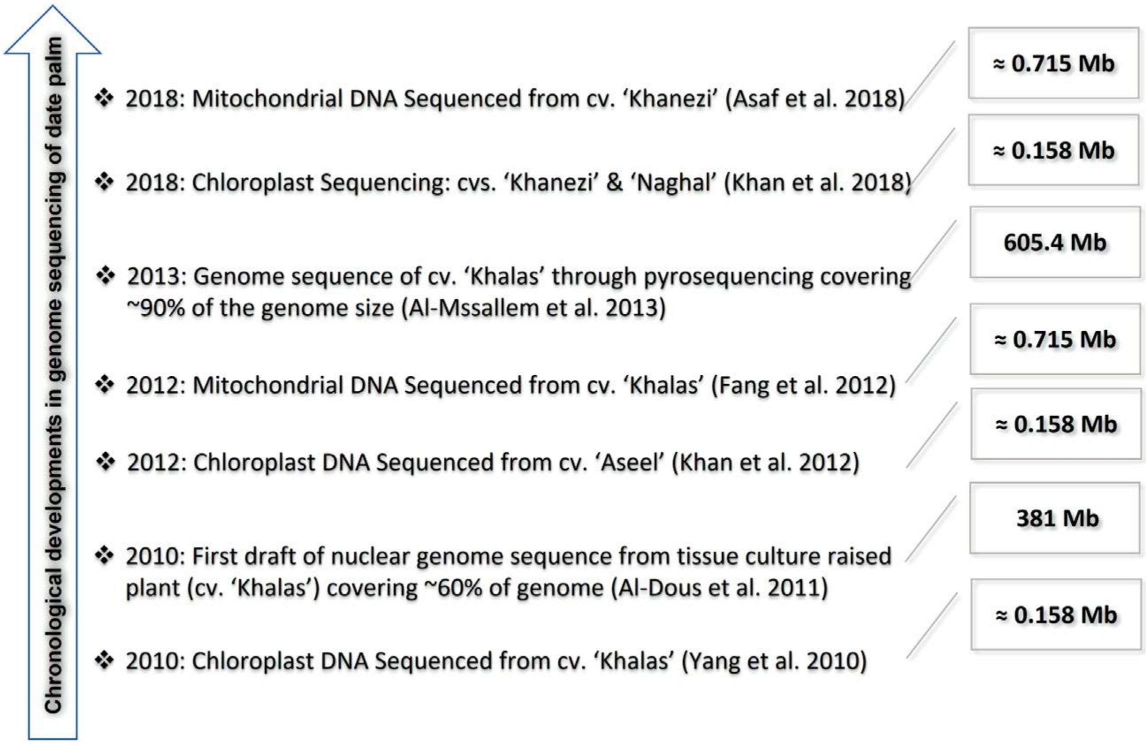


Figure 2.4 Advances in genome sequencing of the date palm (Rahman *et al*., 2022).

2.5. Date palm conservation

Preservation of the genetic diversity represented in all plant ecosystems of the world has become a major issue of international concern. The importance of plant germplasm is universally accepted as a resource of common heritage to be used freely by all humankind in the maintenance, development and improvement of food, feed, fiber and industrial crops. There is a need to share genetic resources and to assume responsibility to conserve and preserve plant germplasm for the future. Hence, the conservation of date palm genetic resources has become a critical issue for the development of date palm production and food security particularly in desert and semi-desert areas (Zehdi-Azouzi *et al*., 2015). Therefore, countries which hold significant amounts of genetic diversity of date palm have a great responsibility to conserve and safe guard date palm germplasm to utilize for genetic improvement and development of crop cultivars for domestic and foreign markets. Conventionally, germplasms are maintained in the form of seeds because they occupy a relatively small space and can be stored for many years. They can be easily dried and packed for transmission to the introduction centres and gene banks. The limitations of this method however are the loss of viability of seeds with the passage of time and their destruction by pathogens and pest attack; discrete clones cannot be maintained except for apomictic species and it is not applicable to vegetatively propagated crops (Kaviani, 2011). On the other hand, the cost of maintaining a large proportion of the available genotypes of these plants in nurseries or fields is extremely high and there is a risk of material being lost as a result of environmental hazards.

Date palm is a dioecious fruit tree that is vegetatively propagated through offshoots. Thus, its germplasm cannot be stored or handled easily using conventional means. The most common method to preserve the genetic resources of date palm is by *in situ* conservation method (Jain, 2011; Bekheet and Taha, 2013). However, there are several problems with the field gene bank **(**Bekheet and Taha, 2013). The collections are exposed to natural disasters and attack by pests and pathogens. Moreover, *in situ* conservation is labour intensive and costly. In addition, distribution and exchange from field gene-bank is difficult because of the vegetative nature of the material and the greater risk of disease transfer.

There is a need to alternative methods of propagation and conservation of date palm cultivars. Plant tissue culture is one of a method for multiplication and storage of date palm germplasm. Molecular biology techniques are also great interest for identifying and characterization cultivars (Jain, 2011; Khan *et al*., 2011). *In vitro* conservation involves the maintenance of explants in a sterile and pathogen free environment. Miniaturization of explants allows reduction in space requirements and consequently labor cost for the maintenance of germplasm collections (Bekheet, 2013; El-Bahr *et al.*, 2016).

There are two main methods of *in vitro* conservation of plant germplasm. First is by reducing growth which is achieved by modifying the culture medium or low temperature incubation (El-Ashry *et al*., 2013; Cruz-Cruz *et al*., 2013). *In vitro* storage under slow growth conditions delays the necessity for sub culturing and consequently allows efficient utilization of labor year round (El-Bahr *et al.,* 2016; Cruz-Cruz *et al*., 2013; Jain, 2011). Second, cryopreservation is storage between-79 and -196°C, the low extreme being the temperature of liquid nitrogen **(**Mortazavi *et al*., 2010**;** Jain, 2011; Cruz-Cruz *et al*., 2013). In this technique, cryoprotectant treatment is given before plunging the tissue in the liquid nitrogen for preventing ice crystal formation in the tissue in order to avoid any damage to the tissue that may adversely affect plant regeneration upon thawing of cryo-stored material (Jain, 2011). The common cryoprotectants are polyethylene glycol (PEG), glucose, and dimethylsulfoxide (DMSO).

Therefore, tissue culture is considered as an alternative method for date palm conservation that can eliminate the obstacles of field conservation. With the development of *in vitro* culture techniques- organogenesis, somatic embryogenesis and embryo rescue–plant regeneration has already been accomplished. Thus, these have made possible short - and long-term storage of *in vitro* cultures, large-scale multiplication of plants of elite genotypes including date palm cultivars (Jain, 2011). Several groups have engaged to optimize *in vitro* conservation protocol of date palm cultivars using *in vitro* regeneration techniques.

2.6. Reproduction and adaptation of date palm

Mechanism of reproduction

Date palms are reproduces sexually by seeds and asexually using suckers and tissue culture techniques. Reproduction of date palms by seed is much faster and easier than using the offshoot and other vegetative propagation methods. Propagation by seeds commonly produces 50% progenies of each male and female plant. Consequently, this situation could enhance the levels of genetic diversity within populations, especially with natural pollination (Zaid and Wet, 2002). Propagation by offshoots is the only method to maintain the true to type of date palm cultivars (Abul-Soad and Mahdi, 2020). These offshoots are produced from axillary buds at the base of the trunk during the juvenile life of the palm. Offshoots are cut with a chisel from the mother plant and transplanted into a nursery adjusted suitable environment in order to induce well root systems. After one year, young date palms are transplanted to their permanent place. The right time of transplantation depends on the development of the root system as well as on the number of palm leaves (10 to 12 are recommended) (Bekheet, 2013; Johnson *et al*., 2013).

The offshoots propagation system has also many limitations such as slow propagation rate, transmission of disease-causing pathogens and insects and production of offshoots in a limited number for a certain period in the life time of a young palm tree (Eke *et al*., 2005; Rad *et al*., 2015), especially most date palms produce 10-20 offshoots during the life spans of cultivars (Bekheet, 2013). Moreover, some cultivars do not produce offshoots at all and also rooting of these offshoots is very difficult (Eke *et al.,* 2005; Rad *et al*., 2015). In order to overcome such kinds of problems plant tissue culture is an alternative method for vegetative propagation of plants including date palm.

Date palm is micro-propagated by somatic embryogenesis (plantlets formed from embryogenic callus) and organogenesis which gives plantlets without passing through the callus phase. Several research studies have been attempted through organogenesis and somatic embryogenesis *in vitro* propagation of date palm cultivars (Eke *et al*., 2005; Eshraghi *et al*., 2005; Taha *et al*., 2007; Alkhateeb, 2008; Gabr and Abd-Alla, 2010; Al-Kharyi, 2010; Al-Khalifah and Shanavaskhan, 2012; Khan and Bi Bi, 2012; Bekheet, 2013; Mazri and Meziani, 2013; Jazinizadeh *et al*., 2015; Rad *et al*., 2015; Diab, 2015; Hassan *et al*., 2021).

Mechanism adaptation

When plants exposed to different abiotic and biotic stresses they have developed different morphological, biochemical, and physiological adaptation mechanisms to survive or adapt to unfavorable conditions. Among plants date palm is one of the desert plants that subjected to high UV radiation, drought, salinity, high temperature. Date palms can mitigate the effects of these stresses with diverse and complex sensing, signaling, and response pathways to determine their capacity of tolerance. Research studies revealed date palms tolerance responses generate by modifying characters like number of pinnae, plant height, adjustment of root/shoot characters, induced leaf senescence, stomatal conductance and demand functions - apparent in the Jmax/Vmax ratio (Elshibli, 2009). Date palms maintain thick, waxy cuticle and pinnately compound leaves covered with many spines, which insulate the tip growing point and reduce evaporation and, the deep root system maximize water uptake, thus traits contribute to plant tolerance for drought stress (Hazzouri *et al*., 2020).

Although, as like other plants date palm respond to salt stress by initiates the production of osmolytes, compatible solutes including proline and improve effects of ROS by increasing the expression of anti-oxidant enzymes and concentrations of anti-oxidant metabolites (Du *et al*., 2019; Du *et al*., 2021Hazzouri *et al*., 2020). Indeed, date palms with high salinity stress can survive by maintaining the ion influx and efflux in the plasma membrane, ion compartmentation in vacuoles, and regulating the osmotic balance the production of suitable solutes such as sugars and sugar alcohols, amines, and amino acids (Hazzouri *et al*., 2020; Ali *et al*., 2024). Yaish and Kumar (2015) reported that date palm varieties can tolerate up to 9 dS m-1 soil salinity, and an excess of Na+ ions accumulated in the leaves of the plants treated with high salt concentrations. Moreover, the enhanced level of compatible N-containing solutes, as well as galactinol and raffinose contributed to prevent oxidative damage to date palm leaves, indicating species-specific physiological responses to climate change (Du *et al*., 2019). Antioxidants and compatible solutes, membrane fatty acids, and phenolic compounds, acting as cell wall constituents and ROS scavengers, play significant roles in date palm leaves in response to changing climate (Du *et al*., 2019).

2.7. Date palm research status in Ethiopia

Date palm is an oldest plant with its long life history of cultivation and production in desert parts of the world. In Ethiopia, date palm plantation began 200 years ago in Northeast part of the country particularly in Afar region along the Awash river bank and spread to Dire Dawa, Somalli and Benishangul-Gumuz (Aregawi Lemlem *et al*., 2018). The date palm production is important for agro-pastoralists to settle in Afar region. Unadvanced date palm agricultural practices and improper postharvest employed by agro-pastoralists produce very low date fruits yield and low quality compared with other date palm producing countries (Aregawi Lemlem *et al*., 2019). The produced date fruits are mainly used for consumption by their own and marketing for nearby towns. Recently, the government of Ethiopia particularly Afar region in collaboration with FAO (Food and Agricultural Organization) date palm farms established at Asayta and Afambo districts and Werer Agricultural Research Center (WARC). The farm includes date palm cultivars collected from Afar region and eastern parts of Ethiopia (Dire Dawa) and early introduced *in vitro* date palm cultivars.

To enhance the number of these desired date palm tree, some attempts made by asexual reproduction using traditional off-shoot propagation system and plant tissue culture techniques. These attempts were unsuccessful due to different constraints such as offshoot inadaptable with the new environment (the problem of inducing roots in suckers), exposure of fungal and endophytic bacterial contaminations for explant, the nature of the plant itself i.e., plant woodiness, high phenol release, and a lack of initial suckers (Nitsuh Aschale, 2022). Some research studies on different aspect of date palm in Ethiopia have been reported (Table 2.1).

Table 2.1 List of research conducted on date palms in Ethiopia

No.	**Research title**	**Study site**	**Reference**
1	Date palm production practices and constraints in the value chain in Afar regional state, Ethiopia	Afambo and Assayta districts	Aregawi Lemlem *et al*., 2018
2	Diversification of livelihoods through date palm production in agro-pastoral areas of afar region, Ethiopia	Afambo and Assayta districts	Aregawi Lemlem *et al*., 2019
3	Survey of date palm (*Phoenix dactylifera* L.) insect pest at Afar region of Ethiopia	Werer Agricultural Research Center and Assayta and Afambo	Zemedkun Alemu and Workishet Taye (2020)

		districts	
4	Genetic diversity and population structure of date palms (*Phoenix dactylifera* L.) in Ethiopia using microsatellite markers	Afambo and Assayta districts, Eerrer Gota district in Somalii region and Dire Dawa	Workia Ahmed *et al*., 2021
5	Genetic diversity assessment in some landraces and varieties of date palm (*Phoenix dactylifera* L.) from Afar Region, Ethiopia, using ISSR markers	Gewane, Assayta and Afambo district	Takele *et al*., 2021
6	Varietal Evaluation of Introduced Date Palm (*Phoenix dactylifera* L.) under Irrigated Condition of Afar Region, Ethiopia	Werer Agricultural Research Center Assayta and Afambo	Yitages Kuma and Niguse Chewaka, 2022
7	Status of Date Palm (*Phoenix dactylifera* L.) Production Practices and Post-Harvest Handling in Afar, Ethiopia: Results of Baseline Survey	Afambo and Assayta districts	Megersa Daba *et al*., 2022a
8	Carbon stock estimation of mixed-age date palm (Phoenix dactylifera L.) farms in northeastern Ethiopia	Afambo and Assayta districts	Mulugeta Betemariyam & Tamiru Kefalew, 2022
9	*In vitro* protocol optimization for mass propagation of elite date palm varieties in Ethiopia: Short communications	Melkawere Research Center and the Afambo (introduced date palm)	Nitsuh Aschale, 2022
10	Evaluation and Registration of Introduced Date Palm (*Phoenix dactylifera* L.) Varieties in Afar Region, Ethiopia	Werer Agricultural Research Center and Assayta and Afambo districts	Megersa Daba *et al*., 2022b
11	Determinants of market participation decision and intensity among date producers in Afar region, Ethiopia: A Double Hurdle Approach	Afambo and Asaiyta districts	Dagmawe Menelek Asfaw *et al*., 2022
12	Evaluation of phenotypic relationships of date palm cultivars at Melka Werer, Ethiopia	Werer Agricultural Research Center	Workia Ahmed *et al*., 2023

Chapter 3

Evaluation of phenotypic relationships of date palm cultivars at Melka Werer, Ethiopia

Abstract

Date palm is one of the oldest perennial trees in Arecaceae family. Date palms are dioecious with a wide range of phenotypic variations. Therefore, evaluation of date palm cultivars using morphological characters is critically necessary for utilization of its genetic resources and improvement and conservation programs. The objective of this study is to assess the phenotypic relationships of date palm cultivars. A total of 45 morphological traits were used and all traits showed mean variations among the cultivars. Principal component analysis on the first component revealed 37% variation in vegetative and reproductive traits data combined together and 29% and 32% of variations was observed in separate vegetative and reproductive morphological traits data, respectively. Among 45 morphological traits, 43 traits exhibited significant differences at $p < 0.05$ in analysis of variance.In addition, 23 traits showed significant variances at $p < 0.05$ in homogeneity of variance analysis among cultivars. Besides, dendrograms were constructed based on combined vegetative and reproductive traits data and in separate vegetative and reproductive traits data. They showed the genetic relationships between date palm cultivars. The result of this study is important as baseline for documenting and further agronomic characterizations of date palm cultivars particularly in Ethiopia.

Key words: *ANOVA, Cultivars, Morphological traits, Principal component*

3.1. Introduction

Date palm (*Phoenix dactylifera* L.) is a monocotyledon dioecious perennial tree that belongs to Arecaceae (Palms) family under the genus of *Phoenix*. It produces edible dietary fruits and parts of a tree having multi-purpose (Al-Khalifah and Shanavaskhan, 2012; Johnson, 2012; El-Far *et al*., 2016).

Perennial crops including date palms have diverse life history and breeding systems unlike annual crops. In date palms phenotypic traits, especially, the reproductive parts have great role to discriminate between cultivars (Simozrag *et al*., 2016; Bedjaoui and Benbouza, 2018). Assessment of diversity and phylogenetic relation of date palm cultivars using morphological data have been reported in Tunisia (Hamza *et al*., 2011; Ouarda*et al*., 2012; Kadri *et al*., 2019; Karim *et al*., 2021), Algeria (Simozrag *et al*., 2016; Bedjaoui and Benbouza, 2018; Abdelkrim *et al*., 2020), Iraq (Khierallah and Azhar, 2016), Egypt (El-Sharabasy and Rizk, 2005; Eissa *et al*., 2009; Ibrahim *et al*., 2014), Sudan (Elsafy *et al.*, 2015), Pakistan (Haider *et al*, 2015; Faqir *et al*., 2018), Iran (Khankahdani and Bagheri, 2019), Saudi Arabia (Al-Khalifah *et al*., 2012), Morocco (Elhoumaizi *et al*., 2002) and Nigeria (Odewale *et al*., 2013).

Date palm cultivars exhibits wide ranges of phenotypic variations due to their out-breeding nature as well as dates are as one of staple fruit that easily distributed commercially throughout the world. These factors enhance the evolutionary diversification and adaptability of palms out of native area. Hence, to enhance and sustain date palm genetic resource utility effective identification of date palm cultivars is critically important at regional and global level. Therefore, the objective of the present study is to assess the phenotypic relationship of early introduced date palm cultivars using morphological traits that are cultivated at Melka Werer research centre, Ethiopia.

3.2. Materials and methods

3.2.1. Description of the study area

Morphological study was conducted at Melka Werer Agricultural Research Centre which is located at 10°9′59N latitude and 40°8′43E longitude in Amibara district of Afar Regional State of Ethiopia. The altitude of this area is 560 meters above sea level and the climate is hot and dry and the mean annual temperature of this area is 29.5°C.

3.2.2. Description of the plant material

The date palm cultivars of this study were recently introduced from England and Israel. Elven date palms cultivars and 45 morphological traits were used (Table 3.1). These cultivars were planted at the same time and equally irrigated from Awash River throughout a year. These cultivars are namely: Saggii, Khalas, Berhee, Ashal Al Hassa, Madjool England, Medjool Israel, Zamlli, Shishi, Khyara, Khadrawy and Jarvis (male). These cultivars were planted with 10 meters gaps between rows as well as palm trees. Morphological data using their vegetative and reproductive characters were collected from 11 date palm cultivars and each cultivar has four replications (4 trees used). One frond per tree and four fronds per cultivar and a total of 44 fronds were used to measure leaf traits; three fruits per tree and 12 fruits per cultivar and a total of 120 fruits from 10 cultivars (females) were collected to study phenotypic variation of date fruits.

Table 3.1 Morphological traits and their codes that were used for analysis

Vegetative traits	Code	Unit	Reproductive traits	Code	Unit
Trunk height	T1	Cm	Peduncle length from its base to the first spikelet	T26	Cm
Trunk circumference	T2	Cm	Peduncle width at the first spikelet	T27	Cm
Frond length	T3	Cm	Ramified bunch's part length	T28	Cm
Frond width at the middle	T4	Cm	Spikelet's length at the bunch's bottom	T29	Cm
Rachis length (from petiole to terminal leaflet base)	T5	M	Spikelet's length at the bunch's middle	T30	Cm
Rachis length (spiny part)	T6	Cm	Spikelet's length at the bunch's top	T31	Cm
Rachis length (1st Leaflet to terminal leaflet base)	T7	Cm	number of fruits per cluster (spikelet)	T32	G
Rachis thickness	T8	Cm	weight of fruits per cluster (spikelet)	T33	G
petiole length	T9	Cm	number of cluster (spikelet) per bunch	T34	Count
Petiole width	T10	Cm	weight of date fruits per bunch	T35	Kg
Number of pinnae (right side)	T11	Count	average weight of date fruits per tree	T36	Kg
Number of pinnae (left side)	T12	Count	No. Bunch per tree	T37	Count
Basal pinnae length	T13	Cm	Fruit weight	T38	G
Basal pinnae width	T14	Cm	Fruit length	T39	Mm
Median pinnae length	T15	Cm	Fruit width	T40	Mm
Median pinnae width	T16	Cm	Pulp weight	T41	G
Apical pinnae length	T17	Cm	Pulp thickness	T42	Mm
Apical pinnae width	T18	Cm	Seed weight	T43	G
Terminal leaflet length	T19	Cm	Seed length	T44	Mm
Terminal leaflet width	T20	Cm	Seed width	T45	Mm
Last terminal leaflet number	T21	Count			
Angle on both sides of the terminal leaflets	T22	degree			
Spine number	T23	Count			
Middle spine width	T24	Cm			
Middle spine length	T25	Cm			

3.3. Morphological data analyses

Data for 45 morphological traits’ were used to calculate mean values, analysis of variance (ANOVA) using SPSS software version 28.0.1. The homogeneity of variances (HOV) of date palm cultivars was calculated using Levene’s test in SPSS software to confirm these traits had equal variance or not among the cultivars. Data for vegetative and reproductive traits were used separately to create dendrogram and Principal component analysis (PCA) biplot loading to show the relationship between date palm cultivars. PCA of biplot loading was performed to detect correlation between morphological traits and date palm cultivars using "ward" method in R package. On PCA biplot loading, small angle between any two vectors indicates a strong positive correlation between variables and large angle between any two vectors indicates negative association between two variables. Besides, vectors (arrows) on same directions and their length on PCA quadrant were showed strongly correlated variables and vice versa. Thus, these vectors discriminated and classified the variables on PCA axes (Ginanjar *et al*., 2017).

3.4. Results

3.4.1. PCA analysis and morphological relations of cultivars

In this study, mean values of all morphological traits showed variations between date palm cultivars (Table 3.2).

Table 3.2 Mean value of 45 morphological traits of 11 date palm cultivars

	Date palm cultivars										
Traits	**Behee**	**Khalas**	**Medjool England**	**Medjool Israel**	**Shishi**	**Zamlli**	**Ashal Al Hassa**	**Khyara**	**Saggii**	**Khardawy**	**Jarvis – male**
T1	207.3 ± 10.5	165.5 ± 24.7	165.5 ± 24.7	160.3 ± 27.2	256.0 ± 52.2	230.5 ± 74.1	193.0 ± 20.3	160.8 ± 34.3	204.0 ± 16.1	122.3 ± 19.1	142.0 ± 57.0
T2	238 ± 9.85	232.3 ± 10.0	221.3 ± 37.0	171.3 ± 28.7	256.3 ± 52.2	266.3 ± 25.6	248.5 ± 20.9	202.5 ± 9.6	255.0 ± 23.8	205.0 ± 10.0	230 ± 35.6
T3	463.5 ± 8.4	424.8 ± 31.6	349.0 ± 32.1	326.5 ± 31.1	360.6 ± 16.7	421.5 ± 69.0	398.3 ± 55.1	479.8 ± 9.4	419.0 ± 22.4	361.3 ± 26.3	353.0 ± 109.3
T4	89.3 ± 4.3	91.3 ± 3.8	103.5 ± 11.7	101.5 ± 8.2	97.5 ± 4.2	86.0 ± 4.9	86.5 ± 5.8	82.8 ± 3.6	116.6 ± 9.1	77.3 ± 2.9	98.5 ± 11.7
T5	435.5 ± 10.6	411.0 ± 31.2	331.8 ± 28.5	306.3 ± 31.0	342.3 ± 18.6	379.8 ± 72.9	364.0 ± 26.5	453.3 ± 11.6	391.5 ± 25.4	340.8 ± 27.5	321.0 ± 116.2
T6	104.5 ± 10.8	113.3 ± 13.4	84.0 ± 16.6	88.3 ± 18.8	91.8 ± 13.6	83.0 ± 132.8	97.0 ± 8.7	98.0 ± 11.9	76.0 ± 13.6	90.0 ± 10.2	73.5 ± 31.5
T7	293.3 ± 14.0	267.5 ± 22.8	250.8 ± 53.1	208.5 ± 19.1	232.3 ± 9.7	277.8 ± 40.0	248.3 ± 16.7	314.5 ± 7.9	290.5 ± 23.4	221.0 ± 22.7	233.5 ± 86.2
T8	10.1 ± 1.0	9.0 ± 0.4	8.5 ± 0.6	8.5 ± 1.0	10.0 ± 0.0	9.5 ± 0.6	10.0 ± 0.0	8.5 ± 0.6	10.0 ± 0.0	6.3 ± 0.5	9.0 ± 1.2
T9	170.3 ± 8.5	157.0 ± 14.9	123.3 ± 14.2	118.0 ± 16.0	128.5 ± 13.5	143.8 ± 30.1	150.0 ± 40.1	165.3 ± 12.7	128.5 ± 19.7	139.5 ± 11.5	119.5 ± 23.4
T10	13.1 ± 1.7	11.5 ± 1.1	11.3 ± 2.3	9.8 ± 1.3	14.1 ± 0.8	13.3 ± 1.7	14.3 ± 0.8	13.0 ± 2.3	14.0 ± 1.1	10.0 ± 0.5	11.0 ± 1.5
T11	100.5 ± 5.4	96.0 ± 2.1	84.0 ± 4.5	81.8 ± 7.5	89.5 ± 3.5	93.8 ± 15.6	94.8 ± 5.1	109.8 ± 5.3	106.3 ± 3.9	90.0 ± 1.4	68.5 ± 25.6
T12	103.3 ± 5.9	96.5 ± 1.0	86.0 ± 2.2	84.3 ± 6.4	89.5 ± 4.9	93.3 ± 15.6	93.5 ± 3.1	110.8 ± 5.1	107.5 ± 3.7	92.0 ± 1.3	69.0 ± 25.2
T13	51.3 ± 2.6	52.8 ± 1.7	59.8 ± 6.1	61.0 ± 11.5	48.6 ± 3.9	46.0 ± 4.8	42.3 ± 1.5	48.0 ± 3.5	67.3 ± 2.0	36.8 ± 1.3	52.3 ± 12.4
T14	2.1 ± 0.4	2.8 ± 0.6	2.1 ± 0.6	2.1 ± 0.4	2.1 ± 0.3	2.3 ± 0.6	2.0 ± 0.3	0.8 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	2.0 ± 0.3
T15	47.5 ± 1.3	44.5 ± 1.3	50.8 ± 3.3	49.5± 0.6	46.0 ± 3.0	47.0 ± 1.4	40.5 ± 1.7	38.5 ± 2.6	56.3 ± 3.3	39.0 ± 0.0	52.0 ± 5.8
T16	3.4 ± 0.6	3.4 ± 0.4	3.3 ± 0.2	3.4 ± 0.5	3.8 ± 0.4	3.8 ± 0.4	3.4 ± 0.2	2.9 ± 0.2	2.9 ± 0.4	2.6 ± 0.2	3.1 ± 0.2
T17	40.3 ± 6.1	31.5 ± 3.3	35.3 ± 4.6	37.5 ± 1.7	38.8 ± 5.1	40.8 ± 3.5	38.3 ± 4.2	39.5 ± 3.7	44.5 ± 7.2	30.0 ± 3.8	46.8 ± 8.8
T18	2.4 ± 0.4	2.8 ± 0.2	2.9 ± 0.1	3.0 ± 0.2	3.1 ± 0.5	3.4 ± 0.3	2.7 ± 0.3	2.4 ± 0.4	2.5 ± 0.3	2.2 ± 0.3	3.0 ± 0.2
T19	11.0 ± 0.7	14.2 ± 2.5	16.7 ± 5.0	22.6 ± 1.1	23.5 ± 4.1	26.4 ± 0.9	18.9 ± 1.7	25.8 ± 1.3	29.3 ± 2.6	19.5 ± 2.2	32.9 ± 11.8
T20	1.1 ± 0.3	0.8 ± 0.1	1.2 ± 0.5	1.1 ± 0.5	1.6 ± 0.5	1.3 ± 0.4	1.7 ± 0.5	1.0 ± 0.0	1.1 ± 0.0	1.4 ± 0.4	1.8 ± 0.6
T21	2.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.3	2.5 ± 0.6	3.0 ± 0.0	3.0 ± 0.0	2.75 ± 0.5	3.0 ± 0.0	3.0 ± 0.0
T22	93.3 ± 4.7	81.3 ± 6.3	90.8 ± 6.5	89.5 ± 4.9	83.0 ± 4.4	93.3 ± 4.3	86.0 ± 4.5	91.5 ± 2.4	89.8 ± 2.4	84.8 ± 3.5	82.8 ± 2.1
T23	30.8 ± 1.7	28.8 ± 1.3	29.3 ± 4.6	30.5 ± 2.4	21.0 ± 0.8	24.8 ± 4.2	34.3 ± 1.1	19.8 ± 3.3	24.3 ± 2.8	19.8 ± 0.5	27.0 ± 6.0
T24	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	0.4 ± 0.2	0.3 ± 0.0	0.3 ± 0.1	1.0 ± 0.1
T25	13.4 ± 1.9	18.2 ± 2.6	11.6 ± 3.1	10.8 ± 1.0	13.3 ± 2.7	10.8 ± 0.7	12.7 ± 1.7	13.5 ± 3.6	7.4 ± 0.6	8.9 ± 1.2	15.6 ± 4.9

T26	186.0 ± 9.7	137.0 ± 4.2	65.0 ± 6.4	62.0 ± 28.4	116.3 ± 15.5	132.0 ± 17.8	105.5 ± 19.2	126.0 ± 25.8	131.0 ± 10.1	114.0 ± 7.6	0
T27	8.4 ± 1.6	7.5 ± 0.7	8.6 ± 1.1	8.6 ± 1.8	8.3 ± 1.3	8.5 ± 1.3	9.0 ± 1.4	8.5 ± 2.1	9.3 ± 1.3	6.2 ± 0.5	0
T28	32.0 ± 6.4	17.5 ± 3.7	24.3 ± 6.5	32.8 ± 14.0	22.0 ± 11.1	19.5 ± 4.2	14.5 ± 11.2	30.5 ± 11.1	16.8 ± 1.7	27.3 ± 8.2	0
T29	54.5 ± 9.8	52.8 ± 1.9	49.0 ± 9.0	49.8± 13.9	53.0 ± 5.9	46.5 ± 6.8	48.3 ± 11.3	48.5 ± 13,4	52.0 ± 2.4	38.0 ± 1.8	0
T30	49.3 ± 5.9	45.1 ± 3.6	40.3 ± 8.4	35.0 ± 11.9	50.0 ± 6.5	44.8 ± 8.3	43.8 ± 9.9	39.0 ± 10.0	49.5 ± 4.7	30.5 ± 2.6	0
T31	26.3 ± 3.1	38.3 ± 4.6	32.3 ± 8.3	25.5 ± 7.3	37.8 ± 12.3	29.0 ± 10.9	40.0 ± 9.6	28.5 ± 6.6	40.0 ± 7.1	23.8 ± 2.1	0
T32	11.1 ± 2.5	10.8 ± 3.8	10.2 ± 1.3	7.6 ± 0.6	16.8 ± 7.1	9.2 ± 2.1	15.7 ± 5.0	13.3 ± 4.1	9.1 ± 0.7	15.9 ± 6.8	0
T33	174.0 ± 117.5	210.1 ± 122.8	198.3 ± 115.4	170.5 ± 25.2	1188.5 ± 77.5	200.7 ± 22.0	203.5 ± 104.3	338.3 ± 123.2	176.0 ± 27.2	209.6 ± 108.1	0
T34	66.3 ± 5.9	52.3 ± 9.3	61.3 ± 5.3	68.5 ± 10.5	68.5 ± 9.3	53.3 ± 3.3	66.8 ± 36.3	65.5 ± 15.2	57.3 ± 11.9	52.3 ± 6.4	0
T35	8.4 ± 4.1	3.8 ± 0.6	3.2 ± 1.3	5.5 ± 3.1	3.4 ± 1.4	4.0 ± 0.6	5.8 ± 3.0	6.7 ± 3.6	4.2 ± 1.8	3.4 ± 2.1	0
T36	131.5 ± 89.94	17.5 ± 7.67	22.75 ± 6.46	31.05 ± 25.33	11.75 ± 10.56	34.25 ± 19.36	38.75 ± 26.95	31.5 ± 16.46	38.75 ± 17.73	6.75 ± 3.72	0
T37	17.0 ± 9.1	4.3 ± 3.9	7.8 ± 2.3	4.8 ± 3.5	3.5 ± 3.0	6.8 ± 5.2	5.8 ± 2.5	5.0 ± 0.8	7.0 ± 4.1	2.5 ± 1.9	0
T38	3.6 ± 0.1	3.7 ± 0.7	5.0 ± 2.6	2.2 ± 0.6	2.6 ± 1.1	4.9 ± 1.6	6.2 ± 2.3	7.6 ± 2.2	2.2 ± 0.9	8.6 ± 1.5	0
T39	2.2 ± 0.1	2.2 ± 0.1	3.2 ± 1.2	2.5 ± 0.4	2.5 ± 0.5	3.2 ± 0.3	2.9 ± 0.7	3.7 ± 0.2	3.2 ± 0.4	4.2 ± 0.4	0
T40	1.3 ± 0.03	1.3 ± 0.06	1.3 ± 0.4	1.0 ± 0.3	1.0 ± 0.2	1.6 ± 0.3	1.6 ± 0.2	2.1 ± 0.1	1.4 ± 0.2	2.1 ± 0.5	0
T41	2.9 ± 0.2	3.1 ± 0.7	4.2 ± 2.3	1.5 ± 0.7	1.8 ± 1.1	2.2 ± 1.2	5.5 ± 2.0	6.2 ± 2.7	0.9 ± 0.8	6.6 ± 1.2	0
T42	1.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.2	0
T43	0.6 ± 0.0	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	0.9 ± 0.3	0.8 ± 0.1	0
T44	1.3 ± 0.0	1.3 ± 0.1	1.8 ± 0.5	1.6 ± 0.2	1.8 ± 0.4	2.0 ± 0.2	1.5 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	1.7 ± 0.5	0
T45	0.5 ± 0.02	0.5 ± 0.02	0.5 ± 0.11	0.4 ± 0.03	0.4 ± 0.04	0.8 ± 0.12	0.6 ± 0.2	0.7 ± 0.14	0.7 ± 0.06	0.8 ± 0.20	0

PCA analysis on the first component accounted 37% in combined vegetative and reproductive traits and the second and third components accounted 29% and 32% of variations in the morphological traits of the cultivars, respectively (Table 3.3). Additionally, PCA biplot analysis revealed that correlation of morphological traits and location of cultivars on PCA plane (Figure 3.1). In this study, strongly correlated morphological traits and cultivars had been laid together in the same space and uncorrelated traits and cultivars were located in different space on PCA plane (Figure 3.1 a, b, c). Consequently, PCA biplot loading concept on the above overall morphological traits data analysis showed strongly correlated morphological traits and cultivars were placed on the same PCA plane as follow: rachis length (spiny part), ramified bunch's part length, number of fruits per cluster (spikelet), weight of fruits per cluster (spikelet), number of cluster (spikelet) per bunch, fruit weight, fruit length, pulp weight, pulp thickness, seed weight, seed length and seed width are positively correlated with Khyara and Khadrawy date palm cultivars; rachis length (spiny part), number of cluster (spikelet) per bunch, seed weight and seed length with Khalas and Ashal Al Hassa; frond width at the middle, basal pinnae length, basal pinnae width, median pinnae length, apical pinnae length, apical pinnae width, terminal leaflet length, terminal leaflet width, middle spine width and middle spine length are correlated with Shishi and Jarvis; trunk height , trunk circumference, frond length, rachis length, rachis thickness, petiole width, number of pinnae (left side), angle on both sides of the terminal leaflets, peduncle length from its base to the first spikelet, peduncle width at the first spikelet, spikelet's length at the bottom of the bunch, spikelet's length at middle of the bunch's, spikelet's length at top of the bunch's, weight of date fruits per bunch, average weight of date fruits per tree, number of bunch per tree and fruit width are with Barhee, Zamlli and Saggii (Figure 3.1a).

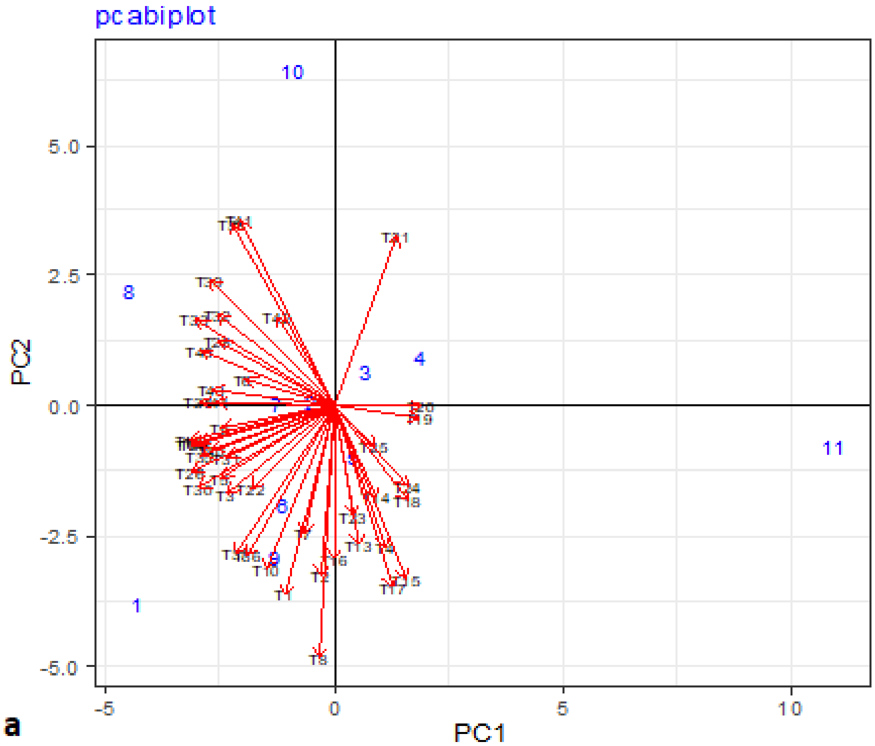
In vegetative trait data analysis, PCA biplot also represented cultivars clustering with similar patterns of traits on PCA quadrant like trunk height , trunk circumference, frond length, rachis length (from petiole to terminal leaflet base), rachis length (1st leaflet to terminal leaflet base), rachis thickness, petiole width, number of pinnae (right side), number of pinnae (left side), angle on both sides of the terminal leaflets and middle spine length are positively correlated with Barhee, Zamlli, Ashal Al Hassa, Saggii; frond width at the middle, basal pinnae length, basal pinnae width, median pinnae length, median pinnae width, apical pinnae length, apical pinnae width, terminal leaflet length, terminal leaflet width, spine number with Shishi and Jarvis; Rachis length (spiny part) and petiole length with Khalas and Khyara (Figure 3.1b).

Table 3.3 Seven principal components of date palm cultivars using morphological traits

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**
Overall traits							
Standard deviation	4.10	2.75	2.40	2.21	1.90	1.51	1.31
Proportion of variance	0.37	0.17	0.12	0.11	0.08	0.05	0.04
Cumulative proportion	0.37	0.54	0.67	0.78	0.85	0.91	0.94
Vegetative traits							
Standard deviation	2.71	2.35	2.06	1.61	1.28	1.13	1.02
Proportion of variance	0.29	0.22	0.17	0.10	0.06	0.05	0.04
Cumulative proportion	0.29	0.51	0.68	0.79	0.86	0.91	0.95
Reproductive traits							
Standard deviation	2.52	2.07	1.73	1.50	1.32	0.94	0.87
Proportion of variance	0.32	0.22	0.15	0.12	0.09	0.45	0.04
Cumulative proportion	0.32	0.53	0.68	0.80	0.91	0.92	0.96

In addition, reproductive traits data in PCA analysis have shown strong correlation between peduncle length from its base to the first spikeletramified bunch's part length, spikelet's length at the bunch's bottom, number of cluster (spikelet) per bunch, weight of date fruits per bunch, average weight of date fruits per tree, number of bunch per tree, fruit width traits and Barhee; peduncle width at the first spikelet, spikelet's length at the bunch's bottom, spikelet's length at the bunch's middle, spikelet's lengthat the bunch's top traits and Khalas, Medjool England, Shishi, Saggii; pulp thickness , seed weight, seed length traits and Medjool Israel and Zamlli; number of fruits per cluster (spikelet), weight of fruits per cluster (spikelet), fruit weight, fruit length, pulp weight, seed width traits and Khadrawy (Figure 3.1c). However, Medjool England, Medjool Israel, Khadrawy and Jarvis cultivars are distantly positioned from the rest cultivars on PCA plane due to variations in almost all vegetative morphological traits (Figure 5.1c) and this also happened similarly in phenogram hierarchial clustering result (Figure 3.2b). In addition, last terminal leaflet number trait was the most significant trait to discriminate Medjool Israel and Medjool England from the rest cultivars in PCA analysis of morphological data (Figure 3.1a-c).

In hierarchial cluster analysis based on overall morphological traits, date palm cultivars were divided mainly into male (Jarvis cultivar) and females (Figure 3.2a). Females were classified into two groups; one group contained Khadrawy, Medjool England and Medjool Israel and the second group was also determined by two sub-clusters. The first sub-cluster included Ashal Al Hassa, Khalas, Saggi, Zamlli, Shishi and Barhee and the second sub-cluster contained only Khyara. The phenogram (Figure 3.1a) revealed that the close genetic relationship between Khalas and Ashal Al Hassa; Zamlli and Saggii; Medjool Israel and Medjool England, while the rest cultivars exhibited genetic variation. Clustering of cultivars based on vegetative traits showed two main categories. The first one also further divided in to two sub-catagories: Ashal Al Hassa, Khalas, Saggi, Zamlli and Shishi were clustered together and Barhee and Khyara represented another sub-category of the first sub-cluster of a phenogram (Figure 3.2b).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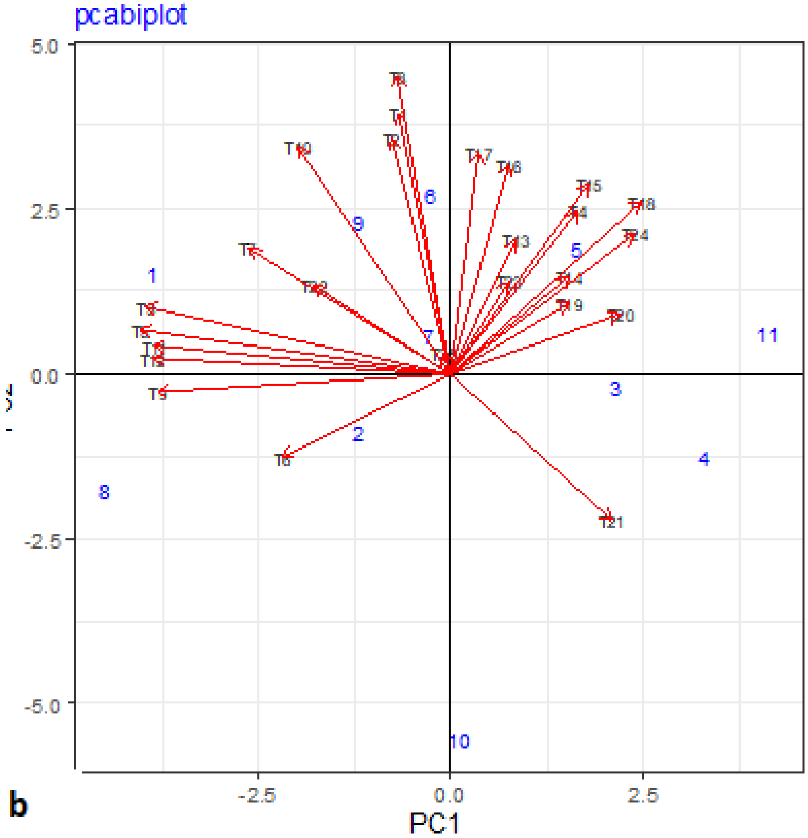
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b

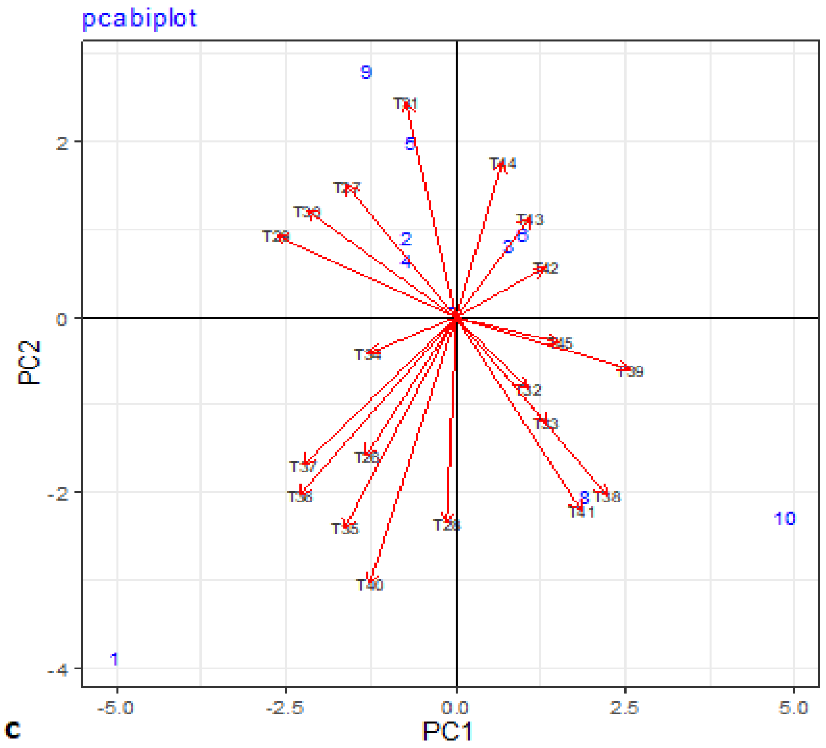
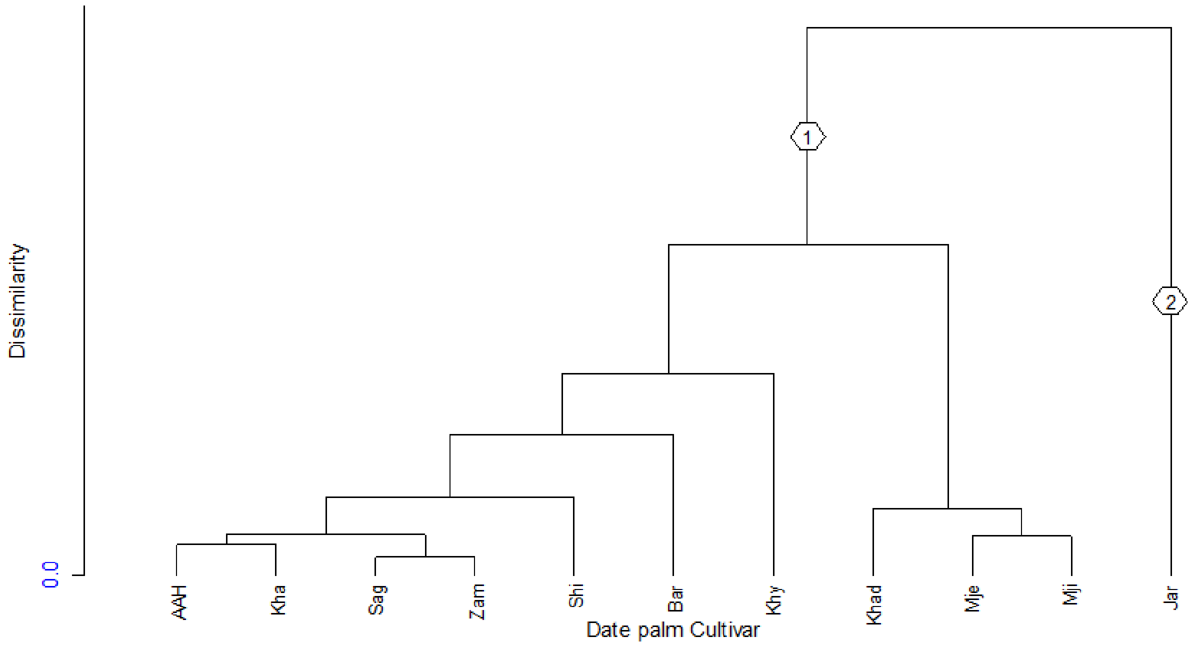


Figure 3.1 PCA biplots showing the correlation of morphological traits and date palm cultivars a) based on the overall morphological data; b) based on vegetative data; c) based on reproductive data. The numbers on PC plane represent date palm cultivars: 1 – Barhee; 2 – Khalas; 3 – Medjool Israel; 4 – Medjool England; 5 – Shishi; 6 – Zamlli; 7 – Ashal Al Hassa; 8 – Khyara; 9 – Saggii; 10 – Khadrawy; 11 – Jarvis (male) and traits are represented by arrows or vectors

The phenogram in Figure 3.2b indicated that there was no variability in their vegetative traits between Barhee and Khyara; Khalas and Ashal Al Hassa; Zamlli and Saggii; Medjool Israel and Jarvis whereas Shishi, Khadrawy and Medjool England showed genetic dissimilarity and shared some vegetative traits with others. In cluster analysis based on reproductive traits two clusters were observed. The first cluster was further divided into two sub-clusters: one sub-cluster contained only Khyara and the second contained Medjool Israel, Medjool England, Khadrawy, Saggii, Zamlli, Khalas, Shishi and Asha Al Hassa while the second cluster comprises only Barhee cultivar (Figure 3.2c).

According to the phenogram in Figure 3.2c, Khalas and Zamlii; Shishi and Ashal Al Hassa; Medjool Israel and Medjool England showed high similarity and they shared most of their reproductive traits.



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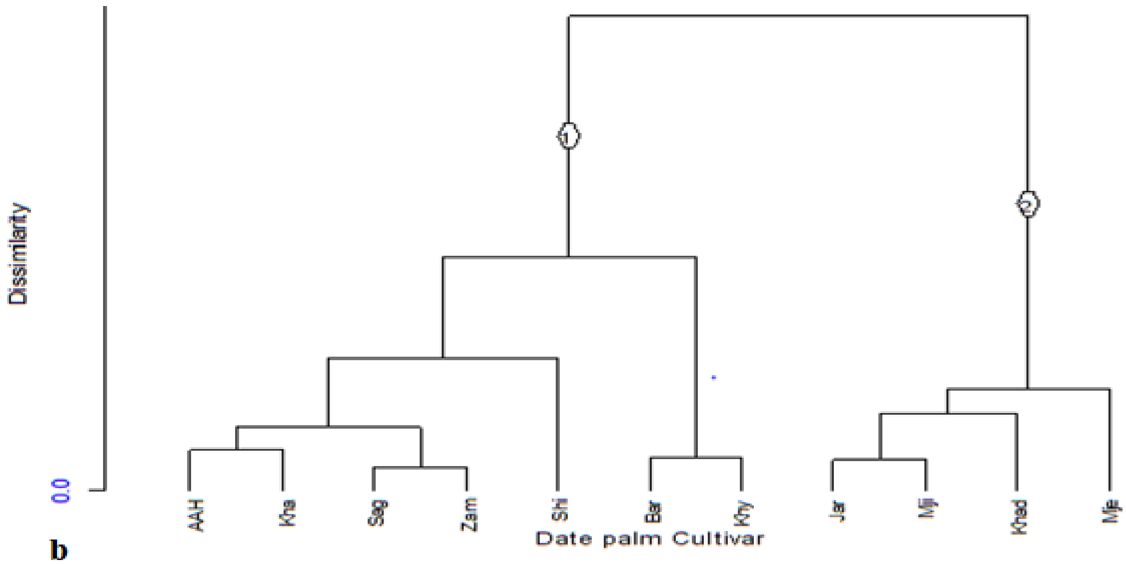

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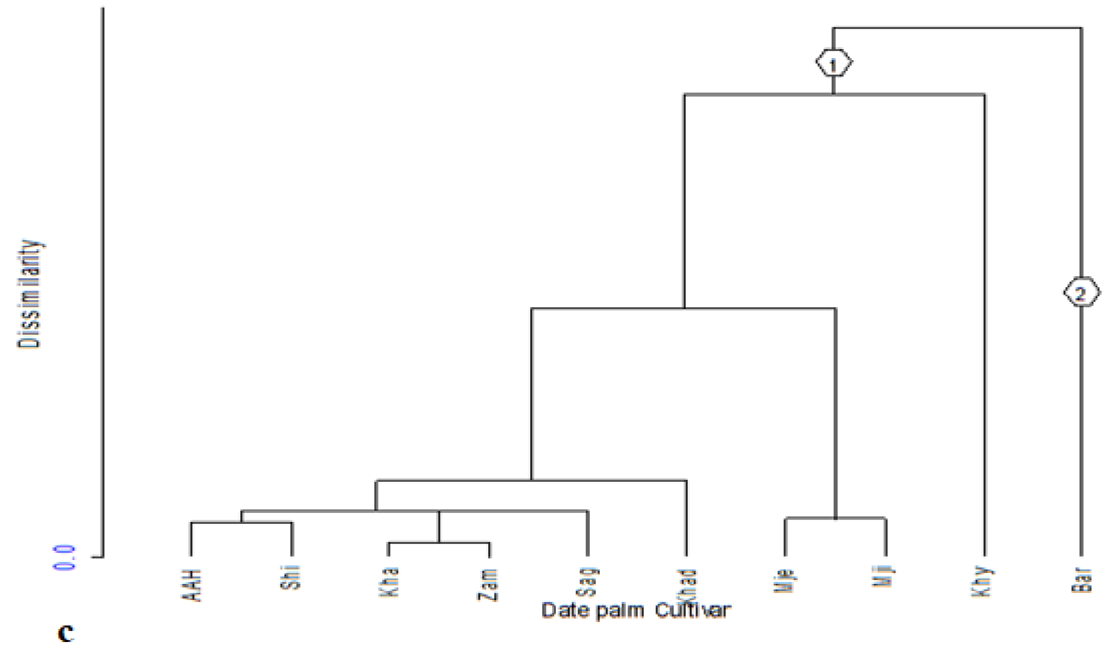


Figure 3.2 Hierarchical clustering of date palm cultivars using morphological data a) based on the traits data; b) based on vegetative data; and c) based on reproductive data that were used in this study. The codes on the tree represent cultivars: AAH – Ashal Al Hassa; Kha – Khalas; Sag – Saggi; Zam – Zamlli; Shi – Shishi; Bar – Barhee; Khy – Khyara; Jar – Jarvis (male); Mji – Medjool Israel; Mje – Medjool England; Khad – Khadrawy.

3.4.2 ANOVA and homogeneity of variances in date palm cultivars

Analysis of variance (ANOVA) detected significant differences among cultivars at $p < 0.05$ or $p < 0.01$ in all morphological traits except traits of rachis length of spiny part and fruit width (Table 3.4). According to Levene's statistics result (Table 3.5) 12 traits as mentioned here: frond width at the middle, petiole length, petiole width, basal pinnae width, median pinnae width, apical pinnae length, apical pinnae width, angle on both sides of the terminal leaflets, middle spine width, peduncle width at the first spikelet, ramified bunch's part length and spikelet's length at the bunch's top showed insignificant differences among date palm cultivars whereas, the rest of 23 traits show significant difference ($p < 0.05$ or $p < 0.01$) among cultivars.

Table 3.4 ANOVA result among 11 date palm cultivars using morphological data

Traits	Sum of Squares	Mean Square	F	Sig.	Traits	Sum of Squares	Mean Square	F	Sig.
Trunk height	63924.1	6392.4	4.6	0^*	Middle spine width	2.94	0.29	13.7	0^*
Trunk circumference	30602.6	3060.2	4.9	0^*	Middle spine length	358.62	35.7	5.58	0^*
Frond length	99677.7	9967.7	4.5	0^*	Peduncle length from its base to the first spikelet	96665.7	9666.6	39.24	0^*
Frond width at the middle	4916.6	491.66	9.8	0^*	Peduncle width at the first spikelet	276.41	27.6	16.02	0^*
Rachis length (from petiole to terminal leaflet base)	91467.2	9146.7	4.1	0.001*	Ramified bunch's part length	3649.91	365.9	5.356	0^*
Rachis length (spiny part)	5547.01	554.71	1.7	0.119	Spikelet's length at the bunch's bottom	9608.23	960.8	13.65	0^*
Rachis length (1st Leaflet to terminal leaflet base)	44162.4	4416.24	4.3	0.001*	Spikelet's length at the bunch's middle	8201.65	820.7	15.08	0^*
Rachis thickness	51.26	5.13	11.7	0^*	Spikelet's length at the bunch's top	6660.68	666.07	11.96	0^*
petiole length	13650.1	1365.01	3.2	0.006*	number of fruits per cluster	890.64	89.064	5.918	0^*
Petiole width	107.28	10.73	5.1	0^*	weight of fruits per cluster	240203	24020.3	3.009	0.008*
Number of pinnae (right side)	5330.7	533.07	5.3	0^*	number of clusters per bunch	15285.7	1528.6	8.062	0^*
Number of pinnae (left side)	5445.2	544.52	5.8	0^*	weight of date fruits per bunch	190.2	19.02	3.498	0.003*
Basal pinnae length	3011	301.1	8.5	0^*	average weight of date fruits per tree	49402.8	4940.3	3.876	0.002*
Basal pinnae width	12.47	1.25	7.8	0^*	No. bunch per tree	742.1	74.2	3.5	0.003*
Median pinnae length	1256.5	125.65	17.	0^*	Fruit weight	259.2	25.92	11.68	0^*

			2						
Median pinnae width	5.86	0.59	4.3	0.001*	Fruit length	48.1	4.81	20.48	0*
Apical pinnae length	1005.7	100.57	3.7	0.002*	Fruit width	43.4	4.34	1.38	0.232
Apical pinnae width	5.45	0.55	5.4	0	Pulp weight	192.2	19.22	9.21	0*
Terminal leaflet length	1743.6	174.36	9.4	0	Pulp thickness	1.3	0.13	10.19	0*
Terminal leaflet width	4.23	0.42	2.2	0.043**	Seed weight	3.3	0.33	16.26	0*
Last terminal leaflet number	4.14	0.41	7.8	0*	Seed length	17.1	1.71	10.41	0*
Angle on both sides of the terminal leaflets	769.91	76.99	4.1	0.001*	Seed width	1.9	0.19	16.96	0*
Spine number	945.18	94.52	9.8	0*					

**Indicates significant value at $p \le 0.01$ and ** indicates significant value at $p \le 0.05$*

Table 3.5 Test of homogeneity of variances (HOV) on morphological traits of 11 date palm cultivars of this study

Morphological traits	**Levene Statistic**	**df 1**	**df 2**	**Sig.**	**Morphological traits**	**Levene Statistic**	**df 1**	**df2**	**Sig.**
Trunk height	2.279	10	33	0.037*	Middle spine width	1.438	10	33	0.207
Trunk circumference	2.138	10	33	0.049**	Middle spine length	3.009	10	33	0.008*
Frond length	6.85	10	33	0*	Peduncle length from its base to the first spikelet	2.231	10	33	0.041**
Frond width at the middle	1.525	10	33	0.175	Peduncle width at the first spikelet	1.432	10	33	0.21
Rachis length (from petiole to terminal leaflet base)	14.257	10	33	0*	Ramified bunch’s part length	1.79	10	33	0.102
Rachis length (spiny part)	2.719	10	33	0.015**	Spikelet’s length at the bunch’s bottom	2.969	10	33	0.009*
Rachis length (1st Leaflet to terminal leaflet base)	2.604	10	33	0.019**	Spikelet’s length at the bunch’s middle	1.851	10	33	0.09

Rachis thickness	5.823	10	33	0*	Spikelet’s length at the bunch’s top	2.004	10	33	0.065
petiole length	1.843	10	33	0.091**	number of fruits per cluster (spikelet)	4.059	10	33	0.001*
Petiole width	1.479	10	33	0.191	weight of fruits per cluster (spikelet)	2.322	10	33	0.034**
Number of pinnae (right side)	14.041	10	33	0*	number of cluster (spikelet) per bunch	6.091	10	33	0*
Number of pinnae (left side)	16.314	10	33	0*	weight of date fruits per bunch	4.247	10	33	0.001*
Basal pinnae length	2.184	10	33	0.045	average weight of date fruits per tree	4.58	10	33	0*
Basal pinnae width	1.835	10	33	0.093	No. Bunch per tree	5.057	10	33	0*
Median pinnae length	6.926	10	33	0*	Fruit weight	2.239	10	33	0.04**
Median pinnae width	1.246	10	33	0.3	Fruit length	3.554	10	33	0.003*
Apical pinnae length	1.124	10	33	0.374	Fruit width	8.269	10	33	0*
Apical pinnae width	1.31	10	33	0.2658	Pulp weight	2.966	10	33	0.009*
Terminal leaflet length	3.801	10	33	0.002*	Pulp thickness	3.049	10	33	0.008*
Terminal leaflet width	2.976	10	33	0.009*	Seed weight	2.533	10	33	0.022
Last terminal leaflet number	22.6	10	33	0*	Seed length	4.288	10	33	0.001*
Angle on both sides of the terminal leaflets	0.764	10	33	0.662	Seed width	4.23	10	33	0.001*
Spine number	5.966	10	33	0*					

*Indicates significant value at $p \le 0.01$ and ** indicates significant value at $p \le 0.05$

3.5. Discussion

For sustainable utilization of plant genetic resources, it is necessary to understand and determine the natural relationships among plant varieties and cultivars based on scientific knowledge and principles. Genetic identification of plant varieties using morphological traits can provide evidences on the bases of their evolutionary relationships. The purpose of this study was to identify and evaluate the relationships of date palm cultivars using morphological markers. In this study, significant variation was presented among date palm cultivars as revealed by PCA, dendrogram, and ANOVA and HOV analysis.

Additionally, all morphological traits showed heterogeneity among date palm cultivars except rachis length of spiny part and fruit width traits which showed insignificant value at $p \leq 0.05$. Bedjaoui and Benbouza (2018) suggested that phenotypic variation was exhibited among Algerian date palm cultivars in both vegetative and reproductive characters in which reproductive characters showed stronger dissimilarity among cultivars than vegetative traits. This result is in agreement with the present study. Additionally, Khalilia *et al*., (2022) and Allam *et al*., (2021) also reported that fruit width showed significant variation between date palm cultivars while this trait revealed contrary result in this study.

According to PCA biplot loading result of the present study, strong and weak correlation of morphological traits was observed for grouping of date palm cultivars on PC planes or quadrants. PCA biplot loading displayed that those vegetative traits: trunk height, trunk circumference, frond length, rachis length (from petiole to terminal leaflet base), number of pinnae (right side) and number of pinnae (left side)were useful traits to separate

Barhee cultivar from the rest date palm cultivars. This finding is consistent with Eissa *et al*. (2009) who reported that Barhee cultivar is distinguished from the rest genotypes mainly by trunk height, trunk circumference, frond length, rachis length (from petiole to terminal leaflet base), number of pinnae.

Additionally, in this study the reproductive traits peduncle length from its base to the first spikelet, ramified bunch's part length, number of cluster (spikelet) per bunch, weight of date fruits per bunch, average weight of date fruits per tree, number of bunches per tree and fruit width were the most useful and correlated parameters to discriminate Brhee cultivar from others. On the other hand, Hammadi *et al*. (2009); Elsafy *et al*. (2015); El - Sharabasy and Rizk (2005); Ouarda *et al*. (2012); Abdelkrim *et al*. (2020) have reported fruit, spine, frond and trunk morphological traits of date palm are the most important parameters for discrimination and identification of cultivars. In general, based on the result of this study we observed that there is variation of morphological traits that briefly distinguish between date palm cultivars particularly for those closely related ones. This implicates there exist high genetic variation among date palm population in Ethiopia. This is a rich genetic wealth that could be seriously studied to harness them for genetic improvement.

In general, as to our knowledge this research is the first work for documenting and identifying the relationships among date palm cultivars in Ethiopia using morphological markers. Consequently, this study could be used to shade light on the understanding on morphological relationships of date palm cultivars and also will assist in selecting cultivars for breeding and improve important agronomic traits of this valuable crop in Ethiopia.

Chapter 4

Genetic diversity and phylogenetic structure of date palms in Ethiopia using inter simple sequence repeat markers

Abstract

Date palm (*Phoenix dactylifera* L.) is a perennial tree with long life span that belongs to Arecaceae family. Assessment of molecular diversity of Ethiopian date palms using DNA markers is critically important on establishing biodiversity information database systems for further conservation and improvement programs. In this study, 113 date palms genotypes were assessed using 10 inter-simple sequence repeat markers. DNA was extracted from young leaves. PCR amplification was conducted using a final volume of 10 µl PCR reaction mixtures. A total of 241 DNA fragments generated by all primers and each primer showed 100% polymorphic loci. The highest (37) and lowest (10) number of bands were produced by (AGG)6 and (AG)10C primers respectively. Genetic diversity estimation parameters showed varied values from each primer and among populations. AMOVA showed 49% and 51% variation within and among population, respectively, and also, 10.55%, 9.17% and 7.68% variations were observed on the first three PCoA axes. PCoA and dendrogram displayed that 113 date palms clustered according to their geographical location. The overall investigation of this was to provide evidences to understand the genetic diversity background of date palms in Ethiopia and helpful for future conservation and improvement programs.

Key words: *Date palms, Ethiopia, Genetic diversity, ISSR markers, Phylogenetic tree*

4.1. Introduction

Date palm (*Phoenix dactylifera* L.) is perennial monocot plant belongs to palm family and has 2n=36 of diploid chromosome set (Al-Mssallem *et al*., 2013). It is one of the most cultivated and utilized edible fruiting tree in North Africa, Middle East and Southern Asia. The origin of this tree is probably in Mesopotamia, currently Iraq, Syria, Kuwait and part of Turkey (Mirbahar *et al*., 2014; Karim *et al*., 2021). Date palms are multipurpose trees that provide food, industrial, commercial, medicinal and ornamental values with great socio-economic impact and agricultural sustainability (Johnson, 2012; El-Far *et al*., 2016). Date fruits are nutritionally rich in carbohydrate containing 70% of sugar like glucose, sucrose and fructose; and also packed essential minerals and vitamins (Aljaloud *et al*., 2020). There is no part that is discarded in a date palm tree. These plant parts are used for different purpose such as trunks to construct houses, hives, bridges and as packing material (Al-Jabri 2014; Fatima *et al*., 2016); terminal buds and young leaves can be cooked as vegetables while rachises are used for paper making (Khiari *et al*. 2011; El-Hadrami and Al Khayri 2012).

The perennial and dioecious natural characteristics of plant cultivars including date palms can provide high diverse phenotypic traits among them. To provide clear understanding on the basis of phylogenetic relationships between cultivars the use of molecular markers is one of the promising alternative tools. Studies on genetic diversity and phylogenetic relationship of date palms have been carried out using dominant and co-dominant molecular markers such as RAPD (Haider *et al*., 2012; Marsafari and Mehrab, 2013; Srivashtav *et al*., 2013; Mirbahar *et al*., 2014), AFLP (Khierallah *et al*., 2011-b; Ibrahim *et al*., 2014), SSR (Elmeer *et al*., 2011; Khierallah *et al*., 2011a; Bodian *et al*., 2012; Racchi *et al*., 2014; Zehdi-Azouzi *et al*., 2015; Yusuf *et al*., 2015; Aljuhani 2016; Moussouni *et al*., 2017; Chaluvadi *et al*., 2018; Ahmed *et al*., 2021), SNP (Hazzouri *et al*., 2015; Mohamoud *et al*., 2019; Faqir *et al*., 2019).

Inter simple sequence repeat marker (ISSR) is one of the widely used molecular markers to asses genetic diversity and population structure of plant species; because the use of this marker is technically easy and low cost to produce multi-locus amplified products ranging from 200 to 2000 base pair in length (Purayil *et al*., 2018; Khankahdani and Bagheri, 2019). Indeed, ISSR is advantage over SSR and AFLP, no need of prior knowledge of flanking sequences (Hamza *et al*., 2012). Genetic diversity of some date palm landraces in Afar region of Ethiopia using ISSR marker have been reported (Takele *et al*., 2021). However, as larger date palm populations are found in the region they used small numbers of accessions. Therefore, the present study is helpful to offer strong baseline on the genetic diversity and population structure of date palms in Ethiopia using ISSR markers along with the findings on SSR markers on the same study sites (Workia Ahmed *et al*., 2021).

4.2. Materials and methods

4.2.1. Plant materials and DNA extraction

At the time of fruiting, a total of 113 date palm samples including early introduced cultivars were used and collected from different location of Afar, Somalia and Dire Dawa regions of Ethiopia (Figure 4.1). Young leaf samples were collected from offshoots and cut into pieces to preserve them in zip-locked plastic bag with the appropriate amount of silica gel. DNA extraction was carried out at Plant Genetics Research Laboratory (PGRL) of Addis Ababa University, Addis Ababa, Ethiopia. About 100 mg dried leaves were powdered by a mixer and miller (Retsch Mixer Mill MM 400) using five 3.2 mm diameter stainless steel beads in a 2-ml sterile centrifuge tube.

Genomic DNA was extracted using a modified 2% cetyl trimethyl ammonium bromide (CTAB) method (Doyle, 1991). One milliliter preheated 2% CTAB extraction buffer containing: 2% CTAB, 100 mM Tris-Base pH 8.0, 25mM Na_2-EDTA, 2M NaCl, 250mg/ml PVP and 2% β-mercaptoethanol were added to the tube containing leaf powder. At the end of extraction and purification procedures DNA pellets are found in the bottom of each centrifuge tube. To dissolve the pellets, 60µl 0.1X TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) buffer containing RNase was added into each centrifuge tube. The quality of DNA was checked using gel electrophoresis in 1% agarose and visualized in Bio-Rad Gel Doc™ EZ System Imager and the concentration of the DNA was measured by using NanoDrop (Thermo Scientific NanoDrop 2000 Spectrophotometer). The DNA with high DNA concentrations was diluted for normalization to a concentration of 100 ng using PCR graded water.

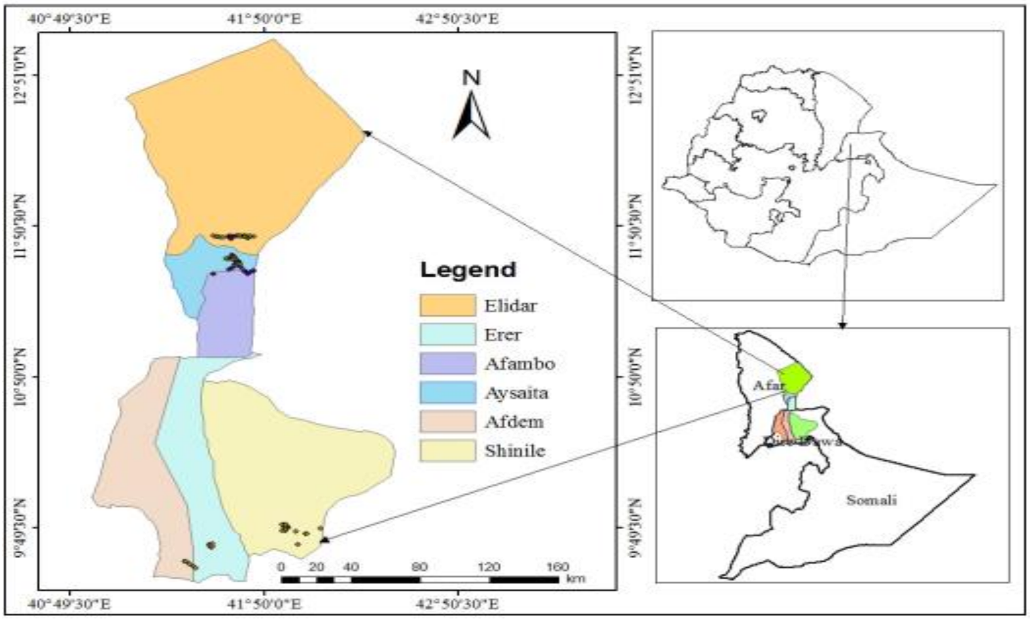
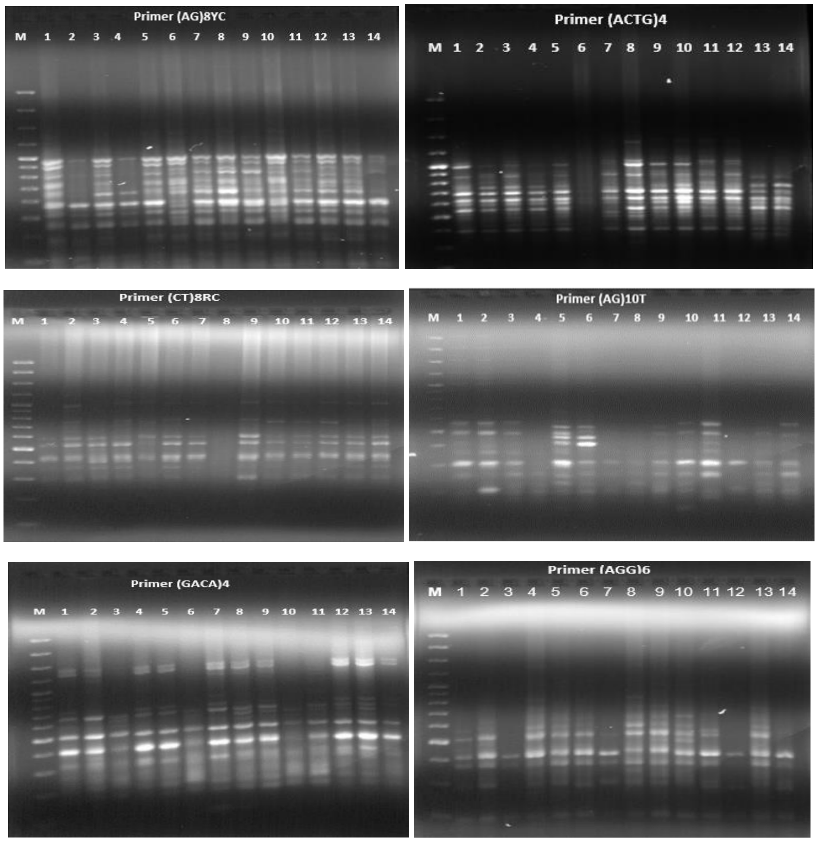


Figure 4.1 Geographical location and distribution of date palm samples used in this study based on GPS system (black dots on map represent location of date palm trees)

4.1.1. PCR amplification and gel electrophoresis

A total of 15 ISSR primers were screened by PCR of which 10 primers were previously used by Karim et al. (2010) and 5 primers by Oumer et al. (2020) (Table 4.1). Among the 15 primers 10 primers were found to be polymorphic and reproducible and used for further PCR amplification. For genomic DNA amplification, each PCR tube contained a final volume of 10 µl PCR reaction mixtures: 100 ng DNA (0.5 µl), 10X PCR buffer (1 µl), 25 mM $MgCl2$ (0.6µl), 10 mM dNTPs mixes (0.2 µl), 20 µM primer (0.5 µl), 6.95 µl of PCR grade water, 5U Taq DNA polymerase (0.25 µl).

The PCR thermal profile was performed as follow: pre-PCR denaturation at 94°C for 5 minutes followed by 35 cycles of denaturing at 94°C for 30 seconds, annealing for 30 seconds at optimized temperature of each primer (Table 4.1), extension at 72°C for 1 min, and a final extension at 72°C for 10 min and the products were stored at 4 ºC until loading on agarose gel for electrophoresis. Gel electrophoresis was done by loading 5 µl PCR products of each sample on 1.5% agarose gel contained 3.0 µl of ethidium bromide for staining and run in 0.5X TBE buffer at a constant voltage of 100 V for 1:00 to 1:30 hours. The ISSR band images were captured under Bio-Rad Gel Doc™ EZ System Imager that was connected to a computer with Image Lab software and stored for data scoring. To estimate the molecular sizes of the resolved DNA fragments, 3µl of a 100 bp DNA ladder (Bio tools) was loaded on a gel along with amplified DNA samples used as a reference DNA marker. Some representative DNA fragment profiles that were amplified by ISSR primers of this study were shown on (Figure 4.2).



Primer (AG)8YC

Primer (ACTG)4

Primer (CT)8RC

Primer (AG)10T

Primer (GACA)4

Primer (AGG)6

Figure 4.2 Examples of amplified DNA fragments profiles of date palms using different ISSR markers.

4.3. Data analysis

4.3.1. ISSR band scoring and data analysis

Band scoring

According to recommended criteria for scoring of DNA bands produced by dominant markers, we excluded bands with weak intensities and those with band sizes <100 bp and band sizes >2000 bp. According to DNA ladder (100 bp), bands with the same base pair size we considered to represent a single locus. Bands obtained were recorded manually as ‘1’ for presence or ‘0’ for absence at the same site of each locus, and ambiguous bands were considered as a missing data and marked as "?". In excel sheet, binary data matrix was assembled with each individual sample in the column and ISSR markers in the row for each primer types and vice-versa according to the software data input format. Each amplified fragment was named by the code of the primers across the row or column followed by their fragment bp sizes.

Table 4.1 ISSR primers screened used in this study

No.	Primer Sequences (5`-3`)	Types of motifs	Annealing Temperature ºC	Range of amplified bands size in base pair
1	(AG)10G	Dinucleotide	52	1000 – 230
2	(AG)10C	Dinucleotide	52	500 – 150
3	(AG)8YC	Dinucleotide	45	1350 – 180
4	(CT)8RC	Dinucleotide	43	1800 – 180
5	(AG)10T	Dinucleotide	57	1100 – 150
6	(CT)10G	Dinucleotide	52	900 – 120
7	(AGG)6	Trinucleotide	55	1600 – 190
8	(GACA)4	Tetranucleotide	45	1500 – 180
9	(ACTG)4	Tetranucleotide	45	1800 – 250
10	(GACAC)4	Pentanucleotide	55	1750 – 200

Note: Y = C or T → (Pyrimidine); R = A or G → (Purine)

4.3.2. Genetic diversity data analysis

To evaluate variances among date palm populations, molecular analysis of variance (AMOVA) and principal coordinate analysis (PCoA) was performed using GenAlEx (genetic analysis in excel) version 6.5 (Peakall & Smouse, 2012). Shannon's information index (I), genetic diversity index (H), different allele number (Na), polymorphic loci percentage, effective allele number (Ne), total number of amplified bands and Ne's genetic identity and dissimilarity was calculated by popgen32 software. The phylogenetic tree was constructed in NTSYS-pc software using SAHN option based on UPGMA method.

4.4. Results

4.4.1. ISSR assay and Polymorphism

Among the screened 15 primers, 10 primers were found to be polymorphic and reproducible (Table 4.2 and Figure 4.1). These 10 primers produced a total of 241 DNA bands with different base pair sizes ranging from 1800 to 120 bp across all studied date palm genotypes and all amplified loci detected by each primer showed 100% of polymorphism (Table 4.2). Each ISSR primer resulted in varied number of DNA bands that ranged from 37 by primer (AGG)6 to 10 by primer (AG)10C and the average number of DNA bands per primer was 24.1. Besides, we evaluated ISSR datasets separately according to types of repeat motifs and anchorage properties of the primers. The number of DNA bands generated by dinucleotide repeat motifs (anchored) primers was 129, by tri-nucleotide repeat motifs was 37, by tetra-nucleotide repeat motifs was 52, by Penta-nucleotide repeat motif was 23 and by unanchored primers was 112 and each primer displayed 100% polymorphic of loci (Table 4.2). The genetic diversity (H) values detected by the primres ranged from 0.165 ± 0.157 ((CT)10G) to 0.328 ± 0.136 ((GACA)4) with total average of 0.281 ± 0.154. The highest Shannon's Information index (I) was exhibited by primer (CT)10G (0.76 ± 0.213), while the lowest was shown by primer (AGG)6 (0.401 ± 0.193) with an average of 0.436 ± 0.195; maximum gene flow was exihabited by primer (CT)10G (value) and primer (AG)8YC (3.066) whereas the lowest was was shown by (AG)10C (0.634).

4.4.2. AMOVA and pair-wise genetic distance comparison

AMOVA analysis was performed to detect percentage of variations among and within populations based on all ISSR primers data and types of ISSR repeat motifs (Table 4.2). All analysis of AMOVA results revealed significant variations among and within populations at $P \geq 0.001$ values (Table 4.3). The AMOVA result obtained from all primers showed 51% and 49% of genetic variations among and within populations respectively. According to types of repeat motifs, the highest variation (63%) was revealed by penta-nucleotide repeat motif primer and the least genetic variations (46%) was presented by tetra-nucleotide repeat motifs primer whereas, within populations the highest genetic variation (57%) was detected by di-nucleotide repeat motifs primers and the least genetic variation (37%) was detected by penta-nucleotide repeat motif primer. Among the nine popuations, narrow Nei's genetic distance (0.1162) was observed between Alasabolo and Mamulae populations while the maximum genetic distance (0.2807) was showen between Errer Gota and Berga popultions (Table 4.5.).

Table 4.2 Ten ISSR markers that produced band polymorphism from 9 date palm populations

Primers	Na	NPL	PPL (%)	Mean ± SD		
				H	I	Nm
(AGG)6	37	37	100	0.249 ± 0.152	0.401 ± 0.193	1.424
(AG)10G	20	20	100	0.326 ± 0.157	0.491 ± 0.194	1.217
(AG)10C	10	10	100	0.305 ± 0.146	0.474 ± 0.146	0.634
(AG)10T	26	26	100	0.259 ± 0.149	0.411 ± 0.188	2.213
(CT)10G	20	20	100	0.165 ± 0.157	0.76 ± 0.213	3.066
(GACAC)4	23	23	100	0.305 ± 0.146	0.469 ± 0.178	1.265
(ACTG)4	28	28	100	0.305 ± 0.156	0.466 ± 0.195	1.772
(GACA)4	24	24	100	0.328 ± 0.136	0.498 ± 0.166	1.116
(AG)8YC	28	28	100	0.321 ± 0.167	0.481 ± 0.206	3.066
(CT)8RC	25	25	100	0.263 ± 0.162	0.413 ± 0.203	2.246
mean	**24.1**	**24.1**	**100**	**0.281 ± 0.154**	**0.436 ± 0.195**	**1.802**
Sum	**241**	**241**				
Based on repeat motif						
Di-nucleotides	129	129	100	0.272 ± 0.162	0.423 ± 0.206	0.674
Tri-nucleotide	37	37	100	0.2493 ± 0.134	0.401 ± 0.173	0.468
Tetra-nucleotide	52	52	100	0.316 ±0.1461	0.481 ± 0.1810	0.473
Penta-nucleotide	23	23	100	0.305 ± 0.1458	0.469 ± 0.178	0.318
Based on anchorage property of nucleotides						
Anchored	129	129	100	0.272 ± 0.1624	0.4231 ± 0.206	0.674
Unanchored	112	112	100	0.2914 ± 0.144	0.4518 ± 0.180	0.433

*Na = number of allele, NPL = number of polymorphic loci, PPL = percentage of polymorphic loci, H = gene diversity, I = Shannon's Information index, Nm = gene flow (Nm = 0.5(1 - Gst)/Gst)

4.1.1. Genetic diversity of date palms

Assessment of genetic diversity levels among date palm populations was performed using ISSR dataset. The overall results for each population on the genetic diversity analysis (Table 4.4), the highest number of polymorphic loci (127) was detected in Legaharae population and the least was detected (87) in Berga population with maximum of 52% and minimum of 36.1% PPL respectively. The mean number of alleles and effective number of alleles ranged from 1.36 ± 0.48 (in Berga) to 1.527 ± 0.50 (in Legaharae) and 1.19 ± 0.32 (in introduced population) and 1.45 ± 0.35 in Mamulae respectively.

Table 4.3 Summery of AMOVA among and within date palm populations at $P \geq 0.001$ values

Source	Df	SS	MS	Est. Var.	%
Using all primers					
Among Pops	8	2370.247	296.281	21.992	51%
Within Pops	104	2158.275	20.753	20.753	49%
Total	112	4528.522		42.744	100%
Using di-nucleotide repeat motif primers					
Among Pops	8	1131.601	141.450	10.341	47%
Within Pops	104	1237.116	11.895	11.895	53%
Total	112	2368.717		22.236	100%
Using tri-nucleotide repeat motif primer					
Among Pops	8	372.170	46.521	3.501	57%
Within Pops	104	277.069	2.664	2.664	43%
Total	112	649.239		6.165	100%
Using tetra-nucleotide repeat motif primers					
Among Pops	8	1131.601	141.450	10.341	54%
Within Pops	104	1237.116	11.895	11.895	46%
Total	112	2368.717		22.236	100%
Using penta-nucleotide repeat motif primer					
Among Pops	8	279.613	34.952	2.663	63%
Within Pops	104	164.723	1.584	1.584	37%
Total	112	444.336		4.247	100%

Table 4.4 Evaluation of genetic relationships of date palm genotypes among populations

Population	Code	Sample size	NPL	PPL (%)	Mean ± SD	
					Na	**Ne**
Alasabolo	As	14	112	46.47	1.47 ± 0.49	1.27 ± 0.36
Bega	Bg	11	87	36.1	1.36 ± 0.48	1.23 ± 0.36
Erer Gota	ErG	14	126	52.28	1.52 ± 0.50	1.29 ± 0.36
Humodoyta	Hd	14	114	47.3	1.47 ± 0.50	1.27 ± 0.36
Kerebuda	Ker	11	101	41.91	1.42 ± 0.49	1.23 ± 0.34
Legaharae	Lg	14	127	52.7	1.53 ± 0.50	1.25 ± 0.34
Mego	Mg	10	93	38.59	1.39 ± 0.49	1.25 ± 0.37
Mamulae	Mm	14	109	45.23	1.45 ± 0.50	1.45 ± 0.35
Early introduce	Intro	11	89	36.93	1.37 ± 0.48	1.19 ± 0.32

*Na = number of allele, Ne = effective number of allele, NPL = number of polymorphic loci, PPL = percentage of polymorphic loci.

Table 4.5 Nei's genetic distance among date palm populations in Ethiopia using 10 ISSR primers

Pop ID	As	Bg	ErG	Hd	Kr	Lg	Mg	Mm	Intro
As	****								
Bg	0.2054	****							
ErG	0.2169	0.2807	****						
Hd	0.1843	0.2668	0.2163	****					
Kr	0.1789	0.2345	0.2450	0.2401	****				
Lg	0.1715	0.2345	0.1482	0.1859	0.1732	****			
Mg	0.1886	0.2554	0.2000	0.2233	0.2302	0.1379	****		
Mm	0.1162	0.2174	0.1951	0.1735	0.1657	0.1280	0.1510	****	
Intro	0.1646	0.2402	0.2090	0.2306	0.1853	0.1470	0.1992	0.1374	****

4.4.3. Principal Co-ordinate analysis

Principal coordinate analysis (PCoA) was done to display the distributions and relationships of date palm genotypes in relation to the first three PCoA axes variation (Figure 4.3). PCoA plane of the first three axes explained 27.4% of the total variation (first axis = 10.55%, second axis = 9.17% and third axis = 7.68%). All date palm genotypes collected from the same site were clustered closely together on the same PCoA. This is similarly observed on the dendrogram of this study. Additionally, PCoA analysis determined organizations of the closest date palm populations such as Legaharae and Mego; Berga and Alasabolo; Mamulae and Kerebuda; Kerebuda and early introduced populations.

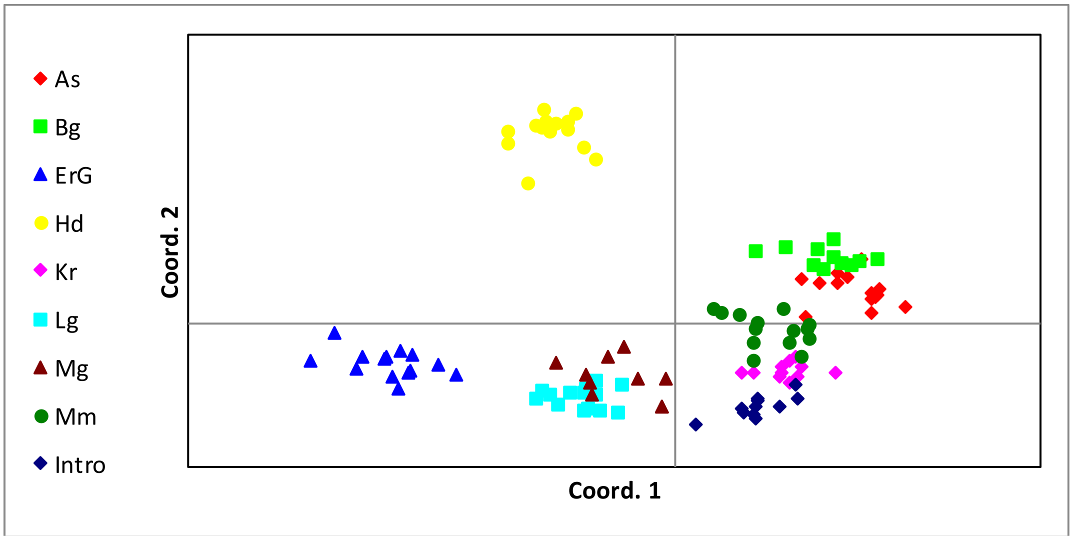


Figure 4.3 Distribution of 113 date palm genotypes of nine date palm populations on PCoA axes based on the genetic distance between date palm individuals and the symbols on PCoA plane with same color and shape represents a single population.

4.4.4. Phylogenetic relationships of date palm populations

UPGMA grouped 113 date palm genotypes of nine populations into two main categories (Figure 4.4). The first main category comprised 102 individuals and these individuals further divided into two sub-clusters. The first sub-cluster of the first main category of a tree further grouped into four containing 50 individuals according to their geographical locations into Alasabolo, Mamulae, early introduced (Humodoyta site) and Berga popuptions. The second sub-cluster of the first main category consists of 52 individuals and assembled into four: Erer Gota, Legahare, Mego and Humodoyta populations based on their collection site. The second main category of a tree contained eleven date palm genotypes of the Kerebuda population. The clustering and genetic relationships of the populations by PCoA and phylogenetic tree analysis of this study are consistent with each other. Nei's coefficient values of genetic similarity pair-wise comparison between populations (Table 4.4) also supported the result of PCoA and phylogenetic analysis.

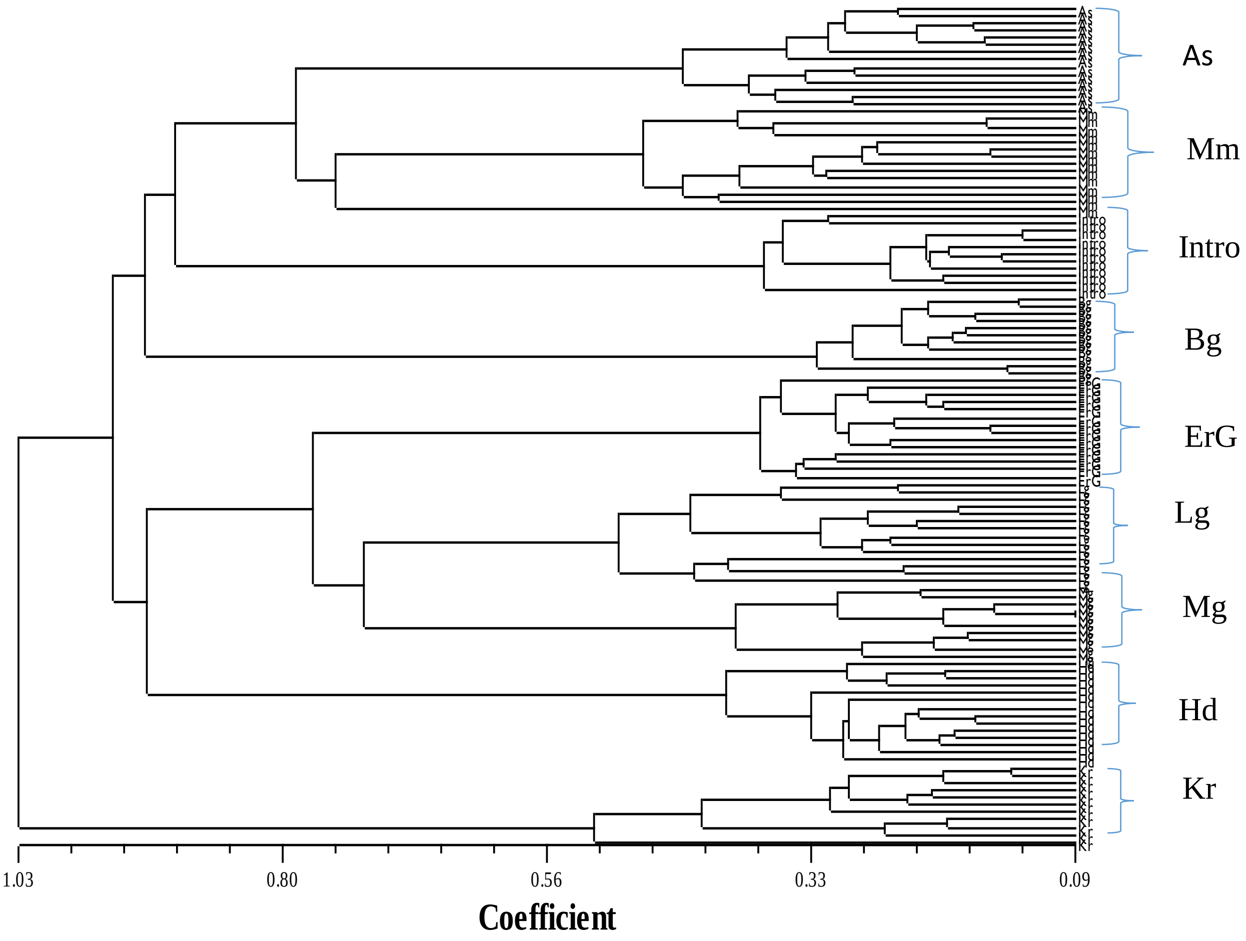


Figure 4.4 Phylogenetic tree analysis result of nine date palm populations using ISSR data, namely: As – Alasabolo, Mm – Mamulae, Intro – early introduced, Bg – Berga, ErG – Erer Gota, Lg – Legahare, Mg – Mego, Hd-Humodoyta, Kr – Kerebuda

4.5. Discussion

4.5.1. Efficiency of ISSR primers on date palms

Development of scientific information on molecular genetic diversity of any crucial crop at national and global level is a baseline for improvement, conservation and sustainable use of that crop. Therefore, the present study mainly focused on the assessment of genetic diversity of Ethiopian date palms using ISSR markers. All ISSR primers that were used in this study produced 100% polymorphic bands and sufficiently detected genetic variation across all date palm genotypes. A total of 241 polymorphic DNA fragments are generated by primers composed of different types of motives: six di-nucleotide motives (129 bands), one tri-nucleotide motif (37 bands), two tetra-nucleotide motives (52 bands) and one penta-nucleotide (23 bands).

Previously, research papers on the investigation of genetic diversity of date palms revealed 100% polymorphism by similar primers: (AG)10C, (AG)10T (Elmeer *et al.*, 2017), (AG)10C, (GACAC)4 and (GACA)4 (Khankahdani and Bagheri, 2019), (AGG)6 and (AG)10T (Karim *et al.*, 2010). On the other hand, Hamza *et al.* (2012) reported percentage of polymorphic bands presented by primers (CT)10G (62.5%), (AG)10T (66.6%) and (AG)10C (60%) as well as Khankahdani and Bagheri (2019) reported no band was amplified by primer by (AGG)6 primer which is in contrast with the present study. Besides, 100% and 80% of polymorphism detected among 17 Iraqi date palms using (GACAC)4, (GACA)4, (AGG)6, (AG)10T and (AG)10C respectively (Khierallah *et al.*, 2014). Indeed, in the current investigation primers (AG)10 and (CT)10T did not show clear amplified DNA bands. (Khankahdani and Bagheri, 2019) have also reported similar results. The result of gene flow (Nm) obtained by all primers was greater than 1 except (AG)10C primer (0.634). The values of Nm >1 interpreted that there is a high level of gene migration between date palm populations and Cui *et al*. (2014) suggested that when the statistical Nm was over 1 sufficient genetic exchange occurred to prevent the genetic differentiation made by genetic drift among populations. Therefore, in this study the genetic flow between date palm populations may be occured by transfer of dates by humans and natural factor like flood. Most of date palm trees that are found in Afar region particularly at Afambo and Asayta grown alongside of Awash River basine. During the rainy season residues of dates eaten by birds and monkeys carried by flood from one place to another. Thus, selling of dates as well as transport of dates residues by flood may be the main factors that cause gene flow in date palm population.

4.5.2. Genetic relationships of date palms in Ethiopia

Nei's genetic identity and genetic distance coefficient values revealed high similarity and low polymorphism between populations (Table 4.4). AMOVA results exhibited genetic differences within and among populations that comparable with other analysis outcomes of this study. Indeed, PCoA and phylogenetic tree results confirmed clustering of date palm individuals according to geographical location and these results are contrary with our previous study using SSR markers (Workia Ahmed *et al*., 2021); this may be due to both markers have different nature on alleles detection and amplification processes.

In this study, high genetic similarity was observed between Alasabolo and Mamulae and Legaharae and Mego populations' based on Nei's genetic similarity coefficient, PCoA and dendrogram. Genetic relation for few individuals from Alasabola and Mamulae as well as between Legaharae and Mego was observed by Workia Ahmed *et al*. (2021). This result partially supports with the current study. Assessment of genetic diversity using ISSR markers for eight samples of date palm landraces collected from different sites at Afar region was reported by Takele *et al*. (2021). These eight samples showed genetic dissimilarity but, do not represent the genetic diversity of date palms from these sites.

Chapter 5

Genetic diversity and population structure of Ethiopian date palms (*Phoenix dactylifera* L.) accessions using microsatellite markers

Abstract

Date palm tree is a special plant that gives eminent contributions in agricultural sustainability and significant socio-economic value. Evaluation of the genetic diversity of date palms at DNA level is very important for breeding and conservation. The objective of this study is to evaluate the genetic diversity and population structure of date palm genotypes using microsstelite markers. In this study, 124 date palm genotypes were amplified using 10 polymorphic microsatellite markers. MPdCIR085 and MPdCIR093 loci showed the highest value of observed and expected heterozygosity, maximum number of alleles and the highest polymorphic information content. A total of 112 of alleles were detected, effective number of alleles with a mean value of 6.61; private alleles ranged from 0.0 - 0.65; observed hetrozygosity ranged from 0.355 - 0.726; expected hetrozygosity varied from 0.669 - 0.906, polymorphic information content with a mean value of 0.809; fixation index individuals relative to subpopulations ranged from -0.066 for locus MPdCIR032 to 0.539 for locus MPdCIR025 while subpopulations relative to total population value ranged from 0.037 (MPdCIR070) to 0.132 (MPdCIR057). All nine accessions, neighbour-joining clustering analysis based on dissimilarity coefficient values were grouped into five major categories while the population structural analysis three groups were formed. Principal coordinate analysis explained 17.33% total of variation in all populations. Generally, the result of this study revealed presence of allele variations and high heterozygosity (> 0.7) in date palm genotypes. The findings of the present study contribute to the genetic improvement and conservation programmes.

Keywords: date palm, genetic diversity, microsatellites, polymorphism, population structure

5.1. Introduction

Date palm tree (*Phoenix dactylifera* L.) is a diploid plant with 2n = 36 chromosome number, a perennial monocotyledonous plant belonging to the Arecaceae family (Mirbahar *et al*., 2014; Flower *et al*., 2019). It is one of the oldest known fruit-bearing tree crops with extensive cultivation and utilization in North Africa and Middle East for at least 5000 years and believed to have originated in Mesopotamia (Bodian *et al*., 2012; Mirbahar *et al*., 2014; Karim *et al*., 2015; Du *et al*., 2020). Date palms have a great socio economic impact and an eminent contribution in agricultural sustainability in many arid and semiarid parts of the world (Kurup *et al*., 2014; Flower *et al*., 2019). It is a multipurpose tree having food, industrial, commercial, medicinal and ornamental values (Johnson, 2012; El-Far *et al*., 2016).

Date palm is distributed throughout the Middle East, North Africa, South Sahel, East and South Africa, and some parts of Europe and USA (Chao and Krueger, 2007; Mirbahar *et al*., 2014; Amom and Nongdam, 2017). It has been introduced to Ethiopia from Middle East countries approximately 200 years ago by Arabian traders (Aregawi Lemlem *et al*., 2018). Cultivation of date palm began in Afar region particularly at Afambo and Asayta and other places nearby Awash River and then spread to Errer Gota and Dire Dawa areas (Hussen, 2010; Ben-Salah, 2015). Date farms of these areas are mainly used for local consumption and income sources through trading in nearby towns. In these areas, different unknown varieties of date fruits are produced which have red and yellow colours with different fruit shapes. In Ethiopia, around 14 known varieties have been introduced from other countries in the last 8 years and have been cultivated in three places: Humodoyta site (Afambo), Asayta and Melka Werer agricultural research center (Melka Werer) for adaptation and improvement program.

Molecular genetic diversity study of date palm varieties has significant contributions in the date palm's genetic improvement and conservation program. To the best of our knowledge, there is no report on the genetic diversity and population structure of date palms in Ethiopia. Molecular genetic diversity studies at DNA level has proven to be the most convenient method for screening variability among plant varieties and determining phylogenetic relationships among plants (Amom and Nongdam, 2017). In other ountries many studies have been conducted to identify date palm genotypes using morphological traits and biochemical markers. However, using these markers alone, detection of genetic variation among genotypes is unreliable because these markers have been influenced by environmental factors and also show low levels of polymorphism (Mirbahar *et al*., 2014; Amom and Nongdam, 2017; Salomon-Torres *et al*., 2017). Different DNA markers have been applied to analyse the genetic relationship of date palm cultivars in many countries such as in Egypt (El-Assar *et al*, 2005; Abd-Alla, 2010), Tunisia (Bodian *et al*., 2012; Zehdi *et al*., 2004), Morocco (Bodian *et al*., 2012), Nigeria (Yusuf *et. al*., 2015), Pakistan (Mirbahar *et al*., 2014) and Syria (Haider *et al*., 2012). It is well known that microsatellite markers are still one of the most powerful molecular tools due to their nature and reproducibility for assessment of genetic diversity, population structure and differentiation. Therefore, the objective of the present research is to assess the genetic diversity and population structure within date palm genotypes collected from different locations in Ethipia using microsatellite markers.

5.2. Materials and Methods

5.2.1. Plant material and DNA extraction

During the time of fruiting, a total of 124 date palm samples both females and males were collected from Afar and Somalia regions of Ethiopia (Figure 4.1.). The collections include introduced date palm varieties (appendix 2.1). Young date palm leaf samples were collected from offshoots, cut into pieces and preserved in silica gel. The dried leaves were ground using liquid nitrogen and acidic sterilised sand. Genomic DNA was extracted using the CTAB method (Doyle, 1991) with some modification made (appendix 3.2). In 100 ml of CTAB buffer (pH 8.0) containing 2% of Cetyltrimethylammonium bromide, 4 ml of 20 mM EDTA, 4 ml of 100 mM Tris-HCl (pH 8.0), 8 g of 1.4 M NaCl and 0.1% β-mercaptoethanol. To test genomic DNA quality, DNA (3 µl) was ran on 1% agarose gel using 0.5X TAE buffer, 90 V, 150 A, 50 W for 30 min set of the gel electrophoresis program and stained with ethidium bromide solution and then illuminated under UV lights, and photographs were captured. DNA concentrations were also determined using a Gene Quant spectrophotometer.

5.2.2. Polymerase chain reaction

A total of 10 SSR primers developed by Billotte *et al*. (2004) were used (Table 5.1) to amplify the isolated DNA. For PCR amplifications, 15 µl PCR reaction mixture containing 100 ng DNA (1 µl), 10X PCR buffer (1.5 µl), 25 mM $MgCl_2$ (1.2µl), 10 mM dNTPs mixes (0.3 µl), 20 µM forward and reverse primer (1 µl), 9.5 µl PCR grade water, and 5U Taq DNA polymerase (0.5 µl) was used. All PCR amplifications were performed in Thermal Cycler (Buio-Rad) with an initial denaturation step at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 1 min, extension at 72ºC for 30 s, final extension at 72ºC for 5 min and holding temperature at 4ºC for infinity. The PCR products were separated using 1.5% agarose gels and stained with ethidium bromide solution and then illuminated under UV lights and the images were captured. The 50bp DNA ladder (Bio tools) was used to estimate the molecular size of DNA fragments of PCR products (Figure 5.1).

Table 5.1 SSR primers used for this study

No	Primer name	Primer sequences	Repeated motif	Base pair size range
1	MPdCIR010	F: ACCCCGGACGTGAGGTG R: CGTCGATCTCCTCCTTTGTCTC	(GA)22	142-162
2	MPdCIR015	F: AGCTGGCTCCTCCCTTCTTA R: GCTCGGTTGGACTTGTTCT	(GA)15	144 -156
3	MPdCIR016	F: AGCGGGAAATGAAAAGGTAT R:ATGAAAACGTGCCAAATGTC	(GA)14	142-166
4	MPdCIR025	F:GCACGAGAAGGCTTATAGT R: CCCCTCATTAGGATTCTAC	(GA)22	220-272
5	MPdCIR032	F: CAAATCTTTGCCGTGAG R:GGTGTGGAGTAATCATGTAGTG	(GA)19	302-320
6	MPdCIR050	F: CTGCCATTTCTTCTGAC R: CACCATGCACAAAAATG	(GA)21	172-260
7	MPdCIR070	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTTGTAGTAT	(GA)17	200-250
8	MPdCIR057	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC	(GA)20	250-310
9	MPdCIR085	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA	(GA)29	166-244
10	MPdCIR093	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG	(GA)16	175-227

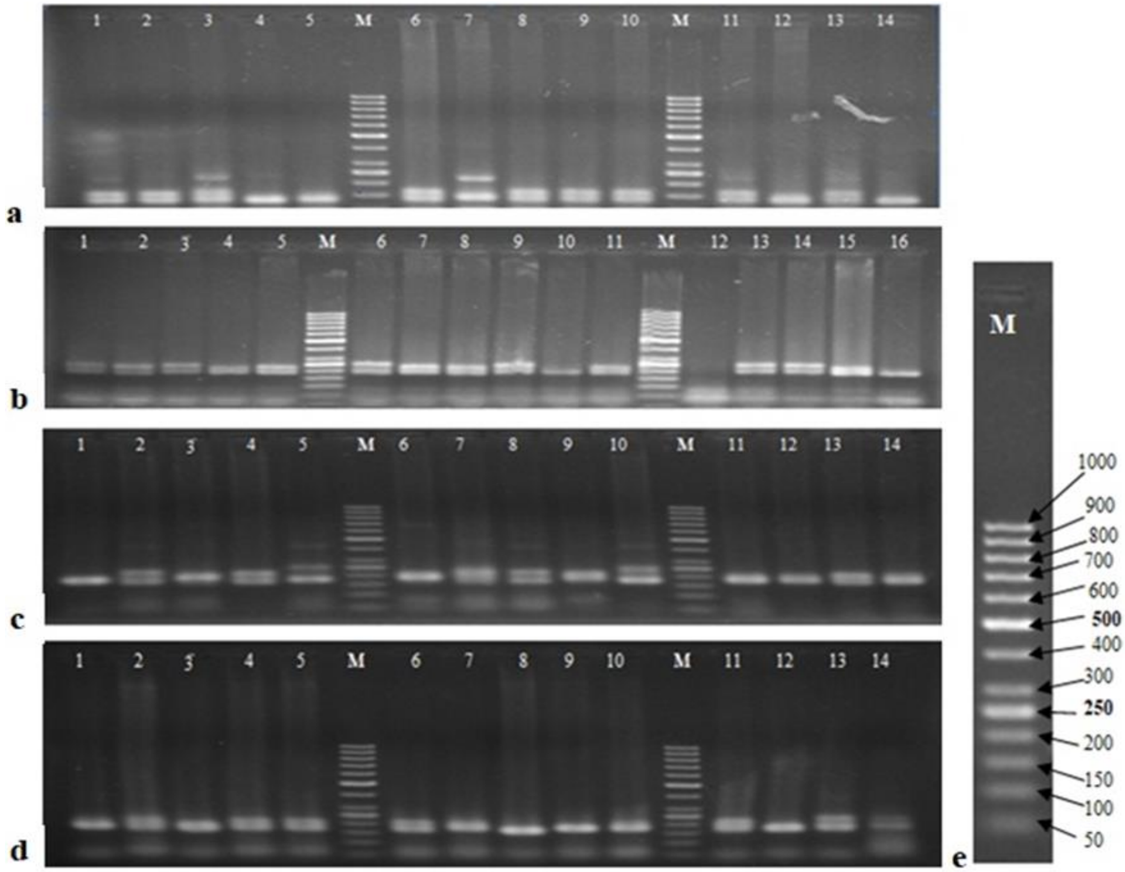


Figure 5.1 Examples of PCR products obtained by the following SSR primers: a) MPdCIR016, b) MPdCIR050, c) MPdCIR085 and d) MPdCIR093 e) M: 50 base pairs molecular weight markers used as a reference for scoring in this study.

5.3. Data Analysis

Amplified DNA fragments produced in each microsatellite locus was recorded manually (appendix 2.4). Genetic diversity parameters i.e. expected hetrozygosity (He), observed hetrozygosity (Ho), observed number of alleles (Na), effective number of alleles (Ne), private alleles per locus, Shannon information index (I), Nm (Gene flow estimated from Fst = 0.25(1 - Fst)/Fst) were calculated by using Popgen32, version 1.31 (Yeh *et al.*, 2009) and GenAlEx, version 6.5 softwares (Peakall and Smouse, 2012). Major allele frequency (MAF) and number of genotypes (NG) per locus was performed using Power Marker software version 3.25. Polymorphic information content (PIC) of each locus was computed by Curves software version 3.0.7 and the fixation index (Fis, Fst, and Fit) were calculated using Arlequin software version 3.5.2.2. Principal coordinate analysis (PCoA) was analysed from distance matrix of each accession using GenAlEx software. Nei's genetic identity and distance was also performed by popgen32 software. Darwin software, version 6.0.21 was used to construct a dendrogram using the neighbour-joining (NJ) algorism based on dissimilarity matrix and by computing bootstraps value over 1000 replicates.

Population genetic structure was analysed based on Bayesian clustering using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) to define the number of clusters in the dataset i.e. ranging from K1 to K10. The admixture ancestry model and correlated allele frequency model were used to perform a Markov chain Monte Carlo simulation algorithm (MCMC). The length of the burn-in period was set to 100,000; MCMC after the burn-in period was set to 200,000, and 10 times was run for each K to estimate K values. Optimal

K value among K groups was determined based on Evanno *et al*. (2005) method using STRUCTURE HARVESTER (Earl and VonHoldt, 2012). The result files obtained from STRUCTURE HARVESTER was also analysed by CLUMPP software (Jakobsson and Rosenberg, 2007) to align the clusters across replicates and to display clusters in each K drawn as coloured box plots.

5.3.1. DAPC and two-locus LD analysis

In addition to STRUCTURE analysis, we performed Discriminant Analysis of Principal Components (DAPC), an assumption-free multivariate clustering method (Jombart *et al.*, 2010) using the R package ‘adegenet’ (Jombart, 2008) to determine genetic structure of date palm genotypes among districts. The optimal number of clusters was inferred using k-means analysis (Jombart, 2008) of principal components (PCs) transformed SSR data and Bayesian Information Criterion (BIC) was used to assess the best supported model i.e. the number and nature of clusters. For the DAPC, eight clusters were chosen because they had the lowest value according to BIC criterion, and 20 PCs were retained. We also calculated Ohta’s variance components of linkage disequilibrium (Ohta, 1982) of D_{IS}^2 (the average disequilibrium within subpopulations), D_{ST}^2 (the contribution to the overall disequilibrium caused by differences in allele frequencies among subpopulations), $D'_{ST}{}^2$ (the variance of the correlation of linkage disequilibrium of one population relative to a total population) and $D'_{IS}{}^2$ (the variance of the correlation of within population relative to that of the total population) using Popgen32, version 1.31.

5.4. Results

5.4.1. SSR marker and its allelic diversity

A wide range of allele variants were observed from each locus (Table 5.2). A total of 112 alleles were amplified with an average of 11.2 from all loci and a minimum (5.0) by MPdCIR032 and a maximum (16.0) number of alleles were detected by MPdCIR085 and MPdCIR093 primers. The mean number of major allele frequency was 0.26; with numbers ranging from 0.16 (MPdCIR085) to 0.37 (MPdCIR016). Number of genotypes per locus ranged from 7 (MPdCIR032) to 47.0 (MPdCIR085). The mean effective number of alleles was 6.61 ranging from 3.0 (for MPdCIR032) to 10.6 (for MPdCIR085). All SSR markers in this study were found to be highly informative with a PIC value ≥ 0.50 of which MPdCIR085 showed the highest PIC (0.90) while MPdCIR032 showed lowest PIC (0.60) with average of 0.81.

Table 5.2 Genetic diversity analysis of 10 polymorphic SSR markers for 124 date palm genotypes

Locus	MAF	NG	Na	Ne	Ho	He	Fis	Fst	Fit	Nm	PIC
MPdCIR010	0.18	40.0	12	7.94	0.67	0.87	0.21**	0.04*	0.24**	3.15	0.86
MPdCIR015	0.28	19.0	7	5.1	0.65	0.80	0.13*	0.89**	0.21**	2.06	0.78
MPdCIR016	0.37	29.0	11	4.94	0.51	0.81	0.36**	0.02	0.37**	3.44	0.78
MPdCIR025	0.31	30.0	13	6.3	0.36	0.84	0.55**	0.08**	0.58**	1.82	0.82
MPdCIR032	0.37	7.0	5	3	0.65	**0.67**	0.03	0.01	0.04	5.11	**0.60**
MPdCIR050	0.24	37.0	11	7.1	0.69	0.86	0.19**	0.01	0.21**	4.52	0.85
MPdCIR057	0.28	30.0	12	5.9	0.47	0.83	0.41**	0.08**	0.45**	1.64	0.81
MPdCIR070	0.30	27.0	9	5.6	0.69	0.82	0.17**	-0.01	0.16**	6.43	0.80
MPdCIR085	0.16	47.0	16	10.6	0.71	**0.91**	0.19**	0.04	0.22**	3.27	**0.91**
MPdCIR093	0.18	41.0	16	9.45	0.73	0.89	0.18**	0.002	0.19**	4.15	0.91
Mean	0.27	30.7	11.2	6.61	0.61	0.83	0.21	0.08	0.26	3.56	0.81
Sum			**112**								

Exact test significant at $*P < 0.05$, $**P < 0.001$

5.4.2. Genetic diversity analysis

In this study, high genetic diversity was observed among nine date palm accessions represented by a total of 124 genotypes. The genetic variation was estimated by observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (Fis, Fst, Fit), are presented in Tables 5.2 and Figure 5.2. The values of observed heterozygosity ranged from 0.36 for the locus MPdCIR025 to 0.73 for the locus MPdCIR093 with mean value of 0.66. The highest expected heterozygosity value of 0.91 for MPdIC085 and the lowest value of 0.71 for MPdCIR032 with a mean of 0.83 for all loci values were observed. In addition, across the population high (0.81) and low (0.73) values of hetrozygosity were observed in date palm populations collected from Mamulae and Kerebuda and also maximum mean number of alleles had been observed in population collected from Mamulae (8.5) and Humodoyta (7.5) and in introduced of date palm population (7.5) (Figure 3.3). Sixteen total private alleles were detected in eight populations and no private allele was observed in Berga population (Figure 5.2).

Population differentiations were also determined by fixation indices (Fis, Fst, and Fit) for each locus with mean value of 0.21, 0.08 and 0.26 respectively. Fixation index (Fis) of individuals of relative to subpopulations ranged from 0.03 for locus MPdCIR032 to 0.55 for locus MPdCIR025 while Fst value ranged from -0.01 (MPdCIR070) to 0.89 (MPdCIR015). Except MPdCIR015, MPdCIR025 and MPdCIR057 all loci showed moderate degree of genotypic differentiation between populations with Fst value of more than 0.05 with significant value at $P < 0.001$. The highest (7.0) and the lowest (4.7) mean values of number of different alleles with a frequency ≥ 5% was in Mego and Legaharae respectively. The mean of number of locally common alleles with a frequency ≥ 5% found in 25% of the total populations ranged from 0.2 (in Errer Gota) to 0.7 (in Mamulae). The mean number of locally common alleles with a frequency ≥ 5% was observed in 50% of the total population with a range of 0.8 (in Kerebuda) to 1.7 (in Mamulae) (Figure 5.2). The genetic distance between date palm populations is presented in Table 5.3. The highest genetic distance (0.64) was observed between Introduced and Kerebuda populations whereas the lowest genetic distance (0.18) was Mamulae and Legaharae populations. AMOVA showed variations 4%, 23% and 73% among populations, among individuals and within individuals respectively (Table 5.4).

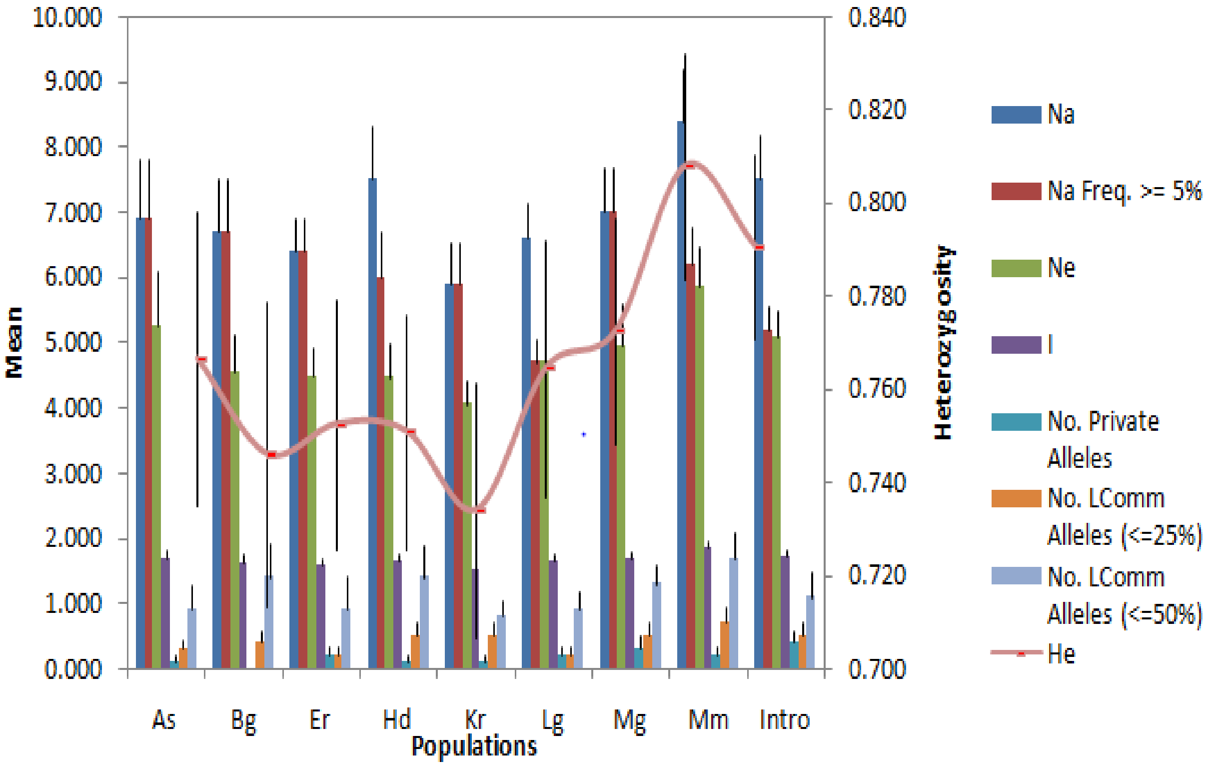


Figure 5.2 Allelic patterns across nine populations of date palm

Table 5.3 Nei's genetic distance between date palm populations

Pop ID	As	Bg	Er	Hd	Kr	Lg	Mg	Mm	Intro
As	****								
Bg	0.3149	****							
Er	0.2819	0.2027	****						
Hd	0.2485	0.2579	0.2906	****					
Kr	0.5382	0.5448	0.5369	0.6244	****				
Lg	0.3058	0.2454	0.2664	0.3174	0.4546	****			
Mg	0.4686	0.5124	0.5220	0.4728	0.2863	0.3722	****		
Mm	0.2546	0.2802	0.3227	0.2965	0.2491	0.1791	0.2222	****	
Intro	0.2909	0.4011	0.4020	0.2989	0.6403	0.3947	0.5843	0.3384	****

Table 5.4 Summary of AMOVA obtained from SSR data

Source	Df	SS	MS	Est. Var.	%
Among Populations	8	74.394	9.299	0.159	4%
Among Individuals	115	575.037	5.000	0.968	23%
Within Individuals	124	380.0	3.065	3.065	73%
Total	247	1029.431		4.191	100%

5.4.3. Clustering and population genetic structure

All genotypes separated into five major clusters (Figure 5.3a) and one population is represented by the same colour as coded in number list (Figure 5.3b) and similarly displayed in a dendrogram. In Cluster 1 twenty eight, cluster 2 thirty four, cluster 3 twenty three, cluster 4 twenty five, cluster 5 fourteen individuals were grouped. Cluster 1, 2, 3, and 5 were further sub-divided into two sub-clusters while cluster 4 was divided into three sub-clusters. All sub clusters further subdivided into sub groups which represented a clade composed of mixed genotypes of the populations. Half of the genotypes of each

Introduced and Humodoyta populations were found together in cluster 1 whereas other clusters contained mixed genotypes from different populations. PCoA analysis revealed 17.33% total variation explained by the first 3 axes (Figure 5.4a). The distribution of genotypes on PCoA graph is not separately clustered depending on their accessions; instead they showed intermixing of genotypes among populations and also similarly displayed on NJ tree. In population STRUCTURE analysis, date palm genotypes from all accessions were segregated into three subpopulations in Bayesian clustering model using STRUCTURE software version 2.3.4 with the criterion of maximum membership probabilities based on Evanno *et al*. (2005) method i.e. the delta K value had the highest peak, at K = 3 (Figure 5.4b). Using STRUCTURE output CLUMPP displayed light blue, orange and violet colours of bar plots (Figure 5.4c) and revealed admixture structure in each date palm population regardless of the accessions. In this study, PCoA, NJ tree and population structure analysis have shown similar results on the distribution and clustering of genotypes (Figure 5.4a and Figure 5.4a, c) were accord with the result in population STRUCTURE.

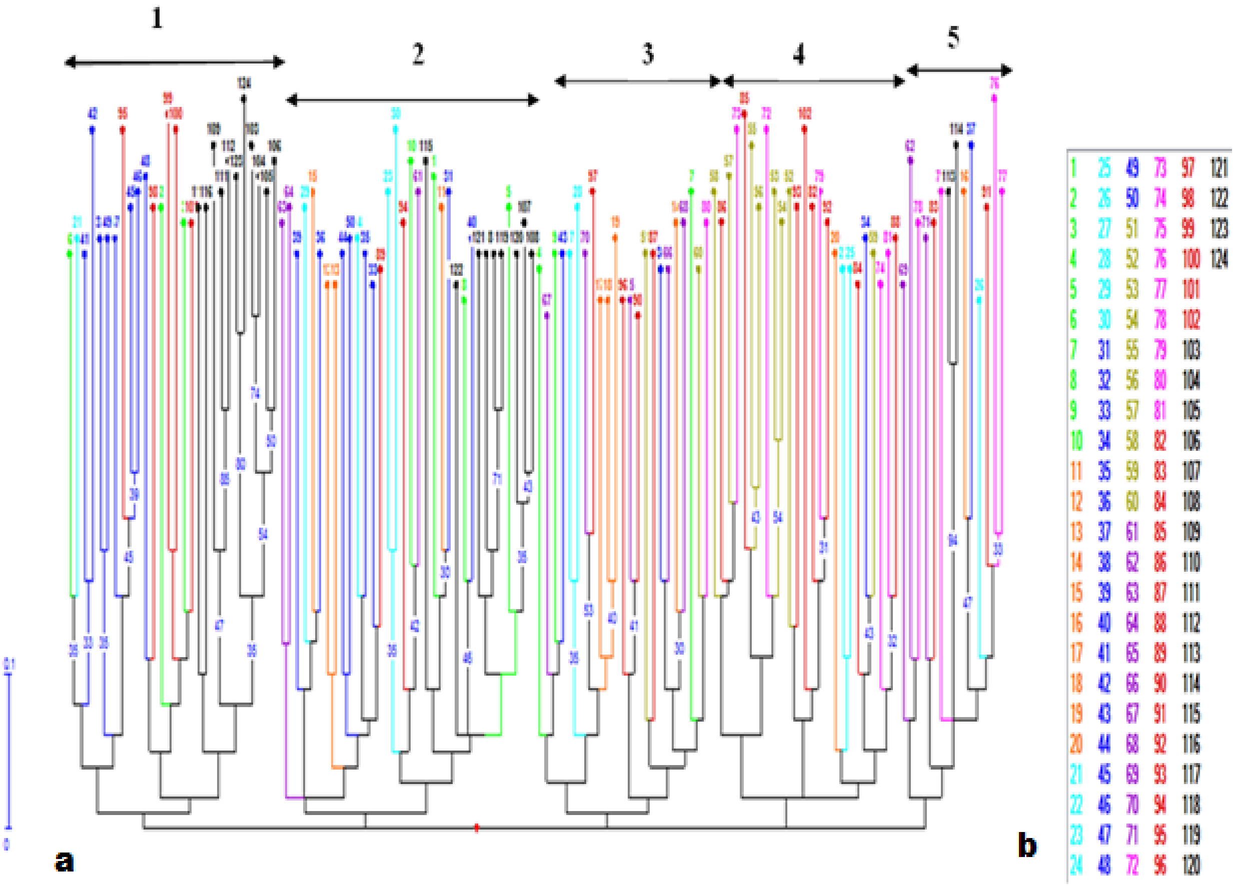


Figure 5.3 Phylogenetic tree analysis result using SSR date **a)** A dendrogram based on dissimilarity matrix using NJ method indicated with bootstrap support ≥ 30 and **b)** List of sample number for each population which are displayed on a tree based on colour labelling i.e. 1 - 10 is Alasabolo group, 11 - 20 is Berga group, 21 - 30 is Errer Gotta group, 31 - 50 is Humedeyta group, 51 - 60 is Kerebuda group, 61 - 71 is Legaharae group, 72 - 81 is Mego group, 82 - 102 is Mamulae group and 103 - 124 is early Introduced group

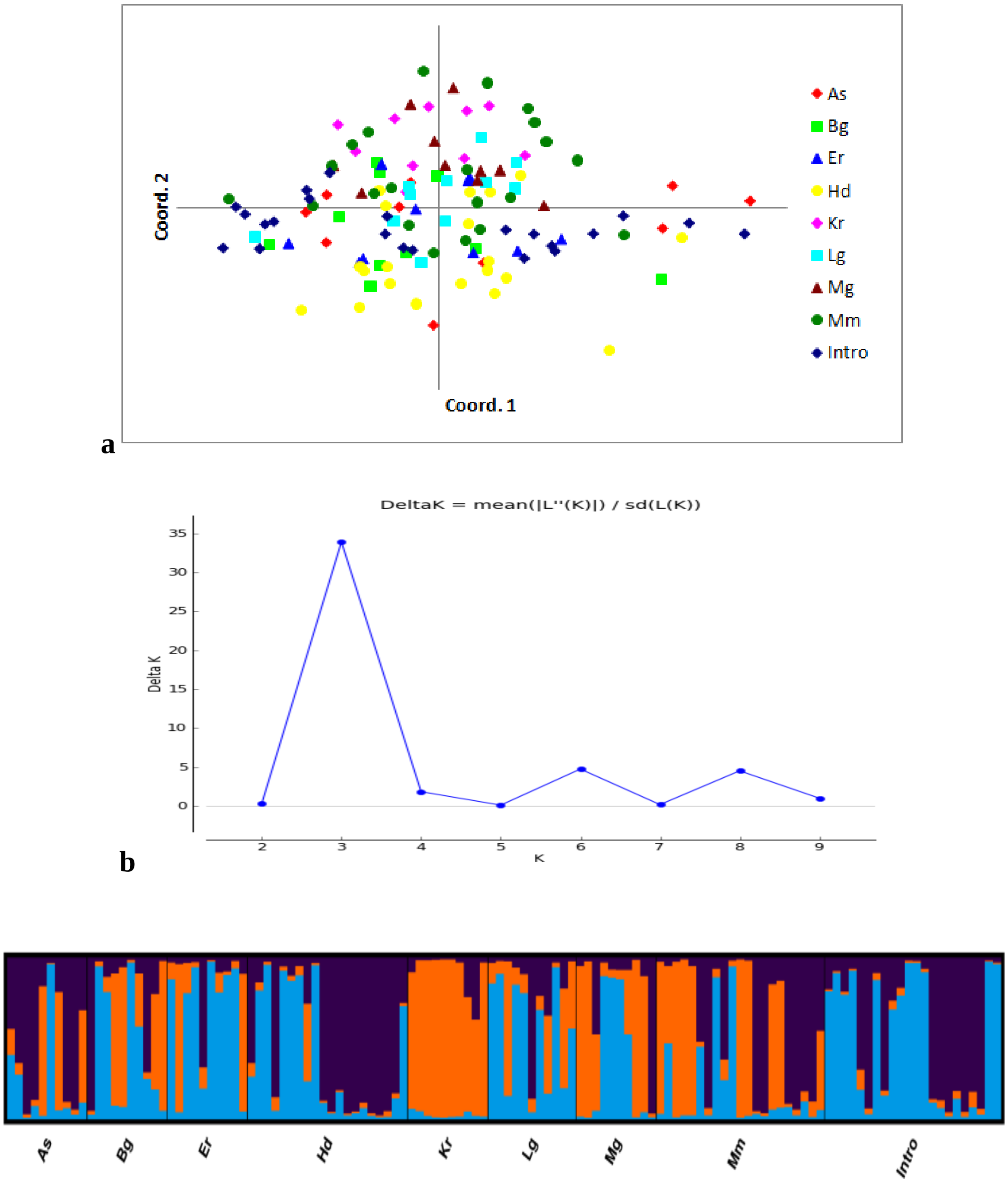


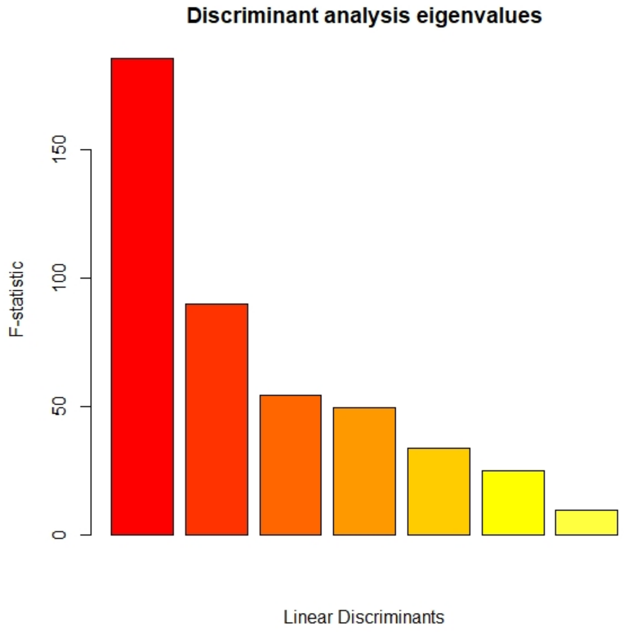
Figure 5.4 PCoA and population STRUCTURE analysis results from SSR data **a**) Two-dimensional plot of Principal coordinates analysis of 124 date palm genotypes represented by nine populations **b**) Estimation of population using ΔK = mean (|L''(K)|) / sd(L(K)) with cluster number (K) ranged from 1 to 10 and K = 3 is the optimal K value based on Evanno *et al*. (2005) method **c**) Results of STRUCTURE analysis based on microsatellite data and estimation of genetic structure of the nine populations using K = 3. Each population is represented by a vertical bar and separated by a black line, partitioned into coloured segments representing the proportion of the individual's genome in the K clusters.

5.4.4. Discriminate Analysis of Principal components and two-locus linkage disequilibrium

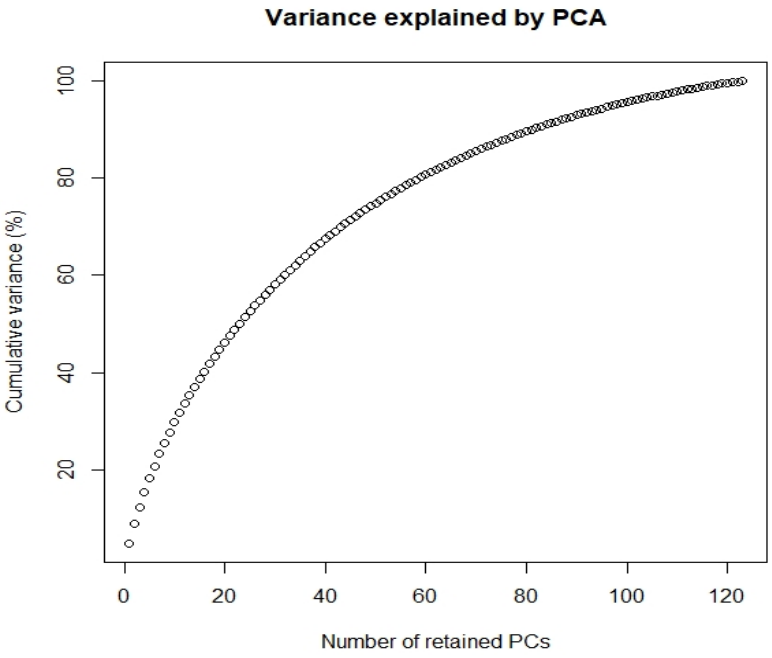
Using Bayesian model based approach the membership probabilities of each date palm individual for the different groups were obtained from DAPC and the results of DAPC analysis. The DAPC separated date palm genotypes into eight clusters based on the first two linear discriminants (Figure 5.5). The DAPC analyses revealed that all of early introduced date palm genotypes except one individual were represented by two genetic clusters (DAPC cluster 4 and 6). This result suggests that these date palm cultivars are genetically distinct groups from others.

Besides, the NJ tree pattern showed that these introduced date palms are predominantly found in sub-cluster of the major cluster 1 and 2 of NJ tree; this result is consistent with the DAPC cluster 4 and 6. The DAPC cluster 1 and 3 only contained 21 and 23 individuals from Afambo and Asayta district respectively. While, the DAPC cluster 2, 5, 7 and 8 represented by mixed individuals from all districts; i.e. DAPC cluster 2 contained a total of 18 individuals: 3 from Afambo, 12 from Asayta and 3 from Shinile; DAPC cluster 5 comprises a total of 13 genotypes: 3 from Afambo, 1 from Asayta, 1 from Errer, 7 from Shinile and 1 introduced cultivar and DAPC cluster 7 consisted 6 individuals from Afambo, 1 individual from Asayta, 9 individuals from Errer Gota; the DAPC cluster 8 included 7 genotypes from Afambo, 4 genotypes from Asayta and 1 genotype from Shinile districts.

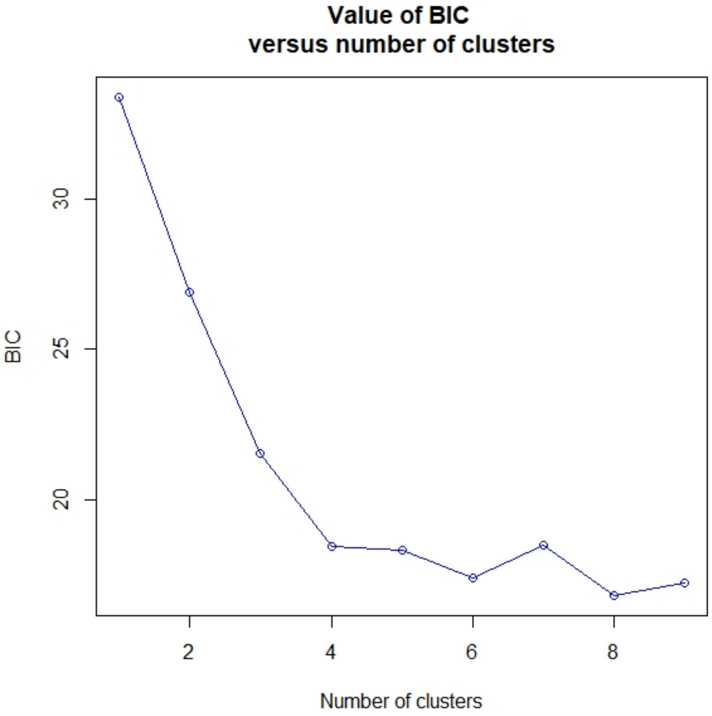
In general, the DAPC result showed that individuals were slightly admixed structure as compared to population STRUCTURE and NJ results. The overall average variance of the disequilibrium of the individual compared to the total populations D_{IT}^2 was 0.04681. A total average of $D'_{IS}{}^2$ (0.0435) was larger than $D'ST^2$ (0.0033). Besides, the average value of D_{ST}^2 (0.0265) was greater than the average of D_{IS}^2 (0.0201). From a total pairs of loci, only 30 pairs of loci showed the dual relationships of $D_{ST}^2 > D_{IS}^2$ and $D'_{IS}{}^2 > D'ST^2$ (Table 5.5). This relation in pairs of loci indicated nonrandom association of the SSR alleles at particular variable loci was mainly caused by limited migration and random process or genetic drift (Ohta, 1982a Ohta, 1982b). However, the rest of 15 pairs of loci showed the dual relationships of $D_{ST}^2 < D_{IS}^2$ and $D'_{IS}{}^2 < D'ST^2$.



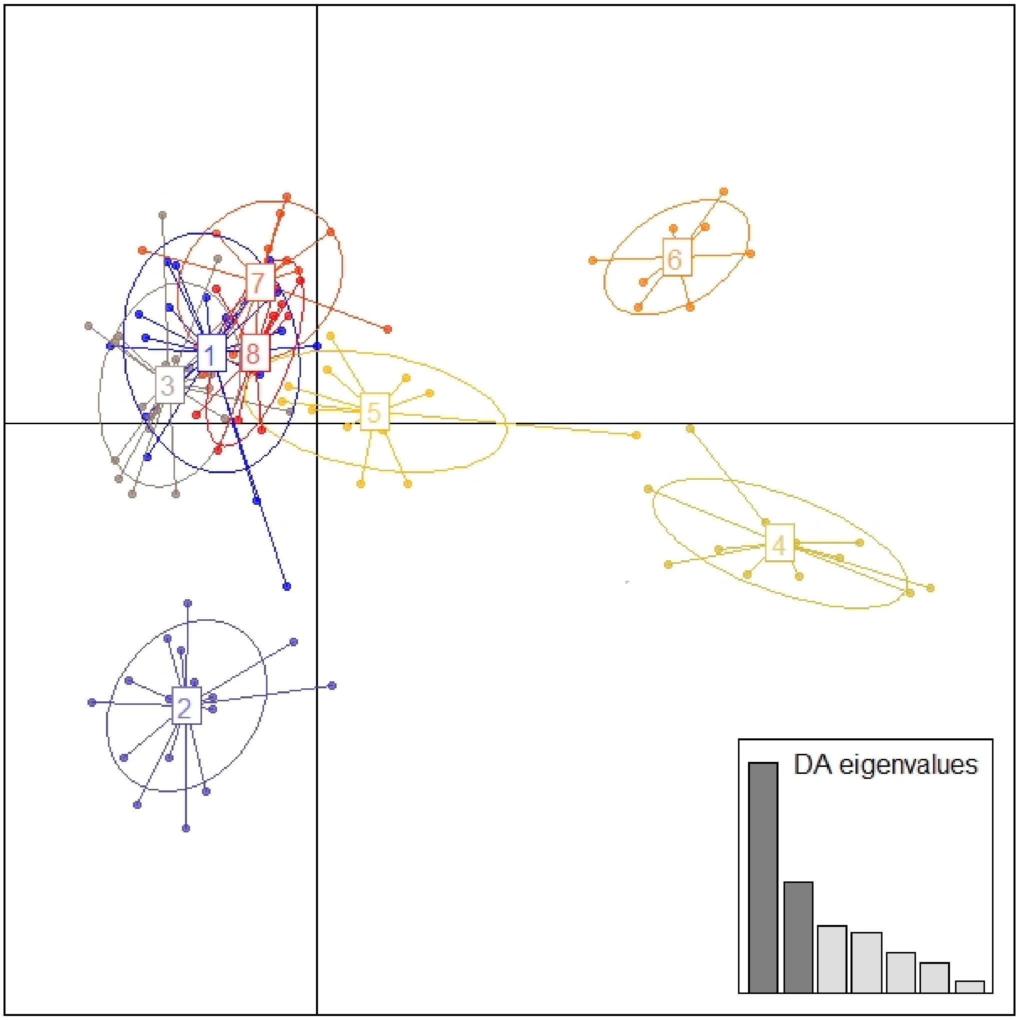
a)


Variance explained by PCA
Cumulative variance (%)
20
40
60
80
100
0
20
40
60
80
100
120
Number of retained PCs

b)


Value of BIC
versus number of clusters
BIC
20
25
30
2
4
6
8
Number of clusters

C)



d)

Figure 5.5 Bayesian model-based clustering of date palms among districts: **a**) number of retained principal components, **b**) value BIC and number of clusters, **c**) discriminate analysis of eigenvalues, d) discriminant analyses of principal components (DAPC) for 124 date palm genotypes. The axes represent the first two linear discriminants (LD); each circle represents a cluster, and each dot represents an individual. Numbers represent the different subpopulations identified by DAPC analysis

Table 5.5 Ohta's two-locus analysis of linkage disequilibrium of 10 SSR markers

Locus A - Locus B	(DIT)^2	(DIS)^2	(D'IS)^2	(DST)^2	(D'ST)^2
MPdCIR010 - MPdCIR015	0.04751	0.01856	0.04314	0.03147	0.00437
MPdCIR010 - MPdCIR016	0.04730	0.02264	0.04363	0.02487	0.00367
MPdCIR010 - MPdCIR025	0.05556	0.02375	0.05144	0.03162	0.00412
MPdCIR010 - MPdCIR032	0.04203	0.01575	0.03886	0.02806	0.00317
MPdCIR010 - MPdCIR050	0.04232	0.02251	0.03863	0.01832	0.00369
MPdCIR010 - MPdCIR057	0.05966	0.02227	0.05514	0.03522	0.00452
MPdCIR010 - MPdCIR070	0.04375	0.02477	0.03905	0.01906	0.00470
MPdCIR010 - MPdCIR085	0.04356	0.02536	0.03866	0.01964	0.00490
MPdCIR010 - MPdCIR093	0.03898	0.02103	0.03474	0.01743	0.00423
MPdCIR015 - MPdCIR016	0.05277	0.01889	0.04908	0.03436	0.00369
MPdCIR015 - MPdCIR025	0.06567	0.01806	0.06213	0.04474	0.00355
MPdCIR015 - MPdCIR032	0.05207	0.01177	0.05071	0.03777	0.00136
MPdCIR015 - MPdCIR050	0.04054	0.01585	0.03838	0.02464	0.00216
MPdCIR015 - MPdCIR057	0.06444	0.02086	0.06164	0.04634	0.00280
MPdCIR015 - MPdCIR070	0.04272	0.01720	0.04057	0.02553	0.00214
MPdCIR015 - MPdCIR085	0.04669	0.01901	0.04294	0.02665	0.00375
MPdCIR015 - MPdCIR093	0.04104	0.01604	0.03844	0.02402	0.00260
MPdCIR016 - MPdCIR025	0.05557	0.02307	0.05120	0.03331	0.00437
MPdCIR016 - MPdCIR032	0.03057	0.01013	0.02922	0.02532	0.00136
MPdCIR016 - MPdCIR050	0.04109	0.02180	0.03874	0.01827	0.00235
MPdCIR016 - MPdCIR057	0.05689	0.02261	0.05336	0.03698	0.00353
MPdCIR016 - MPdCIR070	0.03739	0.02041	0.03304	0.01797	0.00435
MPdCIR016 - MPdCIR085	0.04812	0.02370	0.04499	0.02179	0.00313
MPdCIR016 - MPdCIR093	0.04131	0.02302	0.03758	0.01799	0.00373
MPdCIR025 - MPdCIR032	0.05555	0.01314	0.05354	0.04143	0.00201
MPdCIR025 - MPdCIR050	0.04742	0.01989	0.04459	0.02653	0.00283
MPdCIR025 - MPdCIR057	0.07457	0.02512	0.06850	0.04683	0.00608
MPdCIR025 - MPdCIR070	0.05081	0.02290	0.04671	0.02691	0.00411
MPdCIR025 - MPdCIR085	0.05249	0.02404	0.04776	0.02734	0.00473
MPdCIR025 - MPdCIR093	0.05400	0.02914	0.04803	0.02467	0.00597
MPdCIR032 - MPdCIR050	0.02965	0.01014	0.02870	0.02053	0.00095
MPdCIR032 - MPdCIR057	0.05998	0.01488	0.05680	0.04419	0.00317
MPdCIR032 - MPdCIR070	0.02851	0.01240	0.02729	0.01746	0.00121
MPdCIR032 - MPdCIR085	0.04159	0.01533	0.03965	0.02655	0.00194
MPdCIR032 - MPdCIR093	0.03874	0.01532	0.03682	0.02203	0.00191
MPdCIR050 - MPdCIR057	0.05406	0.02282	0.05089	0.02983	0.00317
MPdCIR050 - MPdCIR070	0.03817	0.02197	0.03593	0.01418	0.00223
MPdCIR050 - MPdCIR085	0.03777	0.02190	0.03560	0.01652	0.00218
MPdCIR050 - MPdCIR093	0.03628	0.02347	0.03318	0.01443	0.00310
MPdCIR057 - MPdCIR070	0.05421	0.02165	0.05067	0.03015	0.00353
MPdCIR057 - MPdCIR085	0.05418	0.02473	0.04909	0.02969	0.00509
MPdCIR057 - MPdCIR093	0.05341	0.02510	0.04830	0.02515	0.00511
MPdCIR070 - MPdCIR085	0.03828	0.02089	0.03571	0.01675	0.00257
MPdCIR070 - MPdCIR093	0.03174	0.01794	0.02913	0.01411	0.00261
MPdCIR085 - MPdCIR093	0.03734	0.02203	0.03446	0.01512	0.00288
Overall Average	0.04681	0.02009	0.04348	0.02648	0.00332

5.5. Discussion

5.5.1. SSR markers for allelic diversity of Ethiopian date palms

The purpose of the present study is to determine the genetic diversity and relationship among date palm genotypes and populations using SSR markers. All of the SSR primers used in this study demonstrated PIC value ranging from 0.509 to 0.899. These are considered to be highly informative markers and the occurrence of allele variations among populations. Similary results were reported by (Khierallah *et al*., 2011; Zehdi-Azouzi *et al*., 2015). A total of 112 alleles were detected from 124 date palm individual samples. This exhibits the presence of high genetic diversity within date palm populations. In this study, number of alleles/ locus varied from 5 to 16 and maximum number of alleles (16) was amplified by MdPCIR085 and MdPCIR093 loci. According to Bodian *et al*. (2011) maximum number of alleles (11) was detected by MdPCIR050; ranging between 4 and 11, whereas Aljuhani (2016) reported number of alleles ranged from 6 to 15 per locus and maximum (15) number of alleles amplified by primer MdPCIR015.

5.5.2. Ethiopian date palm diversity and cluster analysis

The current study showed high genetic diversity among Ethiopian date palm populations may be due to high heterozygosity (> 0.72) caused by the common sextually reproduction systems of date palms being used than asextually reproduction (Workia Ahmed, personal observation). This is also supported by (Ohta, 1982a; Ohta, 1982b) gametes with favorable combinations of alleles would increases in every population. In this study, NJ tree and principal coordinate analysis showed clear genetic relationship between unknown date palm varieties and early introduced date palm cultivated varieties. The dendrogram generated five major clusters.

Generally, in this study there was no clear separation among genotypes according to their geographical locations. In cluster one, eight individuals from Humodoyta *kebele*, five individuals from Mamulae *kebele*, three individuals from Alassabolo *kebele* and one individual from Errer Gota were clustered together with these early introduced date palm cultivated varieties (i.e. Sagaii, Jarvis, Khadrawy, Khalas, Medjool Israel and Khayra). Others cultivated varieties (Mdjool England, Shish, Zamli and Ashal Al Hassa) were found in cluster two with mixed individuals from Legaharae, Berga, Humodoyta, Error Gota, Alassabolo and Mamulae populations. Barhee cultivated variety was only found in cluster five incorporated with individuals from other populations. Half of date palm genotypes from Kerebuda population mainly observed in sub-cluster of a clade four categories. This result also supported results in PCoA and population STRUCTURE analysis i.e., intermixing distributions of genotypes on principal component axis and admixture structures. The present result of PCoA showed certain match with the previous studies (Zehdi *et al*., 2004; Chaluvadi *et al*., 2018) whereas contrary with other reports (Khierallah *et al*., 2011; Racchi *et al*., 2014; Zehdi-Azouzi *et al*., 2015; Aljuhani, 2016).

5.5.3. Model-based population structure analysis

According to model-based clustering for genetic structure of date palm individuals, three genetically distinctive subpopulations showed the absence of match with their collection sites. The highest mean similarity score was estimated at K = 3, the most probable clustering of populations was observed at this K level and showed admixture structure among populations. This result displays the date palm populations that have a common genetic background and also they shared common alleles among them. The most differentiated population in this study was observed in Kerebuda population, which showed fewer admixtures than the other populations.

Generally, the structure result had shown shared ancestry between unknown date palm genotypes and early introduced intro date palm cultivars. The admixture result of the present study at K = 3 is in agreement with other reportes (Zehdi-Azouzi *et al*., 2015; Aljuhani, 2016; Moussouni *et al*., 2017). Similarly, He, *et al*., (2017) reported three differentiated date palm memberships at K = 3 using different SSR as well as four date palm groups at K = 4 using SNPs. In both markers they reported the admixture population structure of date palms at best K value of each cluster that was collected from different parts of the world. Intermixing clustering result of date palm genotypes in structure analysis of the present study to some extent coincides with the previous finding at different five K groups that were studied using different SSR primers (Flowers *et al*., 2019; Chaluvadi *et al*., 2018).

The DAPC result of this study exhibited genetic differentiation between groups, while overlooking within-group variation and achieved the best discrimination of individuals into pre-defined groups. We confirmed maximum likelihood-based clustering results using DAPC method that is considered free of Hardy-Weinberg and linkage disequilibrium assumptions. DAPC approach rely on discriminant functions that seeks to maximize the diversity between clusters while minimizes within-cluster diversity

(Jombart *et al*., 2010). Due to this reason DAPC mostly assigned individuals to single cluster (Jombart *et al*., 2010). DAPC was suggested an alternative method used to identify and describe clusters of genetically related individuals by analysing complex genetic data and detecting admixed individuals by determining the probability that each individual belonged to each cluster (Aljuhani, 2016; Miller *et al*., 2020).

Overall, the AMOVA result of expected hetrozygosity revealed high genetic differentiation within date palm genotypes and the outcome of NJ tree, PCoA and structure analysis of this study is dominated by admixture structure patterns among populations. This might be due to the codominant nature of microsatellite markers that contributed to detectionof high allele's variations per locus as well as high heterozygosity within date palm genotypes. On the other hand, date palms are dioecious and cross and wind- pollinated plant species. This nature of the palms facilitates broaden genetic variations within date palm genotypes. In the case of cross-pollinating species, it is obvious that within plant populations maintain high levels of genetic diversity due to their breeding system (LoveLess and Hamrick, 1984; Charlesworth, 2006; Rao and Hodgkin, 2002). Moreover, at date palm collection sites of the current study, there was no integrated management system related to propagation for date palms, i.e. the plants are like wild (Workia Ahmed, personal communication). Consequently, this situation could increase the probability of date palms breeding from seeds rather than offshoots. The date palms germinated by seeds have dioecious nature so that it is expected to increase the genetic distance between date palm genotypes; also low rate of gene flow within the population contribute to exhibited high genetic diversity within populations rather than among populations.

Chapter 6

The genetic relationships of date palm cultivars using single nucleotide polymorphism markers

Abstract:

Date palm (*Phoenix dactylifera* L.) is a perennial fruiting tree which belongs to Arecaceae family under the genus *Phoenix*. The nature of date palms is dioecious and long-lived with a wide range of phenotypic variations. Characterization of the phylogenetics relationships of date palm cultivars using molecular markers is critically important for conservation and exploitation of genetic resources. The objective of this study is to evaluate the genetic relationship of date palm cultivars using SNPs markers. In this study, DNA extracted from 15 date palm cultivars and four SNPs primer pairs were used for PCR amplification. DNA fragments amplified by PDSNP07 (~576bp), PDSNP010 (~581bp), PDSNP017 (~500bp) and PDSNP032 (~6860bp) primers were checked using 3% agaros gel electrophoresis and then purified and sequenced commercially. Phylogenetic tree, nucleotides contents and pairwise distance were conducted from a total of 1578 positions of nucleotides of SNPs sequenes of 15 cultivars. For phylogenetic tree analysis four corresponding reference sequences of date palm cultivars from NCBI database was used and the phylogenetic tree showed two major groups and further divided into many clusters. From 15 cultivars of this study, the sum of total sequences of AT and GC by PDSNP07 (6219 bases), by PDSNP10 (6301 bases), by PDSNP17 (7187 bases) and by PDSNP32 (7235 bases) were obtained. Among cultivars, Ashal Al Hassa contained the highest (175.7) and Medjool England contained lowest

(163.3) GC percentage. While the highest AT percentage was 236.7 (Medjool England) and the least AT percentages was 224.3 (Ashal Al Hassa). In pair-wise distance analysis result, the least value was 0.009 (Barhee and Jarvis) and the highest value was 0.494 (Shishi and Ashal Al Hassa). Therefore, presence of genetic variations among cultivars and identification of their phylogenetic relationship is important in documenting for further research studies and development of date palm conservation program in Ethiopia and elsewhere.

Key words: Cultivars, *Phoenix dactylifera*, Phylogenetic relationships, Polymorphism, SNPs Markers

6.1. Introduction

Date palm (*Phoenix dactylifera* L.) is a monocotyledon dioecious perennial tree that belongs to Arecaceae (Palms) family in the genus *Phoenix*. Cultivation and germplasm exchange of this crop is commonly known for long time in arid and semi-arid parts of the world. Research results indicated that date palm is most probably native in Mesopotamia (Karim *et al*., 2012; Mirbahar *et al*., 2014). This tree has many values including food, industrial, commercial, medicinal and ornamental uses (Johnson, 2012; El-Far *et al*., 2016). Date fruits are nutritionaly rich which contains sugars and other essencial minerals and vitamins (Al-Khalifah and Shanavaskhan, 2012) and different parts of date palm are also used for different purposes including trunks to construct houses, hives, bridges and as packing material (Al-Jabri, 2014). The terminal buds and young leaves are cooked as vegetables while rachises are used for paper making (Khiari *et al*., 2011; El-Hadrami and Al Khayri, 2012).

Ethiopia has date palm cultivation history around 200 years in Afar region particularly at Afambo and Asayta and other places like Errer Gota and Dire Dawa areas (Lemlem *et al*., 2019). According to Hussen (2010) and Ben-Salah (2015), Afar societies have made date palm plantations at Awash delta and Afambo places of Afar region of Ethiopia. Addtionally, a moment ago Ethiopia has imported 14 *in vitro* date palm varieties for production improvement and planted at Afambo (Humodoyta site), Asayta and Melka Werer (Melka Werer agricultural research centre). Date palms have long life history and out-breeding systems due to that morphological traits are vulnerable to environmental factors and can not provide reliable results during the study of genetic variations and identifications among cultivars (Khanam *et al*., 2012; Mirbahar *et al*., 2014).

Therefore, study of genetic variation using molecular markers is the best method for offering clear genetic information and phylogenetic bases of plant species. In date palm, many research studies have been reported on evaluation of genetic diversity and phylogenetic relationships using RAPD (Haider *et al.*, 2012; Marsafari and Mehrab, 2013; Srivashtav *et al*., 2013; Khierallah *et al*., 2014; Mirbahar *et al*., 2014), ISSR (Zehdi *et al*., 2004; Karim *et al*., 2010; Hamza *et al*., 2012), AFLP (Rhouma *et al*., 2007; Khierallah *et al*., 2011-b; Ibrahim *et al*., 2014), SSR (Khierallah *et al*., 2011a; Elmeer *et al*., 2011; Zehdi-; Bodian *et al*., 2012; Racchi *et al.*, 2014; Yusuf *et al*., 2015; Azouzi *et al*., 2015; Aljuhani 2016; Moussouni *et al*., 2017; Chaluvadi *et al*., 2018; Ahmed *et al*., 2021;), SNP (Hazzouri *et al*., 2015; Mohamoud *et al*., 2019; Faqir *et al*., 2019).

Selection and identification of plant varieties using SNP markers is preferred in plant breeding and other plant research programs as relatively dominant and co-dominant markers. This is because of SNPs in plants have high genomic abundance, locus specificity, co-dominance, have potential for high throughput analysis and lower genotyping error rates (Adu *et al*., 2019; Zhang *et al*., 2020). Therefore, the objective of the present study is to investigate the phylogenetic relationship of date palm cultivars in Ethiopia using SNPs markers.

6.2. Materials and methods

6.2.1. Plant materials and DNA extraction

Sample collection for SNPs analysis was conducted in Humodoyta Kebele specifically named Humodoyta *in vitro* date palm adaptation farm and this area is found at Afambo district in Afar regional state of Ethiopia. The alttitude ranges from 330 to 350 meters above sea level and mean annual rainfall of 122 mm with bimodal rainy seasons. Additionally, a total of four unknown date palm genotypes collected from Afambo, Asayta, Dire Dawa (Legaharae) and Errer Gota that were grown as wild were included in the study (Table 6.1). Young leaf samples were collected and cut into pieces and preserved in silica gel.

Table 6.1 Date palm cultivars and their collection site

No	Name of cultivars	Region	Collection place	Coordinate
1	Barhee	Afrar	Afambo - Humodoyta	N11^{O}31.241’ , E041^{O}34.228’
2	Khalas	Afrar	Afambo - Humodoyta	N11^{O}31.202’ , E041^{O}34.233’
3	Medjool Israel	Afrar	Afambo - Humodoyta	N11^{O}31.237’ , E041^{O}34.158’
4	Medjool England	Afrar	Afambo - Humodoyta	N11^{O}31.221’ , E041^{O}34.157’
5	Shishi	Afrar	Afambo - Humodoyta	N11^{O}31.209’ , E041^{O}34.170’
6	Zamlli	Afrar	Afambo - Humodoyta	N11^{O}31.205’ , E041^{O}34.154’
7	Ashal Al Hassa	Afrar	Afambo - Humodoyta	N11^{O}31.205’ , E041^{O}34.154’
8	Khyara	Afrar	Afambo - Humodoyta	N11^{O}31.196’ , E041^{O}34.244’
9	Saggii	Afrar	Afambo - Humodoyta	N11^{O}31.200’ , E041^{O}34.254’
10	Khadrawy	Afrar	Afambo - Humodoyta	N11^{O}31.191’ , E041^{O}34.238’
11	Jarvis – male	Afrar	Afambo - Humodoyta	N11^{O}31.244’ , E041^{O}34.192’
12	Unknown	Afar	Afambo	N11^{O}30.891’ , E041^{O}33.564’
13	Unknown	Dire Dawa	Legaharae	N09^{O}35.722’ , E041^{O}53.609’
14	Unknown	Somalae	Errer Gota	N 09^{O}33.182’, E041^{0}23.149’
15	Unknown	Afar	Asayta	N11^{O}30.981’, E041^{O} 29.687’

The dried leaves were ground using liquid nitrogen and acidic sterilized sand. Genomic DNA was extracted using CTAB method (Doyle, 1991) in 100 ml CTAB buffer (pH 8.0) containing 2% CTAB, 4 ml of 20 mM EDTA, 4 ml of 100 mM Tris-HCl (pH=8.0), 8g of 1.4 M NaCl and 0.1% β-mercaptoethanol. Each DNA sample quality was tested using 1% agarose gel electrophoresis and DNA concentrations were checked using a Gene Quant spectrophotometer.

6.2.2. SNPs primers, PCR amplification and sequencing

Four primers pair reported by Faqir *et al*., 2019 (Table 6.2) were used. PCR was performed in 20 µl total volume containing 50 ng DNA template (3 µl), 10X PCR buffer (2.5 µl), 2.5 mM dNTPs (2 µl), 25 pM of the forward and reverse primer (1 µl), 5.0 units Taq DNA Polymerase (0.5 µl) and ddH2O (11 µl). The thermal PCR condition was set to an initial denaturation at 94°C for 1 min followed by 35 cycles denaturation at 94°C for 30s, annealing for 40 sec using annealing temperature of each primer, extension at 72°C for 30s, final extension at 72°C for 5 min and holding period at 4^oC. The PCR product was checked in gel electrophoresis using 3% agaros before purification and sequenced commercially by DNA Sequencing Services (Macrogen, Inc. Seoul, Korea). The forward and reverse DNA sequence data of each sample was assembled separately using DNA Dragon DNA Sequence Contig Assembler Software version 1.5.2 (www.sequentix.de) and after assembling and editing the sequences copied the consensus sequences to be used for data analysis.

Table 6.2 SNPs primers sequences, their expected band sizes and annealing temperature (Tm)

Primer name	**Primer sequences**	**Expected band size (bp)**	**Tm (°C)**
PDSNP07	F 5’-TGTAAAACGACGGCCAGTGGCACCATTGAGGACTTTGG-3’	576	62
	R 5’-CAGGAAACAGCTATGACCGCCGGTTGCTCTCTAGATCC-3’		
PDSNP10	F 5’-TGTAAAACGACGGCCAGTACTTTGTGGCATTTGGTTCG-3’	581	59
	R 5’-CAGGAAACAGCTATGACCGCTTGTCAGACAGCAATTAGG-3’		
PDSNP17	F 5’-TGTAAAACGACGGCCAGT GCCGAACTAGCCTCCATACC-3’	500	61
	R 5’-CAGGAAACAGCTATGACCTGCTTGACCCAACTTCAACC-3’		
PDSNP32	F 5’-TGTAAAACGACGGCCAGTTGAAGGAGCAAAGGAGATGG-3’	686	60
	R 5’-CAGGAAACAGCTATGACCTGCTGTGTCAAACTCGGAAG-3’		

6.3. SNPs data Analyses

For each cultivar the consensus sequences of all four primers pairs were joined into a single concatemeric sequences and multiple alignments were generated in Molecular Evolution Genetic Analysis (MEGA) software version 7 and used for phylogenetic analysis. Genome reference sequences for this study used one Khalas cultivar sequence taken from website (http://qatarweill.cornell.edu/research/date palm Genome/download.html) and, one khalas, Barhee and Khuzii cultivars sequences obtained from NCBI blast nucleotide search tool. The analysis involved 19 nucleotide sequences including reference sequences. All positions containing gaps and missing data were eliminated. The phylogenic tree was constructed using UPGMA method in MEGA7 (Kumar *et al*., 2016).

The UPGM analysis involved a total of 1578 positions of nucleotides of SNPs sites as final dataset in 15 date palm cultivars sampled for this study and also included corresponding reference sequences of Khanizi, Barhee BC4 and two Khalas cultivars. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al*., 2004). Nitrogen bases composition and pair-wise distance matrix were also assessed using Mega software.

6.4. Results

6.4.1. Genotypic association of cultivars

In phylogenetic analysis two major groups were identified (Figure 6.1). In the first group Barhee, Jarvis, Medjool Israel, Khalas, Ref sequ cv Khalas 2, Ref sequ cv Khanizi, Ashal Al Hassa and unknown cultivars collected from Asayta, Errer Gota and Dire Dawa were clustered together. The second group contained Ref sequ cv Khalas 2, Ref sequ cv Barhee BC4, Zamlli, Medjool England, Shish, Khyara, Saggii, Khadrawy and unknown cultivar collected from Afambo.

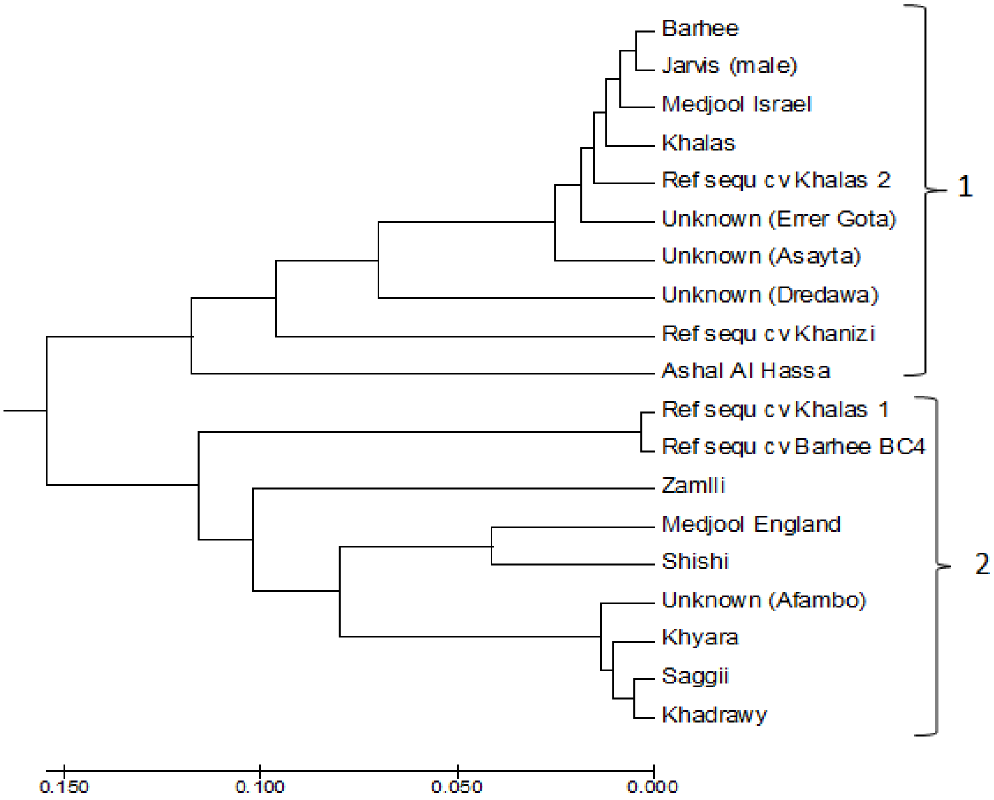


Figure 6.1 Phylogenetic tree showing relationships between date palm cultivars of the present study and corresponding references of date palm cultivars based on their genome SNP sequences data.

6.4.2. Sequence characteristics and pairwise distance of cultivars

The sum of total sequences of AT and GC from all cultivars by PDSNP07 (6219 bases), by PDSNP10 (6301 bases), by PDSNP17 (7187 bases) and by PDSNP32 (7235 bases) was obtained. Among cultivars, Ashal Al Hassa contained the highest (175.7) and Medjool England contained the lowest (163.3). The average AT content was 51.2% (PDSNP07), 62.9% (PDSNP10), 56.5% (PDSNP17) and 62.5% (PDSNP32), while the average GC content was 48.8 % (PDSNP07), 37.1% (PDSNP10), 43.5% (PDSNP17) and 37.5% (PDSNP32). Additionally, there was significant and non-significant differences on AT and GC content in each cultivar observed and; the AT content ranged from 50.4% (PDSNP07) to 64.2% (PDSNP10) whereas the GC content ranged from 35.8% (PDSNP10) to 49.6% (PDSNP07) (Table 6.3). According to the pair-wise distance matrix result the least value was 0.009 (Barhee and Jarvis) and the highest value was 0.494 (Shishi and Ashal Al Hassa (Table 6.4). Additionally, among 15 date palm cultivars similarity was observed between Barhee and Jarvis (0.009) and Saggii and Khadrawy (0.010). The pair-wise distance values of 0.494, 0.488 and 0.402 were observed between Barhee and Ashal Al Hassa, Ashal Al Hassa and Zamlli, unknown cultivar collected from Afambo and Ashal Al Hassa respectively. This genetic similarity and differentiation events of these cultivars were also supported in phylogenetic tree result (Figure 6.1) as well as AT and GC content.

Table 6.3 AT and GC content of date palm cultivars amplified by SNPs primers

Cultivars	PDSNP07			PDSNP010			PDSSNP017			PDSNP032		
	Percentage		Total sequence	Percentage		Total sequence	Percentage		Total sequence	Percentage		Total sequence
	AT	GC		AT	GC		AT	GC		AT	GC	
Saggii	51.8	48.2	407	64	36	411	56.2	43.8	470	62.8	37.2	479
Khalas	50.5	49.5	402	62.4	37.6	410	55.6	44.4	471	63.1	37	481
Ashal Al Hassa	50.4	49.6	409	54.9	45.1	448	56.6	43.4	470	62.4	37.6	481
Medjool Islael	50.5	49.5	402	63.9	36.1	410	56.1	43.9	474	62.6	37.4	481
Jarvis male	50.5	49.5	402	64	36	417	56.2	43.8	479	63.6	36.4	472
Barhee	50.5	49.5	402	63.9	36.1	410	56.1	43.9	478	63.8	36.2	472
Khyara	52	48	406	62.2	37.8	463	55.9	44.1	481	62.6	37.4	481
Medjool England	52.4	47.6	443	64.1	35.9	412	56.4	43.6	468	63.8	36.2	481
Zamlli	52.4	47.6	468	63	37	411	56.5	43.5	483	57.9	42.1	492
Shishi	52.1	48	406	63.7	36.3	410	57.2	42.8	493	63	37	473
Khadrawy	51.8	48.2	407	63.9	36.1	410	56.1	43.9	478	62.6	37.4	481
Unknown (Afambo)	51.8	48.2	415	63.9	36.1	410	56.2	43.8	479	62.6	37.4	486
Uknown (Dreadawa)	50.6	49.4	407	62.4	37.6	436	58.3	41.5	489	61.6	38.4	513
Unknown (Errer Gota)	50.8	49.2	441	63.4	36.6	421	56.4	43.6	477	63	37	479
Unknown (Asayta)	50.5	49.5	402	64.2	35.8	422	58.1	41.9	497	63.4	37.1	483
Total			6219			6301			7187			7235
Average	51.2	48.8	414.6	62.9	37.1	420	56.5	43.5	479.1	62.5	37.5	482.3

Table 6.4 Pair-wise distance among 15 date palm cultivars using genome sequences fragments formed by SNPs primers

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1														
2	0.026													
3	0.015	0.024												
4	0.217	0.245	0.233											
5	0.284	0.315	0.299	0.082										
6	0.300	0.319	0.312	0.216	0.229									
7	0.183	0.201	0.197	0.404	**0.494**	0.488								
8	0.198	0.214	0.204	0.137	0.193	0.203	0.383							
9	0.186	0.209	0.196	0.128	0.182	0.192	0.383	0.017						
10	0.190	0.213	0.200	0.128	0.187	0.193	0.387	0.023	**0.010**					
11	**0.009**	0.024	0.019	0.222	0.292	0.306	0.191	0.202	0.195	0.198				
12	0.193	0.213	0.199	0.131	0.191	0.192	0.386	0.030	0.023	0.028	0.203			
13	0.135	0.150	0.139	0.314	0.361	0.402	0.305	0.307	0.294	0.301	0.139	0.301		
14	0.030	0.038	0.026	0.245	0.315	0.327	0.209	0.217	0.208	0.212	0.040	0.209	0.142	
15	0.042	0.056	0.046	0.259	0.309	0.344	0.223	0.233	0.223	0.228	0.046	0.233	0.139	0.065

Numbers in the first row and column represent date palm cultivars as follow: 1– Barhee, 2 – Khalas, 3 – Medjool Israel, 4 – Medjool England, 5 – Shishi, 6 – Zamlli, 7 – Ashal Al Hassa, 8 – Khyara, 9 – Saggii, 10 – Khadrawy, 11 – Jarvis (male), 12 – unknown (Afambo), 13 – unknown (Dire Dawa), 14 – unknown (Errer Gota), 15 – unknown (Asayta)

6.5. Discussion

Identification of the genetic relationships of date palm cultivars based on scientific techniqes is the most important groundwork for conservation, improvement and sustainable uses of the genetic resources of this crop. Hence, uses of molecular markers are preferable methods for characterization of date palm cultivars. Therefore, the purpose of this study was to identify and evaluate the genetic relationships of Ethiopian date palm cultivars using SNPs markers. Numerous research studies have been conducted on assessing the polymorphism of date palm cultivars using AFLP, RAPD, SSR, ISSR, RLFP and SNPs (Khierallah *et al*., 2011; Sabir *et al*., 2014; Hazzouri *et al*., 2014; Racchi *et al*., 2014; Mathew *et al*., 2015; Faqir *et al*., 2019; Mohamoud *et al*., 2019; Ahmed *et al*., 2021). In this study, we analysed partial SNPs genome sequences of 15 date palm cultivars to determine their genetic relationships particularly for these unknown cultivars which are grown at different locations. The statistical analyses results from SNPs data showed existence of genetic variation among date palm cultivars. The presence of genetic variation among these cultivars is also reported by using microsatellite markers studies (Ahmed *et al*., 2021). Indeed, the SNPs primers PDSNP07, PDSNP010, PDSNP017 and PDSNP032 of the present study have detected polymorphisms among seven Pakistan date palm cultivars based on partial nuclear genome sequences (Faqir *et al*., 2019).

Besides, the present finding of a phylogenetic tree from SNPs data suggest that these unknown date palm cultivars collected from Errer Gota, Asayta and Dire Dawa are closely related with Barhee, Khalas and Jarvis (male) cultivars and also another unknown cultivar collected from Afambo and has shown similarity with Khyara, Saggii and

Khadrawy cultivars. Generally, this research work is the first documenting on the use of morphological markers and partial SNPs genome sequences to identify and characterize date palm cultivars in Ethiopia. Consequently, this study could be used to give clear understanding on genetic relation of date palm cultivars and also will assist in selecting of cultivars for breeding and future genetic improvement of this valuable crop especially in Ethiopia.

Chapter 7

Conclusions and Recommendations

Date palm tree besides as staple food sources it has a wide range of industrial, medicinal, ecological and socio-economic benefits for societies in arid and semi-arid part of the world. For sustainable utilization of the date palms genetic resources, developing of strategies for germplasm conservation, genetic improvement and breeding programs of this tree is necessary and hence, assessing the genetic diversity and identification of the phylogenetic relationship between date palm genotypes using scientific knowledge and principles is the first step to initiate these programs. Based on the gathered information on genetic diversity of Ethiopian date palms at national (in Ethiopian Ministry of Agriculture, Ethiopian Biodiversity Institute and Ethiopian Forestry Institute) and international levels there was no any documented evidences about date palm diversity in Ethiopia.

In this study attempts were made on evaluation of genetic diversity and phylogenetic relationships of date palm genotypes and populations using DNA markers i.e. microsatellite, inter simple sequence repeats, single nucleotide polymorphisms and morphological markers. Results obtained from molecular data analysis of AMOVA, PCoA/PCs, Nei's genetic pair wise comparison, phylogenetic tree, population structure and DPAC have shown clear phylogenetic relationships and polymorphisms in between and among date palm genotypes. Morphological data analysis outcomes also showed the phylogenetic relationships of these 11 date palm cultivars. In conclusion, the finding of this study will provide good genetic databases of date palms for national and international gene banks and also help to understand the distribution and genetic diversity of date palms in Ethiopia. Besides, the results of this study could be used as a baseline for future studies in developing systems for conservation programs, genetic improvement and introduction of gene exploitations of date palms in Ethiopia.

To get more detailed information on the genetic diversity and population structure of date palm in Ethiopia, studies based on mitochondrial and chloroplasts DNAs using SSR, SNPs, ISSR and other markers might be needed. The out-breeding nature of date palms could enhance diversification of this crop. Therefore, further research should be carried out in detail on characterization of date palm fruits using morphological and molecular markers to select agronomic traits in its germplasm gene pools for crop production improvement. In this study, only four SNPs primers were used for partial sequencing of cultivars. Thus, additional SNPs primers or whole genome sequencing should be needed to get detailed information on genetic differentiation of the studied cultivars. Lastly, high heterozygosity was observed between and among date palm genotypes based on the molecular markers of the present study. So, further investigation shouldbe required on sex differentiation using sex-determining primers.

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Appendixies

Appendix 2.1 Data information of date palm samples in Ethiopia during the time of sample collection and cods that are used for analysis

Region	District	Kebele/name of site/	Plant ID during collection	Altitude	Coordinates	Genotype ID for analysis and dendrogram	Pop ID for analysis
Afar region	Afambo	Alassabolo	As1-male	344m	N11^{O}31. 381’, E041^{O}32. 399’	1	As
Afar region	Afambo	Alassabolo	As2	348m	N11^{O}31.401’ , E041^{O}32.493’	2	As
Afar region	Afambo	Alassabolo	As3 – male	352m	N11^{O}31.427’, E041^{O}32.494’	3	As
Afar region	Afambo	Alassabolo	As4	346m	N11^{O}31.252’ , E041^{O}32.534’	4	As
Afar region	Afambo	Alassabolo	As5	349m	N11^{O}31.247’ , E041^{O}32.550’	5	As
Afar region	Afambo	Alassabolo	As6	348m	N11^{O}31.241’ , E041^{O}32.554’	6	As
Afar region	Afambo	Alassabolo	As7	347m	N11^{O}31.236’ , E041^{O}32.567’	7	As
Afar region	Afambo	Alassabolo	As8	347m	N11^{O}31.234’ , E041^{O}32.570,	8	As
Afar region	Afambo	Alassabolo	As9	350m	N11^{O}31.226’ , E041^{O}32.558’	9	As
Afar region	Afambo	Alassabolo	As10	350m	N11^{O}31.261’ , E041^{O}32.597’	10	As
Afar region	Asayta	Berga	Bg1-male	351m	N11^{O}31.641’ , E041^{O}27.557’	11	Bg
Afar region	Asayta	Berga	Bg2	352m	N11^{O}31.642’ , E041^{O}24.550’	12	Bg
Afar region	Asayta	Berga	Bg3	357m	N11^{O}31.606’ , E041^{O}27.539’	13	Bg
Afar region	Asayta	Berga	Bg4-male	355m	N11^{O}31.597’ , E041^{O}27.601’	14	Bg
Afar region	Asayta	Berga	Bg5	360m	N11^{O}31.601’ , E041^{O}27.621’	15	Bg
Afar region	Asayta	Berga	Bg6	355m	N11^{O}31.597’ , E041^{O}27.601’	16	Bg
Afar region	Asayta	Berga	Bg7	352m	N11^{O}31.597’ , E041^{O}27.584’	17	Bg
Afar region	Asayta	Berga	Bg8	354m	N11^{O}31.616’ , E041^{O}27.547’	18	Bg

Afar region	Asayta	Berga	Bg9	360m	N11^{O}31.598’ , E041^{O}27.592’	19	Bg
Afar region	Asayta	Berga	Bg10-male	351m	N11^{O}31.592’ , E041^{O}27.681’	20	Bg
Somalae National region	Erer Gota	Ererr	Er1	1112.215	N 09^{O}33.127’, E 041^{0}23.218’	21	Er
Somalae National region	Erer Gota	Ererr	Er2	1139.952	N 09^{O}32. 641’, E 041^{0}23.592’	22	Er
Somalae National region	Erer Gota	Ererr	Er3-male	1137.209	N 09^{O}32.592’, E 041^{O}23.619’	23	Er
Somalae National region	Erer Gota	Ererr	Er4	1139.647	N 09^{O}32.650’, E 041^{0}23.607’	24	Er
Somalae National region	Erer Gota	Ererr	Er5	1117.702	N 09^{O}33.055’, E 041^{0}23.308’	25	Er
Somalae National region	Erer Gota	Ererr	Er6	1113.434	N 09^{O}33. 133’, E 041^{O}23. 210’	26	Er
Somalae National region	Erer Gota	Ererr	Er7	1120.14	N 09^{O}33.025’, E 041^{O}23.347’	27	Er
Somalae National region	Erer Gota	Ererr	Er8	1106.119	N 09^{O}33.182’,E 041^{0}23.149’	28	Er
Somalae National region	Erer Gota	Ererr	Er9-male	1110.996	N 09^{O}33.122’, E 041^{O}23.221’	29	Er
Somalae National region	Erer Gota	Ererr	Er10	1133.856	N 09^{O}32.648’, E 041^{O}23.598’	30	Er
Afar region	Afambo	Humodoyta	Hd1	393m	N11^{O}30.994’ , E041^{O}34.362’	31	Hd
Afar region	Afambo	Humodoyta	Hd2	392m	N11^{O}31.152’ , E041^{O}34.828’	32	Hd
Afar region	Afambo	Humodoyta	Hd3	392m	N11^{O}30.825’ , E041^{O}33.632’	33	Hd
Afar region	Afambo	Humodoyta	Hd4	392m	N11^{O}30.876’ , E041^{O}34.071’	34	Hd
Afar region	Afambo	Humodoyta	Hd5	396m	N11^{O}30.941’ , E041^{O}34.098’	35	Hd
Afar region	Afambo	Humodoyta	Hd6	392m	N11^{O}30.975’ , E041^{O}33.794’	36	Hd

Afar region	Afambo	Humodoyta	Hd7	396m	N11^{O}30.891’ , E041^{O}33.564’	37	Hd
Afar region	Afambo	Humodoyta	Hd8	396m	N11^{O}30.923’ , E041^{O}33.929’	38	Hd
Afar region	Afambo	Humodoyta	Hd9	393m	N11^{O}30.868’ , E041^{O}33.568’	39	Hd
Afar region	Afambo	Humodoyta	Hd10-male	396m	N11^{O}30.885’ , E041^{O}33.571’	40	Hd
Afar region	Afambo	Humodoyta	Hd11-male	396m	N11^{O}30.854’ , E041^{O}33.659’	41	Hd
Afar region	Afambo	Humodoyta	Hd12-male	393m	N11^{O}30.891’ , E041^{O}33.691’	42	Hd
Afar region	Afambo	Humodoyta	Hd13	392m	N11^{O}30.795’ , E041^{O}33.546’	43	Hd
Afar region	Afambo	Humodoyta	Hd14	393m	N11^{O}30.795’ , E041^{O}33.553’	44	Hd
Afar region	Afambo	Humodoyta	Hd15	392m	N11^{O}30.975’ , E041^{O}33.794’	45	Hd
Afar region	Afambo	Humodoyta	Hd16	392m	N11^{O}30.924’ , E041^{O}33.921’	46	Hd
Afar region	Afambo	Humodoyta	Hd17	392m	N11^{O}30.924’ , E041^{O}33.921’	47	Hd
Afar region	Afambo	Humodoyta	Hd18	396m	N11^{O}30.932’ , E041^{O}33.924’	48	Hd
Afar region	Afambo	Humodoyta	Hd19	393m	N11^{O}30.949’ , E041^{O}34.335’	49	Hd
Afar region	Afambo	Humedeyta	Hd20	392m	N11^{O}30.919’ , E041^{O}34.384’	50	Hd
Afar region	Asayta	Kerebuda	Kr1	356m	N11^{O}31.910’ , E041^{O}26.802’	51	Kr
Afar region	Asayta	Kerebuda	Kr2-male	355m	N11^{O}31.867’ , E041^{O}26.823’	52	Kr
Afar region	Asayta	Kerebuda	Kr3	353m	N11^{O}32.255’ , E041^{O}26.963’	53	Kr
Afar region	Asayta	Kerebuda	Kr4	355m	N11^{O}31.867’ , E041^{O}26.823’	54	Kr
Afar region	Asayta	Kerebuda	Kr5	353m	N11^{O}32.420’ , E041^{O}26.847’	55	Kr
Afar region	Asayta	Kerebuda	Kr6	353m	N11^{O}32.252’ , E041^{O}26.938’	56	Kr
Afar region	Asayta	Kerebuda	Kr7-male	358m	N11^{O}32.248’ , E041^{O}26.951’	57	Kr
Afar region	Asayta	Kerebuda	Kr8	354m	N11^{O}31.940’ , E041^{O}26.802’	58	Kr
Afar region	Asayta	Kerebuda	Kr9	354m	N11^{O}32.262’ , E041^{O}26.947’	59	Kr

Afar region	Asayta	Kerebuda	Kr10	354m	N11^{O}31.913’ , E041^{O}26.812’	60	Kr
Somalae National region	Shinlie	Legaharae	Lg1	1205m	N09^{O}35.957’ , E041^{O}53.160’	61	Lg
Somalae National region	Shinlie	Legaharae	Lg2	1209m	N09^{O}35.916’ , E041^{O}53.240’	62	Lg
Somalae National region	Shinlie	Legaharae	Lg3	1207m	N09^{O}35.903’ , E041^{O}53.222’	63	Lg
Somalae National region	Shinlie	Legaharae	Lg4	1207m	N09^{O}35.947’ , E041^{O}53.162’	64	Lg
Somalae National region	Shinlie	Legaharae	Lg5-male	1208m	N09^{O}35.904’ , E041^{O}53.212’	65	Lg
Somalae National region	Shinlie	Legaharae	Lg6	1206m	N09^{O}35.870’, E041^{o}203’	66	Lg
Somalae National region	Shinlie	Legaharae	Lg7	1211m	N09^{O}35.950’ , E041^{O}53.188’	67	Lg
Somalae National region	Shinlie	Legaharae	Lg8	1218m	N09^{O}35.788’ , E041^{O}53.418’	68	Lg
Somalae National region	Shinlie	Legaharae	Lg9	1207m	N09^{O}35.722’ , E041^{O}53.609’	69	Lg
Somalae National region	Shinlie	Legaharae	Lg10	1207m	N09^{0}35.879’ , E041^{O}53.265’	70	Lg
Somalae National region	Shinlie	Legaharae	Lg11	1205m	N09^{O}35.953’ , E041^{O}53.183’	71	Lg
Afar region	Afambo	Maego	Mg1	353m	N11^{O}30.180’ , E041^{O}36.189’	72	Mg
Afar region	Afambo	Maego	Mg2-male	358m	N11^{O}30.112’ , E041^{O}36.566’	73	Mg
Afar region	Afambo	Maego	Mg3-male	355m	N11^{O}30.153’ , E041^{O}36.474’	74	Mg
Afar region	Afambo	Maego	Mg4	354m	N11^{O}30.072’ , E041^{O}36.530’	75	Mg
Afar region	Afambo	Maego	Mg5	358m	N11^{O}30.157’ , E041^{O}26.649’	76	Mg
Afar region	Afambo	Maego	Mg6	355m	N11^{O}30.148’ , E041^{O}36.461’	77	Mg

Afar region	Afambo	Maego	Mg7	354m	N11^{O}30.244’ , E041^{O}36.407’	78	Mg
Afar region	Afambo	Maego	Mg8	354m	N11^{O}30.228’ , E041^{O}36.237’	79	Mg
Afar region	Afambo	Maego	Mg9	353m	N11^{O}30.183’ , E041^{O}36.183’	80	Mg
Afar region	Afambo	Maego	Mg10	353m	N11^{O}30.183’ , E041^{O}36.183’	81	Mg
Afar region	Asayta	Mamulae	Mm1	352m	N11^{O}30.993’, E041^{O}29.993’	82	Mm
Afar region	Asayta	Mamulae	Mm2	346m	N11^{O}30.994’ , E041^{O}29.808’	83	Mm
Afar region	Asayta	Mamulae	Mm3	350m	N11^{O}30.994’ , E041^{O} 29.986’	84	Mm
Afar region	Asayta	Mamulae	Mm4-male	400m	N11^{O}30.965’, E041^{O}29.436’	85	Mm
Afar region	Asayta	Mamulae	Mm5	400m	N11^{O}30.979’ , E041^{O}29.590’	86	Mm
Afar region	Asayta	Mamulae	Mm6	406	N11^{O}30.995’ , E041^{O}29.994’	87	Mm
Afar region	Asayta	Mamulae	Mm7	352m	N11^{O}30.977’, E041^{O}29.383’	88	Mm
Afar region	Asayta	Mamulae	Mm8	347m	N11^{O}30.991’, E041^{O}29.894’	89	Mm
Afar region	Asayta	Mamulae	Mm9	349m	N11^{O}30.993’, E041^{O}29.770’	90	Mm
Afar region	Asayta	Mamulae	Mm10	353m	N11^{O}30.995’ , E041^{O} 29.886’	91	Mm
Afar region	Asayta	Mamulae	Mm11	356m	N11^{O}30.958’ , E041^{O}29.946’	92	Mm
Afar region	Asayta	Mamulae	Mm12-male	350m	N11^{O}30.969, E041^{O} 29.490’	93	Mm
Afar region	Asayta	Mamulae	Mm13	349m	N11^{O}30.959’ , E041^{O}29.501’	94	Mm
Afar region	Asayta	Mamulae	Mm14	347m	N11^{O}30.984’, E041^{O}29.672’	95	Mm
Afar region	Asayta	Mamulae	Mm15	351m	N11^{O}30.981’ , E041^{O} 29.687’	96	Mm
Afar region	Asayta	Mamulae	Mm16	346m	N11^{O}30.993’ , E041^{O}29.753’	97	Mm
Afar region	Asayta	Mamulae	Mm17	349m	N11^{O}31.007’ , E041^{O} 29.797’	98	Mm
Afar region	Asayta	Mamulae	Mm18	352m	N11^{O}31.010’, E041^{O}29.813’	99	Mm
Afar region	Asayta	Mamulae	Mm19	352m	N11^{O}31.004’ , E041^{O}29.879’	100	Mm

Afar region	Asayta	Mamulae	Mm20	351m	N11^{O}30.983’ , E041^{O}29.953’	101	Mm
Afar region	Asayta	Mamulae	Mm21	355m	N11^{O}30.985’ , E041^{O} 29.906’	102	Mm
Afar region	Afambo	Humodoyta (Introduced vitro date palm)	Sag1	342m	N11^{O}31.200’ , E041^{O}34.254’	103	Intro
Afar region	Afambo	>>	Sag2	342m	N11^{O}31.200’ , E041^{O}34.254’	104	Intro
Afar region	Afambo	>>	Kha1	340m	N11^{O}31.202’ , E041^{O}34.233’	105	Intro
Afar region	Afambo	>>	Kha2	340m	N11^{O}31.202’ , E041^{O}34.233’	106	Intro
Afar region	Afambo	>>	AAH1	343m	N11^{O}31.205’ , E041^{O}34.157’	107	Intro
Afar region	Afambo	>>	AAH2	343m	N11^{O}31.205’ , E041^{O}34.154’	108	Intro
Afar region	Afambo	>>	MJI-1	347m	N11^{O}31.246’ , E041^{O}34.519’	109	Intro
Afar region	Afambo	>>	MJI-2	345m	N11^{O}31.237’ , E041^{O}34.158’	110	Intro
Afar region	Afambo	>>	Jar-1-male	341m	N11^{O}31.244’ , E041^{O}34.192’	111	Intro
Afar region	Afambo	>>	Jar-2-male	341m	N11^{O}31.244’ , E041^{O}34.192’	112	Intro
Afar region	Afambo	>>	Ber1	343m	N11^{O}31.241’ , E041^{O}34.228’	113	Intro
Afar region	Afambo	>>	Ber2	343m	N11^{O}31.241’ , E041^{O}34.228’	114	Intro
Afar region	Afambo	>>	Khy1	343m	N11^{O}31.196’ , E041^{O}34.244’	115	Intro
Afar region	Afambo	>>	Khy-2	343m	N11^{O}31.196’ , E041^{O}34.244’	116	Intro
Afar region	Afambo	>>	MJE-1	347m	N11^{O}31.246’ , E041^{O}34.159’	117	Intro
Afar region	Afambo	>>	MJE-2	342m	N11^{O}31.221’ , E041^{O}34.157’	118	Intro
Afar region	Afambo	>>	Zam1	340m	N11^{O}31.200’ E041^{O}34.156’	119	Intro
Afar region	Afambo	>>	Zam2	340m	N11^{O}31.200’ E041^{O}34.156’	120	Intro
Afar region	Afambo	>>	Shi1	346m	N11^{O}31.208’, E041^{O}34.156’	121	Intro
Afar region	Afambo	>>	Shi2	344m	N11^{O}31.209’ , E041^{O}34.170’	122	Intro

Afar region	Afambo	>>	Khad1	344m	N11^{O}31.191’ , E041^{O}34.238’	123	Intro
Afar region	Afambo	>>	Khad2	344m	N11^{O}31.191’ , E041^{O}34.238’	124	Intro

Apendix 2.2. Plant DNA Isolation Protocol and gel test

1. Take 50-100 mg of fresh leaf tissue, clean it with distilled water, dry and then place it in autoclaved pestle mortar together with quartz sand
2. Add 700 µl of warm CTAB solution to the powdered sample (open the caps carefully), dissolve the powder and incubate the sample for 30 minutes at 65°C.
3. Centrifuge for 5 minutes at 15000 rpm.
4. Transfer 700µl of the crushed tissue (try to avoid solid material) with a broad mouth tip (the tip can be cut by surgical blade to make its bore wider) to a sterile eppendorf tube.
5. Add 700µl of chloroform and mix thoroughly and centrifuge the tube at 16000 xg for 10 minutes with 26^0C.
6. Transfer 600µl of the supernatant to new fresh eppendorf tube and add 60 (10) µl of 3M sodium acetate (pH 5.2) and mix thoroughly → **1st extraction.**

➔ **Second extraction if required**

- Add new 700µl CTAB buffer to the tissue pellet and stir slightly with a new 1000 µl pipette tip or invert tubes several times to get dissolved,
    - Incubate the sample for 30 minutes at 65°C and centrifuge the tube at 16000 xg for 03 - 05 minutes with 4 - 10^0C.
- Transfer 600µl of the crushed and/or CTAB dissolved tissue (supernatant) to a sterile eppendorf tube and
- Add 700µl of chloroform and mix thoroughly and centrifuge the tube at 16000 xg for 10 minutes and with 26^0C.
    - ➢ The same can carry out for a third extraction.
    - ➢ Each fraction proceeds with step 7 and will treated separately.

7. Add **600**µl of ice cold isopropanol and gently mix by inverting the tubes several times.
    - Place in refregrator (-20 ^{0}C) for 15 – 30 minutes (Sometimes can stay for long)
8. Centrifuge at 16000g for 3 minutes to pellet down the DNA @ 4^0C.

9. Discard the supernatant and wash the DNA by 70% ethanol (1ml) by dissolving the pallet completely in the wash buffer (**vortexing**)
    - Re pellet by centrifuge at 16000 xg for 3 minutes @ 4^0C.
10. Repeat the wash step again and dry the pellet (1:00 to 1:30 hr) (Aspiration) (Upside down the eppendorf under dry and clean napkin paper)
11. Suspend the pellet in 0.1X TE buffer with RNase (100 µl) (**<u>60µl</u>**) (**slight vortexing**)
    - **0.1XTE Buffer + RNase =** 1XTE (1.5ml) + PCR Grade H_2O (13.0 or .5ml) + 10 µl RNase

Test Gel and Gel Electrophoresis

1. Measure 1.0gm agar-agar and dissolve in 100ml 0.5X TAE buffer
    - 100ml 0.5X TAE buffer = 5ml 10X TAE buffer + 90ml ddH_20
2. Prepare 2 µl of loading dye on parafilm paper and/or other similar + 4/5 µl sample DNA
3. Run gel electrophoresis for 15 – 30 minutes (20` is enough) at (90V, 150mA and 50W)
4. Stain in Ethidium Bromide for 15 – 30 minutes
5. Watch the gel on Geno Sens Capture under Trans UV (Tool bar + button on machine)

$C_1 V_1 = C_2 V_2$ → amt of isopropanol = amt. of sample

$70\% \ X \ 100ml = 95\% \ X \ V_2$ → β-mercapto = 20ml/l or 1ml for 50ml CTAB (2%)

$V_2 = \frac{70\% \ X \ 100 \ ml}{95\%} = 73.68$

- <u>CTAB buffer</u> (100 mM Tris-HCl (1.58gm) pH 8.0, 2% (w/v) CTAB (2gm), 20 mM EDTA (0.93gm), 1.4 M NaCl (11.69gm), 4% PVP (2.0gm) and 2% (v/v) β-mercaptoethanol (1.0ml))

Appendx 2.3 List of published papers part of this dissertation

1. Ahmed, W., Feyissa, T., Tesfaye, K. and Farrakh, S. (2021). Genetic diversity and population structure of date palms (*Phoenix dactylifera* L.) in Ethiopia using microsatellite markers. *J. of Genetic Engine and Biotech* **19** (64):2-14. https://doi.org/10.1186/s43141-021- 00168-5
2. Workia Ahmed, Tileye Feyissa, Kassahun Tesfaye and Sumaira Farrakh (2023). Evaluation of phenotypic relationships of date palm cultivars at Melka Werer, Ethiopia. SINET: Ethiop. J. Sci., 46(2): 188-202, DOI: https://dx.doi.org/10.4314/sinet.v46i2.7

Appendx 2.4 microsatellite genotypic data of 124 date palm genotypes using 10 SSR marker in Ethioipia

The first columon is indicate number of population= 9

The first row is indicate loci = 10

	MPdCIR 010		**MPdCIR 015**		**MPdCIR 016**		**MPdCIR 025**		**MPdCIR 032**		**MPdCIR 050**		**MPdCIR 057**		**MPdCIR 070**		**MPdCIR 085**		**MPdCIR 093**	
pop = Alasabolo																				
As1-male	**144**	**144**	**146**	**152**	**148**	**148**	**228**	**228**	**304**	**304**	**174**	**230**	**250**	**300**	**210**	**210**	**172**	**172**	**183**	**189**
As2	**150**	**150**	**146**	**150**	**150**	**150**	**220**	**220**	**302**	**306**	**180**	**220**	**270**	**290**	**200**	**250**	**190**	**200**	**185**	**209**
As3 -male	**150**	**176**	**150**	**150**	**160**	**160**	**220**	**250**	**302**	**306**	**210**	**260**	**270**	**270**	**210**	**200**	**180**	**180**	**187**	**187**
As4	**150**	**180**	**146**	**152**	**148**	**160**	**268**	**268**	**302**	**306**	**210**	**250**	**276**	**280**	**200**	**240**	**180**	**210**	**191**	**197**
As5	**144**	**144**	**150**	**150**	**148**	**148**	**250**	**250**	**304**	**304**	**200**	**240**	**260**	**280**	**210**	**210**	**170**	**170**	**177**	**215**
As6	**146**	**152**	**150**	**150**	**148**	**154**	**228**	**228**	**302**	**306**	**190**	**240**	**276**	**280**	**210**	**250**	**176**	**176**	**197**	**197**
As7	**180**	**180**	**148**	**152**	**146**	**164**	**250**	**250**	**302**	**304**	**230**	**230**	**280**	**280**	**200**	**200**	**160**	**200**	**177**	**181**
As8	**146**	**180**	**150**	**150**	**148**	**148**	**250**	**250**	**302**	**306**	**200**	**240**	**270**	**280**	**200**	**250**	**178**	**210**	**185**	**189**
As9	**150**	**150**	**148**	**152**	**148**	**160**	**232**	**250**	**302**	**306**	**210**	**260**	**280**	**280**	**240**	**240**	**200**	**230**	**187**	**187**
As10	**144**	**152**	**150**	**150**	**148**	**152**	**250**	**250**	**304**	**304**	**190**	**220**	**276**	**280**	**230**	**230**	**168**	**168**	**181**	**225**
pop = Berga																				
Bg1-male	**144**	**148**	**148**	**150**	**148**	**148**	**228**	**268**	**304**	**304**	**190**	**190**	**270**	**280**	**210**	**210**	**172**	**230**	**189**	**225**
Bg2	**150**	**150**	**150**	**152**	**148**	**150**	**226**	**226**	**302**	**306**	**180**	**210**	**280**	**280**	**210**	**220**	**178**	**178**	**185**	**185**
Bg3	**148**	**150**	**148**	**152**	**148**	**150**	**226**	**226**	**302**	**304**	**190**	**190**	**280**	**290**	**210**	**210**	**160**	**178**	**181**	**187**
Bg4-male	**144**	**176**	**148**	**152**	**144**	**160**	**250**	**270**	**302**	**304**	**180**	**180**	**290**	**290**	**200**	**200**	**178**	**230**	**177**	**197**
Bg5	**150**	**180**	**150**	**156**	**144**	**144**	**228**	**228**	**304**	**306**	**200**	**220**	**290**	**290**	**226**	**240**	**198**	**210**	**189**	**215**
Bg6	**152**	**160**	**148**	**152**	**150**	**150**	**260**	**260**	**302**	**306**	**172**	**180**	**280**	**280**	**220**	**250**	**168**	**250**	**197**	**201**
Bg7	**150**	**176**	**146**	**152**	**148**	**148**	**250**	**250**	**302**	**306**	**230**	**230**	**280**	**280**	**210**	**226**	**178**	**250**	**183**	**197**
Bg8	**146**	**146**	**148**	**152**	**148**	**160**	**250**	**250**	**302**	**306**	**190**	**190**	**280**	**280**	**210**	**240**	**178**	**240**	**183**	**201**
Bg9	**148**	**148**	**144**	**152**	**146**	**156**	**250**	**250**	**302**	**306**	**190**	**190**	**280**	**280**	**210**	**226**	**180**	**200**	**183**	**201**
Bg10-male	**148**	**150**	**148**	**152**	**148**	**154**	**250**	**270**	**302**	**306**	**200**	**210**	**290**	**290**	**226**	**226**	**198**	**210**	**177**	**177**
pop = Erer																				
Er1	**146**	**152**	**144**	**146**	**148**	**150**	**228**	**228**	**302**	**306**	**180**	**210**	**276**	**280**	**210**	**240**	**176**	**220**	**189**	**215**
Er2	**148**	**150**	**152**	**152**	**148**	**148**	**220**	**250**	**302**	**306**	**180**	**180**	**260**	**290**	**210**	**120**	**168**	**220**	**181**	**181**
Er3-male	**144**	**152**	**150**	**150**	**148**	**150**	**226**	**250**	**302**	**304**	**220**	**220**	**280**	**280**	**220**	**220**	**168**	**250**	**189**	**189**

Er4	**144**	**150**	**150**	**150**	**148**	**152**	**226**	**260**	**304**	**304**	**180**	**200**	**280**	**280**	**200**	**250**	**168**	**178**	**185**	**209**
Er5	**144**	**150**	**152**	**152**	**148**	**152**	**220**	**230**	**302**	**306**	**200**	**200**	**250**	**290**	**210**	**210**	**168**	**226**	**187**	**209**
Er6	**144**	**160**	**146**	**152**	**148**	**156**	**260**	**260**	**302**	**306**	**200**	**250**	**280**	**280**	**210**	**220**	**176**	**240**	**185**	**197**
Er7	**144**	**150**	**144**	**156**	**148**	**154**	**250**	**260**	**302**	**304**	**190**	**250**	**280**	**280**	**210**	**250**	**198**	**250**	**189**	**189**
Er8	**150**	**150**	**144**	**144**	**150**	**150**	**250**	**270**	**302**	**306**	**180**	**250**	**280**	**280**	**210**	**250**	**198**	**250**	**187**	**187**
Er9-male	**150**	**150**	**152**	**152**	**150**	**150**	**228**	**228**	**304**	**306**	**190**	**240**	**290**	**290**	**226**	**226**	**178**	**250**	**187**	**209**
Er10	**152**	**152**	**150**	**150**	**146**	**146**	**220**	**250**	**302**	**302**	**200**	**220**	**270**	**280**	**220**	**220**	**168**	**230**	**183**	**183**
pop = Humedeyta																				
Hd1	**144**	**144**	**150**	**150**	**148**	**148**	**228**	**228**	**304**	**304**	**190**	**190**	**270**	**290**	**200**	**200**	**168**	**226**	**185**	**189**
Hd2	**146**	**152**	**148**	**148**	**148**	**148**	**228**	**228**	**302**	**306**	**200**	**200**	**276**	**280**	**220**	**220**	**198**	**198**	**185**	**201**
Hd3	**144**	**144**	**150**	**152**	**148**	**154**	**228**	**228**	**302**	**304**	**180**	**240**	**280**	**280**	**210**	**220**	**178**	**240**	**197**	**197**
Hd4	**144**	**150**	**144**	**152**	**148**	**164**	**232**	**232**	**304**	**304**	**200**	**250**	**270**	**290**	**210**	**240**	**198**	**210**	**191**	**225**
Hd5	**150**	**150**	**150**	**150**	**148**	**154**	**228**	**228**	**302**	**304**	**200**	**210**	**280**	**280**	**226**	**250**	**178**	**250**	**187**	**209**
Hd6	**148**	**150**	**150**	**150**	**150**	**152**	**228**	**228**	**304**	**306**	**200**	**210**	**290**	**290**	**220**	**240**	**176**	**226**	**187**	**209**
Hd7	**160**	**174**	**148**	**152**	**148**	**160**	**260**	**260**	**304**	**304**	**172**	**180**	**290**	**300**	**226**	**226**	**168**	**250**	**197**	**201**
Hd8	**150**	**150**	**148**	**152**	**148**	**160**	**250**	**250**	**304**	**306**	**180**	**180**	**280**	**310**	**200**	**200**	**198**	**198**	**197**	**201**
Hd9	**144**	**150**	**152**	**152**	**150**	**150**	**228**	**228**	**304**	**304**	**200**	**210**	**280**	**280**	**210**	**220**	**176**	**226**	**187**	**201**
Hd10-male	**146**	**146**	**150**	**150**	**148**	**148**	**250**	**250**	**302**	**306**	**200**	**240**	**276**	**290**	**250**	**250**	**230**	**230**	**187**	**201**
Hd11-male	**146**	**146**	**148**	**148**	**148**	**148**	**228**	**228**	**302**	**306**	**200**	**200**	**270**	**280**	**210**	**230**	**172**	**200**	**187**	**197**
Hd12-male	**146**	**146**	**148**	**150**	**160**	**160**	**228**	**228**	**306**	**306**	**230**	**260**	**276**	**276**	**210**	**230**	**172**	**200**	**185**	**185**
Hd13	**142**	**142**	**148**	**148**	**148**	**160**	**232**	**232**	**302**	**306**	**210**	**240**	**280**	**280**	**210**	**210**	**198**	**200**	**185**	**197**
Hd14	**146**	**146**	**150**	**150**	**148**	**148**	**220**	**220**	**304**	**304**	**190**	**190**	**280**	**280**	**210**	**210**	**178**	**98**	**197**	**197**
Hd15	**146**	**146**	**148**	**148**	**150**	**152**	**220**	**220**	**302**	**304**	**220**	**230**	**270**	**280**	**210**	**240**	**168**	**200**	**201**	**201**
Hd16	**146**	**180**	**148**	**148**	**160**	**160**	**232**	**232**	**302**	**304**	**220**	**230**	**270**	**280**	**210**	**240**	**178**	**178**	**201**	**201**
Hd17	**146**	**180**	**148**	**148**	**148**	**148**	**220**	**220**	**302**	**304**	**220**	**250**	**270**	**300**	**210**	**230**	**178**	**178**	**197**	**197**
Hd18	**148**	**180**	**146**	**148**	**176**	**176**	**220**	**220**	**302**	**302**	**200**	**240**	**254**	**260**	**210**	**230**	**180**	**180**	**187**	**209**
Hd19	**142**	**142**	**148**	**148**	**148**	**148**	**220**	**220**	**302**	**304**	**200**	**200**	**280**	**280**	**220**	**220**	**178**	**210**	**185**	**201**
Hd20	**146**	**146**	**146**	**146**	**148**	**170**	**228**	**228**	**302**	**302**	**190**	**200**	**280**	**280**	**200**	**240**	**178**	**198**	**185**	**197**
pop = Kerbuda																				
Kr1	**148**	**148**	**148**	**152**	**144**	**144**	**250**	**250**	**302**	**304**	**200**	**200**	**286**	**286**	**210**	**240**	**180**	**210**	**185**	**197**
Kr2-male	**144**	**144**	**146**	**156**	**148**	**154**	**250**	**250**	**304**	**306**	**180**	**180**	**250**	**256**	**200**	**230**	**168**	**198**	**181**	**181**
Kr3	**144**	**152**	**146**	**156**	**146**	**152**	**258**	**258**	**302**	**304**	**210**	**250**	**260**	**260**	**210**	**240**	**168**	**168**	**189**	**209**

Kr4	144	144	146	156	146	152	258	258	302	306	200	200	260	260	210	240	178	200	189	209
Kr5	152	176	152	156	144	144	258	258	302	306	190	210	260	270	220	220	180	200	193	215
Kr6	144	152	152	156	148	156	258	258	304	306	200	200	260	270	220	220	180	200	177	177
Kr7-male	148	160	148	152	148	160	258	258	302	306	180	180	290	290	226	226	180	180	189	215
Kr8	146	146	152	152	148	148	258	258	302	306	172	172	270	270	210	240	180	180	189	215
Kr9	150	189	148	152	148	170	232	232	304	306	200	220	260	260	210	240	198	210	189	189
Kr10	144	144	148	152	146	146	250	250	302	306	200	220	260	260	200	200	178	200	189	197
pop = Legaharae																				
Lg1	144	152	146	152	150	154	250	250	304	304	220	250	276	276	210	220	176	176	181	225
Lg2	144	152	150	156	150	150	228	256	306	306	230	250	276	280	210	220	168	198	205	205
Lg3	150	150	146	152	148	150	258	258	304	304	200	240	250	276	200	220	168	198	181	181
Lg4	146	176	146	152	148	150	258	276	304	304	210	250	250	256	220	250	178	178	187	187
Lg5-male	148	176	146	152	148	160	228	250	304	304	180	200	290	290	210	250	178	178	199	201
Lg6	148	150	148	152	148	160	250	250	304	304	190	190	280	280	200	200	180	180	187	197
Lg7	150	176	148	152	146	160	250	270	302	306	190	240	280	280	220	250	180	200	185	197
Lg8	148	150	148	152	144	156	250	270	304	304	190	240	270	270	200	230	178	230	185	197
Lg9	144	144	146	152	148	150	270	270	302	306	200	200	276	276	210	220	168	226	185	201
Lg10	148	152	144	152	148	148	250	250	302	302	180	180	280	280	200	210	198	198	189	201
Lg11	148	150	146	152	146	156	250	260	302	304	190	230	286	286	210	220	198	198	185	185
pop = Mego																				
Mg1	144	144	150	156	146	146	258	258	302	302	200	250	270	270	210	210	178	178	177	181
Mg2-male	152	176	148	148	148	170	258	258	304	306	180	230	260	270	226	226	180	180	193	215
Mg3-male	142	152	148	152	148	154	228	250	304	306	250	260	260	270	210	240	180	200	187	193
Mg4	176	176	148	152	146	146	260	260	304	306	190	240	276	280	200	240	168	210	185	185
Mg5	142	176	146	152	146	146	260	260	310	318	220	250	290	300	220	250	180	240	197	201
Mg6	142	174	146	152	146	154	260	260	304	304	200	200	270	276	210	220	180	240	185	197
Mg7	144	144	148	152	150	150	228	228	304	306	190	230	260	260	210	250	168	226	171	171
Mg8	150	150	152	156	146	152	250	250	304	306	210	210	260	260	226	230	178	178	183	209
Mg9	144	150	146	152	146	146	232	268	304	306	200	210	260	260	200	200	178	178	187	193
Mg10	142	144	148	152	148	148	220	220	304	306	250	250	260	300	210	230	180	200	191	197
pop = Mamulae																				
Mm1	148	160	152	152	150	150	250	250	304	304	200	210	260	260	220	230	180	200	183	209

Mm2	**148**	**150**	**152**	**152**	**146**	**152**	**260**	**270**	**302**	**306**	**220**	**220**	**270**	**310**	**210**	**240**	**178**	**198**	**185**	**209**
Mm3	**150**	**180**	**152**	**152**	**148**	**148**	**258**	**258**	**304**	**306**	**200**	**210**	**260**	**260**	**210**	**240**	**168**	**198**	**183**	**209**
Mm4-male	**152**	**176**	**150**	**156**	**146**	**146**	**258**	**258**	**304**	**304**	**190**	**200**	**260**	**276**	**220**	**220**	**168**	**168**	**177**	**209**
Mm5	**148**	**176**	**148**	**152**	**148**	**160**	**258**	**258**	**302**	**306**	**180**	**180**	**250**	**270**	**210**	**240**	**178**	**220**	**189**	**189**
Mm6	**144**	**144**	**148**	**152**	**152**	**176**	**232**	**250**	**302**	**304**	**200**	**250**	**280**	**280**	**250**	**250**	**178**	**210**	**197**	**225**
Mm7	**142**	**142**	**148**	**152**	**148**	**148**	**268**	**268**	**304**	**306**	**200**	**200**	**290**	**290**	**210**	**230**	**168**	**210**	**191**	**197**
Mm8	**144**	**160**	**146**	**152**	**148**	**154**	**220**	**228**	**304**	**306**	**240**	**240**	**280**	**280**	**220**	**240**	**178**	**178**	**185**	**193**
Mm9	**148**	**176**	**148**	**152**	**148**	**160**	**250**	**250**	**302**	**304**	**172**	**190**	**290**	**290**	**210**	**240**	**178**	**226**	**199**	**201**
Mm10	**144**	**174**	**146**	**152**	**146**	**150**	**260**	**260**	**304**	**304**	**210**	**250**	**290**	**310**	**210**	**250**	**198**	**210**	**185**	**197**
Mm11	**148**	**152**	**146**	**152**	**146**	**152**	**250**	**250**	**304**	**306**	**200**	**200**	**260**	**260**	**226**	**228**	**180**	**180**	**183**	**209**
Mm12-male	**144**	**172**	**146**	**150**	**148**	**154**	**250**	**250**	**304**	**306**	**230**	**230**	**260**	**260**	**226**	**228**	**198**	**210**	**183**	**209**
Mm13	**142**	**180**	**148**	**148**	**148**	**148**	**250**	**250**	**304**	**304**	**190**	**200**	**276**	**276**	**210**	**210**	**176**	**200**	**187**	**187**
Mm14	**142**	**180**	**148**	**148**	**160**	**160**	**220**	**220**	**304**	**304**	**190**	**220**	**270**	**270**	**210**	**226**	**176**	**200**	**201**	**201**
Mm15	**148**	**176**	**146**	**152**	**148**	**148**	**250**	**250**	**304**	**304**	**190**	**210**	**260**	**290**	**230**	**240**	**178**	**210**	**185**	**189**
Mm16	**150**	**152**	**144**	**152**	**144**	**144**	**250**	**250**	**302**	**302**	**180**	**190**	**276**	**300**	**210**	**240**	**198**	**198**	**183**	**201**
Mm17	**142**	**160**	**146**	**146**	**148**	**148**	**220**	**250**	**302**	**302**	**230**	**240**	**270**	**290**	**210**	**230**	**176**	**190**	**187**	**225**
Mm18	**150**	**150**	**144**	**144**	**170**	**170**	**220**	**220**	**302**	**302**	**190**	**240**	**250**	**270**	**200**	**250**	**180**	**180**	**187**	**225**
Mm19	**146**	**176**	**144**	**144**	**150**	**150**	**232**	**232**	**302**	**302**	**200**	**220**	**250**	**270**	**200**	**200**	**180**	**180**	**197**	**201**
Mm20	**150**	**150**	**144**	**144**	**160**	**160**	**250**	**250**	**304**	**306**	**200**	**250**	**270**	**270**	**200**	**230**	**180**	**180**	**197**	**209**
Mm21	**142**	**180**	**146**	**156**	**150**	**150**	**220**	**250**	**306**	**306**	**200**	**200**	**260**	**310**	**230**	**230**	**210**	**210**	**189**	**209**
pop = Introduced vitro date palms																				
Sag1	**146**	**176**	**146**	**154**	**156**	**160**	**232**	**270**	**306**	**306**	**200**	**240**	**290**	**290**	**200**	**240**	**172**	**220**	**197**	**197**
Sag2	**146**	**160**	**146**	**154**	**150**	**154**	**232**	**270**	**302**	**306**	**200**	**240**	**290**	**290**	**200**	**240**	**172**	**220**	**197**	**197**
Kha1	**148**	**176**	**146**	**154**	**160**	**160**	**250**	**270**	**306**	**306**	**200**	**250**	**280**	**280**	**210**	**240**	**172**	**220**	**197**	**197**
Kha2	**148**	**176**	**146**	**154**	**150**	**156**	**232**	**270**	**302**	**306**	**200**	**250**	**280**	**280**	**200**	**244**	**172**	**220**	**197**	**197**
AAH1	**144**	**160**	**150**	**150**	**148**	**148**	**220**	**250**	**304**	**304**	**180**	**210**	**290**	**300**	**210**	**240**	**172**	**220**	**187**	**209**
AAH2	**144**	**160**	**150**	**150**	**148**	**148**	**220**	**250**	**304**	**306**	**200**	**240**	**290**	**300**	**230**	**240**	**198**	**226**	**187**	**209**
MJI-1	**146**	**176**	**146**	**154**	**152**	**156**	**250**	**250**	**306**	**306**	**172**	**190**	**290**	**296**	**200**	**220**	**170**	**198**	**187**	**209**
MJI-2	**180**	**180**	**146**	**154**	**160**	**160**	**228**	**228**	**302**	**306**	**190**	**190**	**260**	**260**	**210**	**210**	**198**	**226**	**185**	**197**
Jar-1-male	**146**	**180**	**146**	**152**	**152**	**152**	**256**	**270**	**302**	**306**	**190**	**190**	**290**	**296**	**200**	**240**	**198**	**200**	**187**	**209**
Jar-2-male	**146**	**180**	**146**	**146**	**152**	**152**	**228**	**270**	**302**	**306**	**190**	**190**	**290**	**290**	**220**	**240**	**210**	**210**	**187**	**209**

Ber1	146	180	146	152	152	156	228	270	304	304	230	240	280	280	220	226	176	176	185	201
Ber2	146	152	146	154	152	156	260	260	304	304	230	240	280	280	220	230	176	176	185	201
Khy1	148	174	150	150	148	156	260	270	304	304	210	250	250	256	210	250	172	226	185	185
Khy-2	148	174	150	154	160	160	220	232	302	306	200	250	270	270	210	250	198	226	189	209
MJE-1	148	176	150	150	148	148	250	260	302	306	190	240	260	300	210	230	172	226	183	197
MJE-2	148	176	150	150	148	148	220	232	302	306	190	240	260	300	210	210	180	226	185	209
Zam1	144	180	150	152	148	148	256	260	302	306	190	240	260	300	210	230	172	226	183	197
Zam2	144	180	150	150	148	148	220	250	304	304	190	240	260	300	210	210	198	226	187	209
Shi1	150	180	150	156	148	148	258	260	302	306	200	240	290	300	210	230	172	226	187	201
Shi2	146	176	150	156	148	160	220	250	302	304	200	210	290	280	210	210	198	226	187	187
Khad1	146	174	146	152	150	154	232	270	302	306	190	240	276	276	220	250	160	220	185	197
Khad2	146	174	146	154	150	154	262	270	306	306	190	240	276	276	220	250	172	220	191	197