Nat. Volatiles & Essent. Oils, 2021; 8(5): 9974-9994

NVEO
Natural Volatiles & Essential Oils

Morphological and genetic studies for some date palm grown in Siwa Oasis

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Abstract

The present study was carried out during 2020 and 2021 seasons on date palm (*Phoenix dactylifera* L.), Seidi and Frehi cultivars, Meghal 1 and Meghal 2 grown in Siwa Oawsis, Marsa Matruh governorate, Egypt. The trees were cultivated at 6 X 6 meters apart and irrigated by flood irrigation. Five date palms / replicates from each one was chosen randomly where the experiment was composed from twenty date palm trees, which were healthy and similar in vigour and size as possible. Vegetative and fruit samples were taken to perform the measurements to define the morphological relation among the date palms. Thirty fruit from each palm / replicate were taken to measure the fruit physical and chemical characteristics, and the obtained data were analysed statistically by using Randomized complete block design (RCBD). The obtained results showed that the vegetative growth features; frond length and spine area distance, leaf area, as well as number of spines did not give enough indicator to distinguish among date palm trees. Moreover, fruiting characteristics like bunch weight, yield in kg, fruit weight, size, length and diameter, and flesh fruit weight showed that there were obvious variations among the understudy date palms. Besides, fruit chemical characteristics such as the percentages of TSS, total sugars, reduced sugars, tannins and fruit acidity showed that Meghal 1 and Meghal 2 are more related to Seidi cultivar than Frehi date palm cultivar in the two seasons. Besides, DNA barcoding also showed that Meghal 1 and Meghal 2 are closer together and to Seidi date palm cultivar than Frehi cultivar.

Keywords: Date palm, DNA barcoding, Yield, fruit quality, Vegetative growth

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious and perennial tree, which is belongs to Arecaceae family (Elhoumaizi *et al*., 2002). It's an important fruit crop in the Palmae family that covers 3% of the cultivated area of the world. *Phoenix dactylifera* is one of the most important fruit crops in Egypt, where is the first producer for dates in the world, which produce approximately1.590.414 million ton and the cultivated area is 49000.74 ha in 2018 (FAO. 2019). Moreover, it's a monocot, dioecious and can stay for a long time and has a great importance (Hassanzadeh Khankahdani and Bagheri, 2019).

The genetic diversity of date palm cultivars was studied by using analysis of phenotype like leaflet isozymes expression as a genetic marker (Mousawi *et al*., 2001). Wünsch and Hormaza (2002) stated that morphological features are considered very beneficial and good phenotype is required to correlate the genetic markers with the best characters. There are a lot of phenotype-oriented techniques for the differentiation of date palm cultivars like description of growth, flowering, and yield features (Al-Doss and Bacha, 2001) and vegetative and reproductive traits (Salem, *et al*., 2008 and Ahmed *et al*., 2011) have been reported to be successful. Elhoumaizi *et al*. (2002) studied the phenotypic diversity of twenty sex date-palm cultivars (*Phoenix dactylifera* L.) from Morocco depending on the leaf number and width, pinnae number, length and width, and the length and width of spine from the top and bottom. They found that there was an important morphological diversity between the studied cultivars, and this give an

indicator for identification of cultivars before the time of fruiting. It was reported that because the vegetative characteristics can express on the genes, so to differentiate between varieties should be depended on the molecular analysis (Ahmed *et al*., 2006 and Zhao *et al*., 2013). Mohamed Lemine *et al*. (2014) reported that using fruit in morphological measuring was the best tool for the distinguishing between date palm varieties.

Hebert *et al*. (2003) reported that that barcoding of DNA could be used for the identification between species, and the basic idea of the discrimination system is simple, if the sequence variations of the DNA barcode among species is higher than within species, they can be successfully discriminated from one another. Moreover, DNA barcoding is a valuable tool for taxonomists working with Palmae, where it can be used to identify species efficiently and accurately on the basis of a standard region as a marker. In addition, DNA barcoding is a molecular phylogeny way that utilize a short-integrated DNA sequence in a well-known gene to discriminate a plant as associate with particular species (Xiwen, *et al*., 2015). To identify an ideal region in plants, which must be sufficiently variable to differentiate all the species and conserved enough to be minimally variable within species, is nevertheless a challenge (Kress *et al*., 2005 and Liu *et al*., 2010). By using chloroplast DNA barcodes, a great progress has been made in differentiating the plant species (Kress and Erickson, 2007). Moreover, a lot of chloroplast gene regions are usually used as plant barcodes along with *matK* and *rbcL*, which are considered as core barcodes (CBOL Plant Working Group *et al*., 2009). It was stated by (Rougerie *et al*., 2009; Hausmann *et al.*, 2011; Khan *et al*., 2015) reported that DNA barcoding has numerous advantages such as solving a lot of problems of traditional taxonomy, helping to overcome the limitations of morphological characters, accelerating the species identification. Besides, it is a verifiable and reproducible path, efficient, low-cost, and very fast technique, which can work for any stage of plant and identify different plant species.

Therefore, the current study was performed to differentiate and barcoding of some date palms (*Phoenix dactylifera* L.) growing in Siwa Oasis, Marsa Matruh governorate, Egypt, depending on their morphological and genetics characteristics.

MATERIALS AND METHODS

Samples and place of study: The present study was carried out during 2020 and 2021 seasons on date palm trees (*Phoenix dactylifera* L.), Seidi, Frehi, Meghal 1 and Meghal 2 grown in Siwa Oawsis, Marsa Matruh governorate, Egypt. The trees were spaced at 6 X 6 meters apart and irrigated by flood irrigation. Healthy and similar five trees/replicates in their vigor and size were chosen randomly to conduct this experiment where the experiment was composed from twenty date palm trees in total. Vegetative and fruit samples were taken to the laboratory of Plant Production Department at Faculty of Agriculture Saba Basha, Alexandria university to measure the vegetative and fruit parameters to investigate the morphological relation among the four date palm trees. The obtained data were analyzed statistically by using Randomized complete block design (RCBD) in five replicates/samples for each one.

Morphological studies

Vegetative measurements: Frond length (m), number of spines, length of spines area and leaf area (m)
Fruit Yield: was measured at the harvesting time in terms of bunch weight, yield in kg per palm and in ton per hectare.

Fruit quality: At the time of the fruit ripening, samples from 30 fruit/ 30 replicate was randomly collected from each palm/replicate to estimate fruit physical and chemical characteristics.

Fruit physical characteristics: Average fruit weight (kg). Flesh fruit weight (g), seed weight (g) and fruit size (cm^3) were measured, and flesh / fruit ratio was calculated. Fruit length and fruit diameter (cm) were measured by using a Digital Vernier Caliper.

Fruit chemical characteristics:

Total soluble solids percentage in fruit juice (TSS) was determined using hand refractometer and the result was expressed as a percentage. Total and reducing sugars were estimated calorimetrically using Nelson arsenate - molybdate colorimetric method (Nielsen, 2010). Non-reducing sugars were calculated by the calculating the difference between total sugars and reducing sugars. Fruit acidity (%) expressed as malic acid was determined in fruit juice by titrating with 0.1 N sodium hydroxide in the presence of phenolphthalein as an indicator according to A.O.A.C. (2000), and then total soluble solids/acidity ratio was calculated. Soluble tannins content as a percentage from the fresh weight of date pulp was determined according to the method of Julkunen−Titto.14 An aliquot (50 µL) of each extract or standard solution was mixed with 1.5 mL of 4% vanillin (prepared with methanol) and then 750 µL of concentrated HCl were added. The well-mixed solution was incubated at ambient temperature in the dark for 20 min. The absorbance against blank was read at 500 nm. (+)-Catechin was used to make the standard curve (0.05−1 mg/mL). The results were expressed as mg CE/100g FW (El Arem *et al.*, 2012).

Molecular studies:

Genomic DNA Extraction: Genomic DNA was extracted from leaf tissue of all genotypes using the method as described by Saghai-Maroof *et al.* (1984) with modifications according to Irfan *et al.* (2013).

PCR purification: PCR protect was purification by using Mini kit @ i-Ntron Biotechnology according to the manufacturer descriptions.

Polymerase Chain Reaction (PCR) analysis: PCR reaction was conducted to confirm the presence of the genes of interest in the genomic DNA. The reaction was done using oligonucleotide specific primers **(Table 1).**

Table (1): primers name and sequences used in the current study

Name	Primer	Sequence	Tm	GC%
Rbcl	Forward	5'-ATGTCACCACAAACAGAGACTAAAGC-3	57.2	42
	Reverse	5'-GTAAAATCAAGTCCACCRCG-3	52.8	48

Table (2): The reaction conditions were optimized as the following:

Hot star master mix	12.5µl
Forward primer (10 pmol/µl)	1.25 µl
Reverse primer (10 pmol/µl)	1.25 µl
Template DNA (100 ng/µl)	2 µl
dd H_2O	Up to 25 µl

Amplification was carried out in a Techne Flexigene PCR Thermal Cycler programmed for 30 cycles as follows: 94°C/5 minutes (1 cycle); 94°C/45 sec, 50°C/45 sec, 72°C/45 sec (30 cycles); 72°C/7 minutes (1 cycle); 4°C (infinitive).

DNA electrophoresis:

Agarose (1%) was used for resolving the PCR products. 100bp. DNA marker as a standard DNA was used in the present study.

TBE buffer (10X)	
Tris	108 gm
Boric acid	55 gm
0.5 M EDTA (pH 8.0)	40 ml
dd H_2O	Up to 1 L
Gel preparation	
Agrose	1.6%
TBE buffer (0.5X)	80 mL
EtBr (10 µg/µl)	2 µl
Loading buffer	
Bromophenol blue	0.25 gm
Xylene cyanl	0.25 gm
Glycerol (30%)	100 mL
Sample preparation	
PCR product	4 µl
Glycerol (30%)	3 µl

The amplified DNA fragment was separated on 1% (w/v) agarose gel, Candidate PCR band was excised and purified from agarose gel for the subsequent steps of bioinformatics studies procedures using Axy Prep™ DNA Gel extraction kit (Cat. # AP-GX-50) and subsequently sequenced (Alfa Company, USA). The sequencing data were initially checked by NCBI BLAST search, assembled, and edited using EditSeq (DNASTAR, FinchTV). Moreover, these sequences relating to the amplified sequence were submitted to the GenBank (http://www.ncbi.nlm.nih.gov) for getting an accession number.

Conserved regions: Multiple sequence alignments of the PCR products were conducted from different genotypes using the PROMALS server according to Pei and Grishin, (2007), Clustal Omega server as maintained by Sievers *et al*. (2011) and the BIOEDIT software (Hall *et al*., 2011), was used to extract the conserved regions. The PROMALS is up to 30% more accurate compared to the best alignment methods with improvement for distantly related sequences. Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. The BIOEDIT 7.1.5 software is a user-friendly biological sequence alignment editor, and it is intended to provide basic functions for protein sequence editing, alignment, manipulation, and analysis.

Molecular evolutionary and phylogenetic analysis: All available nucleotide sequences relating to the amplified sequence were downloaded from GenBank (http://www.ncbi.nlm.nih.gov). Details on these sequences, including their genotype, were extracted from the GenBank annotations, phylogenetic tree was estimated. The amplified sequences were compiled, analyzed, and aligned with those of similarity sequence obtained from the NCBI GenBank database. The evolutionary history was inferred using the Neighbor Joining method. The process of finding a phylogenetic tree using maximum likelihood involves finding the topology and branch lengths of the tree that will give us the greatest probability of observing nucleotide sequences in our data. So, for Phylogenetic analysis Mafft server (Katoh *et al.*, 2019), Clustal Omega server and MEGA7 software were used.

Statistical analysis: The obtained data were subjected to one-way ANOVA according to (Ott and Longnecker, 2015) and least significant difference (LSD) at 0.05% was used to compare between the means of the treatments.

RESULTS

Morphological studies

Data in Table 3 showed that frond length and spine area distance were greatly differed among the studied date palms, where Seidi cultivar significantly differed from Meghal 1 and Meghal 2, which consequently differed greatly from Frehi date palm cultivar in the two seasons. Moreover, concerning the leaf area the differences among Seidi cultivar and Meghal 1 or Meghal 2 were so slight not enough to be significant, while all of them were obviously differed from Frehi date cultivar in both experimental seasons. Regarding to the number of spines, Seidi cultivar differed statistically from Frehi cultivar, while the differences between both Meghal 1 or Meghal 2 and Seidi or with Frehi were not significant in the first season. Besides, in the second season the differences in the spine numbers were so slight not enough to be significant among all of them.

Table 3: Frond length, spine area distance, length of leaf area and spines number in some date palms cultivated in Siwa Oasis during 2020 and 2021 seasons

Treatments	Frond length (m)	Spine area distance (m)	Leaf area length (m)	Spines number
	2020			
Seidi	4.30a	1.41a	2.89a	27.4a
Meghal 1	4.15b	1.31b	2.84a	25.8ab
Meghal 2	4.20b	1.28b	2.91a	25.2ab
Frehi	3.92c	1.21c	2.71b	23.6b
$LSD_{0.05}$	0.11	0.06	0.12	2.72
	2021			
Seidi	4.30a	1.39a	2.88a	26.2a
Meghal 1	4.16b	1.30b	2.81a	25.2a
Meghal 2	4.17b	1.29b	2.84a	23.8a
Frehi	3.95c	1.21c	2.64b	23.8a
$LSD_{0.05}$	0.12	0.05	0.07	2.60

Means not sharing the same letter(s) within each column, significantly different at 0.05 level of probability

Results in Table 4 demonstrated that bunch weight, yield in kg per palm and yield in ton per hectare were statistically differed in Seidi cultivar from Meghal 1, Meghal 2 and Frehi date palms in both experimental seasons. Furthermore, Meghal 1 date palm also clearly differed from Meghal 2 or Frehi date palms in the two seasons. Additionally, the same parameters were greatly high in Meghal 2 comparing with Frehi cultivar in the two seasons. The results also showed that bunch weight, yield in kg per tree and yield in ton per hectare were higher in the first seasons than that in the second season.

Table 4: Bunch weight, yield in kg per tree and in ton per hectare in some date palms cultivated in Siwa Oasis during 2020 and 2021 seasons

Treatments	Bunch weight (kg)	Yield (kg/tree)	Yield (ton/hectare)
	2020		
Seidi	23.1a	152a	40.43a
Meghal 1	19.4b	130.2b	34.63b
Meghal 2	17.3c	125.4c	33.36c
Frehi	15.2d	120.8d	32.13d
$LSD_{0.05}$	1.40	4.53	1.21
	2021		
Seidi	22.3a	142.8a	37.98a
Meghal 1	18.0b	125.2b	33.30b
Meghal 2	16.0c	1119c	31.65c
Frehi	13.6d	114.2d	30.38d
$LSD_{0.05}$	1.99	4.30	1.14

Means not sharing the same letter(s) within each column, significantly different at 0.05 level of probability

Data in Table 5 cleared that fruit weight, size, length, diameter, and flesh fruit weight were greatly different among Seidi, Meghal 1, Meghal 2 and Frehi date palms in the two seasons. The order from the high to the least was Seidi, Meghal 1, Meghal 2 and Frehi respectively in both experimental seasons. Besides, it was noticed that fruit weight, size, length, diameter, and flesh fruit weight in the date palms under study were higher in the second seasons than the first season. Concerning to the kernel weight, the results showed that it was significantly high in Seidi cultivar than in Meghal 1, Meghal 2, which consequently were obviously different from Frehi date palm cultivar in the two seasons. The differences in kernel weight between Meghal 1, and Meghal 2 date palms were so slight to be significant in both seasons. Regarding to the fruit shape index, the results showed that the differences among Seidi, Meghal 1, Meghal 2 and Frehi date palms in the two seasons were not enough to be significant. Flesh fruit ratio was greatly differed in Seidi, Meghal 1 and Frehi from Meghal 2 in the two seasons, while the differences among Seidi, Meghal 1 and Frehi were not significant.

Table 5: Fruit physical characteristics in some date palms cultivated in Siwa Oasis during 2020 and 2021 seasons

Treatments	Fruit weight (g)	Fruit size (cm^3)	Fruit length (mm)	Fruit diameter (mm)	Kernel weight (g)	Flesh fruit weight (g)	Fruit shape index	Flesh/ fruit ratio
	2020							
Seidi	19.88a	19.06a	4.15a	2.59a	2.45a	17.43a	1.60a	0.88a
Meghal 1	15.77b	14.57b	3.92b	2.40b	2.13b	13.63b	1.63a	0.86a
Meghal 2	13.21c	12.55c	3.71c	2.26c	2.13b	11.09c	1.64a	0.84b
Frehi	10.07d	9.46d	3.23d	1.97d	1.26c	8.81d	1.64a	0.87a
$LSD_{0.05}$	1.53	1.63	0.16	0.1	0.13	1.52	0.10	0.02
	2021							
Seidi	20.10a	19.27a	4.25a	2.7a	2.46a	17.64a	1.57a	0.88a
Meghal 1	16.03b	15.27b	4.03b	2.47b	2.22b	13.81b	1.63a	0.86a
Meghal 2	13.32c	12.75c	3.78c	2.29c	2.50b	11.06c	1.65a	0.83b
Frehi	10.47d	9.95d	3.36d	2.00d	1.36c	9.11d	1.68a	0.87a
$LSD_{0.05}$	1.20	1.41	0.19	0.17	0.2	1.31	0.11	0.02

Means not sharing the same letter(s) within each column, significantly different at 0.05 level of probability

The results listed in Table 6 indicated that the percentages of total soluble solids and reduced sugars in Seidi, Meghal 1 and Meghal 2 date palms were statistically different from Frehi cultivar in the two seasons, while the differences among Seidi and Meghal 1 or Meghal 2 were so slight not enough to be significant in both experimental seasons. The highest values were seen in Seidi cultivar in both experimental seasons over the other studied date palms. Total sugars percentages were obviously high in Seidi, Meghal 1 and Meghal 2 date palms comparing with Frehi in the two seasons. Besides, in the first season, the percentage of total sugars between Seidi and Meghal 1 or Meghal 2 date palms were not significant, but in the second season it was found that Seidi significantly differed from Meghal 2. Regarding to the percentage of non-reduced sugars, the differences among Seidi, Meghal 1, Meghal 2 and Frehi date palms were so slight not enough to be significant in the two seasons. Concerning the percentages of tannins and acidity, it was noticed that the high significant percentages were found in Frehi cultivar comparing with Seidi, Meghal 1, and Meghal 2 in the two seasons. Besides, the differences among Seidi cultivar, Meghal 1, and Meghal 2 were not significant in the two seasons. TSS/acidity ratio was high in Seidi, Meghal 1, and Meghal 2 date palms and greatly differed from that in Frehi cultivar in both seasons. Moreover, the differences among Seidi, Meghal 1, and Meghal 2 were not significant in the first season, while in the second season, its value in Seidi cultivar was greatly different from that in Meghal 1 and Meghal 2.

Table 6: fruit chemical characteristics in some date palms cultivated in Siwa Oasis during 2020 and 2021 seasons

Treatments	TSS %	Total sugars %	Reduced sugars %	Non-reduced sugars %	Tannins %	Acidity %	TSS/ Acidity ratio
	2020						
Seidi	31.75a	26.68a	20.52a	6.16a	0.436b	0.21b	151.87a
Meghal 1	30.92a	25.81a	19.30a	6.50a	0.445b	0.22b	144.38a
Meghal 2	30.79a	26.10a	19.03a	7.07a	0.453b	0.22b	143.30a
Frehi	26.53b	21.57b	15.73b	5.84a	0.476a	0.26a	102.76b
$LSD_{0.05}$	1.71	1.39	1.54	1.24	0.02	0.02	12.96
	2021						
Seidi	32.84a	29.25a	21.99a	7.26a	0.444b	0.20b	163.19a
Meghal 1	30.84a	27.82ab	20.49a	7.33a	0.438b	0.21b	147.06b
Meghal 2	30.58a	27.25b	20.43a	6.83a	0.449b	0.21b	143.15b
Frehi	25.92b	22.20c	15.74b	6.46a	0.474a	0.25a	105.49c
$LSD_{0.05}$	2.52	1.84	1.76	1.05	0.01	0.02	14.78

Means not sharing the same letter(s) within each column, significantly different at 0.05 level of probability

Molecular studies

The main objective of this current study was to amplify and characterize *RbcI* from the chloroplast genome to assess their suitability for the resolution of *Phoenix dactylifera* genotypes. Electrophoretic analysis of PCR products based on partially amplified *RbcI* genes resulted in a single amplified DNA band. As a result of progress in sequencing and technologies of account, DNA sequences have become the highest source of new information for developing our understanding of evolutionary and genetic relation.

Studies in molecular phylogenetics usually treat with evolutionary relations among more clades, while those in population genetics target diversity within and among populations of one species. DNA barcoding is established on the premise that a short-standardized sequence can discriminate single of a species because genetic diversity between species more that within species (Hebert *et al.*, 2003). Since then, DNA barcoding has become greatly important as a tool in taxonomic studies and species delimitation, as well as in the discovery of new (cryptic) species (Hebert *et al.*, 2004). The footprints of comparative sequence analysis are now apparent in almost all areas of the biological sciences, from development to epidemiology (Tibayrenc, 2005). To increase the phylogenetic relation of chosen date palm varieties in this study, *RbcI* intergenic spacer was evaluated for discrimination power to identify date palm varieties. they discuss the role of DNA barcodes in developing the taxonomic enterprise and its potential to supply a contextual framework for both building phylogenies and community genetics.

During evolutionary process, the residues that perform key functional and structural roles tend to be well-preserved by natural selection, i.e., *conserved regions*, other residues that may be less crucial for structure and function tend to mutate more frequently. Therefore, by comparing sequences through alignment, patterns of conservation and variation can be identified. A marked similarity among 2 protein sequences may reflect the fact that they are derived by evolution from the same ancestral sequence, and

the name of this Sequences is called *homologous* and the evolutionary similarity between them is known as *homology*. otherwise, sequences that have a more distant common ancestor will have accumulated many more mutations, and their evolutionary relationship will be less immediately obvious, or even impossible to deduce from sequence alone.

The comparison of amino-acid sequence with another to find the degree of similarity between them is a key step performed through pairwise sequence alignment. Alignments work on the common base that two homologous sequences derived from the same ancestral sequence will have at least some corresponding residues at the congruous positions in the sequence; if corresponding positions in the sequence are aligned, the degree of matching should be greatly significant compared with that of two randomly selected unconnected sequences. Thus, a quantitative measure is required to define the degree of resemblance. In the simplest case, only identical matched residues are counted, which gives a measure known as percentage sequence identity. When protein sequences are being compared, more sophisticated methods of assessing similarity should be used, like scoring scheme. This scoring specifies the substitution matrix and gap penalty used to measure the degree of similarity. There are two different alignments strategies: *global alignment* and *local alignment.* Both alignment strategies, global or local, are based on one of three methods: *dot matrix, dynamic programming and word method*. Each method handles the difference between global and local alignment by an optimization strategy used in aligning similar residues. The classical global pairwise alignment algorithm using *dynamic programming* is the *Needleman–Wunsch* (NW) *algorithm*. While Smith–Waterman (SW) algorithm is the dynamic programming in local alignment. The pairwise sequence alignment is a key stone in database similarity searching and multiple sequence alignment. Sequence database searching algorithms should satisfy three criterions: *sensitivity*, *specificity,* and *speed*. The sensitivity is the ability to find as many correct hits as possible, i.e., true positives. The specificity, also called selectivity, is the ability to exclude incorrect hits, i.e., false positives. The pace is the time it needs to have data from database researches. Development in molecular biology and DNA sequencing techniques has enabled us to characterize the genomes of several organisms rapidly. Analyses of the DNA sequences of different species are providing valuable information about their taxonomy, gene makeup and utilization. The cultivated date palm is the highest agriculturally paramount species of the family of *Arecaceae*. The standard chloroplast DNA barcode for land plants recommended by the Consortium for the Barcode of Life plant working group needs to be evaluated for a wide range of plant species. Therefore, we assessed the potential of the *rbcL* markers for the authentication of the four *Phoenix dactylifera* palms (*Phoenix dactylifera* isolate Meghal 1, *Phoenix dactylifera* isolate Meghal 2, *Phoenix dactylifera* isolate Siwa Oasis SQ01 (Seidi), and *Phoenix dactylifera* isolate Siwa Oasis SQ02 (Freihi) were collected from Siwa Oasis. There is no one universal method to authenticate date palms. In this study, 4 different *Phoenix dactylifera* genotypes were sequenced and analyzed for *rbcL* genes by using bioinformatic tools to establish a cultivar-specific molecular monogram.

Phylogenetic trees were constructed based on the *rbcL* sequences, and the results suggested that *rbcL* can be used for determining the levels of genetic variations and for barcoding. One of the most significant applications of DNA barcoding is to overcome taxonomic obstacles, where it is difficult to identify unknown or wrongly named species in a family with similar morphology.

The quality of the extracted DNA was detected 1% agarose gel electrophoresis as observed in

Figure 1. The results indicated that there is no fragmentation was observed in extracted DNA. The quantity of extracted DNA samples was determined by using Nanodrop Spectrophotometer and the concentration ranged from 30 - 50 ng/µl. The extracted DNA was directly used in PCR amplification as found in **Figure 2** for the *MatK* gene (900 bp).

DNA barcoding is a technique for distinguishing species using short, different, and incorporated DNA regions. The *Rbcl* sequence data obtained from date palms was aligned and subjected to BLASTn using NCBI. Similarity index percentage was checked with P. dactylifera chloroplast complete genome and accession numbers for all the sequences were obtained from GenBank and published under the accession numbers listed in **Table 7.**

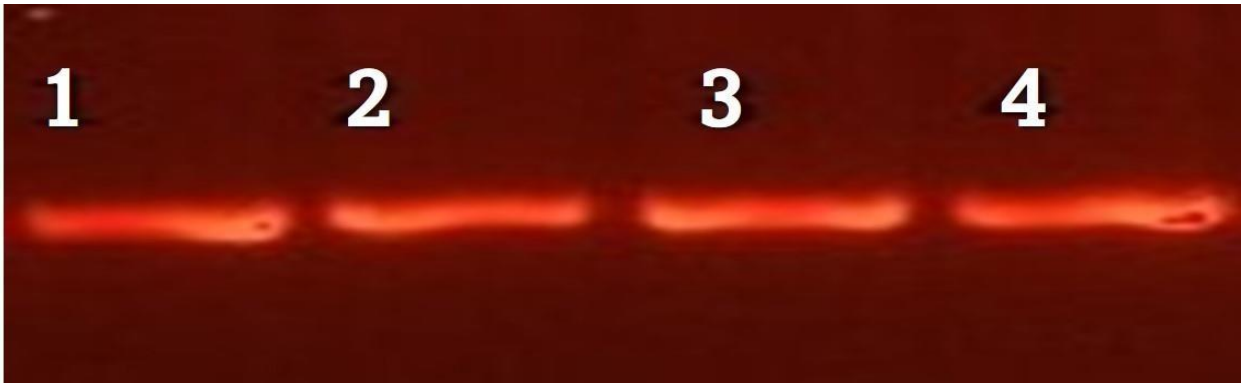

Figure (1) Agarose gel electrophoresis for extracted DNA from samples (1) *Phoenix dactylifera* Meghal 1 (2) *Phoenix dactylifera* Meghal 2, (3) *Phoenix dactylifera* Siwa Oasis *SQ01 (SEIDI),* (4) *Phoenix dactylifera* Siwa Oasis *SQ02 (FREIHI).*

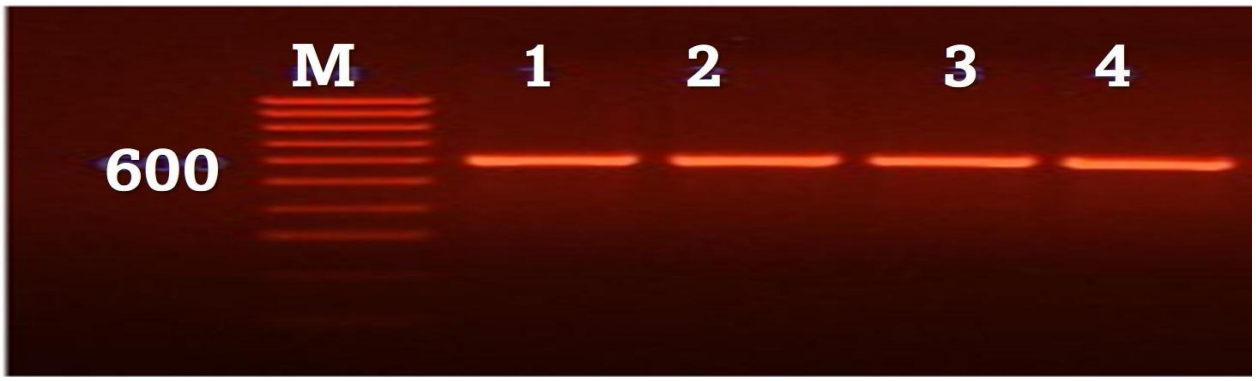

Figure (2) Agarose gel electrophoresis for amplified samples by using the primer *MatK* for (1) *Phoenix dactylifera* Meghal 1 (2) *Phoenix dactylifera* Meghal 2, (3) *Phoenix dactylifera* Siwa Oasis *SQ01 (SEIDI),* (4) *Phoenix dactylifera* Siwa Oasis *SQ02 (FREIHI).*

For Rbcl region, DNA sequence varied from 549 bp for Siwa Oasis SQ01 (Seidi), 559 bp for Siwa Oasis SQ02 (Freihi), 567 bp for Meghal 1 to 600 bp for Meghal 2 (Table 7). In addition, the GC content of the Rbcl sequences varied from 27.87% for Siwa Oasis SQ01 (Seidi), 42.40 for Siwa Oasis SQ02 (Freihi),

42.33 bp for Meghal 2 to 42.68 bp (Table 7). The *Rbcl* barcode exhibited complete PCR success (100%). High-quality sequencing data were obtained for *Rbcl* with a success rate of 83.3%. The haplotype variation (Hd), difference of haplotype, nucleotide variation (Pi), theta (per site) from Eta, mean number of nucleotide variations (K) among all varieties was noticed to be 0.953, 0.00058, 0.62918, 0.69463, 373.73, respectively, in *Rbcl*.

For instance, the nucleotide composition of *Phoenix dactylifera isolate* Meghal 1 was A=28.92%, C = 20.46 %, G = 22.22 % and T = 28.40 %, the G+C = 42.68 % and A+T = 57.32 % in a full length 567 nucleotides with molecular weight (MW) (344009.00 *dalton)*. Furthermore, the nucleotide composition for *Phoenix dactylifera isolate* Meghal 2 was A = 29.50 %, C = 20.17 %, G = 22.17 % and T = 28.17 %, the G+C = 42.33 % and A+T = 57.67 % in a full length 600 nucleotides with M.wt (363994.00 *dalton)*. Otherwise in *Phoenix dactylifera isolate* Siwa Oasis *SQ01 (SEIDI)*, the nucleotide composition was A = 29.14 %, C = 20.40%, G = 22.59 % and T = 28.85%, the G+C=27.87% and A+T = 57.01 % in a full length 549 nucleotides with M.wt (333119.00 *dalton*). Moreover, the nucleotide composition for *Phoenix dactylifera isolate* Siwa Oasis *SQ02 (FREIHI)* was A = 29.34%, C = 20.57%, G = 21.82% and T = 28.26%, the G+C = 42.40% and A+T = 57.60 % in a full length 559 nucleotides with M.wt (*339130.00 dalton*) (**Table 7**).

Consequently, the *in silico* deduced proteins from the *Rbcl* genes of the two studied isolates *Phoenix dactylifera isolate* Meghal 1 and *Phoenix dactylifera isolate* Meghal 2 have the same sequence length (*189)*, amino acid composition (*Ala* to *Tyr)* and Molecular weight (*21144.93* daltons). On other hand, the length of putative proteins of both Phoenix dactylifera isolate Siwa Oasis SQ01 (Seidi) and Siwa Oasis SQ02 (Frehi) were (183,187) respectively with different amino acid composition. Also, the molecular weight of two putative proteins of Phoenix dactylifera isolate Siwa Oasis SQ01 (Seidi) and Siwa Oasis SQ02 (Freihi) were (19540.39, 20300.18 daltons) respectively (**Table 8**).

Table (7): Nucleotide's composition of *RbcL* sequences of *Phoenix dactylifera* trees grown in Egypt using BioEdit sequence Alignment Editor.

Samples	A%	C%	G%	T%	G+C%	A+T%	Length	*(M.wt) Dalton*
Phoenix dactylifera isolate Meghal 1	28.92	20.46	22.22	28.40	42.68	57.32	567	344009.00
Phoenix dactylifera isolate Meghal 2	29.50	20.17	22.17	28.17	42.33	57.67	600	363994.00
Phoenix dactylifera isolate Siwa Oasis *SQ01 (Seidi)*	29.14	20.40	22.59	28.85	27.87	57.01	549	333119.00
Phoenix dactylifera isolate Siwa Oasis *SQ02 (Freihi)*	29.34	20.57	21.82	28.26	42.40	57.60	559	*339130.00*

Table (8): Amino acid composition of putative protein of *Rbcl* barcode gene of Phoenix dactylifera isolate grown in Egypt using BioEdit sequence Alignment Editor.

Amino Acid	*Phoenix dactylifera isolate* Meghal 1	*Phoenix dactylifera isolate* Meghal 2	*Phoenix dactylifera isolate* Siwa Oasis *SQ01 (Seidi)*	*Phoenix dactylifera isolate* Siwa Oasis *SQ01*(Freihi)
Ala A	7.41	7.41	2.19	2.14
Cys C	1.59	1.59	4.37	4.28
Asp D	5.29	5.29	1.09	1.60
Glu E	6.35	6.35	1.09	0.53
Phe F	3.17	3.17	4.92	5.88
Gly G	8.47	8.47	7.10	7.49
His H	1.06	1.06	2.73	2.67
Ile I	3.70	3.70	4.37	4.81
Lys K	7.41	7.41	3.83	4.28
Leu L	8.99	8.99	6.01	8.02
Met M	0.53	0.53	2.73	1.60
Asn N	2.65	2.65	3.83	3.21
Pro P	5.82	5.82	3.83	4.28
Gln Q	2.65	2.65	3.28	3.74
Arg R	5.29	5.29	9.29	9.63
Ser S	4.76	4.76	14.75	14.44
Thr T	8.99	8.99	4.37	3.74
Val V	7.41	7.41	2.73	2.14
Trp W	1.59	1.59	3.28	2.67
Tyr Y	6.88	6.88	6.01	5.88
Length	189	189	183	187
MW (M.wt) Dalton	21144.93	21144.93	19540.39	20300.18

Multiple Sequence Alignment (MSA) using BioEdit Sequence Alignment Editor

The data in **Table (9)** presented the similarity percentage calculated by the BLAST and the phylogenetic affinity based on the plant sequencing using the *RbcL* gene, which showed highly similiters percentages ranged from 99.24 to 100 for all the studied species with others in the GenBank database. For instance, the collected sample *Phoenix dactylifera* Meghal 1 was identical (100%) with different species of the same genera *Phoenix* such as *Phoenix* (*dactylifera, canariensis, reclinate, roebelenii, theophrasti Greuter and paludosa*) on GenBank (100%), while *Phoenix dactylifera* isolate Siwa Oasis SQ01 (SEIDI) has a high percentage of similarity (99.24%). Furthermore, Phoenix dactylifera Meghal 2 showed high similarities with Phoenix (*dactylifera, canariensis, reclinate and flexuosa*) in percentage 99.65% to 100% (**Table 9**). *Phoenix dactylifera* isolate Siwa Oasis SQ02 (Freihi) has a high identity percent (99.88%) with several species such as *Phoenix roebelenii, Phoenix dactylifera* and *Phoenix theophrasti* (**Table 9**).

Table (9) The highly similar species in the Nucleotide Genbank Databases using *rbcL* gene sequences for *Phoenix dactylifera* species grown in Egypt.

ID	*Morphological identification*	*BLAST search match*	*positions in the final dataset*	*BLAST similarity (%)*	*Phylogenetic affinity*
1	*Phoenix dactylifera isolate* Meghal 1	*Phoenix dactylifera* *Phoenix canariensis* *Phoenix reclinata* *Phoenix roebelenii* *Phoenix theophrasti Greuter* *Phoenix paludosa*	539 1359 1359 607 705 1258	100 100 100 100 100 100	*P. dactylifera* Meghal 1
2	*Phoenix dactylifera isolate* Meghal 2	*Phoenix canariensis* *Phoenix dactylifera* *Phoenix reclinate* *Mauritia flexuosa*	442 1411 1401 1445	100 99.83 100 99.65	*P. dactylifera* Meghal 2
3	*Phoenix dactylifera isolate* Siwa Oasis *SQ01 (Seidi)*	*Phoenix dactylifera* *Phoenix canariensis* *Phoenix reclinata* *Phoenix paludosa*	580 1359 1359 541	99.25 99.24 99.24 99.24	*P. dactylifera* Siwa Oasis *SQ01 (SEIDI)*
4	*Phoenix dactylifera isolate* Siwa Oasis *SQ02 (Freihi)*	*Phoenix roebelenii* *Phoenix dactylifera* *Phoenix theophrasti*	539 538 705	98.88 98.88 98.88	*P. dactylifera* Siwa Oasis *SQ02 (FREIHI)*

Considerable variations are detected in all regions including the start and the end of the sequences. The number of substitutions inferred for each nucleotide position was calculated using PAUP and Bioedit. Substitutions occur uniformly across the gene **(Figures 3).** The patterns and magnitudes of nucleotide variation in the *rbcL* gene were evaluated at the genus, family, subclass, class, and division in a comparative study using the 11 complete sequences **(Figures 4).** The nucleotide variation ranged from 1.1% at the intrageneric level. The divergence in *rbcL* sequences took a significant leap above the family level, resulting in a distinct gap between the intra-familiar and inter-familiar variation in the sequences. Multiple sequence alignment shows that, there are variable numbers of Indels and substitution in the gene *rbcL*. The alignment of *rbcL* gene of combined nucleotide sequence shows 29 single nucleotide polymorphisms at a different position in four varieties and the overall mean distance is 0.027. The *Phoenix dactylifera isolate* Meghal 2 has 33 nucleotides more than Meghal 1 at the start of the gene and has many nucleotide substitutions at the end (574, 575, 576, 577, 578, 579, 581, 583, 584, 586, 587, 588, 589). Also, the nucleotide substitutions occur in *Phoenix dactylifera isolate* Siwa Oasis *SQ01 (Seidi)* at (54, 56, 59, 60, 575, 577, 579, 580, 581, 586, 590, 593). In addition, *Phoenix dactylifera* isolate Siwa Oasis SQ01 (Seidi) has nucleotide substitution at multiple sites (474, 536, 540, 562, 565) **(Figures 5).**

Evolutionary analysis and phylogenetic tree of *Phoenix dactylifera* grown in Egypt using RbcLgene

The main objective of this study was to amplify and characterize loci *rbcL* from the chloroplast genome to assess their suitability for the resolution of date palms. These markers demonstrate high polymorphism among the date palms but were inefficient in discriminating them. Genetically, the date palm is highly differing as a result of the presence of numerous cultivars distributed across different habitats (Khan *et al*., 2012). The chloroplast genome has been utilized greatly for the discrimination of different cultivars of date palm in Saudi Arabia and Egypt (Al-Qurainy *et al*., 2011). The cultivar-especially clusters in *rbcL* trees resolved with high bootstrap trust levels (95-100%) by using UPGMA and NJ, while, with ML, the bootstrap levels were 76-100%. ML cleared the same tree topology as UPGMA and NJ with single-locus analysis for date palms improving. Multiple sequence alignment shows that there are variable numbers of Indels in the gene *Rbcl*. Using neighbor joining method and maximum likelihood (**Table 10**), the evolutionary distances for the four *Phoenix dactylifera* were distinguished into individual clades.

In case of maximum likelihood methods, the combined tree showed four clusters or cladograms and they represented as follows: Group I belong to *Phoenix dactylifera* comprising different isolate included Meghal 1 and other genera Sabal. Furthermore, Group II included two isolates of *Phoenix dactylifera* Siwa Oasis *SQ01 (Seidi) and SQ02 (Freihi)* which are closely related to group III comprising two isolates Meghal 2 and DP60. Otherwise, group IV are distant related to another group which comprise only one isolate *Phoenix Paludosa9* (**Figure 3**). In the case of the neighbor joining method, the combined tree showed four groups or cladograms and they represented as follows: Group I belong to *Phoenix dactylifera* comprising different isolates and other genera Sabal. Furthermore, Group II included two isolates of *Phoenix dactylifera* Siwa Oasis *SQ01 (Seidi) and SQ02 (Freihi)* which are closely related to group III comprising two isolates Meghal 2 and *Phoenix Paludosa9*. Otherwise, group IV are distant related to other groups comprising two isolate DP60 *and* Meghal 1 (**Figure 3**). The alignment of *Rbcl* gene of *Phoenix dactylifera* nucleotide sequence revealed 649 variable sites and 359 parsimony sites, the overall mean distance is 2.93 and the estimated Transition/Transversion bias (R) is 0.74.

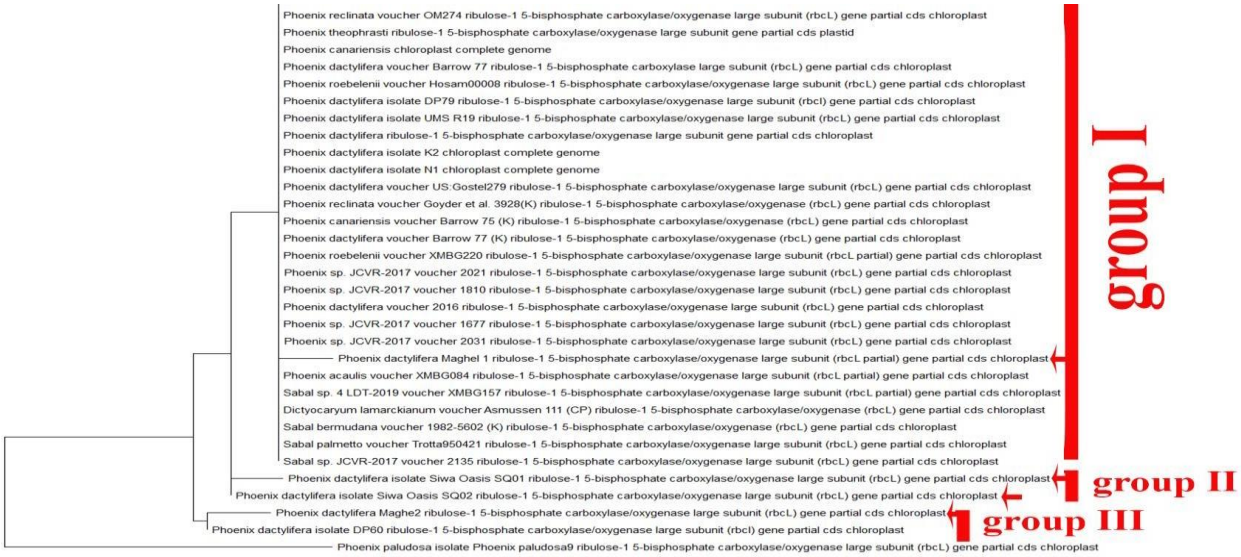

Figure 3: Evolutionary analysis and phylogentic tree of Phoenix dactylifera species grown in Egypt using *RbcL* gene using Maximum Likelihood method.

Maximum Likelihood Estimate of Transition/Transversion Bias

The estimated Transition/Transversion bias (*R*) is 0.74 as found in Table 9. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model [1]. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -5374.618. This analysis involved 35 nucleotide sequences. There was a total of 600 positions in the final dataset. Each entry shows the probability of substitution (r) from one base (row) to another base (column) [1]. For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversions substitutions are shown in *italics*. The nucleotide frequencies are 28.21% (A), 28.58% (T/U), 20.95% (C), and 22.26% (G). This analysis involved 35 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 600 positions in the final dataset (Table 10-13).

Table 10: Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	*7.89*	*5.78*	8.95
T/U	*7.80*	-	10.33	*6.14*
C	*7.80*	14.11	-	*6.14*
G	11.38	*7.89*	*5.78*	-

Table 11: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*12.55*	*9.2*	0.06
T	*12.38*	-	5.11	*9.77*
C	*12.38*	6.97	-	*9.77*
G	0.07	*12.55*	*9.2*	-

Table 12: Estimates of evolutionary divergence between *Rbcl* sequences of samples

	Species	1	2	3	4
1	*Phoenix dactylifera isolate* Meghal 1				
2	*Phoenix dactylifera isolate* Meghal 2	3.14			
3	*Phoenix dactylifera isolate* Siwa Oasis *SQ01 (Seidi)*	3.10	3.10		
4	*Phoenix dactylifera isolate* Siwa Oasis *SQ02 (Freihi)*	2.87	2.99	2.34	0.0

Table 13: Test of the homogeneity of substitution patterns between sequences

	Species	1	2	3	4
1	*Phoenix dactylifera isolate* Meghal 1		000	000	000
2	*Phoenix dactylifera isolate* Meghal 2	1.00		000	000
3	*Phoenix dactylifera isolate* Siwa Oasis *SQ01 (Seidi)*	1.00	1.00		000
4	*Phoenix dactylifera isolate* Siwa Oasis *SQ02 (Freihi)*	1.00	1.00	1.00	

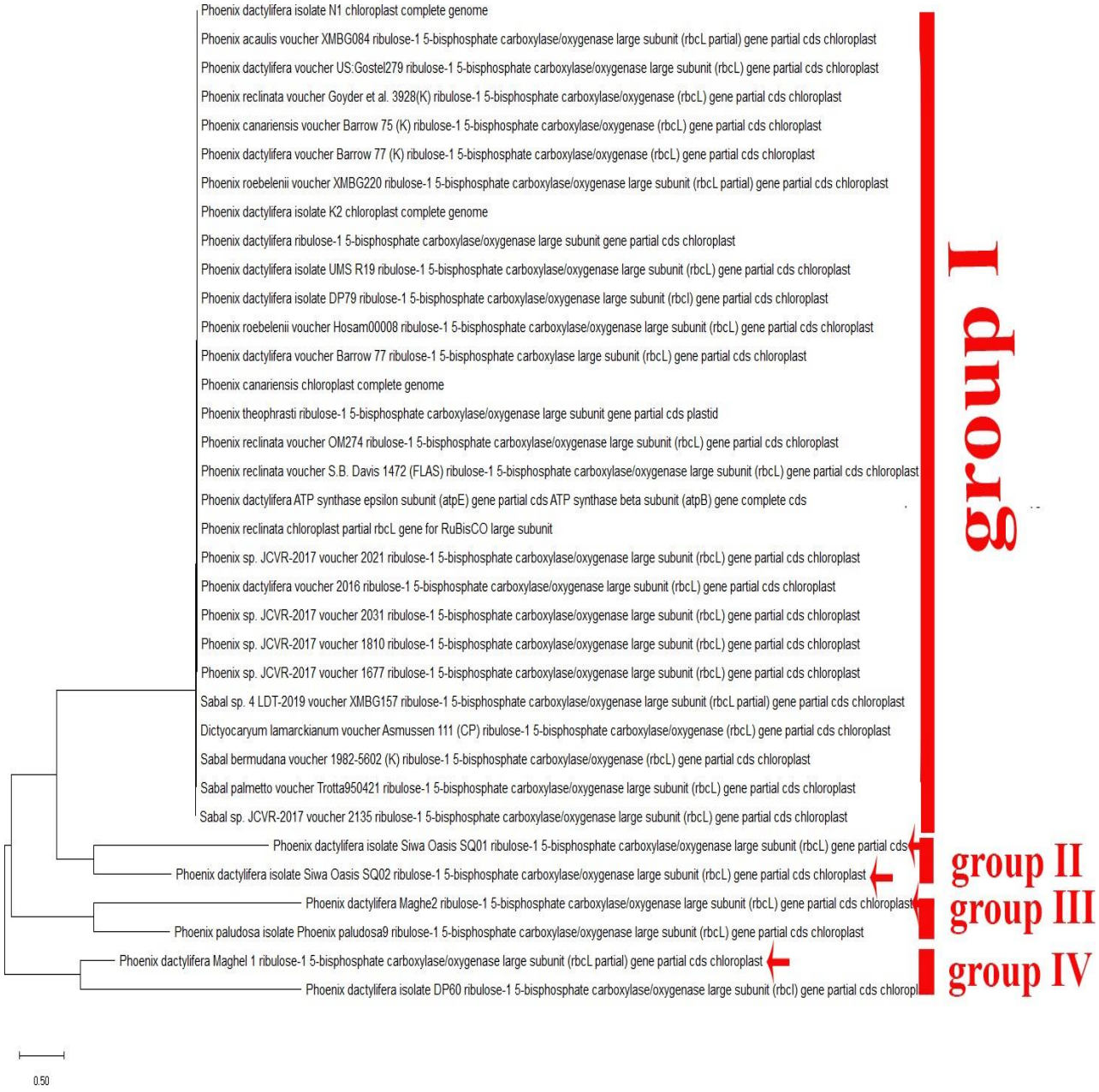

Figure 4: Evolutionary analysis and phylogenetic tree of Phoenix dactylifera species grown in Egypt using *RbcL* gene using the Neighbor-Joining method

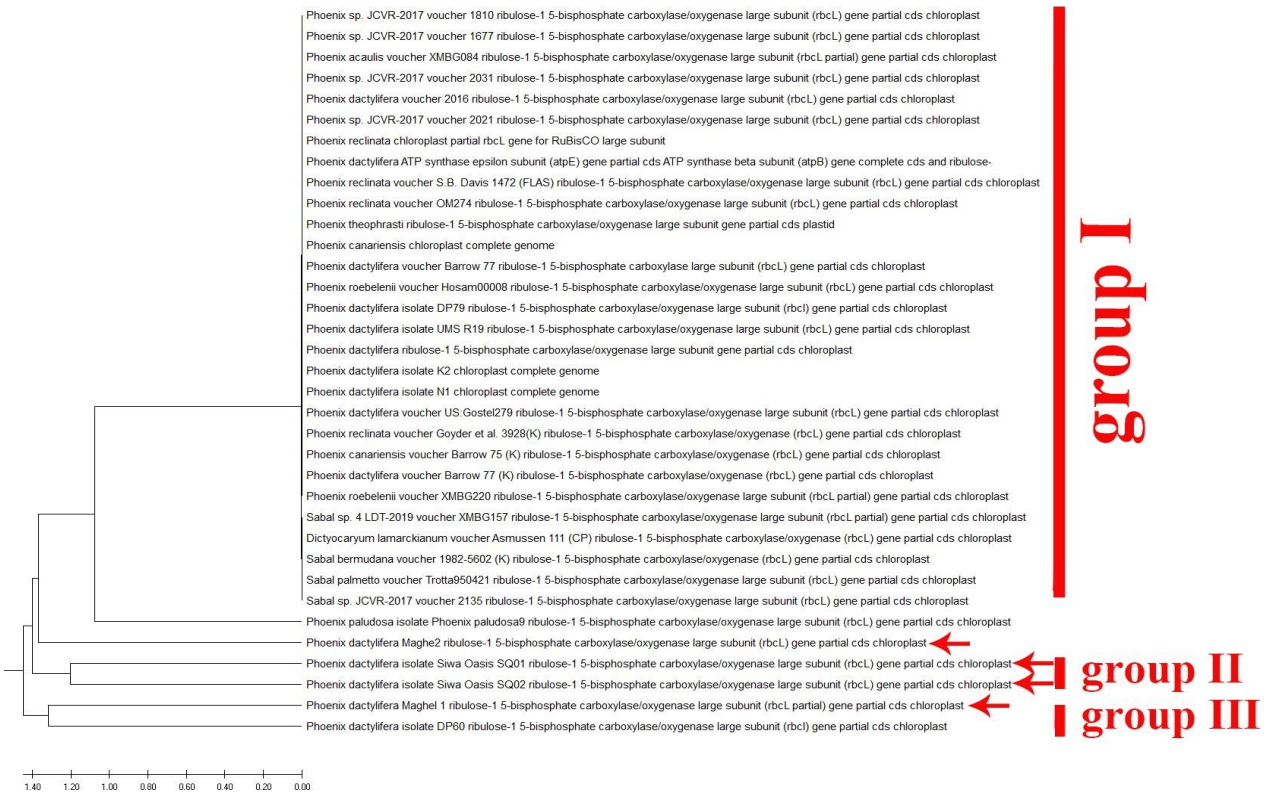

Figure 5: Evolutionary analysis and phylogenetic tree of Phoenix dactylifera species grown in Egypt using *RbcL* gene using the UPGMA method. There was a total of 161 positions in the final dataset.

DISCUSSION

The obtained results showed that it could be depended on vegetative growth and fruiting parameters such as frond length, spine area distance, leaf area length, spines number, bunch weight and fruit yield in kg per tree or in ton per hectare as good tools to discriminate among known and unknown date palms. Moreover, fruit physical and chemical characteristics like fruit weight, size, length, and diameter, kernel weight, Flesh fruit weight, flesh/ fruit ratio, the percentages of TSS, tannins, acidity TSS/acidity ratio, total, reduced and non-reduced sugars as well as DNA barcoding could be used also as effective methods to study the relation among known and unknown date palms. These results were previously confirmed by the forementioned findings of many authors, they stated that there are a lot of phenotype-oriented techniques for the differentiation of date palm cultivars like growth, flowering and yield features (Al-Doss and Bacha, 2001) and description of vegetative and reproductive traits (Salem *et al.*, 2008 and Ahmed *et al.*, 2011). Depending on the measuring of morphological features as leaf number and width, pinnae number, length and width, as well as the length and width of spine from the top and bottom, Elhoumaizi *et al.*, (2002) noticed that there was an important morphological diversity between twenty sex date-palm cultivars (*Phoenix dactylifera* L.) from Morocco and this gave an indicator for identification of cultivars before the time of fruiting. Chloroplast genome sequence data is the basic kind of DNA which is used for barcoding of plant because it has a haploid and stable genetic temple where there is no recombination, and it is mostly uniparentally inherited (Adams and Palmer, 2003 and Xiwen,

et al., 2015). Besides, DNA barcoding has become significantly important for the study of taxonomy, defying the species, and discovering of new species (Hebert *et al*. 2004; DeSalle *et al*., 2005; Hebert and Gregory, 2005; Savolainen *et al*., 2005 and Hajibabaei *et al*., 2007). Ahmed *et al*. (2006) and Zhao *et al*. (2013) stated that because the vegetative characteristics can express on the genes, so to differentiate between varieties should be depended on the molecular analysis. The application of a barcoding accelerates the discrimination and the discovery of species and proceed the investigation of ecologists and taxonomists (Newmaster et al., 2006). Elshibli and Korpelainen (2009) characterized 15 cultivars by using tree and fruit morphology as well as softness characters of fruits, and they noticed that there were high levels of variation among the studied cultivars in both phenotypic. Hammadi *et al*. (2009) reported that the percentage of spanned middle part, apical variation angle, and higher pinnae width at the top leaf, solitary spine percent, and spine length at the middle and major spine angle, thorns length, area of thorns and the leaf length could be considered as a good method to investigate the resemblance or variations between the date palm females between thirty date palm varieties. Moreover, our results were previously confirmed by Kadkhodaei *et al*. (2010), they sated those cultivars and species were differentiated on the foundation of morphological and physiological features that are occasionally hard to discriminate. Additionally, leaf measurements like; thorns length, number of pinna and the length of leaf have shown the resemblance and the variations between the palms (Saker, 2011 and Haider *et al*., 2015). Al-Khalifah *et al*. (2012) stated that some date palm cultivars (*Phoenix dactylifera* L.) have similar known morphological features that complicate cultivar identification and require genetic guide to prove phylogenetic relations. The morphological analysis of fruits of Barhy, Deglet Noor, Hilaliah, Hilwa, Khalas, Makhtomi, Moneifi, Nabtet Ali, Omal Khashab, Rothana, Sabbaka, Shagra, Sukkary, Wannanah date pale cultivars cleared a high standard of variation in length-width ratio, colour, shape of the fruit, and fruit-base. Moreover, morphologic characters showed that the fruit shape is one of the characteristics most affected by genetic diversity. Mohamed Lemine *et al*. (2014) reported that using fruit in morphological measuring was the best tool for the distinguishing between date palm varieties. It could be relayed on ten vegetative features to differentiate among 26 Algerian date palm cultivars such as palm length, rachis thickness, spine number, middle spine width and thickness, middle leaf length and terminal leaf width, and leaflets number to differentiate among cultivars. Moreover, the authors sated those lengths of spikelet parts, fruit weight and length, cavity length, calyx diameter, and width, as well as thickness of seeds (Bedjaoui and Benbouza, 2020).

CONCLUSION

- By depending on the vegetative parameters like frond length, spine area distance, leaf area, length, number of spines, it is difficult to discriminate among unknown and known date palms.
- Fruit weight, size, length and diameter, fruit yield in kg and in ton per hectare showed that there were obvious differences among Seidi and Frehi cultivars, Meghal 1, and Meghal 2 in Siwa Oasis.
- Fruit chemical characteristics such as the percentages of total soluble solids, total and reduced sugars, tannins, and acidity showed that Meghal 1 and Meghal 2 are close to each other and both of them are close to Seidi cultivar than Frehi in the two seasons.
- DNA barcoding emphasized the genetic relation among Meghal 1, Meghal 2 and Seidi more than Frehi.

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