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IDENTIFICATION OF SOME CULTIVARS OF EGYPTIAN DATE PALM (*PHOENIX DACTYLIFERA* L.) USING DNA BARCODING

Hagar A. Hani[1], Gadalla E.G.[2], Haggag S. Zein[1], Shereen Abu El-Maaty[1] and Mona H. Hussein[1]

[1]Genetics Department, Faculty of Agriculture, Cairo University, Egypt.
[2]Central laboratory for Date Palm research and development, Agriculture Research Center, Egypt.

Abstract

Date palm *Phoenix dactylifera* L. species of the *Arecaceae* family, being economically very important, is widely cultivated in the Middle East and North Africa, In the present study, six different cultivars of dates, *viz.* Hayani, Sakkoty, Gondila, Bartamoda, Malkaby and Amhaat were sequenced for *rbcl*, *matK*, *ycf5* and *psbA-trnH* genes and analyzed using bioinformatics tools to establish it's potential as a DNA barcoding of Egyptian date palm culti-vars. The results showed that the chloroplast *matK*, *rbcl*, *ycf5* markers were more informative than the *psbA-trnH* chloroplast DNA marker. Phylogenetic trees were constructed on the basis of the *matK*, rbcl, *ycf5* sequences, and the results suggested that *matK*, *rbcl*, *ycf5* can be used for determining the levels of genetic variation and for barcoding.

Key words: *Phoenix dactylifera*; Barcoding; *rbcl*; *matK*; *ycf5*; *psbA-trnH*.

Introduction

Phoenix dactylifera L. From the family: *Arecaceae* (2n = 36) (Farooq *et al.,* 2012) known as Date palm is one of the most economically substantial fruits in the most Arab countries, Middle East and North Africa. Previously, it has been a main agricultural crop for human life (El-Juhany 2010). During the last years, its cultivation extended in Australia, Pakistan, and other countries (Chao *et al.,* 2007). Whole parts of palm trees were beneficial and used by a human as well as the main product of the palm (dates), which is affluent in protein, vitamins and mineral salts (El-Juhany 2010). also is used as a source of feeding, staple food, farm income, and other products for local desert habitat. Many cultivars of date palm have therapeutic properties so it is used in the traditional medicine. The date fruits contain natural compounds, biologically active antioxidants and antibacterial activities (Biglari *et al.,* 2007; Saafi *et al.,* 2011 and Samad *et al.,* 2016). The distribution of date cultivars in different regions around the world causes the extreme diversity of date palms at the genetic level. The morphological traits of date palm fruits (shape, size, weight, color, aspects of the fruit skin, consistency, texture, etc.) and biochemical markers like isozymes and proteins are highly influenced by environmental changes (Tarroum *et al.,* 2011). The term DNA barcode was first recognized by (Hebert *et al.* 2003), who proposed an identification system for specimens based on "The barcode of life" consortium (Hebert *et al.,* 2004) and has acquired the attention of the scientific community (Chen *et al.,* 2010). The concept of DNA barcoding has become a very progressively efficient tool for species identification, classification of living organisms and taxonomic studies (Pires *et al.,* 2010). It involves sequencing of the short standard region of the DNA sequences approximately 650bp (Meier *et al.,* 2006) that are present in all plants, animals, microbes and viruses (CBOL Plant Working Group 2009, Kress *et al.,* 2007 and Hollingsworth *et al.,* 2010). Mitochondrial CO1 "Cytochrome oxidase 1 "gene-based identification systems, a 648-bp region (Herbert *et al.,* 2004) become as a universal barcode gene at-large PCR products for animal identification (Williams and Knowlton 2001). Historically, DNA barcoding analysis start in palms (Jeanson *et al.,* 2011) obtained a 92% success in species identification by testing a combination of three markers (the plastid *matK* and *rbcL*, and the nuclear *ITS2*). Chloroplast genome is highly conserved among plant species, can be classified into three functional categories

(1) protein coding genes, (2) introns and (3) intergenic spacers; the latter two considered as noncoding regions which are a primary source of data for molecular systematic, phylogeographic studies and DNA barcode for plants (Shew *et al.,* 2007)**.** Chloroplast DNA markers are used for precise identification of all plant species (Chase *et al.,* 2005; CBOL Plant Working Group, 2009; Devey *et al.,* 2009; Fazekas *et al.,* 2009; Chen *et al.,* 2010; Al-Qurainy *et al.,* 2011; Ahmed *et al.,* 2014 and Ahmed *et al.,* 2016). A variety of loci from chloroplast genome including *rbcL*, *rpoB*, *rpo*C1, *ndhJ, accd, psbA -trnH* spacer, and *mat*K, have been tested for a potential DNA barcoding of plants with different degrees of variation and identification ability (Kress *et al.,* 2007; Lahaye *et al.,* 2008; Chen *et al.,* 2010; Hollingsworth *et al.,* 2011 and Ahmed *et al.,* 2014). Other techniques used to identify plant species as a biochemical marker has many restrictions. So there is a pressing requirement for using a precise technique at least for the most economical cultivars with higher trade value (Khan *et al.,* 2012). Subsequently, the major objective of this work was to identify some cultivars of the most economically Egyptian date palm using plastid loci (*rbcl, matK, ycf5,* and *psbA-trnH*) and nuclear locus ITS (Internal Transcripted Spacer) to test it's potential as a barcode for accurate identification of date cultivars.

Materials and methods

Plant materials and DNA extraction

The young leaves of six Egyptian cultivars of date palm (Hayani, Sakkoty, Gondila, Bartamoda, Malkaby and Amhaat) were collected from Palm Research Center in Aswan and Giza, then stored at -80°C. Frozen tissues were immersed in liquid nitrogen and mashed using a sterile mortar and pestle to obtain a fine powder. The total genomic DNA was extracted using CTAB method Keb-LIanes et al. (2002) Fig. 1.

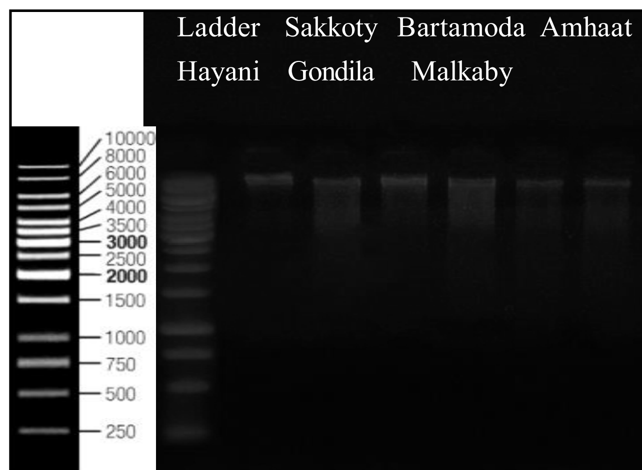


Fig. 1: DNA extraction gel of six date palm cultivars.

Primer design

The sequences of universal primers *ITS* (Internal Transcripted Spacer) region and *ycf5* (the gene that required for Cytochrome biogenesis and encoding an approximately 320-residue protein of unknown function) based on the conserved region of 18S, 5.8S, also from monocotyledons, gymnosperms, mosses, and medical plant species were extracted from GenBank using the keywords "ITS", Internal transcripted spacer, and "*ycf5*" in the annotation. (Chiou *et al.,* 2007; Chen *et al.,* 2010; Pang *et al.,* 2012). As well as the sequence of specific primers rbcl (4-Ribulose-1, 5-Biophosphate Carboxylase/ Oxygenase Large Subunit) gene it catalyzes the carboxylation of ribulose-1, 5-bisphosphate (Dhingra *et al.,* 2004), matK (maturase K The protein it encodes is an intron maturase, a protein that splices introns this protein retains only a well conserved domain X and remnants of a reverse transcriptase domain (Zoschke *et al.,* 2010; YU *et al.,* 2011), and *psbA-trnH* (an intergenic spacer (IGS) a region of non-coding DNA between gene *psbA* and *trnH*). Based on conserved regions of different cultivar of *Phoenix dactylifera* were extracted from Gene bank in FASTA format, multiple sequence alignment was done via Clustal omega>EMBL-EBL followed by manual correction of the alignment sequencing. To reduce error, all sequences were subjected to accurate processing, removed Ns (low-quality sequences with more than 1% nucleotides), the longest and shortest 1% sequences were treated as outliers and excluded, after that the Primer-blast was used to design target-specific primers. (Pang *et al.,* 2012).

PCR amplification and sequencing of candidate DNA Barcodes

A total volume of 25 µL of PCR mixture contained the following: 12.5 µL Taq PCR Master Mix (Qiagen), yielding a final concentration of 200 µM of each deoxynucleotide and 1.5 mM $MgCl_2$, 1 µM of each primer (Humanizing genomics, macrogen), 2 µL genomic DNA, and adjusted the rest with sterile distilled water. PCR amplification was performed using a T100 thermal cycler (BioRad, USA), A long (14 × 11 cm) 1.5% agarose gel in 1X TBE buffer containing 0.25 µg/mL ethid-ium bromide was used for electrophoresis of the PCR products.

The sizes of the PCR products resulting from the primer pairs of the specific barcoding gene were determined using a 100-bp and 1kp ladder (Qiagen). Primers and PCR thermal conditions are listed in Table 1. Purified PCR products were sequenced with the primers used for PCR amplification.

Table 1: Primers and reaction conditions used in this study.

Name of the Gene	Sequence 5'-3'	GC%	Run conditions	Size of the marker in bp
rbcl	F:TACAAATTGACTTATTATACTGACTACG R:CAAGTAATGTCCTTTGATTTCACC	32.26 37.5	94! 5 min,60! 1 min, 72! 1.5 min + 3 sec/cycle, 35 cycles72! 7 min	663
matK	F:TCTTTACATTTATTGCGATTCTTTCTT R:CAATACGGTACAAAATTTAGCTTAGA	29.93 29.63	94! 5 min,59.2! 1 min, 72! 1.5 min + 3 sec/cycle, 35 cycles72! 7 min	636
psbA-trnH	F:ACTTTTGTCTTAGTGTATATGAATCGTTG R:TCTGACCTCCATACTTAGATCGAGATA	31.03 40.74	94! 5 min,64! 1 min, 72! 1.5 min + 3 sec/cycle, 35 cycles72! 7 min	604
ycf5	F:ACTTTAGAGCATATATTAACTC R:ACTTACGTGCATCATTAACCA	27.27 38.1	94! 5 min,53! 1 min, 72! 1.5 min + 3 sec/cycle, 35 cycles72! 7 min	360-394
ITS	F:CCTTATCATTTAGAGGAAGGAG R:TCCTCCGCTTATTGATATGC	40.91 45	94! 5min, 50! 1 min, 72! 1.5 min + 3 sec/cycle, 35cycles72! 7 min	707

Table 2: Sources of samples, voucher information, and GenBank database accession numbers of DNA sequencesof taxa used in the present study.

Cultivar	Sequence characteristics								
	rbcl			matK			ycf5		
	Accession No.	length(bp)	GC%	Accession No.	length(bp)	GC%	Accession No.	length(bp)	GC%
Hayani	MH057656	613	44.6	MG999900	575	31.8	MK190869	354	33%
Sakkoty	MH057657	613	44.5	MG999901	575	31.5	MK190870	354	33%
Gondilia	MH057658	613	44.4	MG999903	575	31.7	MK253293	354	34%
Bartemoda	MH057659	613	44.3	MG999904	575	31.8	MK253294	354	33%
Malkaby	MH057660	613	44.6	MG999905	575	31.9	MK253295	354	33%
Amhaat	MH057661	613	44.3	MG999906	575	31.4	MK253296	354	33%

Data analysis

The *rbcl, matK, ycf5, psbA-trnH* and *ITS* sequences of all date palm cultivars were uploaded to NCBI, and the Basic Local Alignment Search Tool (BLASTn) was used to perform queries for comparison to already-reported sequences in GenBank. All sequences obtained were deposited in GenBank for reference; their accession numbers are provided in Table 2. The sequencing data acquired for all date palm cultivars of the *rbcl*, *matK*, *ycf5*, *psbA-trnH* and *ITS* sequences were aligned using CLUSTAL Omega. Pairwise distance, transitional/ transversional substitutions, and the maximum likelihood substitution matrix were estimated with the MEGA 6.2 software. Phylogenetic trees were inferred by the maximum likelihood (ML), neighbor-joining tree (NJ), and unweighted pair group method with arithmetic mean (UPGMA) methods, which were performed using MEGA 6.2 (Tamura *et al.,* 2013).

Results and Discussion

Efficiency of PCR amplification

The success rates for *rbcl, matK* and *ycf5* sequences were 100%, Results showed that *rbcl, matK* and *ycf5* provided the highest rates, followed by *psbA-trnH* and *ITS*. Fig. 2, while *PsbA-trnH* spacer primers resulted distinct double bands in all cultivars except Gondila, Even with greatly increased annealing temperature, double bands were still present Fig. 3. *ITS* primer designed for

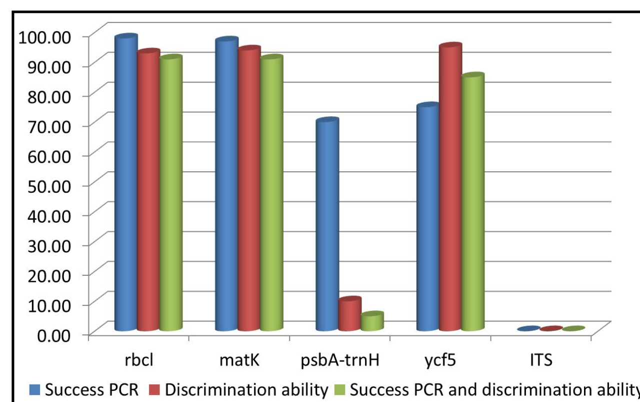


Fig. 2: Properties of five plant loci tested as putative barcodes. Blue bars indicate PCR success; Red bars indicate percent success in discrimination between species of a pair; Green bars indicate PCR success combined with the ability to discrimination between species of a pair.

this study failed to amplify Fig. 4, so *ITS* locus was not included in sequence analysis, PCR amplification *rbcl*, *mat K*, and ycf5 primers showed in fig. 5.

Proposed regions

The primer pairs chosen using for *psbA-trnH*, *ITS*

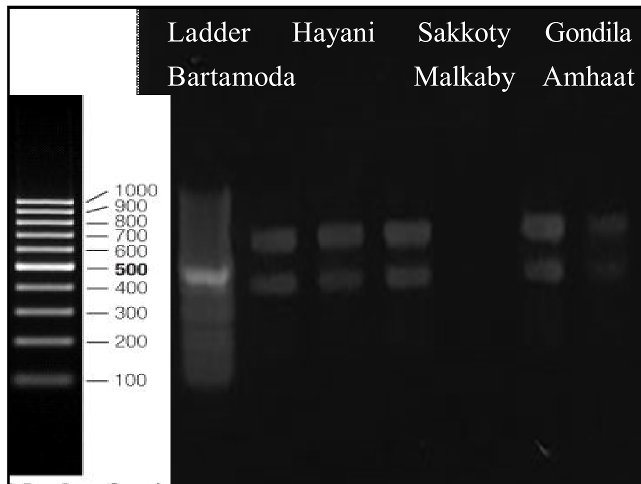


Fig. 3: More stringent reaction conditions were applied by running the amplification with the primer annealing temperature at 59 °C-64 °C, double bands were still evident with *psbA-trnH* primer.

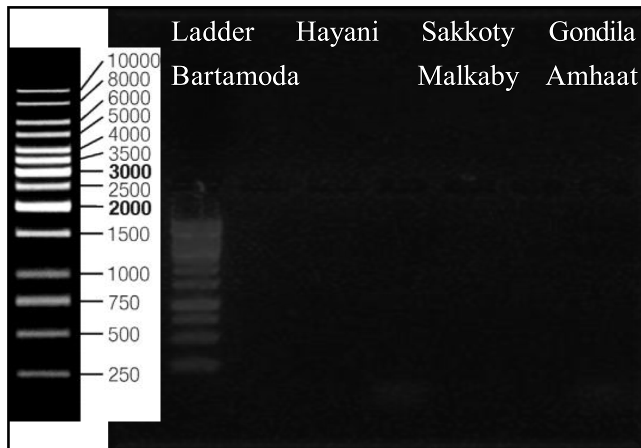
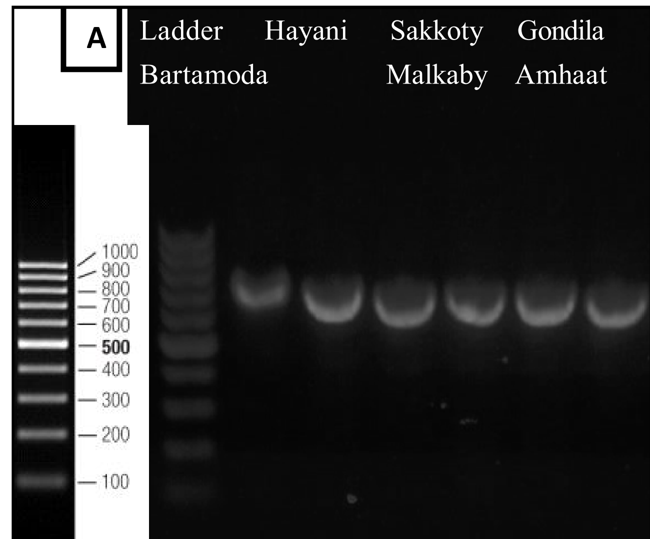
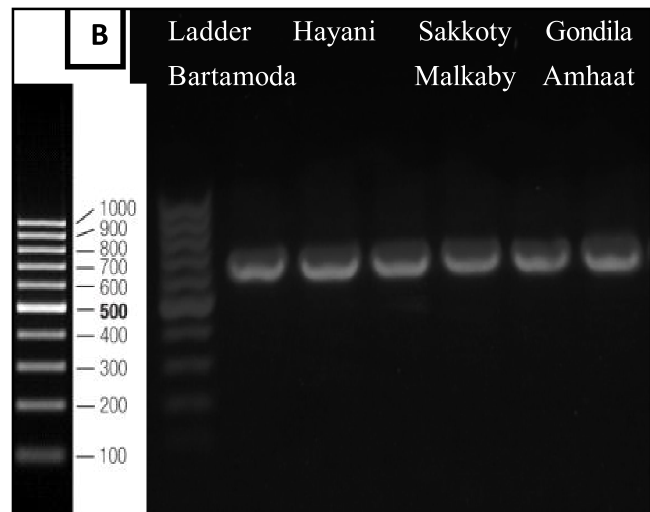


Fig. 4: ITS primer designed for this study failed to amplify, even with running the amplification with different annealing temperature at 50°C-55 °C.





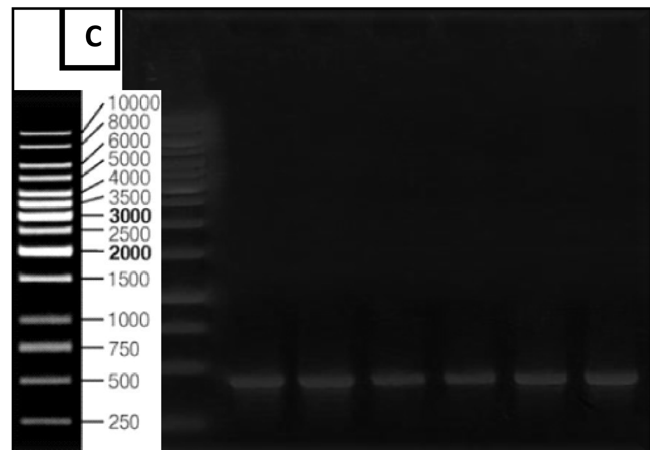


Fig. 5: The products of PCR using published *rbcl*, *matK*, *ycf5* primers, A: *rbcl* primer, B: *matK* primer and C: *ycf5* primer.

did not work well for all cultivars Table 3. Non-specific primer binding resulted in multiple bands or complete lack of amplification. Because the purpose of these experiments was to test the functionality and utility of the proposed barcoding conditions and primers (as per www.kew.org/barcoding) on date palm further analyses were performed only on those primers that successfully generated single products under universal conditions for *Ycf5*, an under specific conditions for *rbcl* and *matK*. First, at 350 bp, the *ycf5* region is short as a result followed by *matK* 575 bp, then *rbcl* 615bp. All loci sequences are relatively easy to amplify resulted in a single amplified DNA band. Second, determination of genetic diversity using multiple sequence alignment for evry gene with 6 sequences and calculating the pairwise distance and overall distance (Tamura *et al.,* 2013) Tables 4, 5, 6 confirmed that the *ycf5, rbcl, matK* regions respectively possess high genetic diversity. Third, according to the BLAST method, for 42345 species, identification accuracies using the *matK, rbcl, ycf5* regions were 98%, 92%, 80% at the species level, respectively. The inclusion

of many closely related species supports the notion that the *rbcl*, *mat*K, ycf5 regions are not only capable of discriminating plant taxa from different plant families but also able to distinguish closely related taxa at the genus and species levels. This finding suggests that, similar to *CO1* in animals, the *rbcl, matK, ycf5* regions in plants are a suitable DNA barcode for *phoenix dactilifera* at different taxonomic levels. These regions have many advantages: a size of a few hundred nucleotides, comparison of relationships from the subspecies to the order levels, double-checking possible sequence errors in alignments directed by secondary structure, etc. Based on this evidence and our own findings, we propose that the *rbcl*, *mat*K, *ycf5* should be a gold standard barcodes for identifying date palm cultivars (chen 2010). In contrast to other studies our study presents a chloroplast non-coding region, *psbA-trnH*, does not amplify well, and amplifies as multiple bands (Whitlock *et al.,* 2010). It is occasionally longer than is currently feasible for a barcode may have mononucleotide repeats that are difficult to sequence accurately (Fazekas *et al.,* 2008; Starr *et al.,* 2009; Spooner *et al.,* 2009) and insertion events, including insertions of other genes (Wang *et al.,* 2008) into the region. Within some groups, *psbA-trnH* is not sufficiently variable to distinguish among closely related species and in others intraspecific variation is high (Sass *et al.,* 2007; Edwards *et al.,* 2008). The current study also evaluated a nuclear region Internal transcripted spacer does not amplify and it is not exist in date palm genome.

Table 3: Amplification success of suggested primer pairs.

Marker	Successful amplification (single bands)	Non-specific amplification (multiple bands)	Used for identification
rbcl	6/6 =100%	0%	Yes
matK	6/6 =100%	0%	Yes
psbA-trnH	5/6 =96%	1/6=4%	No
ycf5	6/6 =100%	0%	Yes
ITS	0%	0%	No

Only markers with near universal amplification success were sequenced and tested for identification.

Table 4: Pairwise distance among the date palm cultivars revealed by *rbcl* locus.

Hayani	Sakkoty	Gondila	Bartemoda	Malkaby	Amhaat
Hayani					
Sakkoty	3.0000				
Gondila	1.0000	2.0000			
Bartemoda	1.0000	2.0000	0.0000		
Malkaby	0.0000	3.0000	1.0000	1.0000	
Amhaat	1.0000	2.0000	0.0000	0.0000	1.0000

Overall average =1.2000

Table 5: Pairwise distance among the date palm cultivars revealed by *matK* locus.

Hayani	Sakkoty	Gondila	Bartamoda	Malkabi	Amhaat
Hayani					
Sakkoty	0.0120				
Gondila	0.0137	0.0069			
Bartamoda	0.0068	0.0120	0.0103		
Malkaby	0.0137	0.0051	0.0086	0.0137	
Amhaat	0.0154	0.0068	0.0069	0.0120	0.0085

Overall average =0.0099

Table 6: Pairwise distance among the date palm cultivars revealed by *ycf5* locus.

Hayani	Sakkoty	Gondila	Bartemoda	Malkaby	Amhaat
Hayani					
Sakkoty	0.0000				
Gondila	3.0000	3.0000			
Bartemoda	0.0000	0.0000	3.0000		
Malkaby	0.0000	0.0000	3.0000	0.0000	
Amhaat	0.0000	0.0000	3.0000	0.0000	0.0000

Overall average =1.0000

Phylogenetic analysis

In this study, the phylogenetic relationships among the date palm cultivars were evaluated using three phylogenetic methods Maximam likelihood ML,Neighbor Joining NJ and unweighted pair group method with arithmetic mean UPGMA. No differences were found between the results of NJ-, ML- and UPGMA-tree based analyses, so the resolution power was calculated based on UPGMA analyses alone. A phylogenetic tree built based on the *rbcl* sequences of the date palm varieties showed genetic distance Fig. 6. This dendrogram supported the cultivars organization into four clusters, a single cultivar Sakkoty was recovered as a single genotype in first cluster, second cluster consisted of Hayani and Malkaby, a single cultivar Gondila was recovered as a single genotype in third cluster and forth cluster included Bartamoda and Amhaat.

A phylogenetic tree built based on the *matK* sequences of the date palm cultivar showed genetic distance Fig. 7. This dendrogram supported the varieties' organization two main clusters, the first cluster include Hayani and Bartamoda, the second cluster had 2 sub-clusters: the first sub-cluster included Gondila and Amhaat, the second sub-cluster included Sakkoty and Malkaby. As for the phylogenetic tree built based on the *ycf5*

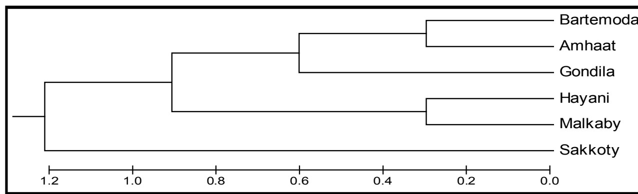


Fig. 6: Phylogenetic tree of date palm cultivars constructed on the basis of *rbcl* sequences using the UPGMA method. Branch length was calculated by Tamura 3-parameter method.

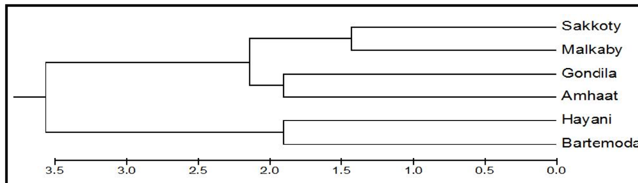


Fig. 7: Phylogenetic tree of date palm cultivars constructed on the basis of *matk* sequences using the UPGMA method. Branch length was calculated by Tamura 3-parameter method.

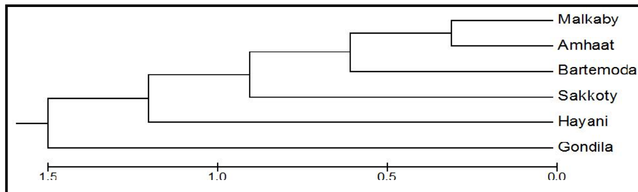


Fig. 8: Phylogenetic tree of date palm cultivars constructed on the basis of *Ycf5* sequences using the UPGMA method. Branch length was calculated by Tamura 3-parameter method.

sequences of the date palm varieties showed genetic distances Fig. 8. This dendrogram supported the cultivar organization into four clusters, the first cluster included a single cultivar Gondila, second cluster consisted of Hayani third cluster consisted of Sakkoty forth cluster had 2 sub-clusters: the first included Bartamoda, the second sub-cluster included Amhaat and Malkaby.

Conclusion

Identification and discrimination is very important to save the quality and performance of cultivars of dates in local markets. The data showed that Egyptian date palm cultivars are identified by three plastied; *matk* , *rbcl* and *ycf5* loci were not only a strong promise as a potential barcode for date palm cultivars but they were also useful for explaining the phylogenetic relationships at this taxonomic level., the *psbA-trnH* region cannot be used as a barcode gene for date palm cultivars.

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