Iraqi Journal of Agricultural Sciences –2026:57(2):440-453
P-ISSN: 0075- 0530/ E-ISSN: 2410-0862
DOI: https://doi.org/10.36103/z8wbmz88

GENETIC VARIABILITY AND MOLECULAR EVOLUTION OF THE IRAQI DATE PALM CULTIVARS USING INTERNAL TRANSCRIBED SPACER 1 (ITS1) REGION OF NUCLEAR RIBOSOMAL DNA

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ABSTRACT

This study was aimed to investigate genetic diversity of nine Iraqi date palm cultivars (*Phoenix dactylifera L.*), two of them were propagated by tissue culture tiqneque. The variation in GC content in the ITS1 region was recorded at (52–53%) with an average of (52.7). The data suggests that transitions have a lower frequency compared to transversions in this region, where the overall bias R for transition/transversion has been estimated at (0.661). The aligned sequences associated with internal transcribed spacer 1 (ITS1) showed intraspecific variation, and the matched sequences enabled the identification of all cultivars studied as haplotypes. Phylogenetic trees constructed using distance-based "neighbour-joining" (NJ) and "maximum parsimony" (MP) techniques provided strong evidence for the independent structure of the considered accessions. Tests of molecular evolution reveal that the internal transcribed spacer 1 (ITS1) evolved in accordance with a precise neutrality paradigm. In a population predicted to be in equilibrium after the 6th generation, suggesting a recent demographic expansion in the Iraqi date palm population.

Keywords: sequence analysis, nucleotide substituted, neighbour-joining, mismatch distribution

Received: 16/9/2023, Accepted: 6/12/2023, Published: 28/02/2026

INTRODUCTION

Classification of date palms (***Phoenix dactylifera L.***) is based on the morphology of the tree and the fruits, whereby the phenotypic traits may not be entirely reliable and could be not directly correlated with the genotypes of date palms. These traits are frequently influenced by environmental conditions or exhibit variation depending on the developmental stage of the date palm (International Plant Genetic Resources Institute (IPGRI)., 2005). Biochemical markers, due to their lack of variation, possess limited utility in distinguishing between different types of palm trees (Akkak *et al*., 2009). In contrast, DNA markers serve as more accurate indicators and could be effectively identify palm varieties based on their genetic makeup. These markers not only determine genetic diversity but also investigate the relationships among different palm species. Consequently, they have been extensively employed in the study of genetic variation in palm trees. Molecular indicators have emerged as a vital tool in the exploration of biodiversity (Jaradat, 2014). Over the past decade, numerous studies have attempted to identify Iraqi date palm cultivars using DNA-based markers, particularly Random Amplified Polymorphic DNA (RAPD) markers (Ali *et al*., 2007; Al-Khateeb & Jubrael, 2006; Bader *et al*., 2007; Jubrael, 2001; Khierallah & Husien, 2013; Khierallah *et al*., 2014), Amplified Fragment Length Polymorphism (AFLP) markers (Jubrael *et al*., 2005; Khierallah, 2007; Khierallah, *et al*., 2007; Khierallah, *et al*., 2008; Khierallah, *et al*., 2011a), microsatellite markers (Khierallah, *et al*., 2011b; Khierallah, *et al*., 2017), Inter-Simple Sequence Repeat (ISSR) markers (Khierallah, *et al*., 2014), and Cleaved Amplified Polymorphic Sequence (CAPS), and PCR Nested (Khierallah, *et al*., 2017). These markers exhibit some adequacy in the

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identification of date palm varieties. As well as more studies by other researchers on palm development. Therefore, we used ITS to determine genetic relationships and study genetic variation between varieties. The nuclear ribosomal DNA (rDNA) is a valuable tool for conducting phylogenetic studies at the level of individual species. It provides multiple advantages in comparison to other portions of the genome. The organisational structure of the nuclear ribosomal DNA (nrDNA), for instance, could be identified by the existence of extremely preserved sequences of tandem units in one or more specific loci. Furthermore, its swift evolution and ease of detection, amplification, and then sequencing Analysing the nrDNA would be a promising approach for generating effective molecular markers that could be used to assess genetic diversity and organisation in higher plants. An internal transcribed spacer (ITS) of DNA is situated among the genes for small-subunit ribosomal RNA (rRNA) and large-subunit (rRNA), either in the chromosome or in the corresponding transcribed region of the polycistronic precursor transcript of nrRNA. The nuclear ribosomal RNA (rRNA) genes are present in numerous copies that are arranged in tandem arrays within the genome of a plant, similar to those found in other eukaryotes (Mainaa *et al*., 2019). The ITS1 region is located among the 18S and 5.8S rRNA genes. In contrast, the ITS2 region is situated among the 5.8S and (26S procaryotic or 28S in eucaryotic) rRNA genes. Multiple copies extending over thousands of units make up the genes that code for ribosomal RNA and spacers. the Intergenic spacers (IGS) along with non-transcribed spacers (NTS), both kinds of non-transcribed DNA sections, are employed to distinguish between these duplicates. Each aggregate of ribosomal genes in eukaryotic organisms is comprised of five main components, with consequently the 5′ external transcribed sequence (5′ ETS), the 18S nrRNA gene, the ITS1 region, the 5.8S nrRNA gene, the ITS2 region, the 28S nrRNA gene, and then the (3′ ETS). The ETS and ITS pieces are excised during the nrRNA maturation as by-products that are non-functional during the maturation and are rapidly degraded. This research presents the genetic examination and molecular progression of nuclear ribosomal DNA (nrDNA) of Iraqi date palm in order to set up genetic relationships between various cultivars.

MATERIALS AND METHODS

Date palm cultivars: Ten date palm cultivars were selected for this study, all the genotypes were propagated through offshoots except of two that were propagated through tissue culture. The chosen fruiting cultivars, listed in alphabetical order, include "Barhi offs", "Khalas offs", "Majhool tc", "Majhool 1 offs", "Majhool 2 offs", "Maktomi offs", "Mir alhajj offs", "Showaithy tc", "Showaithy offs", and "Um alduhan offs". These samples were procured from five distinct locations in Baghdad, and Anbar. Randomly chosen five leaves, measuring range about 15 cm, for each of the ten cultivars. Only trees that were healthy, well-established, and well-characterized, with an age range of 10 to 15 years. Expert assistance was provided by specialists from the Ministry of Agriculture date palm stations, Baghdad University, and Janet Al-Nakheel company Laboratory in Iraq, in order to ascertain the cultivar to which these selected trees belonged.

DNA Extraction : Total genomic DNA was extracted from the entirety of young leaves that were frozen using the **FavorPrep™ Plant Genomic DNA Extraction Mini Kit** (sample size: 20 ~100 mg) from Korea.

Agar's Gel Electrophoresis: The principle of agarose gel electrophoresis revolves around the separation of nucleic acids based on their size and charge. In this method, the visualization of DNA fragments is accomplished by staining the gel with Red safe staining solution, which permeates the DNA bases and subsequently exposing the gel to UV light on a transluminator. The DNA molecular weight marker utilized in this experiment was a 100 base pair ladder. The gel was then subjected to an electrical field of 70 volts and 65 Amps for a duration of 1 hour. The DNA was observed by examining it under a UV transilluminator in 1X TBE buffer.

PCR Amplification of the (ITS1) region: A premix kit for polymerase chain reaction

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(PCR) amplification was employed, which consisted of I-Taq DNA Polymerase with an activity of 2.5U, deoxyribonucleotides (DNTPs) with a concentration of 2.5 mM, reaction buffer with a 10X concentration, and gel loading buffer with a 1X . The PCR amplification mixture for the specific diagnostic gene reaction was conducted in a total volume of 25 µl comprising 1.5 µl of DNA, 5 µl of Taq PCR PreMix, and 1 µl of each primer with a concentration of 10 pmol. Subsequently, 16.5 µl of distilled water was added to achieve a final volume of 25 µl. The primers used for PCR amplification were the universal ITS5 (5′- ATGATAACTCGACGG ACCGC -3′) and ITS2 (5′ TCTTCGAGCCCCCAACTTTC -3′′) primers. The thermal cycling conditions consisted of an initial denaturation phase at 95 °C for a duration of 5 minutes, followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 57 °C for a period of 1 minute, and extension at 72 °C for 1 minute, concluding with a final extension phase at 72 °C for 5 minutes. The amplification process was conducted using a thermal cycler, the (Gene Amp PCR system 9700), which was manufactured by Applied Biosystems. Subsequently, all the PCR outcomes have been separated employing 1.5% agarose gel electrophoresis, and afterwards they have all been stained with red safe dye (Intron Korea). The obtained sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI).

Sequence alignment and analysis: The sequences of the ten annotated accessions were submitted to the National Centre for Biotechnology Information (NCBI), Gen Bank (Accessions numbers, OQ911641.1–OQ911650.1 for the small subunits of 18S nrDNA of the ITS1 region) (Table 1). The DAMBE programmes (Xia, 2017) as well as MEGA version 11.0.13 (Tamura *et al*., 2021) were employed to align and evaluate the nucleotide sequences. The alignment had been manually verified, and a Maximum Composite Likelihood (MCL) methodology was applied to calculate the pairwise sequence difference among cultivars within the ITS1 region (Tamura *et al*., 2004). The GC content for each sequence was calculated online by Biologics Corp. at (https://wwwBiologics Corp.com/tools/GCcontent/)

Phylogenetic trees were created utilising "neighbour joining" (NJ). With 1000 bootstrapping replicates, the "neighbour-joining and maximum parsimony" trees are being constructed. The scores for consistency indexes (CI) as well as retention indexes (RI) have been determined in addition to the homoplasy index. The equation R = [A*G*k1 T*C*k2]/[(A+ G)*(T + C)] was employed to determine the ratio of transitions/transversions (ti/tv), wherein A, G, C, and T stand for each frequency of the 4 nucleotides (Tamura *et al*., 2021). The analysis shows the number of nucleotide substitutions each site found among the sequences. By analysing the aligned sequences using DnaSP program version 5.10.01 (Librado and Rozas, 2009), which proved to identify polymorphism indexes, demographic historical, and haplotype indices (Hd), along with nucleotide substitution ratios, for assessing genetic diversity among cultivars. In the (ITS1) sequences and the diversity (Pi), along with their respective standard deviation. Moreover, the estimation of a mean pairwise nucleotide differences (K) was performed. The examination of selective neutrality was carried out using Tajima's D , and Fu and Li's D* and F* methods. Furthermore, the evaluation of demographic parameters encompassed the analysis, of the mismatch distribution of pairwise sequence differences, the distribution of the allelic frequency at a site (Tajima, 2009), and population size change. The visualization of the genetic relationship among the date palm cultivars, as identified by the detection of haplotypes, was accomplished through network analysis, utilising a (NETWORK) programme version 4.6.1.0 (Bandelt et al., 1999). Additionally, DAMBE version 6 was used to include the scatter chart plot produced by the multivariate PCA (Xia, 2017).

RESULTS AND DISCUSSION

The length of the amplified segment is roughly 600 base pairs, which matches the intergenic area that encompasses the transcribed intergenic spacer 1 (ITS1) (Fig.1).

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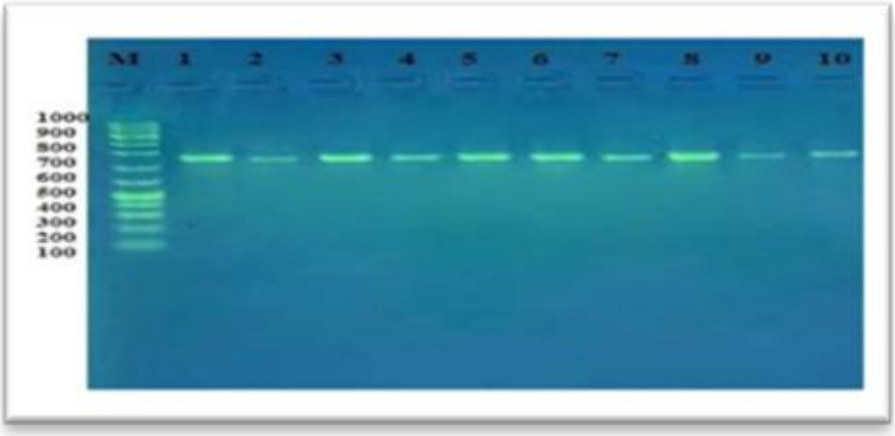

Figure 1. Gel electrophoresis of genomic DNA extraction

Genetic diversity analysis:

Nucleotide composition variance

The obtained sequences revealed variations, specifically in the GC content percentage recorded in the ITS1 region. The range varied from (52%) in "Majhool 1 offs", "Majhool 2 offs", and "Showaithy tc" cultivars to (53%) in all the other cultivars, with a mean of (52.7%) (Figs. 2, 3). Purine bases exhibited a transition/transversion ratio of (K_1 = 1.466), whereas pyrimidine bases had (K_2 = 1.141). All the bases were calculated to have a transition/transversion ratio (R) of (0.661). Table 2 lists the substitutions that were noticed.

Table 1. Iraqi date palm samples accessions, query number, and type of substitution sequence, and percentage of identical

Sample number	Type of substitution	Nucleo tide	Sequence ID with Submission	Sequence ID with compare	identity
Showaithy offs	Transvertion	C\A	ID:OQ911641.1	ID: XR_005510437.1	99%
	Transition	A\G			
Maktomi offs	Transvertion	C\G	ID:OQ911642.1	ID: XR_005510437.1	99%
Barhi offs	Transition	A\G	ID:OQ911643.1	ID: XR_005510437.1	99%
Majhool 1 offs	Transvertion	C\A	ID:OQ911644.1	ID: XR_005510437.1	99%
	Transvertion	G\T			
Majhool 2 offs	Transition	G\A	ID:OQ911645.1	ID: XR_005510437.1	99%
Mir alhajj offs	Transition	C\T	ID:OQ911646.1	ID: XR_005510437.1	99%
	Transition	A\G			
Khalas offs	Transvertion	A\C	ID:OQ911647.1	ID: XR_005510437.1	99%
	Transvertion	T\A			
	Transvertion	T\A			
Um alduhan offs	Transition	T\C	ID:OQ911648.1	ID: XR_005510437.1	99%
	Transvertion	G\T			
	Transition	T\C			
Showaithy tc	Transvertion	C\A	ID:OQ911649.1	ID: XR_005510437.1	99%
	Transition	A\G			
	Transvertion	A\T			
Majhool tc	Transvertion	T\G	ID:OQ911650.1	ID: XR_005510437.1	99%
	Transvertion	C\A			

Note2*: (Majhool 2 offs) cultivar. Means that the cultivar was propagated by offshoot (offs), but the mother was propagated from tissue culture (TC).

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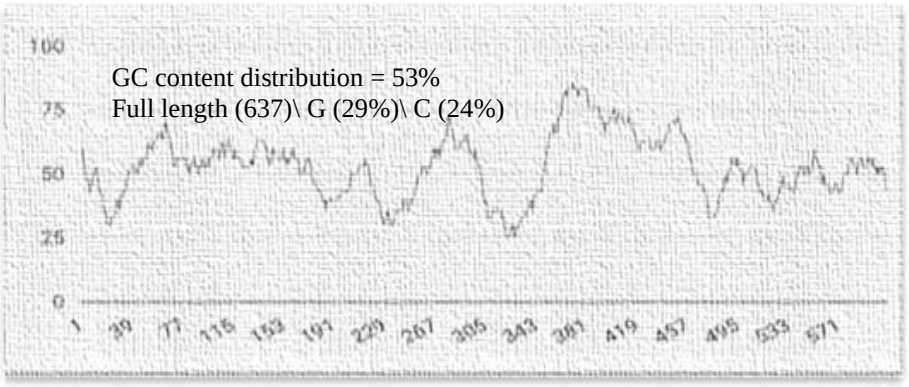

Figure 2. Show the percentage and variation of GC distribution at 53% among rDNA sequences of Iraqi date palm cultivars.

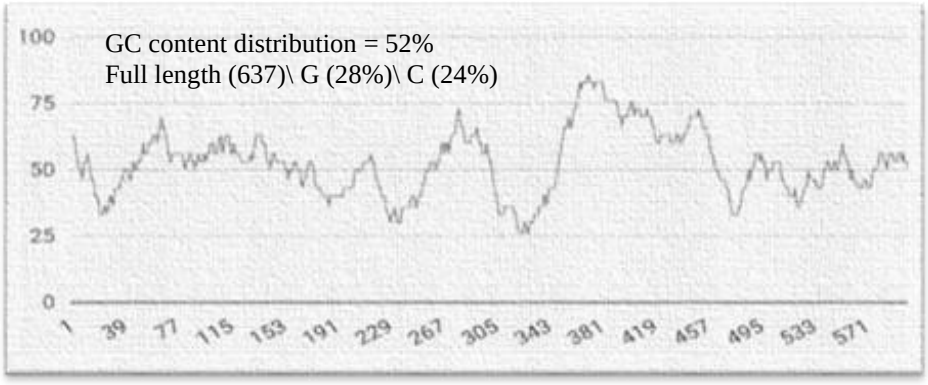

Figure 3. Show the percentage and variation of GC distribution at 52% among rDNA sequences of Iraqi date palm cultivars.

The study demonstrates that within intergenic spacer 1 (ITS1) of the Iraqi date palm, transitions occurred less frequently than transversions. According to (Table 2), transitions involving G/A and C/T transitions are more common than A/G and T/C transitions.

Table 2. Estimated relative frequencies of nucleotide changes in the nrDNA ITS1 region

	A	T	C	G
A	-	***6.91***	***7.33***	**12.81**
T	***7.21***	-	**8.37**	***8.73***
C	***7.21***	**7.89**	-	***8.73***
G	**10.57**	***6.91***	***7.33***	-

NOTE1*:- Each entry in the provided data represents the probability of substitution (r) from one base (row) to another base (column). Transitional substitutions are denoted in bold, while transversional substitutions are shown in italics.

Sequence alignment led to the generation of a matrix consisting of 637 base-pair characters. Within this matrix, 619 sites were found to be conserved, while 18 sites displayed variability. Among the variable sites, two sites were found to be parsimony informative at positions (19, and 64). Additionally, and 16 singleton sites Table (3), show at positions (109, 133, 166, 182, 287, 289, 321, 335, 356, 372, 404, 421, 462, 511, 619, and 620). The frequencies of nucleotides that were detected in the ITS sequences were as follows: 23.88%, 22.90%,24.29%, and 28.93% for A, T, C, and G, respectively.

Table 3. Tests for neutrality and sequence polymorphism performed on (nrDNA).

Iraqi date palm sequences	The value
No. of sequences	10
Length of sequence	637 bp
Monomorphic characters	619
Variability	18
Singleton variable sites	16
parsimony	2
H	10
Variance of (Hd)	0.00200
Pi ± SD	0.00614 ± 0.00066
Hd ± SD	1.000 ± 0.045
Pi(JC)	0.00617
K	3.911
R2	0.0546
Tajima's D	-1.79885 (S)* (P < 0.05)
Fu and Li's D	-1.95079 (NS) (0.10 > P > 0.05)
Fu and Li's F	-2.15603 (NS) (0.10 > P > 0.05)
Fu's Fs statistic	-7.001

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Genetic relations among the ITS1 sequences

The examination of the distance matrices reveals that the average genetic distance was 0.0029, with genetic distances ranging from 0.0016 to 0.0048. The genetic distances of 0.0016 were observed between Barhi offs, Majhool 2 offs, and Maktomi offs cultivars, suggesting a significant resemblance in their ITS1 sequences. Additionally, there exists a level of resemblance among Majhool tc, Majhool 1 offs, Mir alhajj offs, and Showaithy offs cultivars, with a distance of (0.0032), while Um alduhan offs have a distance of (0.0047). However, it is important to note that a higher level of genetic distance was observed in the Showaithy tc cultivar at 0.0048. The Maximum Parsimony (MP) and Neighbour-Joining (NJ) methodologies were applied for creating phylogenetic trees. The parsimony of the evaluation yielded 18 trees, thus identifying the most parsimonious tree at 4 steps. It's noteworthy to take into account that the obtained consistency index (1.000), retention index (1.000), and homoplasy index (0.000) revealed the existence of no homoplasic characters in the set of sequences under evaluation. The neighbour-joining dendrogram grouped Iraqi understudy date palm cultivars into two major groups, as depicted in Fig. 4. Showaithy tc and Showaithy offs cultivars are the only ones included in the first category. The remaining cultivars form the second group, which encompasses Barhie offs, Khalas offs, "Majhool tc, Majhool 1 offs, Majhool 2 offs, Maktomi offs, Mir alhajj offs, and Um alduhan offs cultivars.

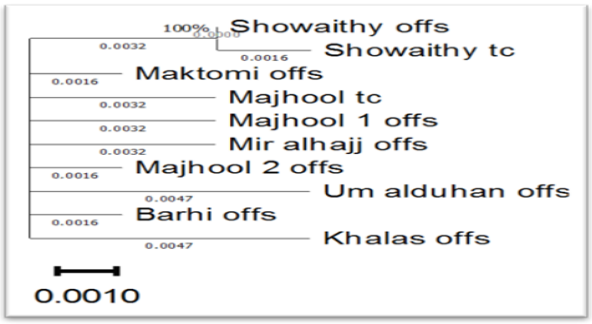

Figure 4. Neighbour joining tree According to the ITS1 sequences of the nrDNA, describe the tree and distance among Iraqi date palm cultivars

Genetic evolve: Tests for the selective neutrality: In order to establish whether the observed diversity patterns in the Iraqi date palm cultivars' nuclear ribosomal DNA sequences significantly departed from a neutral equilibrium model, we used a variety of statistical approaches. to test the null hypothesis by applying both Tajima's and Fu and Li's approaches to test for the selective neutrality of the spotted mutations. Our results showed that Tajima D in the ITS1 region had negative and statistically significant values ($D = -1.79885$ $P < 0.05$). On the other hand, the statistical tests conducted using Fu and Li on the entire sample yielded negative and non-significant values of $D^* = -1.95079$ ($P > 0.10$) and $F^* = -2.15603$ ($P > 0.10$) at the ITS1 region. Both of the two Tajima along with Fu and Li's commonly used tests, which are used for assessing the selective neutrality hypothesis, can be interpreted as indicators of equilibrium among mutations and gene drift in the presence of a limited number of rare mutations identified within the sequences under investigation. In order to provide further clarification regarding the cause of a deviation from neutrality, the Fu statistic is assessed. This particular statistic is more effective in detecting deviations from neutrality as well as in testing for population evolution and recent population expansion. A review of Table (3) and Fig. (5) indicates that the Fu's Fs parameter has revealed significant negative values, which are listed at -7.001. These findings are further supported by Harpending's raggedness and the Ramos-Onsins (Ramos-Onsins and Rosas, 2002) statistic values of ($r = 0.0909$; $R2 = 0.0546$) in the date palm (nrDNA) sequences. The mismatch distributions in the rDNA sequences deviate from selective neutrality. The ITS1 sequences appear to have undergone early evolution. This is also reinforced by the high negative worth of Fu's Fs. Most of the evidence refers to recent population expansion, but at a slow rate. While the mismatch distribution acts as an indication for expansion time.

Mismatch distribution: Analysing the mismatch distribution revealed that the variance in the rDNA's ITS1 sequences indicates a deviation from neutrality (Figs. 6,

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and 7). The allele frequency graph revealed that among all 10 Iraqi date palm cultivars that were evaluated overall, there appeared to be a demographic equilibrium rather than recent population expansion (Fig. 5).

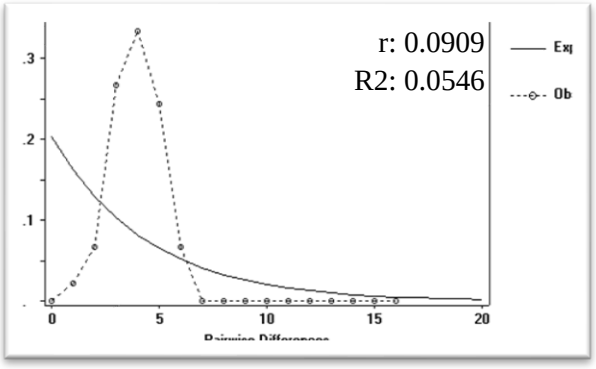

Figure. 5 displays a frequency spectrum of Iraqi date palm rDNA sequences detected in the ITS1 region. The solid lines in the spectrum reflect the distributions noticed under neutrality and equilibrium (mutation drift).

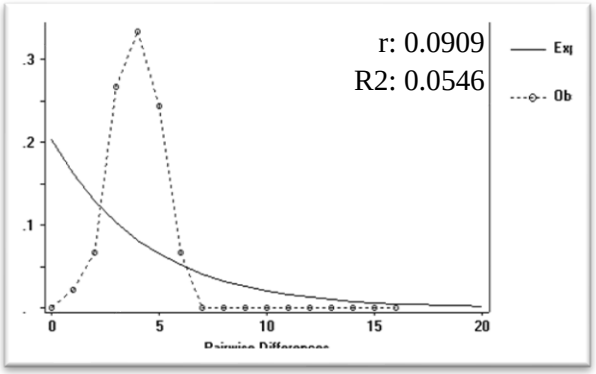

Figure 6. Mismatch distribution of palm population Displaying the observed distribution Expected Values for Population Size Changes with Initial Theta (0.000) and Final Tau (3.733).

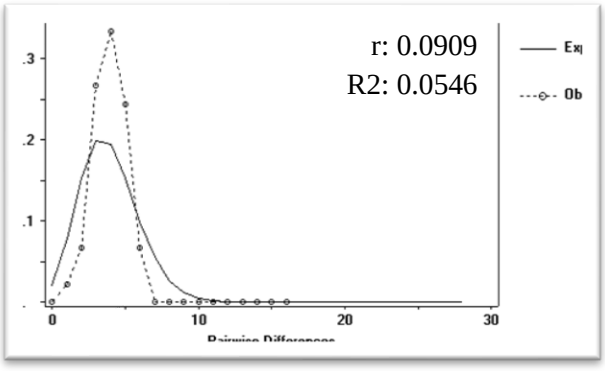

Figure 7. Mismatch distribution of date palm population. Displaying the observed distribution Expected Values for Population Size Changes at the Initial Theta (0.000), Final Theta (1000), and Final Tau (3.733).

Population Size Change of palm

The anticipated number of segregating sites within the 10 sequences has been determined to be Sn(t) at (0.954), with a Sn(t)/al value of (0.337). The average number of pairwise differences was found to be (0.259), or the estimated count of separating sites in two sequences, S2(t). S2(t)/a1 observed to have a ratio of (0.259). Therefore, The total number of segregating sites in the ten cultivars is expected to be in equilibrium with the anticipated number after (0.6N) generations and return the population to equilibrium (Fig. 7).

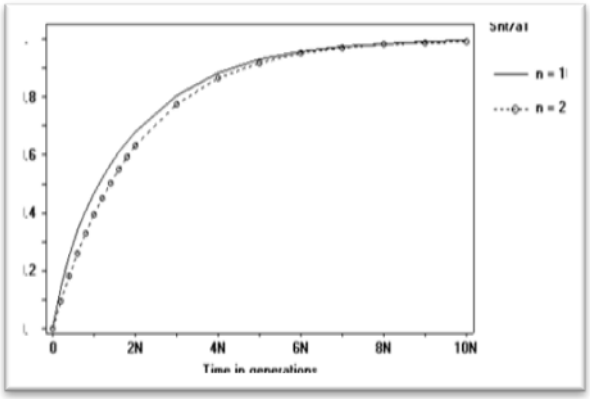

Figure 8. Mismatch in the population's distribution of date palms displaying the distribution of pairwise variations as seen and as predicted employing a population expansion equation at initiate (0.000), final (1000), final tau (3.911), and time (0.6N) generations.

Haplotypes distribution based on ITS1 sequences: The haplotype network displayed in (Fig. 8), which is based on (ITS1) spacer sequence regions, reveals an obvious evolution and demographic expansion of palms associated with an ancestor haplotype. A network demonstrates interconnectivity within its central structure, specifically in the founder haplotype, which is represented by the three cultivars: the Barhi offs, Majhool 2 offs, and Maktomi offs cultivars. which are considered ancestors of other cultivars. Consequently, it can be deduced that the three cultivar haplotypes serve as the sequential progenitors of the remaining sequences that have experienced mutations over the course of evolution. The substitutions within the three cultivars were comprised of two mutants between them, while the remaining cultivars

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were listed in the subsequent manner: four mutants with both Khalas offs and Um alduhan offs, and three mutants with both Majhool tc, Majhool 1 offs, Mir alhajj offs, and Showaithy tc cultivars. Furthermore, the analysis also presents a network that establishes a connection between the haplotype of the Showaithy tc cultivar and the Showaithy offs through a single mutant. Additionally, the hatch marks in (Fig. 9) indicate the number of discovered mutational occurrences, reflecting the chronological age of the Iraqi palm trees.

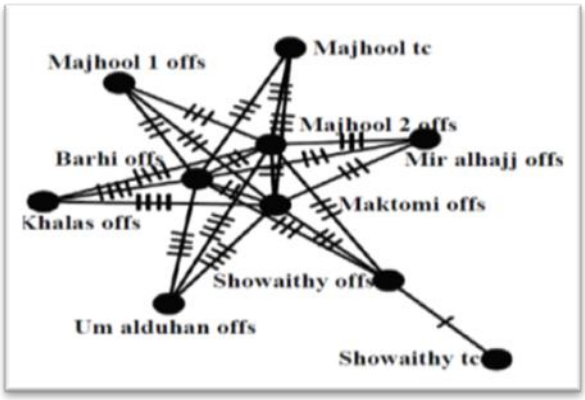

Figure 9. The network of haplotypes estimated from (ITS1) sequences reveals relationships among the 10 cultivars. The hatch marks are proportional to the number of mutations

Principal component analysis

The scatter plot acquired by performing a multiple-variate principal component analysis of the first two elements (PC1 and PC2) from the (ITS1) region indicated that PC1 displayed a variance of 0.1493 with respect to the cultivars as well as an Eigen value of 6.6667, whereas PC2 displayed a variance of 1.0453 and an Eigen value of 6.5173 (Fig.10).

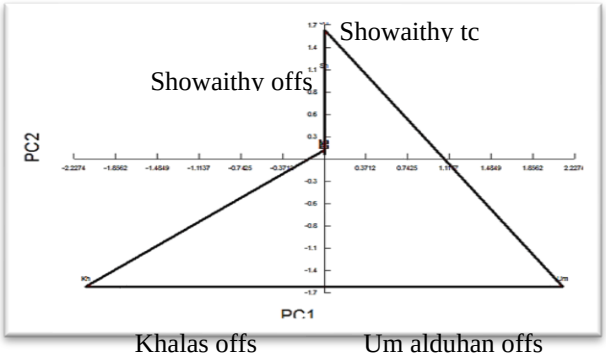

Figure 10. A scatter plot diagram for (principle component analysis), demonstrates the relative distribution of 10 date palm cultivars based on the (ITS1) region

A set of the best 10 Iraqi date palm cultivars has been investigated, and a sequence based on the (ITS1) of the (nrDNA) region was established. The length of the sequence is 637 bp, as well as the nucleotide composition frequencies have been (23.88%), (22.90%), (24.29%), and (28.93%) for A, T, C, and G, respectively. Fewer findings have been documented by Mainaa *et al.* (2019), indicating that the average size of the Tunisian date palm is at (442.7) bp, while the nucleotide proportions are almost equivalent with A accounting for (24.76%), T for (25.67%), C for (27.62%), and G for (21.95%). Similar results, however, have been reported for other plant species, including members of the Asteraceae family, where the ITS region's total length variation varied from (650) to (750) bp. Adenine, thymine, cytosine, and guanine had average frequencies of (25%), (24%), (26%), and (25%), respectively, with a total GC content of (51%) and an AT content of (49%) (Amar *et al*., 2012). In contrast, it was detected by Baraket *et al.* (2013) that the average length of the ITS region created from the species Ficus carica was 697.5 bp, in addition to the nucleotide composition consisting of (19.7%) adenine, (18.6%) thymine, (31.4%) cytosine, and (30.2%) guanine. In accordance with the assessment of the complete ITS sequence in the (Naga King Chilli) by Kehie *et al.* (2016), which found that it had an average length of (620) bp, the nucleotide frequencies for A, T, C, and G were (18.85%), (17.56%), (33.95%), and (29.64%), respectively. As an addition, in angiosperms, the ITS region's sequence length varied between 565 and 700 bp. Most of the observed transition/transversion ratio (R) of (0.661) based on the (ITS1) region demonstrates a similarity to the ratio found in the analysis of the entire (ITS) region of Tunisian Fig was (R) at (0.7) recorded by Baraket *et al*. (2013). It is interesting to note that this ratio is lower than the ratio of 4.375 established over the whole (ITS) region of Tunisian date palms, as reported by Mainaa *et al.* (2019). While the (ti/tv) ratio was reported by Amar *et al.* (2012) to have been (1.43) for the Asteraceae family, in another instance by Kehie *et al.* (2016), the (ti/tv) ratio for *Capsicum* sp. was reported at (3.746).

Iraqi Journal of Agricultural Sciences –2026:57(2):440-453
P-ISSN: 0075- 0530/ E-ISSN: 2410-0862
DOI: https://doi.org/10.36103/z8wbmz88

Furthermore, according to Sharma *et al*. (2002), the ratios for wheat and wild barley were (7.4), and (7.4), respectively. The ratio fell as a result of a fall in the frequency of transition events relative to transversion events. The levels of nucleotide and haplotype diversities observed in the Iraqi date palm with a nucleotide diversity (Pi) of 0.00614 and a haplotype diversity (Hd) of 1.000) appear to be relatively high when compared to the values reported by Mainaa *et al*. (2019) for Tunisian date palm cultivars (Pi = 0.00155, Hd = 0.552). However, the levels of haplotype diversity are similar to those reported, but it appears these observed levels of nucleotide diversity are comparatively low in comparison to other species, such as (*Ficus carica*), as recorded by Baraket *et al*. (2013) with a haplotype diversity (Hd) of (0.996) and a nucleotide diversity (Pi) of (0.072). Additionally, Kehie *et al.* (2016) recorded a haplotype diversity (Hd) of (1) and a nucleotide diversity (Pi) of (0.01499) in *Capsicum* sp.Sequence analysis of the internal transcribed spacer 1 (ITS1) of nuclear ribosomal DNA (nr DNA) reveals a relatively limited extent of genetic variability. In fact, all ten cultivars exhibit observed haplotypes at the examined sequences. An "Average Pairwise Nucleotide Difference" (K) of (3.911) identified within this region demonstrates a depressed level of genetic variation . Although the value is higher compared to the value obtained by Mainaa *et al*. (2019) in the Tunisian date palm (K = 0.686). On the other hand, the values of K reported by Kehie *et al*. (2016) in the *Capiscum* sp. at 9.267 and Baraket *et al.* (2013) in the *Ficus carica* species at 35.34%, indicate a significant degree of polymorphism within the entire ITS sequence for those particular species. The average ITS1 region percentage of genetic variation of the understudy date palm cultivars is estimated to be 5.9% across the full data set. The outcomes acquired from the of Iraq date palm were in complete accordance with the findings reported by Mainaa *et al*. (2019). It was revealed that the level of genetic variance detected in the Tunisian date palm was comparatively less significant when compared to other plant species, specifically, *Juniperus osteosperma*, *Ficus carica*, and *Capsicum* sp. (Terry *et al*., 2000; Baraket *et al*., 2013; Kehie *et al*., 2016), respectively. The dendrogram for Neighbor- Joining, which is based on the internal transcribed spacer 1 (ITS1) region, effectively clustered the date palm cultivars. It appears that the Iraqi date palm cultivars share a common genetic foundation. As a result, this supports the hypothesis of a unique population in Iraq, similar to the situation in Tunisian date palm (2019). These findings align with previous investigations that employed the other molecular markers to examine the Iraqi date palm (Jubrael, 2001; Jubrael *et al*., 2005; Al-Khateeb & Jubrael, 2006; Bader *et al*., 2007; Khierallah, 2007; Khierallah *et al*., 2007; Khierallah *et al*., 2008; Khierallah *et al*., 2011a; Khierallah *et al*., 2013; Khierallah *et al*., 2017). Though, the exploration of a genetic arrangement of the date palm (*Phoenix dactylifera*). The Microsatellite markers were too utilised to evaluate the genetic variety in 30 cultivars of date palm in Iraq (Khierallah *et al*., 2011b; and Khierallah *et al*., 2017). Additionally, (Hamwieh *et al*., 2010) employed eight cultivars of date palm from Iraq to assess the effectiveness of more than 1,000 primer pairs for the "Simple Sequence Repeats" (SSR) marker by analysing the genomic sequencing data. Recently, the "Inter Simple Sequence Repeat" (ISSR) marker was employed to assessment the genetic relations between 17 cultivars of date palm in Iraq (Khierallah *et al*., 2014). This agrees only with cultivars that are propagated from offshoots. Quite the opposite, it is inconsistent with individuals that are propagated by tissue culture, as there were individuals with more altered genetic patterns than others. This seems to be entirely distinct from the prevailing notion that tissue culture results in genetically identical individuals.

Mismatch distribution

Mismatch distributions in rDNA sequences demonstrate a deviation from selective neutrality (Figs. 6, 7). The multimodal character of the mismatch curves does not appear to support the recent population expansion of these sequences (Fig. 4). The ITS sequences appear to reflect an early stage of evolution (Baraket *et al*., 2013). the mismatch

Iraqi Journal of Agricultural Sciences –2026:57(2):440-453
P-ISSN: 0075- 0530/ E-ISSN: 2410-0862
DOI: https://doi.org/10.36103/z8wbmz88

distribution typically manifests as multimodal, whereas in a population that has recently undergone a demographic expansion, it presents as unimodal. The haplotype network established with the nr DNA internal transcribed spacer 1 ITS1 sequences (Fig. 9) demonstrates date palms' population expansion and haplotype over the evolution from an original haplotype. Thus, the conclusion is that a haplotype represents the sequence ancestor of the remaining sequences that have experienced evolutionary mutations. The study also displays a network of relationships between the haplotypes, with a set of hatch marks reflecting the number of mutations. This outcome is in line with the NJ classification tree in (Figure 4), and a scatter plot diagram in (Figure **10).**

Population size change

The anticipated number of segregating sites within the 10 sequences has been determined to be Sn(t) at (0.954), with a Sn(t)/al value of (0.337). The average number of pairwise differences was found to be (0.259), or the estimated count of separating sites in two sequences, designated as S2(t). S2(t)/a1 proved equally reported to have a ratio of (0.259). Therefore, The total number of segregating sites in the ten cultivars is expected to be in equilibrium with the anticipated number after (6^{th}) generations (Fig. 7). Moreover, Tajima (2009), showed that the rate of variation growth is often sluggish, especially when n = 2. To be more precise, it takes 1.4N generations for this number to decrease by half from its greatest value. On the other hand, this method requires just 0.5N generations when n = 100. Additionally, the researcher demonstrated that more segregating sites are created more quickly with larger sample sizes.

Principle component analysis

Principal component analysis revealed two main clusters; the first and second eigen vectors account for 6.66% and 6.51% of the variation, respectively. This analysis agrees with the population structure and dendrogram result in that. As with the other analyses, no clustering based on collection location or propagation methods was observed. Similar to the investigation of Moroccan date palms conducted by Ibrahimi *et al*. (2023). The analysis of the combined data from SSR and DAMD markers revealed that the first three axes were (7.3%), (5.75%), and (4.38%), respectively. The PCA tests plot highlighted the grouping of the Iraqi cultivars with the Tata population. In contrast, Khierallah and Azhar, (2016) demonstrated The study employed by morphological markers to identify a variation of 31.86% for PC1, with an Eigen value of 4.67. Moreover, PC2 displayed a variation of 17.0%, accompanied by an Eigen value of 4.63.

CONCLUSION

During the course of our comprehensive investigations that we conducted, we successfully refuted the alternative claims that were examined, which suggested that tissue culture does not result in genetic variation. On the contrary, we observed a significant increase in genetic diversity, as evidenced by the genetic characteristics. Consequently, it is imperative to undertake further investigations in this domain to ascertain the future implications of these variations on the diversity of the date palm community. However, we are for the diversity that will ultimately facilitate the production of resilient individuals. Subsequently, we worked hard to preserve the sequences of these species in the gene bank so that they would be available for other studies.

ACKNOWLEDGEMENTS

This work was supported by the Department of Biology Graduate Studies, College of Science, University of Baghdad. We would like to thank Dr. Ayyad W. Alshahwany, for offering aid in acquiring specimens and for contributing labour and direction.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

DECLARATION OF FUND

The authors declare that they have not received a fund.

ETHICAL APPROVAL AND ANIMAL WELFARE

This study did not involve human participants or experimental animals. Therefore, ethical

approval and animal welfare approval were not required.

FUNDING

This research received no external funding.

AUTHORS' DECLARATION

The authors confirm that this manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. All figures and tables included in this manuscript are original and prepared by the authors. Any third-party material has been included with the necessary permissions. All authors have read and approved the final version of the manuscript.

AUTHORS' CONTRIBUTION STATEMENT

All authors contributed equally to the conceptualization, methodology, investigation, data analysis, and writing of the manuscript. All authors reviewed and approved the final version of the manuscript.

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Iraqi Journal of Agricultural Sciences –2026:57(2):440-453
P-ISSN: 0075- 0530/ E-ISSN: 2410-0862
DOI: https://doi.org/10.36103/z8wbmz88

التنوع الوراثي والتطور الجزيئي لأصناف نخيل التمر العراقي باستعمال منطقة المباعد الداخلي 1 (ITS1) من الريبوسوم النووي DNA

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المستخلص

تم فحص الحمض النووي الريبوسومي النووي للتحقق من العلاقات الوراثية بين ثمانية أصناف من نخيل التمر العراقي تم تكاثرها بالفروع واثنتين بالزراعة النسيجية لتوضيح تطورها الجزيئي. تم تسجيل التباين في محتوى GC في منطقة ITS بنسبة 52–53٪. تم العثور على التحيز الإجمالي R للانتقال/التبادل ليكون 0.661، مما يشير إلى أن الانتقال أقل تردد من التبادل في هذه المنطقة. سمحت التسلسلات المحاذاة بتحديد جميع الأصناف على أنها أنماط فردية. وقد لوحظ تباين بين الأنواع في تسلسل (ITS1) لنخيل التمر كذلك وجد أن التنوع في الأنماط الفردية والنيوكليوتيدات. قدمت الأشجار التطورية التي تم إنشاؤها باستخدام طرق المسافة (NJ) و (MP) دعمًا كبيرًا للتنظيم المستقل للمدخلات التي تم اختبارها. يوضح تحليل التطور الجزيئي أن المباعد الجيني الريباسي ITS1 قد تطور في ظل نموذج محايد تمامًا.

الكلمات المفتاحية: تحليل التتابع، استبدال النيوكليوتيدات، الانضمام بالجوار، التوزيع غير المتطابق.