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ROSA L. SPECIES IDENTIFICATION USING THE *RBCL* GENE IN KURDISTAN REGION, IRAQ

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SUMMARY

The innovative research employed *rbcL* (ribulose bisphosphate carboxylase) primers in detecting the internal transcribed spacer (ITS) region on the chloroplast cpDNA of five species of the genus *Rosa* L., using the polymerase chain reaction (PCR) and phylogenetic relationship. The species are *R*. *gallica*, *R*. *hemisphaerica*, *R*. *foetida*, *Rosa* x *damascene*, and *R. centifolia*. The results revealed the presence of a major band (1700 bp) in all studied species. Based on a phylogenetic analysis of the *rbcL* gene sequence data, two major clusters were evident in the dendrogram. The species *R. gallica* and *R. hemisphaerica* have a good bootstrap value of 99%. The rest of the three species (*R. centifolia*, *R. foetida*, and *Rosa* x *damascene*) has a bootstrap value ranging between 55%–57%. The study also authenticated the species *R. hemisphaerica* and *R. foetida* were newly recorded species in the international GenBank NCBI (National Center for Biotechnology Information).

Keywords: *Rosa* L. species, chloroplast DNA, identification, ITS, phylogenetic analysis, *rbcL* gene

Key findings: Using the *rbcL* gene of chloroplast (DNA), the study identified five different species belonging to the genus *Rosa* L. (Rosaceae) grown in the Kurdistan Region, Iraq.

INTRODUCTION

The Rosaceae is a vastly prominent family, mostly found in the Northern Hemisphere, warm, and temperate climates (Mabberley, 1997). Heywood (1973) specified the family Rosaceae with 122 genera and 3370 species. Christenhusz *et al*.'s (2017) results authenticated the said family contained 90 genera and 2950 species. Recently, an update

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on this number stated 109 genera and 5819 species in the genus Rosa (Bisby and Ruggiero, 2018). Four subgenera formed the genus, according to traditional classification, i.e., R. subgen.: Hulthemia, Platyrhodon, Hesperhodos, and Rosa (Wissemann, 2003).

The R. subgen Rosa contained most of the species, which further divided into 10 sections. The first three subgenera are monotypic, containing two species, whereas the R. subgen. Rosa includes all remaining species, subdivided into 10 sections: (R. sect. Pimpinelliaeae [DC.] Ser; R. sect. Cinnamomeae [DC.] Ser.; R. sect. Synstylae DC.; R. sect. Indicae Thory; R. sect. Banksianae Lindl.; R. sect. Laevigatae Thory; R. sect. Bracteatae Thory; R. sect. Gallicanae [DC.] Ser.; R. sect. Carolinae Crep.; and R. sect. Caninae [DC.] Ser.). According to Ku and Robertson (2003), this genus only has two subgenera, i.e., R. subgenus Rosa and R. subgenus Hulthemia.

Roses are commonly in gardens, serving as cut flowers. They also serve as materials in medicines. Likewise, they are one of the most considered ornamental woody plants. Roses contribute for preserving livelihoods and improving the environment; therefore, the attention for their greater care and development from plant breeders rises. This produces more cultivars, causing the decrease in their phenotypic, anatomical, and biochemical variations, making it difficult to distinguish them. Consequently, the molecular markers could offer a perspective on comprehending and resolving these variations (Gudin, 2001; Scalliet et al., 2008; Rasheed et al., 2024a).

Previous research indicated DNA indices may provide insight into the evolutionary process of species of the genus Rosa (Rusanov et al., 2005; Zhu et al., 2015; Rasheed et al., 2024b). Therefore, the DNA sequence analysis is a highly considered new approach in studying evolutionary relationship and genetic diversity in crop plants. The eukaryotic cells contain three types of genetic systems, i.e., nuclear, mitochondrial, and chloroplast DNA. The multiple genes in these cell organelles perform important functions in supplying the cell with energy. In plant taxonomic studies, mitochondrial and chloroplast genes are applicable because of the low levels of sequence variations, their ease of application, and high accuracy of results, using a primer to detect the internal transcribed spacer (ITS) region.

The repeated use of the rbcL gene has been successful in numerous studies to understand the plant systems. Recently, the matK gene, which is an abbreviation for maturase K, formerly known as orfK, has also succeeded. The matK gene has a prominent role in chloroplast protein synthesis, gene transcription, energy conversion, and plant development. The said gene has a notable role in determining plant molecular systems due to its rapid development in nucleotides and corresponding amino acid levels. However, studies also confirmed these genes are not dynamic in all plant cells (Kores et al., 2000). Chloroplast genome sequences have proven to be extremely useful in establishing evolutionary relationship among the plant species with closer links to evolution (Daniell et al., 2016).

The systematic use of sequences and molecular data has answered many evolutionary questions and has contributed considerably to the successful classification of plants. In the Rosaceae family, the chloroplast gene investigation has been central in various studies (Li et al., 2020; Hasan, 2023; Alwan and Saeed, 2024), using three sequences of chloroplast DNA (cpDNA), i.e., rp 116 interon, trnL-F, and atpB-rbcL to study the evolutionary relationship in the genus Rosa. They also confirmed the chief role of these genes in evolutionary studies and the identification of molecular markers and codes for the DNA strands for subsequent studies (Liu et al., 2015). Using universally recognized protocols and DNA regions, DNA barcoding compiles an international database of organisms for the purpose of species identification. The DNA barcoding is a reliable method for identifying plant species, and the databases, viz., GenBank, provide the necessary sequences (Hebert et al., 2003; Shehab, 2023).

The current research aimed to use chloroplast DNA in genetic characterization based on sequence data among the species of the genus Rosa. It also sought to compare chloroplast sequence data to identify these species, assess their evolutionary relationship, and know their unique biological variety, as researchers looked for gene sequences in the GenBank at NCBI (Kress and Erickson, 2007).

MATERIALS AND METHODS

Plant samples

The plant taxa used in the presented study relied on five species of the genus Rosa, R. gallica, R. hemisphaerica, R. foetida, Rosa x damascene, and R. centifolia. They were a collection during field trips held in April–June 2023 in the Districts of Duhok, Arbil, and Sulaymania, Kurdistan Region, Iraq.

DNA Extraction

Genomic DNA extraction proceeded from newly grown leaves using a special extraction kit (Favorgen/Biotech CORP prepared). Renewing the concentration and purity of the DNA used the nanodrop device, with the measurement at a wavelength of 260–280 nm (1.6–1.8). The primer rbcL used in the chloroplast gene interaction experiments helped detect the Internal Transcribed Spacer (ITS) region in the chloroplast DNA (Table 1), with the Premix kit obtained from Bioneer Company in 0.5 ml tubes. Its composition appears in Table 2 (Hu et al., 2014).

Polymerase chain reaction (PCR)

The mixture preparation for the master reaction ensued by mixing the components of the PCR premix prepared by Bioneer in an Eppendorf tube, with the following steps: Adding sterile distilled water, then adding the primers for the chloroplast gene (R+F) together for each sample to reach the final volume of 20 µL, considering the reaction's preparation had refrigerated conditions. Later, mixing well the solution in the vortex device ran for three seconds to complete the reaction components, then, placing the tubes in the thermocycler device to conduct the PCR, using the plan provided in Table 3. At the end of reaction time, the tubes' removal from the device continued with 5 microliters of DNA samples withdrawn and placed in a 1.2% agarose gel for 120 min (Mirzaei and Rahmani, 2011).

Table 1. Specific primers and their sequences used in the study.

Primers	Sequence (5'-3')
rbcL F	TGTCACCAAAAACAGAGACT
rbcL R	TTCCATACTTCACAAGCAGC

Table 2. Premix kit.

No.	Components		Concentration	Volume (µL)
1	PCR Premix	Taq DNA Polymerase	1 U	10
		dNTP (dATP, dCTP, dGTP, dTTP)	250 µM	
		Reaction Buffer with 1.5 mM $MgCl_2$	1X	
		Stabilizer and tracking dye	0	
2	Primer		100 P.mol/µL	2
3	D.D.W			4
4	DNA		50 mg/µL	4
				20

Table 3. Plan used for replication in chloroplast gene interactions.

No.	Stage	Temperature (°C)	Time (min)	Cycles
1	Initial denaturation	94	4	1×
2	Denaturation	94	1	
3	Annealing	45	1	35×
4	Elongation	72	2	
5	Final extension	72	12	1×
6	Terminal incubation	4	∞	

DNA sequencing for the chloroplast gene

The sequence of the nitrogenous bases for the Rosa species incurred determination based on the results of the rbcL gene. The results of the PCR for the said gene reached evaluation by sending the primers for the resulting band to the concerned institutions. The sequence of the genes' reading used Psomagen/USA, and matching of We used the BLAST program to compare the gene sequences with the recorded gene sequences in the National Centre for Biotechnology (NCBI).

Statistical analysis

The results of the primers used in the chloroplast gene markers include estimating the resulting band and its molecular sizes that appeared on the agarose gel. Comparing the locations of the bands with the standard size index after converting the results to binary character tables indicated a band presence (1) and a band absence (0). In determining the genetic relationship among the examined Rosa L. species, transforming all characterization data into similarity data used the following equations (Nei and Li, 1979; Yao et al., 2007).

$$\text{Genetic similarity} = 2nxy/nx+ny$$

Where,
nxy: represents the number of common bands between x and y, which represent any two of the studied plants;
nx: represents the total number of bands of x; and
ny: represents the total number of total bands in y.

The estimation of genetic distance among the species utilized the computer program, Similarity of Quantitative, based on the following equation:

$$\text{Genetic distance} = 1 - 2nxy/nx+ny$$

After finding the genetic relationship among the Rosa L. species, a dendrogram construction continued based on the unweighted pair group method for the arithmetic average (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Specific gene of chloroplast DNA

The results came from five Rosa L. species collected from different localities of the Kurdistan Region, Iraq, (R. gallica, R. hemisphaerica, R. foetida, Rosa x damascene, and R. centifolia). Their assessment for identification and classification study showed the use of specific molecular markers, as represented by the specific gene of chloroplast DNA. The use of rbcL gene detected the internal transcribed spacer (ITS) region in chloroplast DNA, which was responsible for the loss of large units of ribulose 1,5-bisphosphate (RuBP), and highly valuable in determining photosynthesis pathways. A major band with a molecular size of 1700bp appeared in all the studied species of Rosa L. This might be due to the primer finding a complementary site on the DNA strand, which led to its binding and replication (Devos and Gale, 1992). The results on detecting the nucleotide sequences of fragments amplified using the rbcL gene for the studied species are available in Figure 1 and Table 4.

Table 4. Sequence of nitrogenous bases for isolates registered in the International GenBank NCBI.

No.	Species	Nucleotide sequence
1	Rosa gallica	AACTGCTCTACCGTAATTCTTAGCGGATAACCCCAATTTAGGTTTAATAGTACATCCCAATAG GGGGCGGCCATACTTGTTCAATTTATCTCTTTCAACTTGGATCCCGTGAGGCGGGCCTTGGA AAGTTTTAACATAAGCAGTAGGGATTCGTAAATCCTCCAGACGTAGAGCGCGCAAGGCCTT GAACCCAAACACATTACCTACAATGGAAGTAAACATGTTAGTAACCGAACCCTCTTCAAAAA GGTCTAGGGGGTAAGCTACATAAGCAATAAATTGACTTTCTTCTCCAGCAACAGGTTCAATG TGGTAGCATCGCCCTTTGTAACGATCAAGACTGGTAAGCCCATCAGTCCATACAGTTGTCCA TGTACCAGTAGAAGATTCCGCAGCTACCGCTGCCCCTGCTTCCTCAGGCGGAACTCCAGGT TGAGGAGTTACTCGAAATGCTGCCAAGATATCAGTATCTTTGGTTTCATAGTCCGGAGTATA ATAAGTCAATTTATAATCTTTAACACCAGCTTTGAATC
2	Rosa hemisphaerica)New Record(	AACTGCTCTACCGTAATTCTTAGCGGATAACCCCAATTTAGGTTTAATAGTACATCCCAATAG GGGGCGGCCATACTTGTTCAATTTATCTCTTTCAACTTGGATCCCGTGAGGCGGGCCTTGGA AAGTTTTAACATAAGCAGTAGGGATTCGTAAATCCTCCAGACGTAGAGCGCGCAAGGCCTT GAACCCAAACACATTACCTACAATGGAAGTAAACATGTTAGTAACCGAACCCTCTTCAAAAA GGTCTAGGGGGTAAGCTACATAAGCAATAAATTGACTTTCTTCTCCAGCAACAGGTTCAATG TGGTAGCATCGCCCTTTGTAACGATCAAGACTGGTAAGCCCATCAGTCCATACAGTTGTCCA TGTACCAGTAGAAGATTCCGCAGCTACCGCTGCCCCTGCTTCCTCAGGCGGAACTCCAGGT TGAGGAGTTACTCGAAATGCTGCCAAGATATCAGTATCTTTGGTTTCATAGTCCGGAGTATA ATAAGTCAATTTATAATCTTTAACACCAGCTTTGAATC
3	Rosa foetida)New Record(	AAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTATAAATTGACTTATTACACTCCGGA CTATGAAACCAAAGATACTGATATCTTGGCAGCATTTCGAGTAACTCCTCAACCTGGAGTTC CGCCTGAGGAAGCAGGGGCCGCGGTAGCTGCGGAATCTTCTACCGGTACATGGACAACTG TATGGACTGACGGGCTTACTAGTCTTGATCGTTACAAAGGGCGATGCTACCACATTGAACCT GTTGCTGGAGAAGAAAGTCAATTTATTGCTTATGTAGCTTACCCCTTAGACCTTTTTGAAGAA GGTTCGGTTACTAACATGTTTACTTCCATTGTAGGTAATGTGTTTGGGTTCAAGGCCTTGCG CGCTCTACGTCTGGAGGATTTACGAATCCCTCCTGCTTATGTTAAAACTTTCCAAGGCCCGC CTCACGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGCCGCCCCCTATTGGGATG TACTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTT
4	Rosa x damascene	AAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTATAAATTGACTTATTATACTCCGGA GTATGAAACCAAAGATACTGATATCTTGGCAGCATTTCGAGTAACTCCTCAACCTGGAGTTC CGCCTGAGGAAGCAGGGGCAGCGGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGT ATGGACTGATGGGCTTACCAGTCTTGATCGTTACAAAGGGCGATGCTACCACATTGAACCTG TTGCTGGAGAAGAAAGTCAATTTATTGCTTATGTAGCTTACCCCTTAGACCTTTTTGAAGAGG GTTCGGTTACTAACATGTTTACTTCCATTGTAGGTAATGTGTTTGGGTTCAAGGCCTTGCGC GCTCTACGTCTGGAGGATTTACGAATCCCTACTGCTTATGTTAAAACTTTCCAAGGCCCGCC TCACGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGCCGCCCCCTATTGGGATGTA CTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTT
5	Rosa x centifolia	AAAGCAAGTGTTGGATTCAAAGCTGGTGTTAAAGATTATAAATTGACTTATTATACTCCGGAC TATGAAACCAAAGATACTGATATCTTGGCAGCATTTCGAGTAACTCCTCAACCTGGAGTTCC GCCTGAGGAAGCAGGGGCAGCGGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTA TGGACTGATGGGCTTACCAGTCTTGATCGTTACAAAGGGCGATGCTACCACATTGAACCTGT TGCTGGAGAAGAAAGTCAATTTATTGCTTATGTAGCTTACCCCCTAGACCTTTTTGAAGAGG GTTCGGTTACTAACATGTTTACTTCCATTGTAGGTAATGTGTTTGGGTTCAAGGCCTTGCGC GCTCTACGTCTGGAGGATTTACGAATCCCTACTGCTTATGTTAAAACTTTCCAAGGCCCGCC TCACGGGATCCAAGTTGAAAGAGATAAATTGAACAAGTATGGCCGCCCCCTATTGGGATGTA CTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTT

Gene registration on NCBI

Based on the results, five isolates growing naturally in the northern regions of Iraq reached registration on NCBI, with identification numbers also provided (Table 5). The sequencing data used developed the phylogenetic tree for comparing various plant samples once uploaded to the GenBank and given accession numbers. In the conduct of phylogenetic analysis, the study engaged the MEGA11 program (Tamura et al., 2021). The phylogenetic tree constructed employed the maximum likelihood approach in the Tamura-Nei model (Tamura et al., 2004). Additionally, running 1000 re-samplings of bootstrap analyses applied the ITS gene and the molecular DNA sequences of the joined genes as input data. The genetic tree based on rbcL gene sequences grouped five species into two main groups. The first group has three different species, including R. centifolia, R. foetida, and R. x damascene, with bootstrap values ranging between 55%–57%. The

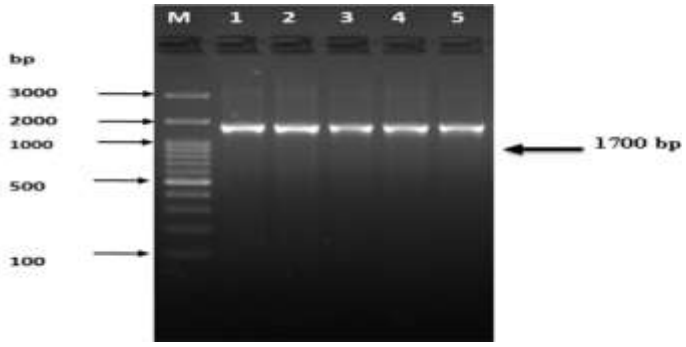

Figure 1. PCR reaction product of the specific gene rbcL chloroplast DNA (cpDNA) for the species studied.

Table 5. Percentage distribution of Rosa species based on partial rbcL gene according to blast in the GenBank of NCBI.

Accession Number	Plant Identified	Query Cover (%)	Identification Number (%)	GenBank Accession Number	Country Identification
OQ843473	Rosa gallica	100	100	NC061271	China
OQ843474	Rosa hemisphaerica	New Record			
OQ843475	Rosa foetida	New Record			
OQ843476	Rosa x damascene	100	100	MG679231	India
		100	100	MG679233	India
		100	100	MG946895	Pakistan
		100	100	OQ735273	China
		100	100	MZ261849	China
		100	100	NC061174	USA
		100	100	MG946863	Pakistan
OQ843472	Rosa x centifolia	100	100	OQ744229	China
		100	100	MZ261886	China

second group had two different species, which included R. gallica and R. hemisphaerica, with a bootstrap value of 99% (Figure 2). Overall, the Rosa species R. gallica and R. hemisphaerica have a good bootstrap value, with the R. foetida and R. hemisphaerica as newly recorded species (Table 5).

From the sequencing data, the dendrogram revealed the Rosa samples collected from Northern Iraq were genetically identical to the samples already included in the NCBI database. Despite inconsistent bootstrap results, comparative analysis of the ITS regions showed the taxa under consideration had comparable evolutionary ties. The relevant studies have confirmed using the specific gene rbcL is one of the most promising and effective methods for molecular characterization, taxonomy of the plants, and the relationship between cultivated and wild species of the Rosaceae family. The specific rbcL gene has been effective in many taxonomic studies. This is because DNA sequence data is one of the most influential tools used to distinguish the evolutionary relationship (Taib et al., 2023).

The similarity in molecular sizes indicates these species were from the same genetic origin, as it also showed the strength of the relationship between different clans of the same origin (Kuhn et al., 1993). Molecular study is helpful in plant classification, particularly in setting the boundaries of species, cultivars, ecological relationship, and evolutionary matters. It is also applicable in the field of applied research in crop breeding to determine the distinct bonds to essential crop traits and develop the genetic maps determining the bonds to select hybrid plants.

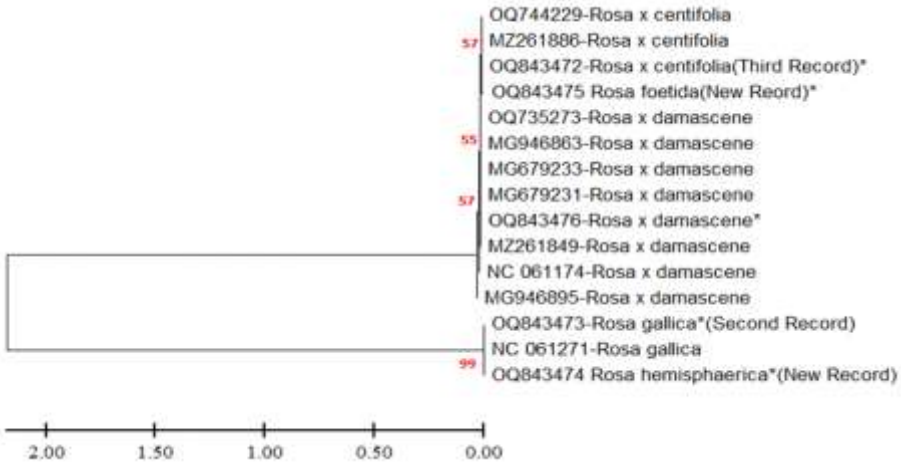

Figure 2. Phylogenic tree of Rosa sp Iraq (*). The phylogenic tree generation employed the Maximum Likelihood approach centered upon the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings.

Phylogenetic study of important plant species is useful to identify the new species (Onstein et al., 2015). Several genes have served for DNA barcode studies, viz., rbcL, mat-K, trnH-psbA, ndhF, trnL-trnF, and ITS individually and integrated (Moylan et al., 2004).

The existing study revealed the phylogenetic relationship of R. Sect. indica thorny with 52 species in the Rosaceae family. The chloroplast gene markers also identified the presence of trnL-F and atpB-rbcL genes to study the evolutionary relationship in the genus Rosa (Liu et al., 2015). Potter et al. (2002) explained in his study of evolutionary relationships in the Rosales using chloroplast genes. They said the use of matK and trnL-trnF genes confirmed it as an effective tool in identifying potential molecular relationships and decoding codes to analyze DNA. More studies are necessary to know about the greater molecular facts on rose species to recognize this genus and draw a correct phylogeny.

The use of rose chloroplast genes provides a complementary tool in characterizing the diversity among the rose cultivars and identifying the DNA. This chloroplast genome sequencing of Rosa spp. contains molecular markers that will make demographic, evolutionary, and related investigations much easier (Li et al., 2020). The detection of chloroplast genome sequences (cpDNA) is a new approach in studying the taxonomic and evolutionary relationships and the biological diversity of plants, in general. It is also highly efficient in deciphering the evolutionary relationship between the genetically related cultivars and species to improve our understanding of evolutions within plant species and genome-wide evolutionary studies (Wang et al., 2014; Daniell et al., 2016).

The application of matK is beneficial in plant taxonomic studies, as it is rapidly evolving and highly variable as a DNA coding site. It also provides clear methodological explanations pivotal in comparative studies in various fields of ecology, molecular taxonomic studies, and evolution in plants, in addition to studying genetic relationship, understanding biodiversity, and plant identification (Payacan et al., 2017). The plastid DNA genome is highly preserved in both structure and gene content (Yang et al., 2014). The genes caused by the chloroplast DNA genome suitable for various studies include phylogeny, evolutionary history of specific population genetics, and DNA barcodes. The standard barcodes for terrestrial plants also depended on two gene sections from the cpDNA genome, i.e., rbcL and matK (Gichira et al., 2017).

With its imperfect coordinated development of many copies, variable alleles from parents, DNA contamination of various species, and certain technological issues, the ITS gene became the first suggestion as a barcode for flowering plants (Kress et al., 2005). Nuclear DNA-ITS and chloroplast DNA sequencing have been effective to achieve phylogenetic resolution at the species level in various genera of angiosperms (Lee and Wen, 2001).

Moreover, numerous obstacles associated with taxonomic limitations emerged among Rosaceae, such as, phylogenetic relationship in the genus Rosa revisited based on rpl16, trnL-F, and atpB-rbcL sequences (Liu et al., 2015). These include incompatibility across species, gene sharing, high hybridization costs, and significant morphological tendency differences (Khatamsaz, 1995; Judd et al., 2002). Hybridization, polyploidy, and complex evolutionary processes are often common in the flora, making it difficult to establish species limits (Rieseberg et al., 2006; Fazekas et al., 2009).

CONCLUSIONS

Five wild specimens of the genus Rosa L. collected from exceptional regions of the Kurdistan Region, Iraq, bore evaluation through phylogenetic relationship. Both the ITS and rbcL (DNA) showed two awesome clusters in the chloroplast dendrogram tree. The first cluster comprised the species R. centifolia, R. foetida, and R. x damascene, while the second group has the species R. hemisphaerica and R. gallica of genus Rosa L.

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