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Molecular classification of Barbeyaceae (*Barbeya oleoides* Schweinf.) using four different DNA barcodes

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Abstract. Despite many efforts to determine the phylogenetic relationship between Barbeyaceae and other families of Rosales, the sister relationship of this family has remained unclear. *Barbeya oleoides*, which is currently the only species in the family Barbeyaceae, and native to Somalia, Ethiopia, and the Arabian Peninsula were collected from Wadi Turbah Zahran northwestern of Al-Baha City, southwestern Saudi Arabia (20°14'N, 41°15'E). To study the sister relationship of Barbeyaceae and the other families of Rosales, the complete chloroplast sequences were used for phylogenetic analysis. In addition, four standard DNA barcodes (the internal transcribed spacer 2 (ITS2), ribulose 1,5-biphosphate carboxylase (rbcL), maturase K (matk), the intergenic spacer region (trnH-psbA)) were used to test for their quality in identifying phylogenetic relationship of the studied families. Sequence analysis of the complete chloroplast sequences showed that Rosales clade has two subclades and clearly discriminated all families within this order. This is the first report of a partial ITS2 locus sequence in *B. oleoides*. The partial ITS2, rbcL, matk, and gene sequences discriminate *B. oleoides* of the Barbeyaceae family from the closely related plant families: Cannabaceae, Rosaceae, Rhamnaceae, Urticaceae, Moraceae, Elaeagnaceae, Dirachmaceae, and Ulmaceae. In contrast, the partial trnH-psbA sequence of *B. oleoides* did not show any homology to the available DNA sequence of plant families in GenBank, suggesting that it is more suitable as DNA barcode for variations within one species.

Keywords: DNA barcoding, phylogenetic analysis, ITS2, rbcL, matK, trnH-psbA.

Abbreviations:

B. oleoides: *Barbeya oleoides* Schweinf.

ITS2: the internal transcribed spacer 2

rbcL: ribulose 1,5-biphosphate carboxylase

matk: maturase K

trnH-psbA: the intergenic spacer region

w/v: wight/volume

IUCN: The International Union for Conservation of Nature

1. INTRODUCTION

Barbeya oleoides Schweinf. (*B. oleoides*) is the only species in the family Barbeyaceae and considered one of the smallest families in the plant kingdom (Dickison and Sweitzer, 1970, Chaudhary, 1999). Barbeyaceae belongs to the order Rosales, which comprises eight families besides Barbeyaceae: Cannabaceae, Dirachmaceae, Elaeagnaceae, Moraceae, Rhamnaceae, Rosaceae, Ulmaceae, and Urticaceae (Angiosperm Phylogeny Group (APG), 1998, 2003, 2009). *B. oleoides* is a small olea-like tree, reaching a height of up to 5 m, and can be found as a bushy shrub. The plant is characterized by dense hairs that cover the lower surfaces of the leaves. It is widely distributed in southwestern Saudi Arabia, including the Al-Baha region and locally known as kathah in Arabic (Dickison and Sweitzer, 1970). In Al-Baha region, the plant was reported only in seven locations between 1454 and 1768 masl on drainage lines facing the southwest and northeast (Namazi et al., 2021).

B. oleoides is considered a medicinal plant in Saudi Arabia and used as a folkloric remedy for the treatment of diseases such as infection, edema, or related inflammatory diseases (Baka, 2010). Few local studies have evaluated the medicinal effects of different plant parts, including leaves and stems (Ahmed et al., 2002; Khojah et al., 2021). The International Union for Conservation of Nature (IUCN) categorized *B. oleoides* as the least concerned taxon. However, it represents a monotypic taxon, implying the importance of taxonomic and phylogenetic studies on this plant (Rana and Ranade, 2009; Sarwar and Araki, 2010).

DNA barcoding is widely used as an effective tool to identify species and to make the obtained data publicly available to help understand, conserve, and utilize biodiversity. DNA barcodes in plants are usually located in the chloroplast genome, either within coding sequences (such as maturase K (matK) and ribulose biphosphate carboxylase (rbcL)) or in intergenic regions (such as the chloroplast trnH-psbA spacer region), or located at nuclear loci (such as the internal transcribed spacer of ribosomal DNA2 (ITS2)) (CBOL Plant Working Group, 2009; China Plant BOL Group, 2011; Li et al., 2015). The combination of these markers is important to achieve the highest discriminatory power and molecular identification of species.

The aim of this study was to investigate the phylogenetic relationship and molecular identification of *B. oleoides* using the whole chloroplast genome and four different barcodes of plant DNA. The sequences of rbcL, matk, and trnH-psbA loci were compared to those available for *B. oleoides* in the NCBI GenBank database; however, the current study is considered the first report on

the amplification and sequencing of the ITS2 region of the *B. oleoides* genome.

2. MATERIALS AND METHODS

2.1 Plant materials and DNA extraction

The plant samples were collected from Wadi Turbah Zahran, northwestern Al-Baha City, southwestern Saudi Arabia (20°14'N, 41°15'E). Total genomic DNA was extracted from 50 mg of fresh leaves using the CTAB extraction method described by Aboul-Maaty and Oraby (2019). The 3× CTAB extraction buffer contained 3% CTAB (w/v), NaCl, 0.8 M Tris-HCl pH 8.0), and EDTA (0.5 M EDTA pH 8.0). Compound 2 was preheated to 65 °C and 3% 2-β-mercaptoethanol was added to 3× CTAB extraction buffer immediately before use. Then, 800 µL of this buffer was added to the plant samples, which were ground into a powder using liquid nitrogen. The mixture was incubated in a water bath at 60–65 °C for 1 h and mixed gently every 20 min by inverting each tube 20 times. After cooling the mixture to room temperature, an equal volume of chloroform was added. The mixture was centrifuged at 13,000 rpm for 15 min at room temperature. The upper aqueous phase was then transferred to a new 1.5-mL Eppendorf tube. NaCl (6 M) with a volume equal to half the volume of the upper aqueous phase and 3 M potassium acetate (1/10 the volume of the upper aqueous phase) were added and simultaneously mixed with ice-cold 100% isopropyl alcohol (approximately two-thirds of the volume of the aqueous phase).

The extracted DNA was quantified using a UV spectrophotometer (NanoDrop 2000 Spectrophotometer; Thermo Scientific, UK).

2.2 PCR amplification and sequencing

The sequences of the primers used for amplification of the investigated regions are shown in Table 1. Amplifications were performed in 50-µL reactions. DNA amplification was carried out using PCR TECHNE (GMI, USA) and the conditions were as follows: pre-denaturation at 94 °C for 3 min followed by 34 cycles of denaturation at 94 °C for 30 s, annealing for 40 s, extension at 72 °C for 50 s, and one cycle of final extension at 72 °C for 5 min. The annealing temperatures for each primer are listed in Table 1. The PCR products were separated by 1.0% agarose gel electrophoresis. For DNA purification, Expin PCR purification kit (Gene ALL, Korea) was used.

Amplicons were sequenced by Macrogen (Seoul, South Korea).

Table 1. List of primers used for the investigated DNA barcoding loci.

DNA locus	Primer name	Primer sequence	Annealing temperature
rbcL	rbcLaF	ATGTCACCACAAACAGAGACTAAAGC	60.6 °C
	rbcLarev	GTA AAAATCAAGTCCACCRG	57.5 °C
matK	matK-KIM1	ACCCAGTCCATCTGGAAATCTTGGTTC	66.1 °C
	matK-KIM3	CGTACAGTACTTTTGTGTTTACGAG	56.8 °C
trnH-psbA	psbAF	CGCGCATGGTGGATT CACAATCC	65.7 °C
	t-rnH2	GTTATGCATGAACGTAATGCTC	54.8 °C
ITS2	ITS-S2F	ATGCGATACTTGGTGTGAAT	55 °C
	ITS4rev	TCCTCCGCTTATTGATATGC	55.8 °C

2.3 Sequence analysis

For the phylogenetic analysis of *B. oleoides*, the resultant sequences were compared with reference sequences in the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>). To query for highly similar sequences, Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used. The retrieved sequences were aligned, trimmed, and analyzed using the MEGA11 program (Tamura et al., 2021). A phylogenetic tree was constructed after finding the best DNA models using MEGA11, and models with the lowest Bayesian Information Criterion (BIC) scores were considered the best model to describe the substitution pattern.

The maximum likelihood method (based on the best model obtained) was used to construct the phylogenetic tree with default parameters, except that the test of phylogeny was modified to the bootstrap method (Felsenstein, 1985). Therefore, the confidence levels for the individual branches of the resulting tree were assessed by the bootstrap test in which 1,000 replicate trees were generated from resampled data, and statistical support for each constructed tree was also provided by pairwise distance estimated using the best-fit model.

To infer phylogenetic relationships within Rosales, the seven plastid genomes were compared to *Barbeya oleoides*. All plastid genome sequences were aligned using MAFFT v7.402 (Katoh and Standley 2013). Maximum likelihood analyses were conducted using raxmlGUI (Silvestro and Michalak 2012) with GTR+G, and 1000 bootstrap replicates.

3. RESULTS

The amplification and sequence success rates of ITS2, rbcL, matK, and trnH-psbA from specimens of *B. oleoides* were 100%. The lengths of the ITS2, rbcL, matK, and trnH-psbA sequences used for the analysis were 356,

555, 783, and 498, respectively. Sequences of the ITS2, rbcL, matK, and trnH-psbA loci were submitted to the NCBI GenBank database under the following accession numbers: OP023315, OP094675, OP094676, and OP094677, respectively. The obtained sequences were used as query sequences in BLAST at NCBI to find similar sequences in the order Rosales. For the ITS2 loci the species included in the analysis are shown in Fig. 1.

The nucleotide sequences of all selected species were analyzed using MEGA11 which revealed that the Tamura 3-parameter model had the lowest BIC scores (Tamura, 1992). As a result, evolutionary history was inferred using the maximum likelihood method and Tamura 3-parameter model (Fig. 1).

In addition, by blasting the sequence of rbcL loci, the most highly similar identity sequences obtained from GenBank were the Rosaceae family, For the rbcL loci the species included in the analysis are shown in Fig 2. The nucleotide sequences of all selected species were analyzed by MEGA11, which revealed that the Jukes-Cantor model is the best model to describe the substitution pattern (Jukes and Cantor, 1969). Therefore, evolutionary history was inferred using the maximum likelihood method and the Jukes-Cantor model (Fig. 2).

The matK loci sequence showed that the most highly similar identity sequences obtained from GenBank were from the Rhamnaceae family. For the matK loci the species included in the analysis are shown in Fig 3. The nucleotide sequences of all selected species were analyzed using MEGA11 which revealed that the Tamura 3-parameter model had the lowest BIC scores (Tamura, 1992). As a result, evolutionary history was inferred using the maximum likelihood method and Tamura 3-parameter model (Fig. 3).

In contrast, the trnH-psbA loci of the investigated *B. oleoides* did not show any similarity to any other plant family in the BLAST search at NCBI, and the present identity to the available data of *B. oleoides* (only two accessions: NC_040984 and MG880221) was 89.39%

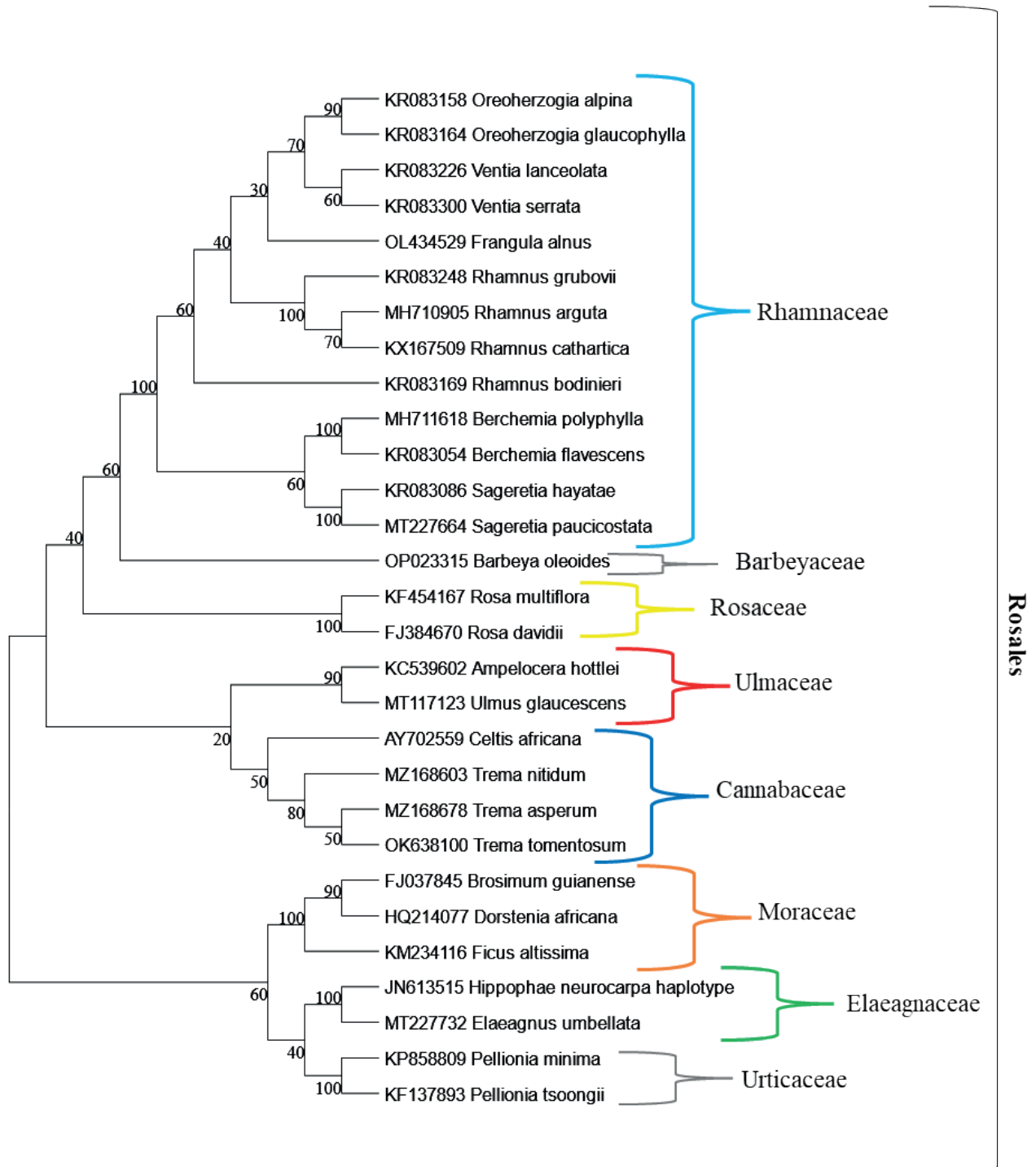


Figure 1 Phylogram based on ITS2 locus for *B. oleoides* and the retrieved species from NCBI using the maximum likelihood tree method. The evolutionary history and phylogenetic relationship were inferred using the maximum likelihood method and the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log-likelihood is shown. The analysis involved 16 nucleotide sequences. The codon positions included were 1st+2nd+3rd+ noncoding. There were 364 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

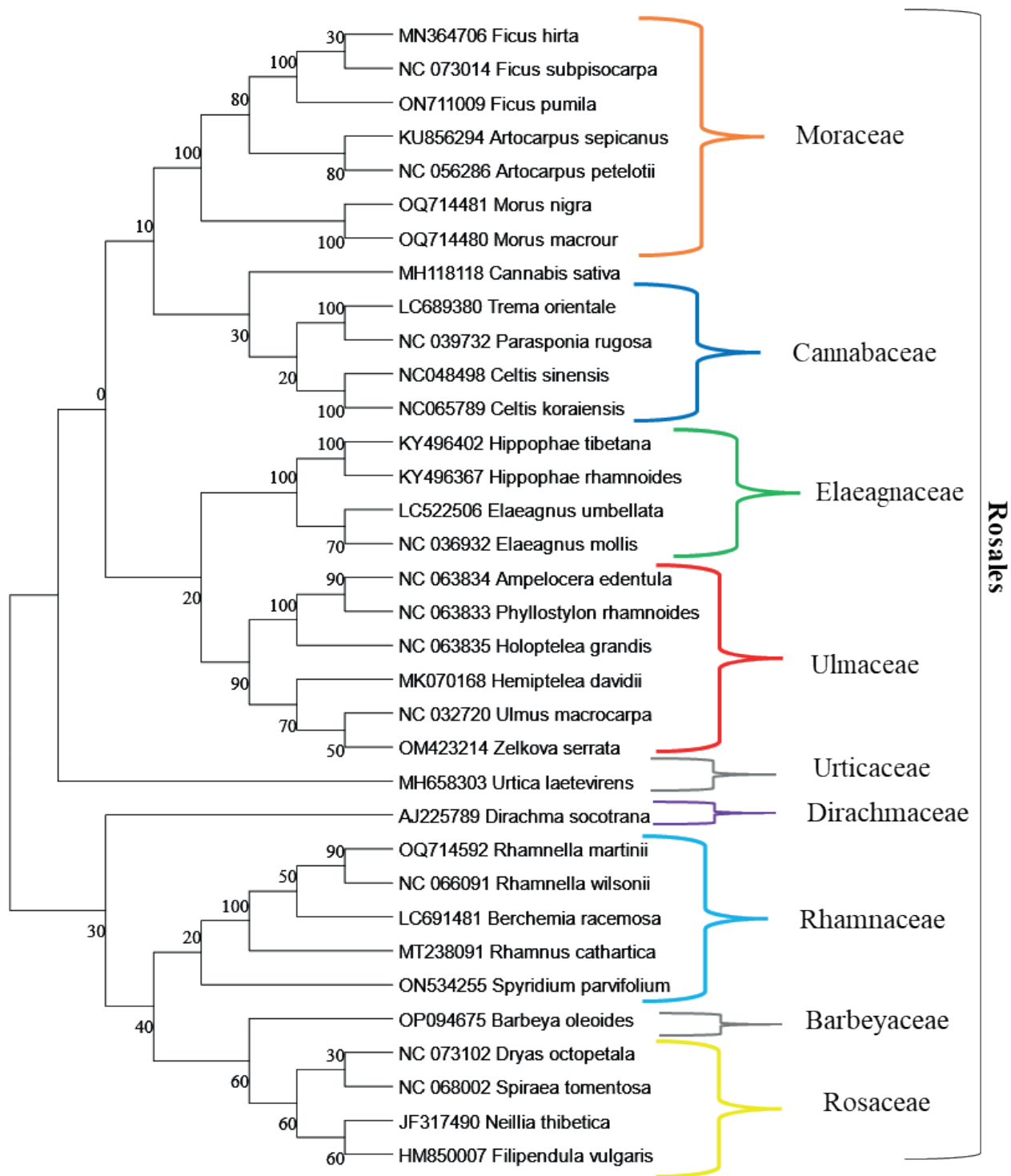


Figure 2 Phylogram based on *rbcL* locus for *B. oleoides* and the retrieved species from NCBI using the maximum likelihood tree method. The evolutionary history and phylogenetic relationship were inferred using the maximum likelihood method and the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log-likelihood is shown. The analysis involved 16 nucleotide sequences. The codon positions included were 1st+2nd+3rd+ noncoding. There were 364 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

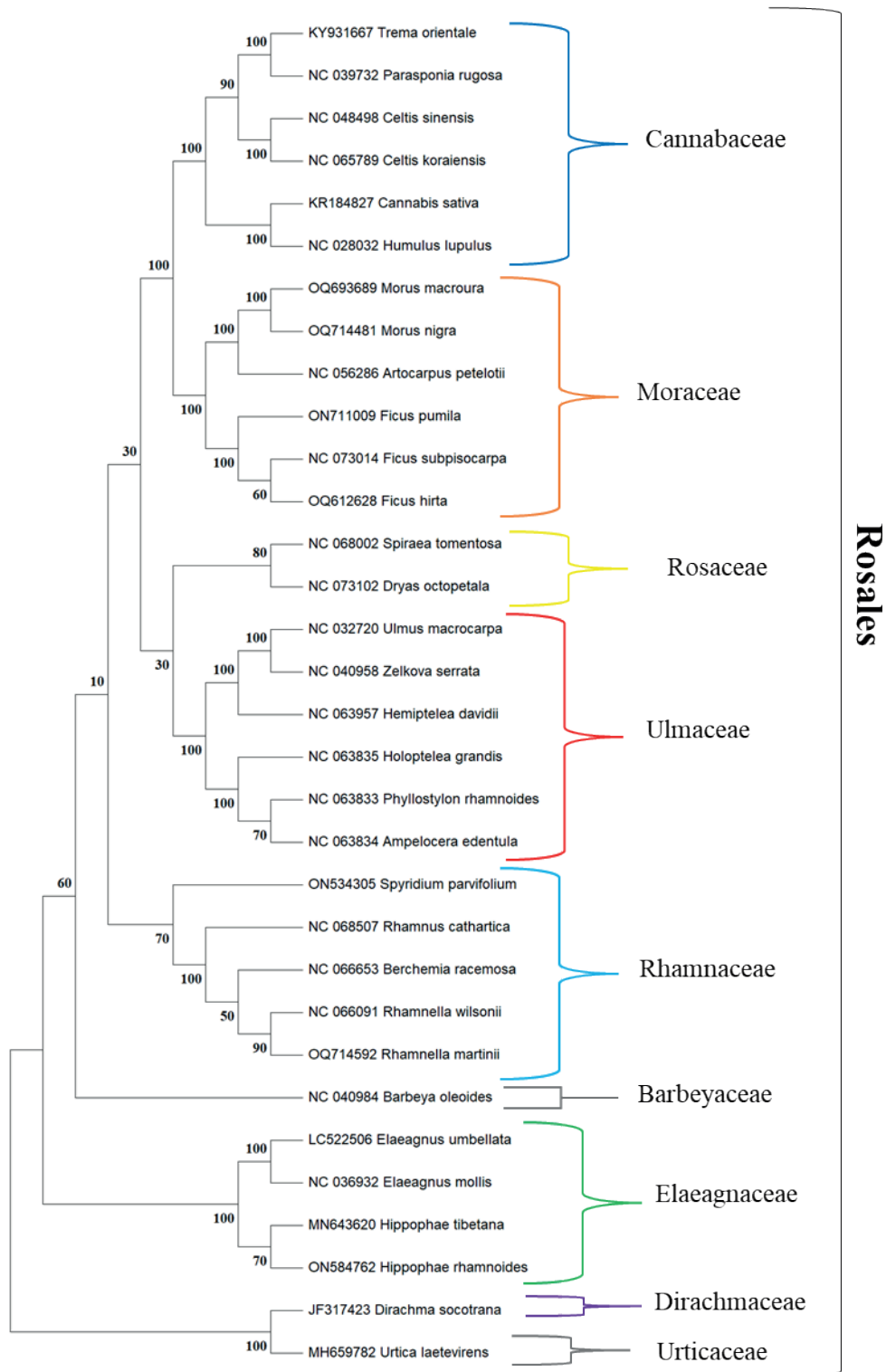


Figure 3 Phylogram based on matK locus for *B. oleoides* and the retrieved species from NCBI using the maximum likelihood tree method. The evolutionary history and phylogenetic relationship were inferred using the maximum likelihood method and the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log-likelihood is shown. The analysis involved 16 nucleotide sequences. The codon positions included were 1st+2nd+3rd+ noncoding. There were 364 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

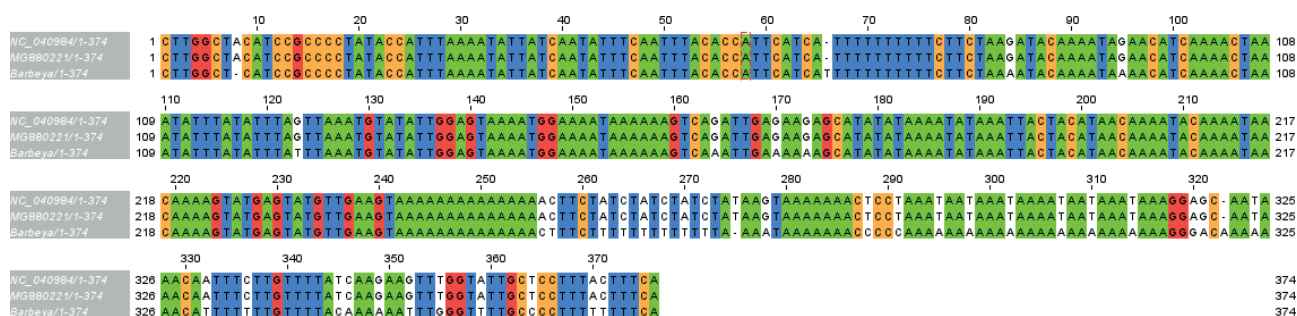


Figure 4 Variable sites between aligned sequence of trnH-psbA locus for *B. oleoides* and the retrieved sequence of *B. oleoides* from NCBI

with a very low E value (E-value = $1e-127$). The length of the aligned sequence was 374 bp and the percentage of variable sites within this sequence was 10% (Fig. 4).

The completed sequences of chloroplast of the families within Rosales (except Dirachmaceae not yet sequenced and instead available plastid sequences were used) were included for phylogenetic analysis using raxmlGUI (Silvestro and Michalak 2012). The result showed that the Rosales clade contains two subclades Fig. 5. The first subclade includes Rhamnaceae, Barbeyaceae, Elaeagnaceae, and Rosaceae. The Second subclade includes Ulmaceae, Cannabaceae, Moraceae, and Urticaceae. However, due to the absence of the completed chloroplast sequence of Dirachmaceae species, the family appeared as an outgroup taxon.

4. DISCUSSION

DNA barcoding is an important tool for species and family identification. Combining nuclear and chloroplast DNA barcodes is a good approach for DNA barcoding in plants (Kress et al., 2010). Not all DNA barcodes exhibit the same performance and efficiency across all plant species (Liu et al., 2011). In 2009, matK and rbcL barcodes were suggested to be the core sequences of plant DNA barcodes, while ITS and trnH-psbA are complementary plant DNA barcodes (CBOL Plant Working Group, 2009). The karyotype characteristic that is commonly utilized in cytotoxic analyses is the count of chromosomes. However, the complete sequencing of *Barbeya oleoides* has not been accomplished, thus preventing the utilization of chromosome number as a means of facilitating its classification. Chen et al. (2010) attempted to develop a practical and standardized tool for authenticating medicinal plants using DNA barcodes. In their study, the phylogeny of 4,800 species from 193 families across seven phyla (angiosperms, gymnosperms, ferns, mosses, liverworts, algae, and fungi) was analyzed, including different DNA barcodes. Their results pro-

posed the use of ITS2 as a core DNA barcode to identify medicinal plants at different taxonomic levels. The results of the ITS2 sequence BLAST and phylogenetic relationship inferred by using the maximum likelihood method discriminate Barbeyaceae from the Cannabaceae family, which is represented by two highly similar genera: *Aphananthe* and *Celtis*. Thus, the power of ITS2 for species identification was confirmed in the current study, and it was also documented to be a useful DNA marker for phylogenetic reconstructions at both the genus and species levels by several studies (Schultz and Wolf, 2009; Schultz et al., 2005). The applicability of different DNA barcode regions for species identification within Rosaceae has been tested, and the results indicate that ITS2 is the best of all loci tested for barcoding Rosaceae (Pang et al., 2011).

The matK barcode alone was not suggested to be a suitable universal barcode. This is due to the low success rate of species identification (Fazekas et al., 2008) and the different discrimination rates in different taxonomic groups; for example, the matK discrimination rate was more than 90% for species in the family Orchidaceae (Kress and Erickson, 2007) but less than 49% in the nutmeg family (Newmaster et al., 2008). In contrast to these previous studies, our study presents a strong case for the matK region as a good DNA barcode for authenticating *B. oleoides* and discriminating the Barbeyaceae family from other closely related families, such as the Rhamnaceae family, which is represented in the analysis by the genera *Ventilago* and *Berchemia*.

Although the rbcL region is not recommended for use as a candidate plant barcode based on several limitations, such as its modest discriminatory power at the species level (CBOL Plant Working Group, 2009; Chen et al., 2010; Fazekas et al., 2008; Lahaye et al., 2008) and the length of the gene, this study evaluated the rbcL barcode, and the results indicate accurate identification of *B. oleoides*. In addition, consistent with the current study, both rbcL and matK barcodes have the ability to discriminate among Cannabaceae, Rosaceae, and Rham-

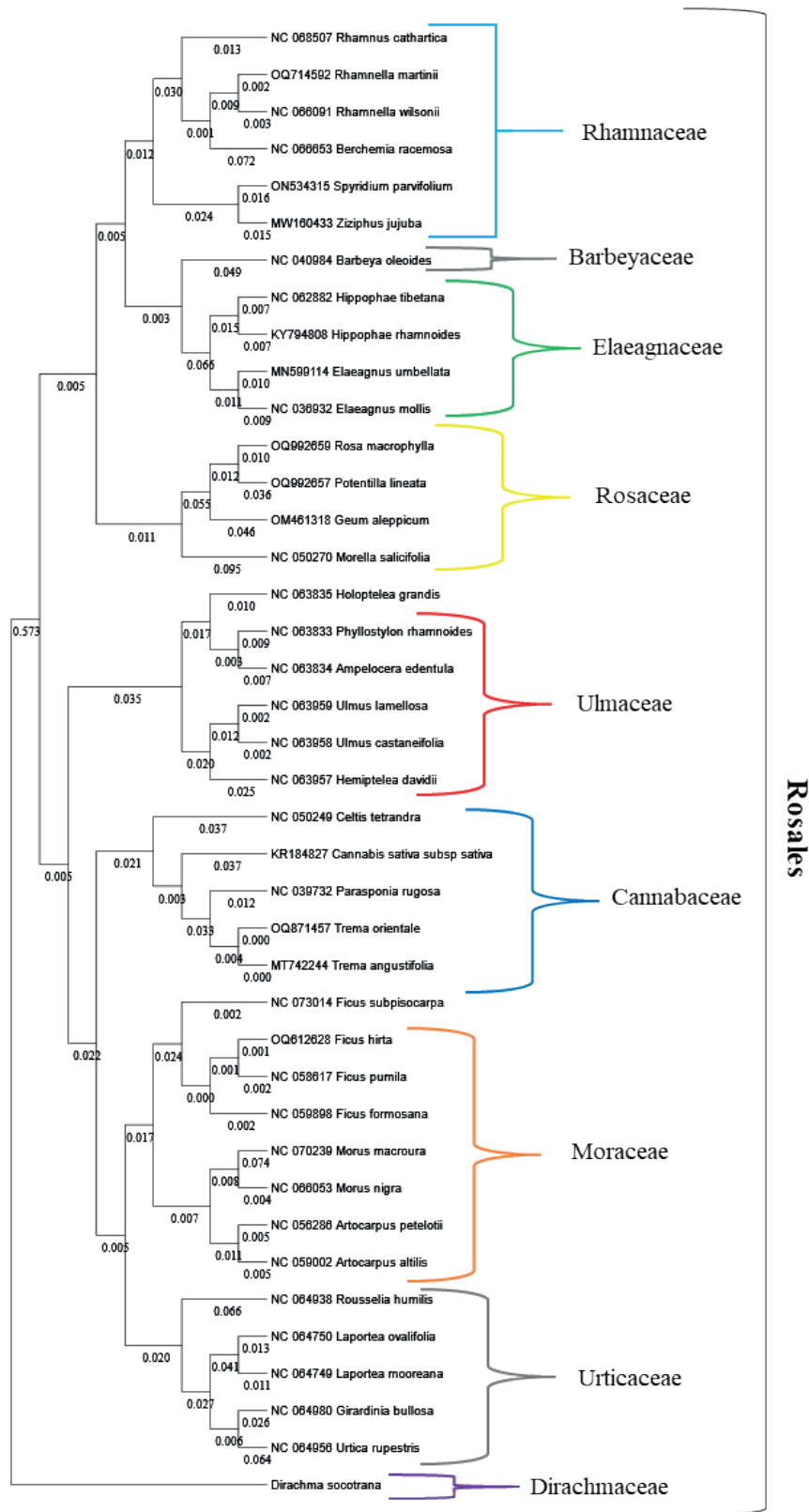


Fig. 5 Phylogenetic relationships of Barbeyaceae family with related families within Rosales based on the whole chloroplast genomes by maximum likelihood (ML) with bootstrap values above the branches.

naceae families within the order Rosales (Muhammad and Siddiqui, 2022).

Although the ITS2, *matK*, *rbcL*, and *trnH-psbA* loci of *B. oleoides* were efficient in the identification of this plant species, only ITS2, *rbcL*, and *matK* could discriminate the Barbeyaceae family from other closely related families. A phylogenetic evolutionary tree was constructed for *B. oleoides* species for each *rbcL*, *matK*, and ITS2, which was supported by the values of nodes on most branches being higher than 90%, indicating that the evolutionary relationships between *B. oleoides* and other closely related families are highly reliable. This result was supported by the ability of the trees constructed to differentiate clearly between the Barbeyaceae family and other closely related families, such as Cannabaceae, Rosaceae, and Rhamnaceae (fig. 1,2,3).

The weak discriminatory power of *trnH-psbA* compared to ITS2 at low taxonomic levels has been widely studied and has been suggested as a complementary barcode for the identification of medicinal plant species (Chen et al., 2010; Kress et al., 2005; Kress and Erickson, 2007). Our results indicated that the *trnH-psbA* locus was efficient in the identification of *B. oleoides*, but failed to distinguish between closely related families. This may be attributed to the high substitution rate within the chloroplast *trnH-psbA* spacer region (Whitlock et al. 2010). Therefore, as the *trnH-psbA* region accumulates more variation, it may offer more resolution at lower levels of taxa such as species and cultivars (Kress et al., 2010; Kress et al., 2005; Kress and Erickson, 2007; Chen et al., 2010).

The complete chloroplast sequence analysis considers a strong and informative tool for phylogenetic relationship analysis (Moore et al. 2007; Do et al. 2013). The analysis showed that the Rosales clade includes two subclades, which agreed with previous studies (Zhang et al. 2011). In addition, it showed that Barbeyaceae is sister to Elaeagnaceae. Other previous studies placed Elaeagnaceae in the same clade with Barbeyaceae, Dirachmaceae and Rhamnaceae (Soltis et al. 2007; Savolainen et al. 2000; Richardson et al. 2000a), hence, confirming the result of completed chloroplast phylogenetic analysis. Additionally, morphological studies confirmed the close relationship of Barbeyaceae, Dirachmaceae, Elaeagnaceae and Rhamnaceae (Richardson et al. 2000b; Wang et al. 2009).

5. CONCLUSIONS

This study is the first to assess molecular marker-based identification and classification of *B. oleoides*. This study produced DNA sequences from ITS2, *matK*, *rbcL*, and *trnH-psbA* barcoding loci in *B. oleoides* collected

from Al-Baha, Saudi Arabia. It is concluded that the three barcode markers, ITS2, *matK*, and *rbcL*, worked reasonably well in the differentiation of Barbeyaceae from closely related families of order Rosales. In addition, the study reported a low resolution of the *trnH-psbA* marker for the identification of families within the order Rosales. Thus, it is suggested to be used to differentiate among variations within *B. oleoides* plants, or variations within one species. Finally, the present study provides data to aid in the correct identification and conservation of this medicinally important plant species.

DATA AVAILABILITY

Sequences of the ITS2, *rbcL*, *matK*, and *trnH-psbA* loci were submitted to the NCBI GenBank database under the following accession numbers: OP023315, OP094675, OP094676, and OP094677, respectively

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