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Original article

Identification and molecular study of medicinal *Plectranthus* species (Lamiaceae) from Saudi Arabia using plastid DNA regions and ITS2 of the nrDNA gene

Widad Saleem Al-Juhani [a,b,*], Kadry Ne Abdel Khalik [a,c]

[a] Biology Department, Faculty of Applied Science, Umm Al-Qura University, 24381 Makkah, Saudi Arabia
[b] Research Laboratories Centre, Faculty of Applied Science, Umm Al-Qura University, 24381 Makkah, Saudi Arabia
[c] Botany Department, Faculty of Science, Sohag University, Sohag 82514, Egypt

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ABSTRACT

Plectranthus is a genus of the Lamiaceae family that includes many species of medicinal and agricultural importance. However, this genus has been the subject of taxonomic debate and contains species that are difficult to distinguish. The present study focused on six *Plectranthus* species commonly found in Saudi Arabia: *P. arabicus*, *P. tenuiflorus, P. barbatus, P. pseudomarrubioides*, *P. asirensis,* and *P. hijazensis*. *P. hijazensis* is endemic to Saudi Arabia. The capacities of five different plastid DNA barcodes (*matK*, *rbcL*, *trnH-psbA*, and *ITS1* and *ITS2* regions of the nrDNA gene) to identify and distinguish between *Plectranthus* species were evaluated. The following analytical methods were used to evaluate the efficiencies of the selected markers: BLAST, inter- and intraspecific distance, barcode gap, secondary structure of *ITS2*, and maximum likelihood (ML) phylogenetic trees. The results demonstrated that the nuclear *ITS2* region can be successfully amplified and sequenced (100%), leading to a strong ability to discriminate between species and a clear barcode gap. Furthermore, there were significant differences in the *ITS2* secondary structure among *Plectranthus* spp. Samples of *Plectranthus* formed monophyletic groups according to species in the ML tree, with high supported values. Our results establish that all *Plectranthus* species in Saudi Arabia can be classified into two groups within the Coleus clade. To our knowledge, this is the first time that local and endemic *Plectranthus* spp. have been identified and compared with *Plectranthus* samples of different geographical origins. Our results confirm the diversity of *Plectranthus* species growing naturally in southwestern Saudi Arabia. In addition, *P. hijazensis,* which is endemic to Saudi Arabia, was determined to be genetically distinct from other *Plectranthus* species and should, therefore, be the focus of future research, in addition to the preservation of the natural environment of these species.

1. Introduction

Plectranthus is one of the most important genera in the Lamiaceae family. The genus has been the subject of taxonomic debate for a variety of reasons, including the strong similarity between species and existence of multiple names for each species. In addition, it is difficult to identify species of genera that are closely related to *Plectranthus* (Musila et al., 2017). Initial classification attempts for *Plectranthus* were based on morphological characteristics, which resulted in many contradictions in the arrangement and number of subgenera and sections belonging to the genus. Morphological taxonomic classification of this tribe has not successfully classified the clades within the sub-tribe (Paton et al., 2004).

Paton et al., (2004) studied the molecular phylogenetics of the Ocimeae tribe based on three plastid DNA regions and argued that the *Plectranthus* genus is paraphyletic, supporting the hypothesis that Ocimeae is of Asian origin. Recently, (Paton et al., 2018) reviewed *Plectranthus* and *Coleus* based on the distribution and medicinal attributes of the phylogenetic plastid genes *trnL-F*,

* Corresponding author.
E-mail addresses: wsjuhani@uqu.edu.sa (W.S. Al-Juhani), knabdelkhalik@uqu.edu.sa (K.N.A. Khalik).
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rps16, and *trnS-G* and showed that the *Plectranthus* genus is distributed within two major clades of *Plectranthus* and *Coleus*.

Seven species of *Plectranthus* have been reported in the Kingdom of Saudi Arabia (KSA) (Collenette, 1999). However, (Chaudhary, 2000) revised these species and accepted only six: *P. arabicus*, *P. cylindraceus*, *P. tenuiflorus*, *P. lanuginosus*, *P. barbatus*, and *P. asirensis*. Recently, (Abdel Khalik, 2016a) recorded a new species, *Plectranthus hijazensis*.

P. asirensis is a Saudi Arabian species used as an antibacterial to treat diaper rash and has antiseptic properties (Abulfatih, 1987). *P. barbatus* grows naturally in Saudi Arabia and is used as a remedy for stomach, intestine, and liver disorders, heart problems, respiratory diseases, and as an insect repellant (Grayer et al., 2010). *P. tenuiflorus,* another local Saudi Arabian species, is used to treat ear infections and as an ornamental plant (Abulfatih, 1987).

Al-Qurainy et al., (2014) compared and identified the relationships among Saudi Arabian *P. asirensis* and *Plectranthus* spp. of different geographical origins using nrDNA-internal transcribed spacer (ITS) and chloroplast barcodes. Abdel Khalik and Osman (2017) studied the genetic diversity among seven species of the genus *Plectranthus* in Saudi Arabia using inter simple sequence repeats (ISSR) and random amplification of polymorphic DNA (RAPD) markers.

Variations in the macro- and micro-morphological features (including seed and pollen shape, size, coat sculpture, and trichome structure, as well as anatomical features of the leaves and stems) of the seven species of *Plectranthus* native to Saudi Arabia have been examined previously (Abdel Khalik, 2016b: Abdel Khalik and Karakish, 2016).

In recent years, there has been worldwide interest in using medicinal plants as a safe alternative to chemical medicines. However, difficulties in accurate identification and unethical practices in the use of these medicinal herbs pose a threat to this potential shift. Thus, the need for reliable verification tools to confirm the identity of these herbs is critical (Zahra et al., 2017). For example, *P. barbatus* and *P. grandis* are closely related species that can be mistaken for one another and used for the same medicinal purposes (Nani et al., 2015).

DNA barcoding as a molecular technique has been recommended as a useful method for species identification (CBOL Plant Working Group, 2009). DNA barcoding is not influenced by external factors or development stage, and DNA can be easily isolated from all tissues (Sucher and Carles, 2008; Seethapathy et al., 2015; Wu et al., 2015; Mishra et al., 2016), thus, providing a basis for species identification at the genetic level (Yu et al., 2017). DNA barcoding uses a short sequence of a standard part of the genome instead of the whole genome to verify the identity of samples. Portions of the *rbcL* and *matK* plastid coding genes have been suggested as barcodes for plant species (CBOL Plant Working Group, 2009) and have become the most-used loci in land plants as the *rbcL* region is highly suitable for amplification and sequencing (Michel et al., 2016; Mohamed, 2016). The nuclear ITS, with relatively strong discrimination power, is complementary to *matK* and *rbcL* in plants (CPBG China Plant BOL Group, 2011). The *ITS2* region, a sub-region of ITS, has been described as a valuable sequence tag for identifying medicinal plants (Chen et al., 2010; Yao et al., 2010; Han et al., 2013). The *ITS2* region is short in length and requires few primers for amplification. In addition, the region contains an abundance of genetic information and is located in the nuclear region, meaning that the *ITS2* region can be used to overcome the issue of failure to amplify the ITS in some species and is suitable for identifying high and low level taxa (Petit and Excoffier, 2009; Naciri et al., 2012; Braukmann et al., 2017).

Overall, Saudi Arabian *Plectranthus* species have not received adequate attention in terms of molecular studies. In the present study, five nuclear regions (*ITS1* and *ITS2* of the nrDNA gene and *matK*, *rbcL*, and *trnH-psbA* of the plastid gene) were chosen as barcodes, and a systematic comparison of different *Plectranthus* specimens local to Saudi Arabia and of different geographical origins was performed. The objectives of the study were a) to determine the performance of DNA barcoding in *Plectranthus* specimens and b) to evaluate the species discrimination powers of different barcodes.

2. Methods

2.1. Study area

The study area included different locations in the Jazan-Fifa Mountains, Baha, and Taif. These areas are located in the Tihama mountain range, which stretches across the southwest of the KSA, with height ranging from approximately 2000 to 12,000 m. The Tihama range is located in the Afro-Alpine vegetation zone, featuring a high plant diversity. Details of sample names, dates, and collection areas are presented in Table 1.

2.2. Plant material

Samples (leaves, flowers, and stems) of six *Plectranthus* species were collected between 2014 and 2018. Voucher specimens were prepared and identified by experts in taxonomy according to Collenette (1999) and Chaudhary (2000) and were deposited at the Herbaria of King Saud University/KSA, Kew and Edinburgh herbarium/UK, and Biology Department, Faculty of Applied Science, Umm Al-Qura University, Mecca/KSA after comparison with voucher specimens. At least three accessions per species were selected for molecular analysis from these voucher specimens.

2.3. DNA extraction

Initially, the following DNA extraction techniques were tested to select the best method: DNeasy Plant Mini Kit extraction (Qiagen/USA) following commercial protocols, traditional CTAB method (Doyle and Doyle, 1987), and modified DNA extraction method of Sahu et al., (2012), as described in Manikandan et al., (2017). The quantity of the extracted DNA was estimated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was tested via 1% agarose gel electrophoresis and ultraviolet light.

2.4. Primer sequences

Different DNA barcodes were amplified using the following primers: a-F (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and a-R (5′-GTAAAATCAAGTCCACCRCG-3′) for *rbcL*; KIM3F (5′-ACCCAGTC CATCTGGAAATCTTGGTTC-3′) and KIM1R (5′-CGTACAGTACTTTTGT GTTTACGAG3′) for *matK*, according to CBOL Plant Working Group (2009); 2F (5′-CGTAGCTACTTCTTCGCAGC-3′) and 5R (5-CCTTAT CATTTAGAGGAAGGAG-3′) for *ITS2*, according to Chen et al., (2010); for *ITS1*; 2F (5′-ATGCGATACTTGGTGTGAAT-3′) and 3R (5′-GACGCTTCTCCAGACTACAAT-3′) and F (5′-ACTGCCTTGATC CACTTGGC-3′) and R (5′-CGAAGCTCCATCTACAAATGG-3′) for *trnH-psbA*, according to (Kress and Erickson (2007).

2.5. DNA amplification and sequencing

DNA regions were amplified according to the method described by Maloukh et al., (2017), using 25 μL reaction volumes containing 12.5 μL Master Mix, 8.5 μL nano-pure water, 1 μL of each primer, and 2 μL DNA. The thermal cycle included an initial denaturation level of 94 °C for 5 min, followed by 94 °C for 45 s. An annealing

Table 1
List of the studied species of *Plectranthus* from Saudi Arabia along with information on sample numbers, locations, and GenBank accession numbers.

Voucher Specimen	Accepted Scientific Name	Place of Collection	Coordinates and Altitude	GenBank Accession Numbers	
				ITS2	*rbcL*
UQU PA1	*Plectranthus arabicus* E.A. Bruce	Jazan, Jabal Fefa, Gardada	17°14′45.1″N 43°05′27.2″E	MN382135	MN381814
UQU PA2				MN382136	MN381815
UQU PA3				MN382137	MN381816
UQU PP4	Plectranthus pseudomarrubioides R.H. Willemse	Jazan, Jabal Fefa	17°14′45.1″N 43°05′27.2″E	MN382138	MN381817
UQU PP5				MN382139	MN381818
UQU PP6				MN382140	MN381819
UQU pH 7	Plectranthus hijazensis Abdel Khalik	Baha, Dam of Medhas	20°01′19.8″N 41°26′00.1″E	MN382141	MN381820
UQU PH8				Fail	MN381821
UQU pH 9				MN382142	MN381822
UQU PB10	Plectranthus barbatus Andrews	Jazan-Jabal Fefa	17°14′45.1″N 43°05′27.2″E	MN382143	MN381823
UQU PB11				MN382144	MN381824
UQU PB12				MN382145	MN381825
UQU PS13	Plectranthus asirensis J.R.I.Wood	Messan, Bani Malek	20°49′56.9″N 41°00′14.1″E	MN382146	MN381826
UQU PS14				MN382147	MN381826
UQU PS 15				MN382148	MN381828
UQU PT16	Plectranthus tenuiflorus (Vatke) Agnew.	Abha, Raydah village	18°12′20.7″N 42°24′35.7″E	MN382149	MN381829
UQU PT17	A synonym of *Plectranthus aegyptiacus* (Forssk.) C. Chr.			MN382150	MN381830
UQU PT18				MN382151	MN381831
UQU PC22		Taif, Jabal Thaqif	21°04′32.5″N 40°18′37.4″E	MN382152	MN381832
UQU PC23				MN382153	MN381833
UQU PC24				MN382154	MN381834

temperature dependent on the type of primer (*ITS1*, 50 °C; *ITS2*, 56 °C; *matK*, *rbcL*, and *trnH-psbA*, 55 °C) was applied for 45 s, followed by 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min, in 40 cycles, using a Mastercycler (Eppendorf Vapo Protect, NY, USA). The quality of the PCR products was confirmed by electrophoresis on 1.5% agarose gel, and products were purified with a 1.0% agarose gel using the QIAquick purification Kit (Qiagen, USA). Sequencing was performed using a genetic analyzer (Applied Biosystem, CA, USA).

2.6. Bioinformatic analysis

Forward and reverse sequences were assembled and edited per sample and per gene in CodonCode Aligner v.8.0.2 (Condon Code Co., USA). The *ITS2* region of each specimen was detected using a hidden Markov model to remove the 5.8S and 28S regions, which may overlap with the *ITS2* region (Keller et al., 2009). A multiple sequence alignment was run for each gene using the Muscle algorithm tool in MEGA 7.0.27 (Kumar et al., 2016). The sequences were manually adjusted, and ambiguous regions were removed.

BLAST was run against known specimens in GenBank. The identification process took place at three levels: family, genus, and species. The query sequences were detected by selecting the highest maximum score and the lowest E-value. Outcomes were classified into three levels, as described in (Meier et al., 2006).

Genetic distances were calculated using the Kimura two-parameter (K2P) model in MEGA v.7.0.27 (Kumar et al., 2016). Inter- and intraspecific distances were calculated as the barcoding gaps by using TaxonDNA v.1.7.8 (Meier et al., 2006). The secondary structures of the *ITS2* sequences were predicted using the *ITS2* ribosomal RNA database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) (Schultz et al., 2005).

Two phylogenetic trees were built in MEGA 7.0.27 (Kumar et al., 2016): a neighbor-joining (NJ) tree and a maximum likelihood (ML) tree. Both trees were created by running 1,000 bootstrap replicates and K2P model of selection. The gamma distributed invariant site (G 1) nucleotide model was used for for ML tree. The number of discrete gamma categories was set as five (Liu et al., 2012; Kumar et al., 2016).

3. Results

3.1. Evaluation Efficiency of DNA barcoding

The preliminary results obtained from the DNA isolation experiments showed that the method of (Manikandan et al., 2017) allowed for the extraction of relatively more and better quality DNA than other methods, and therefore, DNA was extracted from all samples using this method.

The 21 plant specimens for each gene yielded 21 (100%) PCR products for *ITS2* and *rbcL* (Table 2). The lowest percentages of samples yielding PCR products occurred using the *matK* and *trnH-psbA* genes. All 21 (100%) specimens were successfully sequenced for their *rbcL* genes and 20 (95%) specimens for their *ITS2* loci. The *ITS2* region had the highest guanine-cytosine content (Table 2). Thus, two *rbcL* and *ITS2* DNA barcodes were selected for the following analyses.

3.2. Species resolution and barcode analysis

Based on BLAST, 100%, 85.7%, and 28.5% of the *ITS2* sequences were correctly identified at the family, genus, and species levels, respectively (Fig. 1). Furthermore, 100%, 71.4%, and 14.2% of the *rbcL* sequences were successfully identified at the family, genus, and species levels, respectively. E-values were (0) for each gene.

3.3. Inter- and intraspecific distance and barcode gap

The interspecific distances for the *rbcL* and *ITS2* barcode loci are shown in Table 3. The *ITS2* locus showed a minimum interspecific distance of 0.139, while the maximum interspecific distance was 0.000. The *rbcL* loci exhibited a minimum interspecific value of 0.018 and a maximum intraspecific distance of 0.000. Fig. 2 shows that the *ITS2* loci have a wider barcode gap than the *rbcL* loci.

3.4. ITS2 secondary structure

Fig. 3 shows the predicted secondary structures of the *ITS2* sequences used to identify the *Plectranthus* spp. The six species shared a similar *ITS2* morphological structure, with a central ring and four similar helices, namely, I, II, III, and IV. However, there

Table 2
Evaluation of the five DNA barcoding regions of the genus *Plectranthus* used in the current study.

Variable	nrITS regions		cpDNA regions		
	ITS1	*ITS2*	*matK*	*rbcL*	*trnH-psbA*
Number of samples	21	21	21	21	21
Mean and range of GC content (%)	49 (46–50)	67 (71.6–64.5)	35 (34–35.1)	44 (42.1–44.4)	27 (26–28)
Efficiency of PCR amplification (%)	17 (80.9%)	21 (100%)	7 (33.3)	21 (100%)	8 (38)
Success rate of sequencing (%)	7 (33.3%)	20 (95%)	6 (28.5)	21 (100%)	8 (38)
Amplified product length (bp)	~200	~200	~800	~600	~400
Mean and range of sequenced length (bp)	210 (200–270)	250 (210–270)	767 (763–869)	641 (610–658)	414 (310–448)

(GC): Guanine-cytosine content.

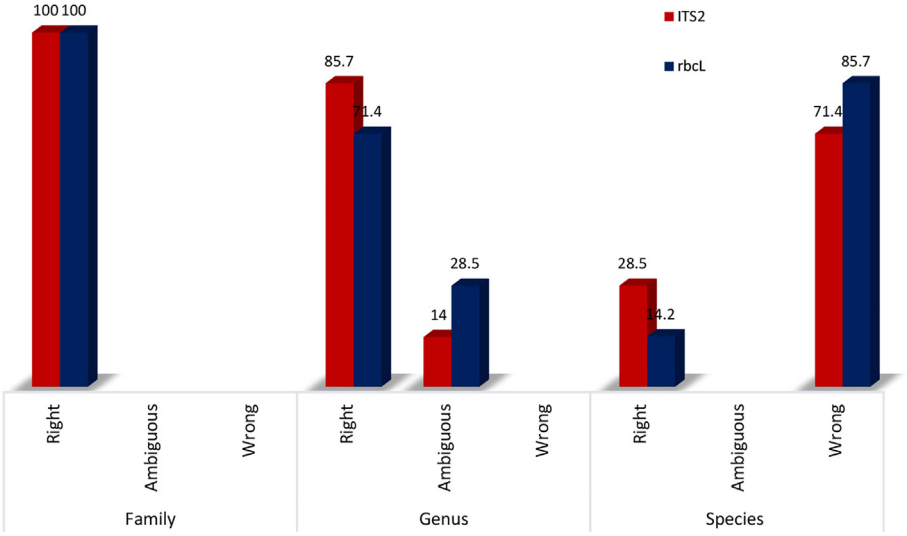

Fig. 1. Successful identification of *Plectranthus* spp., local to the Kingdom of Saudi Arabia at three levels (family, genus, and species) using BLAST and matching sequences drawn from the NCBI database. Values listed in the columns are estimated in percentages (%).

Table 3
Inter- and intraspecific distances for the *rbcL* and *ITS2* barcoding regions used in this study of the genus *Plectranthus* in Saudi Arabia.

Level	*ITS2* locus		*rbcL* locus	
	Inter-specific distance	Intra-specific distance	Inter-specific distance	Intra-specific distance
Mean	0.326	0.00	0.038	0.00
Max	0.231	0.00	0.053	0.00
Min	0.139	0.00	0.018	0.00

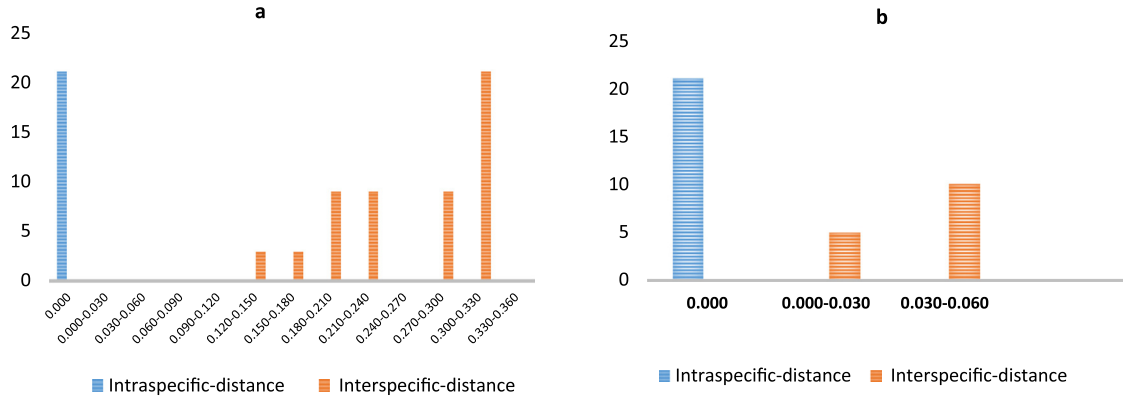

Fig. 2. Barcode gaps for DNA barcodes, a-ITS2 and b-rbcL, based on the K2P distance and six *Plectranthus* spp. local in the Kingdom of Saudi Arabia. Total barcoded sequences for ITS2 = 21, rbcL = 21, three specimens per species.

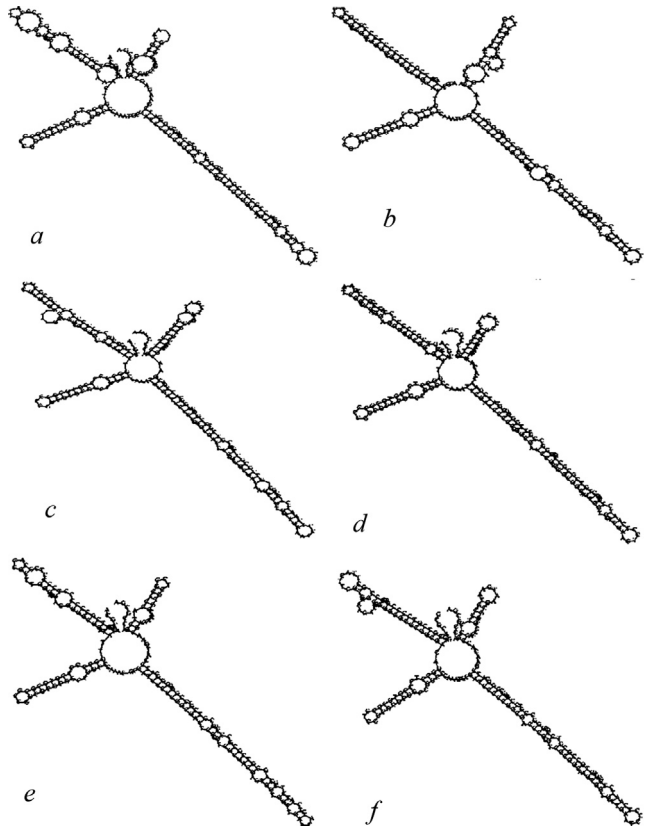

Fig. 3. Observed variation in the secondary structure of *ITS2* barcodes for the identification of *Plectranthus* spp. native to the Kingdom of Saudi Arabia, using an *ITS2* workbench. *a: Plectranthus arabicus. b: Plectranthus barbatus. c: Plectranthus hijazensis. d: Plectranthus pseudomarrubioides. e: Plectranthus asirensis. f: Plectranthus tenuiflorus.*

were variations in the stem loop number, size, and position and degree of angle from the center of the spiral arm of the *ITS2*.

3.5. Phylogenetic trees

The *rbcL* ML tree (Fig. 4) was constructed using 21 Saudi Arabian *Plectranthus* spp. sequences, with accessions numbers MN381814–MN381834. The *ITS2* ML tree (Fig. 5) was constructed using 20 Saudi Arabian *Plectranthus* spp. sequences, with accessions numbers MN82135–MN382154. In addition, 36 *Plectranthus* spp. sequences were downloaded from GenBank representing 8 different countries in Asia, Africa, Australia, and USA.

Overall, ML produced the best trees in terms of forming monophyletic groups for each species and nodes with high support values. Further, the ML-ITS2 tree showed clades with high support values and branches and good separation of species. The ML tree based on the *ITS2* locus data (Fig. 4) divided the sequences into two main clades: I and II. Clade I, with a high supporting value (89%), was divided in three main sub-clades, and clade II was also divided into three main branches. The phylogenetic tree constructed from *rbcL* gene data (Fig. 5) was also split into two clades (I and II). Clade I, with a high support value (89%), was divided into two main branches. Clade II was also divided into two branches. In general, Figs. 4 and 5 show that some species appeared to be highly related to each other as they always appeared in the same clade. For example, *P. barbatus P. caninus, P. asirensis*, *P. otostegioides,* and *P. lanuginosus* appeared to be highly related, as well as *P. pseudomarrubioides*, *P. ornatus*, *P. montanus*, *P. amboinicus*, *P. parviflorus,* and *P. edulis*. Furthermore, *P. tenuiflorus*, *P. hijazensis,* and *P. arabicus* always appeared together in one clade.

4. Discussion

An initial objective of this project was to identify and discriminate between *Plectranthus* species in Saudi Arabia using a DNA barcoding approach. The present study focused on six *Plectranthus* species native to Saudi Arabia. The analyses included the first sequences obtained from these six *Plectranthus* species de novo.

4.1. DNA extraction and PCR challenges

In the *Plectranthus* genus, there are many issues surrounding DNA isolation and purification, which hinder the acquisition of high-quality nrDNA. These include degradation of DNA owing to endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds such as polyphenols, and presence of secondary compounds that directly or indirectly interfere with enzymatic reactions (Manikandan et al., 2017). In the present study, three methods of DNA extraction were compared, and the method described in (Sahu et al., 2012) was found to show the best performance in terms quantity and quality of DNA extracted and successful PCR product collection, particularly with the *ITS2* and *rbcL* loci. This finding is consistent with that of (Manikandan et al., 2017).

It was observed that in dried, old, or degraded samples, fewer PCR products and less successful sequencing were observed, especially for the *ITS1*, *matK*, and *trnH-psbA* loci. The findings of the present study showed that the *ITS2* and *rbcL* loci represented the highest PCR success rates (100%) as well as the highest sequencing success rates (95% and 100%, respectively; Table 2). These observations are consistent with those of (Al-Juhani, 2019), who reported that the *rbcL* and *ITS2* regions had higher rates of amplification and sequencing success, even when using old, dry, or degraded specimens.

4.2. DNA barcodes and species resolution

Al-Qurainy et al., (2014) studied Saudi Arabian *P. asirensis* and reported the possibility of using nrDNA-ITS and the *rbcL* and *rpoC1* chloroplast loci for DNA barcoding in *Plectranthus* species. The present study showed that, in *Plectranthus*, *ITS2* exhibited a higher discrimination rate at the genus level (85.7%) and species resolution (28.5%) than *rbcL*, which showed a discrimination rate of 71.4% at the genus level and species resolution of 14.2% (Fig. 1). The *ITS2* locus is, therefore, recommended to identify herbal medicinal plants (Chen et al., 2010), for the following reasons: (i) it is located within the nuclear genome, which has a different rate of evolution than the plastid genome, (ii) it provides higher species resolution, and (iii) it has a much shorter sequence, allowing for higher recovery from processed plant materials found within herbal products.

Moreover, a possible explanation for the relatively low species-level identification in the present results is the lack of sufficient reference data; several species of *Plectranthus* do not have reference sequence data available in GenBank. There were no reference sequences available for *P. hijazensis, P. arabicus,* or *P. tenuiflorus,* for most of the markers selected for examination. Therefore, BLAST revealed closely related species but not specific species and was only able to successfully identify to the genus level, with low species resolution. Thus, the voucher specimens of the present study are the first ever sequences for the aforementioned species deposited in GenBank and are publicly available. (Hollingsworth, 2011) argued that species with a single sample are potentially distinguishable (i.e., for successful species identification) if the sequence is unique.

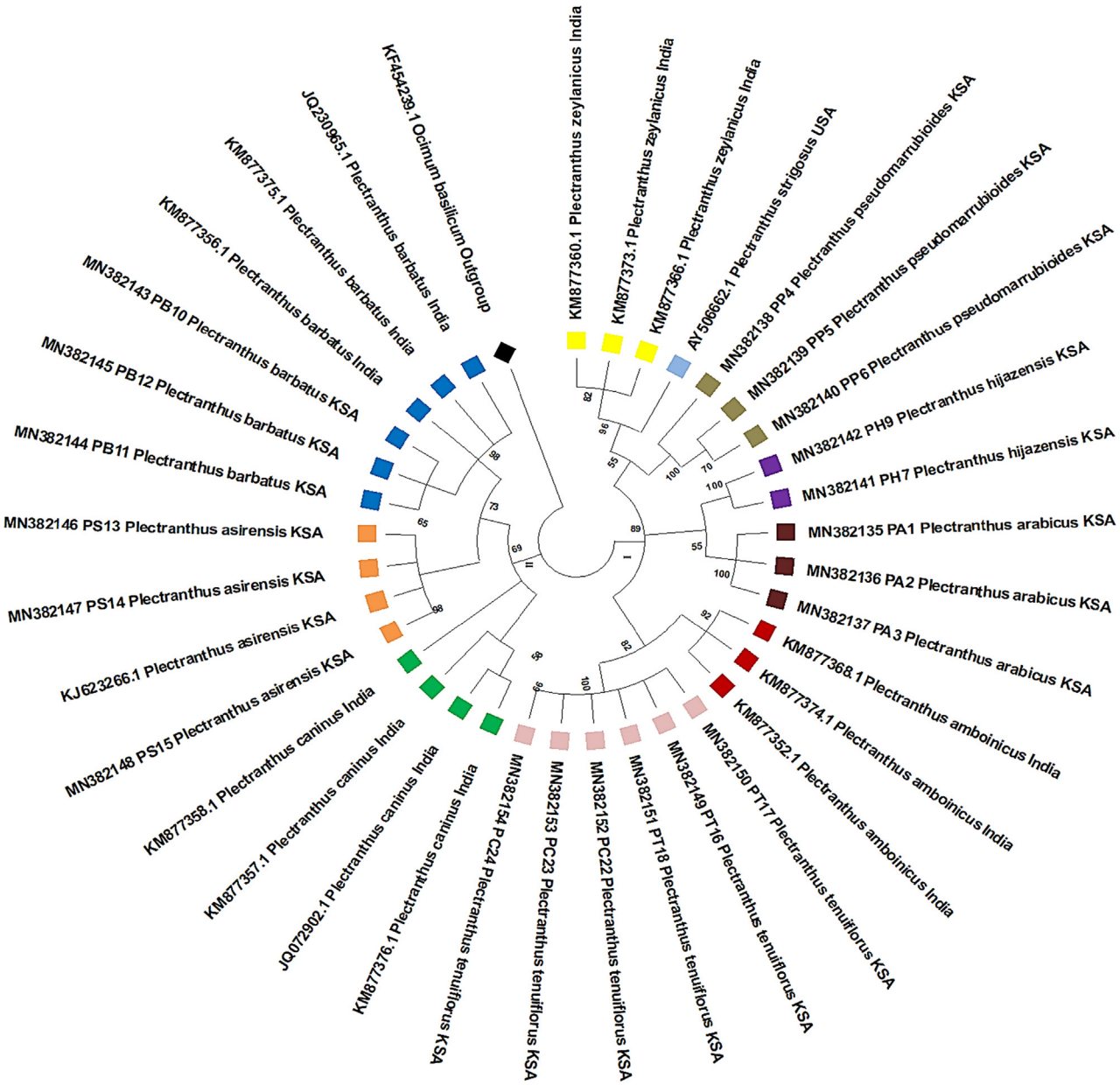

Fig. 4. Maximum likelihood tree of *ITS2* barcodes showing the phylogenetic relationships among *Plectranthus* spp. from the Kingdom of Saudi Arabi and other species of *Plectranthus* originating from different geographic regions. Bootstrap = 1000 replications.

In general, the minimum interspecific distances were greater than the maximum intraspecific distances for the *rbcL* and *ITS2* barcodes (Table 3). The barcoding gap in the *ITS2* region was obvious and larger than that in *rbcL*, where the minimum interspecific distance for *ITS2* was 0.139 and the maximum intraspecific distance was 0.000. Species discrimination is considered successful if the minimum interspecific K2P distance is larger than the maximum intraspecific distance for that species (CPBG China Plant BOL Group, 2011); this condition applies to the samples in the current study as well (Fig. 2).

The secondary structures of the *ITS2* are shown in Fig. 3. Similar *ITS2* secondary structures were observed across all *Plectranthus* spp. However, differences were observed in the morphology of the four helices among species. Variation in the secondary structure of *ITS2* has previously been reported as a tool for identifying species at the molecular morphological characteristics level (Keller et al., 2010). Liu et al. (2019) noted a clear difference between the *ITS2* structures of true "*Gaoben*" samples and the structures of three adulterant species and recommended using *ITS2* as a minibarcode to distinguish between closely or distantly related plant species used in Chinese medicine**.** Hence, the *ITS2* secondary structure could be used to validate *Plectranthus* species identification.

4.3. Phylogeny and taxonomy of Plectranthus spp.

The results of the present study demonstrated that *P. asirensis, P. caninus, P. otostegioides, P. barbatus,* and *P. lanuginosus* were grouped together in clade II of the phylogenetic tree constructed using *ITS2* data and in clade I of the *rbcL* phylogenetic tree. This is also consistent with the observations of (Al-Qurainy et al., 2014) and (Musila et al., 2017), who showed that *P. asirensis*, *P. caninus*, and *P. barbatus* are grouped together in trees built based on the *nrITS* gene, *matK*, and *rbcL*.

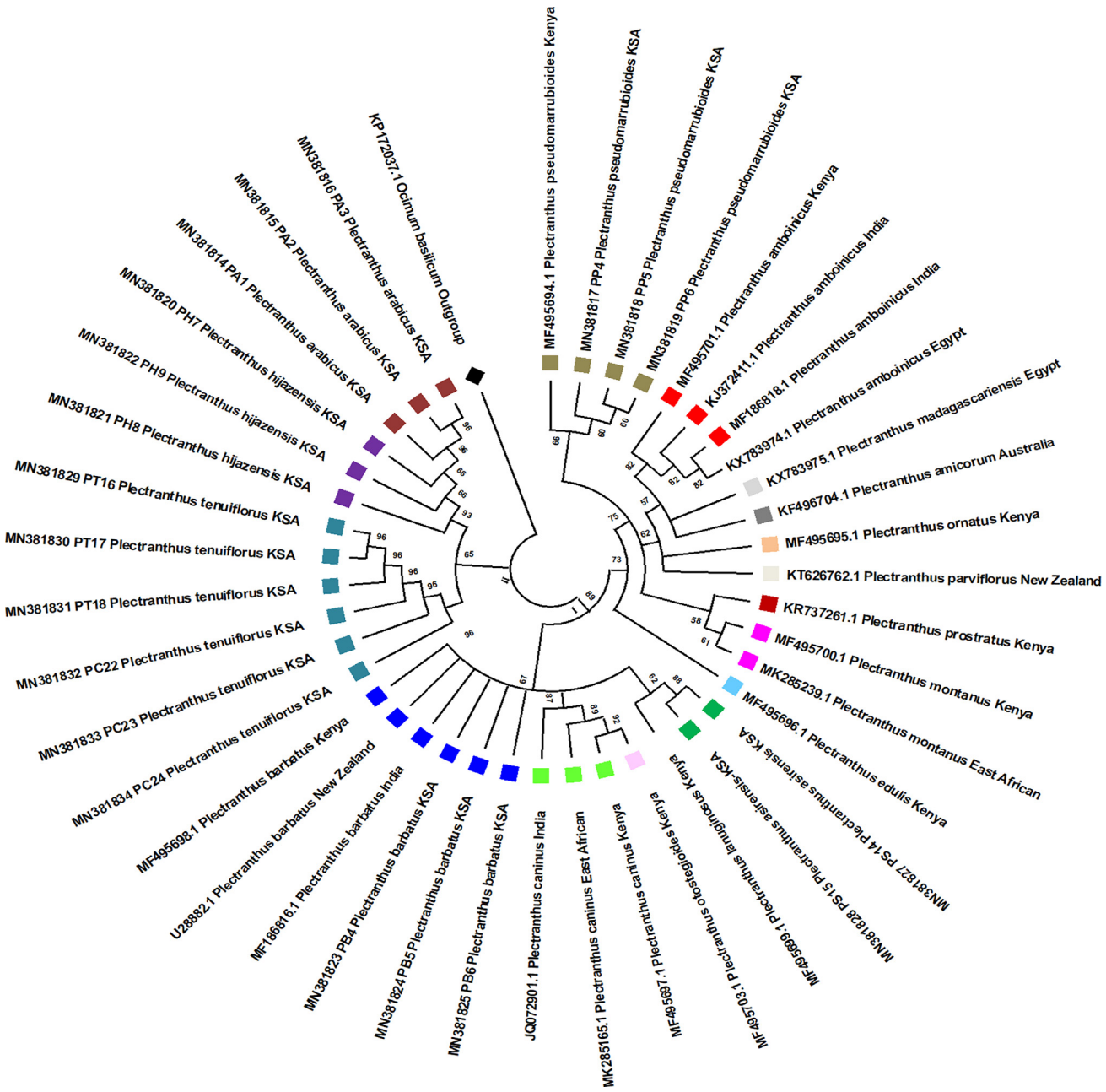

Fig. 5. Maximum likelihood tree of *rbcL* barcodes showing the phylogenetic relationships among *Plectranthus* spp. from the Kingdom of Saudi Arabia and other species of *Plectranthus* originating from different geographic regions. Bootstrap = 1000 replications.

Furthermore, we showed that three species, *P. tenuiflorus, P. hijazensis,* and *P. arabicus*, were always grouped together in the same clade in the trees constructed based on the *ITS2* and *rbcL* loci. This observation broadly supports the findings of (Abdel Khalik and Osman, 2017), who used RAPD and ISSR markers to study *Plectranthus* species native to Saudi Arabia and found that these species are clustered together.

Comparisons with the results of (Paton et al., 2018), who utilized the *trnL-F*, *rps16*, and *trnS-G* plastid genomes, confirmed the validity of the findings of the present study. The species studied herein were classified into two branches within the Coleus clade. Furthermore, our results showed that *P. hijazensis* and *P. asirensis* were separated in two clades with high bootstrap values, which supports the recommendation of (Abdel Khalik and Osman, 2017) to separate these two species into different subgenera.

The findings of the present study support the observations of (Musila et al., 2017), as *P. aegyptiacus (P. tenuiflorus)* was grouped into the same clade as *P. pseudomarrubioides* and *P. amboinicus* in the tree constructed based on the *ITS2* locus. Nevertheless, in the tree based on the *rbcL* data, *P. edulis* was grouped with *P. pseudomarrubioides, P. ornatus, P. montanus, P. amboinicus, P. amicorum, P. madagascariensis,* and *P. parviflorus.*

The present outcomes are consistent with those in previous studies (Frigerio et al., 2019) that have recommended DNA barcoding (*trnH-psbA*, *ITS*, and *rbcL 1-B*) as a useful tool for identifying medicinal and aromatic plants. Our findings also support the observations of Al-Juhani (2019), who showed that *ITS2* and *rbcL* produce species resolutions of up to 77% for 100 species growing on drylands in KSA, most of which are used in traditional medicine. Han et al., (2016) used *ITS2* DNA barcoding to verify and detect

adulterants of 295 herbal medicine species in China, confirming that the *ITS2* region could be used to identify most of the samples (87.7%) and detect adulterants (Han et al., 2016). Furthermore, (Vassou et al., 2016) used the *rbcL* region to create an API-RDBL library of 374 medicinal plants used in the Indian Pharmacopoeia and mentioned the usefulness of *rbcL* DNA barcoding in authenticating herbal drugs and detecting adulterants.

Our results are also in line with the results of (Gao et al., 2010), who highlighted the use of the *ITS2* region to identify 114 samples of the Fabaceae family, which contains many important medicinal plants, and confirmed the power of the *ITS2* region as a tool to authenticate herbal medicines. Furthermore, (Lv et al., 2020) indicated that *ITS2* can identify 98% of medicinal specimens from the Apocynaceae family at the species and genus levels.

Finally, (Le et al., 2020) confirmed the effectiveness of *ITS2* barcoding in detecting, identifying, and classifying the genetic relationships in agricultural varieties of palm used in botanical gardens and orchards. Together, these findings strongly support the use of the *ITS2* region as a tool to distinguish between varieties of *Plectranthus*, considering that several species of *Plectranthus* spp. are used ornamentally and in gardens.

5. Conclusion

This investigation showed that short-region *ITS2* DNA barcodes had strong discriminatory powers. We recommend using *ITS2* as a tool to validate the identity of medical *Plectranthus* species. Molecular processing of the whole-chloroplast genome, as well as next generation sequencing techniques, are promising modern tools that would be fruitful areas for future research into *Plectranthus* spp. Further information on both chloroplast and nuclear genes, in addition to micro-morphological outcomes, could provide more definitive evidence to solve problems and distinguish between closely related species and establish a greater degree of accuracy on the taxonomic status of *Plectranthus* species.

The newly recorded *P. hijazensis* species is endemic to Saudi Arabia and was found to be genetically distinct from the other studied *Plectranthus* species. Therefore, the species should be the focus of future research in different fields of chemotaxonomy, micro-morphology, and scanning electron microscope (SEM) studies. Our result also emphasizes the urgent need to intensify efforts to preserve the local natural diversity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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