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Selection of DNA barcoding loci and phylogenetic study of a medicinal and endemic plant, *Plectranthus asirensis* J.R.I. Wood from Saudi Arabia

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ABSTRACT. Genuine medicinal plant materials are very important for potential crude drug production, which can be used to cure many human diseases. DNA barcoding of medicinal plants is an effective way to identify adulterated or contaminated market materials, but it can be quite challenging to generate barcodes and analyze the data to determine discrimination power. The molecular phylogeny of a plant species infers its relationship to other species. We screened the various loci of the nuclear and chloroplast genome for the barcoding of *Plectranthus asirensis*, an endemic plant of Saudi Arabia. The chloroplast genome loci such as *rps16* and *rpoB* showed maximum similarity to taxa of the same and other genera via BLAST of the National Center for Biotechnology Information (NCBI) GenBank database; hence, they are less preferable for the development of a DNA barcode. However, nrDNA-ITS and chloroplast loci *rbcL* and *rpoC1* showed less similarity via BLAST of the NCBI GenBank database; therefore, they could be used for DNA barcoding for this species.

Key words: DNA barcoding; Chloroplast loci; Nuclear DNA marker; BLAST

INTRODUCTION

Plectranthus is a large and widely spread genus that contains ca. 300 species, which are distributed throughout Asia, Australia, and tropical Africa. The species of this genus are difficult to identify because of their close resemblance and, hence, there is difficulty in the naming of species of several closely related genera such as *Coleus*, *Englerastrum*, and *Solenostemon*. Some species were previously placed in the genus *Plectranthus* but are currently recognized in the more distant genus *Isodon* (Paton et al., 2004). As a result, different names have been given to the same species. Lukhoba et al. (2006) studied the phylogeny of various species of this genus and all species in clade 2; the first clade (members of the genus *Coleus*) is the most studied group taxonomically and economically, and *Coleus* members are more widely spread geographically.

Different groups of phytochemicals have been reported in the genus *Plectranthus*, and each group has potential medicinal values. The important medicinal compounds such as diterpenoids, monoterpenoids, phenolics, and sesquiterpenoids have been reported in *Plectranthus* spp (Lukhoba et al., 2006). This genus has a lot of diversity with regard to its ethnobotanical uses. Diterpenoid compounds extracted from *Plectranthus fruticosus* were tested as antimicrobial agents (Gaspar-Marques et al., 2004). The endemic plant of Saudi Arabia, *Plectranthus asirensis*, also has medicinal values; its extract can be used to treat diaper rash and itching, and it has antiseptic properties (Abulfatih, 1987a,b). The phytochemical analyses of the extracts of *Plectranthus* spp have revealed the presence of abietane diterpenoids and eudesmane sesquiterpene compounds (Orabi et al., 2000; Abdel-Mogib et al., 2002).

P. asirensis is found in the Asir Province of Saudi Arabia; it is known as Shaar-al-Qarood in Arabic. *P. asirensis* is a branched aromatic sub-shrub up to ca. 1.5 m tall. Stems are densely hairy and shaggy, with long hairs at its nodes. Leaves are petiolate, densely pubescent to tomentose, oblong/elliptic, up to 12 cm long, and crenulate-serrulate. Many flowers are verticillasters, distantly arranged in terminal, usually simple, sometimes branched, densely hairy, leafless spikes; bracts are small, colorless, and caduceus. Flowers are deep violet, pedecellate, pedicels, capillary, and spreading. Calyces are small, white, hairy, slightly accrecent; they also have red glands, ca. 5.5 mm long fruit, a glabrous throat or tufts of white hairs; the upper lip is 3.5 x 2.5 mm. The corrolla are 11-15 mm long; the lower lip is deeply boat shaped and sharply deflexed. The stamens and style are exerted; nutlets are reddish brown, subspherical, and sized 1.25 x 1.25 mm (Chaudhary, 2001).

DNA barcoding and phylogenetic study of the endemic plant is very important; they allow us to infer the relationships among other taxa within the same genus and those of other genera. DNA barcodes are short DNA sequences of a standardized portion of the genome that can be used in identification and discrimination of a species. The Consortium for the Barcode of Life (CBOL) has proposed *rbcL* and *matK* as DNA barcodes for plants (CBOL Plant Working Group, 2009). Plant DNA barcoding can be used in ecological evolutionary studies (Hajibabaei et al., 2007; Lahaye et al., 2008; Valentini et al., 2009). Chemotaxonomic markers based on phytochemicals can be used for phylogenetic studies; however, their utility is limited. Molecular phylogeny based on DNA data suggests the evolutionary and biogeography histories of the plants. Therefore, we have chosen the endemic plant *P. asirensis* from Saudi Arabia for the phylogenetic study and selection of DNA barcoding loci from the nuclear and chloroplast genomes for its identification.

MATERIAL AND METHODS

Plant collection

P. asirensis was collected from Jabal Shada, Baha, Saudi Arabia. The plant was identified by a botanist at King Saud University (Figure 1) based on morphological markers. The fresh leaves of *P. asirensis* were collected in silica gel for genomic DNA extraction.

Figure 1. Photograph of *Plectranthus asirensis* taken from the wild condition.

DNA isolation and amplification

For polymerase chain reaction (PCR), pure DNA is important for the amplification of any locus of a gene. We used the modified cetyl trimethyl ammonium bromide (CTAB) method (Khan et al., 2007) for the extraction of high quality genomic DNA. A proportion (0.01 g) of dried leaves was ground in 800 µL extraction buffer (100 mM Tris buffer, pH 8, 25 mM EDTA, 2 M NaCl, 3% CTAB, and 3% polyvinylpyrrolidone). The resultant leaf powder was transferred to a microcentrifuge tube and incubated at 65°C for 20 min, with frequent mixing. The mixture was then cooled to room temperature, and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 12,000 rpm for 10 min. The clear upper aqueous portion was then transferred to a new microfuge tube, a 2/3 volume of ice-cooled isopropanol was added, and the whole mixture was incubated at -20°C for 2 h. For the collection of nucleic acid, the mixture was centrifuged at 10,000 rpm for 10 min. The resultant pellet was washed twice with 80% ethanol and then air-dried under a sterile laminar hood. The nucleic acid was dissolved in sterilized distilled water at room temperature, and the contaminating RNA was removed via 10 mg/mL RNase A for 30 min at 37°C. The DNA concentration and purity were determined by measuring the absorbance of the diluted DNA solution at 260 and 280 nm. Furthermore, the quality of the DNA was determined using agarose gel electrophoresis staining with ethidium bromide.

The purified genomic DNA was used in PCR for the amplification of nr-ITS and chloroplast loci using universal primers. The PCR bead (GE healthcare, Spain) was employed for the amplification of these loci. A single reaction was comprised of 20 µL deionized sterile water, 25 ng DNA per reaction volume, and 10 pmol/µL of each forward and reverse primers. After mixing all of the PCR components, the reaction was set up in the Techne thermal-cycler. PCR was conducted for amplification of the nrDNA-ITS locus at 94°C for 5 min (initial denaturation) followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 1 min, and an extension at 72°C for 1 min, with a final extension step of 72°C for 5 min. The reaction was set up for *rbcL* and *rps16* at 94°C for 5 min for initial denaturation, fragment denaturation at 94°C for 45 s, followed by 30 cycles of annealing at 51°C for 45 s, and an extension at 72 °C for 1 min. Similarly, the PCR was carried out for *rpoB* and *rpoC1* at 94°C for 5 min for initial denaturation, fragment denaturation at 94°C for 45 s, followed by 40 cycles of annealing at 53°C for 40 s, and an extension at 72°C for 1 min. The PCR products were purified according to manufacturer instructions (SolGent PCR Purification Kit) prior to sequencing. The amplified PCR product was directly sequenced at Macrogen Inc., South Korea using the dye terminator chemistry.

Phylogenetic analyses

The nrDNA-ITS and chloroplast sequences of *P. asirensis* were subjected to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm our sequences from related genera available in the GenBank database. The sequences of concern for *P. asirensis* were retrieved from the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the study of its phylogeny. All retrieved sequences were edited and assembled using BioEdit v7.0.9.0 (Hall, 1999) prior to construction of the phylogeny. Some sequences were edited manually, with adjustments as needed. All characters were treated as equally weighted and unordered, and the gaps were regarded as missing data. The branch support was evaluated using 1000 bootstrap (BS) replications with random sequence addition, equal weighting, and TBR branch swapping, holding one tree at each replicate. The nrITS sequence generated from *P. asirensis* in the present study has been submitted to the GenBank database (accession No. KJ623266).

RESULTS AND DISCUSSION

P. asirensis (family: Lamiaceae) is an important medicinal and endemic plant of Saudi Arabia. Phylogenetic study and barcoding are very important for this species because the other species in this genus have potential secondary metabolites. DNA-based molecular markers are more reproducible when compared to other markers for identification and authentication. Nuclear and chloroplast DNA markers are currently used for the development of DNA barcoding and phylogenetic studies. We amplified nuclear and chloroplast gene loci from *P. asirensis* for the selection of DNA barcode loci and phylogenetic analysis. The nrDNA-ITS of *P. asirensis* showed 97% similarity via BLAST at the National Center for Biotechnology Information (NCBI) GenBank database. However, chloroplast loci of *P. asirensis* viz., *rps16*, *rbcL*, *rpoB*, and *rpoC*1 showed 99, 95, 99, and 97% similarity, respectively, to the sequences of taxa available in the GenBank database. The *rps16* and *rpoB* of *P. asirensis* showed maximum similarity (99%) to the other taxa in the GenBank database and, therefore, are less preferable for DNA barcoding in *P. asirensis*. However, the *rps16* gene showed less similarity to the other taxa available

in the GenBank database [studied in *Senecio asirensis* (family: Asteraceae)] (Khan et al., 2013). The other chloroplast loci, *rbcL* and *rpoC1*, had low similarities to the other species; therefore, they can be used for DNA barcoding in this species. Sequence variation is very important for the development of DNA barcodes at the species level to facilitate plant identification with relative ease. However, all loci of the chloroplast and nuclear genomes are not used for a single plant because some gene loci have more similarities at the species level and are difficult to identify.

The concerned sequences of the same genus were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). The nuclear and chloroplast loci (ITS1, 5.8S RNA, and ITS2) and *rps16* were used for the phylogenetic analysis because the concerned species of this genus were available in the GenBank database. The sequence alignment was performed using the CLUSTAL X v1.81 (Thompson et al., 1997). The size of the nrDNA-ITS and chloroplast loci *rps16*, *rbcL*, *rpoB*, and *rpoC1* were 579, 825, 623, 471, and 560 bp, respectively. The ends of all sequences were confirmed using the GenDoc software (Nicholas and Nicholas, 1997) before reconstruction of the phylogeny. The phylogenetic tree was reconstructed among the various species of *Plectranthus* using MEGA5 (Tamura et al., 2011) with the maximum likelihood method (Jones et al., 1992). The phylogenetic relationship was assessed among the 5 species of *Plectranthus* with 2 outgroups (i.e., *Asterohyptis seemannii* and *Eriope exaltata*) using nrITS and, similarly, 22 species of the *Plectranthus* with 2 outgroups (i.e., *Castilleja tenuis* and *Bignonia aequinoctialis*) using *rps16* gene loci. There were a total of 345 and 698 positions in the final dataset when the nrDNA-ITS and *rps16* gene loci were used for phylogenetic analysis. *P. asirensis* had a close relationship to *P. caninus* and *P. coeruleus* at a BS level of 53% using the nrDNA-ITS and *rps16* loci, respectively (Figures 2 and 3). Thus, based on both loci, *P. asirensis* showed a close relationship to the other species as shown in the figures (1 and 2) based on the sequence similarity that was also found in the GenBank database. The *rps16* intron region has been used for the assessment of sequence variation and phylogenetic study among the accession of *Morus* spp. Thus, all accessions of *Morus* spp clustered according to sequence similarity (Yuhua et al., 2011). We are in the process of collecting population samples of *P. asirensis* for an assessment of sequence variations in the nrDNA-ITS and the *rps16* gene. Furthermore, we can develop a DNA barcode for this species based on sequence variation in these loci, which will help in the identification of this species. These data would also be helpful in the conservation of the species using biotechnological approaches because endemic plant species are important genetic resources of any nation.

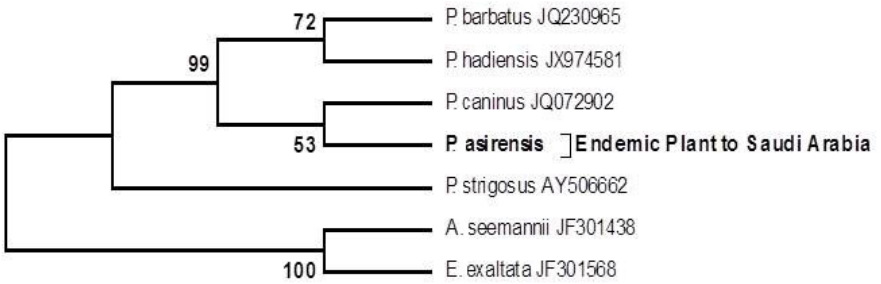

Figure 2. Phylogenetic trees produced among *Plectranthus* spp using the *nrITS* gene (ITS1 + 5.8rRNA + ITS2). Numbers above the branches show maximum likelihood bootstrap values.

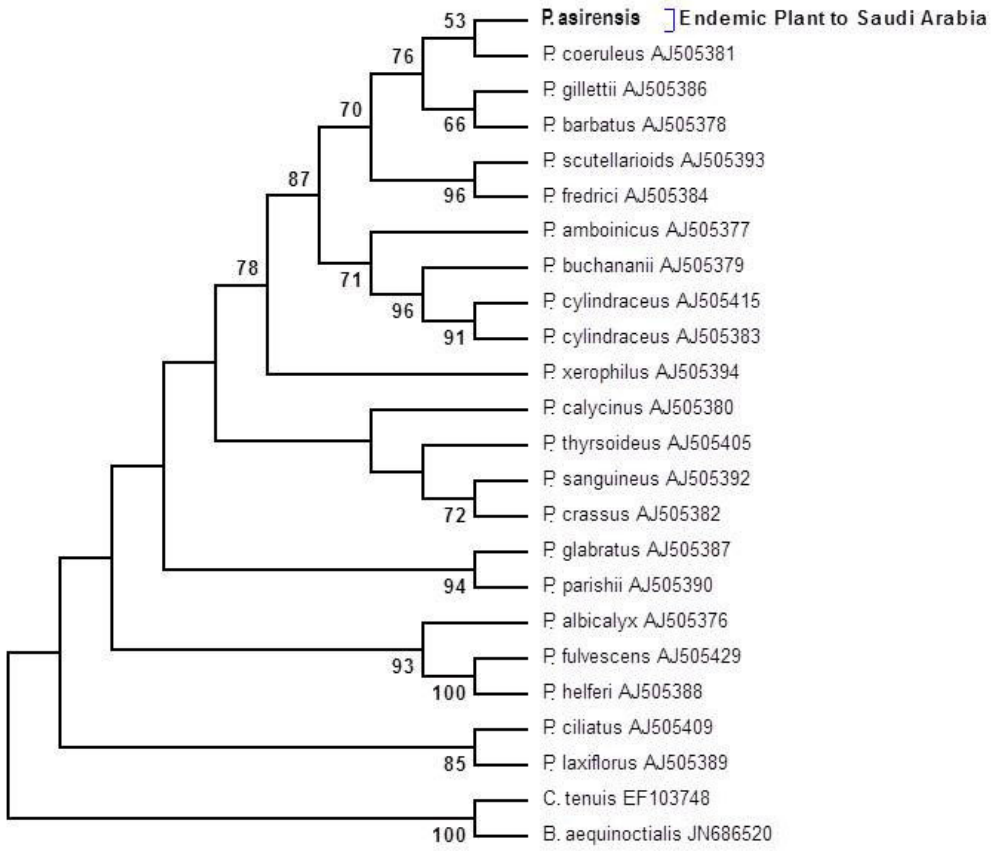

Figure 3. Phylogenetic tree produced among the *Plectranthus* spp using the chloroplast locus *rps16*. Numbers above the branches indicate maximum likelihood bootstrap values; bootstrap values <50% were deleted.

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