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Unraveling systematic inventory of *Echinops* (Asteraceae) with special reference to nrDNA ITS sequence-based molecular typing of *Echinops abuzinadianus*

M.A. Ali[1], F.M. Al-Hemaid[1], J. Lee[2], A.A. Hatamleh[1], G. Gyulai[3] and M.O. Rahman[4]

[1]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[2]Department of Environment and Forest Resources, Chungnam National University, Yuseong-gu, Daejeon, Republic of Korea
[3]Institute of Genetics and Biotechnology, St. István University, Gödöllo, Hungary
[4]Department of Botany, University of Dhaka, Dhaka, Bangladesh

Corresponding author: M.A. Ali
E-mail: alimohammad@ksu.edu.sa

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ABSTRACT. The present study explored the systematic inventory of *Echinops* L. (Asteraceae) of Saudi Arabia, with special reference to the molecular typing of *Echinops abuzinadianus* Chaudhary, an endemic species to Saudi Arabia, based on the internal transcribed spacer (ITS) sequences (ITS1-5.8S-ITS2) of nuclear ribosomal DNA. A sequence similarity search using BLAST and a phylogenetic analysis of the ITS sequence of *E. abuzinadianus* revealed a high level of sequence similarity with *E. glaberrimus* DC. (section *Ritropsis*). The novel primary sequence and the secondary structure

of ITS2 of *E. abuzinadianus* could potentially be used for molecular genotyping.

Key words: *Echinops abuzinadianus*; Asteraceae; Endemic species; Saudi Arabia; Internal transcribed spacer

INTRODUCTION

The genus *Echinops* L. (subtribe Echinopsinae of Cynareae, family Asteraceae) consists of ca. 120 species (Bobrov, 1997; Susanna and Garcia-Jacas, 2007) that are distributed in tropical Africa, the Mediterranean basin, temperate regions of Eurasia, Central Asia, Mongolia, and north-eastern China, with most occurring in the Caucasus and the Middle East (Jäger, 1987; Sánchez-Jiménez et al., 2010). The key taxonomic characteristics of the Cynareae, such as the pappus and the type and density of the indumentum on the stems, leaves, and phyllaries, cannot easily be used to distinguish between *Echinops* species (Mozaffarian, 2006; Sánchez-Jiménez et al., 2010).

In Saudi Arabia, *Echinops* L. is represented by 10 species: *E. abuzinadianus* Chaudhary, *E. erinaceus* Kit-Tan, *E. glaberrimus* DC., *E. hystrichoides* Kit-Tan, *E. macrochaetus* Fresen, *E. mandavillei* Kit-Tan, *E. polyceras* Boiss., *E. sheilae* Kit-Tan, *E. viscosus* DC., and *E. yemenicus* Kit-Tan. Of these, *E. abuzinadianus*, *E. mandavillei*, and *E. sheilae* are endemic to Saudi Arabia (Chaudhary, 2000).

Since the first report of the utility of internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) in plants (Baldwin, 1992), the nuclear ribosomal ITS region (nrITS) has revolutionized species-level plant phylogenetics, because evolution has generally homogenized sequence variation among the numerous ribosomal DNA copies within an individual, making direct sequencing of this region possible for most systems. This, coupled with the availability of universal primers and elevated substitution rates compared to most chloroplast regions, make it accessible and appropriate for resolving interspecific phylogenetic relationships. Although reliance on nrITS as the sole source of phylogenetic evidence has come under criticism because of certain features of its evolution, it remains the most efficient locus for generating species-level phylogenetic inferences in most plant groups (Ali et al., 2013, 2014). The ITS region has been chosen previously for investigating the molecular phylogeny of *Echinops* (Garnatje et al., 2005; Al-Hemaid et al., 2014) and other genera of Cynareae (Susanna et al., 1999; Vilatersana et al., 2000; Hidalgo et al., 2006; Wang et al., 2005, 2007). The present study aimed to unravel the systematic inventory of Saudi Arabian *Echinops*, with special reference to the nrDNA ITS sequence-based molecular genotyping of the endemic species, *E. abuzinadianus*.

MATERIAL AND METHODS

Taxon sampling

Leaf material of *E. abuzinadianus* was collected from a herbarium specimen housed at the National Herbarium and Genbank, National Agriculture and Animal Resources Research Center, Riyadh, Saudi Arabia, and the taxonomic identification was confirmed after consulting the Flora of Saudi Arabia (Chaudhary, 2000). Information on taxonomic status, protolog

(http://www.theplantlist.org/), distribution (Chaudhary, 2000), and nucleotides (http://www.ncbi.nlm.nih.gov/) was also collected.

Genomic DNA isolation, amplification and sequencing

Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The nrDNA ITS regions were amplified using the primers ITS1 and ITS4 (White et al., 1990). A DNA amplification for 35 cycles was conducted by polymerase chain reaction (PCR). The PCR products were purified using a SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea). Sequencing was conducted using a BigDye® Terminator cycle sequencing kit (Applied Biosystems, USA) and an ABI 3100 Avant capillary sequencer (Applied Biosystems).

Phylogenetic analyses

The sequences were performed by BLAST in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and edited using the ABI Sequence Navigator (Perkin-Elmer/Applied Biosystems). Sequence alignment was performed using Clustal X, version 1.81 (Thompson et al., 1997), and subsequently manually adjusted using BioEdit (Hall, 1999).

The ITS sequences of nrDNA from 18 species of *Echinops* were retrieved from GenBank (Table 1). Two species were chosen as outgroup members [*Brachylaena discolor* DC. from the tribe Tarchonantheae Kostel and *Cardopatium corymbosum* (L.) Pers. from the subtribe Cardopatiinae Less.] according to previous study based on their morphological (Petit, 1988) and molecular characteristics (Susanna et al., 2006; Sánchez-Jiménez et al., 2010) (Table 1). Gaps were treated as missing data, and the generated sequences were submitted to GenBank. The boundaries between the ITS1-5.8S and ITS2 gene for *E. abuzinadianus* were determined according to the span referred to features of *Echinops* nrDNA ITS sequences available in GenBank. The ITS2 sequence was extracted from the complete set of the ITS sequences, and used in secondary structure prediction using tools from the ITS2 database (Koetschan et al., 2012). The aligned data matrix was exported as a nexus file and subsequently analyzed using maximum likelihood (ML) in MEGA5 (Tamura et al., 2011).

RESULTS

Systematic inventory

E. abuzinadianus Chaudhary: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-120839); Protolog: Fl. Kingdom Saudi Arabia 2(3): 198, 418 (2000); Distribution: Saudi Arabia; Type Information: Collector: Chaudhary, Locality: Near Abha, Collection Date: 1981-11-17; GenBank Nucleotide: No record.

E. erinaceus Kit-Tan: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-2642); Protolog: Ann. Bot. Fenn. 32(2): 124 (1995); Distribution: Oman, Saudi Arabia, and South Yemen; GenBank Nucleotide: No record.

E. glaberrimus DC.: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-4375); Protolog: Ann. Sci. Nat., Bot., ser. 2, 2: 260 (1834); Distribution:

Saudi Arabia; GenBank Nucleotide: GU134559 (voucher W2004-13486 tRNA-Leu (trnL) gene and trnL-trnF intergenic spacer, partial sequence; chloroplast), GU116509 (isolate W2004-13486 ITS1-5.8S ribosomal RNA gene, and ITS2, complete sequence).

Table 1. Plant accessions used for the molecular phylogenetic analysis of *Echinops*.

	Taxa	GenBank accession No.
Ingroup		
	sect. *Acantholepis* (Less.) Jaub. & Spach	
1	*E. acantholepis* Jaub. & Spach	AY8262223
	sect. *Chamaechinops* Bunge	
2	*E. fastigiatus* Kamelin & Tscherneva	GU116503
3	*E. humilis* M. Bieb	GU116514
4	*E. integrifolius* Kar. & Kir	GU116517
	sect. *Echinops* L.	
5	*E. freitagii* Rech. f.	GU116504
6	*E. kotschyi* Boiss.	GU116520
	sect. *Hamolepis* R.E. Fr.	
7	*E.hoehnelii* Schweinf	GU116506
	sect. *Hololeuce* Rech. f.	
8	*E. hololeucus* Rech. f.	GU116513
	sect. *Nanechinops* Bunge	
9	*E. gmelini* Turcz.	GU116510
	sect. *Oligolepis* Bunge	
10	*E. cornigerus* DC.	GU116552
11	*E. echinatus* Roxb.	GU116497
12	*E. lipskyi* Iljin	GU116523
	sect. *Phaeochaete* Bunge	
13	*E. longifolius* A. Rich	GU116524
	sect. *Psectra* Endl.	
14	*E. strigosus* L.	AY5386532
	sect. *Ritropsis* Greuter & Rech. f.	
15	*E. dichrous* Boiss. & Hausskn.	GU116495
16	*E. endotrichus* Rech. f.	GU116500
17	*E. glaberrimus* DC.	GU116509
	sect. *Terma* Endl.	
18	*E. exaltatus* Schrad.	GU116501
Outgroup		
19	*Brachylaena discolor* DC.	AY8262363
20	*Cardopatium corymbosum* (L.) Pers.	AY8262383

E. hystrichoides Kit-Tan: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-138093); Protolog: Ann. Bot. Fenn. 32(2): 124 (1995); Distribution: Saudi Arabia and North Yemen; GenBank Nucleotide: GU134570 (voucher BC-Hein3942 tRNA-Leu (trnL) gene and trnL-trnF intergenic spacer, partial sequence; chloroplast), GU116515 (isolate BC-Hein3942 ITS1-5.8S ribosomal RNA gene, and ITS2, complete sequence).

E. macrochaetus Fresen: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-129388); Protolog: Museum Senckenbergianum 3: 69 (1840); Distribution: Saudi Arabia; GenBank Nucleotide: No record.

E. mandavillei Kit-Tan: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-10419); Protolog: Ann. Bot. Fenn. 32(2): 122 (1995); Distribution: Saudi Arabia; GenBank Nucleotide: KJ187107 (ITS-1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS2, partial sequence).

E. polyceras Boiss.: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-34304); Protolog: Diagn. Pl. Orient. ser. 1, 10: 85 (1849) [Mar-Apr 1849]; Distribution: Saudi Arabia; GenBank Nucleotide: No record.

E. sheilae Kit-Tan: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-52766): Protolog: Ann. Bot. Fenn. 32(2): 118 (1995); Distribution: Saudi Arabia; GenBank Nucleotide: No record.

E. viscosus DC.: Taxonomic Status: Synonym of *E. sphaerocephalus* L. (http://www.theplantlist.org/tpl1.1/record/gcc-106683); Protolog: Fl. Germ. Excurs. 856: Distribution: Saudi Arabia; GenBank Nucleotide: No record.

E. yemenicus Kit-Tan: Taxonomic Status: Accepted name (http://www.theplantlist.org/tpl1.1/record/gcc-107827); Protolog*:* Ann. Bot. Fenn. 32(2): 118 (1995); Distribution: North Yemen; GenBank Nucleotide: GU134616 (voucher BC-Hein3806 tRNA-Leu (trnL) gene and trnL-trnF intergenic spacer, partial sequence; chloroplast), GU116548 (isolate BC-Hein3806 ITS1-5.8S ribosomal RNA gene, and ITS2, complete sequence).

Sequence characteristics and phylogenetic analyses

The combined length of the ITS region (ITS1-5.8S-ITS2) in *E. abuzinadianus* was 634 bp (Figure 1). The ITS1 region was 252 bp long (GC content 55%), the 5.8S gene was 164 bp long (GC content 54%), and the ITS2 region was 219 bp long (GC content 53%). A BLAST of the ITS sequence of *E. abuzinadianus* indicated maximum identity (99%) with *E. glaberrimus* (Table 2).

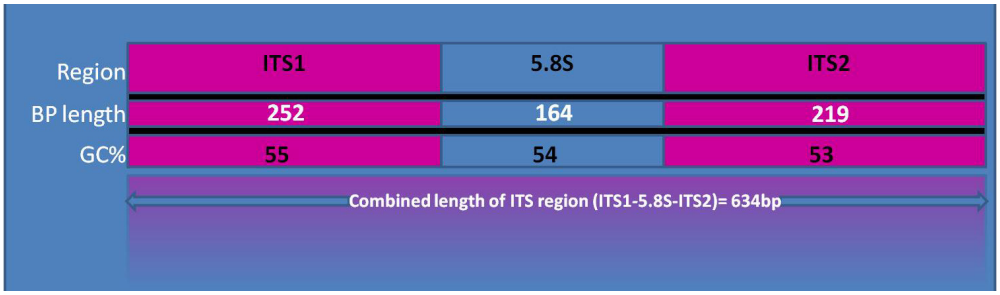

Figure 1. Sequence characteristics of *Echinops abuzinadianus*.

Table 2. Similarity of internal transcribed spacer (ITS) sequences of *Echinops abuzinadianus* according to BLAST.

Taxon	Max. score	Total score	Query cover (%)	Identity (%)	Accession
Echinops glaberrimus	1129	1129	100	99	GU116509
E. spinosissimus	1122	1122	100	99	HE687348
E. yemenicus	1123	1123	100	98	GU116548
E. orientalis	1120	1120	100	98	GU116532
E. chardinii	1120	1120	100	98	GU116490
E. leucographus	1118	1118	100	98	GU116549
E. gaillardotii	1116	1116	100	98	GU116507
E. parviflorus	1114	1114	100	98	GU116533
E. nitens	1114	1114	100	98	GU116529
E. lipskyi	1110	1110	100	98	GU116523
E. viscosus	1110	1110	100	98	AY826283
E. cornigerus	1110	1110	100	98	AY538645
E. griffithianus	1109	1109	100	98	GU116512
E. polygamus	1105	1105	100	98	GU116534
E. leucographus	1105	1105	100	98	GU116522

Parsimony analysis of the entire ITS region resulted in 311 maximally parsimonious trees, with a consistency index of 0.752, a homoplasy index of 0.457, and a retention index of 0.756. There were 526 positions in the final dataset, 101 of which were parsimony-informative. The phylogenetic tree provided clear resolution at the sectional level, with *E. abuzinadianus* nested within the clade of the section *Ritropsis*, which confirms the result of a previous study (Sánchez-Jiménez et al., 2010). The ML analysis results were similar, so only the ML topology is discussed hereafter (Figure 2).

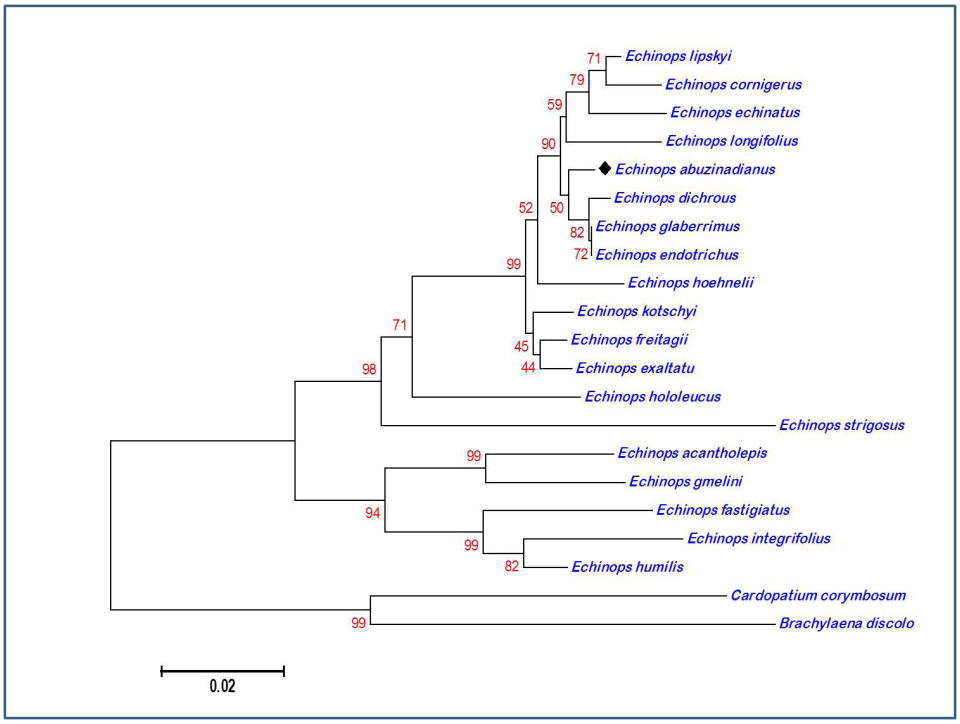

Figure 2. Maximum likelihood tree inferred from analysis of sequence data of the internal transcribed spacer region of nuclear ribosomal DNA.

Molecular typing of *E. abuzinadianus*

The present study revealed that there were eight nucleotide differences between *E. abuzinadianus* and *E. glaberrimus*, at alignment positions 60, 61, 66, 81, 188, 226, 441, 552, and 622 (Figure 3 and Table 3). The nrDNA ITS2 secondary structures of *E. mandavillei* and *E. glaberrimus* were constructed and compared (Figure 4A and B). The secondary structures of nrDNA ITS2 in these two species contained a central ring (primary ring) and four helices (I, II, III, and IV). The ITS2 secondary structures differed in the four helical regions between the two species in stem loop number, size, position, and screw angle. On the basis of the ITS2 secondary structure, *E. abuzinadianus* could be distinguished from allied species or other species of the genus.

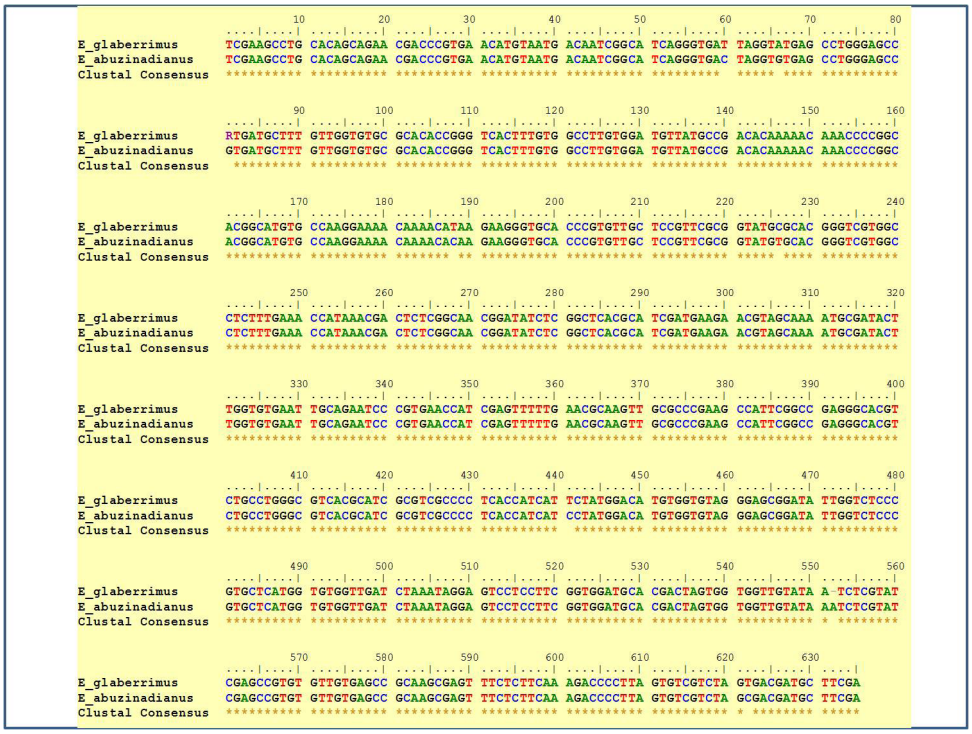

Figure 3. Comparison of ITS sequences of nuclear ribosomal DNA in between *Echinops glaberrimus* and *E. abuzinadianus*. Positions without asterisks in the Clustal line denotes the differences in base pairs in between two sequences.

Table 3. Differences in base pairs between the internal transcribed spacer (ITS) sequences of *Echinops glaberrimus* and *E. abuzinadianus*.

Position in sequence alignment	*Echinops glaberrimus*	*E. abuzinadianus*
60	T	C
66	A	G
81	R	G
188	T	C
226	C	T
441	T	C
552	-	A
622	T	C

A tandem repeat finder (Benson, 1999) was used to detect repeats in the ITS sequences; differences in substitution rate can discriminate functional genes from pseudogenes (Buckler and Holtsford, 1996a,b). The distribution and pattern of nucleotide substitutions in all of the sequences was investigated using Hypermut (Rose and Korber, 2000). This program was originally designed to study the sequence evolution of HIV, and identifies excessive levels of G to A mutations. It assumes that all differences arise from a single substitution, and all substitutions observed in each sequence are compared to the reference sequence, and their physical locations in the sequences are graphically illustrated (Figure 5).

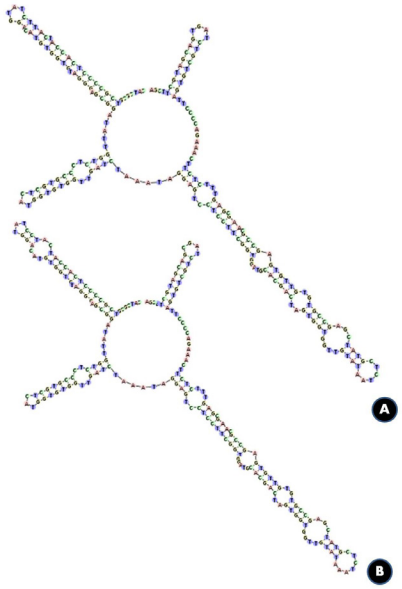

Figure 4. Secondary structures of the ITS2 regions of *Echinops abuzinadianus* (**A**) compared to *E. glaberrimus* (**B**).

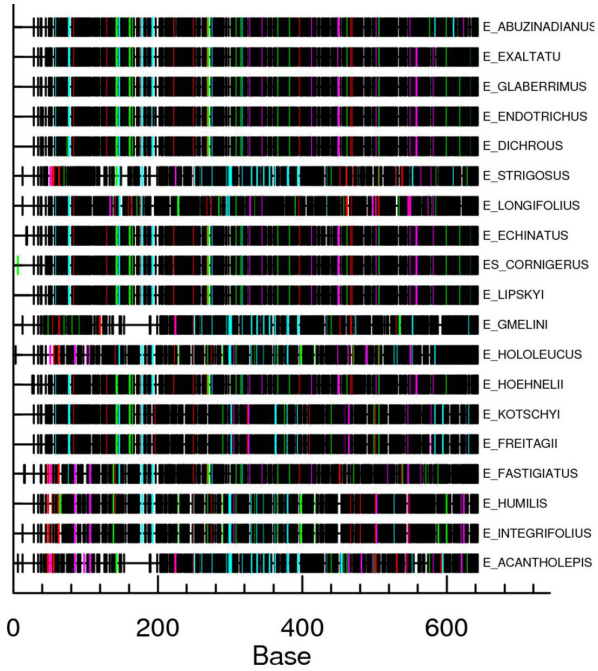

Figure 5. Schematic illustration of the distribution of substitution sites across the ITS region obtained from 20 species of *Echinops*, using *Brachylaena discolor* as reference. red = GG > AG; cyan = GA > AA; green = GC > AC; magenta = GT > AT; black = not G > A transition; yellow = gap.

DISCUSSION

Morphological identification depends on sufficient experience, and can easily be affected by geographical environment and biocoenosis (Rai et al., 2012). DNA barcode technology is widely used, because the genomic sequence is little affected by individual characteristics and developmental stages, and it is relatively a simple procedure (Liu et al., 2011); therefore, DNA barcoding is an effective supplement to traditional/classical morphological methods. Species identification using DNA barcodes has been successfully used across all plant and animal groups. Plant DNA barcoding is now changing over the essence of species identification, and consequently is contributing to the molecularization of taxonomy. DNA barcodes provide practical, standardized, species-level identification tools that can be used for biodiversity assessment, life history and ecological studies, and forensic analysis (Ali and Choudhary, 2011; Ali et al., 2014). Global DNA barcoding efforts have resulted in the formation of the Consortium for the Barcode of Life (CBOL). The Barcode of Life Database (BOLD) contains more than 2.7 million specimen records, with 2 million having barcodes of over 170,000 species (Ratnasingham and Hebert, 2007). In the present study, we used the ITS region of nuclear ribosomal DNA for the DNA barcoding of *E. abuzinadianus*. The primary sequences of ITS, as well as the secondary structures of ITS2, provided sufficient molecular morphological characteristics to distinguish *E. abuzinadianus* from other species of the genus. Recently, a number of studies have suggested that DNA secondary structures are crucial for genomic stability and cellular processes, such as transcription (Bochman et al., 2012; Salvi and Mariottini, 2012). The ITS2 region has also been confirmed as a novel barcode for identifying medicinal plant species (Chen et al., 2010; Gao et al., 2010; Yao et al., 2010; Song et al., 2012); this study has expanded the application of the secondary structure of the ITS2 region as a molecular signature for species identification. The China Plant BOL Group has proposed that ITS/ITS2 should be incorporated into the core barcode for seed plants (Li et al., 2011), and the present study has broadened the application of the ITS2 region to *E. abuzinadianus* in particular.

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