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ORIGINAL ARTICLE

DNA barcodes for the identification of *Anoectochilus roxburghii* and its adulterants

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Abstract

Main conclusion **Chinese medicinal herbs have a similar appearance and are easily confused, complicating identification via traditional methods. This study provided a scientific approach, based on DNA barcoding, to accurately and rapidly identify *Anoectochilus roxburghii* and its adulterants. This technology complements traditional methods of identification of medicinal herbs.**

A comparison of the DNA barcodes matK, psbA-trnH and ITS2 was performed to verify that the ITS2 sequence is an effective marker for rapidly and accurately identifying *A. roxburghii* and its closely related species. Genomic DNA extracted from *A. roxburghii* and its adulterants were used as templates and the ITS2 sequence was amplified using PCR amplification and sequencing. Species identification was conducted using BLAST1 and neighbor-joining trees. The 12 samples were successfully classified into four species based on the ITS2 sequence. The ITS2 sequence length of *A. roxburghii* was 253 bp. The average intra-specific genetic distance of *A. roxburghii* was 0.0021, markedly lower than the inter-specific genetic distance between *A. roxburghii* and its adulterants (0.0380). Our findings illustrate that ITS2 sequence can accurately and efficiently distinguish *A. roxburghii* and its adulterants. In addition, the results provided reference for molecular identification of other Chinese herbal medicine.

Keywords *Anoectochilus roxburghii* · ITS2 · Sequence analysis · Molecular identification

Abbreviations

ITS2	Internal transcribed spacer 2
PCR	Polymerase chain reaction
TBE	Tris-borate-EDTA
NJ	Tree neighbor-joining tree
TLC	Thin layer chromatography
HMM	Hidden Markov model

Introduction

Anoectochilus roxburghii, a member of the Orchidaceae, is a valued plant species in many Asian countries where it is used for ornamental and medicinal purposes (Tseng et al. 2006; Shao et al. 2014a). Because of its unique medicinal properties, such as its notable curative effects of breaking fevers and cooling blood, as well as eliminating dampness and detoxifying, *A. roxburghii* has been called "the king of medicine" (Zhang et al. 2007; Du et al. 2008). The main chemical composition of *A. roxburghii* includes polysaccharides, amino acids, alkaloids, flavonoids and organic acids (He et al. 2004; Zheng et al. 2013; Shao et al. 2014b). It has been used to treat diabetes, tumors, hyperliposis and hepatitis (Chang et al. 2000; Du et al. 2008; Cui et al. 2013). However, because of the plant's demanding

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growth conditions, slow rate of growth, low seed germination rate and long-term excavation, *A. roxburghii* resources have become limited (Luo et al. 2012). Adulteration has been observed because of the supply shortage and high price. Consequently, the rapid and accurate authentication of *A. roxburghii* is required to ensure safety in the medicinal herb trade. *A. roxburghii* and its adulterants vary greatly in pharmacological activities but are similar in profile. Therefore, it is difficult to distinguish *A. roxburghii* based solely on morphological characteristics (Liu et al. 2012). Microscopy and TLC have been used to distinguish *A. roxburghii* from its adulterants to ensure quality and therapeutic effects (Zheng et al. 1997). However, both of the ways are time-consuming and inaccurate because *A. roxburghii* has varied shapes and a complex chemical composition. Therefore, discovering an easy and accurate method for the identification of *A. roxburghii* has attracted attention.

DNA barcoding uses a short DNA sequence from a standard locus as a species identification tool (Hebert and Gregory 2005; Kress et al. 2005; Miller 2007). This method is not affected by species development stage (leaves, seeds, flowers, etc.) and medicine materials (raw herbs or powder) and it has been widely used in identification of medicinal plants and herbs (Han et al. 2008). The third International Barcode of Life Conference (CBOL) presented that matK and rbcL sequences as the international common bar code sequence, ITS/ITS2 sequence and psbA-trnH as complementary sequence. However, DNA barcodes have not been used to identify the medicinal plant *A. roxburghii* or its adulterants. In this study, we chose psbA-trnH, matK and ITS2 as DNA barcodes and discovered that the ITS2 sequence was effective in identifying *A. roxburghii* and its adulterants.

Materials and methods

Plant materials

In this study, all 12 samples were collected from Fujian, Zhejiang, Jiangxi, Guizhou, Guangxi and Taiwan (Table 1), of which eight samples were *A. roxburghii*, two were *Anoectochilus formosanus*, one was *Ludisia discolor* and one was *Goodyera schlechtendaliana*. All plant species were identified by Professor Runhuai Hu (The Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang A & F University). A total of 15 individuals were selected randomly from each of the 12 populations, and about 0.5 g of fresh leaves per plant was collected and immediately dried in silica gel. All samples were stored at −20 °C until being processed. In addition, *Anoectochilus geniculatus, Anoectochilus albolineatus* and *Anoectochilus lylei* ITS2 sequences were downloaded from GenBank.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from silica gel-dried leaves according to the protocol associated with the Plant Genomic DNA Kit (Tiangen Biotech Co, China). For the candidate barcodes, ITS2, psbA-trnH and matK, PCR reaction conditions and universal DNA barcode primers were used (Table 2). PCR amplification of the ITS2 region was carried out in a Peltier Thermal Cycler PTC0200 (BioRad Lab Inc, USA) using approximately 30 ng of genomic DNA as the template in a 25-μl reaction mixture (1× PCR buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of each primer (synthesized by Sangon Co, China), and 1.0 U of Taq DNA Polymerase (TaKaRa). The PCR products were run on a 1.0 % agarose gel in 0.5× TBE buffer and purified with the TIANGel Midi Purification Kit (Tiangen Biotech Co, China). The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were sequenced on an ABI 3730XL sequencer (Applied Biosystems Inc.) using the amplification primers.

Sequence alignment and analysis

Consensus sequences and contig generation were performed by CodonCode Aligner V 2.06 (CodonCode Co, USA). The ITS2 sequences were subjected to HMM model analysis to remove the conserved 5.8S and 26S (or equivalent) rRNA sequences (Keller et al. 2009). The sequences were then aligned using Clustal W (Thompson et al. 1994) and bootstrap tests were conducted using 1000 resampling to assess the confidence of the phylogenetic relationships using MEGA 4.0 (Tamura et al. 2011). Moreover, the bootstrap values were displayed on the branches of the NJ tree. The inter/intra-specific variation of the samples was calculated according to Kress et al. (2005) and Song et al. (2009). Two methods of species identification, including BLAST1 and nearest distance method, were performed as described previously (Ross et al. 2008; Chen et al. 2010).

Results

Amplification and sequencing success

Total genomic DNA was isolated from the samples and the PCR amplification rate of the ITS2 sequences from plants of *A. formosanus* was 100 %. High-quality bidirectional sequences were obtained using the PCR of the ITS2 and psbA-trnH barcodes. The sequencing quality of matK was

Table 1 Plant samples used in the present study

Code	Original accessions	Longitude (E)	Latitude (N)	Source	GenBank accession number
1	*Anoectochilus roxburghii*	117°13′	24°36′	Nanjing, Fujian province	KR815836
2	*Anoectochilus roxburghii*	117°10′	25°44′	Yongan, Fujian province	KR815837
3	*Anoectochilus roxburghii*	116°02′	25°12′	Wuping, Fujian province	KR815838
4	*Anoectochilus roxburghii*	119°12′	27°45′	Qingyuan, Zhejiang province	KR815828
5	*Anoectochilus roxburghii*	120°08′	27°51′	Wencheng, Zhejiang province	KR815829
6	*Anoectochilus roxburghii*	115°11′	25°03′	Anyuan, Jiangxi province	KR815830
7	*Anoectochilus roxburghii*	108°10′	26°14′	Leishan, Guizhou province	KR815831
8	*Anoectochilus roxburghii*	110°20′	22°43′	Beiliu, Guangxi province	KR815832
9	*Anoectochilus formosanus*	120°49′	24°36′	Miaoli, Taiwan province	KR815833
10	*Anoectochilus formosanus*	120°47′	23°51′	Nantou, Taiwan province	KR815839
11	*Ludisia discolor*	117°14′	24°32′	Nanjing, Fujian province	KR815834
12	*Goodyera schlechtendaliana*	119°31′	28°40′	Wuyi, Zhejiang province	KR815835
13	*Anoectochilus geniculatus*	–	–	GenBank	JN166059
14	*Anoectochilus albolineatus*	–	–	GenBank	JN166058
15	*Anoectochilus lylei*	–	–	GenBank	JN166060

Table 2 Primers and reaction conditions used in the present study

Locus	Primer name	Sequences (5′–3′)	PCR conditions
matK	KAF	CTATATCCACTTATCTTTCAGGAGT	94 °C 5 min
	K8R	AAAGTTCTAGCACAAGAAAGTCGA	94 °C 30 s, 45 °C 30 s, 72 °C 1 min, 30 cycles
			72 °C 7 min
psbA-trnH	fwd	GTTATGCATGAACGTAATGCTC	95 °C 4 min
	rev	CGCGCATGGTGGATTCACAATCC	94 °C 30 s, 55 °C 1 min, 72 °C 1 min, 35 cycles
			72 °C 10 min
ITS2	S2F	ATGCGATACTTGGTGTGAAT	94 °C 5 min
	S3R	GACGCTTCTCCAGACTACAAT	94 °C 45 s, 57 °C 50 s, 72 °C 1 min, 35 cycles
			72 °C 10 min

inferior to that of the other two loci, but a complete sequence was still produced by the assembly. The alignment results indicated that the eight populations of *A. roxburghii* and two of *A. formosanus* psbA-trnH sequences did not harbor any variation sites. For these two species, the psbA-trnH sequence was less divergent and thus not suitable for species identification. The matK sequences also lacked a variation site, which indicated that this locus was not suitable to differentiate among the two species. However, all 12 samples were successfully classified into four species based on the ITS2 sequence. For this reason, only the ITS2 barcode was used in further analyses.

Sequence analysis and inter/intra-specific variations

In this study, all 12 ITS2 sequences were obtained from the *A. roxburghii*, *A. formosanus*, *L. discolor* and *G. schlechtendaliana* species. The *A. geniculatus A. albolineatus* and *A. lylei* ITS2 sequence was downloaded from GenBank (JN166059, JN166058, JN166060). All 15 ITS2 sequences were included in the final analysis. The characteristics of these sequences are summarized in Table 3. The length of the ITS2 sequences of *A. roxburghii*, *A. formosanus*, *A. geniculatus*, *A. albolineatus* and *A. lylei* were 253 bp and the average GC content were 48.37, 48.62, 47.83, 47.83 and 48.62 %, respectively. The length of the ITS2 sequences of *L. discolor* and *G. schlechtendaliana* were 250 and 252 bp; their average GC contents were 50.00 and 49.60 %, respectively. 43 nucleotide variation sites were found in the ITS2 sequence, contained 21 single base-point mutations. The point mutations (48.84 %) were either purine substitutions (A by G, and G by A; $n = 9$), or pyrimidine substitutions (C by T, and T by C; $n = 8$), or purine/pyrimidine substitutions (G by T, T by G,

Table 3 Sequence characteristics of the ITS2 barcode of *Anoectochilus roxburghii* and its adulterants

Sequence characteristics	ITS2
Length in *A. roxburghii* (bp)	253
Length in *A. formosanus* (bp)	253
Length in *L. discolor* (bp)	250
Length in *G. schlechtendaliana* (bp)	252
Length in *A. geniculatus* (bp)	253
Length in *A. albolineatus* (bp)	253
Length in *A. lylei* (bp)	253
G+C content range (mean) in *A. roxburghii* (%)	48.37
G+C content range (mean) in *A. formosanus* (%)	48.62
G+C content range (mean) in *L. discolor* (%)	50.00
G+C content range (mean) in *G. schlechtendaliana* (%)	49.60
G+C content range (mean) in *A. geniculatus* (%)	47.83
G+C content range (mean) in *A. albolineatus* (%)	47.83
G+C content range (mean) in *A. lylei* (%)	48.62

T by A and A by T; $n = 4$) (Table 4). When calculated according to the K2P model, the average intra-specific genetic distance (0.0021) is far less than the average inter-specific genetic distance (0.0380). The results demonstrated that the differences between the inter-specific and intra-specific divergences were significant.

Species identification capability of the ITS2 barcode and NJ tree analysis

BLAST1 and the nearest distance as two discrimination methods were used to evaluate the ability of the barcoding sequences to distinguish among species in the given samples. The results showed that ITS2 performed well when using either BLAST1 or distance discrimination method at the species level. The NJ tree is an ideal analytical method that can generate a graphical representation of the ITS2 sequence results, especially when they are closely related. It can effectively determine the power of a given locus combination to discriminate among species (Zhang et al. 2012). In this study, the NJ tree demonstrated that the *A. roxburghii* samples clustered into one clade, whereas *A. formosanus*, *A. geniculatus*, *A. albolineatus*, *A. lylei*, *L. discolor and G. schlechtendaliana* clustered into their own clades (Fig. 1). Thus, the NJ tree clearly distinguished between *A. roxburghii* and closely related species. Overall, this study demonstrated that ITS2 was efficient and effective.

Secondary structure of the ITS2 sequence

To identify the species, we focused not only on the divergence of primary sequences of ITS2, but also on the use of variations in the secondary structures of ITS2. According to the ITS2 database built by Koetschan et al. (2010), the ITS2 secondary structure of *A. roxburghii* and its adulterants contain a central ring and four similar helices: Helix I, II, III, and IV. Moreover, Helix III was relatively longer than the others which were shown in Fig. 2. Secondary structures of different species formed the diversified helices in number, size and loci of loops, degree of angles from the center of the spiral arm. It revealed that their differences were mainly present in Helix I and III after analyzing the ITS2 secondary structure. The secondary structure of *A. formosanus* shows that Helix I was different from the others and the secondary structure of *L. Discolor* is close to *A. roxburghii*, but it can still distinguish them by Helix IV. The secondary structure of *G. schlechtendaliana* contains a small center ring but a big ring in Helix I. Therefore, we could distinguish *A. roxburghii* and its adulterants at the molecular level by comparing ITS2 secondary structures.

Discussion

DNA barcoding using short DNA region to identify *A. roxburghii* and its close species was quick and accurate, which was a more precise identification method at the molecular level. In this study, three DNA regions, ITS2, psbA-trnH and matK, had been recommended as primary DNA barcodes for plants according to the rules of CBOL. Then, we tested the ability of these three DNA regions through PCR amplification to discriminate between *A. roxburghii* and its adulterants, but only ITS2 sequence was capable of being used to distinguish at the inter-specific level to a greater extent than psbA-trnH or matK. DNA barcodes must simultaneously contain enough variability to be used for species identification and adequate conservative regions for the design of universal primers (Stoeckle 2003). The matK sequence belongs to the chloroplast genome sequences, via maternal uniparental inheritance; thus, constructed branch relations often cannot truly reflect the evolutionary direction within the species (Han et al. 2011). Meanwhile, the matK sequences amplified success rate is very low and the psbA-trnH sequences lack a variation site so we have not chosen them for further experiments. However, in the identification of other species, it should utilize three different DNA barcodes. The results indicated that ITS2 sequences proved to fully comply with these requirements as a new technical means and basis.

New methods for identifying traditional Chinese medicines should focus on accuracy, digitization, repeatability, simplicity and practicality (Zhao et al. 2013). The DNA sequence identification technique has important significance comparing with the traditional methods. Our

Table 4 Variable sites in *Anoectochilus roxburghii* and its adulterants

Site	6	24	28	33	35	38	41	42	44	50	51	52	54	55	74	78	81	82	90	105	106	107
A. roxburghii (Nanjing)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A
A. roxburghii (Yongan)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A
A. roxburghii (Wuping)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A
A. roxburghii (Qingyuan)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A
A. roxburghii (Wencheng)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	C	T	A	C	C	C	A	A
A. roxburghii (Anyuan)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	C	T	A	C	C	C	A	A
A. roxburghii (Leishan)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	C	T	A	C	C	C	A	A
A. roxburghii (Beiliu)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A
A. formosanus (Miaoli)	A	A	G	G	A	G	C	T	T	T	C	G	C	T	T	T	A	C	C	C	A	A
A. formosanus (Nantou)	A	A	G	G	A	G	C	T	T	T	C	G	C	T	T	T	A	C	C	C	A	A
L. discolor (Nanjing)	A	G	G	A	T	G	C	G	T	C	G	A	T	C	T	C	G	T	C	C	A	G
G. schlechtendaliana (Wuyi)	G	A	T	A	G	G	T	T	C	C	G	G	T	T	T	C	A	C	C	T	G	A
A. geniculatus (GenBank)	A	A	G	G	A	T	C	T	T	T	G	G	C	T	T	T	A	C	T	C	A	A
A. albolineatus (GenBank)	A	A	G	G	A	T	C	T	T	T	G	G	C	T	T	T	A	C	T	C	A	A
A. lylei (GenBank)	A	A	G	G	A	G	C	T	T	T	G	G	C	T	T	T	A	C	C	C	A	A

Site	122	145	153	160	168	183	186	192	193	197	198	210	215	217	228	229	230	231	237	243	248
A. roxburghii (Nanjing)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Yongan)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Wuping)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Qingyuan)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Wencheng)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Anyuan)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Leishan)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. roxburghii (Beiliu)	G	–	A	T	A	C	T	G	G	A	A	A	T	A	T	A	A	A	C	A	C
A. formosanus (Miaoli)	G	–	A	T	A	C	T	G	G	A	A	G	T	A	T	A	A	A	C	A	C
A. formosanus (Nantou)	G	–	A	T	A	C	T	G	G	A	A	G	T	A	T	A	A	A	C	A	C
L. discolor (Nanjing)	G	–	T	T	G	T	T	A	A	T	C	C	T	T	T	–	–	–	T	G	C
G. schlechtendaliana (Wuyi)	A	A	T	T	G	T	C	G	G	T	G	G	A	A	C	A	–	–	A	G	T
A. geniculatus (GenBank)	G	–	A	A	A	C	T	G	G	A	A	G	T	A	T	A	A	A	C	A	C
A. albolineatus (GenBank)	GG	–	A	A	A	C	T	G	G	A	A	G	T	A	T	A	A	A	C	A	C
A. lylei (GenBank)	G	–	A	T	A	C	T	G	G	A	A	G	T	A	T	A	A	A	C	A	C

43 nucleotide variation sites were found in the ITS2 sequence, contained 21 single base-point mutations. The point mutations (48.84 %) were either purine substitutions (A by G, and G by A; $n = 9$), or pyrimidine substitutions (C by T, and T by C; $n = 8$), or purine/pyrimidine substitutions (G by T, T by G, T by A and A by T; $n = 4$)

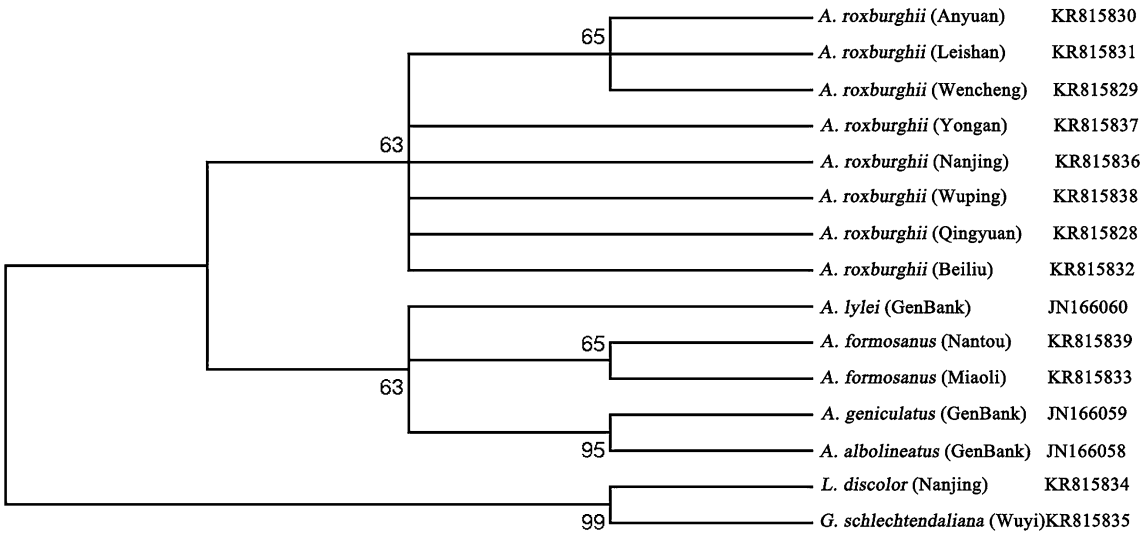

Fig. 1 The NJ tree of *Anoectochilus roxburghii* and its closely related species with the ITS2 sequences. The bootstrap scores (1000 replicates) are shown (≥50 %) for each branch

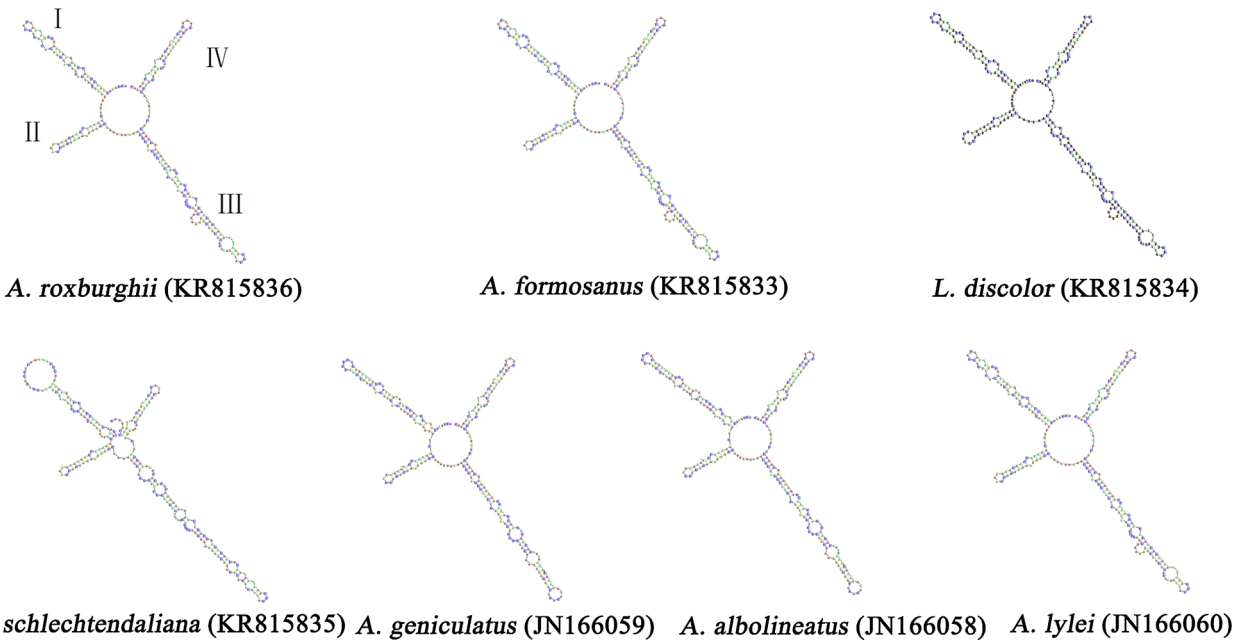

Fig. 2 The secondary structure of ITS2 of *Anoectochilus roxburghii* and its adulterants. Their differences were mainly present in Helix I and III, so we could distinguish them at the molecular level

results highlighted the advantages of using the ITS2 sequence as a DNA barcode from three aspects: first, the ITS2 sequence provides a precise and simple method for the identification of medicinal herbs as a complement to traditional identification methods. Second, it provides an important reference value in the exploration of the relationships between different species of rare herbs. Third, DNA sequencing technology can be applied to control the quality of herbal sources. Several studies used DNA barcoding methods to identify Chinese herbal medicine, such as *Dendrobium officinale Kimura et Migo* (Zhang et al. 2013), *Ajuga ciliata Bunge* (Han et al. 2008) and *Paeoniae Radix Rubra* (Sun et al. 2011), have verified the stability and conservation of the ITS2 sequence. The current study was the first to use a DNA barcode molecular identification technology to distinguish *A. roxburghii* and its adulterants and this study expanded the application of the ITS2 sequence to the medicinal plant field and assured drug safety on clinical use. In conclusion, ITS2 as a DNA barcode would broaden our understanding of plant resource

classification and phylogenetics. We could collect and sort out the related data about ITS2 for further experiments and build a DNA library of species classification and evolutionary information. Therefore, this technology still has much room for development and prospects.

Conclusion

In summary, we confirmed that the ITS2 sequence can be used as a standard DNA barcode to distinguish *A. roxburghii* and its adulterants at the molecular level. Cluster analysis using the ITS2 barcode in *A. roxburghii* was basically consistent with traditional plant morphology. Also, this study not only broadened the application of the ITS2 sequence in the medicinal field, but also provided an efficient method to solve classification problems in a wide range of plant at the species levels.

Author contribution statement TW Lv, QS Shao designed research and wrote the paper, HZ Wang performed research, RD Teng and MY Li helped in collection of plant materials and WS Zhang, LL Zhang helped in data analysis. All authors read, reviewed and approved the manuscript.

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