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DNA barcoding and rapid identification of the precious herb Herba Anoectochili

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[ABSTRACT] Herba Anoectochili is a commonly used medicinal material. However, its adulteration is a serious concern. Due to the similar morphological characteristics of Herba Anoectochili and its adulterants, traditional identification techniques often fail to distinguish between them accurately, which is not conducive to the circulation management and safety of the medicinal materials. To improve the distinction between Herba Anoectochili and its adulterants accurately, this study identified 41 Herba Anoectochili and its adulterant samples based on the ITS2 sequence. Sequence characteristics, Basic Local Alignment Search Tool (BLAST) application, genetic distance, construction of phylogenetic tree, secondary structure prediction, and other methods showed the ITS2 sequence to accurately identify Herba Anoectochili from its adulterants. Furthermore, in this study, we designed a specific primer, based on the ITS2 sequence, and established a real-time PCR detection system for the rapid, sensitive, and specific identification of the original plant of Herba Anoectochili. Compared to DNA barcoding technology, this method has shorter detection time, stronger specificity, and higher sensitivity, which lays the foundation for the rapid identification of Herba Anoectochili.

[KEY WORDS] Herba Anoectochili; ITS2; DNA barcode; Real-time PCR; Rapid identification

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Introduction

Herba Anoectochili, dry whole grass of *Anoectochilus formosanus* and *Anoectochilus roxburghii* (Orchidaceae), is a traditional and precious Chinese herbal medicine widely used in China [1]. It is also widespread in the tropical regions of India, through the Himalayas, south-east Asia, and Indonesia up to New Caledonia, Hawaii. In China, it grows in the wild across Fujian, Zhejiang, Jiangxi, Guangdong, Guangxi, and Taiwan Provinces, although it is mainly cultivated in the Fujian Province [2]. Recent pharmacological research revealed that Herba Anoectochili can regulate blood glucose and blood lipids, reduce blood pressure, protect the liver, manifest anti-inflammatory, analgesic, diuretic, sedative, and anti-oxidative effects, and improve osteoporosis [3-9] owing to its various pharmacologically active compounds such as flavonoids, polysaccharides, volatile compounds, alkaloids, terpenoids, steroids, and glycosides [2, 8, 10-11]. Its rare resources and powerful effects make the wild growing Herba Anoectochili very expensive. In 1990, the Fujian Provincial Government promulgated the document No. 18 (1990), under "Fujian Regulations on the Protection and Management of Wild Medicinal Materials Resources in Fujian Province", and listed Herba Anoectochili as a "provincial-level protected wild medicinal species". The scarcity of resources and associated high prices have led to the emergence of many Herba Anoectochili adulterants in the market; the major ones being *Ludisia discolor* and *Goodyera schlechtendaliana* [12]. All the four species belong to the family Orchidaceae, which makes it difficult to accurately distinguish between them, especially when they are dried and have lost most of their morphological characteristics. However, the chemical composition and content are highly divergent between Herba Anoectochili and its adulterants. Therefore, the Chinese herbal medicine market urgently needs a rapid, effective, and simple identification

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method to guarantee market standardization and clinical safety of Herba Anoectochili.

Accurate identification of herbal medicines guarantees safe medication. The efficacy and safety of Chinese herbal medicines is interfered due to the confusion of original species and lack of identification methods [13]. DNA barcoding is a technique that uses a standard DNA sequence as a marker to achieve fast, accurate, and automated species identification. It has been a research hotspot in taxonomy and identification, in recent years, showing broad application prospects in species identification [14]. It can be used to identify species at the genetic level, regardless of their own traits and external factors. DNA barcoding can generate repeatable and stable results *via* standardized experimental protocol, which makes it suitable for the identification of TCM and beneficial for alleviating the lack of qualified identification talents and. Researchers have proposed different sequences and sequence combinations as barcodes for identifying plants. Among them, Chen *et al* [15] established the DNA barcoding system for identifying herbal medicine products based on the ITS2 and *psb*A-*trn*H barcodes, which have been widely used.

Real-Time PCR (RT-PCR) merges the polymerase chain reaction chemistry with that of fluorescent reporter molecules in order to monitor the yield of amplified products during each cycle of the PCR reaction. Basically, the qPCR instrument consists of a thermal cycler with an integrated excitation light source [a lamp, a laser, or light emitting diode (LED)], a fluorescence detection system or fluorimeter, and appropriate software that displays the recorded fluorescence data as a DNA amplification curve; for the latter, a dsDNA intercalating dye or fluorophore-labeled probe is required to be added to the reaction mixture [16-17]. TaqMan probes are oligonucleotides, containing a donor fluorescent moiety at the 5′-end and an acceptor fluorescent moiety at the 3′-end that quenches the fluorescence emitted from the donor molecule due to their close proximity. During the extension phase, the bound hydrolysis probe is degraded by the 5′–3′-exonuclease activity of DNA polymerase, generating fluorescence from the donor. This process is repeated in each cycle without interfering with the exponential synthesis of the PCR products [18].

In this study, ITS2 sequence was used to identify Herba Anoectochili and its adulterants. Specific primers and probes were designed according to ITS2 sequence and verified by RT-PCR, eventually providing a scientific basis and guarantee for the rapid and accurate identification of Herba Anoectochili and its drug safety.

Materials and Methods

Sampling

In this study, 27 samples of *A. roxburghii*, 5 samples of *A. formosanus*, 6 samples of *L. discolor*, and 3 samples of *G. schlechtendaliana* were obtained through wild-harvesting and purchase from medicinal materials market. All samples were identified by Professor XIONG Fu-Liang of Wuhan University of Technology. The voucher specimens and materials were kept in the Chinese Medicine Resources and Molecular Identification Laboratory of Wuhan University of Technology. In addition, 4 ITS2 sequences of *A. roxburghii*, 4 ITS2 sequences of *A. formosanus*, 3 ITS2 sequences of *L. discolor* and 2 ITS2 sequences of *G. schlechtendaliana* were downloaded from the GenBank database. A total of 54 test samples were involved in this study, as shown in Table 1.

Table 1 Plant samples used in the present study

Species	Sample ID/GenBank accession number	Source
Goodyera schlechtendaliana	HM021571; KY508554	\
Goodyera schlechtendaliana	BYL201901; BYL201902; BYL201903	Hangzhou, Zhejiang
Anoectochilus roxburghii	HNJ201601; HNJ201604; HNJ201605; HNJ201606; HNJ201608; HNJ201609; HNJ201610; HNJ201612; HNJ201615; HNJ201616; HNJ201617; HNJ201618; HNJ201620; HNJ201623; HNJ201627; HNJ201628;	Yong'an, Fujian
Anoectochilus roxburghii	HNJ201619; HNJ201625; HNJ201629; HNJ201630; HNJ201631; HNJ201632; HNJ201633; HNJ201636; HNJ201639;	Zhangzhou, Fujian
Anoectochilus roxburghii	HNJ201634; HNJ201635	Longyan, Fujian
Anoectochilus roxburghii	KR815831; KR815830; KR815837; KR815838;	\
Anoectochilus formosanus	HNJ201607; HNJ201613; HNJ201621; HNJ201622; HNJ201638;	Yong'an, Fujian
Anoectochilus formosanus	KR815839; KR815833; GQ396668; GQ328777;	\
Ludisia discolor	KR815834; KY508564; KY745866	\
Ludisia discolor	XYL201701	Baoting, Hainan
Ludisia discolor	XYL201702; XYL201703	Wuzhishan, Hainan
Ludisia discolor	XYL201901; XYL201902; XYL201903	Haikou, Hainan

Methods

DNA extraction

DNA from Herba Anoectochili and its adulterants were extracted using the standard operating procedure of DNA barcoding [19]. After wiping the herbs and original plant surfaces with alcohol-soaked cotton, 20–30 mg of each sample was taken and ground with a high-throughput tissue grinder (50 Hz, 120 s). gDNA of each sample was extracted using the

Plant Genomic DNA Kit (Tiangen Biotech Co., China), and its integrity was examined by electrophoresis in a 1.0% agarose gel.

PCR amplification and sequencing

ITS2 sequences of the samples were amplified using universal primers, PCR reaction conditions and amplification procedures [20] have been listed in Table 2. The PCR reaction system was of 25 μL, including 12.5 μL of PCR mix, 1 μL of 2.5 $\mu mol \cdot L^{-1}$ forward primer, 1 μL of 2.5 $\mu mol \cdot L^{-1}$ reverse primer, and approximately 20–100 ng of template DNA. Amplified products were identified by 1.0% agarose gel electrophoresis, and sequenced in both directions on a 3730xl sequencer (Applied Biosystems, United States).

Data analysis

CodonCode Aligner V7.0.1 (CodonCode Co., USA) was used to assemble the sequences [21]. Considering the hidden Markov model-based HMMer annotation method, the 5S and 28S ends were removed and the ITS2 sequence obtained [22]. Sequence alignment was performed, analyzed intraspecific and interspecific genetic distance of test materials based on K2P (kimura 2-parameter) model, clustering tree constructed in MEGA6.0 [23], and 1000 bootstrap replicates were considered to test the support for each branch. The ITS2 secondary structures of Herba Anoectochili and its adulterants were predicted according to the ITS2 database and its website (http://its2.bioapps.biozentrum.uni-wuerzburg.de/).

Table 2 Information about the primers and PCR and RT-PCR reaction conditions

Regions	Primers	Sequences (5′-3′)	PCR reaction condition
ITS2	P3	YGACTCTCGGCAACGGATA	94 °C, 5 min
	E4	RGTTTCTTTTCCTCCGCTTA	94 °C, 30 s; 56 °C, 30 s; 72 °C, 45 s 40 cycles
			72 °C, 10 min
	AF-F	TCGACACCAATTGCCCAGTA	95 °C, 15 s; 60 °C, 20 s; 72 °C, 20 s, 40 cycles
	AF-R	ATCAGCCATGAATGTGTACATT	
	AF-P	TGCACACTTGTGCGACGGGT	

RT-PCR amplification primer and probe design

Primer and probe design was performed using the primer designing tool website (https: //www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) based on the ITS2 sequence of Herba Anoectochili. A pair of specific primers, and a probe, was designed for the non-conserved region of the original species of Herba Anoectochili (Fig. 1). Among them, the 5′ end of AF-P was labeled with a fluorescent reporter group FAM, and the 3′ end was labeled with a quenching group Eclipse. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.. The primers, probe and the reaction conditions have been listed in Table 2.

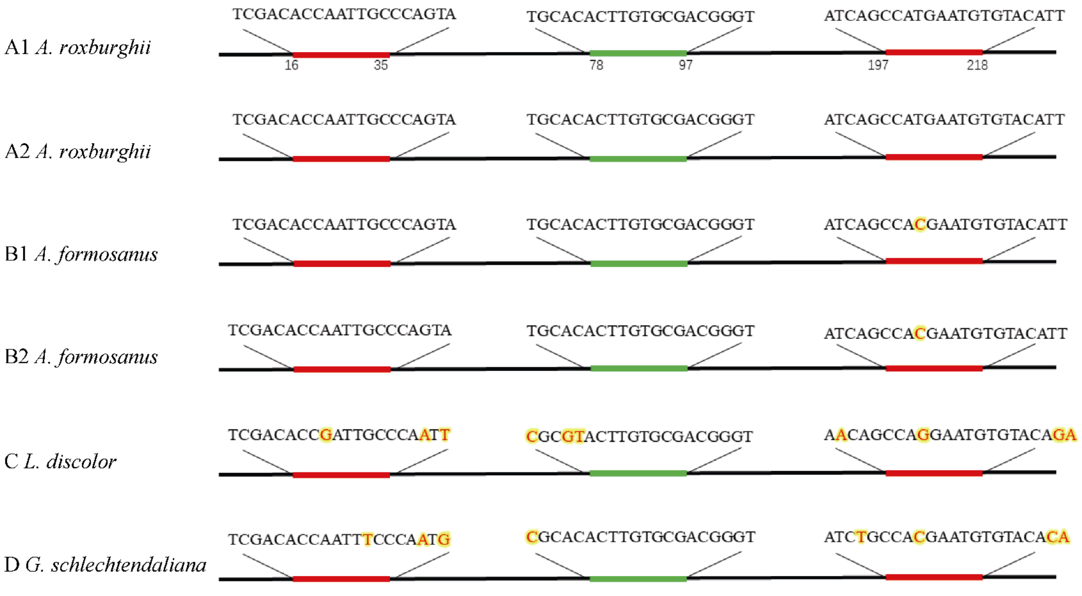


Fig. 1 primers and probe specific position in the alignment of sequences. Red: primers; Green: probe; Red letters in yellow background: specific sites

Specificity of RT-PCR amplification

Using the DNA of Herba Anoectochili and its adulterants as a template, PCR amplification was performed according to the manufacturer's instructions of TransStart Probe qPCR SuperMix (TransGen Biotech Co., Ltd.). The reaction system (20 μL) was prepared referring to the kit instructions, including 10 μL of 2× TransStart Probe qPCR SuperMix, 0.4 μL of the forward and reverse primers, 0.4 μL of the fluorescent probe, and volume adjusted to 20 μL with ddH_2O. Three replicates for each sample were prepared, and the amplification acquisition curve was obtained using Qiagen Corbett Rotor-Gene Q. Data collected were analyzed using Rotor-Gene ScreenClust HRM Software and data quality was determined by fluorescence amplification curves and thresholds (Ct).

Results

DNA barcoding analysis

Length of the ITS2 sequence of *A. roxburghii*, *A. formosanus*, *L. discolor*, and *G. schlechtendaliana* was 253 bp, 253 bp, 250 bp, and 252 bp, respectively. The GC content of all samples ranged from 48.22% to 50%, the average GC content being 48.94% (Table 3). The ITS2 sequences of the original plants of Herba Anoectochili, *A. roxburghii* and *A. formosanus,* were approximately as long as those of *L. discolor* and *G. schlechtendaliana.* Simultaneously, their GC content difference was not significant.

Table 3 Length of the ITS2 sequence and GC content

Species	Length of the ITS2 sequence (bp)	GC content (%)
A. roxburghii	253	48.00–48.62
A. formosanus	253	48.62
L. discolor	250	50
G. schlechtendaliana	252	49.60

SNP site analysis

The length of aligned sequences was 254 bp. In this study, two haplotypes A1 and A2 among *A. roxburghii* samples, two haplotypes B1 and B2 among *A. formosanus* samples, one haplotype C among *L. discolor* samples, and one haplotype D among *G. schlechtendaliana* samples were found. The SNP sites of each sample compared to the sequence of A1 are shown in Table 4. There were 24 SNPs and 3 deletions from 229 bp to 231 bp between A1 and C. There were 25 SNPs, 1 insertion at 145 bp, and 2 deletions from 229 bp to 230 bp between A1 and D. This finding verified that the SNP sites between Herba Anoectochili (*A. roxburghii*) and its adulterants (*L. discolor* and *G. schlechtendaliana*) were significant enough to distinguish between them.

Table 4 SNP site information for all samples

Haplotype	SNP											
	6	24	28	33	35	41	42	44	50	51	52	54
A1	A	A	G	G	A	C	T	T	T	G	G	C
A2	*	*	*	*	*	*	*	*	*	*	*	*
B1	*	*	*	*	*	*	*	*	*	*	*	*
B2	*	*	*	*	*	*	*	*	*	C	*	*
C	*	G	*	A	T	*	G	*	C	*	A	T
D	G	*	T	A	G	T	*	C	C	*	*	T
	55	74	78	81	82	105	106	107	122	254	153	168
A1	T	C	T	A	C	C	A	A	G	-	A	A
A2	*	T	*	*	*	*	*	*	*	*	*	*
B1	*	T	*	*	*	*	*	*	*	*	*	*
B2	*	T	*	*	*	*	*	*	*	*	*	*
C	C	T	C	G	T	*	*	G	*	*	T	G
D	*	T	C	*	*	T	G	*	A	A	T	G
	183	186	192	193	197	198	210	215	217	228	229	230
A1	C	T	G	G	A	A	A	A	A	T	A	A
A2	*	*	*	*	*	*	*	*	*	*	*	*
B1	*	*	*	*	*	*	G	*	*	*	*	*
B2	*	*	*	*	*	*	G	*	*	*	*	*
C	T	*	A	A	T	C	C	*	T	*	-	-
D	T	C	*	*	T	G	G	T	*	C	-	-
	231	237	243	248								
A1	A	C	A	C								
A2	*	*	*	*								
B1	*	*	*	*								
B2	*	*	*	*								
C	-	T	G	*								
D	*	A	G	T								

*: shows the same base with line 1; -: shows deletion; red letter in yellow background: SNP sites in primers and probe

Inter/intra-specific genetic distance analysis

The intra-specific genetic distance of *A. roxburghii* and *A. formosanus* varied from 0 to 0.004, whereas that of *L. discolor* and *G. schlechtendaliana* was 0. As shown in Table 5, the inter-specific genetic distance between *A. roxburghii* and *A. formosanus* varied from 0.004 to 0.012, whereas that between *A. roxburghii* and *L. discolor* varied from 0.099 to 0.104. The inter-specific genetic distance between *A. roxburghii* and *G. schlechtendaliana* varied from 0.104 to 0.109. Similarly, the inter-specific genetic distance between *A. formosanus* and *L. discolor* varied from 0.099 to 0.104 while that between *A. formosanus* and *G. schlechtendaliana* also varied from 0.099 to 0.104. This data showed the maximum inter-specific genetic distance between *A. roxburghii* and *A. formosanus* (0.012) was far less than the minimum inter-specific genetic distance between the two original plants, *A. roxburghii* and *A. formosanus*, and the adulterants, *L. discolor* and *G. schlechtendaliana* (0.099). As a result, Herba Anoectochili and its adulterants could be distinguished by the inter/intra-specific genetic distance.

Table 5 Inter/intra-specific genetic distance of all sample

Species	*A. roxburghii*	*A. formosanus*	*L. discolor*	*G. schlechtendaliana*
A. roxburghii	0–0.004			
A. formosanus	0.004–0.012	0–0.004		
L. discolor	0.099–0.104	0.099–0.104	0	
G. schlechtendaliana	0.104–0.109	0.099–0.104	0.108	0

Phylogenetic analysis

With *Lilium brownie* being an out-group, an NJ tree was constructed based on the ITS2 sequence of all samples (Fig. 2). All the authentic Herba Anoectochili, *A. roxburghii* and *A. formosanus*, constituted a monophyletic clade with support value of 59%. The adulterants *L. discolor* and *G. schlechtendaliana* were clustered into another branch with a bootstrap value of 75%, and each of them was clustered into a robust monophyletic branch (bootstrap value 100% and 99%, respectively). Cluster analysis results demonstrated that the ITS2 sequence can effectively distinguish between Herba Anoectochili and its adulterants.

Secondary structure prediction

The ITS2 secondary structures of Herba Anoectochili and its adulterants were predicted, as shown in Fig. 3. All samples showed a typical one-ring and four-helix structure, with each helix having stem-ring structures of different sizes and numbers. The ITS2 secondary structure of *A. roxburghii* was similar to that of *A. formosanus*. The difference across haplotype A1 (*A. roxburghii*), C (*L. discolor*), and D (*G. schlechtendaliana*) was significant. First, the main ring structure of A1 was simple while that of C and D was more complicated. Secondly, the helix lengths were different across the three. Finally, the size and type of the stem-ring structure of each helix were inconsistent. The results together showed that Herba Anoectochili and its adulterants could be distinguished by the secondary structure of ITS2 sequence.

RT-PCR specificity test

The real-time fluorescent PCR reaction was conducted according to the reaction system and conditions of the test, using the total DNA from *A. roxburghii*, *A. formosanus*, *L. discolor*, and *G. schlechtendaliana* as the template, and specificity of the primer for the original plant of Herba Anoectochili was determined (Table 6). Under the premise of effective amplification of primers (Ct < 35), only the specific curves of *A. roxburghii* and *A. formosanus* DNA were amplified, and no amplification curve was observed in other samples (Fig. 4).

Discussion

In recent years, with the wide application of Herba Anoectochili in many fields such as medicine, health care, beauty, and drinking supplies, the demand for Herba Anoectochili in domestic and foreign markets has been rising, and the market gap has been increasing every year [24]. Currently, adulterants are being sold in the market, using similarly shaped *L. discolor* and *G. schlechtendaliana*. Authentication of herbs has always presented a great challenge. While authentication was originally carried out by organoleptic (i.e., appearance, smell, and taste) and microscopic methods (i.e., examining and analyzing the shape and content of various plant cells or particles under a microscope), these were heavily dependent on personal expertise and could sometimes be imprecise. With the advent of chemical profiling techniques, herbs have been authenticated by the detection of secondary metabolites. These chemical methods have played an indispensable role in quality control and standardization of herbal drugs, although they are time-consuming and need human expertise and specialized equipment [25]. DNA molecular identification techniques rely on DNA fragments that reflect differences across biological individuals, population, or species genomes, and are not limited by environmental changes. These are beneficial for the identification of Chinese herbal medicine varieties. [26].

Samples of *A. roxburghii* and *A. formosanus* in this study were mainly collected from Fujian Province, the main area producing them. Adulterant *L. discolor* was mainly collected from Hainan Province while *G. schlechtendaliana* was collected from Zhejiang Province. However, the samples also included the sequences of Herba Anoectochili and adulterants, as reported by other researches [27]. According to the "Guidelines for DNA Molecular Identification of Chinese Herbal

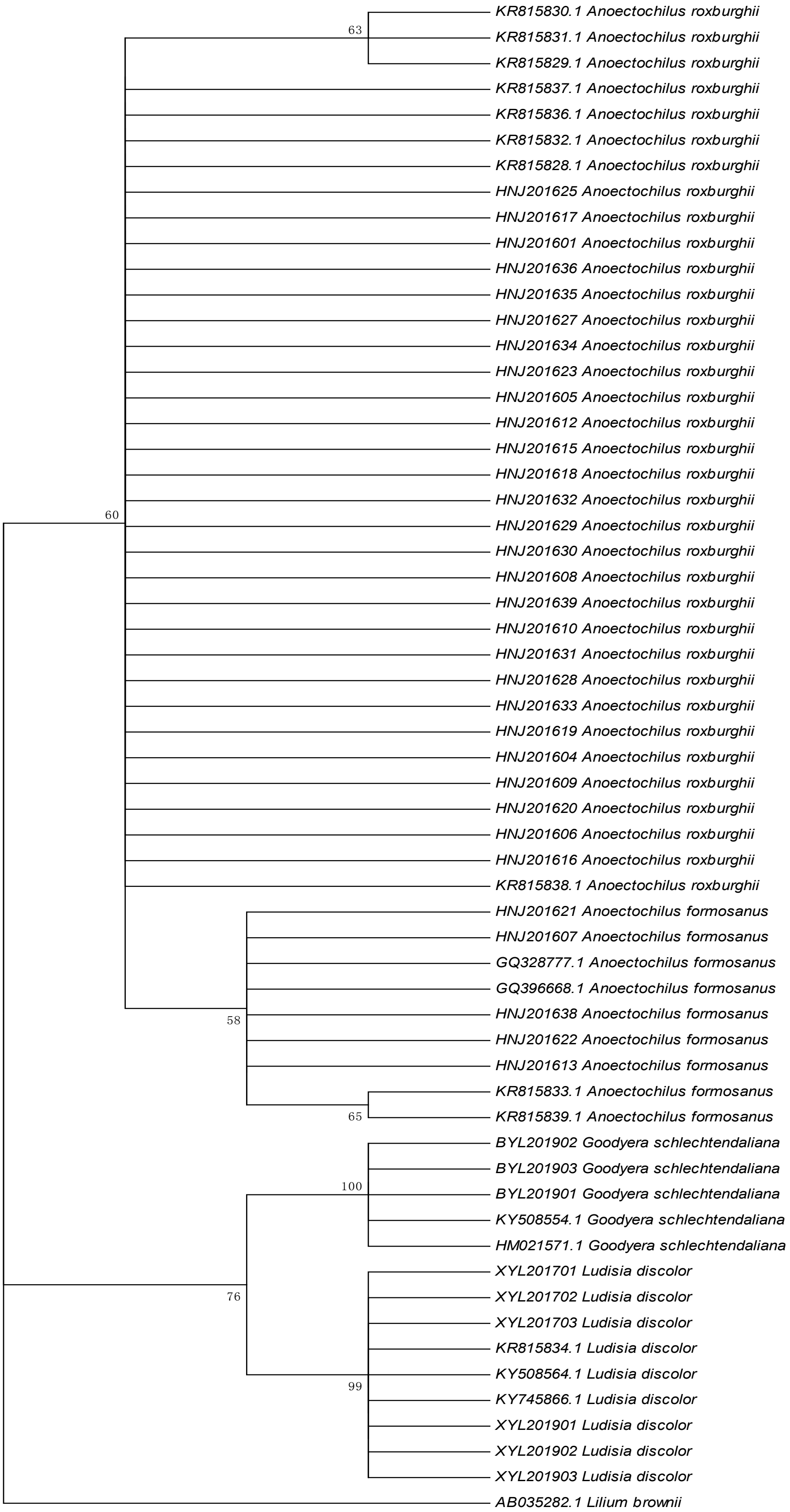
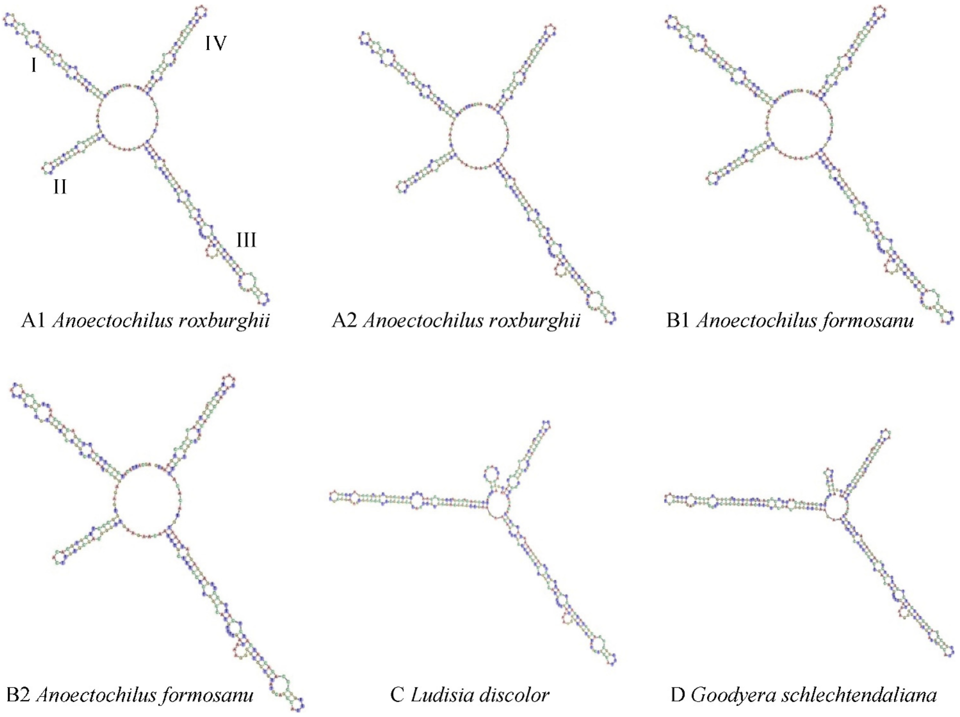


Fig. 2 NJ tree based on ITS2 sequences of Herba Anoectochili and its adulterants. Bootstrap 1000 repetitions; only branches with support ≥ 50% shown



A1 *Anoectochilus roxburghii* A2 *Anoectochilus roxburghii* B1 *Anoectochilus formosanu*

B2 *Anoectochilus formosanu* C *Ludisia discolor* D *Goodyera schlechtendaliana*

Fig. 3 The ITS2 secondary structures of Herba Anoectochili and its adulterants

Table 6 Results of specificity verification test

Species	Ct	Result determination
A. roxburghii	21.46	Genuine
A. formosanus	23.67	Genuine
L. discolor	N	Adulterant
G. schlechtendaliana	N	Adulterant

*N: None

Medicines" published in the 2015 edition of the Chinese Pharmacopoeia, Herba Anoectochili and its adulterants were identified, and the maximum K2P genetic distance between the original species of Herba Anoectochili (0.012) was found to be far less than the minimum K2P genetic distance (0.099) between Herba Anoectochili and its adulterants. It was also found from the NJ tree that Herba Anoectochili is separately clustered into a single branch, which was clearly distinguishable from the adulterants. The ITS2 secondary structure of Herba Anoectochili and its adulterants also had differences in stem-ring size and structure. The results together indicated Herba Anoectochili to be effectively identified by the ITS2 barcode sequence. After comparing 40 ITS2 sequences in a hyper variable region of *A. roxburghii* and *A. formosanus*, a pair of specific PCR primers and TaqMan probes were designed and verified by RT-PCR reaction. The specific curves of Herba Anoectochili DNA were amplified while its adulterants were not. This finding indicated that the primers designed in this study were specific enough to identify Herba Anoectochili rapidly and accurately. Finally, Herba Anoectochili and its adulterants could be identified specifically and rapidly.

Conclusions

This study used DNA barcode technology to distinguish

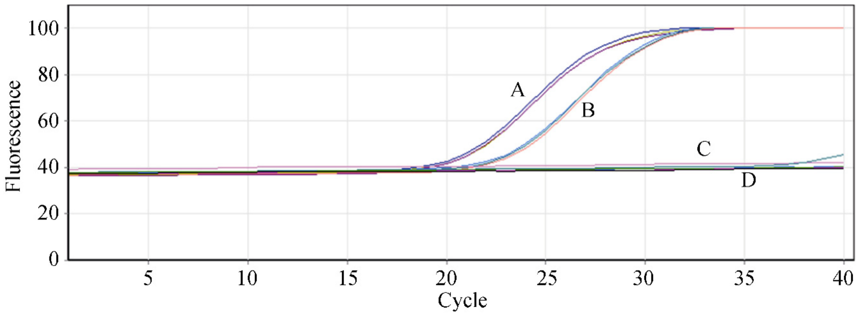


Fig. 4 Amplification curves of Herba Anoectochili and its adulterants. A: *A. roxburghii*; B: *A. formosanus*; C: *L. discolor*; D: *G. schlechtendaliana*

between the precious herb Herba Anoectochili and its adulterants, thereby promoting the clinical medication safety of Herba Anoectochili, and providing a rapid identification technology for protection of the endangered plant Herba Anoectochili. At the genetic level, it laid the foundation for the molecular identification and classification system for research and resource development, and utilization of Herba Anoectochili and related species.

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