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Authentication of traditional Chinese medicinal herb "Gusuibu" by DNA-based molecular methods

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ABSTRACT

Ensuring the authenticity of raw materials used as herbs is a key step prior to producing medicines. "Gusuibu" is a traditional Chinese medicine for the treatment of bone diseases. *Drynaria roosii* Nakaike is the botanical origin of "Gusuibu". However, many "Gusuibu" adulterants which are morphologically similar, have been widely used in China. It is important to develop DNA-based markers to efficiently distinguish authentic "Gusuibu" from adulterants. In this study, 21 chloroplast genomes from seven species including *D. roosii* and six "Gusuibu" adulterant species were sequenced. The chloroplast genomes of *D. roosii*, *D. sinica* Diels, *D. bonii* Christ, *D. delavayi* Christ, *D. quercifolia* (L.) J. Sm., *D. propinqua* (Wall.) J. Sm., and *Pseudodrynaria coronans* (Wall.) Ching were 154,181 bp, 151,711 bp, 151,542 bp, 151,709 bp, 151,570 bp, 152,442 bp, and 151,466 bp in length, respectively. Phylogenetic analysis indicated that whole chloroplast genomes could be used to distinguish *D. roosii* from adulterants and between each adulterant. Comparing chloroplast genome sequences, 12 protein-coding genes and eight intergenic sequences with high divergence in chloroplast genomes were identified to exploit specific DNA barcodes and sequence characterized amplified region (SCAR) markers. One specific DNA barcode and three SCAR markers, which were available to distinguish *D. roosii* from adulterants, were developed and four primer pairs were designed. The primer pairs for amplification of DNA barcode and SCAR markers designed in this study will be useful for economically and effectively distinguishing *D. roosii* from adulterants, and for guaranteeing the quality, safety, and effectiveness of "Gusuibu" herbs.

1. Introduction

"Gusuibu" is a traditional Chinese medicine which has been extensively used in the treatment of bone injuries such as bone fracture, osteoporosis and arthritis, and also in the treatment of inflammation, hyperlipidemia, arteriosclerosis, and so on (Chang et al., 2003; Jeong et al., 2005; Li et al., 2011; Lin et al., 2002; Ma et al., 1996; Sun et al., 2004; Wong and Rabie, 2006). In the Pharmacopoeia of the People's Republic of China, "Gusuibu" is limited to the dried rhizome of *Drynaria roosii* (ChPC, 2015), which contains the active ingredient naringin (≥ 0.5% by dry weight). *D. roosii*, belonging to the family Polypodiaceae, is an epiphytic, epilithic perennial pteridophyte with a fleshy rhizome (Zhang et al., 2013). In China, many other fern species were also named as "Gusuibu", including five *Drynaria* species *D. sinica, D. bonii, D. delavayi, D. quercifolia,* and *D. propinqua*, and even other species *Pseudodrynaria coronans* and *Davallia formosana* (Zou et al., 2011). The distribution of some species was overlapping. It was common for "Gusuibu" to be misused or mixed used by people and pharmaceutical company. However, only traces of naringin were detected in rhizomes of *D. quercifolia* and *P. coronans*, and no naringin was detected in the other five species (Li et al., 2003; Du et al., 2002). Hence, except for *D. roosii*, all other species were treated as adulterants of "Gusuibu" (Zou et al., 2011). There are two speculative reasons for "Gusuibu" adulterants to be widely used. First, all the adulterants are epiphytic pteridophytes with a fleshy rhizome which is very similar to *D. roosii* in morphology. It is difficult to distinguish the closely related species by local people due to lack of plant knowledge. Second, no cultivated "Gusuibu" was available, so the herbs of "Gusuibu" almost were

Abbreviations: SCAR, sequence characterized amplified region; IRs, inverted repeats; LSC, large single-copy region; SSC, small single-copy region; tRNA, transfer RNA; rRNA, ribosomal RNA; PCGs, protein-coding genes; IGS, intergenic sequence; Pi, nucleotide diversity

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collected from wild resources. With the rapid increase in the consumption, resources have been decreasing. In the herb market, dried rhizomes of adulterants were intentionally sold as these are difficult to be distinguished from the rhizomes of *D. roosii* with the unaided eye. Indiscriminate application of these herbal medicines can cause inconsistent therapeutic effects and even unforeseen side effects.

Recently, many researchers have succeeded to some extend in distinguishing the dried rhizome of *D. roosii* from adulterants by microscopic or chromatographic methods (Liu and Liu, 2003; Zhou et al., 1996). However, the anatomical traits sometimes vary within the same species due to external factors and it is not possible to discriminate, using the phytochemical profiles, between closely related species that share similar morphological characteristics or chemical profiles (Han et al., 2016). Also the technologies are complicated and costly, which resulted in low accuracy and efficiency in authenticating herbs. Molecular genetic tools based on DNA sequence variations have been used for species identification. In recent years, DNA-based molecular methods have been developed to distinguish authentic herbs from adulterants (Chen et al., 2014; Michel et al., 2016; Xiong et al., 2018). DNA barcoding, relying on short and standard DNA sequences, is an effective tool for rapidly and accurately identifying plant species. Due to sufficient variation between species but minimal variation within species, many single-locus such as *matK, rbcL, trnH-psbA*, *ITS*, and *trnL-F*, and combined multi-locus such as *rbcL + matK,* and *matK + atpF-atpH + psbK-psbI* were widely used in the identification of plants grown on the land (Cabelin and Alejandro, 2016; Fazekas et al., 2008; Li et al., 2012; Saarela et al., 2013; Tnah et al., 2019). Xue and Xue (2008) used *trnL-trnF* spacer region with real-time scorpion PCR method to authenticate the *D. roosii* from related adulterants. However, no primers were universal and the real-time PCR method was costly. Meanwhile, detection of adulterants using *D. roosii* species-specific scorpion was a failure. It is a common limitations for the mentioned traditional barcodes, including the lack of universal primers, the low PCR success rate and the amplification of pseudogenes. Due to the low evolution rate and certain information on sequence, chloroplast genome was proposed as potential alternative to traditional DNA barcoding (Li et al., 2015). The size of chloroplast genome ranges between 120 and 160 kb in length in almost all land plants (Tonti-Filippini et al., 2017; Wicke et al., 2011). It's conserved sequence contains about 130 genes, with a typical circular quadripartite structure comprising two identical copies of inverted repeats (IRs), separated by a large single-copy region (LSC) and a small single-copy region (SSC).With the development of next-generation sequencing, the complete chloroplast genome sequence has been successfully used as a plant super-barcode to distinguish closely related species in some taxa (Wang et al., 2018a; Meng et al., 2018). On the contrary, chloroplast genome data can still be costly and requires a complicated bioinformatics process before being used as super-barcode, including short DNA fragments assembly and genome annotation. Therefore, super-barcode is effective but still has some limitations.

An ideal DNA barcode should be easily retrievable with a universal primer pair, an appropriately short sequence length to facilitate DNA extraction and amplification, and exhibit a barcode gap between intraspecific and interspecific divergences. Since no universal plant barcode works across all plant species, specific DNA barcodes and markers for a target group could be presented as an ideal option that might be effective and economical to identify a target plant (Chase and Fay, 2009; Chen et al., 2010). Specific barcodes and markers could be exploited from the chloroplast genome by comparing the chloroplast genome sequences of the target group. With alignment of the chloroplast genome sequences of target taxa, universal or species specific primers can be designed to differentiate authentic medicine from adulterants. Recently the sequence characterized amplified region (SCAR) marker from chloroplast genome was proved to be a power DNA marker to differentiate closely related species(Kim et al., 2016; Kiran et al., 2010; Moon et al., 2017).

In this study, 21 chloroplast genomes of seven species including *D. roosii*, *D. sinica, D. bonii, D. delavayi, D. quercifolia, D. propinqua*, and *Pseudodrynaria coronans* were sequenced using the Illumina HiSeq X-Ten platform. Comparison of the chloroplast genome structure revealed genetically divergent regions in the seven species. Phylogenetic analysis suggested that the chloroplast genome was available as super-barcode to distinguish "Gusuibu" origin species from adulterants. Furthermore, one specific DNA barcode and three SCAR markers were developed to authenticate "Gusuibu" origin species, and universal and species specific primer pairs were designed. The results will be valuable for the quality control of "Gusuibu" herbs.

2. Materials and methods

2.1. Plant materials, DNA extraction and sequencing

Plant materials including leaves and rhizomes were collected from native habitats and dried using silica gel. Voucher specimens were deposited in the herbarium of the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (Supplementary Table 1). Total genomic DNA was extracted using modified cetyltrimethyl ammonium bromide method (Allen et al., 2006). From each purified sample of total DNA, 0.5 μg was fragmented to construct short-insert (300–500 bp) libraries following the manufacturer's manual (Illumina) and then used for sequencing. The DNA samples were indexed by tags and pooled together in one lane of a Genome Analyzer (Illumina HiSeq X-Ten) for sequencing at BGI-Shenzhen.

2.2. Chloroplast genome assembly and annotation

The paired-end reads were filtered using GetOrganelle pipeline (https://github.com/Kinggerm/GetOrganelle) to get plastid-like reads, and then the filtered reads were assembled using SPAdes version 3.10 (Bankevich et al., 2012). The genome was automatically annotated using CpGAVAS (Liu et al., 2012), and then adjusted using Geneious version 9.1.7 (Kearse et al., 2012). The tRNAscan-SE program (Lowe and Chan, 2016) was used to confirm the tRNA genes. The chloroplast sequence generated in this study was submitted to GenBank (accession number: MK761229-MK761240, MK761242-MK761248, MK789652, MK789653) (Table 1 and Supplementary Table 2). The circular genome maps of all the 21 plastomes were obtained using the Organellar Genome DRAW (OGDRAW) tool (Lohse et al., 2013)

2.3. Genome comparison

mVISTA was used to analyze the divergence in the Shuffle-LAGAN mode among seven species with annotation of *D. roosii* as a reference (Frazer et al., 2004). Sliding window analysis was conducted to determine the nucleotide diversity of the chloroplast genome using DnaSP version5 (Librado and Rozas, 2009).

2.4. Phylogenetic analysis

The 21 whole genome matrix was aligned using MAFFT version 3.73(Katoh and Standley, 2013), and then manually edited using Geneious version 9.1.7 (Kearse et al., 2012). The best-fitting model of nucleotide substitutions was determined according to the Akaike Information Criterion in jModeltest version 2.1.10 (Darriba et al., 2012). The GTR + I + G model was used in both. The maximum likelihood method was used to construct the phylogenetic tree in MEGA7, with 1000 bootstrap replicates (Kumar et al., 2016).

2.5. Development of specific DNA barcodes and SCAR markers

Primer pairs for amplification of genes and SCAR with high divergence were designed using Primer Premier 6 (Supplementary Table 3). The genomic DNA of individual plants from eight species was PCR

Table 1
The characteristics of the chloroplast genomes of seven species.

Characteristic	*D.delavayi*	*D.sinica*	*D.quercifolia*	*D.propinqua*	*P.coronans*	*D.bonii*	*D.roosii*
Squence number	Dd1	Ds1	Dq1	Dp1	Pc1	Db1	Dr1
Accession number	MK761239	MK789652	MK761242	MK761247	MK761244	MK761229	MK761232
Total chloroplast genome (bp)	151,709	151,711	151,570	152,428	151,472	151,505	154,181
Coverage(x)	497	511	385	82	80	190	104
LSC region(bp)	80,931	80,939	80,750	80,935	80,830	80,682	85,897
IR region (bp)	24,582	24,574	24,586	24,975	24,618	24,595	23,424
SSC region(bp)	21,614	21,624	21,648	21,543	21,406	21,633	21,436
coding(bp)	88,551	88,790	88,522	89,174	88,652	88,502	87,894
non-coding(bp)	63,158	62,921	63,048	63,254	62,820	63,003	66,287
Number of genes	115	115	113	114	114	113	113
rRNA genes	4	4	4	4	4	4	4
tRNA genes	27	27	26	26	26	26	26
Protein-coding genes	84	84	83	84	84	83	83
GC content (%)	40.80%	40.80%	40.60%	40.80%	40.70%	40.60%	40.90%
LSC region	39.60%	39.63%	39.33%	39.49%	39.42%	39.29%	39.68%
IR regions	44.40%	44.40%	44.40%	44.60%	44.50%	44.50%	45.10%
SSC region	37.00%	36.80%	36.70%	36.90%	36.80%	36.60%	36.60%

LSC, Large single copy; SSC, Small single copy; IR, Inverted repeat; tRNA, transfer RNA; rRNA, ribosomal RNA.

amplified in a 20 µl volume using 10 pmol primers. The PCR products were verified by gel electrophoresis on a 1.5% agarose gel. To develop specific DNA barcodes, amplification was performed according to the following conditions: 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 6 min. The PCR products were sequenced directly using Sanger method. The sequence alignment and phylogenetic analysis of gene fragments were performed using MEGA7, with 1000 bootstrap replicates. To develop SCAR markers, amplification was performed using species-specific primers (Supplementary Table 3)according to the following conditions: BRF/BRR and S30 F/S30R, 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 50 s, and a final extension at 72 °C for 6 min; LDF/LDR, 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 6 min.

3. Results

3.1. Chloroplast genome organization of different species

To differentiate *D. roosii* from adulterants, 21 chloroplast genomes of 7 species including *D. roosii* and 6 adulterants species of "Gusuibu" were sequenced. *De novo* assembly generated the circular chloroplast genome of five species, including *D. roosii*, *D. sinica, D. bonii, D. delavayi,* and *D. quercifolia*, and single circular sequence of two species including *D. propinqua and P. coronans* were completed after gap filling. The obtained chloroplast genomes ranged from 151,466 bp to 155,348 bp in size and 80× to 794× coverage. *D. roosii* has the largest chloroplast genome (154,163 bp-154,187 bp) (Table 1, Fig. 1, and Supplementary Table 2) which was consistent with previous reported result (Sun et al., 2017). All of the chloroplast genomes had the conserved quadripartite structure found in most land plants consisting of a LSC region with a size of 80,569 bp–85,911 bp, a SSC region with a size range of 21,406 bp–21,648 bp, and a pair of IRs (including IRA and IRB) each with the size range of 23,422 bp– 24,975 bp. The total GC content ranged from 40.6% to 41.0%. The GC contents of the LSC and SSC regions of all the seven species were lower than that of the IRs regions (44.4%–45.1%) because of the higher GC content in the four duplicated ribosomal RNA (rRNA) genes.

All the seven plastomes were highly conserved in gene order and intron number, but were divergent to some extent in gene content. The number of total genes annotated from chloroplast genomes of seven species ranged from 113 to 115. The chloroplast genomes of *D. roosii* encoded 113 genes including 83 protein-coding genes (PCGs), 26 transfer RNA (tRNA) genes and four rRNA genes. The seven species shared 113 identical genes (Table 2). Two genes presented divergence among species. Four species, including *D. propinqua, P. coronans, D. delavayi*, and *D.sinica*, contained an *rps16* gene but other species did not. Except for *D. delavayi*, and *D.sinica*, no species contained *trnI-AAU* gene. In all the chloroplast genomes, five PCGS (*psbA, rps7, rps12, ycf2*, and *ndhB*), six tRNA (*trnR-ACG, trnA-UGC, trnI-GAU, trnH-GUG, trnN-GUU,* and *trnM-CAU*) genes, and four rRNA (*rrn4.5, rrn5, rrn16,* and *rrn23*) genes were duplicated. Except for *ndhB* and *trnM-CAU*, which were located in LSC region, all of the duplicated genes were located in IR regions. Nine intron-containing genes were investigated including six single-intron genes (*atpF, rpoC1, rpoC2, rpl2, ndhB* and *ndhA*) and three two-intron genes (*clpP, ycf1 and ycf3*). Fifteen pseudogenes were identified including five (*rps2, rps3, rps4, rps7* and *rps11*) for small subunit ribosomal, three (*rpl2, rpl21* and *rpl22*) for large subunit ribosomal, two (*rpoC1* and *rpoB*) for DNA-dependent RNA polymerase, one (*petA*) for cytochrome b–f complex, and four other (*infA, ycf2, ycf3* and *ndhF*) pseudogenes.

3.2. Comparative chloroplast genomic analysis

To identify divergent regions among species, sequence identity plots of the chloroplast genomes of seven species were generated with the annotation of *D. roosii* chloroplast genome as a reference. The results showed that the seven chloroplast genomes shared highly conserved identity (Fig. 2). *D. roosii* shared over 97% identity with other species (Supplementary Table 4). In the chloroplast genomes, LSC and SSC regions were more divergent than IRs regions. Furthermore, non-coding regions were more divergent than coding regions, the highly divergent non-coding regions among the seven chloroplast genomes appeared in intergenic sequence (IGS), such as *trnS-psb30, atpH-atpI, matK-chlB, psbM-petN,* and *ndhB-trnR*, and in introns of genes *ycf3, trnT-CGU*. For the coding regions, the relatively divergent regions were *matK, chlB, rbcL,* and *psbB* (Fig. 2). For further understanding the nucleotide diversity (Pi) at the sequence level, the DNA polymorphism among the seven species was calculated. The results showed that single copy regions were more variable than the IR regions (Fig. 3). A total of 87 IGS regions and 50 coding regions showed Pi values greater than 0.01. Genic regions were more conserved than the IGS region. It was predicted that all the above divergent regions could be candidates to develop molecular markers and barcoding for future phylogenetic analyses of *Drynaria* species and species identification of "Gusuibu".

3.3. Phylogenic analysis of chloroplast genomes

To determine if the entire chloroplast genomes could be used as a

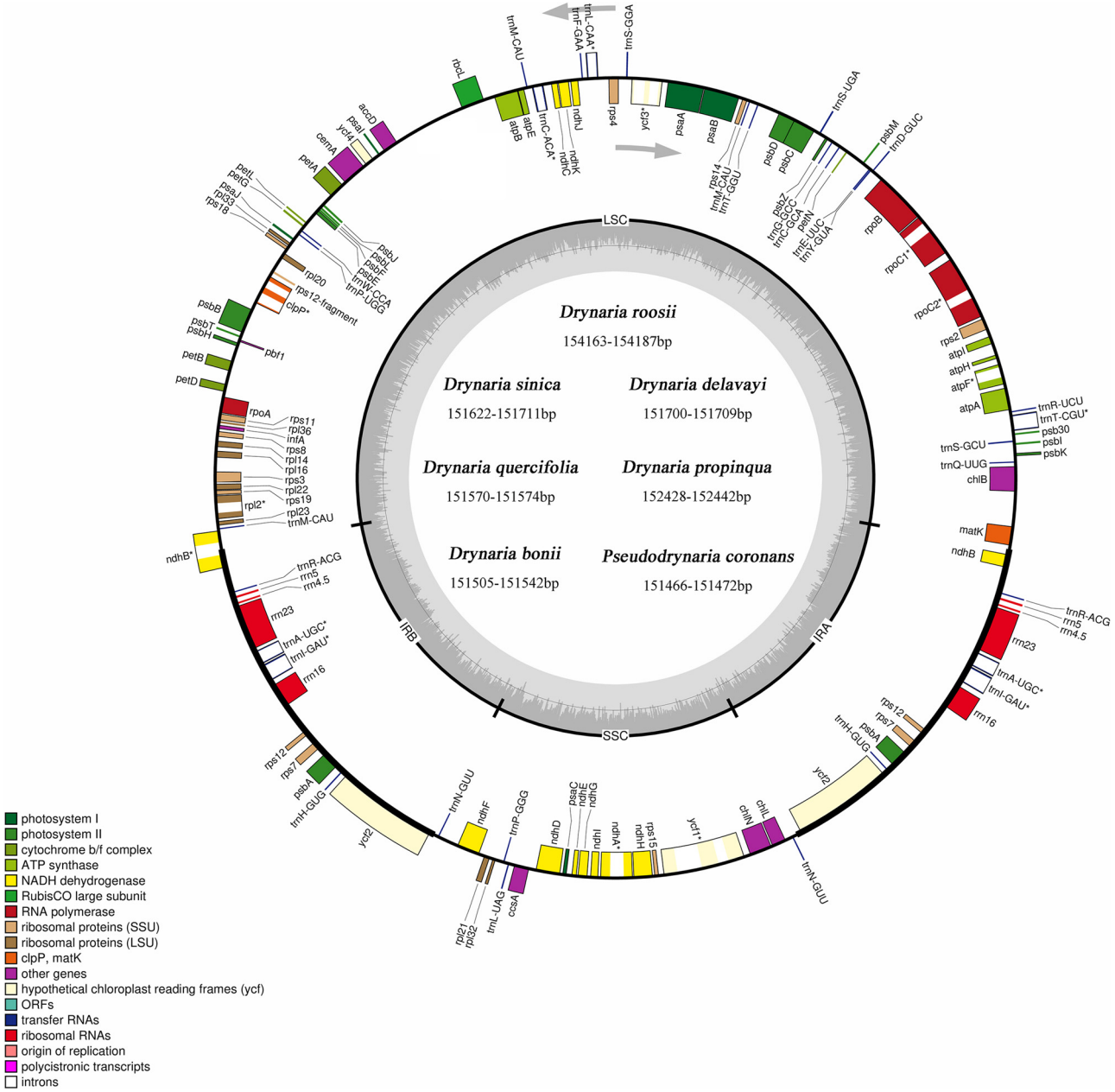

Fig. 1. Chloroplast genome map of seven species. Genes lying outside the circle are transcribed in the counter clockwise direction, while those inside are transcribed in the clockwise direction. The colored bars indicate different functional groups. The darker gray area in the inner circle denotes GC content while the lighter gray corresponds to the AT content of the genome. LSC: large single copy, SSC: small single copy, IR: inverted repeat.

super-barcode to distinguish *D. roosii* from adulterants, the phylogenetic analysis of 22 chloroplast genomes from seven species, including 21 chloroplast genomes sequenced in present study and one chloroplast genome downloaded from NCBI (Sun et al., 2017), was performed. The phylogenic tree showed that all nodes had bootstrap values of 100% (Fig. 4). Individual plants from the same species were clustered in a monophyletic clade with 100% bootstrap value. At the same time, the phylogenetic relationship showed that species *D. propinqua* and *P. coronans, D. delavayi* and *D. sinica, D. quercifolia* and *D. bonii* formed a clade respectively, and *D. roosii* had a relative far genetic distance with other species

3.4. Authentication of D. roosii from adulterants using specific DNA barcode

The coding regions are more conserved and stable than the noncoding regions for the development of DNA barcodes. Nine genes *matK, rbcL, psaB, psbB, chlB, ndhA, ycf1, ycf3,* and *atpB,* with highly divergent regions and high Pi values were selected to identify the DNA barcode. Based on the sequences from chloroplast genomes, primer pairs were designed to amplify and sequence the genes fragments with high distinguishability among the seven species. However, except for primers of *chlB* and *rbcL* genes, other primers presented low PCR amplification efficiency (Supplementary Table 2). Further sequencing showed that *chlB* presented low sequencing success rate. Only the *rcbL* gene fragment was amplified with 100% amplification and sequencing efficiency from 21 individual plants. Sequence alignment showed that there were six nuclear acid sites that were specific for *D. roosii* in a 500 bp fragment (Fig. 5). Furthermore, 23 individual plants including six *D. roosii,* two *D. bonii*, three *D. propinqua*, three *P. coronans*, three *D. sinica*, three *D. delavayi*, two *D. quercifolia*, and one *Davallia formosana* were used to test the primers and sequences of *rbcL* gene fragment.

Table 2
A list of genes found in the chloroplast genomes of seven species.

Category	Grope of Genes	Name of Genes
Other genes	Acetyl-CoA-carboxylase genes	*accD*
	c-type cytochrom synthesis gene	*ccsA*
	Envelop membrane protein genes	*cemA*
	Protease clp genes	*clpP*[b]
	Translational initiation factor genes	*infA*[c]
	Maturase genes	*matK*
Genes for photosynthesis Subunits	ATP synthase	*atpA*[c], *atpB*, *atpE*, *atpF*[a], *atpH*, *atpI*[c]
	protochlorophyllide reductase	*chlB*[c], *chlL*[c], *chlN*
	NADH-dehydrogenase	*ndhA*, *ndhB*[a, d], *ndhC*, *ndhD*, *ndhE*, *ndhF*[c], *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
	cytochrome b/f	*petA*, *petB*, *petD*[c], *petG*, *petL*, *petN*
	photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	photosystem II	*psbA*[d], *psbB*, *psbC*, *psbD*, *psbE*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*, *psb30*
	rubisco	*rbcL*
Self replication	Large subunit of ribosome	*rpl2*[c], *rpl14*, *rpl16*, *rpl20*, *rpl21*[c], *rpl22*[c], *rpl23*, *rpl32*, *rp36*
	DNA dependent RNA polymerase	*rpoA*, *rpoB*[c], *rpoC1*[a,c], *rpoC2*[a,c]
	Small subunit of ribosome	*rps2*[a,c], *rps3*[c], *rps4*[c], *rps7*[c,d], *rps8*, *rps11*[c], *rps12*[d], *rps14*, *rps15*, *rps16*[e], *rps18*, *rps19*
	rRNA Genes rrn	*rrn4.5*[d], *rrn5*[d], *rrn16*[d], *rrn23*[d]
	tRNA Genes trn	*trnC-GCA*, *trnM-CAU*, *trnC-ACA*[a], *trnF-GAA*, *trnL-CAA*[a], *trnS-GGA*, *trnM-CAU*, *trnT-GGU*, *trnS-UGA*, *trnG-GCC*, *trnC-GCA*, *trnE-UUC*, *trnY-GUA*, *trnD-GUC*, *trnR-UCU*, *trnT-CGU*[a], *trnS-GCU*, *trnQ-UUG*, *trnR-ACG*[d], *trnA-UGC*[a,d], *trnI-GAU*[a,d], *trnH-GUG*[d], *trnN-GUU*[d], *trnL-UAG*, *trnP-GGG*, *trnM-CAU*, *trnP-UGG*, *trnW-CCA*, *trnI-AUU*[e]
Unkown function	Conserved open reading	*ycf1*[b], *ycf2*[c,d], *ycf3*[b,c], *ycf4*

[a] contains one intron.
[b] contains two introns.
[c] shows pseudogenes.
[d] shows genes duplicated in the IRs regions.
[e] *D.roosii*, *D.quercifolia*, *D.bonii* did not contain *rps16* and *trnI-AAU*. *D.propinqua* and *P.coronans* did not contain *trnI-AAU*.

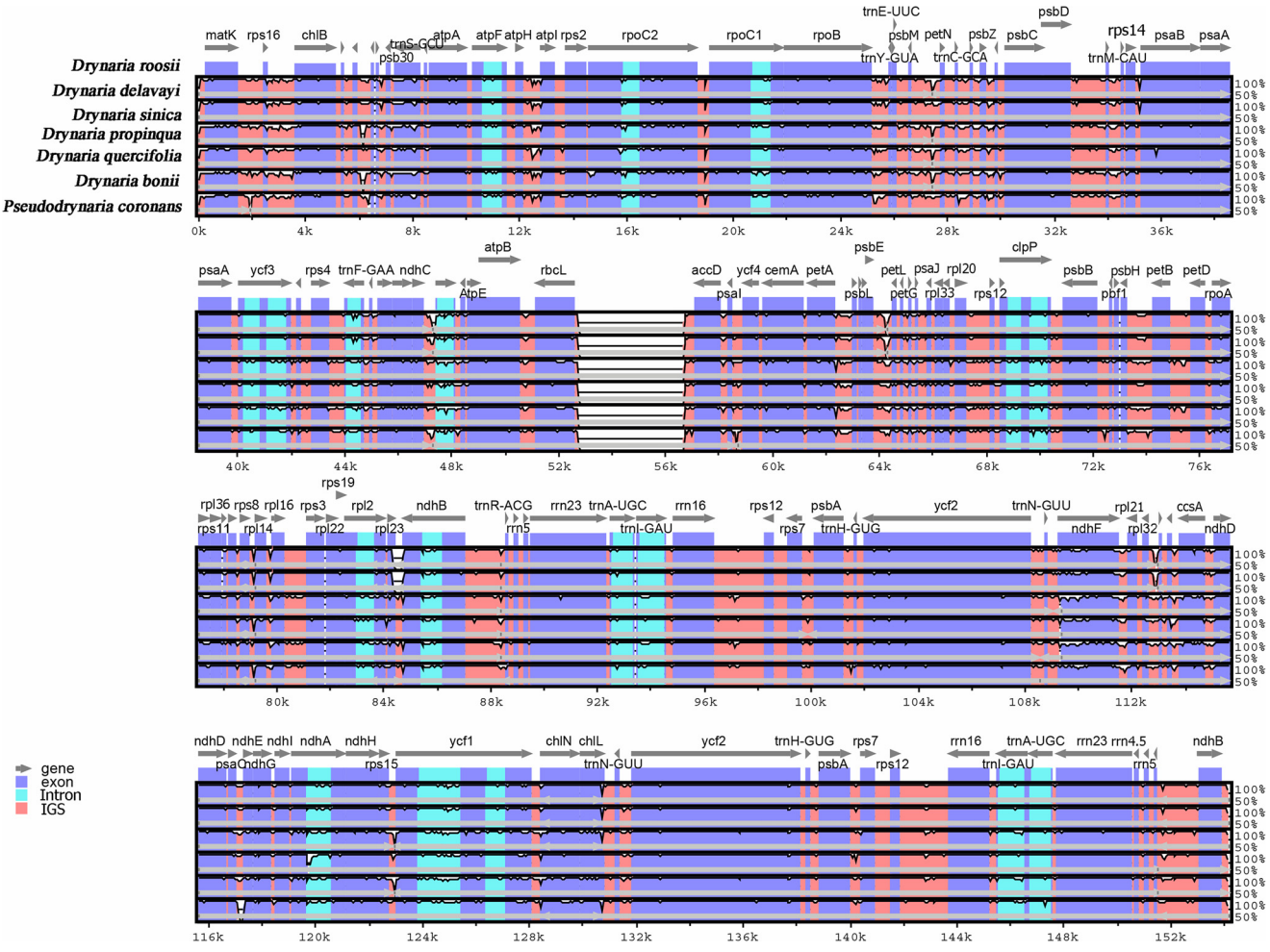

Fig. 2. Comparison of seven chloroplast genomes using *D. roosii* annotation as a reference. The vertical scale indicates the percentage of identity, ranging from 50 to 100%. The horizontal axis indicates the coordinates within the chloroplast genome. Genome regions are color-coded as exons, introns and intergenic spacer (IGS).

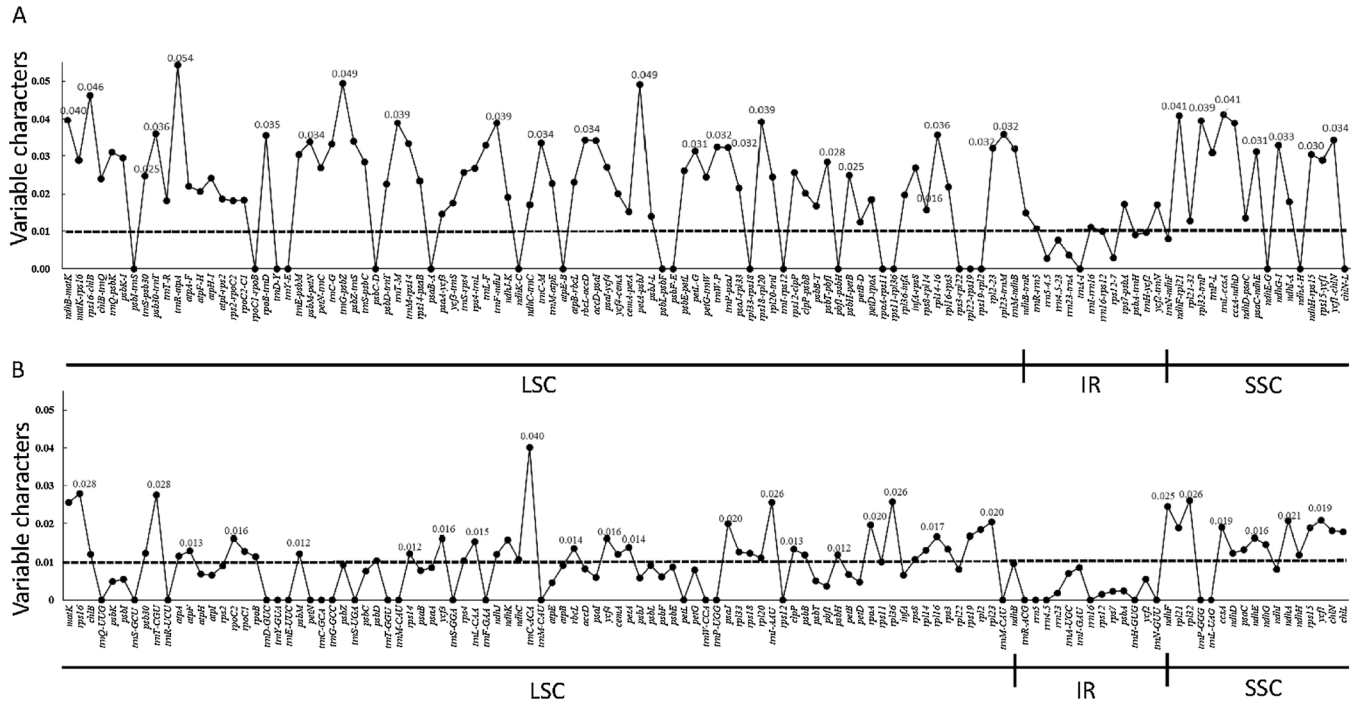

Fig. 3. The nucleotide diversity (Pi) values of the chloroplast genomes among seven species. A. Pi values of IGS, B. Pi values of coding genes.

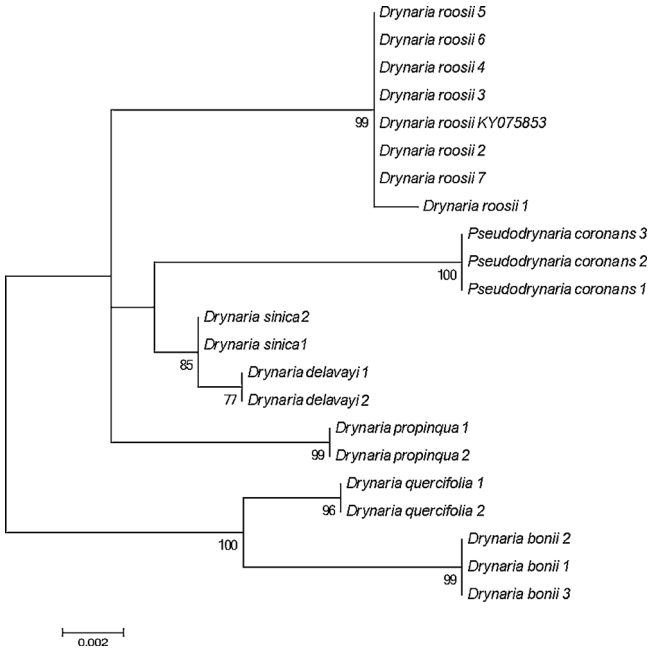

Fig. 4. Maximum likelihood (ML) phylogenetic tree inferred from the whole chloroplast genomes of 22 individual plants including 21 sequenced in this study and one downloaded from NCBI (KY075853).

Phylogenic tree showed that individual plants from the same species were always clustered into one clade except for individual plants from species *D. sinica* and *D. delavayi* (Fig. 6). *D. formosana* had genetic distance far from other species.

3.5. Authentication of D. roosii from adulterants using SCAR markers

Molecular characterization by SCAR markers allows effective and reliable authentication and discrimination of herbs from the adulterants. Based on the divergence and Pi values, three PCGs (*ycf2, rps16,* and *psbZ*) and eight IGS regions (*rbcL-accD, rpl14-rpl16, psbA-trnH, matK-chlB, trnS-psb30, atpH-atpI, trnA-ycf2*, and *ndhB-trnR*) with indels were selected to develop SCAR markers to distinguish *D. roosii* from adulterants. Primer pairs were designed to amplify the specific amplicon (Supplementary Table 3). Only in three IGS regions (*trnS-psb30, rbcL-accD, and ndhB-trnR*), a *D. roosii* specific DNA band was amplified (Fig. 7). The *trnS-psb30* region of *D. roosii* chloroplast genome had a 223 bp deletion region (Supplementary Fig.1), and had a higher Pi value (0.025) (Fig. 3). Based on the sequence alignment, the universal primer pair S30 F/S30R for the seven species was designed according to the conserved sequences located on the upstream and downstream of the deletion region. Amplified products were 388 bp in all individual plants of *D. roosii* and about 600 bp in other species (Fig. 7A). The *ndhB-trnR* region of *D. roosii* chloroplast genome had a 6 bp insertion region (Supplementary Fig. 2). Primer pair RBF/RBR was designed based on the sequence of *D. roosii*. The 6 bp insertion region located on the 3' end of forward primer RBF. PCR amplification resulted in a 104 bp DNA fragment with the expected SCAR amplicon size from individual plants of *D. roosii*, whereas no PCR product was observed for other seven species (Fig. 7B). In the LSC region of *D. roosii* chloroplast genome, there was a 3969 bp long fragment insertion between genes *rbcL* and *accD* (Fig. 2). Based on the insertion sequence, primer pair LDF/LDR was designed to amplify the SCAR of *D. roosii*. The primer amplified 1733 bp amplicons from *D. roosii* individual plants, but no amplification from other seven species individual plants (Fig. 7C).

4. Discussion

In China, traditional identifications of herbs usually relied on appearance, smell, or taste of herbs, which was performed by experienced people; but it was sometimes imprecise and is difficult to train a person to acquire the appropriate skills. Recently, microscopic methods and chemical analysis have been used to identify herbs through precise instruments such as microscopes and liquid chromatography-mass spectrometry. However, these methods were complicated and expensive; moreover, it was impossible to discriminate between closely related species that shared similar morphological characteristics or chemical profiles. With the development of molecular techniques, DNA-

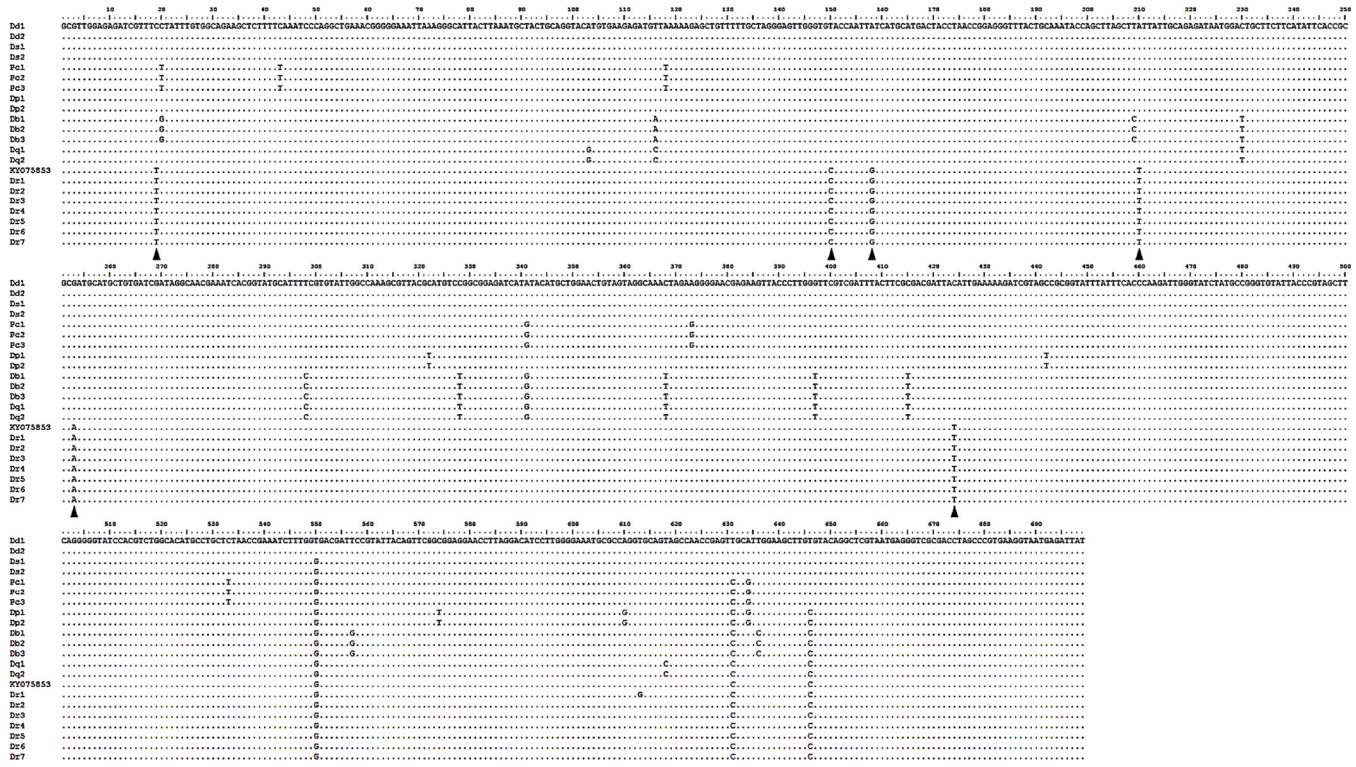

Fig. 5. Alignment of *rbcL* fragment sequences of seven species. Sequences were aligned using ClustalW in MEGA 7, and alignment was edited using BioEdit (version 7.0.9). The triangle indicated the specific nuclear acid sites in *D. roosii*.

based authentication was proved to be more reliable and effective (Mishra et al., 2016). DNA barcodes, chloroplast genomes, and SCAR markers were exploited in species authentication of different target herbal groups (Li et al., 2015; Park et al., 2018a,b; Park et al., 2019; Sheidai et al., 2019; Wang et al., 2018b).

Traditional DNA barcodes mainly derived from chloroplast loci were not suitable for all plant taxonomic group. Whole chloroplast genome was proposed to be used as super-barcode instead of traditional DNA barcodes to identify plant species (Nock et al., 2011; Wu et al., 2010; Li et al., 2015). Due to the rapid development of sequencing technique and bioinformatics, chloroplast genome could be completed inexpensively and easily. To date, many chloroplast genomes were reported to be used in species identification of some target taxa group (Wang et al., 2018a, 2017; Zhou et al., 2018). However, it is still unclear whether chloroplast genomes can be used as a DNA super barcode in all plant groups especially in pteridophyte. Apart for one complete chloroplast genome sequence of *D. roosii* reported in literature (Sun et al., 2017), there are no other complete chloroplast genome sequences available for “Gusuibu” and its adulterants species. To ensure the authenticity of “Gusuibu”, more complete chloroplast genome sequences from different individuals of *D. roosii* and its closelyrelated species were needed to exploit DNA barcodes and markers by comparative genome analysis. In the present study, the chloroplast genomes of the botanical origin of “Gusuibu” *D. roosii* and six adulterants species were analyzed (Fig. 1). The sequence identity of chloroplast genomes was high among species (Supplementary Table 4). Phylogenic analysis showed that every species formed a monophyletic clade with 100% bootstrap value (Fig. 4), which suggested that the whole chloroplast sequences were available super barcode for authenticating *D. roosii* from adulterants. Meanwhile the phylogenic tree also showed that *D. delavayi* and *D. sinica* had closer phylogenetic relationship, and so did *D. quercifolia* and *D. bonii* (Fig. 4). It is possible that the chloroplast genome sequences may be useful for phylogenetic investigations of *Drynaria* genus species. Furthermore, alignments of the chloroplast genome sequences discovered some genes with higher divergence and Pi values which were useful in potentially identifying the specific DNA barcodes (Figs. 2, 3 and 4). However, except for *rbcL*, all genes were unavailable to develop DNA barcodes due to low PCR amplification efficiency, low sequencing success rate, or less specific nucleotide sites (Supplementary Table 3). Although whole *rbcL* gene was 1428 bp in length, the 700 bp fragment amplified by primer pair rbcLF/rbcLR contained six specific nucleotide sites in *D. roosii* (Fig. 5). Phylogenic analysis showed that the *rbcL* gene fragment was an ideal DNA barcode to distinguish *D. roosii* from adulterants, which was also tested in 23 other individual plants. Since *D. formosana*, which belongs to the family Davalliaceae, is used as “Gusuibu” adulterant, it was also tested in this study. However, the results showed that *rbcL* gene fragment was unavailable to distinguish *D. sinica* and *D. delavayi*. Meanwhile intra-species variation existed in the *rbcL* gene fragment of some species (Fig. 6).

Besides DNA barcodes, SCAR markers were widely used in the discrimination of genuine medicinal herbs from adulterants (Moon et al., 2017). Using SCAR markers is a fast, reliable, inexpensive and easy process to conduct in any laboratory. SCAR markers can be detected only through three sequential experiments including DNA extraction, PCR amplification, and gel electrophoresis, and even without DNA extraction through Tissue-direct PCR and gel electrophoresis (Kiran et al., 2010; Li et al., 2010). SCAR markers usually were generated from polymorphic regions in genomes. In chloroplast genome, indels existed in some divergent regions of not only intragenic but also intergenic regions and introns. Species-specific SCAR primers located at suitable position within or flanking the unique indels may be designed to identify the species. In the present study, 13 indels were detected according to the chloroplast genome alignment among seven species (Supplementary Table 3). However only one universal and two species-specific SCAR primer pairs were obtained to amplify three intergenic regions. The three primer pairs designed according to the sequence of *D. roosii* chloroplast genome can amplify a specific DNA band in *D. roosii* (Fig. 7). The primer pairs BRF/BRR and LDF/LDR amplified one band only in *D. roosii* (Fig. 7B and C), and primer pair S30 F/S30R amplified one band in *D. roosii* shorter than that in other species

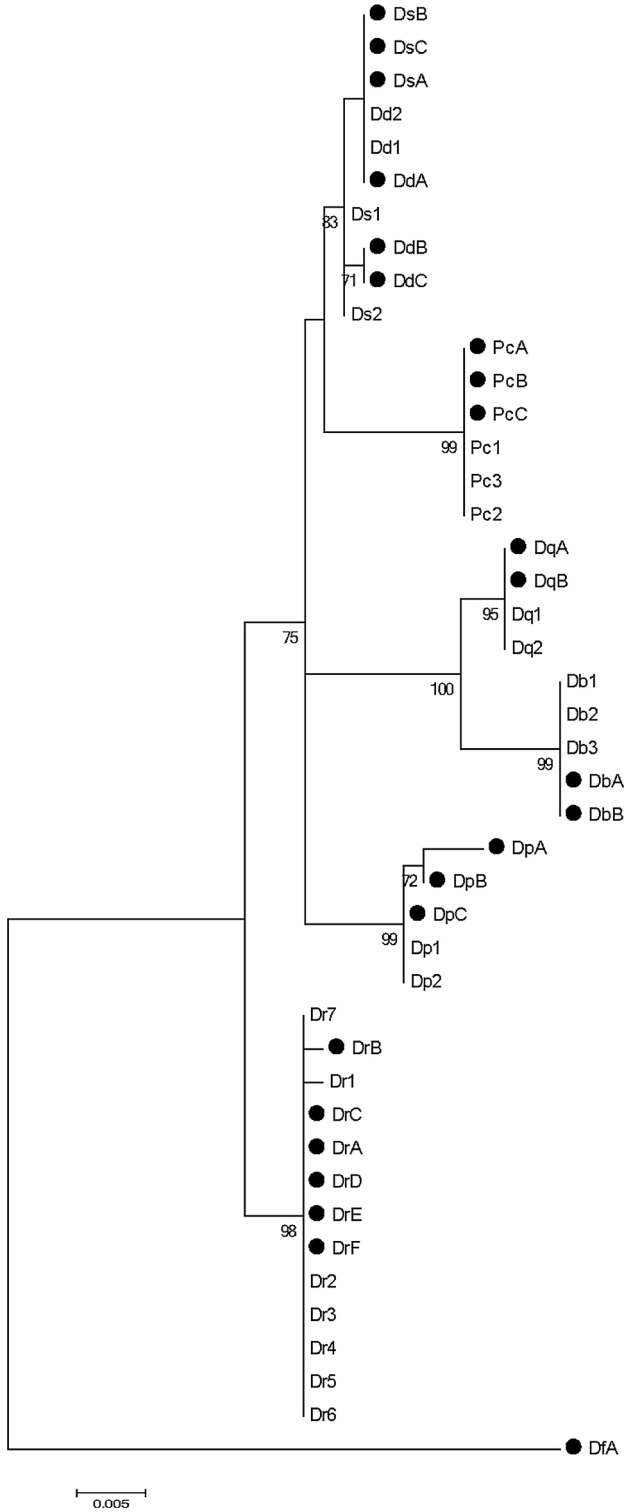

Fig. 6. Maximum likelihood (ML) phylogenetic tree inferred from the *rbcL* fragments of 44 individual plants including 21 used to sequence chloroplast genome in this study and 23 used to test the DNA barcode. The black dot indicated the tested individual plants including one *Davallia formosana*.

(Fig. 7A). The botanical origin of "Gusuibu" can be differentiated from adulterants with specific DNA bands. In summary, the results provided three DNA-based molecular methods for authentication of "Gusuibu" origin. Among the three methods, SCAR markers probably are the best tool and whole chloroplast is the most complicated tool for herb authentication. In practice, the source of herbs is diverse, and it is possible

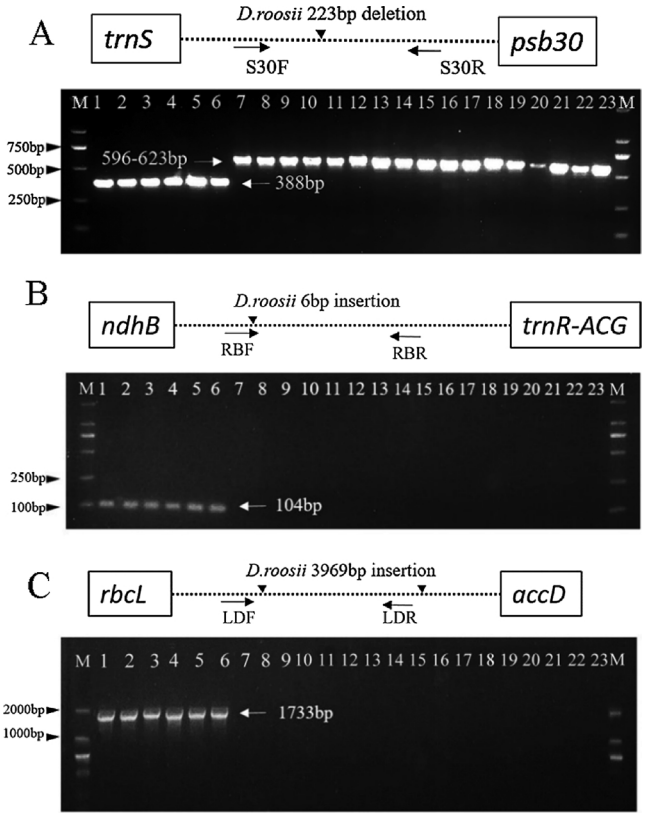

Fig. 7. The gel images of chloroplast DNA of eight species amplified using SCAR marker primers. Twenty-three individual plants were used to test the SCAR markers in this study using (A) S30 F/S30R, (B) RBF/RBR, and (C) LDF/LDR primer pairs. 1–6, *D. roosii* (DrA-DrF); 7–8, *D. bonii* (DbA-DbB); 9–11, *D. propinqua* (DpA-DpC); 12–14, *Pseudodrynaria coronans (PcA-PcC); 15*–17, *D. sinica* (DsA-DsC); 18–19, *D. quercifolia* (DqA-DqB); 20–22, *D. delavayi* (DdA-DdC); 23, *Davallia formosana* (DfA).

that a combination of different molecular methods is needed to precisely authenticate the origins of herbs.

5. Conclusions

D. roosii is the only origin of "Gusuibu", but it is difficult to distinguish the dried rhizome of *D. roosii* from adulterants. In this study, 21 complete chloroplast genomes from seven species, including *D. roosii*, and six adulterant species were obtained. The whole chloroplast sequences were available to authenticate *D. roosii* from adulterants. Furthermore, based on the chloroplast genomes, one specific DNA barcode *rbcL* gene fragment and three SCAR markers, which can successfully authenticate *D. roosii* from adulterants, were identified, and two universal and two specific primer pairs were designed. The results could be useful for guaranteeing the quality, safety, and effectiveness of "Gusuibu" herbs.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111756.

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