Postprint: Study on the Origin of Cultivated Aconitum vilmorinianum Based on Complete Chloroplast Genome Analysis

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Abstract

To investigate the influencing factors on the quality of cultivated Radix Aconitum Vilmoriniani, a toxic medicinal material used in Yunnan traumatology, the chloroplast genomes of samples from 10 different cultivation bases were sequenced using the Illumina HiSeq 4000 high-throughput sequencing platform. Following assembly and annotation of the sequencing data, bioinformatics tools were employed to analyze the chloroplast genome characteristics and construct phylogenetic trees. The results indicated that: (1) The full length of the chloroplast genomes from the 10 cultivars ranged from 155,744 to 155,937 bp, with the large single-copy and small single-copy regions measuring 86,363–86,548 bp and 16,921–17,007 bp, respectively, and the inverted repeat region spanning 26,170–26,236 bp; all were annotated with 131 genes. (2) Sequence analysis identified 60–73 SSR loci, and comparative genomic analysis revealed that the chloroplast genomes of the 10 cultivars exhibited a certain degree of expansion, with variable hotspot regions such as trnK-UUU-trnQ-UUG identified therein. (3) Phylogenetic analyses based on both datasets demonstrated that JS-1-4, QJ-1-2, LX-1-3, and LJ-3-2 were closely related to Aconitum vilmorinianum; LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2 were closely related to A. austroyunnanense; in the phylogenetic tree constructed based on the complete chloroplast genome sequence, LJ-4-3 showed close affinity with A. delavayi, and LJ-1-2 with A. duclouxii; whereas in the phylogenetic tree constructed based on protein-coding sequences, LJ-4-3 was closely related to A. episcopale, and LJ-1-2 to A. contortum. The findings reveal that the cultivation of Radix Aconitum Vilmoriniani suffers from an objective problem of mixed provenance, primarily involving two species—A. vilmorinianum and A. austroyunnanense—with certain cultivation bases also adulterated with other Aconitum species, which may constitute one of the factors contributing to quality instability in cultivated Radix Aconitum Vilmoriniani.

Full Text

Complete Chloroplast Genome Analysis Based Study on Origins of Cultivated Radix Aconitum Vilmoriniani

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Abstract

To investigate the factors influencing the quality of cultivated Radix Aconitum Vilmoriniani, a toxic medicinal material used in traumatology in Yunnan Province, we sequenced the chloroplast genomes of samples from ten different cultivation bases using the Illumina HiSeq 4000 high-throughput sequencing platform. Following assembly and annotation of the sequencing data, we analyzed the chloroplast genome characteristics using bioinformatics tools and constructed phylogenetic trees. The results revealed: (1) The complete chloroplast genomes of the ten cultivated varieties ranged from 155,744 to 155,937 bp in length, with large single-copy (LSC) regions of 86,363–86,548 bp, small single-copy (SSC) regions of 16,921–17,007 bp, and inverted repeat (IR) regions of 26,170–26,236 bp. All genomes contained 131 annotated genes. (2) Sequence analysis identified 60–73 SSR loci. Comparative genomic analysis revealed moderate expansion in the chloroplast genomes of the ten cultivated varieties, with variation hotspots identified in regions such as trnK-UUU-trnQ-UUG. (3) Phylogenetic analyses based on two datasets consistently showed that JS-1-4, QJ-1-2, LX-1-3, and LJ-3-2 were closely related to *Aconitum vilmorinianum*; LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2 were closely related to *A. austroyunnanense*. In the phylogenetic tree based on complete chloroplast genome sequences, LJ-4-3 was closely related to *A. delavayi*, while LJ-1-2 showed close affinity to *A. duclouxii*. In contrast, the tree based on protein-coding sequences placed LJ-4-3 near *A. episcopale* and LJ-1-2 near *A. contortum*. These findings demonstrate that cultivated *A. vilmorinianum* suffers from objective problems of mixed germplasm sources, primarily comprising two plant species—*A. vilmorinianum* and *A. austroyunnanense*—with other *Aconitum* species also present in individual cultivation bases. This mixed sourcing may be a contributing factor to the unstable quality of cultivated Radix Aconitum Vilmoriniani.

Keywords: cultivated Radix Aconitum Vilmoriniani, chloroplast genome, sequence characteristics, phylogenetic analysis, species identification

Introduction

Aconitum vilmorinianum Kom., commonly known as Huangcaowu, is a perennial herb belonging to the genus *Aconitum* (Ranunculaceae), series *Volubilia*. It is distributed in central Yunnan, Sichuan (Huili), and western Guizhou, typically growing in mountain thickets at altitudes of 2,100–2,500 m. The medicinal part is the root, which is highly toxic and contains various diterpenoid alkaloids such as yunaconitine. The plant exhibits pharmacological effects including dispelling wind and cold, promoting blood circulation to relieve pain, and detoxifying swelling. It serves as a primary raw material for famous Yunnese traumatological medicines such as Yunnan Red Medicine, Huli Powder, and Baibao Pellet, holding extremely high medicinal value. The demand for Huangcaowu is substantial, initially relying mainly on wild resources. However, over-exploitation has led to a sharp reduction in distribution areas and depletion of wild resources. In recent years, artificial cultivation of *A. vilmorinianum* has been successfully achieved, with large-scale cultivation in Luxi, Jianshui, Gejiu, Dongchuan, Luquan, Lijiang, Qiaojia, and other regions of Yunnan, effectively solving the resource supply problem. Nevertheless, our analysis of the main toxic components in medicinal materials from several cultivation bases revealed unstable content of yunaconitine and even greater fluctuations in another characteristic component, vilmorrianine A. As a toxic *Aconitum* medicinal material, stable quality is particularly crucial for Huangcaowu, making research into quality-influencing factors necessary.

The source of Huangcaowu medicinal material was defined in the 1974 and 1996 editions of the *Yunnan Provincial Drug Standards* as the tuberous root of either *A. vilmorinianum* or *A. austroyunnanense* (both Ranunculaceae), designated as Caowu (Radix Aconiti Vilmoriniani). When the 2005 edition of the *Yunnan Provincial Standards for Chinese Medicinal Materials* was formulated, only *A. vilmorinianum* was included as the botanical source, and the drug name was revised to Huangcaowu (Radix Aconitum Vilmoriniani). The *Flora Yunnanica* (Kunming Institute of Botany, Chinese Academy of Sciences, 2000) documented that *A. vilmorinianum* and *A. austroyunnanense* are morphologically very similar, with flowering periods in August–October and October, respectively. Both species have tuberous roots used medicinally and are employed by local people to treat wind-cold-damp bi syndrome, stroke paralysis, and traumatic injuries, with essentially identical uses. We observed that cultivated Huangcaowu from different regions exhibits several root phenotypes: yellow skin with yellow core, purple skin with yellow core, and purple skin with purple core, with slight variations in floral morphology. Through investigation, we learned that some planting sources consist of wild specimens collected near cultivation bases without clear botanical identification. This suggests that cultivated Huangcaowu may suffer from mixed germplasm sources, consequently leading to unstable medicinal quality. The genus *Aconitum* contains numerous species with blurred interspecific boundaries and similar morphology that is difficult to distinguish. Environmental factors cause intraspecific variations in plant morphology, flower color and shape, root number, and root skin color. Traditional methods relying on morphological, physicochemical, and microscopic identification cannot accurately differentiate *Aconitum* species. Therefore, molecular biological approaches are essential for clarifying the botanical origin of Huangcaowu to ensure medicinal quality from the source.

Chloroplasts are vital organelles for photosynthesis and metabolic activities in most higher plants and some algae and prokaryotes. As semi-autonomous organelles, they possess their own genome—the chloroplast genome. Chloroplast genomes contribute significantly to screening molecular markers for medicinal plant species and deciphering phylogenetic relationships among closely related plant groups. In recent years, next-generation sequencing technology has developed rapidly with decreasing costs, leading to increasing numbers of published chloroplast genomes and driving rapid advances in molecular biology research. Previous studies have used internal transcribed spacer (ITS) sequences and psbA-trnH sequences to construct phylogenetic relationships of *Aconitum* species, which basically explained relationships at the subgenus level. However, some results indicated that classification below the subgenus level was inconsistent with traditional taxonomy. Chloroplast genomes can serve as DNA super-barcodes for accurate plant species identification. Although Fan et al. (2022) constructed a phylogenetic tree of *Aconitum* using complete chloroplast genome sequences, most species of the genus were not included.

In this study, we sequenced, assembled, and annotated the complete chloroplast genome sequences of ten cultivated *A. vilmorinianum* samples, revealing their basic sequence and structural characteristics. We also analyzed the phylogenetic relationships of these ten cultivated varieties by combining them with 32 published *Aconitum* chloroplast genome sequences. Through these analyses, we aimed to address the scientific question of whether mixed germplasm sources exist in Huangcaowu cultivation and whether this contributes to quality instability. This research will clarify the germplasm source issues of cultivated Huangcaowu and provide data for botanical identification, standardized cultivation, and rational development and utilization of this medicinal plant.

Materials and Methods

1.1 Experimental Materials Cultivated *A. vilmorinianum* samples were collected from cultivation bases including Ganhaizi Sayingpan in Luquan County, Santang Township in Luxi County, Yaoshan Town in Qiaojia County, and Yongning Township in Ninglang County. Healthy plants were selected, and fresh young leaves were collected and preserved with silica gel for subsequent experiments.

Table 1 Collected information of ten cultivated varieties

Sample ID	Collection location	Longitude	Latitude (N)	Altitude (m)
DC-2-2	Tangdan Town, Dongchuan	130°2	26°9	-
GJ-1-3	Duimen Mountain, Gejiu City	103°11	23°21	-
JS-1-4	Puxiong Township, Jianshui County	103°4	23°29	-
LJ-1-2	Tai’ an Township, Lijiang City	100°5	26°47	-
LJ-3-2	Tai’ an Township, Lijiang City	100°6	26°48	-
LJ-4-3	Tai’ an Township, Lijiang City	100°5	26°44	-
LQ-1-3	Ganhaizi Sayingpan, Luquan County	102°30	26°3.5	-
LX-1-3	Santang Township, Luxi County	103°48.5	24°26.7	-
NL-1-3	Yongning Township, Ninglang County	100°48.2	27°26	-
QJ-1-2	Yaoshan Town, Qiaojia County	103°6.7	27°9.6	-

1.2 Chloroplast DNA Extraction and Sequencing Total genomic DNA was extracted from fresh young leaves using the plant genomic DNA extraction kit from Bio Teke. DNA purity was assessed using a NanoDrop 2000 micro-spectrophotometer (Thermo Fisher Scientific, USA), and agarose gel electrophoresis was used for DNA quality assessment. Qualified samples were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for DNA library construction and sequenced on the Illumina HiSeq 4000 high-throughput sequencing platform.

1.3 Chloroplast Genome Assembly, Annotation, and Physical Map Construction Raw sequencing data were filtered using the NGS QC Toolkit software (Patel & Jain, 2012) with default parameters to remove low-quality reads and obtain clean reads for assembly. Using the published chloroplast genome sequence of *A. vilmorinianum* (NC_{038094}) as a reference, clean reads from the ten cultivated varieties were assembled de novo with NOVOPlasty software (Dierckxsens et al., 2017) with a k-mer value of 39. Annotation was performed using the online tool GeSeq (Tillich et al., 2017) and manually corrected in Geneious R11.1.5 software (Kearse et al., 2012). Chloroplast genome sequences were imported into the OGDRAW-Draw online tool (Greiner et al.,

2019) to generate physical maps. The fully annotated cpDNA sequences were submitted to GenBank under accession numbers: DC-2-2 (OP227195), GJ-1-3 (OP227196), JS-1-4 (OP227197), LJ-1-2 (OP227198), LJ-3-2 (OP227199), LJ-4-3 (OP227200), LQ-1-3 (OP227201), LX-1-3 (OP227202), NL-1-3 (OP227203), and QJ-1-2 (OP227204).

1.4 Chloroplast Genome Characteristic Analysis Simple sequence repeats (SSRs) in each sequence were detected using the online tool MISA (Beier et al., 2017) with parameters set as follows: mono-nucleotide SSR $\geq$ 10, di-nucleotide SSR $\geq$ 5, tri-nucleotide SSR $\geq$ 4, tetra-nucleotide SSR $\geq$ 3, penta-nucleotide SSR $\geq$ 3, and hexa-nucleotide SSR $\geq$ 3.

1.5 Chloroplast Genome Comparative Analysis The IRscope online tool (Amiryousefi et al., 2018) was used to analyze boundary differences among the four regions of the chloroplast genomes from the ten cultivated varieties. Using *A. vilmorinianum* as the reference sequence, whole chloroplast genome alignments of *Aconitum* species were performed using mVISTA (Frazer et al., 2004) with the shuffle-LAGAN mode. Nucleotide diversity (Pi) of the ten cultivated varieties was calculated using DnaSP software (Rozas et al., 2017) with a step size of 200 bp and window length of 600 bp.

1.6 Phylogenetic Analysis To explore the evolutionary relationships among the ten cultivated varieties and their phylogenetic positions within *Aconitum*, 32 published *Aconitum* chloroplast genome sequences were downloaded from NCBI. Additionally, one *Consolida* species (*C. orientalis*, NC_{047292}) and one *Delphinium* species (*D. anthriscifolium*, MK253461) were selected as outgroups for phylogenetic tree construction (Table 2). Whole-sequence alignment was performed using MAFFT v7.0 (Katoh & Standley, 2013). Both maximum likelihood (ML) and Bayesian inference (BI) methods were employed to enhance result reliability. ML trees were constructed using PhyloSuite (Zhang et al., 2020) with 1,000 bootstrap replicates. Bayesian trees were also constructed using PhyloSuite, running 2,000,000 generations with sampling every 1,000 generations based on the Markov chain Monte Carlo (MCMC) algorithm, using random trees as starting trees. The first 25% of samples were discarded as burn-in, and the remaining samples were used to construct the majority-rule consensus tree when the average standard deviation dropped below 0.01.

Table 2 Published species list and information for phylogenetic tree construction

Genus	Chinese name	Latin name	GenBank accession
Aconitum	马耳山乌头	*A. vilmorinianum*	NC_{038094}
Aconitum	伏毛铁棒锤	*A. hemsleyanum*	NC_{038095}
Aconitum	-	*A. delavayi*	NC_{038097}
Aconitum	-	*A. forrestii*	MZ959044

Genus	Chinese name	Latin name	GenBank accession
Aconitum	-	*A. episcopale*	NC_{038096}
Aconitum	-	*A. ramulosum*	OM289059
Aconitum	-	*A. bulleyanum*	OK323949
Aconitum	-	*A. ouvrardianum*	OM289057
Aconitum	滇南草乌	*A. austroyunnanense*	MN635745
Aconitum	-	*A. pendulum*	NC_{053848}
Aconitum	-	*A. flavum*	NC_{056280}
Aconitum	宾川乌头	*A. duclouxii*	OM328070
Aconitum	-	*A. stylosum*	OM328071
Aconitum	苍山乌头	*A. contortum*	NC_{038098}
Aconitum	-	*A. ciliare*	NC_{031420}
Aconitum	-	*A. jaluense* subsp. *jaluense*	KT820668
Aconitum	-	*A. japonicum* subsp. *napiforme*	KT820670
Aconitum	-	*A. kusnezoffii*	NC_{031422}
Aconitum	-	*A. carmichaelii*	NC_{030761}
Aconitum	-	*A. volubile*	KU556690
Aconitum	-	*A. chiisanense*	NC_{029829}
Aconitum	-	*A. austrokoreense*	KT820663
Aconitum	-	*A. monanthum*	NC_{031423}
Aconitum	-	*A. coreanum*	NC_{031421}
Aconitum	-	*A. pseudolaeve*	NC_{035892}
Aconitum	-	*A. finetianum*	NC_{036358}
Aconitum	-	*A. longecassidatum*	NC_{035894}
Aconitum	-	*A. angustius*	NC_{036357}
Aconitum	-	*A. reclinatum*	MF186593
Aconitum	-	*A. sinomontanum*	NC_{036359}
Aconitum	-	*A. barbatum* var. *puberulum*	KT964698
Gymnaconitum	-	*G. gymnandrum*	NC_{033341}
Consolida	-	*C. orientalis*	NC_{047292}
Delphinium	-	*D. anthriscifolium*	MK253461

Note: "—" indicates that the Chinese species name cannot be found in the electronic Flora Reipublicae Popularis Sinicae.

Results

2.1 Basic Structural Features and Classification of Complete Chloroplast Genomes Similar to most angiosperm chloroplast genomes, the ten cultivated varieties exhibited a typical quadripartite circular structure with a total length ranging from 155,744 bp (LJ-1-2) to 155,937 bp (DC-2-2) [Figure 1: see original paper]. Each genome consisted of a large single-copy (LSC) region, a small single-copy (SSC) region, and a pair of inverted repeats (IRs). The IR regions measured 26,170–26,236 bp, the LSC regions 85,453–86,548 bp, and the SSC regions 16,921–17,007 bp. All ten chloroplast genomes were highly conserved, showing similar size and structure with a uniform GC content of 38.1% and obvious AT bias. Annotation results (Table 3) revealed consistent gene content and order across all samples, each containing 131 genes comprising 85 protein-coding genes (PCGs), 37 tRNA genes, and 8 rRNA genes.

Table 3 Basic information of chloroplast genome of ten cultivated varieties

Sample	Whole genome length (bp)	LSC length (bp)	SSC length (bp)	IR length (bp)	Total GC content (%)	Number of genes	PCGs	tRNAs	rRNAs
JS-1-4	155,879	86,548	16,921	26,170	38.1	131	85	37	8
QJ-1-2	155,879	86,548	16,921	26,170	38.1	131	85	37	8
LX-1-3	155,879	86,548	16,921	26,170	38.1	131	85	37	8
LJ-3-2	155,879	86,548	16,921	26,170	38.1	131	85	37	8
LJ-4-3	155,879	86,548	16,921	26,170	38.1	131	85	37	8
LQ-1-3	155,879	86,548	16,921	26,170	38.1	131	85	37	8
GJ-1-3	155,879	86,548	16,921	26,170	38.1	131	85	37	8
NL-1-3	155,879	86,548	16,921	26,170	38.1	131	85	37	8
DC-2-2	155,937	86,548	16,921	26,170	38.1	131	85	37	8
LJ-1-2	155,744	86,363	17,007	26,236	38.1	131	85	37	8

The 131 genes could be classified into four functional categories: (1) transcription and translation-related genes, (2) photosynthesis-related genes, (3) other genes, and (4) genes of unknown function. Transcription and translation-related genes included ribosomal protein subunit genes, RNA polymerase genes, rRNA genes, and tRNA genes, with tRNA genes being the most numerous. Photosynthesis-related genes comprised photosystem I genes, photosystem II genes, NAD(P)H oxidoreductase genes, cytochrome b/f complex genes, ATP synthase genes, and the large subunit of ribulose-1,5-bisphosphate carboxylase, with photosystem genes being the most abundant. Other genes totaled six, while unknown function genes numbered eight. Among these, 21 genes were duplicated, including two large ribosomal subunits (rpl2, rpl23), two small ribosomal subunits (rps7, rps12), four rRNA genes (rrn4.5, rrn5, rrn16, rrn23), seven tRNA genes (trnA-UGC, trnI-CAU, trnI-GAU, trnL-CAA, trnN-GUU, trnR-ACG, trnV-GAC), one NADH dehydrogenase (ndhB), and five unknown function genes (ycf1, ycf2, ycf3, ycf4, ycf15) (Table 4).

Table 4 Functional classification of chloroplast genome genes in ten cultivated varieties

Function	Gene category	Gene name	Number
Self-replication	Large subunit of ribosome	rpl2($×2), rpl14, rpl16, rpl20, rpl22, rpl23(×2), rpl33, rpl36|11|||S UGC(×2), trnC− GCA, trnD − GUC, trnE − UUC, trnF − GAA, trnfM − CAU, trnG − GCC, trnG − UCC, trnH − GUG, trnI − CAU(×2), trnI − GAU(×2), trnK− UUU, trnL − CAA(×2), trnL− UAA, trnL − UAG, trnM − CAU, trnN − GUU(×2), trnP− UGG, trnQ − UUG, trnR − ACG(×2), trnR− UCU, trnS − GCU, trnS − GGA, trnS − UGA, trnT − GGU, trnT − UGU, trnV − GAC(×2), trnV− UAC, trnW − CCA, trnY − GUA|37||Photosynthesis|PhotosystemI|psaA, psaB, psaC, psa CoAcarboxylasesubunit|accD|1|||C− typecytochromesynthesis|ccsA|1||Unknownfunction|Hypothet	8

2.2 SSR Analysis of Chloroplast Genomes A total of 60 (LJ-1-2) to 73 (JS-1-4) SSR loci were identified in the chloroplast genomes of the ten cultivated varieties [Figure 2: see original paper]. These included 28–37 mono-nucleotide SSRs, 13–16 di-nucleotide SSRs, 6–9 tri-nucleotide SSRs, 6–9 tetra-nucleotide SSRs, and 2–5 penta-nucleotide SSRs. No hexa-nucleotide SSRs were detected. Mono-nucleotide repeats accounted for the largest proportion (44.4%–51.5%). The shared SSR motifs across all ten samples included mono-nucleotide repeats A/T and C/G, di-nucleotide repeats AT/TA, tri-nucleotide repeats AAT/ATT and ATC/ATG, tetra-nucleotide repeats AAAG/CTTT, AAAT/ATTT, and AATG/ATTC, and penta-nucleotide repeats AATAT/ATATT. The distribution of SSRs varied among the ten cultivated varieties, with 534 SSRs in LSC regions, 88 in SSC regions, and 42 in IR regions. The proportional distribution differed among samples: LSC region (78.6%–83.1%), SSC region (10.8%–14.7%), and IR region (5.6%–8.6%).

2.3 IR Boundary Expansion and Contraction Analysis Comparison of IR boundaries in the ten chloroplast genomes revealed lengths ranging from 155,744 bp (LJ-1-2) to 155,937 bp (DC-2-2) [Figure 3: see original paper]. IR regions measured 26,127 bp (DC-2-2) to 26,170 bp (JS-1-4, QJ-1-2, LX-1-3, LJ-4-3), LSC regions 86,496 bp (LJ-1-2) to 86,680 bp (DC-2-2), and SSC regions 16,921 bp (LJ-3-2) to 17,007 bp (LQ-1-3, GJ-1-3, NL-1-3). All samples showed similar LSC, SSC, and IR region sizes with highly conserved boundaries between IR and SC regions, though slight variations existed among samples. The rps19 gene was located 63 bp from the IRb region in all ten samples. Except for LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2, which had rpl2 lengths of 1,486 bp, all other samples had rpl2 lengths of 1,490 bp. The ndhF gene was positioned 113–119 bp from the IRb/SSC boundary. The ycf1 gene spanned both SSC and IRa regions in all samples, with most of the gene (4,012–4,027 bp) located in the SSC region and 1,286 bp in the IRa region. The IRa/LSC boundary was situated between rpl2 and trnH genes in all ten samples, with trnH located 120–126 bp from the IRa/LSC boundary.

2.4 Chloroplast Genome Alignment Analysis Whole-genome alignment of the ten cultivated varieties using mVISTA with *A. vilmorinianum* as reference revealed highly conserved genomes, with IR regions being more conserved than coding regions, which were in turn more conserved than non-coding regions. Several highly variable regions were identified, primarily in intergenic spacers: trnK-UUU-trnQ-UUG, trnC-GCA-trnT-GGU, trnT-UGU-trnL-UAA, atpH-atpI, trnP-UGG-psaJ, rbcL-accD, ycf4-cemA, rpl16-rps3, and genes rpl20, ycf1, and ndhF [Figure 4: see original paper].

Sliding window analysis of nucleotide diversity in highly variable regions revealed values ranging from 0 to 0.00915. Regions with nucleotide diversity >0.006 included intergenic spacers trnY-GUA-trnE-UUC, ccsA-ndhD, petD-rpoA and genes trnS-GCU, ycf1, rpl16, and ndhF. The ccsA-ndhD intergenic spacer showed the highest variation with a nucleotide diversity of 0.00915. High-variation sites were mainly located in LSC and SSC regions, with no highly variable fragments detected in IR regions [Figure 5: see original paper], consistent with mVISTA analysis results.

2.5 Phylogenetic Analysis of *Aconitum* Species To investigate relationships among cultivated varieties from different cultivation bases, phylogenetic trees were constructed using two datasets (complete chloroplast genome sequences and protein-coding gene sequences) and two methods (ML and BI), incorporating the ten cultivated varieties, 32 published *Aconitum* sequences, and two outgroup species [Figure 6: see original paper]-[Figure 7: see original paper]. All results showed that 42 *Aconitum* species clustered into one major clade, with the entire genus divided into three subgenera (*Gymnaconitum*, *Lycoctonum*, and *Aconitum*), with *Gymnaconitum* as the basal group. All ten cultivated varieties belonged to subgenus *Aconitum*. Topological differences existed between trees based on different datasets. The complete chloroplast genome-based trees showed: LJ-1-2 clustered with *A. pendulum* and two other species (branch a, 99/1 support), showing close affinity to *A. duclouxii*; LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2 clustered with *A. austroyunnanense* (branch b, 100/1 support); LJ-4-3 clustered with *A. delavayi* and *A. forrestii* (branch c, 100/1 support), closely related to *A. delavayi*; JS-1-4, QJ-1-2, LX-1-3, and LJ-3-2 clustered with *A. vilmorinianum* and *A. hemsleyanum* (branch d), showing close relationship with *A. vilmorinianum*. In contrast, the protein-coding gene-based trees showed: LJ-1-2 clustered with *A. contortum* (branch a, 88/1 support); LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2 clustered with *A. austroyunnanense* (branch b, 100/1 support); LJ-4-3 clustered with *A. episcopale* and *A. delavayi* (branch c, 74/1 support), closely related to *A. episcopale*; JS-1-4, QJ-1-2, LX-1-3, and LJ-3-2 clustered with *A. vilmorinianum* (branch d).

Discussion

Medicinal material quality is influenced by both internal and external factors, primarily botanical origin and environmental conditions. As a crucial Yunnanese traumatological medicine and highly toxic medicinal material, germplasm confusion in Huangcaowu cultivation may cause differences in chemical composition types and contents, thereby affecting quality stability and posing significant safety risks. Current research on Huangcaowu has focused on chemical components, pharmacological effects, and pharmacognostic identification, but no reports exist on chloroplast genomes of cultivated varieties. This study employed high-throughput sequencing and bioinformatics to investigate complete chloroplast genome characteristics and phylogenetic relationships of ten cultivated varieties.

The results revealed similar chloroplast genome sizes among the ten cultivated varieties, all exhibiting typical, highly conserved quadripartite structures comprising one LSC region, one SSC region, and a pair of IR regions. Genome length variation was only 190 bp, with uniform GC content of 38.1% and obvious AT bias. All genomes contained 131 genes, consistent with other *Aconitum* species studies, indicating slow chloroplast genome evolution and relative conservation within the genus.

Chloroplast SSRs are widely used in genetic diversity analysis and molecular marker-assisted breeding due to their unlimited biological abundance, high polymorphism, easy replication, and maternal inheritance. This study identified 60–73 SSR loci in the ten cultivated varieties, with mono-nucleotide SSRs being most abundant, followed by di- and tri-nucleotides. A/T repeats dominated mono-nucleotides, AT/TA dominated di-nucleotides, and AAT/ATT dominated tri-nucleotides, consistent with previous studies on *A. vilmorinianum* from Wuding County, Yunnan. These SSR loci provide candidate molecular markers for genetic studies of Huangcaowu.

Although chloroplast genomes are highly conserved, gene structure and size varied slightly at IR/SC boundaries among the ten cultivated varieties. LSC, SSC, and IR regions showed minor size differences of 86,496–86,680 bp, 16,921–17,007 bp, and 26,127–26,170 bp, respectively, indicating moderate expansion. IR regions were more conserved than LSC and SSC regions, likely due to gene conversion correcting mutations in duplicated genes, thereby reducing variation frequency. The size and distribution of rps19, ndhF, ycf1, and trnH genes at the four boundaries showed minor differences among samples. High-variation regions identified by mVISTA and DnaSP included trnK-UUU-trnQ-UUG, trnY-GUA-trnE-UUC, trnC-GCA-trnT-GGU, trnT-UGU-trnL-UAA, atpH-atpI, trnP-UGG-psaJ, rbcL-accD, ycf4-cemA, rpl16-rps3, and genes rpl20, ycf1, and ndhF. These findings align with previous studies by Park et al. (2017) and Meng et al. (2018). While fragments such as ndhF-trnL, psbA-trnH, psbD-trnT, and trnT-trnL have been used in *Aconitum* phylogenetic studies, they have not fully resolved interspecific relationships. These newly identified variation hotspots may serve as novel DNA barcodes for species identification and phylogenetic studies of *Aconitum*.

Compared to traditional barcoding fragments, complete chloroplast genome sequences provide higher resolution for clarifying lower-level phylogenetic relationships. Our phylogenetic trees based on two datasets and two methods consistently divided 42 *Aconitum* species into three major clades (subgenera *Aconitum*, *Lycoctonum*, and *Gymnaconitum*), with subgenera *Aconitum* and *Lycoctonum* as sister groups and *Gymnaconitum* as basal. All ten cultivated varieties belonged to subgenus *Aconitum*, but only four (JS-1-4, QJ-1-2, LX-1-3, and LJ-3-2) were closely related to *A. vilmorinianum*. Four others (LQ-1-3, GJ-1-3, NL-1-3, and DC-2-2) were closely related to *A. austroyunnanense*. The two Lijiang samples showed inconsistent placement: LJ-4-3 was allied with *A. delavayi* (series *Ambigua*) in the whole-genome tree but with *A. episcopale* (series *Volubilia*) in the PCG tree; LJ-1-2 was related to *A. duclouxii* (series *Bullatifolia*) in the whole-genome tree but to *A. contortum* (series *Stylosa*) in the PCG tree. This suggests that non-coding regions may also play a role in plant phylogeny. Except for the confused sources in Lijiang, cultivated varieties from other regions fell into two main groups: *A. vilmorinianum* and *A. austroyunnanense*, indicating that current cultivation primarily involves these two botanical sources.

Furthermore, our results showed intermingling of some groups from series *Volubilia*, *Ambigua*, *Stylosa*, *Bullatifolia*, and *Brachypoda*, inconsistent with the classification in *Flora Reipublicae Popularis Sinicae*. Similar results were reported by Zhang et al. (2003), suggesting potential issues with morphology-based classical taxonomy. Given the high phenotypic variability of *Aconitum* species during growth, classification based solely on phenotypic characteristics is clearly insufficient, which may explain the mixed germplasm sources during domestication and cultivation. Although most *Aconitum* species have been sequenced, phylogenetic studies remain limited. Our inferred relationships provide important information for species identification and evolutionary studies, but more comprehensive phylogenetic analyses incorporating more species and additional technical approaches are needed for a more complete and realistic evolutionary framework.

In conclusion, this study demonstrates from a chloroplast genome perspective that cultivated Huangcaowu in major Yunnan cultivation bases indeed suffers from objective mixed germplasm problems, primarily involving *A. vilmorinianum* and *A. austroyunnanense*. Since current quality standards only recognize *A. vilmorinianum* as the botanical source, germplasm standardization should be the first priority for addressing quality issues. Whether *A. austroyunnanense* should also be included as an acceptable source requires comprehensive evaluation based on comparative studies of their chemical constituents and pharmacological activities.

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Note: Figure translations are in progress. See original paper for figures.