Chloroplast genome sequencing and comparative analysis of six medicinal plants of Polygonatum

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Abstract

The genus Polygonatum boasts abundant germplasm resources and comprises numerous species. Among these, medicinal plants of this genus, which have a long history, have garnered attention of scholars. This study sequenced and analyzed the chloroplast genomes of six species of Polygonatum medicinal plants (P. zanlanscianense, P. kingianum, P. sibiricum, P. cyrtonema, P. filipes and P. odoratum, respectively) to explore their inter-specific relationships. The sequence length (154, 578–155, 807 bp) and genome structure were conserved among the six Polygonatum species, with a typical tetrad structure. The genomes contain 127–131 genes, containing 84–85 protein-coding genes, 37–38 transfer RNA genes, and 6–8 ribosomal RNA genes. The genomes contained 64–76 simple sequence repeats (SSRs) and 36–62 long repetitive sequences. Codon bias patterns tended to use codons ending in A/T. In thirty types of codons with RSCU $>$ 1, 93.3% ended in A/T of the six species. Twenty-one highly variable plastid regions were identified in the chloroplast genomes of the six medicinal plants. In addition, phylogenetic analysis of these and other 53 Polygonatum chloroplast genomes showed that P. cyrtonema, P. odoratum and P. filipes were clustered on one large clade, whereas P. kingianum and P. zanlanscianense were clustered on other clades. P. sibiricum is a monophyletic group and our tree supports the classification of P. sibiricum as an independent clade. This study provides a novel basis for intragenus taxonomy and DNA barcoding molecular identification within the genus Polygonatum medicinal plants.

Chloroplast genome sequencing and comparative analysis of six medicinal plants of *Polygonatum*

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K E Y W O R D S: *Polygonatum* , chloroplast genome, interspecific relationships, DNA barcoding molecular identification, phylogenetic

1 | INTRODUCTION

Polygonatum Mill. is the largest genus in the Polygonateae tribe.*Polygonatum* Mill. comprises 70 species widely distributed across the temperate Northern Hemisphere (Gong et al., 2023). Of these, 37 species and one variety had records of medicinal use and the rhizome was the most commonly used part of the plant. Many *Polygonatum* plants are highly effective in treatment age-related diseases, diabetes, lung diseases, coughs, fatigue, and feebleness in clinical practice (Zhao et al., 2018). *Polygonatum kingianum* , *P. sibiricum* ,*P. cyrtonema* and *P. odoratum* contain various active ingredients, such as polysaccharides, steroidal saponins, and alkaloids which are collected from the Chinese Pharmacopoeia as the official source of Polygonati rhizoma and Polygonate odorati rhizome (Luo et al., 2022). Polysaccharides from *P. filipes* and *P. zanlanscianense* , as recorded by provincial standards, can significantly inhibit the formation of intermediate diabetes products (Zhao et al., 2020).*Polygonatum* is a diverse and widely distributed plant.*Flora of China* records that it is distributed in many provinces of China, including Heilongjiang, Jilin, Liaoning, Hebei, Shanxi, Shaanxi, Nei

Mongol, Ningxia, Gansu, Henan, Shandong, Anhui, Zhejiang, and Sichuan. Among them, *P. cyrtonema* is mainly distributed in the Yangtze River Basin, whereas *P. kingianum* occurs in southwestern China (Guo et al., 2022). In addition, the presence of multiple medicinal plants of *Polygonatum* make it difficult to identify. The phyllotaxis is usually an important basis for distinguishing *Polygonatum* plants (Xia et al., 2022), which can be mainly classified into alternate leaf types and verticillate leaf types according to the phyllotaxis characteristics; for example,*P. cyrtonema* , *P. filipes* , and *P. odoratum* are alternate leaf types and *P. sibiricum* , *P. kingianum* , and*P. zanlanscianense* are verticillate leaf types. The rhizome morphology of *Polygonatum* plants is also diverse, with *P. sibiricum* having a "Jitou-type" and an atypical "Jitou-type".*P. cyrtonema* have three types, "Jiang-type", "Cylinder-type", and "Baiji-type", with variations in the composition of the rhizomes of the different types (Hu et al., 2022). The indiscriminate use of different species may cause crucial effects on the patient. Therefore, establishing an effective identification method to distinguish between the medicinal species of *Polygonatum* , is necessary. DNA barcoding and molecular markers have been used for accurate species delimitation and phylogenetic relationship inference in*Polygonatum* (Jiao et al., 2018; Lee et al., 2021). However, species identification using traditional DNA barcoding techniques is limited and does not allow for the accurate identification of closely related species (Newmaster et al., 2008). The chloroplast genome sequence has been proposed as a superbarcode for species authentication (Li et al., 2015). The chloroplast genomes of medicinal plants such as*Atractylodes* (Wang et al., 2021), *Gentiana* (Zhao et al., 2022), *Peucedanum* (Sun et al., 2023) and *Tripterygium* (Xu et al., 2024) have been studied one after another and their intragenus evolutionary relationships have been explored. Studies of the chloroplast genomes of *Polygonatum* spp. have also been reported. The genus *Polygonatum* is a monophyletic group comprising three sections (*sect. Sibirica* , sect. *Polygonatum* , and*sect. Verticillata*) (Xia et al., 2022).*Heteropolygonatum* , *Disporopsis* , *Maianthemum* , and*Disporum* are sister groups to *Polygonatum* within Polygonateae (Wang et al., 2022). During the long evolutionary process of the genus, the leaf type of this group evolved from verticillate leaves to alternate leaves. The construction of a phylogenetic tree based on the chloroplast genome showed that *P. kingianum* can be clearly distinguished from other species in the verticillate leaf group, providing a reliable means for the accurate identification of *P. kingianum* (Shi et al., 2023). However, these studies did not focus on sequencing or analyzing the chloroplast genomes of medicinal plants of the genus *Polygonatum* . Consequently, we sequenced and analyzed the whole chloroplast genomes of six medicinal species of *Polygonatum* to enrich the understanding of genome characteristics, screen mutational hotspots, and SSRs for authentication of *Polygonatum* medicinal plants and elucidate the phylogenetic relationship of *Polygonatum* medicinal plants.

2 | MATERIALS AND METHODS

2.1 | Plant materials collection and DNA extraction

This study collected six medicinal plants of *Polygonatum* from different regions (Figure S1, Table S1). The species was confirmed and identified and all voucher specimens were stored at the Chinese Materia Medica Resource Center, Anhui University of Chinese Medicine (Hefei, China). Healthy and fresh leaves were chosen to extract the complete genomic DNA using a plant DNA mini kit (Plant DNA Kit D3485, Omega Bio-Tek, Guangzhou, China). The purity, integrity, and concentration of the DNA were checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1.0%(w/v) agarose gel electrophoresis (Wu et al., 2021). The concentration of DNA samples that meet the requirements of chloroplast genome sequencing [?] 20 ng/μL; the total amount of samples [?] 100 ng; $OD_{260/280}$ = 1.8–2.2, and high-quality DNA was used to construct gene libraries (Zhu et al., 2018).

2.2 | Chloroplast DNA sequencing, assembly and annotation

Genesky Biotechnologies Inc. (Shanghai, China) was commissioned to use Illumina HiSeq 4000 to randomly sequence the chloroplast genomes of each DNA sample from *Polygonatum* plants. Genomic DNA was frag-

mented after quality control and the adaptor was ligated to construct the library. To obtain high-quality sequencing data and improve the accuracy of subsequent bioinformatic analyses, quality control and filtering of the original offline data must be performed. For example, excluding sequences containing more than 3 N bases, eliminating sequences with less than 60% of high-quality bases (Phred score [?] 20), eliminating low-quality bases at the 3' end, and removing the sequences with lengths less than 60 bp. Assembling clean reads at the contig level. According to the reference near-source species, metaSPAdes software (Nurk et al., 2017) was used for genome assembly, and the assembly results were analyzed and corrected to determine whether the ring was formed, correct the contig direction, and determine the initial base position. The chloroplast genomes were annotated using CPGAVAS2 software (Shi et al., 2019). GenBank files were drawn into a gene circle map using GeSeq (https://chlorobox.mpimp-golm.mpg.de/geseq.html) (Tillich et al., 2017). The sequence data and gene annotation information were uploaded to the National Center for Biotechnology Information (NCBI) database.

2.3 | Structure analysis of the chloroplast genomes

SSRs, also called microsatellites or short tandem repeats (STRs), are tandem repeats of DNA segments composed of 1–6 base pairs widely used in genetic analysis as molecular markers. The SSR sites of each sample genome were detected using the online software MISA (Beier et al., 2017) (https://webblast.ipk-gatersleben.de/misa/), with the minimum repeat parameters set at ten repeat units for mononucleotide, five repeat units for dinucleotide, four repeat units for trinucleotide, three repeat units for tetranucleotides, pentanucleotides, and hexanucleotides (Wang et al., 2022). Forward, palindromic, reverse, and complementary repeats, were predicted using the REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer), the parameters are set to hamming distance = 3, maximum computed repeats = 5, 000 bp, minimal repeat size = 30 bp (Kurtz et al,2001). Codon usage of the chloroplast genomes of six medicinal plants of*Polygonatum* was investigated using the relative synonymous codon usage (RSCU) module in the Python CAI package. In gene translation, the frequency of synonymous codons corresponding to each amino acid is discrepant; that is, some synonymous codons are applied more frequently than others (Parvathy et al., 2022). The RSCU value represents relative synonymous codon usage. For RSCU=1, codon usage without preference; RSCU $>$ 1, codon usage frequency is higher than expected; and RSCU $<$ 1, codon usage frequency is lower than expected (Sharp et al., 1986). Microsoft Office Excel and TBtools (Chen et al., 2020) were used to convert statistical data into visual graphs.

2.4 | Comparison of the chloroplast genomes

The expansion and contraction of inverted repeat regions in the chloroplast genome may lead to changes in genome length. Using CPJSdraw software (Xu et al., 2024), we detected the inverted repeat (IR) boundary regions by comparing the locations of the coding genes. Sequence alignment of the whole chloroplast genome was performed using the online tool mVISTA (Fernández-Jiménez et al., 2021) (http://genome.lbl.gov/vista/index.shtml) in shuffle-LAGAN mode. DnaSP software (Rozas et al., 2017) was used to calculate the nucleotide diversity based on sliding window analysis, setting the window length to 600 bp and the step size to 200 bp. To investigate the presence of selective pressure on the chloroplast protein-coding genes among*Polygonatum* , we used *P. zanlanscianense* as a reference, and the coding sequences were used to calculate ka, ks values using KaKs_Calculator2 (Wang et al., 2010).

2.5 | Phylogenetic tree construction of *Polygonatum* medicinal plants

Six medicinal plants of *Polygonatum* and other medicinal plants of*Polygonatum* downloaded from the NCBI were used for phylogenetic analysis, whereas *Dioscorea aspersa* and *Dioscorea alata* were set as outgroups. A total of 59 chloroplast complete sequences were aligned using MAFFT (Katoh et al., 2013) and trimmed using TrimAL (Capella-Gutiérrez et al., 2009). The best-fit model according to Bayesian information criterion was K3Pu+F+I+I+R4, which was calculated using ModelFinder (Kalyaanamoorthy et al., 2017). An IQ-TREE

(Nguyen et al., 2015) phylogenetic tree was constructed based on the whole chloroplast sequences using the PhyloSuite platform (Zhang et al., 2020). The tree is displayed on the iTOL (Letunic et al., 2021) website (https://itol.embl.de/).

3 | Results

3.1 | Basic characteristics of chloroplast genomes

This study obtained the complete chloroplast genome sequences of six medicinal plants of *Polygonatum* : *P. zanlanscianense* ,*P. kingianum* , *P. sibiricum* , *P. cyrtonema* , *P. filipes* and *P. odoratum* . The length of the complete chloroplast genome sequences ranged from 154, 578 bp (*P. odoratum*) to 155, 807 bp (*P. kingianum*), with an average length of 155, 429 bp. The*Polygonatum* chloroplast genome has a typical quadripartite structure, including large single-copy (LSC), small single-copy (SSC), and a pair of IR regions. The length of the LSC ranged from 83, 527 bp (*P. odoratum*) to 84, 626 bp (*P. kingianum*). In the SSC regions, the chloroplast genome of *P. sibiricum* was the shortest (18, 415 bp) and *P. kingianum* was the longest (18, 529 bp). The IR lengths ranged from 26, 297 bp for *P. odoratum* to 26, 415 bp for *P. zanlanscianense* . GC content was unevenly distributed across the four parts of the chloroplast genome. The total GC content of the chloroplast genome was 37.7% and the GC contents in the LSC, SSC, and IR regions were 35.7–35.8 %, 31.5–31.6 %, and 43 %, respectively (Figure 1, Table 1). Differences were observed in the number of chloroplast genome genes among the six *Polygonatum* species. *P. kingianum* and*P. filipes* encoded 127 and 130 genes, respectively, whereas the other species encoded 131 genes. These genes comprised 84–85 protein-coding genes, 37–38 transfer RNA genes and 6–8 ribosomal RNA genes. The IR regions included seven protein-coding genes (*rpl2* ,*rpl23* , *rps12* , *rps19* , *rps7* , *ndhB* , and*ycf2*), 11 tRNA genes (*trnG-UCC* , *trnE-UUC* ,*trnM-CAU* , *trnH-GUG* , *trnI-CAU* , *trnL-CAA* ,*trnV-GAC* , *trnI-GAU* , *trnA-UGC* , *trnR-ACG* , and*trnN-GUU*), and all four rRNA-coding genes. A total of 22 genes in the chloroplast genomes of the six *Polygonatum* species contained introns *ycf3* and *clpP* each containing two introns.*rps12* , *trnK-UUA* , *rps16* , *trnG-UCC* ,*trnT-CGU* , *atpF* , *rpoC1* , *trnL-UAA* ,*trnV-UAC* , *petB* , *petD* , *rpl16* , *rpl2* ,*ndhB* , *trnI-GAU* , *trnE-UUC* , *trnA-UGC* , and*ndhA* contained a single intron. *trnT-CGU was annotated only in P. kingianum, which lacked rrn4.5, rpoC1and trnV-UAC.* The*ndhB* gene of *P. kingianum* did not contain introns.*The ycf2* and *ycf1* genes of *P. zanlanscianense* also contained two introns, *ycf2* gene of *P. sibiricum* and*P. cyrtonema* contained one intron, and *P. kingianum* ,*P. filipes* and *P. odoratum* had no introns. Gene and intron losses occurred during the evolution of the six medicinal plants of*Polygonatum* (Table 2). TABLE 1 Basic chloroplast characteristics of six medicinal plants of*Polygonatum* .

Characteristics	*P. zanlanscianense*	*P. kingianum*	*P. sibiricum*	*P. cyrtonema*	*P. filipes*	*P. odoratum*
Total size (bp)	155,663	155,807	155,572	155,618	155,333	154,578
LSC length (bp)	84,417	84,626	84,475	84,451	84,279	83,527
IRa length (bp)	26,415	26,326	26,341	26,371	26,300	26,297
IRb length (bp)	26,415	26,326	26,341	26,371	26,300	26,297
SSC length (bp)	18,416	18,529	18,415	18,425	18,454	18,457
Total genes	131	127	131	131	130	131
Protein coding genes	85	84	85	85	84	85
tRNA genes	38	37	38	38	38	38
rRNA genes	8	6	8	8	8	8
Overall GC content(%)	37.7	37.7	37.7	37.7	37.7	37.7
GC content in LSC(%)	35.7	35.7	35.7	35.8	35.7	35.8
GC content in IRa (%)	43	43	43	43	43	43
GC content in IRb (%)	43	43	43	43	43	43
GC content in SSC(%)	31.6	31.5	31.6	31.6	31.6	31.6

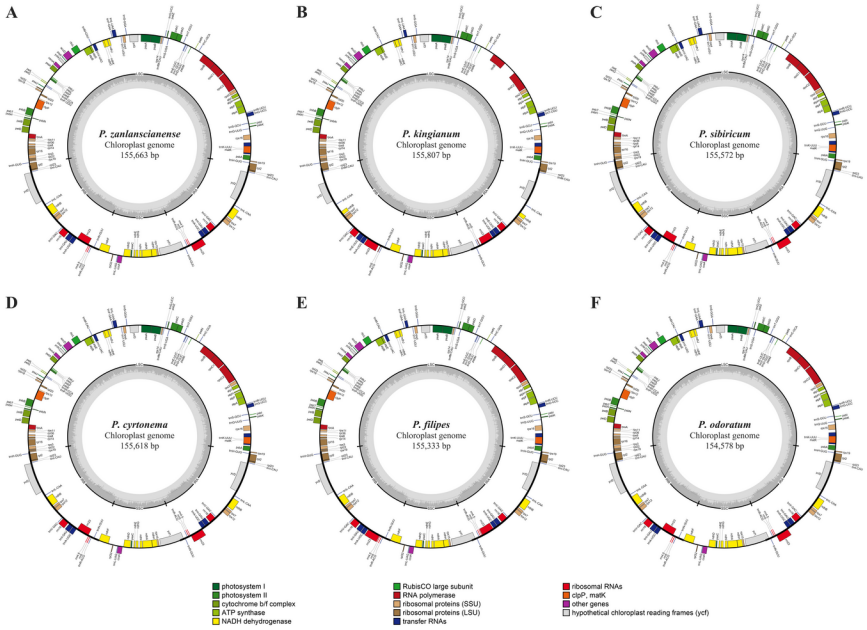

FIGURE 1 Circular maps for chloroplast genome of six medicinal plants of*Polygonatum* . (A) *P. zanlanscianense* , (B) *P. kingianum* , (C) *P. sibiricum* , (D) *P. cyrtonema* , and (E)*P. filipes* , (F) *P. odoratum* . Genes inside and outside the loop are transcribed in a clockwise and anti-clockwise direction. Genes with diverse functions are represented using different colors.

3.2 | Simple sequence repeats and interspersed repeats sequences analysis

The MISA program was used to identify the SSRs in our study. The total number of SSR sites in the six *Polygonatum* chloroplast genomes was 64 (*P. cyrtonema*) –76 (*P. kingianum*), comprising mononucleotide, dinucleotide, trinucleotide, tetranucleotide, and pentanucleotide repeats; only hexanucleotide repeats were found in*P. cyrtonema* (Figure 2A). Among the SSR sites, the number of mononucleotide repeats was the highest, totaling 242. A/T repeat motifs accounted for the largest proportion, ranging from 97.56% to 100% and*P. kingianum* , *P. filipes* and *P. odoratum* had C/G mononucleotide repeat motifs. A total of 93 dinucleotide repeats were found, with AT/TA accounting for 80%. Trinucleotide repeat sequences were constant in *P. odoratum* , *P. filipes* , and *P. cyrtonema* , whereas ATT and ATA were absent in the other three*Polygonatum* species. The tetranucleotide and pentanucleotide repeat sequences in the chloroplast genomes of six *Polygonatum* species were similar. Among them, only *P. sibiricum* had AAAT tetranucleotide repeat motifs and *P. kingianum* had one more AATA than the other species (Tables 3 and S2–S7).

A total of 268 repetitive sequences were identified in this study. Palindromic repeats accounted for the largest proportion (51.12 %), followed by forward repeats (36.07 %) and reverse repeats (2.99 %). No complementary repeats were found in the chloroplast genomes of the six*Polygonatum* species (Figure 2B). Among these types of*Polygonatum* , *P. zanlanscianense* had significantly longer repetitive sequences than the other species, with 62. *P. sibiricum* (49), *P. cyrtonema* (46), *P. kingianum* (39),*P. filipes* (36), and *P. odoratum* (36). Most species were mainly distributed in the IR region. Among them, the number and distribution of all repeat types in *P. filipes* and *P. odoratum* were almost the same. The distribution area was mainly in the LSC region and could be distinguished from the others (Tables S8–S13) and the repeat motif size of *Polygonatum* was mainly concentrated in the range of 30–39 bp (Figure S2).

TABLE 2 List of genes found in the chloroplast genomes of six medicinal plants of *Polygonatum* .

Gene Category	**Gene Name**
Ribosomal protein (LSU)	*rpl33, rpl20, rpl36, rpl14, rpl16*, rpl22, rpl2*,* $^{(\times 2)}$*, rpl23*$^{(\times 2)}$*, rpl32*
Ribosomal protein (SSU)	*rps12*,* $^{(\times 2)}$*, rps16*, rps2, rps14, rps4, rps18, rps11, rps8, rps3, rps19*$^{(\times 2)}$*,*
RNA polymerase	*rpoA, rpoB, rpoC1*, rpoC2*
Protease	*clpP**
Maturase	*matK*
Ribosomal RNA	*rrn16*$^{(\times 2)}$*, rrn23*$^{(\times 2)}$*, rrn5*$^{(\times 2)}$*, rrn4.5*$^{(\times 2)}$
Transfer RNA	*trnK-UUU*, trnQ-UUG, trnS-GCU, trnT-CGU*, trnG-UCC*,*$^{(\times 2)}$*, trnR-U*
Photosystem I	*psaB, psaA, psaI, psaJ, psaC*
Photosystem II	*psbA, psbK, psbI, psbM, psbD, psbC, psbZ, psbJ, psbL, psbF, psbE, psbB, p*
NADH dehydrogenase	*ndhJ, ndhK, ndhC, ndhB*,* $^{(\times 2)}$*, ndhF, ndhD, ndhE, ndhG, ndhI, ndhA*, n*
ATP synthase	*atpA, atpF*, atpH, atpI, atpE, atpB*
cytochrome b/f complex	*petN, petA, petL, petG, petB*, petD**
hypothetical chloroplast reading frames (*ycf*)	*ycf3*, ycf4, ycf2*,* $^{(\times 2)}$*, ycf1**
Rubisco large subunit	*rbcL*
other gene	*accD, cemA, ccsA*

* indicates the intron-containing genes,$^{(\times 2)}$ indicates that the gene has two copies.

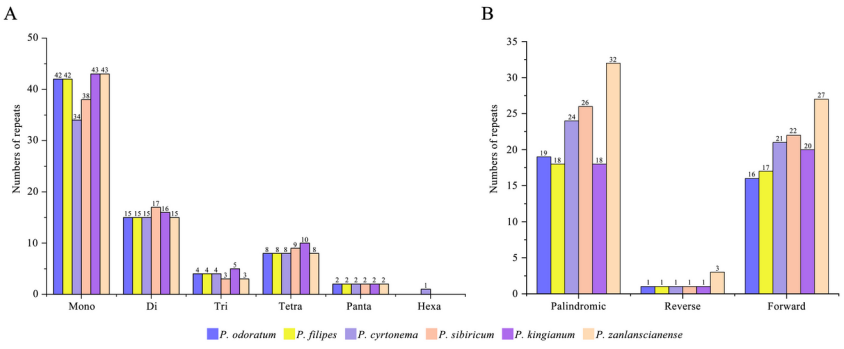

FIGURE 2 SSRs(A) and long repetitive sequences (B) analysis of chloroplast genomes of six *Polygonatum* species.

TABLE 3 Type and amounts of SSRs in the chloroplast genomes of six*Polygonatum* species.

SSR type	**Repeat unit**	*Polygonatum zanlanscianense*	*Polygonatum kingianum*	*Polygonatum sibiricum*
Mononucleotide	A/T	43	42	38
	C/G	0	1	0
Dinucleotide	AT/AT	6	6	7
	GA/TC	3	3	3
	TA/TA	6	7	7
Trinucleotide	ATA	0	0	1
	ATT	0	1	0
	CAG	1	1	1
	TAA/TTA	2	3	1
Tetranucleotide	AAAT	0	0	1

SSR type	Repeat unit	*Polygonatum zanlanscianense*	*Polygonatum kingianum*	*Polygonatum sibiricum*
	AATA	2	3	2
	AATG /CATT	2	2	2
	ATTG	1	2	1
	GAAT	1	1	1
	TTAA	1	1	1
	TTGA	1	1	1
Pentanucleotide	CGAAA /TTTCG	2	2	2
Hexanucleotide	ATAGTA	0	0	0
Total		71	76	69

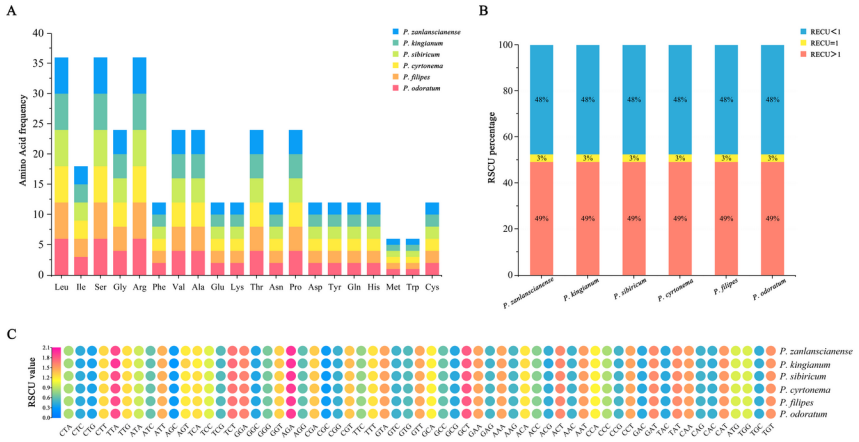

FIGURE 3 Analysis of amino acids and codon bias among six medicinal plants of *Polygonatum* . (A) Frequency of amino acids in the chloroplast genomes of six *Polygonatum* . (B) RSCU percentage analysis of codons in chloroplast genomes. (C) Heat-map of the RSCU values among six *Polygonatum* .

3.3 | Statistics of codon usage

The total number of codons in the chloroplast genome of the six medicinal plants of *Polygonatum* in protein-coding sequences was 23, 381 (*P. kingianum*) to 26, 036 (*P. odoratum*), containing 61 codons encoding 20 amino acids (termination codons were not incorporated in the statistics). Amino acids are encoded by 2–6 synonymous codons, most of which are not Met and Trp. Leu was encoded by the highest number of codons, accounting for 10.3%, whereas Cys was encoded by the lowest number of codons, accounting for 1.2% (except*P. kingianum* accounting for 1.1%). The RSCU value can be used to detect a synonymous codon usage bias. Except for Met and Trp (RSCU = 1), which do not show codon usage bias, most amino acid codons have usage bias. Thirty types of codons were found with RSCU > 1 in the six medicinal plants of *Polygonatum* , of which 28 were A/T-ending codons. Only the TTG codon encoding Leu and the TCC codon encoding Ser ended with G/C, indicating that A/T bases were preferred and G/C bases were not preferred. A comprehensive analysis of the histogram and heat map of codon usage showed that codon usage of the six species was consistent. The analysis of RSCU values provided data for studying the evolution and gene expression of *Polygonatum* (Figure 3, Table S14).

3.4 | IR borders comparison

During plant chloroplast genome evolution, the IR regions are accompanied by contraction and expansion and some genes enter the IR or SC regions. The IR/SC boundaries and their adjacent genes in the six medicinal plants of *Polygonatum* were compared using CPJSdraw. As shown (Figure 4), the total sequence length and IR region length of the chloroplast genome between species were relatively conserved and the genotypes of the IR/SC borders were essentially the same. Genes*rpl22* , *rps19* , *trnN* , *ndhF* , *ycf1* and*psbA* were present at the IR boundaries. The front ends of*rps19* genes of *P. zanlanscianense* , *P. kingianum* ,*P. cyrtonema* , *P. filipes* and *P. odoratum* were 13 or 17 bp away from the IRb boundary, whereas in *P. sibiricum* , the*rps19* gene front ends coincided with the LSC/IRb boundary. In the LSC/IRa boundary, the end of the *rps19* gene of *P. sibiricum* coincides with the LSC/IRa boundary and is also different from other species. *rpl22* was completely situated in the LSC region and was 27–34 bp away from the LSC/IRb boundary, *P. sibiricum* was 47 bp. In six medicinal plants of *Polygonatum, ndhF* gene was prolonged to the IR by 22–34 bp. The *ycf1* gene spans the junction between the SSC and IRa. The *pbsA* gene is located downstream of the junction of LSC and IRa, 87–91 bp from the boundary.

3.5 | Sequence divergence and high variation regions analyses

The mVISTA online tool was used to globally align the chloroplast genomes of these *Polygonatum* species, with *P. zanlanscianense* as the reference and the sequence differences between their genomes were compared (Figure 5). In comparison, the chloroplast genome sequences of six *Polygonatum species* were generally conserved. From the position of sequence differences, the rRNA gene region (blue part) was highly conserved, the non-coding region (red part) was more variable than the conserved protein coding region (purple part). The variation of LSC region and SSR region was greater than that of IR region and the difference was greater in LSC region, followed by SSR region. In addition, DnaSP software was used to determine the nucleotide diversity of the chloroplast genome of six medicinal*Polygonatum* plants and to identify mutation hotspots (Figure 6). The results showed that the Pi values of *Polygonatum* was 0–0.02633 and the high-variation regions were mainly concentrated in the LSC and SSC regions. Twenty-one genic regions with high Pi values (Pi [?] 0.01) were considered hotspots. Among them, 11 genic regions were located in the LSC region, namely *psbA* , *trnK-UUU* ,*psbI-trnS-GCU* , *trnS-GCU* , *rpoB* , *trnL-UAA* ,*trnF-GAA* , and *psbJ* ; among them, 10 genic regions were located in the SSC region, namely *rpl32* , *trnL-UAG* ,*ccsA* , *ccsA-ndhD* and *ycf1* . These hotspots provide a reference for the subsequent molecular identification of*Polygonatum* medicinal plants to identify potential chloroplast DNA barcodes.

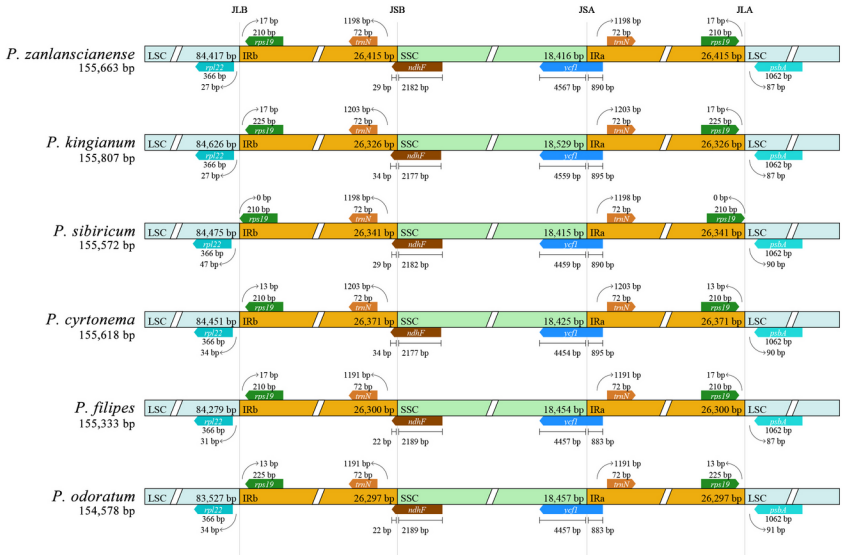

FIGURE 4 Alignment of LSC, SSC, and IR regions boundary of chloroplast genomes of six medicinal plants of *Polygonatum* .

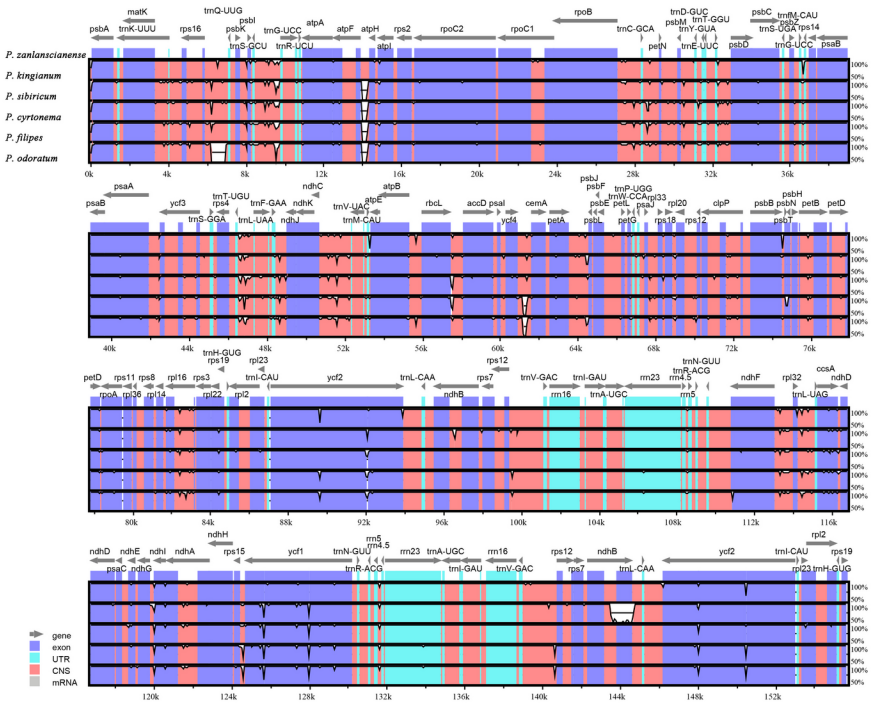

FIGURE 5 Sequence alignment among six *Polygonatum* , with *P. zanlanscianense* as a reference. The y-axis represents the percent identity within 50-100%. Genome regions are color coded as genes (grey arrow), protein coding (purple), RNA coding genes (blue), and non-coding sequences (red).

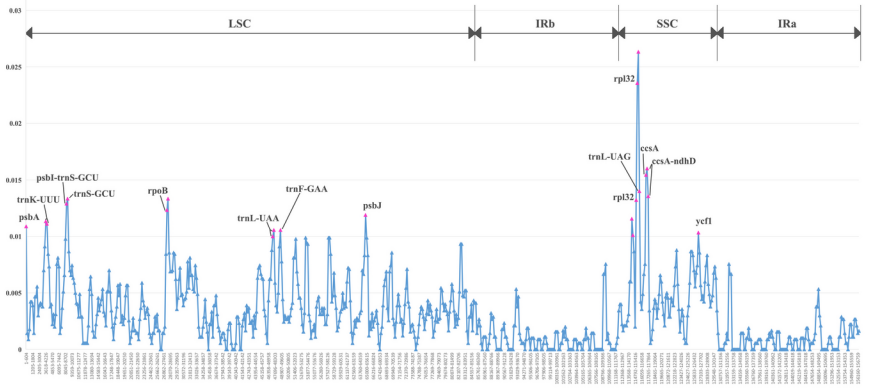

FIGURE 6 Sliding window analysis of the whole chloroplast genomes of six medicinal plants of *Polygonatum* for nucleotide diversity (Pi). X-axis: position of the window. Y-axis: nucleotide diversity of each window. Red signs are the selected high variation regions.

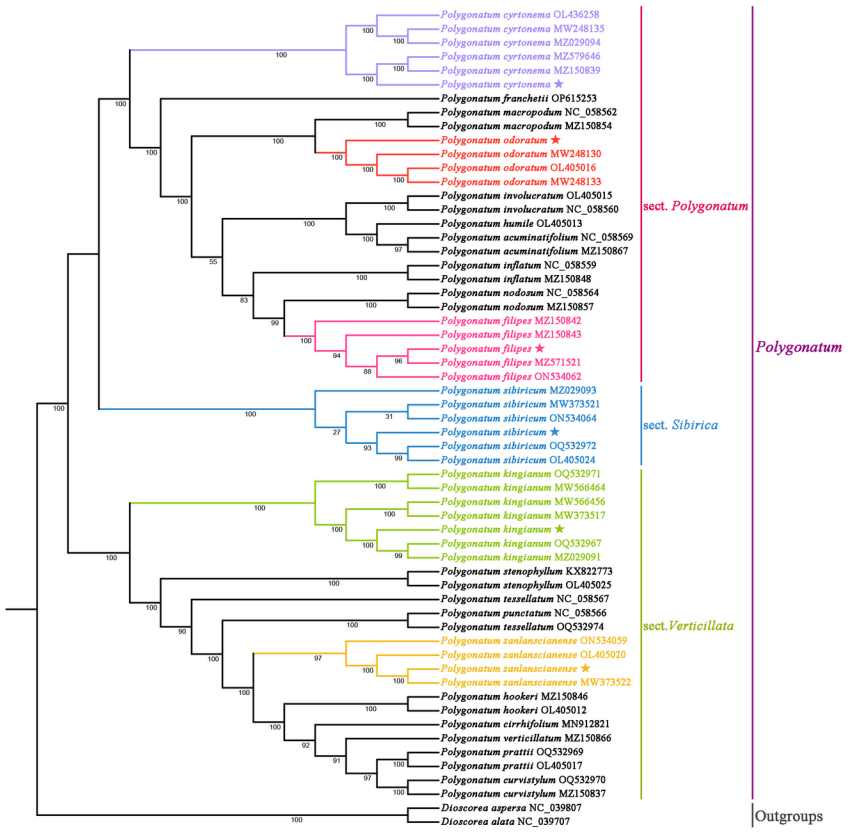

FIGURE 7 Phylogenetic tree constructed with the whole sequences of the chloroplast genomes of the above 59 species.

3.6 | Selective pressure analysis

We calculated the Ka/Ks values of *Polygonatum* protein-coding genes (PCGs) based on KaKs_Calculator2. The genes subject to positive selection are *matK* , *ndhA* , *petB* , and *ycf2* , with a maximum Ka/Ks value of 1.41. The genes that were positively selected can be categorized into five groups according to their functions: (1) maturation enzyme gene *matK* ; (2) photosynthetic system genes *ndhA* and *petB* ; and (4) the *ycf2* gene with unknown function. The remaining genes, all with Ka/Ks values less than 1, were subjected to purifying selection, with *atpI* being the lowest at 0.048 (Figure S3).

3.7 | Phylogenetic analysis

Phylogenetic trees were constructed using the maximum likelihood (ML) approach based on 59 complete chloroplast genome sequences of*Polygonatum* medicinal plants published in the NCBI database (*Dioscorea aspersa* and *Dioscorea alata*) to illustrate the genetic relationships of these species. The results showed that the two outgroups were distinct from *Polygonatum* . The nodes of the phylogenetic tree in this study had a high support rate and strong reliability for the phylogenetic analysis (Figure 7, Table S15). In the phylogenetic trees, all alternate leaf types of *Polygonatum* species were clustered on one large clade and species with verticillate leaves were divided into *sect. Sibirica* and *sect. Verticillata* group, confirming that

the leaf type affects the classification of the genus. Of these, *sect. Polygonatum* and *sect. Sibirica* clustered together and exhibited a sister relationship with *sect. Verticillata.* Among the six*Polygonatum* species, *P. sibiricum* formed a monophyletic group (*sect. Sibirica*) with 100 % support. *P. cyrtonema* was the earliest clade to differentiate, followed *P. odoratum* , *P. filipes* and other related species clustered into a sister clade in *sect. Polygonatum* . *P. kingianum was* the earliest *species differentiated from sect. Verticillata. P. zanlanscianense* and other verticillate leaf type species of *Polygonatum* clustered into sister clades with *P. kingianum. These results are consistent with those of the previous studies.*

In addition, we downloaded the chloroplast genomes of six other*Polygonatum* species from the database of the National Center for Biotechnology Information (NCBI) to explore the chloroplast genome affinities of *Polygonatum* in different regions. The phylogenetic results showed that *P. cyrtonema* was collected in Anhui Province and had the closest relationship with *P. cyrtonema* (MZ579646, MZ150839) stemming from Zhejiang Province and a relatively distant relationship with Hunan (OL436258), Beijing (MW248135), and Jiangxi (MZ029094). *P. zanlanscianense* (from Yunnan Province) was closely related to Guizhou Province (MW373522), followed by Hubei Province (OL405020) and Hunan Province (ON534059).

4 | DISCUSSION

4.1 | Chloroplast genome structure analysis of *P. kingianum*

In the present study, the chloroplast genome sequences of six medicinal plants of *Polygonatum* were analyzed. According to the assembly annotation results, the chloroplast genomes of *Polygonatum* , similar to most angiosperms, exhibit a classical tetrad structure and are closed-circular double-stranded DNA molecules (Daniell et al., 2016). The complete genome length ranged from 154, 578 to 155, 807 bp and the lengths of the LSC, SSC, and IR regions were relatively conserved, with no obvious contraction or expansion. However, the chloroplast genome of *P. kingianum* is the largest and its measured genome size is usually above 155, 700 bp (Guo et al., 2022; Zhang et al., 2023). *P. zanlanscianense* chloroplast genome was the next largest and it was statistically found that the genome length of the verticillate leaf types of *Polygonatum* was typically larger than that of the alternate leaf types (Table S15).*Polygonatum* chloroplast genome encodes 127–131 genes. *P. kingianum* encodes 127 genes, comprising 84 protein-coding genes, 37 transfer RNA genes, and 6 ribosomal RNA genes. Compared to other species, *P. kingianum* lacks *rpoC1* , *trnV-UAC* and*rrn4.5* genes, which is consistent with the results of previous studies (Wang et al., 2022). The length and composition of the chloroplast genome of *P. kingianum* differ from those of other species, which may be related to the limited geographic growth environment of *P. kingianum* .

SSRs are widely distributed in chloroplast genomes and can be used for species identification and genetic diversity analyses (Jiang et al., 2018). A total of 64–76 SSRs are present in the chloroplast genomes of six *Polygonatum* species (Zhang et al., 2023; Yan et al., 2023). The number of SSRs in *Polygonatum* is comparable to that in*Peucedanum* (Liu et al., 2022), *Adonis* (Nyamgerel et al., 2023), and *Paeoniaceae* (Cai et al., 2023). The A/T and AT/TA repeat sequences account for the largest number of mono- and dinucleotide repeat sequences, respectively, influencing the overall GC content of genomes (Gichira et al., 2019).

4.2 | Relatively conserved chloroplast genomes in*Polygonatum*

In the chloroplast genomes of angiosperms, rRNA genes are generally located in the IR region, which to some extent makes the IR region more conserved than the LSC and SSC regions, making the IR region the most conserved region in the chloroplast genome. However, during the evolution of this species, the IR boundary has undergone contraction and expansion, affecting the length of the chloroplast genome (Li et al., 2013; Zhang et al., 2016). The length of *Pelargonium hortorum* chloroplast genome is 217, 942 bp owing to the extreme expansion of its IR region (Chumley et al., 2006); the IR region length of *Pinus thunbergii* is shortened to only 495 bp, and the chloroplast genome length is 119, 707 bp (Wakasugi et al., 1994); whereas the IR of*P. hortorum* is generally absent (Liu et al., 2020). The IR boundary region of the

chloroplast genomes of the six *Polygonatum* medicinal plants is relatively conserved and does not undergo significant expansion or contraction (Zhang et al., 2023; Yan et al., 2023). However, the *rps19* gene of *P. sibiricum* overlapped with the LSC/IR boundary and differed from that of the other five species, which was hypothesized to be related to the evolution of*P. sibiricum* .

The results of mVISTA and nucleotide diversity analyses indicated that the chloroplast genomes of the six *Polygonatum* species were highly similar. The highly variable regions of the chloroplast genomes of *Polygonatum* species were mainly concentrated in the LSC and SSC regions, and 21 highly variable Pi-fragments were screened. Among them, *psbI-trnS-GCU* , *psbJ* , *rpl32* , *trnL-UAG* ,*ccsA* , *ndhD* , and *ycf1* were screened as candidate markers. This provides a reference for subsequent molecular identification of *Polygonatum* species to identify potential chloroplast DNA barcodes.

The selective pressure analysis revealed that *matK* , *ndhA* ,*petB* , and *ycf2* genes were subject to positive selection, of which *ndhA* and *petB* were photosynthetic system genes.*Polygonatum* plants are mainly distributed in the understory, thickets or shady areas of mountain slopes, and adaptation to sunlight stress may be an important genetic basis for the evolution of adaptations at the chloroplast level in *Polygonatum* (Zhang et al., 2023).

4.3 | Species of Polygonatum form three major groups

The genus *Polygonatum* is rich in germplasm resources and contains various species that are difficult to identify owing to their similar morphology. The classification of this genus has long been controversial and is a concern for taxonomic treatment. The genus *Polygonatum* was first established by Miller (Zhao et al., 2014). Baker (Baker et al., 1875) divided *Polygonatum* into three groups,*Alternifolia* , *Verticillata* and *Oppositifolia,* according to the characteristics of phyllotaxis. However, species of*Polygonatum* differ in morphological characteristics, such as perianth and bracts, in addition to differences in leaf type.*Flora of China* synthesized these characteristics and divided the genus into eight groups. Tamura subdivided the genus into two sections:*Polygonatum* and *Verticillata* (Tamura et al., 1993). To date, botanists have not reached a unified view of the classification of *Polygonatum* species based on their morphological characteristics. With the development of molecular biology, phylogenetic studies of species based on chloroplast genome sequences have provided novel perspectives for solving plant affinities. Previous phylogenetic studies of *Polygonatum* chloroplast genomes have supported the classification of this genus into three groups: *sect. Verticillata* , *sect. Polygonatum* , and *sect. Sibirica* , where *sect. Polygonatum* and *sect. Sibirica* and *sect. Verticillata* is a sister species of *sect. Polygonatum* +*sect. Sibirica* (Floden et al., 2018). *Sect. Verticillata* contains *Polygonatum* species with verticillate leaf types,*sect. Polygonatum* contains alternate leaf types, and *sect. Sibirica* usually has only one species, *P. sibiricum* (Qin et al., 2024). Consistent with previous findings, the chloroplast genome phylogenetic tree of *Polygonatum* used in the present study was divided into three clades: *sect. Verticillata* , *sect. Polygonatum* , and *sect. Sibirica* . *P. kingianum* and*P. zanlanscianense* clustered in the verticillate leaf taxon*sect. Verticillata* . *P. cyrtonema* , *P. filipes* and*P. odoratum* clustered into the alternate-leaf taxon *sect. Polygonatum* and *P. sibiricum* clustered as a separate monophyletic group, similar to sect. *Sibirica (* Meng et al., 2014*).*

Studies have shown that geographical factors have a certain effect on the chloroplast genome (Yang et al., 2022). P. cyrtonema, for example, *is widely distributed in China; however, it is mainly distributed in the middle and lower reaches of the Yangtze River (Zhang et al., 2024). At the same time, the southern part of Anhui and the eastern part of Zhejiang Province are in the middle and lower reaches of the Yangtze River. The Dabie Mountains and Southern Anhui Mountains in southern Anhui is suitable altitude for the growth of P. cyrtonema* (Liu et al., 2023). *Beijing and Hunan Province are farther away from this region and have different climatic environments; therefore, it is speculated that those may be geographically isolated from the P. cyrtonema in the Yangtze River Basin. Therefore, the phylogenetic tree showed that the P. cyrtonema from Anhui was more closely related to the P. cyrtonema from Zhejiang. We believe that geographical factors influence the phylogeny of Polygonatum species and this requires further exploration.*

5 | Conclusions

This study utilized the Illumina Hiseq platform to obtain the complete chloroplast genomes of six medicinal species of the genus*Polygonatum* . The chloroplast genomes were relatively conserved in terms of length, gene content, and genomic structure. A certain pattern was observed in genome length among species. *Polygonatum* spp. with verticillate leaves were larger than those with alternating leaves. The genes encoded by *P. kingianum* appeared to be lost compared to those of the other species. Twenty-one highly variable loci were screened as markers for the candidate identification of*Polygonatum* species. The phylogeny of the species in the genus was studied based on chloroplast genome sequences and the results supported the classification of *Polygonatum* into three groups:*sect. Verticillata* , *sect. Polygonatum* , and *sect. Sibirica* . This study provides a basis for the molecular identification, phylogenetic evolution, and genetic diversity of *Polygonatum* .

AUTHOR CONTRIBUTIONS

Jinchen Yao: Conceptualization(equal); methodology(equal); data curation and writing-original draft preparation(equal). **Zhaohuan Zheng:** Conceptualization(equal); software(equal); data curation and writing-original draft preparation(equal). **Tao Xu:** Formal analysis(equal); investigation(equal); resources(equal); visualization(equal). **Duomei Wang:** Methodology(equal); software(equal); visualization(equal); supervision(equal).**Jingzhe Pu:** Validation(equal); investigation(equal); formal analysis(equal). **Yazhong Zhang:** Conceptualization(equal); writing—review and editing(equal); project administration(equal); funding acquisition(equal). **Liangping Zha:** Conceptualization(equal); writing—review and editing(equal); project administration(equal); funding acquisition(equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study are available in the GenBank repositories; the accession numbers are OQ928151, OQ928152, OQ928153, OQ928154, OQ928155, and OQ928156.

CONSENT FOR PUBLICATION

Not Applicable.

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