The complete chloroplast genome of *Glycyrrhiza uralensis* Fisch. isolated in Korea (Fabaceae)

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ABSTRACT: The chloroplast genome of *Glycyrrhiza uralensis* Fisch was sequenced to investigate intraspecific variations on the chloroplast genome. Its length is 127,689 bp long (34.3% GC ratio) with atypical structure of chloroplast genome, which is congruent to those of *Glycyrrhiza* genus. It includes 110 genes (76 protein-coding genes, four rRNAs, and 30 tRNAs). Intronic region of *ndhA* presented the highest nucleotide diversity based on the six *G. uralensis* chloroplast genomes. A total of 150 single nucleotide polymorphisms and 10 insertion and deletion (INDEL) regions were identified from the six *G. uralensis* chloroplast genomes. Phylogenetic trees show that the six chloroplast genomes of *G. uralensis* formed the two clades, requiring additional studies to understand it.

Keywords: chloroplast genome, Fabaceae, *Glycyrrhiza uralensis*, intraspecific variation, nucleotide diversity, phylogenetic analysis

Glycyrrhiza uralensis Fisch. is distributed mainly in Central Asia, Mongolia, and China (Hayashi and Sudo, 2009). Together with two sister species, *Glycyrrhiza glabra* L. and *Glycyrrhiza inflata* Batalin, *G. uralensis* is one of important traditional Chinese medicine used as sweetener (Rizzato et al., 2017), and has long been cultivated in China and Korea (Hayashi and Sudo, 2009; Park et al., 2020a). Because *G. uralensis* is recognized as the best species among three *Glycyrrhiza* species with recent decrease of production of *G. uralensis* in inner Mongolia, the closely related species of *G. uralensis* have been considered as alternative species (Lee et al., 2010; Kim et al., 2019a). Due to this importance, multiple number of chloroplast genomes have been sequenced till now (Kang et al., 2018; Jia et al., 2019) and several molecular markers, such as simple sequence repeats, have been developed (Hayashi et al., 2005; Lee et al., 2019; Hantemirova et al., 2020). Moreover, various useful compounds, including antibacterial and antithrombotic

compounds, have been identified from *G. uralensis* (He et al., 2006; Kwon et al., 2010; Lee et al., 2010; Tao et al., 2012; Adianti et al., 2014). In addition, natural hybrids of *Glycyrrhiza* were reported (Chen et al., 2020) and some cultivars through hybridization were developed (Lee et al., 2017). Here, we completed the chloroplast genome of *G. uralensis* cultivated in Korea to investigate intraspecific variations on chloroplast genomes and its intraspecific phylogeny.

Materials and Methods

Plant material

We collected the *G. uralensis* in Godo 9-gil, Iksan-si, Jeollabuk-do, in Korea (36°00'14.45"N, 127°03'49.47"E). A voucher and isolated DNA was deposited in the InfoBoss Cyber Herbarium (IN, the voucher number IB-01093).

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DNA extraction and chloroplast genome determination

Total DNA was extracted from fresh root by using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Genome sequencing was performed using NovaSeq6000 at Macrogen Inc., Korea, resulting in 15.8 Gbp raw reads. *De novo* assembly was done by Velvet v1.2.10 (Zerbino and Birney, 2008) and GapCloser v1.12 (Zhao et al., 2011). Assembled sequences were modified and confirmed by BWA v0.7.17 (Li, 2013) and SAMtools v1.9 (Li et al., 2009). All analyses were conducted in the Genome Information System (GeIS; <http://geis.infoboss.co.kr/>) used in the previous studies (Burn et al., 2020; Kim et al., 2021).

Genome annotation was conducted based on another chloroplast genome of *G. uralensis* (MT120790) with Geneious Prime 2020.2.4 (Biomatters Ltd., Auckland, New Zealand). A circular map of *G. uralensis* chloroplast genome was drawn using OGDRAW v1.31 (Greiner et al., 2019).

Identification of intraspecific variations

Single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were identified from the multiple sequence alignment of the six *G. uralensis* chloroplast genomes conducted by MAFFT 7.450 (Katoh and Standley, 2013) with 'Find variations/SNPs' implemented in Geneious Prime 2020.2.4 (Biomatters Ltd.). In addition, multiple sequence alignments from eleven *Glycyrrhiza* species were used in the same way (Table 1). Moreover, pair-wise alignments of the selected chloroplast genomes (Table 1) were also utilized in the same way. This method has been used in the previous studies investigating intraspecific variations on organelle genomes

(Choi et al., 2021; Park et al., 2021c, 2021d). INDEL region was defined as the continuous INDELs.

Nucleotide diversity analysis of chloroplast genomes

We calculated nucleotide diversity in the six chloroplast genomes of *G. uralensis* using the method proposed by Nei and Li (1979) with the Perl script. Nucleotide diversities were scanned along the genome with 500-bp window size and 200-bp step size for overlapped sliding windows. Our script for nucleotide diversity calculation has been utilized in the previous studies (Kim et al., 2019b; Lee et al., 2020a, 2020b, 2020c; Park et al., 2021e; Park and Xi, 2021).

Phylogenetic analysis

Maximum-likelihood (ML) and Bayesian inference (BI) phylogenetic trees were constructed based on the multiple sequence alignment of 57 *Glycyrrhiza* chloroplast genomes by MAFFT v7.450 (Katoh and Standley, 2013). The ML tree was reconstructed in MEGA X with 1,000 bootstrap repeats (Kumar et al., 2018). In the ML analysis, a heuristic search was used with nearest-neighbor interchange branch swapping, TVM + F + R4 model, and uniform rates among sites. All other options used the default settings. The posterior probability of each node was estimated by BI using MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001) plug-in implemented in Geneious Prime 2020.2.4 (Biomatters Ltd.). The HKY85 model with gamma rates was used as a molecular model. A Markov chain Monte Carlo algorithm was employed for 10,000,000 generations, sampling trees every 200 generations, with four chains running

Table 1. List of number of intraspecific variations identified from chloroplast genomes of the twelve *Glycyrrhiza* species including *G. uralensis*.

Species	No. of SNPs	No. of INDEL regions	INDEL coverage (bp)	Alignment length (bp)
<i>G. uralensis</i> Fisch.	150	10	75	126,304
<i>G. aspera</i> Pall.	43	22	79	127,890
<i>G. astragalina</i> Gillies ex Hook. & Arn.	95	44	475	127,201
<i>G. echinata</i> L.	11	8	15	128,072
<i>G. foetida</i> Desf.	13	25	166	127,857
<i>G. glabra</i> L.	102	61	623	128,132
<i>G. glandulifera</i> Waldst. & Kit.	29	24	77	128,000
<i>G. inflata</i> Batalin.	25	35	162	127,960
<i>G. lepidota</i> (Nutt.) Pursh	1163	281	2339	128,805
<i>G. pallidiflora</i> Maxim.	49	39	676	127,790
<i>G. squamulosa</i> Franch.	48	20	125	127,788
<i>G. triphylla</i> Fisch. & CAMEy.	100	38	205	127,856

simultaneously. Trees from the first 2,500,000 generations were discarded as burn-in.

Results and Discussion

The chloroplast genome of *G. uralensis* (GenBank accession: MZ066516) isolated in Korea is 127,689 bp long, which is shorter than those of typical plant chloroplast genomes. This is caused by lack of one of inverted repeat regions in the typical plant chloroplast genomes (Fig. 1), which is a common

phenomenon in genus *Glycyrrhiza* (Kang et al., 2018). However, *Abrus* chloroplast (GenBank accession: MT328396), neighbor genus of *Glycyrrhiza* (Hayashi et al., 1998), presents a typical structure of chloroplast genome, indicating that this atypical structure is specific to *Glycyrrhiza*. It contains 110 genes consisting of 76 protein-coding genes (PCGs), four ribosomal RNAs (rRNAs), and 30 transfer RNAs (tRNAs) (Fig. 1).

A total of 150 SNPs and 10 INDEL regions covering 75 bp were identified from the six *G. uralensis* chloroplast genomes. Eighteen out of 76 PCGs, including *matK*, *atpB*, *ndhK*, *psaB*,

Fig. 1. Circular map of chloroplast genome of *Glycyrrhiza uralensis* isolated in Korea. Gene shown outside are transcribed clockwise, and inside the circle are transcribed counter clockwise. Genes are color-coded to distinguish different functional groups. The dark grey and the light grey plot in the inner circle correspond to the GC content and AT content, respectively. Inside chloroplast genome, the three arcs of which colors are grey, light green, and light blue indicate large single copy (LSC), small single copy (SSC), and inverted repeat (IR) regions.

psbC, *rpoB*, *rpoC1*, *rpoC2*, *atpF*, *atpA*, *rpl20*, *psbB*, *rps3*, *ycf2*, *ycf1*, *ndhL*, *ccsA*, and *ndhF*, cover at least one SNPs. The number of PCGs containing at least one SNP is slightly larger than that of *Chenopodium album* L. (Park et al., 2021b); however, the ratio of non-synonymous to synonymous SNPs is 15:19, which is typical in angiosperms. The genes containing

non-synonymous SNPs, *matK*, *atpB*, *ndhK*, *rpoC1*, *rps3*, *ycf1*, and *ccsA*, can be a candidate to develop *Glycyrrhiza*-specific molecular marker to evaluate their generic background.

To evaluate the numbers of SNPs and INDELs identified from *G. uralensis* chloroplast genomes, we investigated intraspecific variations of eleven *Glycyrrhiza* species (Table 1) using the

Fig. 2. Scattered plot of number of single nucleotide polymorphisms (SNPs) and insertion and deletion (INDEL) coverage (bp) identified from the twelve *Glycyrrhiza* species and nine species. X-axis is number of SNPs and Y-axis is INDEL coverage. Blue dot indicates the number of SNPs and INDEL coverage identified from *G. uralensis*, orange dots were from eleven *Glycyrrhiza* species, and grey dots were from those of nine plant species identified based on pair-wise alignment of complete chloroplast genomes.

Table 2. List of number of intraspecific variations identified from chloroplast genomes of the various plant species.

Family	Species	No. of SNPs	No. of INDEL regions	INDEL coverage (bp)	Alignment length (bp)	Reference
Asteraceae	<i>Chrysanthemum zawadskii</i> Herbach.	182	71	426	151,359	Baek et al. (2021), Hongmei et al. (2021)
		108	45	251	151,221	
Brassicaceae	<i>Arabidopsis thaliana</i> L.	55	10	36	154,485	Park et al. (2020b)
		116	6	474	154,938	
		10	12	33	154,492	
		50	4	14	154,478	
		56	44	120	154,550	
		97	8	482	154,945	
		62	7	25	154,491	
		33	6	22	154,485	
		39	49	132	154,559	
		121	10	479	154,952	
		94	2	460	154,938	
		86	46	570	155,012	
		57	8	19	154,492	
		60	49	119	154,561	
		37	44	110	154,552	

Table 2. Continued.

Family	Species	No. of SNPs	No. of INDEL regions	INDEL coverage (bp)	Alignment length (bp)	Reference
Orchidaceae	<i>Goodyera schlechtendaliana</i> Rehb. F.	200	59	511	154,607	Oh et al. (2019a, 2019b)
		842	130	1,779	154,964	
		740	95	1,470	154,820	
		514	110	2,133	155,322	
		700	112	1,366	154,665	
		597	72	1,065	154,525	
		445	29	415	154,119	
		864	123	2,045	155,027	
		282	86	1,060	154,261	
		163	43	652	154,067	
Rosaceae	<i>Pyrus ussuriensis</i> Maxim.	121	61	781	160,727	Cho et al. (2019)
Fagaceae	<i>Fagus multinervis</i> Nakai	2	2	2	158,350	Park and Oh (2020), Yang et al. (2020)
		2	3	3	158,350	
		0	0	0	158,348	
Schisandraceae	<i>Illicium anisatum</i> L.	20	17	114	146,939	Park et al. (2019a)
Theaceae	<i>Camellia japonica</i> L.	25	2	2	156,976	Park et al. (2019c, 2019d)
		25	2	2	156,972	
		25	0	0	156,971	
		8	7	36	157,004	
		8	9	38	157,005	
		33	9	38	157,005	
		78	60	644	157,110	
		78	61	645	157,111	
		103	61	645	157,111	
Campanulaceae	<i>Campanula takesimana</i> Nakai	80	61	671	157,139	Park et al. (2021a)
		33	39	662	169,737	
Poaceae	<i>Zoysia matrella</i> (L.) Merr.	28	56	91	135,894	Lee and Park (2021)

available complete chloroplast genomes. The numbers of SNPs ranged from 11 to 1,163 and those of INDEL regions were from 8 to 281 (15 to 2,339 bp in length) (Fig. 2). Many of numbers of intraspecific variations of the other plant species showed similar or smaller than those of *G. uralensis* except *Goodyera schlechtendaliana* Rehb. F. (Fig. 2, Table 2). It indicates that the number of SNPs of *G. uralensis* is in the middle, but INDEL coverage is relatively small. However, genetic diversity of chloroplast genomes should be investigated further based on well-designed sampling strategy considering with the number of samples and their locations together.

Nucleotide diversity of six *G. uralensis* chloroplast genomes

was calculated to investigated hotspot of nucleotide diversity. Average nucleotide diversity was 0.0002216 (Fig. 3A) which is higher than those of *A. thaliana* (0.000017) isolated from several countries (Park et al., 2020b) and *C. album* (0.0000625) isolated in the Korean Peninsula (Park et al., 2021b). This high intraspecific nucleotide diversity may be caused by different genetic backgrounds of *G. uralensis*, in which accessions are separated in two independent clades in the phylogenetic tree (Fig. 3A). Interestingly 5' region of *atpB* contained three non-synonymous SNPs (Fig. 3B), which is congruent to the studies presenting that *atpB* is one of positive selected genes (Xie et al., 2018; Lee and Park, 2021; Zhang et al., 2021). In addition,

Fig. 3. Nucleotide diversity calculated from the six *G. uralensis* chloroplast genomes. **A.** X-axis indicates coordination of chloroplast genome and Y-axis indicates nucleotide diversity of each window. Average nucleotide diversity was displayed as dark blue color and labels on graph indicate nearby gene names of the peak of which nucleotide diversity is above 0.001. **B.** Enlarged diagram shows multiple sequence alignment in 5' region of *atpB*. **C.** Enlarged diagram shows multiple sequence alignment in 5' region of *ndhA* intron.

intron region of *ndhA* also displayed high nucleotide diversity, caused by one big insertion region from MT120791 (Fig. 3C). This phenomenon was also observed in various chloroplast genomes, such as *A. thaliana* (Park et al., 2020b), *Viburnum amplificatum* Kern (Park et al., 2020c), and *Coffea arabica* L. (Park et al., 2019b). Furthermore, intergenic regions of *petN-trnC*, *trnC-rpoB*, *atpI-atpH*, *atpA-trnG*, *trnQ-accD*, *accD-psaI*, *psbE-petL*, and *trnR-trnL* presented high nucleotide diversity (Fig. 3A), which can be used for developing molecular markers to distinguish *G. uralensis* populations or cultivars.

Fifty-seven *Glycyrrhiza* chloroplast genomes with one outgroup species of *Abrus pulchellus* subsp. *cantoniensis* (Hance) Verdc., were used for constructing phylogenetic trees. Both phylogenetic trees display that six *G. uralensis* are clustered into the two clades including one *G. glabra* (GenBank accession: MG736059) with high supportive values of ML and BI trees except one low bootstrap value of ML (Fig. 4). Phylogenetic position of *G. uralensis* is congruent to the previous phylogenetic

studies (Hayashi et al., 1998, 2000; Liu et al., 2019; Duan et al., 2020). In addition, one *G. glabra* chloroplast (GenBank accession: MG736059) was found in the clade of *G. uralensis* (Fig. 4). One of possibility of this chloroplast can be misidentification because *G. glabra* was in the same clade with *G. inflata* in the previous studies (Hayashi et al., 1998, 2000; Liu et al., 2019; Duan et al., 2020). Moreover, two *G. uralensis* chloroplast genomes (GenBank accessions: KU862308 and MN199032) isolated in Korea and China (Jia et al., 2019), respectively, were clustered separately (Fig. 4), suggesting that geographical origin of *G. uralensis* might not be related to these two clades due to its cultivation in East-Asian countries (Yamamoto et al., 2003; Hayashi et al., 2005). Interestingly, non-synonymous SNPs identified only in the two *G. uralensis* chloroplast genomes (GenBank accessions: KU862308 and MN199032) were found in *atpB* (Fig. 3B) as well as *ndhK*, *rpoC2*, and *ycf1*. In addition, synonymous SNPs from those chloroplast genomes were also identified in *psbA*, *psbC*, *rpoB*,

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The authors declare that there are no conflicts of interest.

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