The chloroplast genome of the *Iris japonica* Thunberg (Butterfly flower) reveals the genomic and evolutionary characteristics of *Iris* species

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ABSTRACT

*Iris japonica* Thunberg is one of the horticultural species belonging to the *Iris* genus and Iridaceae family. Previous studies have revealed its hepatoprotective activity and ornamental values. However, little genetic and genomic information about this species is available. Here, to decipher the chloroplast genome and reveal its evolutionary characteristics, we sequenced, *de novo* assembled, and comprehensively analyzed the chloroplast genome of *I. japonica*. The genome was 152,453 bp in length and displayed a circular structure with a large single-copy region, a small single-copy region, and two inverted repeat regions. It contained 131 genes, including 85 protein-coding genes, eight ribosomal RNA genes, and 38 transfer RNA genes. We also identified 23 microsatellite repeat sequences, 34 tandem repeat sequences, and 60 dispersed repeat sequences in the chloroplast genome of *I. japonica*. Sequence divergence analyses of the chloroplast genomes of 20 *Iris* species revealed that the top four most highly variable regions were ndhC-trnV-UAC, rpl22-rps19, rps16-trnQ-UUG, and trnG-UCC-trnR-UCU. Phylogenetic analysis showed that *I. japonica* was most closely related to *I. tectorum*. This study reported a new chloroplast genome of *I. japonica* and performed comparative analyses of 20 *Iris* chloroplast genomes. The results would facilitate the evolutionary research and development of molecular markers for *Iris* species.

1. Introduction

The butterfly flower (*Iris japonica* Thunberg) belongs to the Iridaceae family and is native to Japan and China. The butterfly flower has ornamental value for its beautiful butterfly-shaped flowers called ‘Hudie Hua’ in China. Moreover, *I. japonica* is a medicinal plant for treating bronchitis, internal injuries, rheumatism, and swelling. The butterfly flower has many biological activities, such as antioxidant, anti-mutagenic, anti-angiogenic, anti-inflammatory, and hypoglycemic functions (Xu et al. 2021). Although many studies about the medicinal value of *I. japonica* have been conducted, no work has studied its genetic and genomic information, limiting the species identification and evolutionary analysis of *I. japonica*.

Complete chloroplast genomes are circular, linear, or polycyclic double-stranded DNA molecules, with a length mostly around 120–160 kb (Bock 2015). The complete chloroplast genome sequence can be used for the development of DNA barcodes, the determination of phylogenetic relationships, and the identification of patterns of gene loss and adaptive changes that optimize photosynthesis (Olejniczak et al. 2016).

Recently, many chloroplast genomes have been deciphered due to the advanced genome sequencing technologies and bioinformatics tools. One study sequenced and assembled the chloroplast genomes of 14 Korean-native *Iris* species (Kang et al. 2020). The 14 *Iris* chloroplast genomes were compared, and Bayesian phylogenetic trees were constructed. Five other studies reported the sequencing and assembly of the chloroplast genomes of *Iris sanguinea* (Lee et al. 2017), *Iris domestica* (Ai et al. 2019), *Iris loczyi* (Choi et al. 2020), *Iris tectorum* (Liu et al. 2020), and *Iris lactea* var. *chinensis* (Cai et al. 2021). Additionally, one assembly of the chloroplast genome of *I. japonica* was available in Genbank (NC_060499.1). However, a detailed analysis of this assembly was not reported.

Here, we sequenced and assembled the chloroplast genome of *I. japonica* and conducted a comparative analysis with 19 other *Iris* species. Our study presented the very first detailed analysis of the *I. japonica* chloroplast genome. The results will lay a solid foundation for the future development of *I. japonica*-based medicine.

2. Materials and methods

2.1. Plant materials and DNA extraction

We collected the fresh leaves of the *I. japonica* plants from Guangxi Province, China (E104°26′, N20°54′). The voucher...
specimen (IMPLADS2021009040) and its DNA sample (IMPLADS2021009040_dna01) were deposited at the Herbarium of Institute of Medicinal Plant Development (IMD), China (http://www.implad.ac.cn/, Haimei Chen, e-mail: hmcchen@implad.ac.cn). We extracted the total DNA of one I. japonica sample using the plant genomic DNA kit (Tiangen Biotech, Beijing, Co., Ltd.). Total DNA was separated with electrophoresis in 1.2% agarose gels and analyzed with the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) to assess quality and purity. Our study, including sample collection, was conducted in compliance with relevant institutional, national, and international guidelines and laws.

2.2. Plastome sequencing, assembly, and annotation

The 1 μg total DNA of I. japonica was used to construct the sequencing libraries using the TrueSeq DNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA) following the manufacturer’s instructions and the libraries were sequenced on the Illumina NovaSeq PE150 instrument (Illumina Inc., San Diego, CA, USA) (Caporaso et al. 2012).

The genome was de novo assembled using NOVOPlasty (version 3.8.3) with the default parameters (Dierckxsens et al. 2017). The chloroplast genome of l. japonica was annotated with CPGAVAS2 (version 2.0) web service (Shi et al. 2019). The circular gene map of the chloroplast genome was drawn using the cpgavas2 web server (http://47.96.249.172:16019/analyser/view). The raw data, final genome assembly sequence, and annotated information were deposited in CNCB-NGDC, with the accession numbers SRR18908135 and GWHBISG01000000.

2.3. Comparative genome analysis

The complete chloroplast genome sequences of 20 Iris species were downloaded from the GenBank database (Supplementary Material, Table S1), and the l. japonica chloroplast genome was obtained in this study (GWHBISG01000000). We conducted the comparative analysis of these 20 genomes using mVISTA with the ShuffleLAGAN mode (Brudno et al. 2003). The genes around the border of the IR, LSC, and SSC regions of the 21 Iris species were analyzed using IrsScope online software (Amiryousefi et al. 2018) (https://irscope.shinyapps.io/irapp/). The genetic distance of the intergenic spacer (IGS) was calculated using the distmat program from EMBOSS (v6.3.1) with the Kimura 2-parameter (K2P) evolutionary model (Rice et al. 2000). We also compared our new chloroplast genome with the one deposited in the Genebank (NC_060499) using SeqMan software (version7.1.0).

2.4. Selective pressure analysis of protein-coding genes

The selective pressure of the protein-coding genes (PCGs) in the chloroplast genome was analyzed based on nucleotide substitution rate variation (Song et al. 2020). To detect the genes of the l. japonica chloroplast genome that were under selection, we extracted 71 common PCGs among the 20 Iris chloroplast genomes, performed multiple sequence alignment using MAFFT (v7.313), and constructed a maximum likelihood (ML) tree using IQTREE (v1.6.10) (Nguyen et al. 2015). The selective pressure of PCGs was analyzed using the aBSREL (adaptive branch-site random effects likelihood) model implemented in Hypho software (v2.2.4) (Smith et al. 2015).

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the chloroplast genomes of 33 Iris species, and Crocus cartwrightianus (NC_041459) was selected as the outgroup. We used the PhyloSuite (v1.1.16) (Zhang et al. 2020) to extract the coding sequences of 66 common PCGs (atpA, atpB, atpE, atpF, atpH, ccsA, cemA, infA, matK, nadA, ndhB, ndhC, ndhE, ndhF, ndhG, ndhJ, ndhK, petA, petB, petD, petG, petL, petN, psaA, psaB, psaC, psaI, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbl, psbM, psbT, rbcL, rpl14, rpl16, rpl20, rpl22, rpl23, rpl32, rpl33, rps16, rpoA, rpoB, rpoC1, rpoC2, rps2, rps3, rps4, rps7, rps8, rps11, rps12, rps14, rps15, rps16, rps18, ycf1 and ycf2) of the chloroplast genome of these 34 species. Then the coding sequences of the 66 shared PCGs were aligned using MAFF (v7.313) (Katoh et al. 2002). The phylogenetic tree was constructed using the ML method implemented in IQ-TREE (v1.6.8) with the TVM + F + I + G4 nucleotide substitution model (Smith et al. 2015). Finally, we assessed the reliability of the phylogenetic tree by the bootstrap test with 1000 replications and visualized the final tree using iTOL (https://itol.embl.de/) (Letunic and Bork 2019).

3. Results

3.1. Genome organization and compositions

The chloroplast genome of l. japonica presented a typical circular DNA molecule with a total length of 152,453 bp (Figure 1). It had a conserved tetrapartite structure, including an LSC region, an SSC region, and a pair of IR regions, with lengths of 83,252, 18,489, and 25,356 bp, respectively. The chloroplast genome of I. japonica (NC_060499) were aligned using mVISTA to compare with the one deposited in the Genebank (NC_060499) obtained in this study, and the genetic distance of the intergenic spacer (IGS) was calculated using the distmat program from EMBOSS (v6.3.1) with the Kimura 2-parameter (K2P) evolutionary model (Rice et al. 2000). We also compared our new chloroplast genome with the one deposited in the Genebank (NC_060499) using SeqMan software (version7.1.0).

3.2. Comparative analysis of 20 Iris chloroplast genomes

The chloroplast genome sequences of 21 species including the 19 species listed in Table S1 (Supplementary Material), I. japonica (GWHBISG01000000) obtained in this study, and I. japonica (NC_060499) were aligned using mVISTA to
identify the variations in nucleotide sequence. The results showed that some highly variable regions were detected, including the protein-coding regions of ycf1, petB, and petD and the IGS regions of rps16-trnQ-UUG, rpoB-trnC-GCA, ndhC-trnV-UAC, rpl33-rps18, and rpl22-rps19 (Figure 2). The alignment of the chloroplast genomes of the 14 other species is shown in Figures S2 and S3 (Supplementary Material).

3.3. Ir structure analysis of 20 Iris species

IR boundary analysis of 21 complete chloroplast genomes of Iris species showed that most had the same boundary structure (Figure 3). For the 20 species except for I. japonica, both rpl22 and rps19 genes were located around the border area of the LSC and IRb regions. By contrast, the rps19 gene of I. japonica (GWHBISSG01000000) was present in the LSC region. Besides, the ndhF and ycf1 genes of I. tectorum, I. japonica, and I. domestica were located at the border area of IRb and SSC. The ycf1 gene of most species spanned the SSC and IRb regions. The psbA genes were found at the border area of LSC and IRa.

3.4. Hypervariable region identification and selective pressure analysis

To find highly variable regions among the 20 Iris chloroplast genomes, we calculated the genetic distance of IGS regions
using the K2P model. K2P values of \textit{ndhC-trnV-UAC}, \textit{rpl22-rps19}, \textit{rps16-trnQ-UUG}, \textit{trnG-UCC-trnR-UCU}, \textit{rpl36-infA}, \textit{trnC-GCA-petN}, \textit{rpoB-trnC-GCA}, \textit{rpl33-rps18}, \textit{rps19-trnH-GUG}, \textit{trnH-GUG-rpl2}, \textit{rpl2-trnH-GUG}, and \textit{psbK-psbl} were high at 27.05, 19.79, 15.97, 11.69, 10.45, 8.25, 7.95, 6.42, 6.35, 6.12, 6.12, and 6.05, respectively (Supplementary Material, Figure S4). The variations in these IGS regions were large, which suggested that these regions could be used to develop potential molecular markers for species discrimination of \textit{Iris} species.

Figure 2. Sequence alignment of seven chloroplast genome of \textit{Iris} species using mVISTA and chloroplast genome of \textit{I. lactea} (NC_056175) as reference. The top arrow shows transcription direction, blue color indicates protein-coding regions, pink color shows non-coding sequences and light green indicates tRNAs and rRNAs. The x-axis represents the coordinates in the cp genome while y-axis represents percentage identity within 50–100%.
Using the aBSREL model, we found that the genes \textit{rpoC2} and \textit{ycf1} were positively selected in \textit{I. japonica}. Moreover, the genes \textit{ycf2}, \textit{ndhG}, and \textit{rpl20} were positively selected in other \textit{Iris} species. These genes may be related to the adaptation of these \textit{Iris} species to diverse environmental conditions (Supplementary Material, Table S3).

### 3.5. Phylogenetic analysis

In this study, the chloroplast genome sequence of \textit{I. japonica} (GWHBISG01000000) was aligned with 32 previously published genome sequences. The 32 published genome sequences are the chloroplast genome sequences of \textit{I. sanguinea}.
(Lee et al. 2017), I. missouriensis (Joyce et al. 2018), I. speculatrix (Kang et al. 2020), I. loczyi (Choi et al. 2020), I. x hollandica, I. rossii (Kang et al. 2020), I. odaesanensis (Kang et al. 2020), I. koreana (Kang et al. 2020), I. minutoaurea (Kang et al. 2020), I. lactea (Kang et al. 2020), I. rutenica (Kang et al. 2020), I. setosa (Kang et al. 2020), I. hippolyti, I. tectorum, I. dichotoma (Kang et al. 2020), I. laevigata (Kang et al. 2020) and I. pseudacorus (Kang et al. 2020), I. lactea var lactea (Cai et al. 2021), I. japonica (NC_060499), I. domestica, I. cedreti (Volis et al. 2022), I. lycotis (Volis et al. 2022), I. gatesii, I. nigricans (Volis et al. 2022), I. atrorufusca (Volis et al. 2022), I. haynei (Volis et al. 2022), I. atropurpurea (Volis et al. 2022), I. mariæ (Volis et al. 2022) and I. germanica. Moreover, I. japonicum was sister to moderately-supported clade containing I. tectorum and I. hippolyti. (Figure 4). During this analysis, we found that another sequence from the same species was released in GenBank (NC_060499.1). Phylogenetic analysis suggested that the two sequences were highly similar. Sequence comparison showed 254 variations between the two sequences (Supplementary Material, Table S4).

4. Discussion
In this study, we sequenced the chloroplast genome of I. japonica and conducted comparative and evolutionary analyses of the chloroplast genomes with 19 other Iris chloroplast genomes. Overall, the I. japonica chloroplast genome was similar to those of the 19 other Iris species.

Notably, comparative analysis found some variations in genome organization. Different gene contents were noted at the border areas of the IR and LSC/SSC regions among the 21 Iris species. The rps19 of only one species was present in the LSC region. However, the duplicated rps19 of other 20 species were located in the IRa and IRb regions. This type of junction based on the different positions of rps19 was also found in the chloroplast genomes of Aphelandra knappiae and Blepharis ciliaris, which caused by the contraction and expansion in the IR regions (Alzahrani et al. 2020).

Highly variable regions in the chloroplast genome can serve as the DNA barcodes for evaluating phylogeny (Dong et al. 2012) and taxa classification (Dong et al. 2014). On the basis of genetic distances, some highly variable regions were observed in the 20 Iris chloroplast genomes, including ndhC-trnV-UAC, rpl22-rps19, rps16-trnQ-UUG, trnG-UCU-trnR-UCU, rpl36-infA, and trnC-GCA-petN. In the future, we will test the possibility of distinguishing these Iris species using molecular markers developed from these regions.

As described previously, only one comprehensive analysis of the phylogenetic relationships was reported. In that study, 14 Korean Iris species were separated into four clades (Kang et al. 2020). Here, we conducted phylogenetic analysis using 20 Iris
species, including the 14 reported species. Our study included a systematic analysis with six additional *Iris* chloroplast genomes. In our analysis, the 14 species reported previously were also clustered into four clades. For the newly added five species, the sister relationships between *I. loczyi* and *I. speculatrix*, and between *I. tectorum* and *I. japonica* were observed. In summary, the results obtained from this study will help us unveil the chloroplast genome evolution of *Iris* species.

**Ethics statements**

*I. japonica* was not listed as a national and provincial key protected wild plant in China nor a threatened species on the IUCN Red List. Therefore, no specific permissions or licenses were needed for the sampling of *I. japonica* for research purpose according to the regulations of the People’s Republic of China on the protection of wild plants. During the sampling process, we followed the local sampling guideline to ensure no substantial harm to the collecting individual.

**Author contributions**

B.W. and HMC conceived the study. XYZ and HYY performed the data analysis and drafted the manuscript. B.W. critically reviewed the article. All authors approved the final version of the manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability statement**

The sample was stored at the Herbarium of the Institute of Medicinal Plant Development, Beijing, China, with the voucher numbers Implad201808209. The chloroplast genome sequence and the related annotations were available at https://ngdc.cncb.ac.cn/gwh in the Genome Warehouse in China National Genomics Data Center [38] with accession number GWHBISG01000000. The raw sequencing data for the Illumina platforms were deposited in NCBI-NR (https://www.ncbi.nlm.nih.gov/sra/?term=NCBI-NR). The accession numbers of Bioproject, Biosample, and GSA are PRJCA009243, SAMC781189, and CRA007000, respectively.

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