Integrated analysis of three newly sequenced fern chloroplast genomes: Genome structure and comparative analysis

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Abstract

Background: Some ferns have medicinal properties and are used in therapeutic interventions. However, the classification and phylogenetic relationships of ferns remain incompletely reported. Considering that chloroplast genomes provide ideal information for species identification and evolution, in this study, three unpublished and one published ferns were sequenced and compared with other ferns to obtain comprehensive information on their classification and evolution.

Materials and Methods: The complete chloroplast genomes of Dryopteris goeringiana (Kunze) Koidz, D. crassirhizoma Nakai, Athyrium brevifrons Nakai ex Kitagawa, and Polystichum tripterum (Kunze) Presl were sequenced using the Illumina HiSeq 4,000 platform. Simple sequence repeats (SSRs), nucleotide diversity analysis, and RNA editing were investigated in all four species. Genome comparison and inverted repeats (IR) boundary expansion and contraction analyses were also performed. The relationships among the ferns were studied by phylogenetic analysis based on the whole chloroplast genomes.

Results: The whole chloroplast genomes ranged from 148,539 to 151,341 bp in size and exhibited typical quadripartite structures. Ten highly variable loci with parsimony informative (Pi) values of > 0.02 were identified. A total of 75–108 SSRs were identified and only six SSRs were present in all four ferns. The SSRs contained a higher number of A + T than G + C bases. C-to-U conversion was the most common type of RNA editing event. Genome comparison analysis revealed that single-copy regions were more highly conserved than IR regions. IR boundary expansion and contraction varied among the four ferns. Phylogenetic analysis showed that species in the same genus tended to cluster together with and had relatively close relationships.

Conclusion: The results provide valuable information on fern chloroplast genomes that will be useful to identify and classify ferns, and study their phylogenetic relationships and evolution.

Keywords

Chloroplast genome, Fern, Phylogenetic tree, RNA editing, Simple sequence repeat
1 | INTRODUCTION

Ferns are the most evolved of the spore-forming plants, and some have medicinal properties (Chen et al., 2007). The genus Dryopteris (Dryopteridaceae, comprising 225–300 species) is considered ideal for studying diversification, hybridization, and polyploidy in ferns (Sessa et al., 2012). D. goeringiana and D. crassirhizoma, which originated in the northeastern region of China are distributed across Russia, Japan, and North Korea. The rhizome and petiole residues of D. crassirhizoma are used in traditional Chinese herbal medicine to eliminate heat and toxins, promote blood circulation, and treat blood stasis (Z. Zhao et al., 2007). Polystichum (Dryopteridaceae) is one of the most abundant genera of ferns and commonly occurs in lowlands and montane to alpine areas (Zhang, 2012); it contains 500 species, with 208 species known in China (Zhang & Barrington). Athyrium Roth (Athyriaceae), the lady-fern genus, contains approximately 220 described species (Ran Wei & Zhang, 2016). Athyrium brevifrons is often used as a wild vegetable in northeastern China because of its high nutritional value. Because only a small proportion of ferns have been identified and classified, additional studies are needed.

With the development of next-generation sequencing (NGS) technology, the details of the most subtle nuclear gene components in eukaryotic cells have become clearer, and the study of cytoplasmic organelle genomes has also been facilitated in a more straightforward and time-saving way (Ruiz-Ruano et al., 2018). This is especially true for chloroplasts, which are involved in many biochemical metabolism processes, including amino acid, sugar, lipid, vitamin, starch, and pigment synthesis; sulfate reduction; and nitrogen. Most chloroplasts convert light energy into chemical energy via photosynthesis, making chloroplasts indispensable for plants (Bausher et al., 2006; Jarvis & Soll, 2001; Leister, 2003). Compared with nuclear genomes, chloroplast genomes are more highly conserved in terms of gene order, gene content, and substitution rate (Green, 2011; Helena, 2004; Ruhlman & Jansen, 2014; Wolfe et al., 1987; J. H. Xu et al., 2015). Chloroplast genomes have a typically circular structure with one large single-copy (LSC) region, one short single-copy (SSC) region, and two inverted repeat (IR) regions, ranging from 120 to 170 kilobases in length (Downie & Palmer, 1992). Owing to the absence of recombination and maternal transmission, the chloroplast genomes are helpful for tracing source populations (McCauley et al., 1996; Small et al., 2004). They have become a valuable and ideal resource for species identification, population genetics, plant phylogenetics, and genetic engineering considering their similar structures, highly conserved sequences, and stable maternal heredity (Nock et al., 2014). However, gain and loss of genes, gene content duplication, and gene order rearrangements appear to be phylogenetically and species informative (Bausher et al., 2006; Green, 2011; J. H. Xu et al., 2015).

An increasing number of chloroplast genomes have been reported in recent years, especially because NGS has become cheaper and faster. The chloroplast genomes of many plants have been sequenced, including those of bryophytes (M Park et al., 2018; P. Wolf & Karol, 2012), lycophytes (Guo et al., 2016; Tsuji et al., 2007), monophytes (Logacheva et al., 2017; Lu et al., 2015; Ruiz-Ruano et al., 2018; R. Wei et al., 2017; P. G. Wolf et al., 2011), and spermatophytes (Sun et al., 2016; J. H. Xu et al., 2015). As one of the largest group of vascular plants, approximately 2,129 species of ferns are present in China (Z. et al., 2013), of which only 60 have been reported.

D. crassirhizoma, D. goeringiana, A. brevifrons, and P. tripteront studied in the present study are relatively distributed in Heilongjiang Province, China, and all have certain antibacterial effects or edible value. These ferns are also the research hotspots of domestic ferns. Comparing the differences between the chloroplast genomes through the genetic relationship at different levels provides theoretical support for further development and utilization. In the present study, the complete chloroplast genomes of D. goeringiana, A. brevifrons, and P. tripteront were sequenced for the first time. We performed comparisons of the genomes and IR boundary expansion and contraction. Simple sequence repeats (SSRs), highly variable loci, and RNA editing events were also investigated in the four ferns. A phylogenetic tree was constructed based on the chloroplast genomes of almost all ferns reported thus far. The present study was conducted to achieve the following objectives: a) to sequence and report the chloroplast genomes of D. crassirhizoma, D. goeringiana, A. brevifrons, and P. tripteront; b) to compare the chloroplast genomes of eight fern species to identify useful DNA barcodes for plant identification and evolution analysis; and c) to identify a more comprehensive phylogenetic relationship among ferns.

2 | MATERIALS AND METHODS

2.1 | Materials

Wild specimens of D. crassirhizoma, A. brevifrons, D. goeringiana, and P. tripteront were collected from Maer Mountain, Maershan Town, Shangzhi City, Heilongjiang Province (N 45°17′51.45′′, E127°36′00.03′′), China (Figure 1). The four species were identified by Ruifeng Fan from the Heilongjiang University of Chinese Medicine. Voucher specimens were deposited in the Northeast Agricultural University Herbarium with the collection numbers 2018–21 (D. crassirhizoma), 2018–22 (A. brevifrons), 2018–32 (D. goeringiana), and 2018–33 (P. tripteront).

2.2 | Chloroplast DNA extraction and sequencing

Fresh leaves were collected, immersed in liquid nitrogen immediately, and stored at ~80°C prior to DNA extraction. We isolated chloroplast DNA using an improved extraction method (McPherson et al., 2013) and evaluated its quality and quantity using a NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and a Qubit® 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA), respectively. Samples that had a total amount of >1 μg DNA and OD260/280 of 1.8–2.0 were used for library preparation.
Libraries were constructed with 1 μg of chloroplast DNA according to the Illumina TruSeq™ Nano DNA Sample Prep Kit (Illumina Inc, San Diego, USA) protocol. The libraries were sequenced using the Illumina HiSeq 4,000 platform (Biozeron Co., Ltd., China) (Borgström et al., 2011).

2.3 | Genome assembly and annotation

Prior to assembly, low-quality reads were removed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then, the chloroplast genomes were assembled in three steps as follows (Cronn et al., 2008): Clean reads were assembled into contigs using SOAPdenovo 2.04 (Luo et al., 2012), clean reads were mapped to the contigs for assembly and optimization using SOAPGapCloser 1.12 (Q.-Y. Zhao et al., 2011), and redundant sequences were removed.

Gene comparisons, predictions, and combinations were performed using Genewise (https://www.ebi.ac.uk/Tools/psa/genewise/), AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/), and EVidenceModeler version 1.1.1, respectively. Protein-coding genes, tRNA genes, and rRNA genes were predicted using the DOGMA tool (Wyman et al., 2004). The assembled chloroplast genomes were then BLASTed against a series of databases, including Clusters of Orthologous Groups (Tatusov et al., 2003), Swiss-Prot (Magrane, 2011), Gene Ontology (Ashburner et al., 2000), and Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al., 2004; Minoru et al., 2006). Circular chloroplast genome maps of D. crassirhizoma, A. brevifrons, D. goeringiana, and P. tripteran were constructed using Organellar Genome DRAW 1.2 (Lohse et al., 2007).

2.5 | Nucleotide diversity analysis

We measured the parsimony informative (Pi) characters per-site values to identify the most variable chloroplast genes using MAFFT 7.123b (http://mafft.cbrc.jp/alignment/software/) and Variscan 2.0 (http://www.ub.es/softexol/variscan). Pi values were calculated with a step size of 200 bp and a slide window of 300 bp. Loci with a Pi value of > 0.20 were considered as highly variable regions.

2.6 | RNA editing

The RNA editing events were counted in four ferns.

2.7 | Comparison analysis

Genome comparison analysis was conducted for D. crassirhizoma, A. brevifrons, D. goeringiana, and P. tripteran and for the additional ferns Cyrtomium devexiscapulae, D. decipiens, Lepisorus clathratus, and Polypondium glycyrrhiza using mVISTA (http://genome.lbl.gov/vista/mvista/about.shtml) in Shuffle-LAGAN mode. We compared IR boundary expansion and contraction in all the above-mentioned ferns, except P. glycyrrhiza.

2.8 | Phylogenetic analysis

MAFFT v7.149 was used to align the cpDNAs sequences under default parameters (Katoh et al., 2005), and the alignment was trimmed by Gblocks_0.91b to remove low-quality regions with the parameters: -t = d -b4 = 5 -b5 = h (Castresana, 2000). The maximum-likelihood (ML) methods were performed for the genome-wide phylogenetic analyses using PhyML 3.0 (Guindon et al., 2010), respectively. Nucleotide substitution model selection was estimated with jModelTest 2.1.10 (Darriba et al., 2012) and smart model selection in PhyML 3.0. The model general time-reversible (GTR)+I + G was selected for ML analyses with 1,000 bootstrap replicates to calculate the bootstrap values of the topology. The results were treated with iTOL 3.4.3 (Letunic & Bork, 2016). Adiantum capillus-veneris and Myriopteris (Cheilanthes) lindheimeri were included as outgroup species.
3 | RESULTS

3.1 Chloroplast DNA sequencing and genomic features

Overall, 26,981,820–34,833,094 paired-end (2 × 150 bp) raw reads were obtained from the four ferns (Table 1). The Q30 ranged from 89.06% to 91.52%.

The genome size ranged from 149,468 bp (D. crassirhizoma) to 151,341 bp (A. brevifrons). The chloroplast genomes had a circular assembly and exhibited a typical quadripartite structure (Figure 2), including one LSC region (82,384–82,799 bp), one SSC region (21,600–21,708 bp), and two IR regions (22,040–22,682 bp) (Table 1). The overall G + C content was 42.40%–43.76%.

Each fern genome was composed of 89 protein-coding genes, eight rRNA genes, and 35–38 tRNA genes. After removing the duplications, 84 protein-coding genes, four rRNA genes, and 31–33 tRNA genes remained (Tables 1 and 2). The type and number of tRNA genes were distinct in the LSC, SSR, and IRs regions (Table 3). trnN-GUU was present only in the SSC region of A. brevifrons. trnI-GAU was absent only in the IRb region of P. tripteron. A total of 14 genes contained introns: 11 genes (atpF, matK, ndhA, ndhB, petA, petB, petD, rpl16, rpl2, rpoC1, rpoB, and rps16) contained one intron and three genes (clpP, rps12, and ycf3) contained two introns (Table 4). Notably, matK in P. tripteron contained two introns, whereas matK in the other three species contained only one intron. rps12, with one exon in the LSC region and the other two in the IR regions, was considered a trans-spliced gene separated by two introns.

| TABLE 1 | The summary of sequencing data and complete chloroplast genomes of D. crassirhizoma, A. brevifrons, D. goeringiana, and P. tripteron |
|----------|-------------------------------------------------|
| Items    | Species                                         |
|          | D. crassirhizoma | A. brevifrons | D. goeringiana | P. tripteron |
| Sequecing| Total Reads (Mb) | 3.064        | 3.483          | 2.698        | 2.972        |
|          | Total Bases (G) | 4.627        | 5.260          | 4.074        | 4.488        |
|          | Q30 (%)        | 89.06        | 89.07          | 90.97        | 91.52        |
| Genome   | Total length (bp) | 149,468     | 151,341       | 148,947      | 148,539      |
|          | GC content (%) | 43.19        | 43.76          | 43.12        | 42.40        |
| LSC      | Length (bp)    | 82,504       | 82,459         | 82,384       | 82,799       |
|          | Percentage (%) | 55.20        | 54.49          | 55.31        | 55.74        |
| SSC      | Length (bp)    | 21,600       | 21,708         | 21,623       | 21,660       |
|          | Percentage (%) | 14.45        | 14.34          | 14.52        | 14.58        |
| IR       | Length (bp)    | 22,682       | 23,588         | 22,471       | 22,040       |
|          | Percentage (%) | 15.18        | 15.59          | 15.09        | 14.84        |
| Protein-coding genes | Total gene number | 89          | 89             | 89           | 89           |
|          | Duplicated gene number | 5          | 5              | 5            | 5            |
|          | Single gene number | 84         | 84             | 84           | 84           |
| tRNA genes | Total gene number | 36          | 38             | 38           | 35           |
|          | Duplicated gene number | 3          | 5              | 6            | 4            |
|          | Single gene number | 33          | 33             | 32           | 31           |
| rRNA genes | Total gene number | 8           | 8              | 8            | 8            |
|          | Duplicated gene number | 4          | 4              | 4            | 4            |
|          | Single gene number | 4           | 4              | 4            | 4            |

Note: LSC, large single copy; SSC, short single copy; IR, inverted repeats; tRNA, transfer RNA; rRNA, ribosomal RNA. D. crassirhizoma, Dryopteris goeringiana (Kunze) Koidz; A. brevifrons, Athyrium brevifrons Nakai ex Kitagawa; D. goeringiana, Dryopteris crassirhizoma Nakai; P. tripteron, Polystichum tripteron (Kunze) Presl.
3.2 | SSR analysis

Five types of SSRs were identified, including mononucleotides, dinucleotides, trinucleotides, tetranucleotides, and pentanucleotides, with a total of 75–108 SSRs in the four species (Figure 3a and Table S1). There were 55–90 mononucleotides, 8–12 dinucleotides, 2–3 trinucleotides, 8–9 tetranucleotides, and 0–2 pentanucleotides (Figure 3b-f). The types of SSRs of *D. crassirhizoma* were similar to those of *D. goeringiana*, but were more varied, with (ATAA)², (ATCT)¹, and (TTTA)¹ also detected (Figure 3e and Table S1). Only six types of SSRs were simultaneously present in the four ferns: A, C, G, T, AT, and AGAT. The SSRs were composed of a higher number of A + T (63.26%) bases than G + C bases (36.74%; Figure 3g).

3.3 | Nucleotide diversity analysis

The chloroplast genome contains numerous variable nucleotides, which are usually recognized as valuable DNA barcoding regions for resolving closely related species or genera. In the present study, variable loci were identified in the four species, with *Pi* values ranging from 0.0000 to 0.2778 (*rpl16*) (Figure 4 and Table S2). Ten loci with
**Table 2** The common gene list of *D. crassirhizoma*, *A. brevifrons*, *D. goeringiana*, and *P. tripteron*

| Category                     | Gene names                                      |
|------------------------------|-------------------------------------------------|
| Photosynthesis               | Subunits of photosystem I psaA psaB psaC psal psaj |
|                              | Subunits of photosystem II psbA psbA-D2 psbB psbC psbD psbE psbF psbH psbl psbj psbk psbl psbM psbn psbt psbZ |
|                              | Subunits of NADH dehydrogenase ndhA ndhB ndhB-D2 ndhC ndhD ndhE ndhF ndhG ndhH ndhl ndhJ ndhK |
|                              | Subunits of cytochrome b/f complex petA petB petD petG petL petN |
|                              | Subunits of ATP synthase atpA atpB atpE atpF atpH atpl |
|                              | Large subunit of Rubisco rbcL |
| Self-replication             | Large subunits of ribosome rpl14 rpl16 rpl2 rpl20 rpl21 rpl22 rpl23 rpl32 rpl33 rpl36 |
|                              | Small subunits of ribosome rps11 rps12 rps12-D2 rps14 rps15 rps16 rps18 rps19 rps2 rps3 rps4 rps7-D2 |
|                              | DNA-dependent RNA polymerase rpoA rpoB rpoC1 rpoC2 |
|                              | Ribosomal RNAs rm16 rm23 rm4.5 rm5 |
| Other genes                  | Maturase matK |
|                              | Protease clpP |
|                              | Envelope membrane protein cema |
|                              | Acetyl-CoA carboxylase accD |
|                              | C-type cytochrome synthesis gene ccsA |
|                              | Translation initiation factor infA |
|                              | Proteins import function ycf2 ycf2-D2 |
|                              | Proteins with unknown functions ycf1 ycf12 ycf3 ycf4 |

Note: tRNA varied in four species and are listed in Table 3.

**Table 3** List of genes distributed in different regions of *D. crassirhizoma*, *A. brevifrons*, *D. goeringiana*, and *P. tripteron* chloroplast genomes

| Items                          | species                        |
|--------------------------------|--------------------------------|
|                                | *D. crassirhizoma* | *A. brevifrons* | *D. goeringiana* | *P. tripteron* |
| Common tRNA genes              | trnA-UGC trnC-GCA trnD-GUC trnE-UUC trnF-GAA trnG-GCC trnH-GUG trnI-CAU trnL-UAA trnL-UAG trnM-CAU trnN-GGU trnP-GGG trnP-UAG trnQ-UUG trnR-ACG trnR-UCU trnS-GCU trnS-GTA trnS-UGA trnT-GGU trnV-UAC trnW-CCA trnY-GUA trnM-CAU | | | |
| varied tRNA genes in LSC region| trnG-UCC | trnG-UCC-1 trnG-UCC-2 | trnG-UCC-1 trnG-UCC-2 | trnG-UCC-1 trnG-UCC-2 |
| varied tRNA genes in SSC region| trnN-GUU | – | – | – |
| varied tRNA genes in IR region | trnN-GUU × 2 trnL-GAU × 2 | trnN-GUU × 2 trnL-GAU × 2 | trnN-GUU × 2 |

Note: – represents no gene. LSC, large single-copy; SSC, short single-copy; IR, inverted repeats; tRNA, transfer RNA. *D. crassirhizoma*, *Dryopteris goeringiana* (Kunze) Koidz; *A. brevifrons*, *Athyrium brevifrons* Nakai ex Kitagawa; *D. goeringiana*, *Dryopteris crassirhizoma* Nakai; *P. tripteron*, *Polystichum tripterum* (Kunze) Presl.

**Table 4** The length of introns in *D. crassirhizoma*, *A. brevifrons*, *D. goeringiana*, and *P. tripteron*

| Introns              | *D. goeringiana* | *A. brevifrons* | *D. crassirhizoma* | *P. tripteron* |
|----------------------|------------------|-----------------|-------------------|----------------|
| matK-CDS2_matK-CDS1  | 12               | 12              | 12                | 12             |
| matK-CDS3_matK-CDS2  | –                | –               | –                 | 482            |
| rps16-CDS2_rps16-CDS1| 822              | 797             | 807               | 799            |
| atpF-CDS2_atpF-CDS1  | 728              | 704             | 728               | 720            |
| rpoC1-CDS2_rpoC1-CDS1| 695              | 701             | 696               | 691            |

(continues)
TABLE 4 (Continued)

| Introns                  | D. goeringiana | A. brevifrons | D. crassirhizoma | P. tripteran |
|--------------------------|----------------|---------------|------------------|--------------|
| ycf3-CDS3_ycf3-CDS2      | 625            | 625           | 626              | 620          |
| ycf3-CDS2_ycf3-CDS1      | 731            | 729           | 731              | 731          |
| petA-CDS1_petA-CDS2      | 12             | 12            | 12               | 12           |
| clpP-CDS3_clpP-CDS2      | 555            | 585           | 555              | 572          |
| clpP-CDS2_clpP-CDS1      | 705            | 701           | 705              | 717          |
| petB-CDS1_petB-CDS2      | 788            | 795           | 789              | 818          |
| petD-CDS1_petD-CDS2      | 89             | 638           | 89               | 651          |
| rpl16-CDS2_rpl16-CDS1    | 768            | 758           | 766              | 775          |
| rpl2-CDS2_rpl2-CDS1      | 723            | 724           | 725              | 746          |
| rps12-D2-CDS2_rps12-D2-CDS3 | 577           | 578           | 577              | 577          |
| ndhA-CDS2_ndhA-CDS1      | 943            | 962           | 944              | 978          |
| rps12-CDS3_rps12-CDS2    | 577            | 578           | 577              | 577          |
| Total length (bp)        | 9,350          | 9,899         | 9,339            | 10,478       |

Note: D. crassirhizoma, Dryopteris goeringiana (Kunze) Koidz; A. brevifrons, Athyrium brevifrons Nakai ex Kitagawa; D. goeringiana, Dryopteris goeringiana Nakai; P. tripteran, Polystichum tripteran (Kunze) Presl. CDS, coding sequences.

FIGURE 3  SSRs analyses in the four fern chloroplast genomes. (A) the number of different SSR types detected in the four genomes; (B) the number and types of identified mononucleotide SSR in the four genomes; (C) the number and types of di-nucleotides SSRs in the four genomes; (D) the number and types of trinucleotides SSR in the four genomes; (E) the number and types of tetrancleotides SSR in the four genomes; (F) the number and types of pentanucleotide SSR in the four genomes; (G) the contents of A, T, C, and G in four ferns. SSR, simple sequence repeats.
a Pi value of > 0.2, which were mainly located at SC regions, were considered highly variable loci: trnM-CAU, trnE-UUC, psbZ, trnN-GUU, trnI-CAU, rpl21, psbM, rpl32, trnV-UAC, and rpl16.

3.4 | RNA editing

RNA editing is defined as the post-transcriptional modification of precursor RNAs to alter their nucleotide sequences through the insertion and deletion, or specific substitution of nucleotides to introduce or remove start or stop codons or yield functional RNA species (Tsudzuki et al., 2001). In the present study, a total of 268 RNA editing events were identified in the four chloroplast genomes: 85 in D. crassirhizoma, 55 in A. brevifrons, 50 in D. goeringiana, and 78 in P. tripteron. C-to-U conversion (120 events, 44.8%) was the most prevalent RNA editing event, followed by U-to-C (103 events, 38.4%), A-to-G (36 events, 13.4%), and G-to-A (9 events, 3.4%).

3.5 | Genome comparison

The additional ferns L. clathratus, D. decipiens, C. devexiscapulae, and P. glycyrrhiza were selected for genome comparison analysis. Among the chloroplast genomes of these species, that of L. clathratus was the largest (156,998 bp), whereas that of P. glycyrrhiza was the smallest (129,221 bp). The chloroplast genomes ranged from 148,974 bp to 150,987 bp within Dryopteris genus, and the difference between the largest and smallest genomes was only 2,013 bp. However, this difference was 10-fold in Polypodium (22,120 bp). Additionally, rpoC2, rpoB, psbC, pasA, rbcl, ycf2, ycf1, and ndhB were identified to be divergent among these chloroplast genomes (Figure 5). The sequences in the IR regions were more highly conserved than those in the LSC and SSC regions.

3.6 | IR boundary contraction and expansion

IR boundary expansion and contraction were the main reasons for the differences in genome size, although IR regions were more highly conserved than LSC and SSC regions. The genes trnI, ndhF, chlL, and ndhB were located at LSC/IRb, IRb/SSC, SSC/IRa, and IRa/LSC boundaries, respectively (Figure 6). trnI was located in the LSC region, 40–112 bp away from the LSC/IRb boundary. ndhF crossed the IRb/SSC boundary, with 2,201–2,240 bp within the SSC region, except D. goeringiana. chlL crossed the SSC/IRa boundary, with 47–67 bp within the IRa region, except D. goeringiana. Notably, D. goeringiana presented an opposed gene order in the IRb/SSC and SSC/...
iRa regions compared with the other ferns, with chlL crossing the IRb/SSC region and ndhF crossing the SSC/iRa region. ndhB was located in the LSC region, 299–376 bp from the IRa/LSC boundary. However, ndhB extended into the LSC region of L. clathratus.

### 3.7 | Phylogenetic analysis

The phylogenetic tree (Figure 7) shows that 35 nodes had support values of > 90%, and 27 nodes had support values of 100%. The fern species in the same genus were clustered together to a certain degree. D. crassirhizoma and D. goeringiana were clustered and were shown to have a close relationship with D. decipiens. P. tripteris was identified as a sister species of C. devexiscapulae. Notably, A. brevifrons formed a single clade with A. sinense instead of P. glycyrrhiza (Figure 7).

### 4 | DISCUSSION

The chloroplast genome provides information that is valuable for species identification, population genetics, plant phylogenetics, and genetic engineering. In the present study, three previously unpublished and one published fern were sequenced and compared with other species. The chloroplast genomes, which were 148,539–151,341 bp in length in the present study, were within the limit of fern chloroplast genomes (131,760–181,684 bp) reported by Gao et al., (2018) and Ruiz-Ruano et al., 2018). The ferns in the present study had a typical four-junction region structure (F. Liu & Pang, 2016; Mira Park et al., 2018). Variables were usually present in the LCS and SSC regions, and expansion and contraction were noted in the IR region (Asaf et al., 2017). Gao et al., (2018) and Lu et al., (2015) reported that intergenic sequences were extended, but overlapping genes were reduced in fern chloroplast genomes. Thus,
sequence utilization is more specific, and genes are more independent. In the present study, we noted the absence of some genes in the ferns (trnI-GAU was absent in P. tripteron), and that tRNA genes were more diverse than protein-coding and rRNA genes, which possibly play an essential role in fern evolution.

Other factors that may promote evolution are introns within genes, boundary divergence, mutations, SSRs, and RNA editing events. Genes are interrupted by introns in the major groups of organisms. One-intron genes vary among species, whereas clpP, rps12, and ycf3 have been found to be two-intron genes (Brouard et al., 2016; S. Liu et al., 2017; Ting Wang et al.,.., 2018); these findings were consistent with our observations, except for P. tripteron. One additional gene, matK, contained two introns in P. tripteron. matK is a useful biomarker for phylogenetic analysis in plant classification because its sequence evolution is faster than that of other chloroplast genes (Selvaraj et al., 2008). Notably, the two-intron gene matK and the absence of trnI-GAU may provide valuable evidence regarding molecular evolution of P. tripteron.

Although highly conserved, the expansion and contraction of IR regions are responsible for variations in chloroplast genome size and rearrangement (Raubeson et al., 2007; Yang et al., 2010), thereby promoting genomic evolution (Daniell et al., 2016; Logacheva et al., 2017). D. fragrans has a genome loss of 4,033 bp in the IR region, resulting in a longer SSC region and shorter IRs regions than those in D. crassirhizoma and D. goeringiana (Gao et al., 2018). Moreover, D. fragrans was found to have dispersed gene distribution and extended sequence lengths caused by more intergenic sequences. In the present study, chlL and ndhF were located at the SSC/IRa and IRb/SSC boundaries and extended into the IR regions with different lengths. All these ferns exhibited relatively similar IR boundary characteristics, except D. goeringiana, which presented an opposite gene order in the SSC/IRa and IRb/SSC junctions compared with another six fern species in the present study and another study (L. Xu et al., 2019). Further research is needed to determine whether and how this different gene order influences the evolution of D. goeringiana.

The nucleotide diversity analysis also demonstrated that the IR regions contained fewer variable loci than the SC regions. Additionally, genes with Pi values of > 0.20 were mainly located in the SC regions. None of the intron-containing genes (atpF, clpP, matK, ndhA, ndhB, petA, petB, petD, rpl16, rpl2, rpcC1, rps12, rps16, and ycf3) had a Pi value of > 0.20, except rpl16. Intron-containing genes are more highly conserved than exon-containing genes only in the chloroplast genome. In other words, higher variability was found
in exon-containing genes, which provided more valuable information for species evolution. This result was consistent with that in fern plastomes (R. Wei et al., 2017).

SSRs, which serve as valuable molecular genetic markers, are widely used for population genetics (Doorduin et al., 2011; He et al., 2012) and plant genotyping (Ai- Hong et al., 2011; Jianhua et al., 2012). We can conclude from the present study that the number and types of SSRs are conserved within genera because D. crassirhizoma and D. goeringiana, which belong to the same genus, demonstrated high similarity in the number and types of SSRs. The types of SSRs differ widely among genera, such as that observed between A. brevifrons and P. tripteron. SSRs are usually composed of a higher number of A + T bases than G + C bases (Dai- Yong Kuang et al., 2011), which was in agreement with our observations. However, D. fragrans has higher G + C content than A + T content in SSRs. Taking the environment into consideration, Gao et al. speculated that the high G + C content in SSRs might help D. fragrans cope with substantial temperature differences (Gao et al., 2018). Vast differences in SSRs were noted among D. fragrans, D. crassirhizoma, and D. goeringiana, which belong to the same genus. We can speculate that the types of SSRs types are associated more with the surrounding environment than with the genus. This could explain the considerable differences among SSRs within the same species. The G + C content was similar among the ferns in the present study (42.40%– 43.76%). Ferns have higher G + C content (37.87 ± 1.56), angiosperm (37.71 ± 1.10), bryophyte (33.12 ± 4.16), and green algae (32.47 ± 6.07) (Kwon et al., 2020). The high G + C content of ferns helps them to survive in more environments than other plants. The unparalleled G + C content might be explained by the high level of RNA editing in the organelles (Smith, 2009). It has been reported that fern and hornwort chloroplast genomes have evolved a higher number of RNA editing events than spermatophyte chloroplast genomes, in which only 30–40 RNA editing sites are typically present (Masanori et al., 2003; P. G. Wolf et al., 2004). In the present study, most editing events were C- to- U conversions, which was consistent with that reported in D. fragrans (Gao et al., 2018). It has been reported that the high number of C-to-U conversions developed in the early stages of vascular plant evolution (Koichiro et al., 2008). We can conclude that C-to-U RNA editing events might be essential for the rapid evolution of ferns.

Chloroplast genome data are valuable for resolving species definitions because organelle-based "barcodes" can be established for certain species and then applied to reveal interspecies phylogenetic relationships (Jun-Bo Yang et al., 2013). The phylogenetic relationships of A. brevifrons, D. goeringiana, and P. tripteron have been rarely studied before chloroplast genome data became available. This study evaluated the chloroplast genomes of 43 fern species, almost all the ferns reported thus far, in the phylogenetic analysis. We found that D. crassirhizoma and D. goeringiana were closely related to D. decipiens.
P. tripteron was identified as a sister species of C. devexiscapulae. Interestingly, D. decipiens and C. devexiscapulae were found to be clustered into one branch in a study by Wei et al. (R. Wei et al., 2017). These two species were reported to be closely related to D. crassirhizoma by Xu et al. (L. Xu et al., 2019). Based on the results of the present study, we can speculate that D. crassirhizoma, D. goeringiana, D. decipiens, P. tripteron, and C. devexiscapulae are closely related.

5 | CONCLUSIONS

The complete chloroplast genomes of D. goeringiana, A. brevilfrons, and D. crassirhizoma were sequenced for the first time. We also demonstrated that ferns have a higher G + C content and a higher number of C-to-U RNA editing events than other plants. The genomic characteristics and variations provide valuable information for understanding the evolution and phylogeny of ferns.

ETHICS STATEMENT

None of the species are endangered, protected, or personally owned. This research was authorized by the Institute of Natural Resources and Ecology.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

AUTHOR CONTRIBUTION

Ruifeng Fan: Conceptualization (equal); Funding acquisition (lead); Writing-original draft (lead); Writing-review & editing (equal). Wei Ma: Methodology (lead); Visualization (equal); Writing-review & editing (supporting). Shilei Liu: Software (lead); Writing-review & editing (supporting). Qingyang Huang: Conceptualization (equal); Funding acquisition (supporting); Resources (lead); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The sequences of the complete chloroplast genomes of D. crassirhizoma, A. brevilfrons, D. goeringiana, and P. tripteron were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and under the accession numbers of MN712463, MN712464, MN712465, and MN712466, respectively.

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Additional supporting information may be found online in the Supporting Information section.

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