Phylogenetic analysis based on single-copy orthologous proteins in highly variable chloroplast genomes of *Corydalis*

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*Corydalis* is one of the few lineages that have been reported to have extensive large-scale chloroplast genome (cp-genome) rearrangements. In this study, novel cp-genome rearrangements of *Corydalis pinnata*, *C. mucronate*, and *C. sheareri* are described. *C. pinnata* is a narrow endemic species only distributed at Qingcheng Mountain in southwest China. Two independent relocations of the same four genes (*trnM-CAU-rbcL*) were found relocated from the typically posterior part of the large single-copy region to the front of it. A uniform inversion of an 11–14-kb segment (*ndhB-trnR-ACG*) was found in the inverted repeat region; and extensive losses of *accD*, *clpP*, and *trnV-UAC* genes were detected in all cp-genomes of all three species of *Corydalis*. In addition, a phylogenetic tree was reconstructed based on 31 single-copy orthologous proteins in 27 cp-genomes. This study provides insights into the evolution of cp-genomes throughout the genus *Corydalis* and also provides a reference for further studies on the taxonomy, identification, phylogeny, and genetic transformation of other lineages with extensive rearrangements in cp-genomes.

*Corydalis* DC. is a large and diverse genus, with ~786 species, within the family Papaveraceae (http://www.worldfloraonline.org/downloadData [accessed 9 December 2021]). Plants belonging to the genus *Corydalis* are distributed in the Hengduan Mountains and Qinghai–Tibet Plateau and adjacent areas¹. The structures of the some recognized *Corydalis* chloroplast genomes (cp-genomes) have undergone a series of genetic rearrangements, such as pseudogenization or the loss of genes, to adapt to drastic changes in the environment¹–⁴. *Corydalis pinnata* is a narrow endemic species in China and is only distributed along the streams of Qingcheng Mountain in southwest China at altitudes between 1300 m a.s.l. and 1400 m a.s.l. Consequently, this species must also have undergone a unique genetic shift.

Most of the *Corydalis* plants have potential as medicinal agents due to their therapeutic effects against hepatitis, tumors, cardiovascular diseases, and pain⁵,⁶, but some species are toxic⁷. As one of the most taxonomically challenging plant taxa, the genus *Corydalis* has extremely complex morphological variations because of typical reticulate evolution and intense differentiation during evolution⁸, which has hampered understanding of the identification, taxonomy, and utilization of members of this genus.

Chloroplasts are common organelles with an essential role in the photosynthesis of green plants⁹. The cp-genome is an ideal research model for studying molecular identification, phylogeny, species conservation, and genome evolution because of its conservative structure¹⁰,¹¹. The increasingly wide application of the cp-genome super-barcode in identification make the development of new cp-genome resources urgent and significant¹²,¹³. Cp-genome rearrangements can also be useful as a phylogenetic marker because they lack homoplasy and are easily identified¹⁴–¹⁶. Although some genetic rearrangements of *Corydalis* cp-genomes have been reported¹², the pattern, origin, evolution, and phylogenetic relationship of cp-genome rearrangements in *Corydalis* remain unclear because of a lack of sufficient genetic resources. In the present study, three species of the genus *Corydalis* from Qingcheng Mountain, including a narrow endemic species, were identified based on their cp-genomes. In addition, 12 *Corydalis* cp-genomes from the National Centre for Biotechnology Information (NCBI) database were included in the rearrangement analysis to represent all five subgenera of *Corydalis* and cover most of the

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distribution areas. The structural characteristics, repeat sequences, and cp-genome rearrangements were documented, and phylogenetic trees based on single-copy orthologous proteins were analyzed. The aim of the study was to assess structural variation and provide valuable resources for identification and classification of members of the genus Corydalis.

Results
DNA features of three Corydalis cp-genomes. Cp-genomes of three species of the genus Corydalis were sequenced; the three species were Corydalis pinnata, C. mucronata, and C. sheareri. The sizes of the three newly sequenced Corydalis cp-genomes ranged from 158,399 bp (C. pinnata) to 161,105 bp (C. sheareri) (Table 1). The guanine+cytosine (G+C) contents of the three genomes were 39.6%–40.47%. The three species each had a cp-genome with typical angiosperm quadripartite structure: a large single-copy (LSC) region, a small single-copy (SSC) region, and a pair of inverted repeats (IRs: IRA and IRB). The lengths of the LSC, SSC, and IR regions of the three newly sequenced Corydalis cp-genomes were 87,573–90,438, 23,778–23,322 and 23,778–25,209 bp, respectively (Table 1). After annotation, the sequences of the whole cp-genome sequences of the three Corydalis plants were submitted to the NCBI database; GenBank accession numbers are supplied in Table 1. The C. pinnata cp-genome was taken as an example and a physical map of the cp-genome was created according to the annotation results using OrganellarGenomeDRAW (OGDraw) (Fig. 1). A total of 115–117 unique genes, comprising 80–83 protein-coding genes, 28–30 tRNA genes, 4 rRNA genes, and 4–6 pseudogenes, were present in the three newly sequenced Corydalis cp-genomes (Table 1). In total, seven genes were pseudogenized in one or more Corydalis cp-genomes, and three genes (accD, clpP, and trnV-UAC) were lost in the three newly sequenced Corydalis cp-genomes (Supplementary Table 1).

Chloroplast genome structure rearrangement. Seventeen cp-genomes were included in the syntenic comparisons by Mauve alignment (Fig. 2), including 15 Corydalis cp-genomes, a representative of Papaveroideae cp-genomes (Macleaya microcarpa), and a sister in the Ranunculales cp-genomes (Euptelea pleiosperma) to represent a typical angiosperm quadripartite cp-genome structure. More than 30 locally collinear blocks (LCBs) were identified in the Corydalis cp-genomes, from which 15 rearrangements were deduced (Fig. 2).

A total of 16 relocation blocks were identified in the 15 Corydalis cp-genomes. Block 1 (approximately 6 kb) of 10 Corydalis cp-genomes contained 4–5 tRNA genes (trnL-UAA, trnK-UUU, trnI-GAU, trnG-UGC, and trnA-UGC). The eight protein-coding genes with introns were rps12, rpoC1, rpl2, paf1, ndhB, ndhA, atpF, and ycf2. Two of the 13 intron-containing genes had two introns (trnA and paf1); the remainder of the genes contained only one intron. The trnH-UUU gene contained the largest intron (2474–2488 bp), which contained the whole matK gene. Similar to other angiosperms, the gene rpl2 in the three Corydalis cp-genomes resulted from trans-splicing activity. The 5’ end of rpl2 lay in the LSC region, and the 3’ end was located in the IR region (Supplementary Table 2).

Table 1. Summary of genome structure, and gene content of the three newly sequenced Corydalis cp-genomes.
Comparison of genomic variation in the three newly sequenced *Corydalis* cp-genomes and *C. edulis* cp-genome. Previous studies reported a marked IR region expansion in some *Corydalis* cp-genomes; the IR region expanded into the simple sequence repeat (SSR) region and led to IR–SSC boundary variations\(^1,2\). In the present study, three newly sequenced *Corydalis* cp-genomes were compared with the *C. edulis* cp-genome, which exhibited a typical angiosperm quadripartite cp-genome structure (Fig. 3). The location of the IR region in the three newly sequenced *Corydalis* cp-genomes was relatively conservative (Fig. 3). In these three species, *rps19* was located in the LSC region, and *ndhF* was in the SSC region. The coding region of *rpl2* was in the IR region of the *C. pinnata* cp-genome but spanned the LSC and IRa regions of the *C. mucronata* and *C. sheareri* cp-genomes; therefore, the IRb/LSC boundary (the 5’ end was lost) region created a pseudogene.

The *C. edulis* cp-genome was used as a reference to ascertain differences in the genomic sequences of the three newly sequenced *Corydalis* cp-genomes (Fig. 4a,b). The rearranged regions exhibited higher variability compared with the other regions of the four *Corydalis* cp-genomes studied (Fig. 4a). Similar to other cp-genomes of angiosperms, most of the protein-coding genes were highly conserved, except for the large variation in the protein-coding genes of some genes (e.g., *rps19*, *rpl22*, *ycf1* and *ycf2*), intron regions (*paf1*, *ndhA* and *rpl2*), and intergenic regions (*trnQ-UUG-psbK*, *psbK-psbI*, *atpF-atpH*, *atpH-atpI*, *rpoB-trnC-GCA*, *trnC-GCA-petN*, *trnT-GGU-psbD*, *trnE-UUC-trnT-GGU*, *trnD-GUC-trnY-GUA*, *psaA-pafI*, *pafI-trnS-GGA*, *rps4-trnT-UGU*, *trnT-UGU-trnL-UAA*, *trnR-ACG-trnL-CAA*, and *trnN-GUU-ndhB*) among the chloroplast genomic sequences with a higher degree of variation. Such higher-resolution loci have the potential to be used as barcodes in species identification.

Analyses of long repetitive sequences and SSRs. Interspersed repeated sequences (IRRs) with a repeat unit length of ≥ 39 bp were evaluated in the chloroplast genomes of *C. pinnata*, *C. mucronata*, and *C. sheareri*. These repeats comprised only forward and palindromic repeats and lacked reverse and complementary repeats that are common in other species. Fifty IRRs were found, and among these, the sequence lengths in *C. pinnata*, *C. mucronata*, and *C. sheareri* were 40–49, > 80, and ≤ 49/≥ 80 bp, respectively. The IRR analyses of the chloroplast genomes are shown in Fig. 5a–c.

**Figure 1.** Gene map of the chloroplast genome of *C. pinnata*. Genes within the circle are transcribed clockwise, and those outside are transcribed counterclockwise. Genes belonging to different functional groups are colour coded. The dark grey in the inner circle corresponds to DNA G+C content, and the light grey corresponds to A+T content.
In total, 46 SSRs were found in *C. pinnata*, including 38 mononucleotide repeats, 1 dinucleotide repeat, and 5 trinucleotide repeats; 51 SSRs were identified in *C. mucronate*, including 43 mononucleotide repeats, 1 dinucleotide repeat, 5 trinucleotide repeats, and 2 pentanucleotide repeats; and 46 SSRs were found in *C. sheareri*, including 35 mononucleotide repeats, 1 dinucleotide repeat, 5 trinucleotide repeats, 2 tetranucleotide repeats, and 3 hexanucleotides (Fig. 5d).

**Phylogenetic analyses.** Using concatenated single-copy orthologous proteins to resolve phylogenetic relationships could avoid rearrangement-misled phylogenetic tree reconstruction and provide more reliable evolutionary framework compared with using several specific genes. Therefore, the predicted proteome was used in the phylogenetic analyses rather than the whole cp-genome sequence. Based on 31 single-copy orthologous proteins conserved in 27 species with *E. pleiosperma* as the outgroup, a maximum-likelihood (ML) phylogenetic tree was reconstructed to illuminate the evolutionary history of the compared species (Fig. 6). The ML tree had three major clades: the Fumarioideae clade, Papaveroideae clade, and the clade with the rest of the Ranunculales family members. *Corydalis* constituted a monophyletic sub-clade nested within the Fumarioideae clade. All lineages within *Corydalis* were strongly supported. The three newly sequenced *Corydalis* cp-genomes, namely, *C. pinnata* (Sect. Mucronatae), *C. mucronata* (Sect. Mucronatae), and *C. sheareri* (Sect. Asterostigmata), were closely related.
Discussion

Although the three newly sequenced Corydalis cp-genomes from the same geographic region belong to two different subgenera of Corydalis, the sizes and structures of their LSC, IR, and SSC regions, as well as their total genomes, are highly similar. This includes similar gene losses, inversions, and relocations (Fig. 1 and Supplementary Table 1), which are common features in the Corydalis cp-genomes and are considered to be responsible for the variation in cp-genome sizes.

The loss of three genes (accD, clpP, and trnV-UAC) is a synapomorphic characteristic in the Corydalis cp-genomes (Supplementary Table 1). Xu et al. speculated that the loss of the accD gene occurred before divergence of the genus Corydalis. However, in the present study, the accD gene was found in the cp-genomes of a few species of the subgenus Rapiferae (Supplementary Table 1), which indicated that the loss event happened after divergence of the genus Corydalis. The exact time of the loss event should be further explored by gathering more information on Corydalis cp-genomes. The accD gene is relocated to the nucleus in some species, such as some members of the family Campanulaceae. The pseudogenization or loss of 11 chloroplast ndh genes that encode NADH dehydrogenase subunits only occurred in a few species of the genus Corydalis (C. conspersa, C. davidii, C. adunca, and C. inopinata; Supplementary Table 1). Strikingly, these species are all located in high-altitude areas (1000–5200 m a.s.l.). Therefore, extreme changes in the environment may result in gene deletions or pseudogenization; this phenomenon has been observed in other species. Further studies are required to determine whether or not the pseudogenization or loss of ndh genes will affect photosynthesis in those plants.

The chloroplast genome, as a photosynthetic organelle, is highly conserved in terms of structure, gene content, and arrangement. Large-scale rearrangement exists only occasionally in a few lineages, such as Campanulaceae, Ranunculaceae, Geraniaceae, Fabaceae, Oleaceae, Asteraeae, Plantaginaceae, Euphorbiaceae, and Poaceae. In the present study, rearrangement predominantly occurred in 16 regions (blocks 1–16, Fig. 2) of Corydalis plants, which determine the diversity in Corydalis cp-genomes. Repeat sequences may contribute to structural variations in relatively stable rearrangement regions. Relocation only occurred in the LSC region of the Corydalis cp-genomes, and inversion only occurred in the IR and SSC regions (Fig. 2). This suggested that the patterns of relocation and inversion were regulated in different ways. In addition, blocks 1–16 are likely active rearrangement regions because they have various rearrangement patterns. C. hsiaowutaishanensis (subg. Corydalis), C. adunca (subg. Cremnocapnos), C. Saxicola, and C. fangshanensis (subg. Sophorocapnos) all underwent the inversion of blocks 10–16, but the inversion boundaries of C. hsiaowutaishanensis expanded into block 9, suggesting that the inversion of blocks 9–16 in C. hsiaowutaishanensis was an independent event. Furthermore, some species from different subgenera have the same relocation or inversion pattern, such as the three Corydalis plants (C. pinnata, C. mucronate, and C. sheareri) collected from Qingcheng Mountain in the current study. Although they represent two subgenera, these three species have an almost identical relocation/inversion pattern in their cp-genomes (Fig. 2). Moreover, blocks 5–7 underwent at least two inversions in C. tomentella; blocks 5–7 initially inverted independently and then inverted with blocks 3, 4, and 8. This active rearrangement suggested that relocation or inversion in Corydalis cp-genomes might be affected by the geographical environment.
Figure 4. Comparative analyses of genomic differences in the chloroplasts of *C. pinnata*, *C. mucronata*, *C. sheareri* and *C. edulis*. (a), Sliding window analyses of the entire cp-genome. (b), Alignment visualisation of the chloroplast genome sequences of *C. pinnata*, *C. mucronata*, *C. sheareri* and *C. edulis* using mVISTA. Grey arrows and thick black lines indicate genes and their orientation. Purple bars indicate exons, blue bars represent untranslated regions, pink bars represent non-coding sequences, and grey bars denote mRNA. The similarity among the chloroplast genomes is shown on a vertical scale ranging from 50 to 100%.
Loss of introns and/or genes is instrumental in the regulation of gene expression and can control gene expression temporally and in a tissue-specific manner. The regulation mechanisms of introns for gene expression in plants and animals have been reported. However, the implications or link between gene expression and intron loss for *Corydalis* have not been published. Further experimental work on the roles of introns in *Corydalis* is therefore essential and should prove interesting. Highly variable DNA barcodes play an important role in species identification and phylogenetic analyses. In the current study, protein-coding genes (*rps19*, *rpl22*, *ycf1*, and *ycf2*), intron regions (*paf1*, *ndhA*, and *rpl2*), and the intergenic regions (*trnQ-UUG-psbK*, *psbK-psbl*, *atpF-aptH*, *atpH-atpl*, *pob-trnC-GCA*, *trnC-GCA-petN*, *trnF-GGU-psbD*, *trnE-UUC-trnF-GGU*, *trnD-GUC-trnY-GUA*, *psaA-pafI*, *pafI-trnS-GGA*, *rps4-trnI-UGU*, *trnI-UGU-trnL-UAA*, *trnR-ACG-trnL-CAA*, and *trnN-GUU-ndhB*) exhibited some extent of variation and have great potential as DNA markers (Fig. 4b).

*Cp*-genomes have made marked contributions to the phylogenetic studies of angiosperms and to resolving the evolutionary relationships within phylogenetic clades. However, active rearrangement in *Corydalis* cp-genomes may mislead the reconstruction of species phylogenetic relationships based on DNA sequence of cp-genomes. Phylogenetic reconstruction of the genus *Corydalis* was previously explored with DNA barcoding or relatively conserved nucleotide fragments in cp-genomes. However, deep relationships remained poorly resolved by this phylogenetic approach applying a few plastid markers. Some studies reported that the protein-coding genes shared by all taxa could be used to reconstruct a phylogeny. However, single-copy genes (SCGs) have subsequently emerged as candidates for phylogenetic analysis because paralogues are derived from duplication events other than speciation events and should therefore be discarded from phylogenetic analyses. Therefore, the 31 single-copy orthologous proteins in all 27 cp-genomes were used to reconstruct the phylogeny of the genus *Corydalis*. Three distinct clades were defined by high bootstrap values (Fig. 6) in the resulting phylogenetic tree, which is consistent with previous studies based on molecular markers. This indicated that the application of the single-copy orthologous proteins of cp-genomes can improve the resolution of the phylogeny and taxonomy of the genus *Corydalis*. Findings from the study also provide a reference for the taxonomy and identification of other plants with extensive rearrangement in cp-genomes.
Conclusions

The cp-genomes of three species of the genus Corydalis (C. pinnata, C. mucronata, and C. shearer) from the Qingcheng Mountain in southwest China, including a narrow endemic species (C. pinnata), were characterized. The cp-genomes of the three species exhibited a large-scale rearrangement, including the relocation of four genes (trnM-CAU-rbcL) in the LSC region, the inversion of an 11–14-kb segment (ndhB-trnR-ACG) in the IR region, and the loss of three genes (accD, clpP, and trnV-UAC). The three Corydalis cp-genomes showed high similarity in terms of genome size, gene classes, gene sequences, rearrangement pattern, and distribution of repeat sequences. In addition, the structural alignment of 17 Corydalis cp-genomes with the typical chloroplast genomic structure of angiosperms (E. pleiosperma) revealed a frequent and extensive large-scale rearrangement in the Corydalis cp-genomes. Among them, the relocation of two blocks (trnM-CAU-rbcL and rps16) frequently appeared in the LSC region, and the inversion of four blocks (rps23-trnL-CAA, ndhB-trnR-ACG, trnN-GUU, and ndhA-ycf1) frequently appeared in the IR and SSC regions. The extensive large-scale cp-genome rearrangement may mislead phylogenetic analysis based on cp-genomes. Single-copy orthologous proteins of cp-genomes were therefore used to reconstruct the phylogeny of the genus Corydalis. This method was concluded to have good prospects for elucidating the phylogeny and taxonomy of Corydalis and could potentially be employed for the phylogenetic analysis of other lineages with extensive rearranged cp-genomes in future studies. Findings from this study provide a reference for further studies on the taxonomy, identification, and evolution of the genus Corydalis.

Materials and methods

Plant collection and sampling. The aboveground parts of the three plant species were collected from Qingcheng Mountain, Sichuan Province, China (C. shearer, location: E 103°32'49” N 30°54'5", altitude: 720 m a.s.l.; C. mucronate, location: E 103°28'35” N 30°28'35”, altitude: 980 m a.s.l.; C. pinnata, location: E 103°25'27” N 30°65'5", altitude: 1350 m a.s.l.). The voucher specimens were deposited in the herbarium of the College of Pharmacy, Chengdu University of Traditional Chinese Medicine, China (deposition numbers: C. shearer, CDCM0005283; C. mucronate, CDCM0005284; C. pinnata, CDCM0005285). The collection of samples conformed to the management provisions of the List of State-protected Wild Plants and was approved by the National Forestry and Grassland Administration of China (Supplementary Fig. 1). The specimens were identified by Professor Guihua Jiang.
DNA sequencing, assembly and validation of the chloroplast genome. A modified cetyltrimethylammonium bromide (CTAB) method was used for DNA extraction and the NEBNext Ultra DNA Library Prep Kit for Illumina sequencing was used for 500-bp paired-end library construction. A shotgun library (250 bp) was constructed according to the manufacturer’s (Vazyme Biotech, Nanjing, China) instructions. Sequencing was accomplished with the X10 Ten platform (Illumina, San Diego, CA, USA) using the double terminal sequencing method (pair-end 150)90. Total raw data from a sample was approximately 10.0 G, and > 300 million paired-end reads were attained.

Raw data were filtered by Skewer-0.2.2 2272. The resulting reads were used for genome assembly by GetOrganelle version 1.7.573. Another assembly for each species of the genus Corydalis was performed by ABYSS with C. edulis as the reference to confirm the GetOrganelle assemblies. The draft genome was used to map clean reads by BWA version 0.7.174, and then clean reads were filtered using SAMtools version 1.775. Mapping was visualized by IGV version 2.10.0 76 to check the concatenation of contigs. Furthermore, junction splicing sites were verified with polymerase chain reaction (PCR) and Sanger sequencing. All of the contigs were aligned to the reference cp-genome of C. edulis with MUMmer version 4.0 77. Finally, the sequences were extended and gaps were filled with SSPACE-3.078.

Gene annotation and sequence analyses. Sequence annotation was achieved by Flann version 1.1.270 using the cp-genome of C. conspersa as a reference and some manual correction. BLAST and Apollo80 were used to check the start and stop codons and the intron/exon boundaries with the cp-genome of C. conspersa as a reference sequence. Complete cp-genome sequences were submitted to the NCBI. A physical map of the cp-genomes was generated with Organellar Genome OGDraw81 (http://ogdraw.mpimp-golm.mpg.de/).

Genome structure analyses. To determine synteny and identify possible rearrangements, 19 cp-genomes were compared using Mauve 2.4.082 with the “progressiveMauve” algorithm, including 17 Corydalis cp-genomes, the cp-genome of Macleaya microcarpa (NC_039623) representing Papavaeoidae, and the cp-genome of Euptelea pleiosperma (NC_029429) representing a typical angiosperm cp-genome. The Mauve result was then manually modified to show the notable rearrangements. The cp-genomes of species of the genus Corydalis were completed by mVISTA83 (Shuffle-LAGAN mode) using the genome of C. edulis as the reference. Tandem Repeats Finder84 was used to detect tandem repeats, forward repeats, and palindromic repeats as tested by REPutter85. SSRs were detected by Misa.pl86 using search parameters of mononucleotides set to ≥ 10 repeat units, dinucleotides ≥ 8 repeat units, trinucleotides and tetranucleotides ≥ 4 repeat units, and pentanucleotides and hexanucleotides ≥ 3 repeat units.

Phylogenetic analyses. Twenty-seven cp-genomes were used to reconstruct a phylogenetic tree. First, single-copy orthologous proteins were extracted by OrthoFinder version 2.3.887. Next, genes were aligned by MUSCLE version 3.8, and then the best-fit models of amino acid substitution were estimated by ProtTest version 8.2.1289 including tree robustness assessment using 1000 replicates of rapid bootstrap with the HIVb+I+G+F substitution model based on the results of ProtTest.

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Author contributions
G.H and H.X.Y conceived and designed the research framework; X.F. and J.C.G. collected and identified the sample; X.M.Y and F.H. performed the experiments; Y.L. and N. analyzed the data; X.M.Y and F.H. wrote the paper; C.L. and J.J.D. and H.X.Y. made revisions to the final manuscript. All the authors have read and approved the final manuscript.

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The authors declare no competing interests.

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