Chloroplast genome features of an important medicinal and edible plant: *Houttuynia cordata* (Saururaceae)

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Abstract

*Houttuynia cordata* (Saururaceae), an ancient and relic species, has been used as an important medicinal and edible plant in most parts of Asia. However, because of the lack of genome information and reliable molecular markers, studies on its population structure, or phylogenetic relationships with other related species are still rare. Here, we de novo assembled the complete chloroplast (cp) genome of *H. cordata* using the integration of the long PacBio and short Illumina reads. The cp genome of *H. cordata* showed a typical quadripartite cycle of 160,226 bp. This included a pair of inverted repeats (IRa and IRb) of 26,853 bp, separated by a large single-copy (LSC) region of 88,180 bp and a small single-copy (SSC) region of 18,340 bp. A total of 112 unique genes, including 79 protein-coding genes, 29 tRNA genes, and four rRNA genes, were identified in this cp genome. Eighty-one genes were located on the LSC region, 13 genes were located on the SSC region, and 17 two-copy genes were located on the IR region. Additionally, 48 repeat sequences and 86 SSR loci, which can be used as genomic markers for population structure analysis, were also detected. Phylogenetic analysis using 21 cp genomes of the Piperales family demonstrated that *H. cordata* had a close relationship with the species within the *Aristolochia* genus. Moreover, the results of mVISTA analysis and comparisons of IR regions demonstrated that the cp genome of *H. cordata* was conserved with that of the *Aristolochia* species. Our results provide valuable information for analyzing the genetic diversity and population structure of *H. cordata*, which can contribute to further its genetic improvement and breeding.

Introduction

Piperales, which show discrete vascular bundles in the stem and threefold flower parts [1], are placed in the Magnoliids clade, which is an early evolutionary branch in the angiosperm tree [2]. The category of Piperales has changed in different ways over time. According to the recent APG IV system, Piperales includes three families (Piperaceae, Aristolochiaceae and
Saururaceae), 17 genera, and more than 4,000 species, most of which belong to the Piperaceae family [3]. Saururaceae is the smallest family in Piperales, including only four genera and six species, most of which are aromatic herbs with creeping rhizomes [3–5].

_Houttuynia cordata_, the single member of the _Houttuynia_ genus in the Saururaceae family, is widely used as a leafy vegetable and medicinal herb throughout much of Asia [6]. Its characteristic extract, houttuynin, has been proven to have diverse pharmacological effects including anticestodal [7], antibacterial [8], and antiviral activity [9]. Moreover, extracts of _H. cordata_ have had important functions in improving the immune system of patients with severe acute respiratory syndrome (SARS) [10]. Although _H. cordata_ is the only species in the _Houttuynia_ genus of the Saururaceae family, its populations from different regions vary widely at chromosome numbers (2n = 24–128) and polyploidy levels [6, 11]. This probably resulted from the prevailing cytomixis and meiotic abnormalities that occur during microsporogenesis [11].

Almost all previous studies on this plant have focused mainly on its physiological and biochemical properties [7, 9, 10, 12, 13], whereas few studies were aimed at deciphering the genetic diversity, population structure, and taxonomic status of _H. cordata_ in Piperales. This is mostly likely due to the limited genome information and development of markers.

Chloroplasts have their own circular genome and play a vital role in photosynthesis, physiology and development in most plants. For most angiosperms, chloroplast (cp) genomes typically range in size from 120 to 170 kilobase pairs (kb) [14]. Compared to the nuclear genome, the cp genome is more conserved in terms of gene size and content, genome structure, and linear order of the genes [15]. Generally, the cp genome has a quadripartite cycle, comprising a pair of inverted repeat regions (IRA and IRB) that are separated by one large single-copy (LSC) region and one small single-copy (SSC) region. Previous studies have demonstrated that, compared to the nuclear genome, fewer substitutions of nucleotides and rearrangements of genome structure occur in the cp genome [16, 17], making it an ideal model to decipher genome evolution and phylogenetic relationships in complex angiosperm families [18, 19].

The development of next-generation sequencing technologies has provided highly efficient, low-cost DNA sequencing platforms that produce large volumes of short reads [20]. Moreover, the third-generation sequencing technology, PacBio sequencing platform, otherwise called single-molecule real-time (SMRT) sequencing technology, generates long reads with lengths of up to 30 kb that can easily close the gaps in current reference assemblies through extended repetitive regions. Already, thousands of cp genomes have been completely revealed. Recently, two short communications have reported the length and gene contents of cp genomes in _H. cordata_ [21, 22]. However, a comprehensive analysis of this cp genome has not yet been performed.

In the present study, we _de novo_ assembled the complete cp genome of _H. cordata_, a quadripartite cycle of 160,226 bp, through a combination of PacBio and Illumina sequencing platforms. The rates of synonymous (Ks) and non-synonymous (Ka) substitutions for shared common genes between _H. cordata_ and related species were also calculated. Phylogenetic analysis using 21 Piperales species demonstrated that _H. cordata_ had a close relationship with the species within the _Aristolochia_ genus. Our results provide valuable information for future research in genetic improvement, population genetics, and population diversity of _H. cordata_.

### Materials and methods

#### Plant materials and sequencing

The clones of _H. cordata_ were collected from Guiyang Huaxi Wetland Park, China, and were grown in a glasshouse at Guizhou Normal University. The intact chloroplasts of _H. cordata_ were initially enriched from 5 g of fresh leaves as described by Okegawa and Motohashi [23].
with minor modifications. Briefly, 5 g fresh leaves excluding petioles and veins were homogenized in 400 mM sorbitol, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 2 mM EDTA, 10 mM NaHCO$_3$, 0.5% (w/v) bovine serum albumin (BSA), 5 mM ascorbate, and 20 mM Tricine–NaOH (pH 8.4). After centrifugation at 3000 g for 5 min, the pellet was gently suspended in 400 mM sorbitol, 5 mM MgCl$_2$, 2.5 mM EDTA, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH (pH 7.6). The intact chloroplasts were further purified using 40% (v/v) Percoll (Zhuoyue, Beijing Baiaolaibo, China, http://www.baiaolaibo.com/). Isolated intact chloroplasts were suspended in 3 mM MgCl$_2$ and 25 mM HEPES–KOH (pH 7.6), followed by centrifugation at 10,000 g for 3 min. Then, the cp DNA was isolated from the chloroplasts using the DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. After assessing the quality and quantity of the cp DNA, one DNA library with 20 kb insertion using the PacBio Sequel platform (PacBio, USA) and one DNA library with 450 bp insertion using the Illumina HiSeq X Ten platform (Illumina, USA) were sequenced based on the manufacturer’s instructions. All the raw data, including short Illumina reads and long PacBio reads used in this study are available at the BIG Sub (http://bigd.big.ac.cn), under the accession number: CRA002843.

**Genome assembly and gene annotation**

The 150 bp paired-end reads were generated by the Illumina HiSeq platform. After removing sequencing adapters and low-quality reads with QC values less than 20%, the clean reads were firstly compared to the complete cp genome of the related Aristolochia tubiflora (GenBank accession: NC_041455) using BLASTn software [24] (E-value: 10–6) to select cp genome sequences. These selected Illumina reads were then de novo assembled into scaffolds using SOAP denovo v2.04 (http://soap.genomics.org.cn/soapdenovo.html) with a K-mer value of 51. Low-quality PacBio reads (minimum read length of 500 bp and minimum read quality of 0.80), were removed from the raw data. Then the filtered PacBio reads were error-corrected to remove single nucleotide insertion/deletions using Illumina short reads by the PacBioToCA module of the Celera Assembler with default parameter settings ( -length 500, -partitions 200) [25]. Afterwards, the corrected PacBio reads were used to gap-fill the scaffolds by PBjelly (https://sourceforge.net/projects/pb-jelly/) [26] with all PacBio reads >8kb to generate a circular cp genome map. Frame-shift errors were manually corrected during gene prediction.

The protein-coding genes and noncoding genes (tRNAs and rRNAs) of *H. cordata* cp genome were annotated using Dual Organellar GenoMe Annotator (DOGMA, http://dogma.ccbb.utexas.edu/) with default parameters [27]. Moreover, the intron and exon boundaries of protein-coding genes were manually modified. Similarly, the tRNA genes were verified using the tRNAscan-SE 1.23 program (http://lowelab.ucsc.edu/tRNAscan-SE/). The circular gene map of *H. cordata* cp genome was given by OGDraw v1.2 [28]. Finally, the complete *H. cordata* cp genome was deposited into GenBank (accession number: MN413197).

**Repeat sequence and simple sequence repeat detection**

The web service of REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer/, [29]) was employed to identify repeat sequences according to the following parameters: minimal repeat size to 30; maximum computed repeats to 50; and hamming distance to 10. Match direction included forward, palindrome, reverse, and complement repeat types. To detect simple sequence repeats (SSRs), MISA (https://webblast.ipk-gatersleben.de/misa/, [30]) was used with minimal repeat numbers set at ten, six, four, three, three, and three for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively.
**Codon usage analysis**

Codon usage bias is a universal feature of all genomes and has been proposed to regulate translation dynamics such as translation efficiency and accuracy, as well as protein folding [31]. To further analyze *H. cordata* cp genome evolution, the CodonW1.4.2 program (http://downloads.fyxm.net/CodonW-76666.html) was employed to calculate the synonymous codon usage of protein-coding genes with default settings.

**Phylogenetic analysis**

The 20 complete cp genomes (S1 Table) from Aristolochiaceae and Piperaceae families downloaded from NCBI together with *H. cordata* cp genome were used to construct their phylogenetic relationship. Only the homologous CDs were used to construct the phylogenetic relationship to reduce data redundancy, such as that done in the study of *Rhododendron delavayi* [32]. A total of 75 homologous CDs (coding gene sequence) were employed in this study, including *psbB*, *psbZ*, *ndhF*, *petG*, *rps18*, *ropB*, *petN*, *psbA*, *psaJ*, *ccsA*, *ropC1*, *rbcL*, *psbM*, *ndhG*, *rps19*, *rpl2*, *psbL*, *psaC*, *rps3*, *matK*, *psbE*, *rpl14*, *petA*, *rpl33*, *psaA*, *rpl36*, *ndhK*, *ndhJ*, *psbK*, *atpI*, *psbF*, *psbI*, *rps2*, *rpl32*, *atpH*, *psbN*, *accD*, *psaB*, *rps11*, *atpA*, *rps14*, *infA*, *psaI*, *ycf1*, *rps4*, *atpE*, *psbl*, *rpl16*, *rps15*, *rpl20*, *roA*, *ndhD*, *psbH*, *ropC2*, *ndhI*, *ndhA*, *ndhH*, *rps16*, *atpB*, *petD*, *psbC*, *ndhb*, *petB*, *atpF*, *rps7*, *rpl23*, *psbD*, *clpP*, *rpl22*, *ndhE*, *petL*, *psbT*, *ycf2*, *rps12*, and *ndhC*, were used to determine the phylogenetic relationship. Phylogenetic trees were then constructed using the maximum likelihood (ML) method with 1000 bootstrap replicates by MEGA7 [33].

**Whole cp genome sequence comparisons**

To provide comprehensive knowledge of cp sequence divergence, the *H. cordata* cp genome was compared to four cp genomes from the *Aristolochia* genus. The divergence of the LSC/IRB/SSC/IRA boundary regions among these related species was visualized by IRscope (https://irscope.shinyapps.io/irapp/) based on the annotations of their available cp genomes in GenBank. Additionally, the mVISTA program (http://genome.lbl.gov/vista/mvista/submit.shtml) was also used to compare the whole cp genome divergence among these related species.

**Synonymous and nonsynonymous substitution rate calculations**

To determine synonymous (Ks) and non-synonymous (Ka) substitution rates, we performed pairwise comparisons of the 79 protein-coding genes between *H. cordata* cp genome and four close Aristolochia species. Pairwise alignments of the common genes among species were carried out by MAFFT [34], and the Ka/Ks ratios were calculated with KaKs_calculator 2.0 [35] using the default parameters for plant plastid code.

**Results**

**Chloroplast genome assembly and genome features**

The Illumina sequencing platform gave rise to 3,763 Mb of raw data. After trimming, 3,390 Mb of clean reads with a Q20 value of 95.59% was obtained. The PacBio platform generated 137,091 subreads with an average length of 4,871 bp and an N50 length of 7,337 bp (S2 Table). Both Illumina reads and PacBio subreads were used to construct the complete cp genomes of *H. cordata*.

The *H. cordata* cp genome showed a typical quadripartite cycle of 160,226 bp, comprising a pair of inverted repeats (IRa and IRb) of 26,853 bp, which were divided by a large single-copy (LSC) region of 88,180 bp and a small single-copy (SSC) region of 18,340 bp (Table 1). Regarding GC content, the IR regions showed the highest GC content of 43.03%, followed by 36.81%...
in the LSC region, whereas the SSC region exhibited the lowest GC content of 32.15%. The overall GC content of the cp genome was 38.36%. In total, 112 unique genes were identified in the \textit{H. cordata} cp genome, including 79 protein-coding genes, 29 tRNA genes, and four rRNA genes (Table 1). Furthermore, out of 112 unique genes, 82 and 13 genes were found in the LSC and SSC regions, respectively; while 17 genes, including six protein-coding genes, seven tRNAs, and four rRNAs were duplicated in the IR regions (Fig 1). Among the 112 unique genes, 18 genes (comprising 11 protein-coding genes and seven tRNA genes) had one intron, and only two genes (\textit{clpP} and \textit{ycf3}) harbored two introns (Table 2).

**Repeat sequence and Simple Sequence Repeat (SSR) detection**

In this study, a total of 48 repeat sequences with lengths ranging from 30 bp to 69 bp were detected, including 27 forward repeats and 21 palindromic repeats, whereas no reverse or complement repeats were identified (S3 Table; Fig 2A). Among these repeats, 14 were 30–39 bp in length, 11 were 40–49 bp, 14 were 50–59 bp, and nine were 60–69 bp (Fig 2A). All the repeats were found within seven protein-coding genes (\textit{ccsA}, \textit{petA}, \textit{petN}, \textit{psaB}, \textit{rpl32}, \textit{ycf2}, and \textit{ycf3}) (Fig 2B). Out of 30 repeats (62.5%), 15 forward repeats and 15 palindromic repeats, were contributed by \textit{ycf2}, which is essentially a pseudogene. Most paired repeats (36 repeats, 75%) were located in the same genes; however, 12 repeats, that were all forward types, were seen in different genes (S3 Table).

In total, 86 SSR loci of 17 different types with a length of at least 10 bp were also detected by MISA (Table 3). Among the SSR loci, mononucleotide repeats were the most abundant with 68 SSR motifs (79.07%) of only two types (A/T). Eleven tetrancleotide repeats representing 10 different types and four trinucleotide repeats representing three different types (TTA/TAT/
ATA) were identified. The dinucleotide repeats were only TA type with two motifs, and the pentanucleotide repeats (TCTTT) was observed only once. No hexanucleotide repeats were observed, and the longest SSR was a tetranucleotide repeat (TTTA) of 24 bp in length.

Fig 1. Gene circular map of the complete H. cordata cp genome. Genes on the outside and inside of the circle are transcribed in clockwise and counterclockwise directions, respectively. Genes belonging to different functional groups are color coded. Color intensity corresponds to GC content. The SSC, LSC, and inverted repeat regions (IRA and IRB) are indicated.

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Table 2. Summary of assembled gene functions of *Houttuynia cordata* Thunb cp genome.

| Category for genes | Genes function | Genes |
|--------------------|----------------|-------|
| Genes involving in photosynthesis | Subunits of photosystem | psaA, psaB, psaC, psaL, psbl, psbB, psbD, psbE, psbH, psbI, psbK, psbL, psbM, psbN, psbZ, psbT; |
| | Subunits of cytochrome b/f complex | petA, petB, petD, petG, petL, petN |
| | Large subunit of Rubisco | rbcL |
| | Subunits of ATP synthase | atpA, atpB, atpF, atpH, atpI, atpE |
| | Subunits of NADH-dehydrogenase | ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK, |
| Self-replication | Ribosomal RNA genes | rrs5, rrs4.5, rrs23a, rrs16a |
| | Transfer RNA genes | trnL-CAA, trnL-UAG, trnP-UAG, trnW-CCA, trnM-CAU, trnV-UAC, trnF-GAA, trnL-UAA, trnT-UGU, trnS-GGA, trnF-MAU, trnG-GCC, trnS-UGA, trnT-GGU, trnY-GUA, trnD-GUC, trnC-GCA, trnR-UCU, trnG-UCC, trnS-GCU, trnQ-UGU, trnK-UUU, trnH-GUG, trnL-CAU, trnV-GAC, trnL-GAU, trnA-UGC, trnR-ACG, trnN-GUU |
| | Small subunit of ribosome | rps11, rps12, rps14, rps15, rps16a, rps18, rps19, rps2, rps3, rps4, rps8, rps9 |
| | Large subunit of ribosome | rpl2, rpl14, rpl16, rpl19, rpl22, rpl23a, rpl32, rpl33, rpl36 |
| | DNA-dependent RNA polymerase | rpoA, rpoB, rpoC1b, rpoC2 |
| Other genes | Maturase | matK |
| | Envelope membrane protein | cemA |
| | Subunit of acetyl-CoA | accD |
| | C-type cytochrome synthesis gene | ccsA |
| | Translational initiation factor | infA |
| | Protease | clpP |
| Functionally unknown genes | Conserved open reading frames | ycf1, ycf2a, ycf3a, ycf4 |

The letters indicate the gene with two copies, harboring one intron and two introns, respectively.

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Fig 2. Distribution of repeat sequences in *H. cordata* cp genome. A, Number of forward and palindromic types of repeats with different size in length; B, Number of repeats within seven genes.

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Codon usage analysis

The coding sequence of 79 protein-coding genes gave rise to 22,816 codons. Among these, the leucine codons were the most biased, with a frequency of 10.36%, whereas the cysteine codons had the lowest usage frequency of only 1.11% (S4 Table; Fig 3A). To gain knowledge of synonymous codon usage bias of *H. cordata* cp genome, we also calculated the relative synonymous codon usage (RSCU) value. The results showed that the RSCU values of 31 codons were greater than 1, indicating these codons were preferentially used. Among these preferential codons, the majority of codons ended with A (13 of 31) or U (16 of 31) except UUG and UCC (S4 Table; Fig 3B).

Table 3. Summary of simple sequence repeats in *Houttuynia cordata* cp genome.

| Repeats Unit | Types | Number | Largest Repeats (bp) |
|--------------|-------|--------|----------------------|
| 1            | T     | 40     | 15                   |
|              | A     | 28     | 15                   |
| 2            | TA    | 2      | 16                   |
| 3            | TTA   | 1      | 12                   |
|              | TAT   | 1      | 12                   |
|              | ATA   | 2      | 12                   |
| 4            | AATG  | 1      | 12                   |
|              | ATCT  | 1      | 12                   |
|              | ATTA  | 1      | 12                   |
|              | CAAA  | 1      | 12                   |
|              | CATT  | 1      | 12                   |
|              | CACT  | 1      | 12                   |
|              | TATG  | 1      | 12                   |
|              | TCAA  | 1      | 12                   |
|              | TTTA  | 2      | 24                   |
|              | TTTC  | 1      | 12                   |
| 5            | TCTTT | 1      | 15                   |
| Total        |       | 17     | 86                   |

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![Fig 3. Percentage of amino acids of *H. cordata* cp genome and the ending patterns of biased usage codons (RSCU>1). A, Detail information of amino acids used in *H. cordata* cp genome. B, The A- and U-ending patterns were the most popular in biased usage codons.](https://doi.org/10.1371/journal.pone.0239823.g003)
Phylogenetic analysis and whole cp genome sequence comparisons

To analyze phylogenetic relationships among the Piperales family, we downloaded 20 complete cp genomes covering four genera (S1 Table) from NCBI to construct the phylogenetic trees. To reduce data redundancy, 75 homologous CDs of all 21 cp genomes were used to generate phylogenetic tree by the Maximum Likelihood method with 1000 bootstrap replicates. The phylogenetic tree generated 19 nodes, and *Asarum sieboldii* (NC_037190) formed the outgroup. Out of 19 nodes, 18 nodes bootstrap values were \( \geq 92\% \). Generally, the phylogenetic tree showed that each genus (*Aristolochia*, *Piper*, and *Chloranthus*) constituted a monophyletic group. *H. cordata* together with the species of *Aristolochia* formed a subgroup with bootstrap values of 100%, indicating that they shared a more closer relationship (Fig 4).

Additionally, mVISTA analysis was performed to evaluate divergence in the genome sequences between *H. cordata* and *Aristolochia* species with reference to the annotation of *H. cordata*. The results revealed that all selected cp genomes showed generally high similarity.

![Fig 4. Phylogenetic relationship analysis of 21 Piperales species based on the shared common protein-coding sequence.](https://doi.org/10.1371/journal.pone.0239823.g004)
However, several minor inserted regions and one large inserted region beyond 1 kb were observed (Fig 5), indicating that the *H. cordata* cp genome underwent evolutionary divergence. Overall, the coding regions were more conserved than the non-coding regions and the obviously divergent genes were *ycf2*, *rpl14*, *rpl19*, *atpH*, and *rpl22*.

To gain a more comprehensive comparison, the IR regions that were believed to contribute to the size variation of the cp genome were also compared among these five species (Fig 6). The results showed that, compared to IRB/SSC and SSC/IRA junctions, the distribution of the genes in the LSC/IRB and IRA/LSC border regions were relatively conserved. The *rps19* and *rpl2*, and *trnH* genes were distributed at the LSC/IRB and IRA/LSC junction in all five cp genomes, differing only in the distance of three genes to the junction. For SSC/IRA boundaries, the *ycf1* gene located on the SSC region had a 169 bp, 171 bp, and 1431 bp extension to the IRA region in *A. contorta*, *A. debilis*, and *H. cordata*, respectively. However, a 26 bp and 36 bp distance of the *ycf1* gene to the boundary were found in *A. tagala* and *A. tubiflora*, respectively. For IRB/SSC boundaries, the *ndhF* gene located on the SSC region was either near or

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Fig 5. Alignment of the cp genomes of *H. cordata* and four closely related species. The alignment was performed by mVISTA with *H. cordata* as a reference. Local collinear blocks within each alignment are indicated by the same color and linked.
overlapping the boundary in *A. tagala*, *A. tubiflora*, and *H. cordata*, whereas in *A. contorta* and *A. debilis*, the gene was replaced by the *ycf1* gene located on IRB.

**Synonymous (Ks) and non-synonymous (Ka) substitutions rate analysis**

The Ka/Ks ratio has been considered as well-recognized marker for assessing genome evolution and selection pressure affecting genes [36, 37]. The Ka/Ks ratio of the pairwise common 79 protein-coding genes between *H. cordata* cp genome and four related *Aristolochia* species was calculated (S5 Table). All Ka/Ks values of common genes were below 1 in all four comparisons. Of 79 homologous CDs, 76 CDs (96.20%) had a Ka/Ks value below 0.5 in all tested comparisons, and only the *ycf2* gene always had a Ka/Ks value above 0.5. Overall, the average Ka/Ks value of all common genes was 0.149. Additionally, 31 common genes (44.93%) had an average Ka/Ks value below 0.1, suggesting that these genes have undergone strong purifying selection pressures in *H. cordata* cp genome.

**Discussion**

*H. cordata*, known for its use as a vegetable and medicinal for functions, is a member of the Saururaceae family, which is believed to be an ancient and relic family [38]. The Saururaceae, Piperaceae, and Aristolochiaceae families together form the Piperales, which is an early diverging lineage [3, 38]. Because of the lack of a related cp genome, we employed the combination of PacBio and Illumina sequencing platforms, an effective strategy for the assembly of cp genome without a reference [39, 40] to *de novo* assemble the *H. cordata* cp genome. The complete cp genome of *H. cordata* reported here showed a typical quadripartite cycle of 160,226 bp in length, which was consistent with a previous report of cp genome of *H. cordata* [22]. However, unlike the previous study, we found one more protein-coding gene and one less tRNA were obtained in the present study. Another short communication also reported a complete cp genome of *H. cordata* 161,090 bp in length, harboring 81 protein-coding genes [21]. These studies indicate that the cp genome of *H. cordata* has undergone divergence whether it is more or less. Because the two reports could not provide detailed information on gene contents and genome information, the divergent hot spots in cp genome of *H. cordata* could not be detected. We also noticed that the cp genome of *H. cordata* obtained in the present study was comparable with that of published *Aristolochia* (Aristolochiaceae) species (159,308–160,520 bp; S1 Table) [41, 42], *Piper* (Piperaceae) species (159,909–161,721 bp) and *Saruma henryi*
This finding indicates that cp genomes of the Piperales species were conserved in length. However, the cp genome of *H. cordata* was slightly larger than that of *Passiflora edulis* (151,406 bp) at length [43]. Moreover, the gene content between *H. cordata* and *P. edulis* cp genome was much more divergent, where one genes (*trnE-UUC*) were missing in *H. cordata* cp genome, whereas six genes (including *rpl20*, *rpl22*, *rps7*, *rps16*, *infA*, and *accD*), were missing in *P. edulis* cp genome. The variable IR region and boundary construction in SSC/IR and LSC/IR have been considered as the main driving force for the length variation of angiosperm cp genomes [44]. As shown in Fig 6, the size of the IR region and the adjacent genes in the boundaries of *H. cordata* were similar to those of the four selected *Aristolochia* species. Only minor shifts of these adjacent genes occurred within the boundaries.

Repeat sequences are believed to play an important role in genome rearrangements and sequence variations through illegitimate recombination and slipped-strand mispairing [45, 46]. Forty-eight repeat sequences of 30–69 bp were detected in *H. cordata* cp genome. Furthermore, among the reported *Aristolochia* species, 38–138 repeats were identified [41, 42], suggesting that repeat sequences were variable between lineages, which can be used as genomic markers for phylogenetic analysis [42, 47]. Intriguingly, the largely pseudogene, *ycf2* contributed nearly two-thirds (30 of 48) of the repeats. Similar results were observed in the cp genomes of *Haberlea rhodopensis*, *Vernicia fordii* and *Nasturtium officinale* [40, 48, 49], indicating that *ycf2* was the most variable in cp genome. A total of 86 SSR motifs of 17 different types were observed in *H. cordata* cp genome, fewer than those of the reported *Aristolochia* species (ranging from 95 to 156) [41, 42]. Among these SSRs, mononucleotide repeats with the A/T type were the largest in number, similar to the results of previous studies [40–42, 50, 51]. This is most likely due to high proportions of polyadenine (polyA) and polythymine (polyT) in the cp genome. These results suggest that SSRs reshape the cp genome and are powerful tools for identifying the genetic diversity among different species.

**Conclusion**

Herein, the complete *H. cordata* cp genome was *de novo* assembled by integrating the Illumina and PacBio platforms. The *H. cordata* cp genome showed a typical quadripartite cycle of 160,226 bp, which comprised 79 protein-coding genes, 29 tRNA genes, and four rRNA genes. A total of 48 repeat sequences and 86 SSR loci were identified, which could be used for marker development as well as phylogenetic and population studies in *H. cordata*. Moreover, codon usage analysis revealed that the Leu codon ending with A/U was preferentially utilized. The phylogenetic tree of 21 Piperales species, constructed based on homologous protein-coding genes, demonstrated that *H. cordata* had a close relationship with *Aristolochia* species. In summary, this research lays a foundation for future phylogenetic studies on Piperales species and provides useful information for the genetic improvement and breeding of *H. cordata*.

**Supporting information**

S1 Table. List of the cp genome of 16 Piperales species used for phylogenetic analysis. (XLSX)

S2 Table. Summary of de novo sequencing of cp genome of *H. cordata* Thunb. (XLSX)

S3 Table. Detail information of repeat sequence of *H. cordata* cp genome. (XLSX)

S4 Table. Codon usage analysis of protein coding genes of *H. cordata* cp genome. (XLSX)
S5 Table. Synonymous (Ks) and non synonymous (Ks) substitution rate of homologous coding genes between *H. cordata* and other four closed related species. (XLSX)

**Author Contributions**

**Data curation:** Qun Feng.

**Formal analysis:** Qun Feng.

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**References**

1. Burger WC. The Piperales and the monocots. Bot Rev. 1977; 43: 345–393. [https://doi.org/10.1007/BF02860716](https://doi.org/10.1007/BF02860716).

2. Cai Z, Penaffor C, Kuehl JV, Leebens-Mack J, Carlson JE, Claude WD, et al. Complete plastid genome sequences of Drimys, Liriodendron, and Piper: implications for the phylogenetic relationships of magnolids. BMC Evol Biol. 2006; 6: 77. [https://doi.org/10.1186/1471-2148-6-77](https://doi.org/10.1186/1471-2148-6-77).

3. The Angiosperm Phylogeny Group. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Bot J Linn Soc. 2016; 181: 1–20. [https://doi.org/10.1111/boj.12385](https://doi.org/10.1111/boj.12385).

4. Thien LB, Ellgaard EG, Devall MS, Ellgaard SE, Ramp PF. (1994). Population structure and reproductive biology of *Saururus cernuus* L. (Saururaceae). Plant Spec Biol.1994; 9: 47–55. [https://doi.org/10.1111/j.1442-1984.1994.tb00082.x](https://doi.org/10.1111/j.1442-1984.1994.tb00082.x).

5. Xu Z, Deng M. Saururaceae. In Identification and Control of Common Weeds: Volume 2. 2017; 107–114. Springer, Dordrecht. [https://doi.org/10.1007/978-0-94-1157-7_18](https://doi.org/10.1007/978-0-94-1157-7_18).

6. Oginuma K, Sato H, Kono Y, Chen S, Zhou Z, Peng CI, et al. Intraspecific polyploidy of *Houttuynia cordata* and evolution of chromosome number in the Saururaceae. Chromosome Bot. 2007; 2: 87–91. [https://doi.org/10.3199/iscb.2.87](https://doi.org/10.3199/iscb.2.87).

7. Yadav R, Agarwala M. Phytochemical analysis of some medicinal plants. J Phytol.2011; 3: 12. [http://updatepublishing.com/journal/index.php/jp/article/view/2737](http://updatepublishing.com/journal/index.php/jp/article/view/2737).

8. Kim GS, Kim DH, Lim JJ, Lee JJ, Han DY, Lee WM, et al. Biological and antibacterial activities of the natural herb *Houttuynia cordata* water extract against the intracellular bacterial pathogen salmonella within the RAW 264.7 macrophage. Biol Pharm Bull. 2008; 31: 2012–2017. [https://doi.org/10.1248/bpb.31.2012](https://doi.org/10.1248/bpb.31.2012). PMID: 18981565.

9. Chiow KH, Phoon MC, Putti T, Tan BK, Chow VT. Evaluation of antiviral activities of *Houttuynia cordata* Thunb. extract, quercetin, quercetin and cinanserin on murine coronavirus and dengue virus infection. Asian Pac J Trop Med. 2016; 9: 1–7. [https://doi.org/10.1016/j.ajptm.2015.12.002](https://doi.org/10.1016/j.ajptm.2015.12.002). PMID: 26851778.

10. Lau KM, Lee KM, Koon CM, Cheung CS, Lau CP, Ho HM, et al. Immunomodulatory and anti-SARS activities of *Houttuynia cordata*. J Ethnopharmacol. 2008; 118: 79–85. [https://doi.org/10.1016/j.jep.2008.03.018](https://doi.org/10.1016/j.jep.2008.03.018). PMID: 18479853.

11. Guan JZ, Wang JJ, Cheng ZH, Liu Y, Li ZY. Cytomixis and meiotic abnormalities during microsporogenesis are responsible for male sterility and chromosome variations in *Houttuynia cordata*. Genet Mol Res. 2012; 11: 121–30. [http://dx.doi.org/10.4238/2012.January.17.2](http://dx.doi.org/10.4238/2012.January.17.2). PMID: 22290472.
12. Shingnaisui K, Dey T, Manna P, Kalita J. Therapeutic potentials of *Houttuynia cordata* Thunb. against inflammation and oxidative stress: A review. J Ethnopharmacol. 2018; 220: 35–43. https://doi.org/10.1016/j.jep.2018.03.038 PMID: 29605674

13. Lee SG, Kang H. Ameliorative effect of *Houttuynia cordata* Thunb (Saururaceae) leaf extract in loperamide-induced constipation in rats. Trop J Pharm Res. 2019; 18: 1727–1732. http://dx.doi.org/10.4314/ tjpr.v18i8.23.

14. Downie SR, Palmer JD. Use of chloroplast DNA rearrangements in reconstructing plant phylogeny. Molecular systematics of plants. Springer: Boston, MA, 1992. pp. 14–35. https://doi.org/10.1007/978-1-461-35276-7-2.

15. Wicke S, Schneeweiss GM, Depamphilis CW, Müller KF, Quandt D. The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. Plant Mol Biol. 2011; 76: 273–297. https://doi.org/10.1007/s11103-011-9762-4 PMID: 21424877

16. Duchene D, Bromham L. Rates of molecular evolution and diversification in plants: chloroplast substitution rates correlate with species-richness in the Proteaceae. BMC Evol Biol. 2013; 13: 65. https://doi.org/10.1186/1471-2148-13-65 PMID: 23497266

17. Smith DR. Mutation rates in plastid genomes: they are lower than you might think. Genome Biol Evol. 2015; 7: 1227–1234. https://doi.org/10.1093/gbe/evv069. https://doi.org/10.1093/gbe/evv069 PMID: 25869380

18. Huang H, Shi C, Liu Y, Mao SY, Gao LZ. Thirteen *Camellia* chloroplast genome sequences determined by high-throughput sequencing: genome structure and phylogenetic relationships. BMC Evol Biol. 2014; 14: 151. https://doi.org/10.1186/1471-2148-14-151 PMID: 25001059

19. Walker JF, Zanis MJ, Emery NC. Comparative analysis of complete chloroplast genome sequence and inversion variation in Lasthenia burkei (Madieae, Asteraceae). Am J Bot. 2014; 101: 722–729. https://doi.org/10.3732/ajb.1400049 PMID: 24699541

20. Chui S. S., Hwan A. D., Jin K. S., Youngseok L., Tae-Jin O., Eun L. J., et al. Advantages of single-molecule real-time sequencing in high-gc content genomes. Plos One. 2013; 8(7):e88824. https://doi.org/10.1371/journal.pone.0088824 PMID: 23949349

21. Yu F, Liu Y, Zhang RY. The complete chloroplast genome of *Houttuynia cordata* Thunb. (Family: Saururaceae). Mitochondrial DNA Part B. 2019; 4: 4004–4005. https://doi.org/10.1080/23802359.2019.168105.

22. Yang XL. Characterization of the complete chloroplast genome of *Houttuynia cordata* Thunb and phylogenetic relationships. Mitochondrial DNA Part B. 2020; 5: 605–606. https://doi.org/10.1080/23802359.2019.1710604.

23. Okegawa Y, Motohashi K. Chloroplastic thioredoxin m functions as a major regulator of Calvin cycle enzymes during photosynthesis in vivo. The Plant J. 2015; 84: 900–913. https://doi.org/10.1111/tpj.13049 PMID: 26468055

24. Chen Y, Ye W, Zhang Y, Xu Y. High speed BLASTN: an accelerated MegaBLAST search tool. Nucleic Acids Res. 2015; 43: 7762–7768. https://doi.org/10.1093/nar/gkv784. https://doi.org/10.1093/nar/gkv784 PMID: 26250111

25. Koren S, Schatz MC, Walenz BP, Martin J, Howard JT, Ganapathy G, et al. Hybrid error correction and de novo assembly of single-molecule sequencing reads. Nat Biotechn. 2012; 30: 693. https://doi.org/10.1038/nbt.2280.

26. English AC, Richards S, Han Y, Wang M, Vee V, Qu J, et al. Mind the gap: upgrading genomes by high-throughput sequencing with Pacific Biosciences RS long-read sequencing technology. PLoS One. 2012; 7: e47768. https://doi.org/10.1371/journal.pone.0047768 PMID: 23185243

27. Wyman SK, Jansen RK, Boore JL. Automatic annotation of organellar genomes with DOGMA. Bioinformatics. 2004; 20: 3252–3255. https://doi.org/10.1093/bioinformatics/bth352 PMID: 15180927

28. Lohse M, Drehsel O, Bock R. OrganellarGenomeDRAW (OGDRAW): a tool for the easy generation of high-quality custom graphical maps of plastid and mitochondrial genomes. Curr Genet. 2007; 52: 267–274. https://doi.org/10.1007/s00294-007-0161-y PMID: 17957369

29. Kurtz S, Choudhuri JV, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R. REPuter: the manifold applications of repeat analysis on a genomic scale. Nucleic Acids Res. 2001; 29: 4633–4642. https://doi.org/10.1093/nar/29.22.4633 PMID: 11713313

30. Beier S, Thié T, Munch T, Scholz U, Mascher M. MISA-web: a web server for microsatellite prediction. Bioinformatics 2017; 33:2583–2585. https://doi.org/10.1093/bioinformatics/btx198. https://doi.org/10.1093/bioinformatics/btx198 PMID: 28398459
31. Yu CH, Dang Y, Zhou Z, Wu C, Zhao F, Sachs MS, et al. Codon usage influences the local rate of translation elongation to regulate co-translational protein folding. Mol Cell. 2015; 59: 744–754. https://doi.org/10.1016/j.molcel.2015.07.018. PMCID: 4468063.

32. Li H, Guo Q, Li Q, Yang L. Long-reads reveal that Rhododendron delavayi plastid genome contains extensive repeat sequences, and recombination exists among plastid genomes of photosynthetic ericaceae. PeerJ. 2020; 8(238): e9048.

33. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054. PMCID: 27004904.

34. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30: 772–780. https://doi.org/10.1093/molbev/mst010. PMCID: 3239690.

35. Wang D, Zhang Y, Zhang Z, Zhu J, Yu J. KaKs_Calculator 2.0: a toolkit incorporating gamma-series methods and sliding window strategies. Genom Proteom Bioinf. 2010; 8: 77–80. https://doi.org/10.1016/S1672-0229(10)60008-3.

36. Yang ZH, Nielsen R. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. Mol Biol Evol. 2000; 17: 32–43. https://doi.org/10.1093/oxfordjournals.molbev.a026236. PMCID: 1672004.

37. Guo Y, Liu J, Zhang J, Liu S, Du J. Selective modes determine evolutionary rates, gene compactness and expression patterns in Brassica. The Plant J. 2017; 91: 34–44. https://doi.org/10.1111/tpj.13541. PMCID: 28332757.

38. Isnard S, Prosperi J, Wanke S, Wagner ST, Samain MS, Trueba S, et al. Growth form evolution in Piperales and its relevance for understanding angiosperm diversification: an integrative approach combining plant architecture, anatomy, and biometrics. Int J Plant Sci. 2012; 173:610–639. https://doi.org/10.1086/665821.

39. Wu HY, Ji XY, Yu WW, Du YZ. Complete mitochondrial genome of the stonelily Cryptoperia stillifera Svec (Plecoptera: Peltoperlidae) and the phylogeny of Polynoepeptera insects. Gene. 2014; 537: 177–183. https://doi.org/10.1016/j.gene.2013.12.044. PMCID: 2442485.

40. Yan C, Du J, Gao L, Li Y, Hou X. The complete chloroplast genome sequence of watercress (Nasturtium officinale R. Br.): Genome organization, adaptive evolution and phylogenetic relationships in Cardamineae. Gene. 2019; 699: 24–36. https://doi.org/10.1016/j.gene.2019.02.075. PMCID: 30849538.

41. Zhou T, Yang Y, Hu Y, Zhang X, Bai G, Zhao G. Characterization of the complete chloroplast genome sequence of Lepidium meyenii (Brassicaceae). Conservation genetics resources. 2017; 9: 405–408. https://doi.org/10.1007/s10067-017-0695-3.

42. Li X, Zuo Y, Zhu X, Liao S, Ma J. Complete chloroplast genomes and comparative analysis of sequences evolution among seven Aristolochia (Aristolochiaceae) medicinal species. Int J Mol Sci. 2019; 20: 1045. https://doi.org/10.3390/ijms20051045.

43. Cauz-Santos L. A., Munhoz C. F., Nathalie R., Stephane C., Santos A. A., Penha H. A., et al. The chloroplast genome of Passiflora edulis (passifloraceae) assembled from long sequence reads: structural organization and phylogenomic studies in malpighiales. Frontiers in Plant Science. 2017; 8: 334.

44. Kim KJ, Lee HL. Complete chloroplast genome sequences from Korean ginseng (Panax schinseng Nees) and comparative analysis of sequence evolution among 17 vascular plants. DNA Res. 2004; 11: 247–261. https://doi.org/10.1038/dnares11.14.247. PMCID: 15500250.

45. Bauscher MG, Singh ND, Lee SB, Jansen RK, Daniell H. The complete chloroplast genome sequence of Citrus sinensis (L.) Osbeckvár ’Ridge Pineapple’ : organization and phylogenetic relationships to other angiosperms. BMC Plant Biol. 2006; 6: 21. https://doi.org/10.1186/1471-2229-6-21. PMCID: 17010212.

46. Jansen RK, Cai Z, Raubeson LA, Daniell H, Depamphilis CW, Leebens-Mack J, et al. Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. Proc Natl Acad Sci. 2007; 104: 19369–19374. https://doi.org/10.1073/pnas.0709121110. PMCID: 18048930.

47. Nie X, Lv S, Zhang Y, Du X, Wang L, Biradara SS, et al. Complete chloroplast genome sequence of a major invasive species, crofton weed (Ageratina adenophora). PLoS one 2012; 7: e36869. https://doi.org/10.1371/journal.pone.0036869. PMCID: 32606302.

48. Ivanova Z, Sabliok G, Daskalova E, Zahananova G, Apostolova E, Yahubyan G, et al. Chloroplast genome analysis of resurrection tertiary relict Haberlea rhodopensis highlights genes important for desiccation stress response. Front Plant Sci. 2017; 8: 204. https://doi.org/10.3389/fpls.2017.00204. PMCID: 28265281.

49. Li Z, Long H, Zhang L, Liu Z, Cao H, Shi M, et al. The complete chloroplast genome sequence of tung tree (Vernicia fordii); organization and phylogenetic relationships with other angiosperms. Sci Rep. 2017; 7: 1–11. https://doi.org/10.1038/s41598-016-0028-x. PMCID: 28127051.
50. Du X, Zeng T, Feng Q, Hu L, Luo X, Weng Q, et al. The complete chloroplast genome sequence of Yellow Mustard (Sinapis alba L.) and its phylogenetic relationship to other Brassicaceae species. Gene. 2020; 144340. https://doi.org/10.1016/j.gene.2020.144340 PMID: 31923575

51. Gao C, Deng Y, Wang J. The complete chloroplast genomes of Echinacanthus Species (Acanthaceae): phylogenetic relationships, adaptive evolution, and screening of molecular markers. Front Plant Sci. 2018; 9: 1989. https://doi.org/10.3389/fpls.2018.01989 PMID: 30687376