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PLASTOME REPORT

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Characterization of the complete chloroplast genome of *Helicteres hirsuta* Lour. 1790 (Helicteriodeae: Malvaceae)

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ABSTRACT

Helicteres hirsuta Lour. 1790 is a precious medicinal plant species, especially for treating chronic liver diseases. Genomic data on *H. hirsuta* are limited. Therefore, this current study aimed to characterize the chloroplast genome of *H. hirsuta* and reconstruct the phylogenetic relationship among Helicteroideae taxa. Consequently, the complete chloroplast genome of *H. hirsuta* was 163,404 bp in length and contained 113 unique genes (79 protein-coding genes, 30 tRNA genes, and four rRNA genes). Notably, two introns of *clpP* gene of *H. hirsuta* were lost in comparison to that of other Helicteroideae species. The phylogenetic tree based on chloroplast genomes of eleven Helicteroideae species revealed that *H. hirsuta* was closely related to *Reevesia* species. In conclusion, our study described the first complete chloroplast genome of *H. hirsuta*, which is essential for tracing evolutionary history in the Helicteroideae subfamily.

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Introduction

Helicteres Pluk. ex L. 1753, a genus belonging to the Helicteroideae subfamily of Malvaceae, is characterized by stamens and pistils on an androgynophore, united sepals, and oblong fruits with hairs (Turner 1995). In Vietnam, twelve species and one variety of *Helicteres* were reported, in which two novel endemic *Helicteres* species (i.e. *H. binhthuanensis* V.S.Dang 2020 and *H. daknongensis* V.S.Dang & D.T.Bui 2020) have been recently described (Dang et al. 2020; Hoang et al. 2020). *Helicteres hirsuta* Lour. 1790, a species within this genus, has been recognized for its efficacy as a folk medicine in treating cirrhosis and liver cancer (Loureiro 1790; Thang Hoang et al. 2021). Several studies have investigated extracts derived from the leaves, stems, and roots of *H. hirsuta*, which contain phenolic, flavonoid, and saponin compounds. These compounds are recognized for their diverse bioactivities, including antioxidant, antimicrobial, anticancer, antidiabetic, and against malaria (Pham et al. 2017; Quang et al. 2020; Hieu et al. 2021; Nhat et al. 2022). Recent studies have screened the chemical composition of *H. hirsuta*, identified isolated compounds and secondary metabolites, and explored their biological activities (Nguyen et al. 2019; Tra et al. 2019; Le and Hoang 2020; Wang et al. 2021).

Chloroplast (cp) genomes have been used as "super-barcodes" in inference of evolutionary phylogeny of angiosperms due to their distinctive features of highly conserved structure, small size, and a non-homologous recombination (Wu et al. 2021; Zhang et al. 2021). Until now, the absence of cp genomic data for *Helicteres* has limited the research of the evolution and phylogenetic relationships of this genus and related taxa. Therefore, in this study, the sequenced and characterized complete chloroplast genome of *H. hirsuta* was reported. The results of this study will provide a valuable genomic resource that can facilitate future investigations into the identification of species, population genetics, and evolutionary history of *H. hirsuta*.

Materials and methods

Fresh and healthy leaf sample was collected from a single *H. hirsuta* individual from Tay Ninh province, Vietnam (011°20′29.9" N, 106°17′33.0" E) (Figure 1). The specimen of *H. hirsuta* was deposited at NTT Hi-Tech Institute under the voucher number NTTU-20231101-P023 (contact person: Dr. Hoang Dang Khoa Do, email: dhdkhoa@ntt.edu.vn). Fresh leaves were stored in silica gel at room temperature at NTT Hi-Tech Institute, Nguyen Tat Thanh University for further experiments. No permissions are needed during the collection of *H. hirsuta* samples.

Genomic DNA was extracted from dried leaves using the modified CTAB protocol (Porebski et al. 1997). Subsequently, the Genomic Purification Kit was used to purify DNA samples before being used to prepare sequencing libraries using NEBNext Ultra II DNA Library Prep kit (#E7103, New England Biolabs). Approximately 5 Gb of data of paired-end reads

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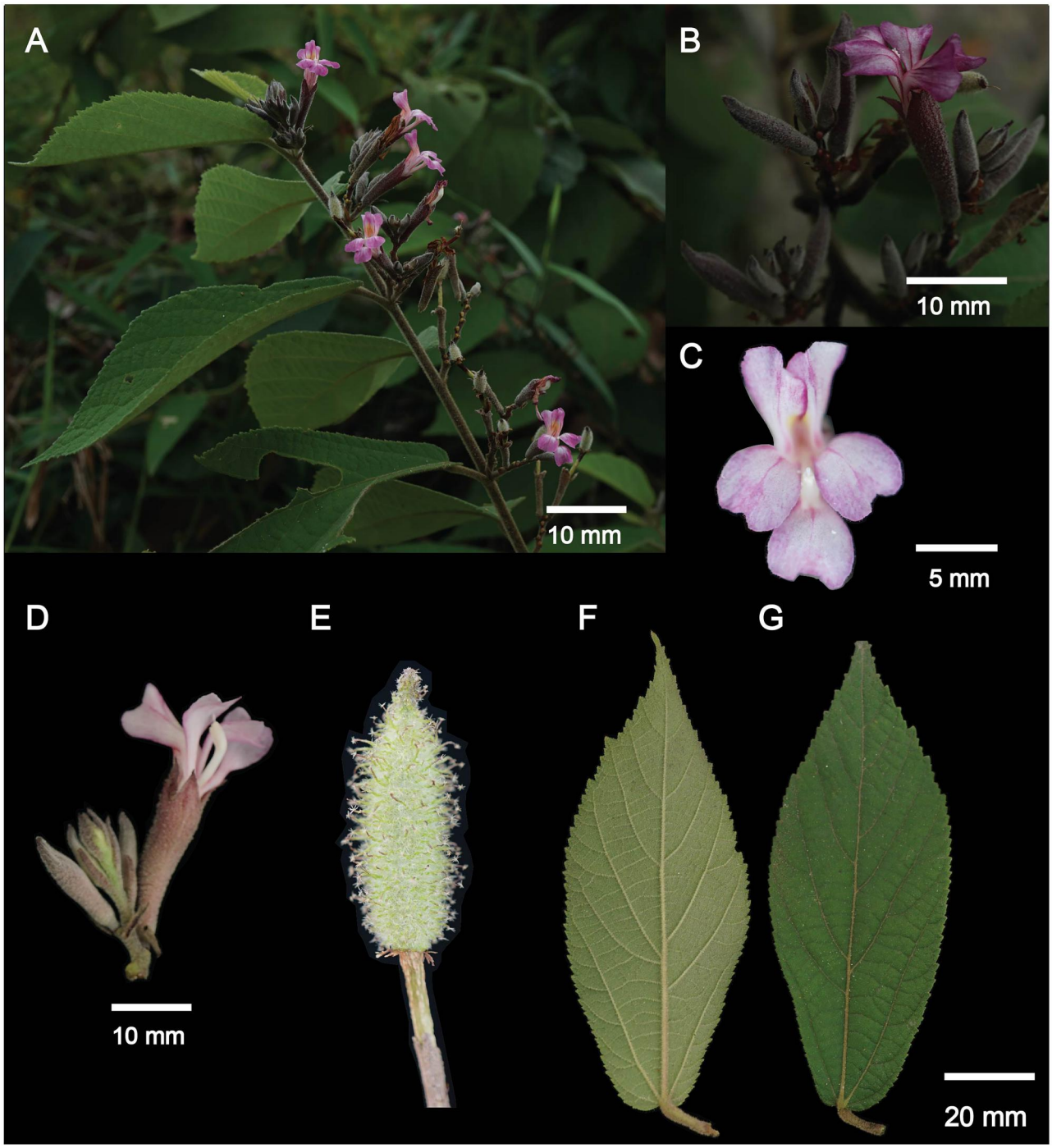

Figure 1. Morphological characters of *Helicteres hirsuta*. (A, B) – inflorescence, axillary or terminal, cymose 2–5 flowers; (C, D) – flower, short pedicel, calyx tubular to campanulate, densely villous to hirsute, calyx lobes 5, unequal, 5 petals, unequal in length, whitish pink or purplish, darker at base of limb, lower 3 petals slightly longer than upper pair; (E) – fruits, ovoid to ellipsoid, with 5 longitudinal lobes, densely villous, apex short-beaked, black when mature; (F) – underside of leaves, and (G) – upperside of leaves, blades narrowly lanceolate to narrowly oblanceolate, abaxially densely yellow brown puberulent. Photograph credits: Nguyen Hoang Danh (A – G).

(150 bp) was generated using the Illumina MiniSeq platform at KTest Science Co Ltd. (HCMC, Vietnam).

GetOrganelle v1.7.7.0 (Jin et al. 2020) and NOVOPlasty v.4.3.1 (Dierckxsens et al. 2016) were used to *de novo* assemble the cp genome of *H. hirsuta*. Both tools used *Reevesia pycnantha* Y.Ling 1951 (NC_059003) as a reference sequence. To calculate coverage depth of cp genome, all raw reads were re-mapped to the assembled chloroplast genome and visualized using Geneious Prime v2023.2.1. The annotation process was conducted using GeSeq tool (Tillich et al. 2017). The gene content was manually checked using Geneious Prime v2023.2.1. The circular cp genome map was illustrated using OGDRAW v.1.3.1 (Greiner et al. 2019).

Fifteen complete cp genomes of Helicteroideae species were downloaded from GenBank database (https://www.ncbi.nlm.nih.gov/), including twelve *Reevesia* taxa and three *Durio* species. One Tilioideae species (*Tilia oliveri* Szyszył. 1890, NC_028590) was used as an outgroup. To reconstruct the

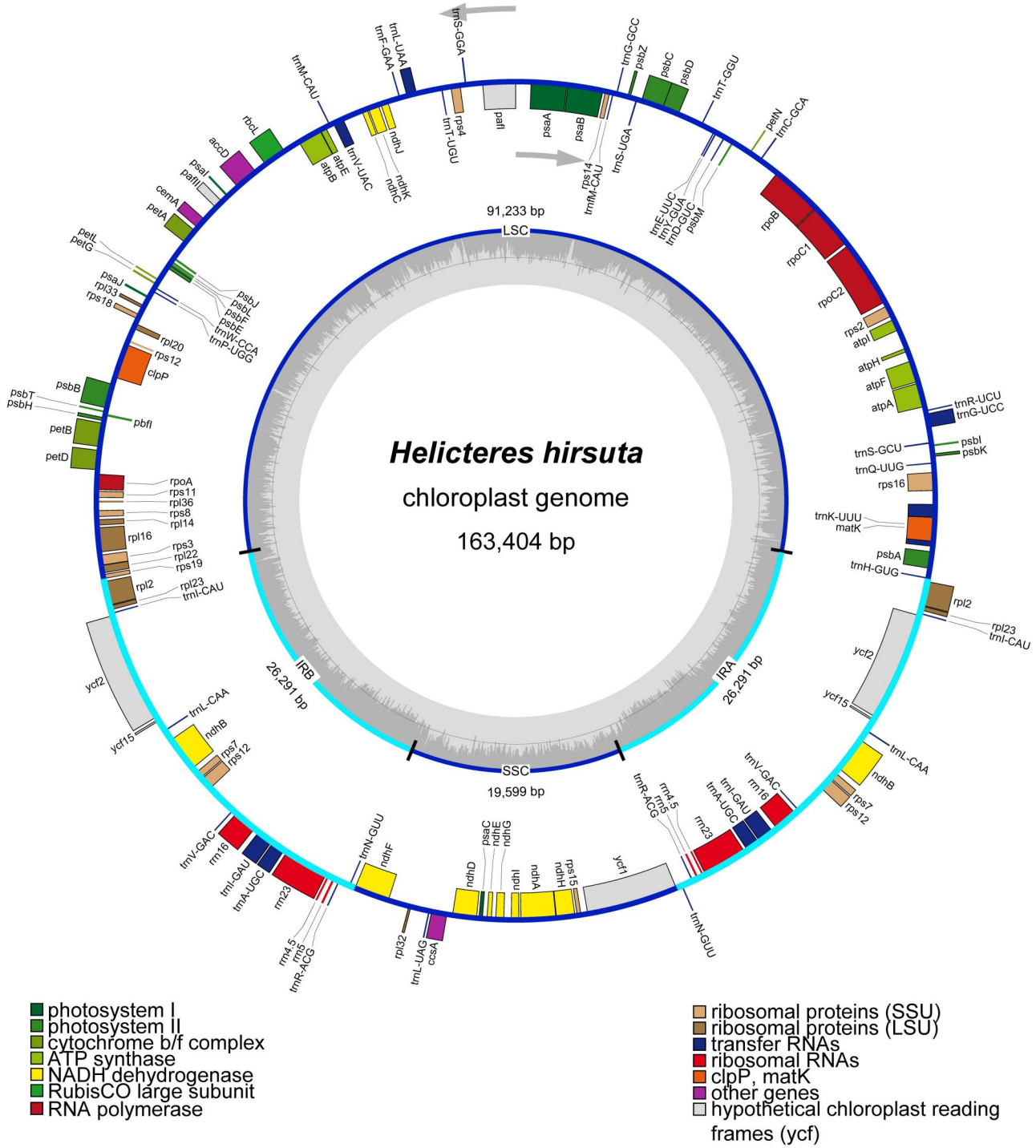

Figure 2. Chloroplast genome map of *Helicteres hirsuta*. In the inside circle, the sky-blue line represents a pair of IR regions, and the dark blue line illustrates LSC and SSC regions. An arrow inside the circle indicates transcriptional direction (clockwise) of genes which are inside the circle. An arrow outside the circle indicates transcriptional direction (counterclockwise) of genes which are outside the circle. The light grey line inside the circle represents the AT content, and the dark grey area illustrates the GC content. At the bottom, the genes of different functional groups are illustrated with various colors.

phylogenetic tree, 79 protein-coding regions were extracted and aligned using Geneious Prime v2023.2.1. Then, jModeltest 2 was employed to estimate the best model of nucleotide substitution. For reconstructing phylogenetic tree, Bayesian inference (BI) tree was performed by MrBayes v.3.2.7a (Huelsenbeck and Ronquist 2001) and maximum likelihood (ML) tree was constructed using IQ-TREE v2.2.2.6 (Nguyen et al. 2015). In ML analysis, IQ-TREE was used with 1,000 bootstrap replicates under the TMV + INVGAMMA model (Posada 2008). The BI analysis was conducted with 1,000,000 generations. The resulting ML and BI trees were modified and visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

The chloroplast genome of *H. hirsuta* was 163,404 bp in length with a 71× depth of coverage (Figure S1). The final assembled and annotated *H. hirsuta* cp genome was

Table 1. List of genes was annotated in the chloroplast genome of *Helicteres hirsuta*.

Functional category	Groups of genes	Name of genes	No. of genes
Self-replication	Ribosomal RNAs	*rrn4.5*[1], *rrn5*[1], *rrn16*[1], *rrn23*[1]	4
	Transfer RNAs	*trnA-UGC*[1,3], *trnC-GCA*, *trnD-GUC*, *trnE-UUC*, *trnF-GAA*, *trnG-UCC*[3], *trnG-GCC*, *trnH-GUG*, *trnI-GAU*[1,3], *trnK-UUU*[3], *trnL-CAA*[1], *trnL-UAA*[3], *trnL-UAG*, *trnfM-CAU*, *trnM-CAU*[1], *trnM-CAU*, *trnN-GUU*[1], *trnP-UGG*, *trnQ-UUG*, *trnR-ACG*[1], *trnR-UCU*, *trnS-GCU*, *trnS-GGA*, *trnS-UGA*, *trnT-GGU*, *trnT-UGU*, *trnV-GAC*[1], *trnV-UAC*[3], *trnW-CCA*, *trnY-GUA*	30
	Large units of ribosome	*rpl2*[1, 3], *rpl14*, *rpl16*[3], *rpl20*, *rpl22*, *rpl23*[1], *rpl32*, *rpl33*, *rpl36*	9
	Small units of ribosome	*rps2*, *rps3*, *rps4*, *rps7*[1], *rps8*, *rps11*, *rps12*[1,4], *rps14*, *rps15*, *rps16*[3], *rps18*, *rps19*[1]	12
	RNA polymerase	*rpoA*, *rpoB*, *rpoC1*[3], *rpoC2*	4
	Translational initiation factor	*infA*[2]	1
Genes for photosynthesis	Subunit of photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*, *pafI*[4], *pafII*	7
	Subunit of photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *pbfI*, *psbM*, *psbT*, *psbZ*	15
	Subunit of cytochrome	*petA*, *petB*[3], *petD*[3], *petG*, *petL*, *petN*	6
	Subunit of ATP synthases	*atpA*, *atpB*, *atpE*, *atpF*[3], *atpH*, *atpI*	6
	Large unit of Rubisco	*rbcL*	1
	Subunit of NADH dehydrogenase	*ndhA*[3], *ndhB*[1,3], *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*	11
Other genes	Maturase	*matK*	1
	Envelope membrane protein	*cemA*	1
	Subunit of acetyl-CoA	*accD*	1
	C-type cytochrome synthesis gene	*cssA*	1
	ATP-dependent protease subunit P	*clpP*	1
	Component of TIC complex	*ycf1*	1
	Hypothetical proteins and conserved reading frames	*ycf2*[1]	1

Note: [1]duplicated gene in IR region; [2]pseudogene; [3]genes containing single intron; [4]genes containing two introns.

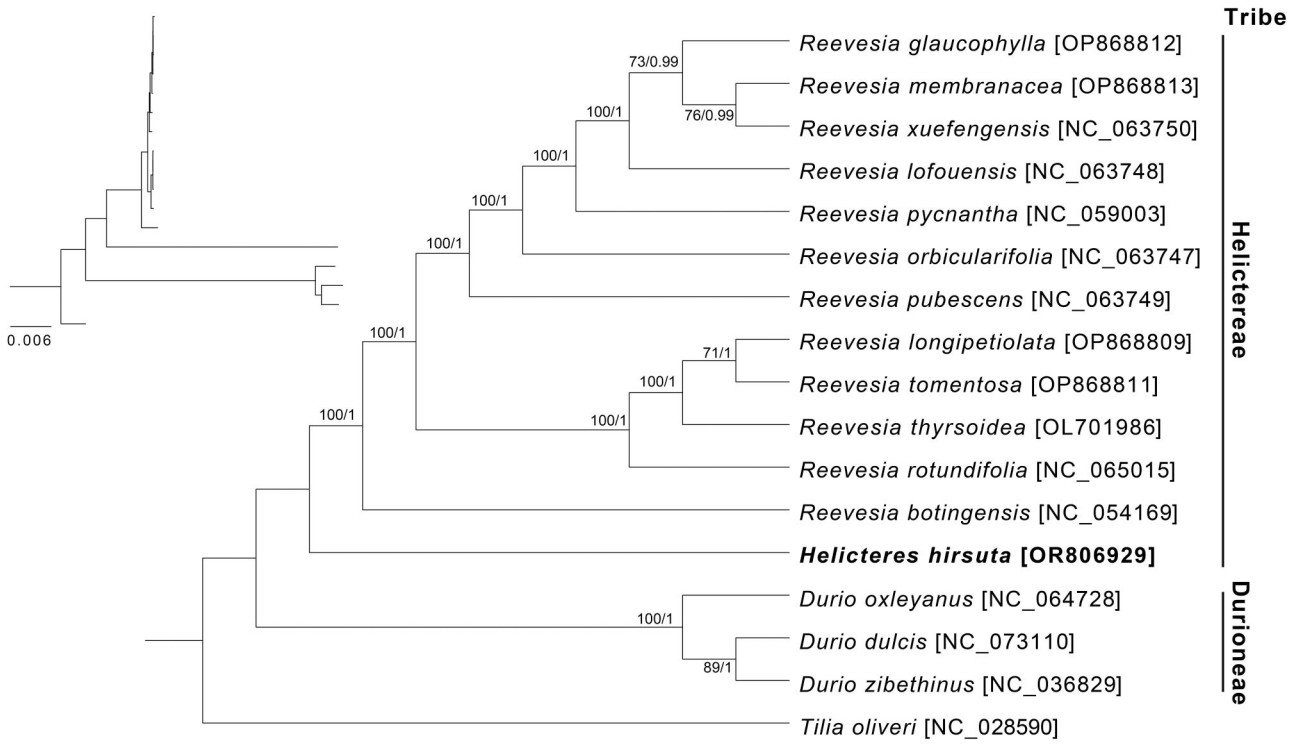

Figure 3. Phylogenetic trees were constructed with 79 protein-coding genes of 16 Helicteroideae species cp genomes using ML and BI methods. Node labels indicate the posterior probability and bootstrap values. The scale bar represents nucleotide substitutions per site. Sequence of *Tilia oliveri* cp genome was used as an outgroup and sequence of *Helicteres hirsuta* cp genome represented by bold colors. The sequences used for tree construction are as follows: *D. oxleyanus* (NC_064728, (Wong et al. 2022)), *D. zibethinus* (NC_036829, (Wang et al. 2021)), *H. hirsuta* (OR806929, this study), *R. botingensis* (NC_054169, (Wang et al. 2021)), *R. lofouensis* (NC_063748, unpublished), *R. orbicularifolia* (NC_063747, unpublished), *R. pubescens* (NC_063749, unpublished), *R. pycnantha* (NC_059003, unpublished), *R. rotundifolia* (NC_065015, unpublished), *R. thyrsoidea* (OL701986, (Wang et al. 2021)), *R. xuefengensis* (NC_063750, unpublished), *T. oliveri* (NC_028590, (Cai et al. 2015)), *Reevesia glaucophylla* (OP868812, Geng et al. 2023), *Reevesia membranacea* (OP868813, Geng et al. 2023), *Reevesia lognipetiolata* (OP868809, Geng et al. 2023), *Reevesia tomentosa* (OP868811, Geng et al. 2023), and *Durio dulcis* (NC_073110, unpublished).

uploaded to GenBank under accession number OR806929. The cp genome exhibited a typical quadripartite structure, comprising a pair of inverted repeat (IR) regions (26,291 bp) separated by a large single-copy (LSC) region (91,223 bp) and a small single-copy (SSC) region (19,599 bp) (Figure 2). The GC content percentages of the LSC, SSC, and IR regions were 34.4%, 31.8%, and 42.3%, respectively. The overall GC content of cp genome was 36.6%.

In *H. hirsuta* cp genome, 113 unique genes were annotated, consisting of 79 protein-coding genes (PCGs), 30 tRNA

genes, and four rRNA genes (Table 1). Of those 113 genes, 18 genes were duplicated, primarily due to their presence in the IR regions, including seven PCGs, seven tRNA genes, and four rRNA genes. Nine PCGs (i.e. *atpF, ndhA, ndhB, petB, petD, rpl2, rpl16, rpoC1,* and *rps16*) comprised single intron, while *pafI* contained two introns (Figure S2). In addition, six tRNA genes (i.e. *trnA-UGC*, *trnG-UCC, trnI-GAU, trnK-UUU, trnL-UAA,* and *trnV-UAC*) contained single intron. The *rps12* gene was a trans-splicing gene (Figure S3). Notably, the *clpP* gene of *H. hirsuta* was not composed of two introns and three exons compared to other available cp genomes of Helicteroideae species.

Based on current sampling size, both ML and BI trees inferred from 79 protein-coding genes exhibited high resolution with strongly supported values (bootstrap $\geq$ 99 and posterior probability $=$ 1) (Figure 3). The monophyly of Helictereae tribe was revealed with high support values. Additionally, *H. hirsuta* was sister to the clade formed by *Reevesia* taxa.

Discussion and conclusion

The cp genome of *H. hirsuta* was successfully sequenced and assembled in this study. The structure and gene content of cp genome was similar to other published cp genomes in the Malvaceae family (Wang et al. 2022; Percy et al. 2023; Tan et al. 2023). Interestingly, we found a loss of two introns of *clpP* gene in cp genome of *H. hirsuta*. Previously, mutations on *clpP* gene were observed and revealed different intron losses and RNA-editing sites (Williams et al. 2019). Additionally, the loss of one or two introns of *clpP* was reported in species of Fabaceae, Oleaceae, Passifloraceae, and Pinaceae (Lee et al. 2007; Dugas et al. 2015; Cauz-Santos et al. 2017; Ni et al. 2017). In Helicterodieae, the loss of two introns of *clpP* was only found in *Helicteres*, but not in the other selected *Reevesia* and *Durio* species used in this study, suggesting a specific alternation in chloroplast genome of this genus. Phylogenetic analysis revealed a close relationship between *Helicteres* and *Reevesia* genera, which was also found in previous studies using *nrITS* and *ndhF* sequences (Alverson et al. 1999; Nyffeler and Baum 2000). However, for elucidating phylogeny among *Helicteres* and related taxa, more samples within Helicterodieae and Malvaceae should be included. In conclusion, this study provided the first complete cp genome in *Helicteres* which can be a reference sequence for further studies on chloroplast genomes. Additionally, the current data on chloroplast genome revealed the relationship between *Helicteres* and related species, which is useful for future phylogenetic studies in the Malvaceae family.

Author contributions

Collected sample, HDKD and HDN; methodology, MTV, HDKD and HDN; experiments, HDN; writing original draft preparation, HDN and HDKD; writing review and editing MTV and HDKD. All the authors have read and agreed to the published version of the manuscript.

Disclosure statement

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

Ethical approval

No ethical approval was required for this study because this study includes no human, animal, or endangered plant samples, and the sampling site was not in the natural reserve. No permissions are needed during the collection of *Helicteres hirsuta* samples.

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Data availability statement

The sequence of *Helicteres hirsuta* chloroplast genome is available in GenBank at https://www.ncbi.nlm.nih.gov/under accession number OR806929. The BioProject, SRA, and Bio-Sample numbers were PRJNA1044269, SRR26928593, and SAMN38378229, respectively.

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