The complete chloroplast genome sequence of *Helwingia himalaica* (Helwingiaceae, Aquifoliales) and a chloroplast phylogenomic analysis of the Campanulidae

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**ABSTRACT**

Complete chloroplast genome sequences have been very useful for understanding phylogenetic relationships in angiosperms at the family level and above, but there are currently large gaps in coverage. We report the chloroplast genome for *Helwingia himalaica*, the first in the distinctive family Helwingiaceae and only the second genus to be sequenced in the order Aquifoliales. We then combine this with 36 published sequences in the large (c. 35,000 species) subclass Campanulidae in order to investigate relationships at the order and family levels. The *Helwingia* genome consists of 158,362 bp containing a pair of inverted repeat (IR) regions of 25,996 bp separated by a large single-copy (LSC) region and a small single-copy (SSC) region which are 87,810 and 18,560 bp, respectively. There are 142 known genes, including 94 protein-coding genes, eight ribosomal RNA genes, and 40 tRNA genes. The topology of the phylogenetic relationships between Apiales, Asterales, and Dipsacales differed between analyses based on complete genome sequences and on 36 shared protein-coding genes, showing that further studies of campanulid phylogeny are needed.

**INTRODUCTION**

Complete chloroplast genome sequences (plastomes) have been very useful for understanding phylogenetic relationships in angiosperms at the family level and above, and have been used to resolve previously recalcitrant nodes (Barrett et al., 2016). However, there are currently large gaps in the coverage of orders and families. Within the ‘very large, very old, and very widespread’ subclass Campanulidae (Beaulieu, O’Meara & Donoghue, 2013; also known as ‘Asterids II’), complete chloroplast genome sequences are currently...
available only for 74 species (out of c. 35,000), in six families (out of 29) and four orders (out of seven). Within the campanulid order Aquifoliales, plastome sequences are currently known only from the large, monogeneric family Aquifoliaceae (Yao et al., 2016).

*Helwingia* is the only genus in the campanulid family Helwingiaceae. It includes four species distributed in eastern Asia, from the Himalayas to Japan: *Helwingia chinensis* Batalin, *H. himalaica* Hook. f. & Thomson ex C.B. Clarke, *Helwingia japonica* (Thunb.) F. Dietr., and *Helwingia omeiensis* (W.P. Fang) H. Hara & S. Kurosawa (The Plant List, 2013; Wu, Raven & Hong, 2005). On current evidence, Helwingiaceae is sister to the Neotropical monogeneric family Phyllonomaceae (The Angiosperm Phylogeny Group, 2016), with which it shares an inferior ovary, epiphyllous inflorescence, and epigynous disc nectary (Ao & Tobe, 2015). These two small, highly disjunct, families are in turn sister to the near-cosmopolitan, but also monogeneric, family Aquifoliaceae.

Chloroplasts originated from free-living cyanobacteria via endosymbiosis and contain their own genome, which is circular and 76–217 kb in length (Hinsinger & Strijk, 2015; Zhang & Gao, 2016). Because of its abundance in plant cells and ease of sequencing, chloroplast DNA (cpDNA) has been widely utilized in studies of plant taxonomy and evolution (Kress et al., 2005; Kress & Erickson, 2007; Newmaster, Fazeas & Ragupathy, 2006; Chase et al., 2007; Taberlet et al., 2007). The small size, single unit, haploid nature, and highly conserved genomic structure of cpDNA also make it useful for species identifications (Yang et al., 2013). Moreover, the many copies per cell mean that usable fragments of the chloroplast genome are more likely to survive in dried herbarium specimens than are nuclear sequences, making direct comparisons with the genome of the type specimen potentially possible (Xu et al., 2015).

The Helwingiaceae's current position in the order Aquifoliales, subclass Campanulidae (The Angiosperm Phylogeny Group, 2016), came after previous placements in the Cornaceae (Cronquist, 1981; Cronquist, 1988) and Araliaceae (Hutchinson, 1964; Hutchinson, 1973), and was based on molecular phylogenetic studies using *rbcL* (Morgan & Soltis, 1993), 18S rDNA and *rbcL* (Soltis & Soltis, 1997), and *ndhF* (Olmstead et al., 2000). Sequencing the chloroplast genome will facilitate the development of additional chloroplast markers for identification and phylogenetic studies within the family, as well as providing a basis for future studies on the phylogenetics and biogeography of the order Aquifoliales. Beaulieu, Tank & Donoghue (2013) suggest that the initial divergence within this order took place in Australasia in the Cretaceous, with an early expansion into South America and Asia where *Phyllonoma* and *Helwingia*, respectively, persist today, while *Ilex* has spread more widely. In the absence of a fossil record for the two small families, a higher resolution phylogeny is needed to assess this hypothesis. The wider phylogenetic relationships among campanulid orders have been investigated in several studies, using chloroplast markers only (Beaulieu, Tank & Donoghue, 2013; Wikström et al., 2015) or combined with nuclear ribosomal genes (ITS, 18S or 26S) (Tank & Donoghue, 2010; Beaulieu, O'Meara & Donoghue, 2013; Magallón et al., 2015), but not yet with complete chloroplast genomes.
Here, we first explore the structure of the chloroplast genome in the Helwingiaceae using *H. himalaica*. We then investigate the phylogenetic relationships in the Campanulidae by using the complete genome sequences and the protein-coding genes shared between *H. himalaica* and other published genomes.

**MATERIALS AND METHODS**

*Helwingia himalaica* is distributed from Nepal through northern India to southwestern China. Plants materials used in this study were intact, fresh, young leaves collected in Bingzhongluo county of Yunnan province (28.015306°N, 98.607944°E). The specimen has been deposited in the herbarium of the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (HITBC). Total genomic DNA was extracted from fresh leaves using a modified CTAB method (*Doyle, 1987; Yang, Li & Li, 2014*). Each amplification was performed in 25 μL of a reaction mixture containing 1×PrimeSTAR GXL buffer (10 mM Tris-HCl (pH 8.2), 1 mM MgCl2, 20 mM NaCl, 0.02 mM EDTA, 0.02 mM DTT; 0.02% Tween 20, 0.02% Nonidet P-40, and 10% glycerol); 1.6 mM of dNTPs, 0.5 μM of each primer; 1.25 U of Prime-STAR GXL DNA polymerase (TAKARA BIO INC., Dalian, China), and 30–100 ng of DNA template. The amplification was conducted using 94 °C for 1 min, 30 cycles of 98 °C for 10 s and 68 °C for 15 min, followed by a final extension step at 72 °C for 10 min. The purified Polymerase chain reaction (PCR) product was fragmented and used for constructing the short-insert (500 bp) libraries according to the manufacturer’s manual (Illumina). DNA of each sample was then indexed by tags and pooled together in one lane in an Illumina Hiseq 2000 to sequence (*Yang, Li & Li, 2014*).

Raw reads were filtered by quality control software NGSQCToolkit v2.3.3 (*Patel & Jain, 2012*) to obtain high quality Illumina data (cut-off value for percentage of read length = 80, cut-off value for PHRED quality score = 30) and vector- and adaptor-free reads. Filtered reads were assembled into contigs in CLC Genomics Workbench v.8 (http://www.clcbio.com) by the de novo method using a k-mer of 63 and a minimum contig length of 1 kb. Outputted contigs were aligned with the chloroplast genome of the asterid *Camellia yunnanensis* (GenBank accession number: KF156838), which was the most similar genome identified via BLAST (http://blast.ncbi.nlm.nih.gov/), and ordered according to the reference genome. Genes in the assembled chloroplast genome were predicted using Dual Organellar GenoMe Annotator (DOGMA) (*Wyman, Jansen & Boore, 2004*). The chloroplast genome was assembled using aligned contigs in Geneious v. 8.1.7 (http://www.geneious.com, *Kearse et al., 2012*). Junctions between large single-copy (LSC)/inverted repeats (IRs) and small single-copy (SSC)/inverted repeats (IRs) were validated by Sanger sequencing of PCR-based products (Table S1).

The assembled genome was annotated using the DOGMA database (*Wyman, Jansen & Boore, 2004*), then manually edited for start and stop codons. Genome maps were drawn in OGDraw v.1.2 (*Lohse et al., 2013*). The annotated chloroplast genome has been submitted to GenBank (accession number: KX434807). REPuter was used to detect and assess repeats, including forward match, reverse match, complement match, and palindromic match repeats (*Kurtz et al., 2001*). Phobos v3.3.12 was used to detect simple
sequence repeats (SSRs) under default parameters (Mayer, Christoph, Phobos 3.3.11, 2006–2010; http://www.rub.de/spezzoo/cm/cm_phobos.htm). Mauve v. 2.4.0 was used for determining the chloroplast genome rearrangements among the campanulid families (Darling et al., 2004).

A matrix of chloroplast genome sequences, including *H. himalaica*, 36 other campanulid species, and *Coffea arabica* as an outgroup (EF044213 in GenBank) (Table 1), was aligned using MAFFT (Katoh & Standley, 2013) and manually edited where necessary. These 37 campanulid species represent all families and major clades within the Campanulidae that had complete chloroplast genome sequences in GenBank. Unambiguously aligned DNA sequences were used for phylogeny construction. Phylogenies were constructed by maximum likelihood (ML), Bayesian Inference analyses (BI), and maximum parsimony (MP).

ML analyses were conducted in RAxML version 8.2.8 (Stamatakis, 2014), using the GTACAT approximation. Convergence of the bootstrap was tested in RAxML using a posteriori bootstrapping analysis. BI analysis was conducted using MrBayes version 3.2.6 (Ronquist et al., 2012) and the best substitution model (‘TVM+G’) tested by AIC in jModelTest version 2.1.10 (Darriba et al., 2012). Four independent Markov Chain Monte Carlo algorithms were calculated for 10,000,000 generations and sampled every 1,000 generations. Potential Scale Reduction Factor (PSRF) values were used to determine convergence in BI using MrBayes version 3.2.6. All PSRF values were 1, indicating that these analyses converged. The first 25% of calculated trees was discarded as burn-in and a consensus tree constructed using the remaining trees. MP analysis was conducted in PAUP version 4.0a150 (http://people.sc.fsu.edu/~dswofford/paup_test/), using the heuristic searches with tree bisection-reconnection (TBR) branch swapping and the ‘Multrees’ option in effect. Bootstrap analysis was conducted with 1,000 replicates with TBR branch swapping.

In addition, 36 protein-coding genes (Table 1) shared across all the 37 campanulid species were selected to build the phylogeny. ML analyses were conducted in RAxML version 8.2.8 (Stamatakis, 2014), using the GTACAT approximation. Convergence of the bootstrap was tested in RAxML using a posteriori bootstrapping analysis. BI analysis was conducted using MrBayes version 3.2.6 (Ronquist et al., 2012) and the best substitution model (‘GTR+I+G’) tested by AIC in jModelTest version 2.1.10 (Darriba et al., 2012). Methods for phylogeny construction using the 36 protein-coding genes follow the description above. PSRF values were used to determine convergence in BI using MrBayes version 3.2.6. All PSRF values were 1, indicating that these analyses converged. The first 25% of calculated trees was discarded as burn-in and a consensus tree constructed using the remaining trees. MP analysis was conducted in PAUP version 4.0a150 (http://people.sc.fsu.edu/~dswofford/paup_test/), using the heuristic searches with TBR branch swapping and the ‘Multrees’ option in effect. Bootstrap analysis was conducted with 1,000 replicates with TBR branch swapping.
| Species                   | Accession number | Family     | Order     | Length (bp) | Coding gene | tRNA | rRNA | GC (%) | LSC (bp) | SSC (bp) | IRs (bp) |
|--------------------------|------------------|------------|-----------|-------------|-------------|------|------|--------|----------|----------|----------|
| Angelica acutiloba       | KT963036         | Apiaceae   | Apiales   | 147,074     | 85          | 35   | 8    | 37.5   | 93,367   | 17,573   | 36,134   |
| Anthriscus cerefolium    | GU456628         | Apiaceae   | Apiales   | 154,719     | 85          | 37   | 8    | 37.4   | 84,768   | 17,551   | 52,400   |
| Bupleurum falcatum       | KM207676         | Apiaceae   | Apiales   | 155,989     | 84          | 37   | 8    | 37.7   | 85,870   | 17,518   | 52,601   |
| Crithmum maritimum       | HM596072         | Apiaceae   | Apiales   | 158,355     | 88          | 37   | 8    | 37.5   | 85,230   | 27,993   | 55,986   |
| Daucus carota            | DQ898156         | Apiaceae   | Apiales   | 155,911     | 85          | 43   | 8    | 37.7   | 84,244   | 17,571   | 54,096   |
| Foeniculum vulgare       | KR011054         | Apiaceae   | Apiales   | 153,628     | 85          | 37   | 8    | 37.6   | 86,659   | 17,470   | 49,499   |
| Ligusticum tenuissimum   | KT963039         | Apiaceae   | Apiales   | 158,500     | 88          | 37   | 8    | 37.6   | 84,875   | 17,661   | 55,964   |
| Osstericum grosseserratum| KT852844         | Apiaceae   | Apiales   | 147,282     | 83          | 36   | 8    | 37.5   | 93,185   | 17,663   | 36,434   |
| Petroselinum crispum     | HM596073         | Apiaceae   | Apiales   | 152,890     | 84          | 37   | 8    | 37.8   | 86,116   | 17,508   | 49,266   |
| Tiedemannia filiformis   | HM596071         | Araliaceae | Apiales   | 154,737     | 85          | 37   | 8    | 37.3   | 84,585   | 17,140   | 53,012   |
| Dendropanax dentiger     | KP271241         | Araliaceae | Apiales   | 156,687     | 87          | 37   | 8    | 38.0   | 86,680   | 18,247   | 51,760   |
| Hydrocotyle verticillata | HM596070         | Araliaceae | Apiales   | 153,207     | 85          | 37   | 8    | 37.6   | 84,352   | 18,739   | 50,116   |
| Kalopanax septemlobus    | KC456167         | Araliaceae | Apiales   | 156,413     | 87          | 37   | 8    | 37.9   | 86,467   | 18,118   | 51,828   |
| Panax ginseng            | AY582139         | Araliaceae | Apiales   | 156,318     | 87          | 37   | 8    | 38.1   | 86,114   | 18,070   | 52,134   |
| Ilex delavayi            | KX426470         | Aquifoliace | Aquifoliales | 157,671   | 95          | 40   | 8    | 37.6   | 87,000   | 18,436   | 52,234   |
| Ilex litholia            | KX426465         | Aquifoliace | Aquifoliales | 157,610   | 95          | 40   | 8    | 37.6   | 86,952   | 18,429   | 52,228   |
| Ilex new species         | KX426469         | Aquifoliace | Aquifoliales | 157,611   | 95          | 40   | 8    | 37.6   | 86,948   | 18,434   | 52,227   |
| Ilex polyneura           | KX426468         | Aquifoliace | Aquifoliales | 157,621   | 95          | 40   | 8    | 37.6   | 87,064   | 18,435   | 52,122   |

(Continued)
| Species                  | Accession number | Family            | Order    | Length (bp) | Coding gene | tRNA | rRNA  | GC (%) | LSC (bp)          | SSC (bp) | IRs (bp)          |
|--------------------------|------------------|-------------------|----------|-------------|-------------|------|-------|--------|------------------|----------|------------------|
| Ilex pubescens           | KX426467         | Aquifoliaceae     | Aquifoliales | 157,741     | 95          | 40   | 8     | 37.6   | 87,109 (55.22)   | 18,436   | 52,238 (33.12)  |
| Ilex szechwanensis       | KX426466         | Aquifoliaceae     | Aquifoliales | 157,822     | 95          | 40   | 8     | 37.7   | 87,204 (55.25)   | 18,513   | 52,182 (33.06)  |
| Ilex wilsonii            | KX426471         | Aquifoliaceae     | Aquifoliales | 157,918     | 95          | 40   | 8     | 37.6   | 87,266 (55.26)   | 18,432   | 52,222 (33.07)  |
| Helwingia himalaica      | KX434807         | Helwingiaceae     | Aquifoliales | 158,362     | 94          | 40   | 8     | 37.7   | 87,810 (55.45)   | 18,560   | 51,991 (32.83)  |
| Artemisia frigida        | JX293720         | Asteraceae        | Asterales | 151,076     | 87          | 37   | 8     | 37.5   | 82,740 (54.77)   | 18,392   | 49,944 (33.06)  |
| Aster spathulifolius     | KF279514         | Asteraceae        | Asterales | 149,510     | 87          | 37   | 8     | 37.7   | 81,961 (54.82)   | 17,972   | 49,577 (33.16)  |
| Centaurea diffusa       | KJ690264         | Asteraceae        | Asterales | 152,559     | 90          | 36   | 8     | 37.7   | 83,596 (54.80)   | 18,487   | 50,476 (33.09)  |
| Chrysanthemum indicum    | JN867592         | Asteraceae        | Asterales | 151,129     | 85          | 35   | 8     | 37.4   | 82,885 (54.84)   | 18,376   | 49,868 (33.00)  |
| Cynara coronigera        | KP842707         | Asteraceae        | Asterales | 152,550     | 87          | 37   | 8     | 37.7   | 83,580 (54.79)   | 18,660   | 50,310 (32.98)  |
| Lactuca sativa           | DQ383816         | Asteraceae        | Asterales | 152,772     | 86          | 44   | 8     | 37.5   | 84,105 (55.05)   | 18,599   | 50,068 (32.77)  |
| Lasthenia burkei         | KM360047         | Asteraceae        | Asterales | 150,944     | 67          | 25   | 7     | 37.4   | 82,193 (54.45)   | 18,271   | 50,480 (33.44)  |
| Parthenium argentatum    | GU120098         | Asteraceae        | Asterales | 152,803     | 57          | 17   | 8     | 37.6   | 84,593 (55.36)   | 18,900   | 49,310 (32.27)  |
| Praxelis clematidea      | KP922320         | Asteraceae        | Asterales | 151,410     | 84          | 32   | 8     | 37.2   | 85,311 (56.34)   | 18,559   | 47,540 (31.40)  |
| Adenophora remotiflora  | KP889213         | Campanulaceae     | Asterales | 171,724     | 82          | 37   | 8     | 38.8   | 105,555 (61.47)  | 11,295   | 54,874 (31.95)  |
| Campsinula takesimana    | KP006497         | Campanulaceae     | Asterales | 169,551     | 83          | 36   | 8     | 38.8   | 102,320 (60.35)  | 7,747    | 59,484 (35.08)  |
| Hanabuaya asiatica      | KJ477692         | Campanulaceae     | Asterales | 167,287     | 82          | 37   | 10    | 38.8   | 104,955 (62.74)  | 8,578    | 53,754 (32.13)  |
| Trachelium caeruleum    | EU090187         | Campanulaceae     | Asterales | 162,321     | 83          | 44   | 10    | 38.3   | 100,110 (61.67)  | 7,661    | 54,550 (33.61)  |
| Kolkrwiza amabilis       | KT966716         | Caprifoliaceae    | Dipsacales | 156,875     | 81          | 38   | 8     | 38.4   | 90,137 (57.46)   | 18,846   | 47,892 (30.53)  |
| Lonicera japonica       | KJ170923         | Caprifoliaceae    | Dipsacales | 155,078     | 81          | 39   | 8     | 38.6   | 88,858 (57.30)   | 18,672   | 47,548 (30.66)  |
| Coffea arabica          | EF044213         | Rubiaceae         | Gentianales | 155,189     | 85          | 45   | 8     | 37.4   | 85,164 (54.88)   | 18,207   | 51,818 (33.39)  |

**Note:**
Numbers in parentheses in the LSC, SSC and IRs columns are the percentage of the total length.
RESULTS

Genome features

The total length of the chloroplast genome is 158,362 bp. Its quadripartite structure includes an LSC with 87,810 bp and SSC with 18,560 bp, separated by a pair of IR
regions with lengths of 25,996 bp (Fig. 1). The GC content is 37.7% (see Table 1 in Yao et al., 2016). A total of 102 unique genes were detected in the chloroplast genome, of which 20 were duplicated in IR regions. Totally, 94 protein-coding genes (76 unique) encode proteins acting in processes related to photosynthesis, the genetic system, and some currently unknown functions (e.g. ycf). In addition, 40 genes (26 unique) encode for tRNAs and eight genes for rRNAs (Table 2). All eight rRNA genes are in IR regions. One ycfI gene is a functional pseudogene as it is on the border between the SSC and IRa region. Gene rps19 is outside the IRb region at the LSC-IRb junction and rpl2 is fully included in the IRa region. Five genes (atpF, rpoC1, rpl2, ndhB and ndhA) have one intron and two genes have two introns (ycf3, clpP and rps12).

Repeated sequences and SSR
Thirty repeated sequences were detected, with lengths ranging from 18 to 43 bp and sequence identity more than 90% (Table 3). Among them, 19 repeated sequences were

| Table 2 List of genes in the chloroplast genome of Helwingia himalaica. |
| Category | Groups of gene | Name of genes |
|----------|----------------|---------------|
| Protein synthesis and DNA-replication | Transfer RNAs | trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnP-M-CAU, trnR-GGC, trnG-UCC, trnH-GUG, trnK-UUU, trnL-UAC, trnM-CAU, trnQ-UUG, trnP-GGG, trnP-UGG, trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-UGC, trnV-UAC, trnW-CCA, trnY-GUA, trnA-UGC(×2), trnL-CAU(×2), trnF-GAU(×2), trnL-CAA(×2), trnL-UGA, trnN-GUU(×2), trnR-ACG(×2), trnV-GAC(×2) |
| Ribosomal RNAs | rrs16, rps2, rps14, rps4, rps12(×2), rps11, rps8, rps3, rps19, rps7(×2), rps15 |
| Ribosomal protein small subunit | rpl33, rpl20, rpl36, rpl14, rpl16, rpl22, rpl2(×2), rpl23(×2), rpl32 |
| Ribosomal protein large subunit | rpoA, rpoB, rpoCl, rpoC2 |
| Subunits of RNA polymerase | NADH-dehydrogenase | ndhA, ndhB(×2), ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK |
| Photosynthesis | photosystem I | psaA, psaB, psaC, psaI, psaL |
| Photosystem II | psbA, psbB, psbC, psbD, psbE, psbF, psbG, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, lhbA |
| Cytochrome b/f complex | petA, petB, petD, petG, petL, petN |
| ATP synthase | atpA, atpB, atpE, atpF, atpH, atpI |
| NADH-dehydrogenase | ndhA, ndhB(×2), ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK |
| Large subunit rubisco | rbcL |
| Miscellaneous group | Translation initiation factor | infA |
| | Acetyl-CoA carboxylase | accD |
| | Cytochrome c biogenesis | ccsA |
| | Maturase | matK |
| | ATP-dependent protease | clpP |
| | Inner membrane protein | ccmA |
| Pseudogene unknown function | Conserved hypothetical chloroplast ORF | ycf3, ycf4, ycf2(×2), ycf15(×2), orf42(×2), orf56(×2), ycf1(×2), orf188 |
dispersed in intergenic regions, 10 in genes, and one in introns. There were 16 forward repeats, nine palindromic repeats, three reverse repeats, and two complement repeats, and 21, 2 and 7 repeats were detected in the LSC, SSC and IRs, respectively. A total of 813 SSRs were found, including 289 mononucleotides, 35 dinucleotides and 70 trinucleotides (Fig. S1). In mononucleotide SSRs, thymine and adenine made up 92% (266). In dinucleotide SSRs, we found repeated units consisting of TA/AT and GA/AG, but no GC/CG and TC/CT repeats.

### Table 3 List of repeated sequences in the chloroplast genome of *Helwingia himalaica.*

| Repeat length (bp) | Repeat A start site | Repeat A location* | Repeat A region | Repeat B start site | Repeat B location | Repeat B region | Repeat type** |
|-------------------|---------------------|--------------------|-----------------|---------------------|--------------------|-----------------|---------------|
| 43                | 0 rpl2(trnH-GUG)    | IRA                | 87797           | rps19(rpl2)         | IRb                | P               |
| 30                | 9030 trnS-GCU       | LSC                | 47728           | trnS-GGA            | LSC                | P               |
| 27                | 45989 ycf3 intron2  | LSC                | 124368          | ndfA intron         | SSC                | F               |
| 26                | 43 rpl2(trnH-GUG)   | LSC                | 87772           | rps19(rpl2)         | IRb                | P               |
| 26                | 10811 trnG-GCC(trnR-UCU) | LSC | 10840 | trnG-GCC(trnR-UCU) | LSC | P |
| 26                | 33886 trnT-GGU(psbD) | LSC | 33912 | trnM-CAU(psbD) | LSC | P |
| 26                | 91380 ycf2          | IRb                | 154796          | ycf2                | IRa                | F               |
| 23                | 61775 accD          | LSC                | 61786           | accD                | LSC                | F               |
| 21                | 9036 trnS-GCU       | LSC                | 37766           | trnS-UGA            | LSC                | F               |
| 21                | 37766 trnS-UGA      | LSC                | 47731           | trnS-GGA            | LSC                | P               |
| 21                | 38950 trnM-CAU      | LSC                | 69860           | trnP-UGG            | LSC                | F               |
| 20                | 38564 lhaA(trnG-UCC) | LSC | 38581 | lhaA(trnG-UCC) | LSC | F |
| 20                | 49313 trnT-UGU(trnL-UAA) | LSC | 49333 | trnT-UGU(trnL-UAA) | LSC | F |
| 19                | 385 trnH-GUG(psbA)  | LSC                | 412             | trnH-GUG(psbA)      | LSC                | P               |
| 19                | 6791 rps16(trnQ-UUG) | LSC | 6817 | rps16(trnQ-UUG) | LSC | F |
| 19                | 8756 psbI           | LSC                | 38919           | trnG-UGCC(trnM-CAU) | LSC | P |
| 19                | 10620 trnG-GCC      | LSC                | 38738           | trnG-UGCC           | LSC                | F               |
| 19                | 15636 atpH(atp1)    | LSC                | 15653           | atpH(atp1)          | LSC                | F               |
| 19                | 34034 trnT-GGU(psbD) | LSC | 111456 | arf56(trnR-ACG) | IRb | R |
| 19                | 34034 trnT-GGU(psbD) | LSC | 134727 | trnR-ACG(trnA-UGC) | IRa | C |
| 19                | 53790 ndhC(trnV-UAC) | LSC | 81476 | rpoA | LSC | P |
| 19                | 59571 rbcL          | LSC                | 59590           | rbcL(accD)          | LSC                | F               |
| 18                | 4719 trnK-UUU       | LSC                | 66845           | petA(psbf)          | LSC                | R               |
| 18                | 5785 rps16(trnQ-UUG) | LSC | 34036 | trnT-GGU(psbD) | LSC | R |
| 18                | 6349 rps16(trnQ-UUG) | LSC | 90529 | ycf2 | IRb | F |
| 18                | 6349 rps16(trnQ-UUG) | LSC | 155655 | ycf2 | IRa | P |
| 18                | 9101 trnS-GCU       | LSC                | 37836           | trnS-UGA            | LSC                | F               |
| 18                | 40424 psaB          | LSC                | 42639           | psaA                | LSC                | F               |
| 18                | 40973 psaB          | LSC                | 43197           | psaA                | LSC                | F               |
| 18                | 57793 atpB(rbcL)    | LSC                | 121171          | ndhD(psaC)          | SSC                | C               |

**Notes:**
* rpl2(trnH-GUG) means spacer between rpl2 and trnH-GUG, etc.
** P means palindromic match, F means forward (direct) match, R means reverse match, and C means complement match.
Genome rearrangement in the Campanulidae

Genome alignment among seven species from the seven campanulid families with known chloroplast genomes revealed massive gene rearrangement, especially in the LSC (Fig. 2). Moreover, all four Campanulaceae species had longer genomes and LSCs, and shorter SSCs, compared with other campanulid species (Table 1). The IR in the chloroplast genome of some Apiaceae (Angelica acutiloba, Foeniculum vulgare, Ostericum grosseserratum and Petroselinum crispum) was contractive (Table 1; Fig. 2). Even though the lowest number of coding genes in any campanulid species was 57 (Parthenium argentatum), only 36 coding genes were shared across all the campanulid families (Table 4), Kumar et al. (2009) which indicates many gene losses or gains had occurred. The number of tRNA ranged from 17 (P. argentatum) to 44 (Lactuca sativa and Trachelium caeruleum), while the number of rRNA was usually eight (Table 1).

Figure 2 Gene arrangement map of chloroplast genome alignment of seven representative species from seven campanulid families and Coffea arabica (as a reference) determined by Mauve software (Darling et al., 2004). The polyline in the blocks indicates sequence similarity among these eight species. Line linking gene blocks among the eight species with same colour indicates ortholog. Gene blocks above are transcribed clockwise and those below are transcribed counterclockwise. The coding genes in the 14 main gene blocks are listed under the figure.
Phylogenetic analyses of the Campanulidae

The phylogeny produced from the analysis of 37 complete chloroplast genomes is well-supported, but while the results from ML and BI are congruent, the phylogeny from MP is not (Figs. 3A and 3B). With ML and BI, Aquifoliales are basal, Asterales are the next branch, and the Dipsacales are sister to the Apiales. The six families with multiple species are all well-supported (Fig. 3A). In the MP phylogeny, however, the Dipsacales are sister to the Asterales, and the Apiales are the next branch (Fig. 3B).

The phylogeny based on 36 shared protein-coding genes has a consistent family-level topology in analyses with BI, ML, and MP. The Aquifoliales are still basal, but the Dipsacales are the next branch, and the Asterales are sister to the Apiales (Fig. 4). However, within the Asteraceae, the topology from BI is different from those from ML and MP, and MP also did not resolve the relationships of *Ilex wilsonii* and *Ilex szechwanensis* (Fig. 4C).

**DISCUSSION**

The only other published chloroplast genomes in the order Aquifoliales are for seven species of *Ilex* in the family Aquifoliaceae (Yao et al., 2016). The length of the *Helwingia* genome (158,362 bp) is similar to that of *Ilex* (157,610–157,918 bp) (see Table 1 in Yao et al., 2016). *Helwingia* (94 genes) has two fewer protein coding genes than *Ilex* (96) while both have the same number of tRNA (40) and rRNA (eight) genes.

Minor differences among the published chloroplast genomes are common, including gene loss or gain events, but these do not separate *H. himalaica* from the others. Both *H. himalaica* and *Helianthus annuus* have *ycf15* (Timme, 2009), but *Guizotia abyssinica* does not (Dempewolf et al., 2010). *H. himalaica* and *L. sativa* have *trnE* (Kanamoto et al., 2004) but *H. annuus* and *G. abyssinica* do not. Gene *rps16* has one intron in *G. abyssinica*, *H. annuus*, and *L. sativa*, but none in *H. himalaica*, while gene *ycfβ* has two introns in *H. himalaica*, *G. abyssinica*, and *H. annuus*, but none in *L. sativa*. Gene *ycf15* is between

| Gene | Length (bp) | Gene | Length (bp) | Gene | Length (bp) |
|------|-------------|------|-------------|------|-------------|
| *atpA* | 1,539 | *psaB* | 2,205 | *psbT* | 144 |
| *atpH* | 246 | *psaC* | 246 | *rbcL* | 1,458 |
| *atpI* | 744 | *psaI* | 113 | *rpl14* | 417 |
| *cemA* | 708 | *psaJ* | 135 | *rpl20* | 415 |
| *ndhC* | 363 | *psbA* | 1,062 | *rpl32* | 207 |
| *ndhD* | 1,516 | *psbD* | 1,062 | *rpl33* | 207 |
| *ndhE* | 306 | *psbF* | 120 | *rpl36* | 114 |
| *ndhJ* | 477 | *psbH* | 222 | *rps2* | 747 |
| *petA* | 963 | *psbI* | 111 | *rps4* | 618 |
| *petG* | 114 | *psbK* | 186 | *rps8* | 435 |
| *petL* | 96 | *psbM* | 117 | *rps11* | 418 |
| *psaA* | 2,253 | *psbN* | 132 | *rps18* | 336 |

Table 4 The 36 protein-coding genes shared by the 37 campanulid species and used for construction of the protein-coding gene phylogeny.
ycf2 and trnL-CAA in *H. himalaica*, but between *rps7* and *trnV-GAC* in *H. annuus*. Gene *ndhF* is in the IRb-SSC junction in both *L. sativa* and *H. himalaica*, but in the SSC-IRa junction in *G. abyssinica* and *H. annuus*. The lengths of the published chloroplast genomes for the Campanulaceae range from 162,321 bp (*T. caeruleum, Haberle et al., 2008*) to 171,724 bp (*Adenophora remotiflora, Kim et al., 2016*) and are longer than those of other campanulid species. *Kim et al. (2016)* attribute this longer length to expansion occurring in the IR and LSC regions as well as the gene arrangements.

The many mononucleotide SSRs identified in *H. himalaica* are potentially useful for studies of the evolutionary history of populations (*Khadivi-Khub et al., 2014; Chae et al., 2014*). The dominance of A/T in mononucleotide SSRs in *Helwingia* is similar to

![Figure 3](image-url)
Figure 4  Phylogeny of 37 campanulid species using their 36 shared coding genes. In subgraph (A) numbers near nodes indicate the Bayesian posterior probability. In subgraph (B) numbers near nodes indicate the maximum likelihood bootstrap values for each clade present in the 50% majority-rule consensus tree. In subgraph (C) numbers near nodes indicate the maximum parsimony bootstrap values for each clade present in the 50% majority-rule consensus tree.
other published studies (Huang et al., 2014; Kuang et al., 2011). It has been suggested that repeated sequences play an important role in genomic rearrangement and sequence variation in chloroplast genomes (Huang et al., 2014; Yang et al., 2013). Approximately 63% of repeats were found in intergenic regions which are often also divergent hotspot regions (e.g. Yao et al., 2015; Huang et al., 2014), showing the potential of these regions for the development of new phylogenetic markers for species identification in Helwingia and related genera in the Aquifoliales.

Massive rearrangements in the chloroplast genome have been identified in the Campanulaceae in comparison with other campanulid families (Fig. 2). Except for gene block 1 and 2, most gene blocks in the LSC have been rearranged, including changes in gene order and transcribing direction (Fig. 2). The chloroplast gene rearrangement in Campanulaceae was first identified in T. caeruleum, and inferred as the effects of recombination of repeats or tRNA genes (Haberle et al., 2008). Comparing with other angiosperm chloroplast genomes, more repeats and tRNA genes occurred near rearrangement endpoints in this species. The positive connection between rearrangement and repeated sequences has also been found in other plants, like Arbutus unedo (Martínez-Alberola et al., 2013), Geraniaceae (Weng et al., 2013), Vaccinium macrocarpon (Fajardo et al., 2013) and cupressophytes (Wu & Chaw, 2014). However, the effects of these chloroplast gene rearrangements on plant physical functions still need more study.

The phylogenetic trees based on complete chloroplast genomes are incongruent with those from the protein-coding genes. Aquifoliales are basal in all phylogenetic analyses, but the phylogenetic relationships among the Asterales, Apiales and Dipsacales differ in different analyses (Figs. 3 and 4). The phylogeny based on complete chloroplast genomes using BI and ML methods found that the Apiales are sister to the Dipsacales (Fig. 3A), which agrees with recent phylogenies for this subclass based on other markers (Beaulieu, Tank & Donoghue, 2013; Wikström et al., 2015; Chen et al., 2016; The Angiosperm Phylogeny Group, 2016). However, using the MP method with the same data resulted in a phylogeny with the Asterales sister to the Dipsacales (Fig. 3B). The phylogenies based on protein-coding genes found that the Apiales are sister to Asterales with all three methods, although the topology within the Asteraceae differed between BI and the other two methods (Fig. 4). Three orders (of seven) and 22 families (of 29) in the subclass Campanulidae could not be included in our analyses because there are no published complete chloroplast genomes for these clades, which emphasizes the need for increased coverage of angiosperm orders and families in future studies of chloroplast genomes.

**CONCLUSION**

We report the chloroplast genome of H. himalaica as the first in the Helwingiaceae and the second genus in the Aquifoliales. It has the typical quadripartite circular structure, including an LSC with 87,810 bp and an SSC with 18,560 bp, separated by a pair of IR regions with 25,996 bp. In total, 142 genes were detected in this genome, consisting of 94 protein-coding genes, 40 tRNA, and eight rRNA. Repeated sequences are mainly
distributed in intergenic regions. Comparisons among the available chloroplast genomes within the campanulids reveal massive chloroplast gene rearrangement in the Campanulaceae. The phylogenetic relationships among Apiales, Asterales and Dipsacales were incongruent between phylogenetic results produced from complete chloroplast genomes and the 36 shared protein-coding genes. The topology within Asteraceae also varied, which shows that further studies are still needed in these three orders. The results of this study will facilitate understanding of not only the family Helwingiaceae and its relationships with other taxa in the Aquifoliales, but also phylogenetic relationships within the angiosperms at higher levels.

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Author Contributions
• Xin Yao conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
• Ying-Ying Liu performed the experiments, analyzed the data, reviewed drafts of the paper.
• Yun-Hong Tan performed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
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