Testing the Complete Plastome for Species Discrimination, Cryptic Species Discovery and Phylogenetic Resolution in Cephalotaxus (Cephalotaxaceae)

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Species of Cephalotaxus have great economic and ecological values. However, the taxonomy and interspecific phylogenetic relationships within the genus have been controversial and remained not fully resolved until now. To date, no study examined the efficiency of the complete plastome as super-barcode across Cephalotaxus species with multiple samples per taxon. In this study, we have evaluated the complete plastome in species discrimination and phylogenetic resolution in Cephalotaxus by including 32 individuals of all eight recognized species and five varieties following Farjon’s classification (2010) with multiple samples per taxon. Our results indicated that not all species recognized in recent taxonomic revisions of Cephalotaxus could be distinguished and not all were monophyletic. Based on the plastome phylogeny, a new taxonomic classification for the genus comprising nine species and two varieties, including a cryptic species, was proposed. The phylogeny also resolved all interspecific relationships. Compared to the plastome based classification, standard DNA barcodes, alone or in combination, only recognized a maximum of seven out of the nine species. Moreover, two highly variable single loci, ycf1 and rps16, each alone achieved full species discrimination. With the moderate length of 1079 bp, rps16 is proposed as a specific barcode to discriminate Cephalotaxus species. The super-barcodes and specific barcode candidates will aid in the identification of endangered Cephalotaxus species, and to help focus conservation measures.

Keywords: Cephalotaxus, complete plastome, species discrimination, cryptic species, phylogenetic relationships, standard DNA barcodes

INTRODUCTION

Species delimitation is fundamental in many areas of biology (Duminil and Di Michele, 2009). Traditional species delimitation is based on the analysis of morphological variation, which is, however, affected by a large number of factors, and can result in difficulties in species identification (Hebert et al., 2003). With the development of molecular phylogenetic methods, applications of the “phylogenetic species concept” have increased, which uses monophyly to define species
(de Queiroz, 1998, 2005; Zimmers et al., 2017). DNA barcoding is a technique to identify biological species, parts and products using one or several standardized DNA regions (Hebert et al., 2003; Kress et al., 2005; Hollingsworth, 2011; Hollingsworth et al., 2016). It has been widely used as a molecular tag for species identification and delimitation, and is contributing to rapid scientific progress on diverse fronts (Joly et al., 2014; Kress et al., 2015; Hollingsworth et al., 2016). Although a number of DNA markers were assessed as candidate DNA barcodes for plants, no barcode alone performed as well as COI in animals (Hollingsworth, 2011). Several different combinations of candidate DNA regions were proposed for barcoding plants (Chase et al., 2007; Kress and Erickson, 2007; Pennisi, 2007; Hollingsworth et al., 2009). However, the standard DNA barcodes suggested for flowering plants (rbcL, matK, trnL-trnF, and trnH-psbA) generally do not perform well across closely related species and recently diverged species mainly due to the lack of adequate genetic variation (Hollingsworth et al., 2009; Li et al., 2011; Yan et al., 2015; Coissac et al., 2016).

With the rapid development of next-generation sequencing (NGS) technologies and decreasing sequencing costs, a large amount of genomic data can be rapidly generated to date, thus the concept of DNA barcodes has been expanded (Coissac et al., 2016; Hollingsworth et al., 2016; Tonti-Filippini et al., 2017). The complete plastome was proposed as “ultra-barcode” (Nock et al., 2011; Kane et al., 2012) or “super-barcode” (Yang et al., 2013) for plant species delimitation, being referred to as next-generation DNA barcode (Hollingsworth et al., 2016). Compared to standard DNA barcodes, next-generation DNA barcodes may overcome the inherent limitations of traditional DNA barcodes, as they potentially possess more informative and variable sites, and therefore can enhance species discrimination power and phylogenetic resolution (Kane et al., 2012; Hollingsworth et al., 2016; Ji et al., 2019). Genome skimming is a cost-effective NGS technology to obtain the whole plastid genome (Straub et al., 2012; Ripma et al., 2014). The discriminatory efficiency of the complete plastome in plants has been assessed in several recent studies (e.g., Turner et al., 2016; Fu et al., 2019; Ji et al., 2019; Song et al., 2020). However, very few studies have been undertaken using multiple individuals per species from multiple congeneric species.

The plastid genome is uniparentally inherited in most plants (Birky, 2001; Gitzendanner et al., 2018) with an exception for most conifers such as Cephalotaxaceae, Taxaceae and Pinaceae where it is paternally inherited (Gianordoli, 1974; Mogensen, 1996), and behaves as a single non-recombining locus, providing a strong signal of phylogenetic history (Petit and Vendramin, 2007; Yu et al., 2018). With a highly conserved gene order and absence of recombination, the plastid genome is an ideal target for comparative analysis across land plants (Wicke et al., 2011; Hollingsworth et al., 2016; Gitzendanner et al., 2018). The benefits of the complete plastome have been argued for its utilization in plant species delimitation, although this may not solve delimitation failures resulting from rare biparental plastid inheritance, introgression and hybridization, such as presumed for Salix (Percy et al., 2014), and Calligonum (Song et al., 2020). However, plastome barcoding has shown a great promise for reliably distinguishing closely related species (Kane et al., 2012; Ruhsam et al., 2015; Zhang et al., 2017; Zhi et al., 2018; Fu et al., 2022). In addition, the plastid genome has a large number of evolutionarily informative variation, which has been widely used for phylogenetic reconstruction at deep to shallow levels in land plants (Moore et al., 2010; Yu et al., 2018; Ji et al., 2019; Li et al., 2019, 2021), including conifers (e.g., Fu et al., 2019; Zhang et al., 2019; Ji et al., 2021).

The genus Cephalotaxus Siebold and Zucc. ex Endl. is a small group of 7-10 species of evergreen conifers belonging to family Cephalotaxaceae in a narrow sense (Singh, 1961; Farjon, 1998, 2010) or Taxaceae in a wider sense (Hart, 1987; Price, 1990, 2003; Christenhusz et al., 2011). The plants in this genus are understory trees distributed in eastern Asia, north of the Indo-Chinese peninsula and Himalayas, mainly in China, Korea, Japan, Vietnam, Laos, Thailand, India, and Myanmar (Fu et al., 1999; Farjon, 2010; Lang et al., 2013; Yang et al., 2017; Zhang et al., 2019). The taxonomy of Cephalotaxus is primarily based on morphological characters and geographical distribution of individual taxa (Farjon, 2010; Lang et al., 2011, 2013; Yang et al., 2017). However, the morphological characters used for species delimitation are quite variable and characteristics usually overlap to a high degree between species resulting in a complex and controversial taxonomic history (Farjon, 2010; Lang et al., 2011, 2013; Zhang et al., 2019). For example, Farjon (2010) recognized eight species and three varieties in Cephalotaxus, i.e., C. fortunei Hook. (including C. f. var. fortunei and C. f. var. alpina H.L. Li), C. hainanensis H.L. Li, C. harringtonii (Knight ex J. Forbes) K. Koch (including C. h. var. harringtonii, C. h. var. nana (Nakai) Rehder and C. h. var. wilsoniana (Hayata) Kitam.), C. lanceolata K.M. Feng, C. latifolia L.K. Fu and R.R. Mill, C. mannii Hook.f., C. oliveri Mast., and C. sinensis (Rehd. and E.H. Wilson) H.L. Li. Out of these, six species were recognized in China by Fu et al. (1999), but had different taxonomic treatments. For instance, C. hainanensis was treated as a synonym of C. mannii, and C. harringtonii var. wilsoniana was treated as a variety of C. sinensis (Table 1). Whereas Lang et al. (2013), Zhang et al. (2019) only recognized seven species in recent revisions of the genus but with taxonomic differences (Table 1). Thus, the taxonomy of Cephalotaxus is controversial and has not been resolved satisfactorily.

Intrageneric phylogenetic relationships of Cephalotaxus also remained largely unresolved or disputable in previous studies which did not include all extant lineages or only used a few gene sequences. For example, Zhang et al. (2000) employed AFLP markers to discriminate only four species and four varieties of Cephalotaxus. Hao et al. (2008) investigated the interspecific relationships of Taxaceae and Cephalotaxaceae based on four cpDNA regions and the nrDNA ITS region with a complete sampling of 12 species and two varieties of Cephalotaxus. In their studies, Cephalotaxus latifolia fell on the basal branch sister to the rest of the species resolved as polytomy, with sister relationships of C. griffithii and C. oliveri, C. mannii and C. hainanensis, C. lanceolata and C. fortunei, and a clade C. harringtonia (C. wilsoniana, C. koreana, C. harringtonia cv. fastigiatu). Ji et al. (2021) reconstructed a phylogeny of Cephalotaxus based on 81 plastid protein-coding genes and ten sampled species.
Their interspecific relationships were fully resolved with strong support, but differed with that in Hao et al. (2008) despite both being based on plastid DNA loci. The conflicting relationships of Cephalotaxus in previous studies need to be verified using more robust molecular markers and more species sampled. While misidentification of samples will also lead to erroneous phylogenetic relationships, a correct species identification is fundamental for phylogeny reconstruction.

The species of Cephalotaxus have important economic values and are used as ornamental plants, and also as medicinal plants due to their alkaloids (Abdelkafi and Nay, 2012). A few species are listed as endangered and vulnerable by IUCN1. Thus, a correct taxonomy and accurate species delimitation are crucial to the utilization and conservation of extant Cephalotaxus species. In this study, we performed genome skimming and assembled the complete plastome of 32 samples, including multiple samples for all Cephalotaxus species recognized in past treatments, and covering the genus’ main distribution range. We aimed to: (1) delimit and discriminate the species of Cephalotaxus using the entire plastome and a phylogenetic approach using the taxonomic classification of Farjon (2010) as baseline; (2) to compare these results to those obtained using standard DNA barcodes (matK, rbcL, trnH-psbA, and trnL-trnF); (3) investigate candidates for specific barcodes; (4) based on the plastome tree propose a phylogeny-based species classification; and (5) discuss the importance of correct species identification and classification on conservation assessments for the genus.

TABLE 1 | Comparison of different taxonomic treatments of the genus Cephalotaxus.

| Farjon, 2010 | Fu et al., 1999 | Lang et al., 2013 | Zhang et al., 2019 | This paper |
|-------------|----------------|------------------|------------------|------------|
| 8 species  | 6 species in China | 7 species | 7 species | 9 species |
| C. fortunei | C. fortunei | C. fortunei | C. fortunei | C. fortunei |
| C. fortunei var. fortunei | C. fortunei var. fortunei | C. alpina | C. alpina | C. alpina |
| C. fortunei var. alpina | C. fortunei var. alpina | C. hainanensis | C. hainanensis | C. hainanensis |
| C. hainanensis | (C. mannii) | C. hainanensis | C. hainanensis | C. hainanensis |
| C. harringtonii | C. harringtonii | C. harringtonii | C. harringtonii | C. harringtonii |
| C. harringtonii var. harringtonii | C. nana | C. harringtonii | C. harringtonii | C. harringtonii |
| C. harringtonii var. nana | C. sinensis var. wilsoniana | C. nana | C. nana | C. nana |
| C. lanceolata | C. lanceolata | (C. griffithii) | (C. griffithii) | (C. griffithii) |
| (C. mannii) | (C. mannii) | (C. sinensis) | (C. sinensis) | (C. mannii) |
| C. latifolia | C. latifolia | (C. nana) | (C. nana) | (C. latifolia) |
| C. mannii | C. mannii | (C. harringtonii) | (C. harringtonii) | (C. harringtonii) |
| C. oliveri | C. oliveri | C. oliveri | C. oliveri | C. sp. nov. |
| C. sinensis | C. sinensis | (C. harringtonii) | (C. harringtonii) | (C. harringtonii) |

Species in bold are recognized in each taxonomic classification, the species in bracket indicates that correspond recognized species in other classifications merged into the species.

Illumina Sequencing, Assembly, and Annotation
Total genomic DNA was extracted from ~20 mg of leaf samples by a modified CTAB method (Doyle and Doyle, 1987). Approximately 5 µg of purified genomic DNA was used to construct shotgun libraries with a TruSeq DNA Sample Prep Kit following the manufacturer’s instructions (NEBNext® Ultra IIITMDNA Library Prep Kit for Illumina®). Paired-end sequencing from both ends of 150 bp fragments was performed on an Illumina HiSeq X Ten platform (Illumina, San Diego, CA, United States) at BGI (Wuhan, China) to generate approximately 3 Gb data for each individual sample. Raw reads were filtered to remove adaptors and low quality reads using the NGS QC Toolkit (Patel and Jain, 2012) with default parameters.

The plastomes were de novo assembled from the genome skimming data using the GetOrganelle toolkit (Jin et al., 2020). The complete plastome of C. hainanensis (NC_042392) was used as the reference. Plastid genes were annotated using PGA (Qu et al., 2019) and coupled with manual adjustment in

MATERIALS AND METHODS

Taxon Sampling
To cover the maximum diversity within species, 2-5 individuals from different populations per species and varieties were sampled

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1https://www.iucnredlist.org/search?query=Cephalotaxus&searchType=species

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| Taxon Farjon, 2010 | Taxon (This study) | Locality information | Voucher Sample ID | GenBank accession numbers | No. of reads after trimming | No. of mapped reads | Sequencing coverage (mean) | Plastome size (bp) | GC content (%) |
|-------------------|-------------------|----------------------|------------------|--------------------------|-----------------------------|-------------------|-----------------------------|-----------------|----------------|
| C. fortunei var. alpina | C. alpina | China, Yunnan, Weixi | GLM-092565 C114 | OK138579 | 15355626 | 192666 | 212.9 | 136,048 | 35.10% |
| C. fortunei var. alpina | C. alpina | China, Yunnan, Lanping | LJ-09438 C118 | OK138581 | 17589552 | 188214 | 207.5 | 136,127 | 35.10% |
| C. fortunei var. alpina | C. alpina | China, Yunnan, Shilin | LJ-09396 C113 | OK138578 | 25002738 | 867933 | 956.9 | 136,154 | 35.10% |
| C. fortunei var. fortunei | C. fortunei | China, Yunnan, Guangnan | GLM-06122 C011 | OK138561 | 15545572 | 293789 | 324.4 | 136,543 | 35.10% |
| C. fortunei var. fortunei | C. fortunei | China, Fujian, Jiangle | LIDZ-0723 C018 | OK138562 | 7516066 | 33080 | 150.0 | 136,770 | 35.10% |
| C. fortunei var. fortunei | C. fortunei | China, Gansu, Wenxian | Zhdq-194 C019 | OK138563 | 18982656 | 229359 | 253.1 | 136,807 | 35.10% |
| C. hainanensis | C. hainanensis | Vietnam, Quang Binh, Botrach | WP-1005 C124 | OK138584 | 15758028 | 103779 | 114.0 | 136,679 | 35.00% |
| C. hainanensis | C. hainanensis | China, Hainan, Ledong | GLM-07384 C019 | OK138564 | 10744576 | 44787 | 49.4 | 136,705 | 35.00% |
| C. hainanensis | C. hainanensis | China, Hainan, Ledong | GLM-06821 C021 | OK138565 | 9630812 | 38936 | 42.6 | 137,024 | 35.00% |
| C. harringtonii var. harringtonii | C. harringtonii var. harringtonii | Korea | 20-98 3201 | OK138560 | 7005528 | 91009 | 100.3 | 136,049 | 35.10% |
| C. harringtonii var. harringtonii | C. harringtonii var. harringtonii | Japan | RBGE19687277A C122 | OK138583 | 8423756 | 93116 | 102.6 | 136,152 | 35.10% |
| C. harringtonii var. nana | C. harringtonii var. nana | Korea, Seoul, Backnoon Mt. | K-15 C024 | OK138566 | 11120458 | 65482 | 72.2 | 136,196 | 35.10% |
| C. harringtonii var. nana | C. harringtonii var. nana | Korea, Seoul, Backnoon Mt. | K-11 C030 | OK138567 | 25610494 | 112136 | 123.4 | 136,290 | 35.10% |
| C. harringtonii var. wilsoniana | C. harringtonii var. wilsoniana | China, Taiwan, Yinan | MMO-081382 C120 | OK138582 | 16120144 | 141039 | 155.3 | 136,189 | 35.10% |
| C. harringtonii var. wilsoniana | C. harringtonii var. wilsoniana | China, Taiwan, Xinhu | GLM-103071 C129 | OK138591 | 15792126 | 49311 | 54.3 | 136,308 | 35.10% |
| C. harringtonii var. wilsoniana | C. harringtonii var. wilsoniana | China, Taiwan, Nantou | GLM-103119 C132 | OK138587 | 16437696 | 193905 | 213.8 | 136,218 | 35.10% |
| C. lanceolata | C. lanceolata | China, Yunnan, Gongshan | DLJET-660 C031 | OK138568 | 18634280 | 68790 | 79.9 | 136,496 | 35.00% |
| C. lanceolata | C. lanceolata | China, Yunnan, Gongshan | GLM-082527 C116 | OK138580 | 17375238 | 65088 | 75.6 | 136,497 | 35.00% |
| C. latifolia | C. sinensis | China, Chongqing, Nanchuan | 86 C105 | OK138576 | 16651598 | 79425 | 87.4 | 136,798 | 35.10% |

(Continued)
| Taxon Farjon, 2010 | Taxon (This study) | Locality information | Voucher | Sample ID | GenBank accession numbers | No. of reads after trimming | No. of mapped reads | Sequencing coverage (mean) | Plastome size (bp) | GC content (%) |
|-------------------|-------------------|----------------------|---------|-----------|---------------------------|---------------------------|-------------------|------------------------|-----------------|---------------|
| C. latifolia      | C. sinensis       | China, Chongqing, Nanchuan | 131     | C107      | OK138577                  | 16182814                  | 97845             | 107.7                  | 136,385         | 35.10%         |
| C. mannii         | C. mannii         | China, Yunnan, Puer   | GLM-07382| C038      | OK138569                  | 17845098                  | 121796            | 133.6                  | 136,792         | 35.10%         |
| C. mannii         | C. mannii         | China, Yunnan, Jinghong | GLM-07383| C039      | OK138570                  | 17220756                  | 171004            | 187.7                  | 136,792         | 35.10%         |
| C. mannii         | C. mannii         | China, Yunnan, Hekou, Nanxi | GLM-164317| C133      | OK138588                  | 30387206                  | 95923             | 105.9                  | 136,870         | 35.10%         |
| C. mannii         | C. mannii         | China, Xizang, Motuo  | GLM-07383| C134      | OK138585                  | 16738946                  | 95533             | 104.8                  | 136,788         | 35.10%         |
| C. mannii         | C. mannii         | China, Xizang, Motuo  | GLM-07383| C135      | OK138596                  | 18521626                  | 133823            | 146.8                  | 136,811         | 35.10%         |
| C. oliveri        | C. oliveri        | China, Sichuan, Emei Mt. | 21      | C041      | OK138571                  | 15658890                  | 41015             | 45.7                   | 134,626         | 35.20%         |
| C. oliveri        | C. oliveri        | China, Chongqing, Nanchuan | 84      | C043      | OK138572                  | 15724158                  | 31618             | 35.2                   | 134,601         | 35.20%         |
| C. oliveri        | C. oliveri        | China, Yunnan, Pingbian | GLM-07587| C044      | OK138573                  | 19709362                  | 52593             | 58.5                   | 136,610         | 35.20%         |
| C. sinensis       | C. sinensis       | China, Sichuan, Meigu  | GLM-07750| C052      | OK138574                  | 17683482                  | 117227            | 128.7                  | 136,663         | 35.10%         |
| C. sinensis       | C. sinensis       | China, Hunan, Sangzhi  | ZhangSD-25| C096      | OK138575                  | 25525226                  | 311275            | 342.4                  | 136,535         | 35.10%         |
| C. sinensis       | C. sp. nov.       | China, Guizhou, Anlong | GLM-06029| C032      | OK138589                  | 19382784                  | 36760             | 40.3                   | 136,929         | 35.10%         |
| C. sinensis       | C. sp. nov.       | China, Yunnan, Xichou  | GLM-07336| C037      | OK138590                  | 19043154                  | 271634            | 318.1                  | 137,285         | 35.10%         |
| Torreya taxifolia | Torreya taxifolia | United States, GA, Atlanta Botanical Garden | 20121291| To33      | OK138559                  | 39120638                  | 452291            | 498.8                  | 137,117         | 35.40%         |
| Torreya jackii    | Torreya jackii    | China, Jiangxi, Fuzhou | PVH-LX03233| To43      | OK138558                  | 25767386                  | 451825            | 496                    | 137,117         | 35.50%         |
| Amentotaxus formosana | Amentotaxus formosana | China, Taiwan, Pingtung | MMO-081377| Am10      | OK138557                  | 24414070                  | 450086            | 500                    | 136,361         | 35.80%         |
Geneious v8.0.2 (Kearse et al., 2012). The tRNAs were checked with tRNAscan-SE v2.0.3 (Lowe and Chan, 2016). Final plastid genome map was drawn using OGDRAW (Lohse et al., 2013).

**Data Analysis**

We employed the complete plastome sequences for species delimitation using tree-based and genetic distance methods. To compare the discriminatory power of standard DNA barcode and super-DNA barcode (the whole plastome), the standard DNA barcodes of matK, rbcL, trnH-psbA and trnL-trnF sequences were extracted from the complete plastomes. Besides, DnaSP v6.12 (Rozas et al., 2017) was used to perform a sliding window analysis with a step size of 200 bp and a window length of 800 bp on the alignment of all *Cephalotaxus* species plastomes, to detect the rapidly evolving candidate molecular markers for species delimitation and discrimination. Nucleotide diversity (Pi) was computed for constructing a DNAsp graph.

In tree-based analyses, since some inversions were found in the plastomes of the outgroups (*Torreya jackii*, *T. taxifolia* and *Amentotaxus formosana*) compared with *Cephalotaxus*, we adjusted the plastid genome structure of the outgroups using Draft in the MULAN web server\(^2\) (Ovcharenko et al., 2005) with *Cephalotaxus fortunei* (C011) as the reference sequence to make the gene order and direction collinear across the genome for dataset alignment. Sixteen datasets were constructed in this study: (a) the complete plastomes, (b) six based on the standard DNA barcodes and some of their combinations: matK, rbcL, trnH-psbA, trnL-trnF, matK + rbcL, and matK + rbcL + trnH-psbA + trnL-trnF, (c) eight from selected high variation loci: trnL-trn16, ycf1, chlN-ycf1, clpP-accD, rps16, accD, ycf2, ndhF-trnR, and (d) added the 10 sampled species of Ji et al. (2021) to our dataset and used only the 81 plastid protein-coding genes. The complete plastomes and the DNA loci in each datasets were aligned separately using the program MAFFT v7.221 (Katoh and Standley, 2013) with manual adjustments where necessary and concatenated into data matrices. Phylogenetic analyses for each dataset using maximum likelihood (ML) were performed in RAxML v8.2 (Stamatakis, 2014) under the GTRGAMMA model. The best-scoring ML tree for each dataset was produced with 1,000 bootstrap replicates to provide support values for each node. Taxon with multiple individuals resolved as monophyletic with bootstrap support (BS) over 50% were treated as successfully discriminated against Farjon’s (2010) classification.

To assess the barcoding gap, inter- and intraspecific distances were calculated using the Kimura 2-parameter (K2P) distance in MEGA v10.1.8 (Kumar et al., 2018) for each of the

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\(^2\)http://mulan.dcode.org
Other genes

Maturase
Protease
Envelope membrane protein
Subunit of acetyl-CoA carboxylase
ATP-dependent protease
C-type cytochrome synthesis gene
Open reading frames

DNA barcode

| DNA barcode | No. of sites | No. of variable sites | No. of parsimony informative sites |
|------------|-------------|-----------------------|-----------------------------------|
| complete plastome | 142194 | 3795 (2.67%) | 3467 (2.44%) |
| matK + rbcL + trnH-psbA + trnL-trnF | 2585 | 63 (2.44%) | 59 (2.28%) |
| matK + rbcL | 1479 | 19 (1.28%) | 19 (1.28%) |
| matK | 783 | 9 (1.15%) | 9 (1.15%) |
| rbcL | 696 | 10 (1.44%) | 10 (1.44%) |
| trnH-psbA | 368 | 16 (4.35%) | 16 (4.35%) |
| trnL-trnF | 738 | 28 (3.79%) | 24 (3.25%) |
| trnL-trn16 | 2149 | 472 (2.19%) | 403 (18.75%) |
| ycf1 | 8988 | 534 (5.94%) | 503 (5.60%) |
| chlN-ycf1 | 2066 | 154 (7.45%) | 142 (6.87%) |
| AtP-accD | 1144 | 73 (6.38%) | 69 (6.03%) |
| rps16 | 1079 | 50 (4.63%) | 45 (4.17%) |
| accD | 3330 | 214 (6.43%) | 173 (5.20%) |
| ycf2 | 7620 | 223 (2.93%) | 194 (2.55%) |
| ndhF-trnR | 1633 | 69 (4.23%) | 64 (3.92%) |

Whole Plastome as Super-Barcode for Discriminating Cephalotaxus Species

We obtained an alignment matrix of 142,194 bp, which had 3,795 (2.67%) variable sites including 3,467 (2.44%) parsimony informative (PI) sites (Table 4). The ML tree showed that all the taxon nodes (the node grouping individuals from the same species/variet) had maximum support values (BP = 100%), except for C. harringtonii var. harringtonii (BP = 73%) (Figure 2). Based on the classification of Farjon (2010), five species (C. hainanensis, C. harringtonii, C. lanceolata, C. manni, and C. oliveri) out of eight of his recognized species (62.5%) were successfully discriminated with highest support values, while the remaining three, C. latifolia, C. fortunei, and C. sinensis, were non-monophyletic. Within C. harringtonii, samples of each variety formed a clade respectively, and C. harringtonii var. nana was sister to the other two conspecific varieties. Samples of C. sinensis (C052 and C096) and C. latifolia (C105 and C107) were recovered in a clade (BP = 100%), but neither species was monophyletic. Interestingly, two individuals (C032 and C037) of C. sinensis formed a distinct clade as sister to a clade (BP = 100%) that included samples of C. fortunei, C. sinensis.

RESULTS

Characteristics of Cephalotaxus Plastomes

High-quality complete plastid genomes of all 32 sampled individuals of Cephalotaxus were obtained and assembled into circular molecules (Figure 1 and Table 2). For the 32 individuals,
FIGURE 2 | Phylogenetic tree reconstructed via maximum-likelihood (ML) analysis of the complete plastome data. Numbers above branches indicate the bootstrap support values. In the top left is an ML phylogenetic tree with branch length excluding outgroups. The branch colors correspond with the species recognized in this study.

(C052 and C096) and C. latifolia (Figure 2). Although samples of the two varieties of C. fortunei (C. fortunei var. fortunei, C. fortunei var. alpina) each formed a separate clade, they were not monophyletic (Figure 2). The phylogenetic tree including additionally 10 Cephalotaxus species used in Ji et al. (2021) based on 81 protein-coding genes recovered the same topology with strong support as the complete plastome tree. In this tree, six out of the ten species grouped in their respective species clades. Two species, C. griffithii (= C. lanceolata) and C. nana (= C. latifolia), grouped into our C. fortunei var. alpina clade, and the other two species, C. fortunei and C. alpina, fell in the clade of C. mannii (Supplementary Figure S1).

Four species recognized by Lang et al. (2013), Zhang et al. (2019), i.e., C. griffithii (C031, C 116, C126, and C128), C. hainanensis (C19, C21, C124, C038, C039, and C133), C. harringtonii (C120, C122, C129, C132, 3201, C032, C037, C052, and C096), and C. nana (C024, C030, C105, and C107) that differed in their taxonomic treatment with Farjon (2010), were not resolved as monophyletic by the complete plastome data; only three species (C. fortunei, C. alpina, and C. oliveri) recognized by Lang et al. (2013), Zhang et al. (2019) were identified successfully. Based on the phylogenetic tree of the entire plastid genome, nine species including a cryptic lineage (C. sp. nov. C032/C037) for Cephalotaxus were successfully identified and received high support here (Figure 2), which corresponded well with their geographic distribution and morphology. From here on, we used these species names proposed in this study (Table 1).

All the interspecific relationships within Cephalotaxus were fully resolved with strong support values (>92%) based on the phylogenetic tree using complete plastome data (Figure 2). Cephalotaxus oliveri was on the earliest diverging branch, and C. harringtonii formed the subsequent clade which was sister to the rest of the species. The remaining species formed two clades, i.e., C. lanceolata (C. hainanensis + C. mannii), and C. sp. nov. (C. fortunei (C. alpina + C. sinensis)).

Species Discrimination Based on Standard Plastid DNA Barcodes

Among the four single standard DNA barcodes (rbcL, matK, trnH-psbA, trnL-trnF), trnL-trnF had the highest number of PI sites of 24 (3.25%, the percentage of informative sites in relation to the length of the sequenced fragment), followed by trnH-psbA of 16 (4.35%) and rbcL of 10 (1.44%), with matK showing the lowest number with 9 (1.15%) (Table 4). The combination of matK + rbcL resulted in a matrix of 1479 bp in length, with 19 (1.28%) PI sites, while the combination of the four standard plastid DNA barcodes (matK + rbcL + trnH-psbA + trnL-trnF) had 2,585 bp and 59 (2.28%) PI sites. Based on the tree-based analysis of the six datasets of the standard DNA barcodes, the single barcodes and combination of matK + rbcL distinguished a maximum of four (50%) and five (55.6%) species (trnL-trnF) for the classification of Farjon (2010) and the one proposed in this study respectively. However, the four-barcode combination (matK + rbcL + trnH-psbA + trnL-trnF) resolved five (62.5%) and seven (77.8%) species, for the respective classifications (Figure 3 and Supplementary Figure S2).
Species Discrimination Based on Specific DNA Barcodes

Sliding window analyses for all the newly assembled *Cephalotaxus* plastid genomes identified eight highly variable regions (mutational hotspots) (*trnl-rn16, ycf1, chlN-ycf1, clp-P-accD, rps16, accD, ycf2, ndhF-trnR*) (Figure 4). All eight hypervariable specific loci provided higher (maximally 62.5%) or the same (37.5%: *clp-P-accD* and *ndhF-trnR*) species resolution than any single standard DNA barcode or the combination of *matK* and *rbcL* for the classification of *Farjon* (2010) (Figure 3). Discrimination success was always higher for the specific loci for the classification proposed in the present study. For this classification, out of these eight specific loci, two, *ycf1* and *rps16*, yielded the maximum species discriminatory rate, identical to that of the complete plastome, followed by *trnl-rn16* which identified eight out of nine (89%) species of *Cephalotaxus* (Figure 3 and Supplementary Figure S2).

Barcoding Gaps

The intraspecific and interspecific K2P distance varied among the 16 datasets. Out of these, the four standard DNA barcodes and their combination, plus *ycf2*, had an interspecific distance of zero, and *chlN-ycf1* the highest intraspecific distance with 2.41%. Although there was some degree of overlapping between the minimum interspecific distance and the maximum intraspecific distance of these loci/combinations (Supplementary Table S1), relatively distinct barcoding gaps were identified for the complete plastome, plastid protein-coding genes and *rps16*, *ndhF-trnR* and *ycf2* (Supplementary Figure S3). Out of these, four datasets, i.e., the complete plastome, plastid protein-coding genes, *ycf1*, and *rps16*, showed maximum species resolution.

DISCUSSION

Performance of the Super-, Standard, and Specific Barcodes

With the ever more cost-effective NGS technology, the complete plastid genome assembled from genome skimming data has been used several times as super-barcode with promising potential for plant species delimitation (Kane et al., 2012; Turner et al., 2016; Tonti-Filippini et al., 2017; Fu et al., 2019; Ji et al., 2019; Song et al., 2020). In the present study, compared to the standard
barcodes, the complete plastome showed much higher species discrimination rates, and distinguished five out of the eight (62.5%) species of *Cephalotaxus* recognized by Farjon (2010). Only two (25%) to four (50.0%) species were resolved by any single barcode and the combination of *matK* + *rbcL*, while the combination of all four standard barcodes recovered five species (62.5%), the same as the whole plastome. With view to the nine species proposed here, that all species were highly supported in the phylogeny based on the entire plastomes and the plastid coding genes, the standard DNA barcodes (single barcode and their combinations) only discriminated a maximum of seven (77.8%) of those species (with the combination of *matK* + *rbcL* + *trnH-psbA* + *trnL-trnF*) and with relatively low support values (Figure 3 and Supplementary Figure S2).

Our species discrimination results indicated that the complete plastome can provide more variation to discriminate *Cephalotaxus* species compared to the standard barcodes, which has also been demonstrated in other studies (e.g., Ji et al., 2020; Song et al., 2020; Fu et al., 2022). In addition, the complete plastome approach based on genome skimming can circumvent issues of locus choice, and low PCR and sequencing recovery rate, which are sometimes encountered in standard DNA barcoding studies (Ruhsam et al., 2015; Curci et al., 2016). Therefore, the whole plastome as super-barcode illustrated the benefits of moving beyond standard barcodes in *Cephalotaxus* (Tonti-Filippini et al., 2017; Nevill et al., 2020).

Although the super-barcode effectively distinguished species beyond the standard barcodes, its use may sometimes be limited due to the relatively higher costs and complex analyses of genome skimming data compared to the standard PCR and Sanger sequencing barcode approach. A “specific” barcode is a fragment of DNA sequence that has a sufficiently high mutation rate to enable high species identification within a given taxonomic group beyond the standard DNA barcodes, and a more wider use of plant group-specific barcodes for discriminating closely related species was predicted (Li et al., 2015). In this study, we identified eight highly variable loci from the plastid genomes of *Cephalotaxus*. Among them, although *trnl-trnf16* was the most divergent locus, it achieved only a discriminatory rate of 8 out of 9 species (88.9%) (Figure 3), which implied that the selection of barcoding loci based on maximum sequence divergence may not be in line with maximum species discrimination ability. The two loci *ycf1* and *rps16* showed the maximum species discrimination ability, the same as the complete plastome (Figure 3). Considering the length of *ycf1* (8988 bp), it is not suitable as a hypervariable specific barcode. With a PCR-manageable length of 1079 bp, *rps16* can be proposed here as a specific barcode for discriminating *Cephalotaxus* species. In addition, these identified highly variable loci represent valuable genetic resources for future population genetics and phylogeographic studies.

**Monophyly of Species and Discovery of Cryptic Species**

The inclusion of multiple individuals from a single species sampled is highly important to confirm species delimitation successfully and accurately (Liu et al., 2012; Fu et al., 2022). Therefore, in this study, all species/varieties of *Cephalotaxus* had more than one individual included (two to five individuals per taxon). Samples of five species and all varieties in Farjon’s (2010) taxonomic classification were resolved as monophyletic with strong support in the complete plastome phylogeny, while another three species, *C. latifolia*, *C. sinensis* and *C. fortunei*, were not individually resolved as monophyletic. Samples of *C. latifolia* and *C. sinensis* together formed a highly supported clade, but were not resolved in their respective species monophyly. This indicated that they belonged to the same genetic lineage, and suggested a merging of *C. latifolia* into *C. sinensis*. This is supported by their sympatric distribution and overlapping of some morphological traits. Notably, the two samples (C032 and C037) collected from the karst region of southwest Guizhou and southeast Yunnan, China, identified as *C. sinensis*...
under the classification of Farjon (2010), were recovered in a highly supported isolated clade, suggesting a cryptic species of Cephalotaxus (Figure 2). In a study including more individuals collected from this karst region, in adjacent areas in southwest Guizhou, southeast Yunnan and north Vietnam, standard DNA barcodes already indicated the existence of this cryptic species, though it was not distinguished due to a lack of sufficient sequence variations in the nrDNA ITS region (Gao et al., 2015). The new taxon may be adapted to the special karst habitats and has become a distinct species over time, as also found in Taxus (Liu et al., 2011; Möller et al., 2013, Möller et al., 2020) and Amentotaxus (Gao et al., 2017, 2019) in this region. More detailed morphological study for this group is needed to describe the new species. Cephalotaxus fortunei var. fortunei and C. fortunei var. alpina were each resolved as monophyletic respectively, but the species was not monophyletic. This supports the view that the two varieties are best represented at species rank (Lang et al., 2011, 2013; Zhang et al., 2019).

The 81 protein-coding gene phylogenetic tree that included all ten species of Cephalotaxus used in Ji et al. (2021) showed that only six of the ten species (C. oliveri C. harringtonii, C. wilsoniana, C. hainanensis, C. mannii, and C. sinensis) grouped into the respective species clade. However, four species (C. fortunei, C. alpina, C. griffithii, C. nana) fell into the clade of other species. For instance, two species, C. fortunei (MT555080) and C. alpina (MT555079) grouped together with C. mannii (MT555084) with very short branch length within C. mannii clade, indicating that the samples identified as C. fortunei and C. alpina in Ji et al. (2021) are misidentified and are likely C. mannii. Similarly, C. griffithii (MT555081) and C. nana (MT555085) were placed in the C. fortunei var. alpina clade, hinting they were also misidentified and likely represent C. alpina (Supplementary Figure S1). According to the geographic distribution of the four species, their identification is sound. For example, C. griffithii (MT555081) treated as C. lanceolata in Farjon (2010) was collected from Dulongjiang, Gongshan, Yunnan, its type locality. Cephalotaxus fortunei was from Nanchuan, Chongqing, and C. nana from west Hubei treated as C. latifolia in Fu et al. (1999), Farjon (2010). The unusual placement of the four species might be the result from experimental and/or identification errors.

Cephalotaxus oliveri has a distinct morphology and no taxonomic controversy in all classifications (Farjon, 1998, 2010; Lang et al., 2011, 2013; Zhang et al., 2019), and the species can be discriminated in all barcoding datasets. Our results did not support the merging of C. lanceolata into C. griffithii, C. sinensis into C. harringtonii (Lang et al., 2011, 2013; Zhang et al., 2019), and C. mannii into C. harringtonii (Lang et al., 2013) or C. hainanensis (Zhang et al., 2019), nor supported C. harringtonii var. wilsoniana as a variety of C. sinensis (Fu et al., 1999). Cephalotaxus lanceolata formed a distinct clade separate from C. griffithii (C126 and C128), and morphologically differed from C. griffithii by lanceolate leaves and rounded base. Within C. harringtonii, each of the three varieties and the species itself were resolved as monophyletic. The variety C. harringtonii var. wilsoniana is endemic to Taiwan, China, the other two taxa mainly occur in Japan and Korea. We observed a low sequence variation within the species and samples were missing of C. harringtonii var. nana from Japan. Therefore, we refrained from a decision of whether to raise the varieties to species level until more samples of this species particularly from Japan are studied.

### Congruence With Previous Phylogenetic Relationships

Phylogenetic relationships of Cephalotaxus in previous studies were not fully resolved likely due to a low number of DNA loci used, incomplete sampling, or both (e.g., Cheng et al., 2000; Hao et al., 2008). In this study, the interspecific phylogenetic relationships of Cephalotaxus were fully resolved (Figure 2), and the topology is basically consistent with the phylogeny based on 81 plastid protein-coding genes (Ji et al., 2021), but with some differences in interspecific relationships possibly due to experimental error or misidentification (see above).

In the present study, C. oliveri was on the first diverging branch. C. harringtonii formed the subsequent grade and sister to the rest of the species, which was also recovered in Ji et al. (2021), but such relationship was not recovered by the combination of four plastid loci (matK, rbcL, trnL–F, and psbA–trnH) and nrITS region (Hao et al., 2008). The remaining species formed two clades in this study, i.e., C. lanceolata (C. hainanensis + C. mannii), and C. sp. nov. (C. fortunei (C. alpina + C. sinensis)). The sister relationship of C. hainanensis and C. mannii, and monophyly of C. harringtonii was supported in Hao et al. (2008), but other interspecific relationships were not recovered. While in Ji et al. (2021), C. alpina and C. latifolia (representing C. nana in Ji et al., 2021) were resolved as sister and then as sister to C. sinensis. Another difference is represented by C. fortunei; this species grouped in the clade of C. hainanensis (C. mannii (C. lanceolata + C. fortunei)) being sister to C. lanceolata (with low branch support 75%) with very short branch length (Ji et al., 2021). Such inconsistent relationships were likely either due to species misidentification or experimental errors (see above). Therefore, the use of the entire plastome and multiple samples per species from different populations resulted in a fully resolved and accurate phylogeny of Cephalotaxus.

### Implications for Conservation

Accurate species delimitation and identification are key to proper species management and conservation (Trias-Blasi and Vorontsova, 2015). Because of the morphological similarities between Cephalotaxus taxa, it is often difficult to unambiguously identify species. In this study, the whole plastome as super-barcode in combination with a revised classification enabled an accurate delimitation of taxa, including disputed ones in Cephalotaxus, and suggested the presence of a cryptic species. In this genus, C. lanceolata and C. hainanensis are listed as endangered, C. mannii and C. oliveri are vulnerable in the global IUCN Red List (see text footnote 1). Of these, only C.
oliveri is recognized in all classifications (Table 1). In recent taxonomic revisions of the genus (Lang et al., 2013; Zhang et al., 2019), C. lanceolata and C. mannii were merged into C. griffithii and C. hainanensis respectively, and their conservation rank changed consequently. However, these taxonomic treatments were not supported by our results, and we propose to retain these species. This has conservation consequences. For example, C. lanceolata is a species with extremely small populations which are confined to a small area in northwest Yunnan and the adjacent region of north Myanmar, and it is highly endangered. If C. lanceolata would be treated as a synonym of C. griffithii (Lang et al., 2011, 2013; Zhang et al., 2019), the populations in Yunnan and Myanmar would no longer receive attention for conservation since C. griffithii is not listed as endangered species. Our study also discovered a new cryptic species (C. sp. nov.). Based on IUCN criteria v3.1 (IUCN, 2001), this cryptic species would be assessed as threatened species due to small population size fewer than 2,500 mature individuals, ongoing habitat degradation from surrounding agriculture and forest clearances in this region. While plants of this taxon had always been identified as C. sinensis, a species categorized as Least Concern (LC), and never considered endangered.

CONCLUSION

In this study, we have evaluated the species discrimination efficiency of a super-barcode (the complete plastome), standard DNA and specific DNA barcode using 32 plastomes from all recognized species/varieties in different taxonomic classifications of Cephalotaxus with multiple samples per taxon. Our phylogenetic results based on the complete plastome sequences indicated that nine species, including a cryptic taxon, could be discriminated phylogenetically and the interspecific relationships of Cephalotaxus were fully resolved with strong support. The standard barcodes, alone or in combinations, were unable to discriminate all nine species. Of the eight selected hypervariable specific barcode loci, we found that only ycf1 and rps16 alone were able to distinguish all nine species, and, because of its PCR-convenient length, propose rps16 as a specific barcode. In addition, our result indicates that the inclusion of multiple samples per species from different population is necessary for both plant DNA barcoding and phylogeny studies. The new insights into the species delineation of Cephalotaxus in this study will facilitate their identification and would allow targeted conservation management of the endangered Cephalotaxus species in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession numbers BankIt2499120 AM10 OK138557, BankIt2499138 To43 OK138558, BankIt2499138 To33 OK138559, BankIt2499502 3201 OK138560, BankIt2499502 C011 OK138561, BankIt2499502 C014 OK138562, BankIt2499502 C018 OK138563, BankIt2499502 C019 OK138564, BankIt2499502 C021 OK138565, BankIt2499502 C024 OK138566, BankIt2499502 C030 OK138567, BankIt2499502 C031 OK138568, BankIt2499502 C038 OK138569, BankIt2499502 C039 OK138570, BankIt2499502 C041 OK138571, BankIt2499502 C043 OK138572, BankIt2499502 C044 OK138573, BankIt2499502 C052 OK138574, BankIt2499502 C096 OK138575, BankIt2499502 CI05 OK138576, BankIt2499502 CI07 OK138577, BankIt2499502 C113 OK138578, BankIt2499502 C114 OK138579, BankIt2499502 C116 OK138580, BankIt2499502 C118 OK138581, BankIt2499502 C120 OK138582, BankIt2499502 C122 OK138583, BankIt2499502 C124 OK138584, BankIt2499502 C126 OK138585, BankIt2499502 C128 OK138586, BankIt2499502 C132 OK138587, BankIt2499502 C133 OK138588, BankIt2499536 C032 OK138589, BankIt2499536 C037 OK138590, and BankIt2500849 C129 OK138591.

AUTHOR CONTRIBUTIONS

L-MG and D-ZL designed the experiments. L-MG and MM collected the plant samples. JW, C-NF, and Z-QM performed the experiments and analyzed the data. JW, L-MG, and MM wrote the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.768810/full#supplementary-material

**Supplementary Figure S1** | Maximum Likelihood phylogenetic tree of 42 Cephalotaxus samples including the 10 species used in Ji et al. (2021) based on 81 plastid protein-coding genes with proportional branch lengths. Numbers to the right of nodes indicate bootstrap support values.

**Supplementary Figure S2** | Maximum Likelihood phylogenetic trees of 32 Cephalotaxus samples based on the eight highly variable plastid loci, the four single standard DNA barcode and two combinations of standard DNA barcodes (a. trn-16S, b. ycf1, c. chn-ycf1, d. cipP-accD, e. rps2, f. accD, g. ycf2, h. ndf1-trnR, i. matK + rbcL + trnL-psbA + trnL-trnF, j. matK, k. matK + rbcL, l. rbcL, m. trnH-psbA, n. trn-trnL). Numbers to the right of nodes indicate bootstrap support values.

**Supplementary Figure S3** | Relative distribution of intraspecific and interspecific distances for the datasets of the complete plastome, 81 plastid protein-coding genes, combination of the four standard barcodes, and eight highly variable regions (trn-16S, ycf1, chn-ycf1, cipP-accD, rps2, accD, ycf2, ndf1-trnR). X-axes relate to K2P distances arranged in intervals, and the y-axes correspond to the percentage of occurrences.

**Supplementary Table S1** | Examination of the 15 DNA loci used for discriminating the recognized species of Cephalotaxus in this study.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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