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Abstract

Species delimitation remains a challenge worldwide, especially in highly diverse tropical and subtropical regions. Here, we use an integrative approach that combines morphology, phylogenomics, and species distribution modeling (SDM) to clarify the cryptic differentiation within the enigmatic hemiparasitic love vine *Cassytha filiformis* (Lauraceae) in China and adjacent regions. We generated complete plastid genomes and nuclear ribosomal sequences for diverse samples from across the species range and compared results with previously published plastid data, recovering two well-supported monophyletic clades. Further, the analysis revealed significant differences in two morphological characters and SDM, indicating distinct environmental factors influencing their distributions. Fossil-calibrated analyses to estimate the origins and diversification patterns for the cryptic species gave divergence age estimates corresponding to the Oligo-Miocene; a period of new ecological opportunities associated with the prevailing East Asian monsoon. Multivariate analyses support the conclusion that southern China and adjacent regions have a different, previously

unknown, cryptic lineage of *Cassytha filiformis*. Our study highlights the importance of using multivariate approach to characterize plant species, as well as the significant role that past climatic changes have played in driving speciation in parasitic plants in tropical and subtropical zones.

Keywords

Parasitic angiosperms, species delimitation, morphology, phylogeny, *Cassytha*

1. Introduction

For centuries, systematic biologists have relied on morphological characters for diagnosing and delimiting species, however, speciation is not always accompanied by morphological change (Kenfack, 2011), species boundaries are often ambiguous (Posso-Terranova & Andres, 2018). The true number of biological species is likely to be greater than the current tally of species, most of which are delineated on purely morphological grounds (Bickford et al., 2007). Species delimitation is the act of identifying species-level biodiversity (Carstens et al., 2013) and incorporating cryptic species leads to novel insights regarding biodiversity patterns and processes (Fiser et al., 2018). Many groups from the poles to the equator and in all major terrestrial and aquatic regions include species that are difficult, or sometimes impossible to distinguish morphologically and thus have been classified incorrectly as a single taxon (Knowlton, 1993; Beheregaray & Caccone, 2007; Pfenninger & Schwenk, 2007; Kenfack, 2011). The taxonomic challenge posed by cryptic species has been recognized for nearly 300 years (Bickford et al., 2007), but the advent of the "phylogenetic species concept" gave biologists a new framework for detecting and differentiating morphologically similar species (de Queiroz, 2005).

As such, research on the delimitation of species has increased exponentially with the development of genetic approaches and the use of phylogenetic approaches to define species (Roca et al., 2001; Hebert et al., 2003; Hebert et al. 2004; Lu et al., 2010; Fennessy et al., 2016; Yu et al., 2018; Chai et al., 2022; Wang et al., 2022; Newton, Starrett, Jochim, & Bond, 2023). Similarly, powerful statistical approaches have been proposed to use morphological variation as the criteria for species delimitation (Valcárcel & Vargas, 2010). Accordingly, an

integrative approach should be able to provide the best inferences about species delimitation (Padial & De La Riva, 2010; Posso-Terranova & Andres, 2018).

However, species diversification has been promoted by notable geological and climatic change (Zachos et al., 2001; Sun et al., 2014; Deng et al., 2018; Westerhold et al., 2020) and plant diversity resulting from climate-related events has been observed in diverse plant lineages (Feng et al., 2020; Schmerler et al., 2012). For example, the East Asian flora (EAF) is incredibly rich in species diversity and includes more than 3,000 genera (Chen et al., 2018). The East Asian monsoon (EAM) likely driven by the Tibetan Plateau (TP) growth and global warming (Wu et al., 2022), may promote species speciation in the related regions, particularly in highly diverse tropical and subtropical area.

Many parasitic plant species have at least partly hidden lives and their morphological adaptations can be subtle (Bickford et al., 2007) and as such, cryptic species diversity is likely. Parasitic plants comprise ~4,500 species (1.2% of flowering plants) representing ~280 genera from 20 families (Rubiales & Heide-Jørgensen, 2011; Twyford, 2018). Parasitic plants can be chlorophyllous, photosynthetic hemiparasites or achlorophyllous holoparasites (Irving & Cameron, 2009), but all invade other plants directly via a specialized parasitic organ called the haustorium (Yoder & Scholes, 2010). Hemiparasites are more species-rich and generalist hemiparasites may have a wide host range, often attaching to multiple, diverse, co-occurring plants (Brown et al., 2021; Liu et al., 2023). Parasitic plants have had increased attention over the past three decades (Nickrent, 2020), since they are found in a wide range of ecosystems, including subarctic tundra, heathlands, savanna woodlands, deserts, temperate and tropical forests, as well as agricultural ecosystems (Press & Phoenix, 2005; Shen et al., 2006). However, much less attention has been given to their evolution and any features useful for species delimitation.

The widespread hemiparasitic Lauraceae genus *Cassytha* L. currently contains 19 described species, one variety and four forms (http://www.theplantlist.org/). This genus is controversial and has not been resolved satisfactorily. Morphological characters used for species delimitation in *Cassytha* are often problematic, with overlap between species resulting in a complex and controversial taxonomic history, with many taxa in the genus distinguished by only a few fruit color, shape, and indumentum characters (Weber, 1981, 2007). For example, *Cassytha filiformis* L. strongly resembles other robust-stemmed, racemose species such as *C. pubescens* R.Br., *C. capillaris* Meisn., *C. melantha* R.Br., *C. larsenii* Kosterm., *C.*

flindersii (J.Z.Weber) J.Z.Weber and *C. peninsularis* J.Z.Weber. However, stem and branch indumentum in *Cassytha* can vary from glabrescent to pubescent within the same species, or even a single individual (Weber, 1981, 2007) and such morphological gradients often cause taxonomic confusion in the group (Liu et al., 2021).

C. filiformis is cosmopolitian in tropical and subtropical regions. Although regarded as a serious invasive weed in Cuba, Puerto Rico, and the Chagos Archipelago in the Indian Ocean (Zhang et al., 2022), *C. filiformis* is also exploited for medicines, cosmetics, rope, and cushion making in China, India, Nigeria, and the Pacific Islands (Adamu et al., 2017; Zhang et al., 2022). It is currently the only species reported from China (Li et al., 2008), though Liu et al. (2021) speculated that *C. capillaris* maybe also occur there, based on fruit morphology of some accessions and comparisons with the plastome of an Indonesian sample of the latter (GenBank No. MF939338; Song et al., 2019). However, only a few samples have been sequenced for the genus in China. The distribution range of *C. filiformis* is pantropical whereas *C. capillaris* is mainly distributed in tropical Australia, but with isolated records from Assam, Borneo, the Lesser Sunda Islands, Malulu, New Guinea, and Vietnam (Zhang et al., 2022). These distribution patterns have led to a reconsideration of whether *C. capillaris* is present in China and/or whether samples from there instead represent cryptic taxa within the *C. filiformis*. This study provides an ideal case for assessing the species delimitation in parasitic angiosperms.

Molecular phylogenetic methods have been used to address several long-standing issues in parasitic plant taxonomy and evolutionary biology (Wicke & Naumann, 2018; Nickrent, 2020). However, inter- and intrageneric phylogenetic relationships of *Cassytha* have remained largely unresolved or disputed in previous studies, which relied on few gene sequences (plastid: *mat*K, *psb*A–*trn*H, *trn*K and nuclear regions: *RPB2* and ITS) and sampled few individuals (Rohwer, 2000; Chanderbali et al., 2001; Rohwer & Rudolph, 2005; Wang et al., 2010; Li et al., 2016). Kokubugata et al. (2012) generated *trn*K intron sequences from 50 individuals covering nine species to investigate the intrageneric phylogenetic relationships within *Cassytha*, revealing *C. filiformis* to be paraphyletic. Recent improvements in genomic sequencing technologies provide additional options for generating better-supported phylogenies (Hollingsworth et al., 2016), including complete plastome sequencing and the nuclear ribosomal DNA arrays (nrDNA). As the plastomes of many parasitic plants experience a relaxation of selection and thus elevated rates of base substitution (dePamphilis

et al., 1997), variation may be present at lower taxonomic levels. Therefore 'genome skimming', i.e., low-coverage whole genome sequencing aimed at recovering high copy genomic regions such as plastids, may be informative for exploring evolutionary relationships at the population and species level in *Cassytha*.

In this study, we examine Chinese *Cassytha* samples currently placed into *C. filiformis* as well as several related members of the racemose group to investigate the phylogenetic and evolutionary history of the genus in the region. The study will generate new complete plastome and nrDNA (18S–ITS1–5.8S–ITS2–26S) sequences for *C. filiformis* from a range of populations, combining these with previously published plastid data to investigate phylogenetic relationships, possible divergence dates, combining with geographic and morphological data to investigate cryptic differentiation in *C. filiformis*, and help define potentially overlooked cryptic species.

2. Materials and Methods

2.1 Plant materials and sequencing

Samples of *C. filiformis* were collected from five provinces in China: Fujian, Guangdong, Guangxi, Hainan, and Yunnan (Figures 1B, S1; Tables S1-2). Due to the pantropical distribution of *C. filiformis*, we also collected samples from Japan, Kenya, Laos and Thailand (Figures 1B, S1; Table S1-2). Stems for each individual were dried with silica gel, with vouchers deposited at the Herbarium of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (HITBC), Yunnan, China and identified by morphological and molecular comparisons, as described previously (Liu et al., 2017, 2021, 2022, 2023).

Our data consist of complete plastomes and nrDNA from 52 newly *de novo* genome skims, augmented with four plastomes from GenBank (Table S2). The resulting 56 plastome samples represented three species: 52 *C. filiformis*, with three *C. pubescens* and one *C. melantha* accessions used as outgroups. Genomic DNA from newly sequenced samples was extracted using a modified CTAB method (Doyle & Doyle, 1987) with a Tiangen DNA secure Plant Kit (DP305). The concentration and integrity of DNA were determined by gel electrophoresis and Nanodrop. From each purified sample of total DNA, greater than 1 μg was fragmented to construct shotgun libraries (500 bp insert size) with a TruSeq DNA Sample Prep Kit following the manufacturer's instructions (NEBNext® Ultra IITMDNA Library Prep Kit for Illumina®). Paired-end sequencing with 150 bp reads was performed on an Illumina

HiSeq2000 at BGI, with the aim of generating approximately 6 Gb of data for each sample. Raw reads were filtered to remove adaptors and low-quality reads using the NGS QC Toolkit (Patel & Jain, 2012) with default parameters.

2.2 Assembly, annotation, and comparison

Clean reads were assembled with GetOrganelle (Jin et al., 2020), which uses baiting and iterative mapping to assemble plastomes with minimal manual intervention. This approach integrates SPAdes (Bankevich et al., 2012), Bowtie2 (Langmead & Salzberg, 2012), BLAST+ (Camacho et al., 2009), and Bandage (Wick et al., 2015). The assembled plastomes were annotated using PGA (Qu et al., 2019) and GeSeq (Tillich et al., 2017) and comparison of published *Cassytha* plastomes (Wu et al., 2017; Song et al., 2019; Liu et al., 2021) led us to choose *C. filiformis* MH03 (GenBank No. MT621616) as the plastome reference for assembly and annotation. After annotation, a manual check was undertaken and the missing genes and gene boundaries were verified in Geneious Prime (https://www.geneious.com). The circular map of plastomes was drawn with CHLOROPLOT (https://irscope.shinyapps.io/Chloroplot/) and OGDRAW (Greiner et al., 2019). Assembly of nrDNA sequences provides a separate genomic region for comparative analysis and we recovered the 18S rDNA, ITS1, 5.8S rDNA, ITS2, and 26S rDNA clusters, with MAFFT (Katoh et al., 2019) used for sequence alignment, followed by a manual check using Geneious Prime. The annotated organelle genomes and nrDNA have been submitted to GenBank (accession numbers: OP476276-OP476327 and OP453368-OP453415). Single nucleotide polymorphisms (SNPs) from the plastomes and nrDNA were tried to analyze in STRUCTURE v2.3.4 (Pritchard et al., 2000), setting K from 1–10 with 20 replicates for each K value.

To visualize the extent of divergence between representative plastomes, we compared ten genomes from different tribes of Lauraceae. We choose *Neocinnamomum delavayi* (Lecomte) H.Liu KZ01 [MT621607] as the X-axis, since *Neocinnamomum* has a sister relationship with *Cassytha* (Rohwer & Rudolph, 2005). The tribes Cryptocaryeae, *Cryptocarya hainanensis* Merr. ZF10 [MT621586], Caryodaphnopsideae, *Caryodaphnopsis tonkinensis* (Lecomte) Airy Shaw GLQ08 [MT621583], Perseae, *Phoebe bournei* (Hemsl.) Yang SCH08 [MT621604], Cinnamomeae, *Cinnamomum camphora* (L.) J.Presl KZ05 [MT621650], and Laureae, *Litsea glutinosa* (Lour.) C.B.Rob. ZF03 [MT621605]), as well as different clades within *Cassytha* (choosing *C. pubescens* AZ01, *C. melantha* AZ04, *C. filiformis* MH01 as Type I, and *C. filiformis* MH03 as Type II, since MH01 was identified as *C. capillaris* initially, MH03 is the

reference in this study) were used in mVISTA (http://genome.lbl.gov/vista/index.shtml) (Frazer et al., 2004) in LAGAN mode. In addition, we extracted the plastid *trn*K gene intron from the 56 plastomes using Geneious Prime and compared them to a previously published alignment from the same region across *Cassytha* (Rohwer & Rudolph, 2005; Kokubugata et al., 2012).

2.3 Species discrimination and phylogenetic analyses

We recorded the proportion of species that resolved as monophyletic following phylogenetic analysis. The utility of different datasets for species identification was investigated using the tree-based approach ML (maximum likelihood) and BI (Bayesian inference) methods using IQTREE 2 (Minh et al., 2020) and MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best-fit model for each dataset was determined using ModelFinder (Kalyaanamoorthy et al., 2017), with the best-fit substitution model selected by –TEST using a tree search with 1,000 bootstrap replicates in a single run.

A total of 107 *Cassytha* individuals were used to investigate phylogenetic relationships, representing the 56 newly-extracted complete plastome samples reported here, plus 51 previously published *trn*K sequences covering nine species: *C. filiformis*, *C. capillaris, C. ciliolata* Nees, *C. glabella* R.Br., *C. melantha*, *C. muelleri* Meisn., *C. pergracilis* (Hatus.) Hatus., *C. pubescens*, and *C. rufa* J.Z.Weber adopted from NCBI (Rohwer & Rudolph, 2005; Kokubugata et al., 2012). The plastome is a single linkage unit that traces a single evolutionary history (dePamphilis et al., 1997), so to understand the relationships between plastomes and nrDNA better, further analysis of complete plastome and associated nrDNA (18S–ITS1–5.8S–ITS2–26S) data was undertaken for those 48 individuals for which nrDNA sequences assembly was successful. Discordance analysis of the 48 sequenced organelle genomes and nuclear DNA datasets was performed using ML and phytools was used to compare the resulting ML trees.

2.4 Molecular dating and estimation of divergence times

To calibrate the molecular dating of *Cassytha*, three reliable calibration points were used to constrain the root of Lauraceae, the stem age of *Neocinnamomum* and *Persea* group following Li et al. (2011; 2016) and Huang et al. (2016). Abundant and widespread fossil record of Lauraceae have been reported from the late early to late Cretaceous (e.g., Drinnan et al., 1990; Herendeen et al.,1994; Eklund, 2000; Takahashi et al.,1999, 2014). But unequivocal

fossil for molecular dating is scarce owing to ambiguous traits of the fossils (Li et al., 2011). Here, we adopted three reliable calibration points. Firstly, the molecular dating estimated of the crown node of Laurales (~107.7 Ma) (Doyle et al., 2008; Doyle & Endress, 2010; Massoni et al., 2015), which was supported by the fossil record (Friis et al., 1994). Secondly, the Cretaceous fossil *Neusenia tetrasporangiata* Eklund has well-preserved flower buds and shows a high degree of affinity with the extant taxa of *Neocinnamomum* H. Liu (Eklund, 2000; Atkinson et al., 2015). We use this fossil to date the stem node of *Neocinnamomum* (ca. 83 Ma) (Li et al., 2016). The tribes Perseae and Laureae diverged in the early Eocene (ca. 52 Ma) (Li et al., 2011), which is also supported by early Eocene fossils from Europe and North America (Li et al., 2016). In addition, *Alseodaphne changchangensis* J.H.Jin & J.Z.Li , a perfectly preserved fossil leaf from the late early to early late Eocene coal-bearing series of the Changchang Basin of Hainan Island, China (Li et al., 2009) was used to date the stem age of *Persea* group (Li et al., 2011; Huang et al., 2016; Qin et al., 2023).

Dating analyses were conducted using Markov Chain Monte Carlo (MCMC) methods in BEAST version 2.4 (Bouckaert et al., 2014). For setting the parameters of BEAUti, site model chose the “BEAST model test”, clock model chose “Relaxed Clock Log Normal” and “Yule Model” for speciation. To avoid overestimation of root age, we set parameter of *offset* at 108 Ma in lognormal distribution both with the mean “M” at 0.5 and the standard deviation “S” at 0.6. Two independent MCMC runs were performed with one cold chain and three heated chains for 1,000,000,000 generations and sampled every 10,000 generations. Effective sample sizes (ESSs) >200 for all parameters after the first 100,000 iterations were discarded as burn-in, as determined in Tracer V1.7.2 (Rambaut et al., 2018) and a maximum clade credibility (MCC) tree was generated using TreeAnnotator by setting “Mean heights” for the “Node heights” and visualized using FigTree version 1.4.4 (Rambaut, 2018).

2.5 Species distribution modeling (SDM) and niche overlap

SDM was carried out to predict suitable present climate envelopes for the *C. filiformis* Type I and Type II clades, using the MaxEnt 3.4.1 software package (https://biodiversityinformatics.amnh.org/open_source/maxent/). Sampling was undertaken for *C. filiformis* populations recognised by the FOC (Flora of China), CVH (Chinese Virtual Herbarium: http://www.cvh.ac.cn/class), PPBC (Plant Photo Bank of China: http://ppbc.iplant.cn), POWO (Plants of the World Online: http://powo.science.kew.org), Tropicos (http://www.tropicos.org), and the GBIF (Global Biodiversity Information Facility:

https://www.gbif.org). A total of 118 individuals were collected, covering almost the entire distribution of *C. filiformis* from across China (see Figure S1 and Table S1).

Environmental variables were selected from the Harmonized World Soil Database (HWSD) (FAO/IIASA/ISRIC/ISSCAS/JRC, 2012), Geospatial Data Cloud (http://www.gscloud.cn/sources) and WorldClim 1.4 (WorldClim 1.4 — WorldClim 1 documentation). All variables have a uniform spatial resolution of 2.5 min. To reduce modeling overfitting, we calculated Pearson's correlations among environmental variables. If two variables were highly correlated ($|r| > 0.8$), the one with higher contribution was selected, with 35 predictors (11 climate factors, 6 solar radiation, 4 wind speed, 11 soil factors, 3 topographic factors) used for final modeling (Table S3). The area under the Receiving Operator Curve (AUC) was used to evaluate model performance (Phillips & Dudík, 2008). The ecological niche divergence analyses (PCA-env analysis, niche overlap index, niche equivalence, and niche similarity) were based on the studies of Lin et al. (2021) and Tang et al. (2021). Niche overlap and the null hypothesis test were based on two similarity metrics in 'ecospat' package in R, using 1000 replicates to generate a pseudoreplicated null distribution.

2.6 Hemiparasite morphological observations and statistical analyses

We recorded the collection longitude, latitude, and altitude of all samples in the field. The flowering and fruiting durations were based on herbarium records and verified further by field observations from 2015 to 2023. Morphological characteristics of the two *C. filiformis* morphotypes were recorded following the methods of Liu et al. (2017; 2023), with character selection based on field observations and characters used in previous studies of the genus (Weber, 1981; Weber, 2007; Kokubugata et al., 2012; Liu et al., 2023). Morphological observations and photographs were taken using a Nikon D870 with a Stereo Microscope (Motic SMZ168-BL). Five reproductive morphological characters were documented for specimens using statistical analyses: flower size, inflorescence length, inflorescence thickness, fruit size and fruit shape index (Figure 2). However, as some individuals were sterile when sampled, not every collection could be used for morphological analysis (see Table S4). To determine which traits provided useful information, we examined statistically significant morphological differences using ANOVA. All statistical analyses used to assess differences in morphological characteristics between the two morphotypes were performed with GraphPad Prism 10 (One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 10.0.0 for Windows, GraphPad Software,

Boston, Massachusetts USA, www.graphpad.com). All data are presented as mean ± SD. All comparisons were tested using unpaired two-tailed Student's t-test, with $P \leq 0.05$ considered statistically significant.

3. Results

3.1 Plastome sequencing and general characteristics

Cassytha plastome sequences were completed for 56 individuals: 52 *C. filiformis* (34 Type I, 18 Type II) and two outgroup taxa consisting of three *C. pubescens* and one *C. melantha* sample (Figure 1A; Table S2). *Cassytha* is one of the earliest divergent groups of the inverted repeat-lacking clade (IRLC), which has lost one IR region and most NADH dehydrogenase (*ndh*) genes, *ndh*B, *ndh*C, *ndh*G, *ndh*I, *ndh*J, and *ndh*K, with remnants of some *ndh* regions as pseudogenes (Figure 3). As such this group does not possess the typical quadripartite structure (an LSC, an SSC, and a pair of IRs) of other Lauraceae (Figures S2-3). The *C. filiformis* plastome sizes ranged 114 (Type II) to 115 kb (Type I), but both types contained the same 102 unique genes, including 68 protein-coding genes, 30 tRNA genes, and four rRNA genes, with a GC content of 37% (Figure S2). GenBank accession numbers for all newly sequenced plastomes and nrDNA are reported in supplementary Table S2.

The aligned consensus length of the 56 complete plastomes was 124,798 bp and the corresponding extracted *trn*K gene matrix was 2,594 bp. The analysis of cytonuclear discordance for 48 aligned nrDNA (18S–ITS1–5.8S–ITS2–26S) sequences was 5,200 bp, with a corresponding plastome length of 124,432 bp, with organelle genome sizes very similar between accessions (Table S5). The largest plastome was *C. melantha* AZ04, with 118,123 bp (Table S6). We found plastome size varied by 555 bp across samples, with the two cryptic *C. filiformis* lineages having non-overlapping size ranges: the *C. filiformis* Type I (Figure 1A: Clade 1) plastome being larger (Figure S3 Type I: 114,955–115,158 bp) than Type II (Figure 1A: Clade 2) (Figure S3 Type II: 114,603–114,743 bp) based on the unaligned sequences, due in part to multiple large insertions. For example, there were deletions of up to 287 bp (the brown dashed box in Figure S4) between the gene *rpl*2 and *trnM*-CAU|*trnI*-CAU in Type II relative to Type I. However, there were also unique insertions and polymorphic structural features, such as a 71 bp insertion in individuals collected from Guangxi, Yunnan and Laos (Figure S4).

Variation occurred in the noncoding regions, with some variants also seen in coding *ycf*1 and *ycf*2 genes in comparison to other Lauraceae (Figure 3). Most variants within *C. filiformis* occurred in the noncoding regions, but some were seen in coding genes, including *trn*K, *rps*16, and *clp*P (Figure 3). Synteny and rearrangements have been detected in ten plastomes of Lauraceae, with significant synteny found here within the sampled *Cassytha* species, as well as other Lauraceae (*Cryptocarya hainanensis*, *Neocinnamomum delavayi*, *Caryodaphnopsis tonkinensis*, *Phoebe bournei*, *Cinnamomum camphora*, and *Litsea glutinosa*) (Figure S5).

3.2 Phylogenetic relationships and genetic structure

Phylogenetic relationships among different datasets were analysed and as the consensus trees from the ML and BI analyses were almost identical in their topologies, only the ML consensus tree based on the complete plastomes are presented here (Figure 1A), including with bootstrap support values and posterior probabilities. This tree contains two principal *C. filiformis* clades, with Clade 1 (Figure 1A, Bootstrap support values [BS] = 100%, Posterior probabilities [PP] = 1.00) including most individuals collected from South-East Asia (Laos and Thailand) and South-East China (Guangxi and Yunnan), supported strongly as the sister with Clade 2 (Figure 1A, BS = 100%, PP = 1.00) representing sequences from a range of pantropical regions China (Fujian, Taiwan), Indonesia, Japan, and Kenya. Samples in these two clades also co-occur in some regions, such as Guangdong and Hainan provinces (Figure 1B).

Phylogenetic trees based on nrDNA sequences had a very similar overall topology to the plastome but exhibited minor differences at interior nodes (AM01 and ZJ01, marked with dashed lines in Figure S6). All *C. filiformis* Type II accessions cluster as a monophyletic lineage, but without strong support in the ML and BI analyses, Clade 1 (BS = 80%, PP = 0.97) and Clade 2 (BS = 38%, PP = 0.81) (Figure S6B). The first clade consisted of all South-East Asia and South-East China accessions, while the second clade included all pantropical *C. filiformis* accessions plus two Guangdong and Hainan accessions, based on the phylogeny of plastomes (Figure S6). The monophyly of both clades received full branch support (BS = 100%, PP = 1) in the plastome tree (Figure S6A), while the clades in nrDNA were separated from modest (BS = 80%, PP = 0.97) and weak (BS = 38%, PP = 0.81) supports (Figure S6B).

After combining the *trn*K sequences and relating them to existing morphological characteristics, a few samples collected in China labelled as *C. capillaris* were unrelated to the sequenced Australian individual for this species and were instead nested within *C. filiformis* Type I (Figure 4). In addition, two samples identified as *C. filiformis* were nested with *C. ciliolata* (Figure 4); however, as these two samples were downloaded sequences, their identity cannot be verified easily. For those individuals which we sampled (see Figure 1; Table S2), we rechecked all sequences together with the morphology of our vouchers, herbarium specimens from E, HITBC, KEW and KUN, confirming that the sample labelled *C. capillaris* (MH01, 02, MF939338, SZ01) had been identified incorrectly and belongs to *C. filiformis* Type I (Figures 1, 4, S6).

The aligned matrix of the plastomes contained 124,432 single nucleotide polymorphisms (SNPs) and 5,200 SNPs of the nrDNA are used for the STRUCTURE analyses. The datasets revealed K = 3 is the best estimated value, suggesting that there were three distinct genetic clusters in our *Cassytha* datasets (Figure S7). Under this model, the outgroup samples from the Australian sites (*C. pubescens*: AZ01-03, *C. melantha*: AZ04) formed one cluster, the pantropical sites China (Fujian, Guangdong, Hainan, and Taiwan), Indonesia, Japan, and Kenya formed the second, with the South-East Asia (Laos and Thailand) and South-East China (Guangxi and Yunnan) accessions formed the third cluster. These clusters showed clear subdivisions and evidence of differentiation among samples. Each vertical bar shows the proportional representation of the estimated cluster membership for a single individual. The two forms of *C. filiformis* were separated more clearly based on plastomes than nrDNA. Gene flow (introgression) was detected among the two types, such as in ZJ01, ZH14, LS05, ZH03, LS03, AM01 and JFL01 (Figure S7). Such gene flow may increase the difficulty of recognizing morphological differences between *C. filiformis* Type I and Type II.

3.3 Morphological characteristics

The flowering and fruiting of *C. filiformis* in China were described in our previous study (Liu *et al*., 2023) and based on our observations from 2015–2023, *C. filiformis* blooms and fruits all year round (especially from May to December). We collected the ripened fruit of Type I in May and Type II in August and November, but several Type I individuals did not flower for more than three years.

The inflorescences of the *C. filiformis* samples observed here are spicate (mostly), capitate, or racemose (Figure 2A-i, -ii). The flowers bisexual possess verticillate bracts and bracteoles and are 1–1.5 mm diam. with six tepals in two whorls, nine fertile stamens in three whorls, one whorl of staminodes and a central fertile pistil. The persistent tepals are yellow to white, the outer three triangular, acute, very small with yellow pubescent and ciliate (Figure 2A-iii, -iv, -v); inner three ovate, acute, pubescent outside, glabrous inside, and yellow green to white, (Figure 2A-iii, -iv, -vi; Figure 2B). There are four whorls of stamens with three members in each whorl; the second outer whorl adnate to the tepals (Figure 2A-vi), the others free. Each member of the third whorls bears two lateral yellow glandular appendages (Figure 2A-vii). The anthers of the two outer whorls are introrse while those of the third whorl are extrorse. All anthers are bilocular and show valvular dehiscence (Figure 2A-iv, -vi, -vii). The fourth androecial whorl consists of staminodes (Figure 2A-vii). The ovary is monocarpellary, glabrous, narrow stylar canal, ca. 1.5–2 mm long (Figure 2A-viii).

No obvious morphological differences were seen between the flowers of Type I and Type II and there were no significant differences for inflorescence length, flower size, or fruit size. However, inflorescence thickness was significantly thicker for Type I (Figure 2C, P = 0.0281). Similarly, fruit shape index was also significant different between Type I and II (P=0.0065) (Figure 2C), with the fruits of most Type I ovoid, compared to globose in all Type II samples and some Type I (Figure 2B).

3.4 Estimation of divergence times

Divergence time estimates based on plastomes and nrDNA from BEAST and with the root constrained to 108.05 Ma (plastomes, 95% highest posterior density [HPD]: 108.20–107.06 Ma; Figure 5) and 110.74 Ma (nrDNA, 95% HPD: 110.89–108.93 Ma; Figure S8) are largely consistent with previous studies (Li et al., 2016; Chen et al., 2020). The crown age for *Cassytha* was estimated to be late Eocene: 37.04 Ma (plastomes, 95% HPD: 44.40–32.84 Ma; Figure 5, node 1) and 37.86 Ma (nrDNA, 95% HPD: 45.89–33.06 Ma; Figure S8, node 1). The split between the *C. filiformis* Type I and Type II clades was estimated as Oligocene to early Miocene 23.94 Ma (plastomes: 95% HPD: 34.10–18.74 Ma; Figure 5, node 2) and 29.62 Ma (nrDNA: 95% HPD: 37.39–26.26 Ma; Figure S8, node 2). Type I apparently then diversified during the early Miocene ~18.99 Ma (plastomes: 95% HPD: 23.93–13.31 Ma;

Figure 5, node 3) and ~16.12 Ma (nrDNA: 95% HPD: 25.86–6.28 Ma; Figure S8, node 5). In contrast, Type II appears to have radiated during the middle Miocene ~13.65 Ma (95% HPD: 17.13–6.92 Ma; Figure 5, node 4) based on the plastome sequences. In contrast, the nrDNA result showed that Type II was paraphyletic with two individuals of Type I and the clade radiated in the middle to late Miocene from 9.13 Ma (95% HPD: 4.41–16.37 Ma; Figure S8) to 13.41 Ma (95% HPD: 5.29–18.30 Ma; Figure S8).

3.5 Species distribution modelling and ecological niche divergence

For both the Type I and Type II clades, AUC values for potential distribution modelling were >0.90, indicating strong prediction accuracy, with potential distributions predicted by the model highly compatible with the occurrence point and current distribution predictions generally good representations of the actual distributions for both clades (Figure 6). The distribution of the two clades is influenced by environmental factors, with Type I affected by the Min Temperature of Coldest Month (bio 06) (66.4%, Figure 6A), indicating it is affected by severe fluctuations in ambient temperature. In contrast, the most important factors in shaping the distribution of Type II were Mean Temperature of Coldest Quarter (bio 11, 52.8%) and Temperature Seasonality (bio 04, 20.3%) (Figure 6B) as this taxon occurs within a narrower and warmer temperature range than Type I. However, although Type I is apparently more tolerant of lower temperatures and more severe ambient temperature fluctuations, its more montane habitat and the generally low base temperatures there means it is still vulnerable to prolonged or extreme cold.

Results from the climatic niche analysis of *C. filiformis* are shown in Figure S9. Principal component analysis (PCA) showed that the first two principal components could explain 74.9% of the parameter variables selected by correlation analysis (PC1 = 52.6%, PC2 = 22.3%). Based on the first two principal components, ecological niche dynamics of Type I and Type II within *C. filiformis* have a significant difference in the environmental needs (Figure S9A–C). The pair-wise comparison between the species environmental niche in Type I and Type II rejected the null hypotheses of niche equivalency and the niche similarity test was rejected ($P > 0.05$). The results of the niche equivalence and similarity tests further indicate that the niches of *C. filiformis* Type I and Type II have undergone significant changes during the speciation process (Figure S9D–G). In addition, the predicted occupied niche of

isothermality indicates there are considerable differences between the temperature needs of *C. filiformis* Type I and Type II (Figure S9H).

4. Discussion

4.1 Phylogenetic inference and the discovery of cryptic species

Parasitic plant diversity is often cryptic, as they tend to live hidden lives and often have complex, specialized and/or reduced morphological adaptations for parasitism (Nickrent, 2020). There are relatively few definitive morphological characters for *Cassytha* (Weber, 1981, 2007) and the inter- and intrageneric phylogenetic relationships of *Cassytha* have been disputed in previous studies (Rohwer & Rudolph, 2005; Wang et al., 2010; Kokubugata et al., 2012) and remain largely unresolved. In this study, we used genome skimming to recover the complete plastome and nrDNA array from geographically widespread samples of *C. filiformis*, revealing two well supported clades within the taxon as currently defined (Figures 1A, S6, S7).

Previous phylogenetic studies including *Cassytha* always use *C. filiformis* as a representative species (Chanderbali et al., 2001; Wang et al., 2010; Li et al., 2016; Wu et al., 2017; Song et al., 2019), as it is a widespread pantropical taxon that is easy to collect. However, the samples of *C. filiformis* did not resolve as monophyletic by means of *trn*K sequence (Kokubugata, 2012), partly due to a lack of sequence variation and potential identification errors (Figure 4). In the case of widespread and variable taxa, the inclusion of multiple individuals from different regions can often help improve species delimitation, so the current study covered a wide area, including Chinese islands (Hainan, Taiwan), continental China (Fujian, Guangdong, Guangxi, Yunnan), Australia, Indonesia, Japan, Kenya, Laos, and Thailand (Figures 1B, S1), representing the species distribution across the Old World, but with particularly detailed sampling across China. The South-East Asian and South-East China *C. filiformis* samples formed a highly supported clade (Type I) separate from a pantropical (Type II) clade (Figure 1A) and while these two clades have a partially sympatric distribution in Guangdong and Hainan (Figure 1B), they clearly belong to different genetic lineages. Overlapping distributions between these two types may be the results of population expansion. Multiple clusters were also found at some sites, which may imply gene flow among sites and/or multiple introductions to the same site (Figure S7). The morphological

variation seen here is linked strongly to genetic components, increasing the difficulty of separating these cryptic lineages using morphology.

Although some morphological traits also overlap, inflorescence thickness and fruits shape index help to distinguish the *C. filiformis* Type I and Type II lineages (Figure 2), suggesting that they represent at least two cryptic species. Cryptic taxa within *Cassytha* may have been overlooked, especially in the *C. filiformis* complex, since most *Cassytha* species delimitation is based on morphology and there are relatively few distinguishing characteristics (Weber, 1981, 2007). There is some molecular and anatomical evidence that there are cryptic taxa within some Australian species, including *C. filiformis* (Conran, unpubl. obs.) and this is the subject of genetic and morphological investigations.

Cryptic species are not only limited to *Cassytha* and are seen in other parasites, such as the hemiparasitic Orobanchaceae genera *Phtheirospermum* Bunge ex Fisch. & C.A.Mey. (Yu et al., 2018), *Pedicularis* L. (Liu et al., 2022) and *Euphrasia* L. (Garrett et al., 2022), as well as the holoparasitic *Cuscuta* sect. *Californicae* (Yunck.) Costea & Stefanović (Costea et al., 2020). Cryptic species are important for a number of reasons, not least species conservation, as rare taxa cannot be conserved until species boundaries are established and distributions known. The correct identity of cryptic parasite species is also relevant to more applied areas such as food security, where a lack of taxon-specific knowledge about host preferences and biocontrol measures may inform actions to prevent crop losses (Palomares-Rius et al., 2014). A survey of Chinese *C. filiformis* host plants shows that it grows mainly on trees and shrubs from phylogenetically divergent members of the rosid and asterid eudicot clades, often attacking multiple adjacent hosts simultaneously, and forming extensive colonies (Liu et al., 2023). Future *Cassytha* research should focus on combining genomic and morphological approaches and host preferences, to address the true scale of species diversity in this enigmatic group.

4.2 Plastome divergence

Parasitic plants frequently demonstrate functional reductions in plastid genes and major modifications to plastome structure due to relaxed selection pressure with the transition to (partial) heterotrophy. However, few studies to date have generated plastomes from multiple individuals within and between closely related species, therefore population-level patterns of variation remain unknown. The *C. filiformis* plastomes in this study were around 114 (Type II) to 115 kb (Type I) (Figure S3; Table S6), which is slightly lower than *C. pubescens* (~117

kb) and *C. melantha* (~118 kb), but greatly reduced relative to non-parasitic Lauraceae plastomes (~148–158 kb) and due mainly to the loss of one IR copy, as well as the *ndh* genes (Figures 3, S5). Loss or pseudogenization of *ndh* genes occurs in a range of heterotrophic plant groups, such as *Cuscuta* (McNeal et al., 2007), *Epifagus* Nutt. (dePamphilis & Palmer, 1990), and some mycotrophic orchids (Kim et al., 2015; Barrett et al., 2018). Moreover, similar IR losses have also occurred in non-parasitic gentians (Fu et al., 2021) and legumes (Choi et al., 2019) with a predominantly herbaceous habits, suggesting losses in this gene family may occur readily and not just with transitions to parasitism, possibly as a response to stressful conditions such as low- or variable-light environments (Barrett et al., 2018).

4.3 Cryptic differentiation of Cassytha filiformis complex

The *C. filiformis* complex contains at least two species lineages, strongly supported by the plastome dataset, modest to weak support by the nrDNA dataset (Figures 1, S6), and diagnosable by morphological characters (Figure 2). The prediction of the potential geographical distribution of *C. filiformis* complex in southern China and adjacent regions also found that the potential distribution area of each clade showed clear environmental differences (Figure 6A, B). Phylogenetic relationships between the two lineages were largely consistent between the plastome and nrDNA datasets, but molecular dating was inconsistent between the plastome and nrDNA datasets. Perhaps, the relatively low resolution of the nrDNA tree and the occurrence of gene flow could have led to this phylogenetic conflict. However, heterogeneity between plastome and nrDNA datasets might have also played a contributing role.

Environmental factors can affect the spatial distribution of species, as well as their habitat suitability (Kong et al., 2017; Zhang et al., 2019; Huang et al., 2023). The stability and variability of the East Asian monsoon (EAM) is associated with temperature, wind speed, and surface incoming solar radiation (Xu et al., 2006), with many species groups diversifying rapidly following the establishment of the EAM in southern China during the mid-Miocene (Kong et al., 2017). In this study, the estimated ages for the origin of *C. filiformis* complex and subsequent population-level divergences fell into the range of the East Asian monsoon establishment and intensification. Of the two *C. filiformis* lineages Type I is apparently more tolerant of low temperature and sharp fluctuations in ambient temperature than Type II (Figure 6A, B), so we speculate that Type I is better adapted mountainous environments, while Type II is more suited to warmer, more stable coastal environments, as shown in their

predicted potential geographical distributions (Figure 6C, D). These results suggest that environmental factors (temperature, solar radiation, wind speed, and water) could play an important role in predicting the potential distribution areas of these cryptic taxa, but this requires phylogeographic studies using more dense population samplings and multiple individuals per population.

4.4 Performance of the species distribution modelling, niche divergence and gene flow

A major goal of ecology is in the inspection of niche divergence to explain rapid lineage diversification and mechanisms of morphological evolution across clades (Lin et al., 2021). Species distribution modelling can take the nonlinear relationship between the distribution of species and environmental factors (De Marco Jr et al., 2008; Pecchi et al., 2019; Tang et al., 2021). Generally, climate is considered having a close relationship with species distributions, as well as providing basic information on suitable habitats (Medlock et al., 2013; Uden et al., 2015). Some climate variables may contribute to the cryptic differentiation between *C. filiformis* Type I and Type II, such as min temperature of coldest month (bio 6) and mean temperature of coldest quarter (bio 11) (Figures 6, S9). Our ecological niche model provides a sufficiently accurate estimation for *C. filiformis* Type I and Type II (Figure S9). Based on the PCA-env analysis, there is considerable variation in the niche space of *C. filiformis* between Type I and Type II. The niche overlap results showed high overlap between Type I and Type II ranges (Figure S9), which may provide the chances for exchanging DNA between them. Gene flow (introgression) produced phylogenetic conflict in species delimitation (Chan et al., 2023), potentially shaping the morphological variation and evolution of *C. filiformis* Type I and Type II (Figure S7). For example, if some genome components were less prone to introgression than others, they should be particularly suitable to delimitate species (Petit & Excoffier, 2009). Here the plastome sequences were much more suitable to delimitate *C. filiformis* cryptic types than the nrDNA sequences (Figure S7).

Niche equivalency evaluates whether the environmental conditions differ between communities and niche similarity evaluates the similarity in the relative distributions of environmental conditions over longer periods of time. Tests of equivalence and similarity revealed that *C. filiformis* Type I and Type II differed in their environmental niches (Figure S9). Similarly, the niche similarity results suggest that there is no significant climatic niche conservatism between Type I and Type II, but the predicted occupied niches of isothermality between Type I and Type II are different. In conclusion, underlying genetics and niche

588 divergence may both contribute to the difficulties seen in classifying these two cryptic
589 lineages using morphology.

590 5. Conclusions

591 Our analyses of *C. filiformis*, combining molecular phylogeny, morphology, and
592 distribution patterns, strongly suggest that topological constraints, reinforced by subsequent
593 differential climatic adaptations have resulted in cryptic lineage divergence leading to the
594 formation of two discrete taxonomic entities: Type I: distributed in South-East Asia and
595 South-East China and Type II: distributed in pantropically. Although the Type II entity may
596 well include other cryptic taxa from outside China, a worldwide study of the complex was
597 beyond the scope of the present study.

598 These findings suggest that cryptic diversity in parasitic plants is probably higher than
599 morphology alone would suggest, and that further investigation of widespread and
600 polymorphic taxa may help improve taxon definition and conservation. The study shows that
601 a combination of geographic and climatic factors has played a fundamental role in promoting
602 diversification and evolution of species in the tropical and subtropical zones, and that these
603 processes may give a good instruction for parasitic plant speciation studies.

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Data Accessibility and Benefit-Sharing

GenBank numbers, plastomes OP476276-OP476327 and nrDNA OP453368-OP453415 (see Table S2 for accessions).

Conflict of Interests

The authors have declared that no competing interests exist.

Author Contributions

JL, Z-FL, X-QC, ADT, PMH, conceived and designed the research. Z-FL, S-FZ, X-YZ, J-LH, J-CT, G-DT, S-YQ, XD, LL, H-HM, L-ND, TH, HM, J-HX, C-NC, did the sampling. Z-FL, S-FZ, X-YZ, LH, conducted the experiments, analysed the data. Z-FL wrote the manuscript. ADT, JGC, PMH, JL, X-QC, QW contributed to the revision of the manuscript.

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Figure Legends

Figure 1. Plastomes phylogenetic tree and geographic analyses of *Cassytha filiformis*. (**A**) Phylogenetic tree showing Type I (red) and Type II (blue) clades; Numbers above branches indicate likelihood bootstrap percentages (BS) and Bayesian posterior probabilities (PP). (**B**) Geographic origins of *C. filiformis* worldwide and enlarged view of the collection sites in Southern China. Sample sites are color-coded by red (Type I) and blue (Type II) dots corresponding to the phylogenetic clades. The base map was downloaded from the Standard Map Service System (http://bzdt.ch.mnr.gov.cn; No. GS (2024) 0447)

Figure 2. Morphological characters and statistical analyses of *Cassytha filiformis*. (**A**) Images of inflorescence and flower morphology in the two types of *C. filiformis*. i. the inflorescence of Type I; ii. the inflorescence of Type II; iii. the external structure of the flower; iv. the internal structure of the flower; v. the outer tepals; vi. the inner tepal with the first whorl of stamen; vii. the second to fourth whorls of stamens; viii. ovary. (**B**) Images of fruit in the two types of *C. filiformis* (**C**) Relationship between morphological trait measurements made in reproductive wild-collected herbarium specimens for diverse *C. filiformis*. Trait variation in flowers, inflorescences, and fruits. The black dots indicate each individual measurement. *P* values were determined by using 1-way ANOVA with Tukey's multiple comparisons test. Data are presented in Table S4.

Figure 3 Comparison of the *Cassytha filiformis* complex plastome types with other related species using mVISTA. (**A**) The variation between *Cassytha* and other Lauraceae. (**B**) The variation within *Cassytha*.

Figure 4. Phylogenetic relationships of *Cassytha* based on 107 *trn*K sequences. Phylogenetic tree showing Type I (red), Type II (blue) and outgroup (green) clades. The samples labelled as red were wrongly identified as *C. capillaris* initially; the sample labelled as green were the correct *C. capillaris* collected in Australia; the samples labelled as purple were identified as *C. filiformis* (suspected identification error) but nested with *C. ciliolata*. Numbers above branches indicate likelihood bootstrap percentages (BS) and Bayesian posterior probabilities (PP). Dashes indicates no support.

Figure 5. A simplified maximum clade credibility tree of *Cassytha* from BEAST divergence time analysis. The estimated age of main nodes is presented above the branch. Node bars represent the 95% highest posterior density (HPD) interval. Five key stem/crown nodes (black) were annotated by numbers.

Figure 6. The percentage contribution of important environmental variables for the modern distribution of two types in *Cassytha filiformis* and its distribution pattern of potential habitat suitability in Southern China and adjunct regions. (**A, B**) The cumulative contribution of top five factors for both are more than 90%. (**A**) Bio 6 = Min Temperature of Coldest Month; Wind 12 = Wind speed of December; Bio 11= Mean Temperature of Coldest Quarter; Srad 11= Solar radiation of November; Bio 01= Annual Mean Temperature. (**B**) Bio 11= Mean Temperature of Coldest Quarter; Bio 04 = Temperature Seasonality (standard deviation×100); Bio 01= Annual Mean Temperature; Srad 06= Solar radiation of June; Bio 14 = Precipitation of Driest Month. (**C, D**) The distribution pattern of potential habitat suitability for *C. filiformis* Type I and Type II.

Supporting Information Legends

Figure S1 The collection sites of *Cassytha filiformis* in this study.

Figure S2 The plastome structure of *Cassytha filiformis*.

Figure S3 Structures comparison of the two types of plastome of *Cassytha filiformis*.

Figure S4 Partial sequence alignment of the two types of plastome in *Cassytha filiformis*.

Figure S5 Synteny and rearrangements detected in *Cassytha* and other Lauraceae plastomes using the Mauve multiple-genome alignment program.

Figure S6 The cytonuclear discordance between 48 GetOrganelle assembled plastomes and nrDNA sequences based on ML analyses.

Figure S7 Individual assignment probability barplots from the genetic clustering analysis. K = 3 is the best fit for our data.

Figure S8 A simplified maximum clade credibility tree of *Cassytha* from BEAST divergence time analysis based on the nrDNA.

Figure S9 Niche of *Cassytha filiformis* Type I and Type II under climatic ecological space. (A) The principal component analysis (PCA) of climate variables. **(B)** Contribution of the variable for PC-1. **(C)** Ecological niche dynamics of Type I and Type II within *C. filiformis* in the environmental space described by the first two principal component axes. The colour of blue indicates predicted ecological niche overlap, green indicates predicted ecological niche for Type I, and red indicates predicted ecological niche for Type II; The solid and dashed contour lines illustrate, respectively, 100% and 50% of the available environment. **(D-E)** Histograms of niche equivalency distributions of Type I and Type II, diamond lines represent observed values. **(F-G)** Histograms of niche similarity distributions of Type I and Type II, diamond lines represent observed values. **(H)** Predicted occupied niche of isothermality (bio11) between Type I and Type II within *C. filiformis*.

Table S1 The locations and geographic coordinates of *Cassytha filiformis* in this study.

Table S2 The sequences taxa, locations, geographic coordinates and GenBank accession numbers.

Table S3 Species occurrence data used for species distribution modeling.

Table S4 Some characters subjected to morphological analysis.

Table S5 Comparison of characteristics of different data sets in *Cassytha*.

Table S6 The organelle sizes of different *Cassytha filiformis* individuals.