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Camellia neriifolia and *Camellia ilicifolia* (Theaceae) as separate species: evidence from morphology, anatomy, palynology, molecular systematics

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Abstract

Background The systematic status of sect. *Tuberculata* and its taxonomy have recently attracted considerable attention. However, the different bases for defining the characteristics of sect. *Tuberculata* has led to many disagreements among the plants in this group. *Camellia neriifolia* and *Camellia ilicifolia* have been the subject of taxonomic controversy and have been treated as different species or varieties of the same species. Therefore, it is important to use multiple methods, i.e., integrative taxonomy, to determine the taxonomic status of *C. neriifoli*a and *C. ilicifolia*. This is the first study to systematically explore the taxonomic position of these two plants on the basis of Morphology, Anatomy, Palynology and Molecular Systematics.

Results Extensive specimen reviews and field surveys showed that many differences exist in *C. neriifolia* and *C. ilicifolia*, such as the number of trunk (heavily debarked vs. slightly peeling), leaf type (smooth thin leathery, shiny vs. smooth leathery, obscure or slightly shiny), leaf margin (entire vs. serrate), flower type (subsessile vs. sessile), number of styles (3–4 vs. 3), and sepal (ovate vs. round). Moreover, *C. neriifolia* has a more distinctive faint yellow flower color, and trunk molting was more severe in *C. neriifolia* than that in *C. ilicifolia*. In addition, micromorphological analysis of the leaf epidermis showed that the two species differed in the anticlinal wall, stomatal apparatus, and stomatal cluster, and pollen morphology analyses based on pollen size, germination furrow, and polar and equatorial axes showed that they are both distinct from each other. The results of the phylogenetic tree constructed based on the whole chloroplast genome, protein-coding genes, and ITS2 showed that both *C. ilicifolia* and *C. neriifolia* were clustered in different branches and gained high support.

Conclusions The results combine morphology, anatomy, palynology, and molecular systematics to treat both *C. neriifolia* and *C. ilicifolia* as separate species in the sect. *Tuberculata*, and the species names continue to be used as they were previously. In conclusion, clarifying the taxonomic status of *C. neriifolia* and *C. ilicifolia* deepens our understanding of the systematic classification of sect. *Tuberculata*.

Keywords *Camellia neriifolia*, *Camellia ilicifolia*, Molecular systematics, Separate species, Taxonomic revision

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Background

Camellia L. sect. *Tuberculate* H. T. Chang, which is set up on the basis of "*Camellia tuberculata* Chien", a species with "tuberculate protuberances on the surface of the fruits," is considered to be a specialized group of *Camellia* that retains its original shape (Chang 1981; Min and Zhong 1993). The classification of sect. *Tuberculate* began in 1939, and this section was set up by the famous botanist Prof. Chong-Shu Qian (Chien 1939). Subsequently, many new species within the tea group were gradually discovered (Chang 1981; Min and Zhong 1993; Chang and Ren 1991, 1996; Min 1999). Initially, Sealy divided the genus *Camellia* into 12 sections based on macroscopic morphological studies and field investigations, and *C. tuberculata* was placed under sect. *Heterogenea* Sealy (Sealy 1958). However, Chang (1981) thought that Sealy classification system does not reveal the evolutionary level or relationships of the divided taxa, thus he revised the genus *Camellia* and first proposed the section '*Tuberculate*' on the basis of tuberculate shape of the fruit, and meanwhile, he named the newly discovered *C. ilicifolia* in Guizhou and assigned it to sect. *Pseudocamellia* Sealy Rev. *Camellia neriifolia*, another species in sect. *Tuberculate* described by him in 1984 (Chang 1984), is distinct by its glabrous shoots, cuneate or slightly rounded leaf base, glabrous capsules, verrucose protuberances, and other features in the protologue. It is noteworthy that the type locality of both species is Jinshagou, Chishui County, Guizhou Province. Min et al. revised the genus *Camellia* based on specimen studies, grouping the original 230 species into 119 taxa. He believed that there was an intimate evolutionary relationship between sect. *Pseudocamellia* and sect. *Tuberculate* and moved *C. ilicifolia* from sect. *Pseudocamellia* into sect. *Tuberculate*, and merged the *C. ilicifolia* with the *C. neriifolia* in a combined treatment (Min and Zhong 1993). However, Chang thought that the taxonomic traits of the two capsules show essential differences between *C. neriifolia* and *C. ilicifolia*, therefore he did not agree with Min and Zhong (Chang and Ren 1996). However, the taxonomic positions of these two species have not been accurately determined.

In recent years, Jiang (2010) classified *Camellia* L. based on leaf characteristics, and the findings indicated that the epidermal characteristics of the leaves of *C. neriifolia* and *C. ilicifolia* are similar, supporting the merger of the two. However, molecular phylogenetic of 146 species of plants within *Camellia* conducted by him (Jiang 2017) based on molecular data (*mat*K, *rbc*L, *ycf*1, and *trn*L-F) revealed that *C. neriifolia* and *C. ilicifolia* belong to different groups. Wu (2022) studied the phylogeny and evolution of secondary product metabolism in 166 species of *Camellia* L. using comparative transcriptomics, showed that sect. *Pseudocamellia* and sect. *Tuberculate* converges on the same branch. However, *C. ilicifolia* was not included in this study. In additon, the taxonomic status of *C. neriifolia* and *C. ilicifolia* remains unclear.

During plant evolution, the basic organizational structure and morphological features of plants have remained relatively stable and vary between species because of their genetics and adaptation to the environment (Zhou 2000). The flower, fruit, and leaf characteristics of plants have been widely used to classify Theaceae (Shen et al. 2008; Li et al. 2001; Luo et al. 1999). Pollen is a type of reproductive cell produced during plant reproduction (Hornick et al. 2021), and it has unique morphological features that can reflect the genetic and ecological characteristics of plants (Zhang et al. 2016). In plant classification, pollen features such as morphology, size, and outer wall ornamentation can be used to aid in identification (Fuchs 1967). Pollen ornamentation is diverse in type, complex in outer edge structure, and varies from one type of plant to another (Shi et al. 2022). Its morphology is mainly controlled by genes and is highly genetically conserved, providing a great deal of taxonomic information (Zhang et al. 2022). Previous studies have emphasized the importance of pollen morphology in Theaceae identification. Although sect. *Tuberculate* is a special group in Theaceae, there were few studies on pollen morphology and only sporadic reports on individual species (Wei et al. 1992). At present, there is no comprehensive field practice survey for *C. neriifolia* and *C. ilicifolia*, and their extant specimens are incomplete in terms of flower, fruit, and leaf characteristics. It is difficult to classify them on the basis of specimens, and they remain to be investigated. Chloroplast genome with relatively stable genetic information and high-resolution taxonomic information, thus revealing the evolution, origin, and affinity among species (Huang et al. 2022; Jiang et al. 2022). Therefore, the combination of morphological and phylogenetic analyses can provide more complete and accurate classification results (Lee and Palci 2015). In this study, *C. neriifolia* and *C. ilicifolia* were comparatively analyzed using traditional morphology, anatomy, palynology, and molecular systematics, to clarify the identities of the two taxa.

Material and methods

Materials collection

C. neriifolia and *C. ilicifolia* used in this study were obtained from Chishui City, Guizhou Province (former from Hutou Mountain, Yuanhou, and latter from Jinshagou). Photographs were obtained during the field surveys. The young leaves were preserved in sealed bags and subjected to DNA extraction. The specimens were maintained in the Tree Herbarium of the School

of Forestry, Guizhou University (GZAC) (LZ-20221016, LZ-20221108).

Morphology of plants

The habitats, trunks, branches, leaves, flowers, and fruits of both species were observed in their natural states using morphological methods. Leaf texture, color (both abxial and adaxial of the leaf), shape and shape of the leaf margin, leaf base, and leaf vein characteristics; petal size, number, color, presence or absence of persistent sepals, and number of styles; fruit shape, size, color, and folds of both species; and color and shape of the outer bark of the trunk and branches were observed. Statistical analyses were performed using Excel 2010 (Table S1).

Plant leaf epidermis

Leaves from both plants (used for morphological observations) were cleaned and preserved by immersion in an FAA solution (70% ethyl alcohol, acetic acid, and formaldehyde in the ratio 90:5:5). When leaf observation was required, the leaves were removed from the FAA solution, and the residual solution was washed away with water. Leaf pieces were cut in the midvein region of the leaf according to an area of 0.5 cm × 0.5 cm, placed in 100% sodium hypochlorite, dissociated in a 30 °C thermostatic water bath, and then fished out when the leaf pieces were white. Leaf pieces were removed with tweezers and small razor blades to remove all leaf flesh and obtain a clean leaf epidermis. The clean upper and lower leaf epidermis was fashioned into clinical slices, stained with magenta acetate solution, placed under an optical microscope for careful observation, and the results obtained were kept in the image. The micromorphology of the upper and lower leaf epidermis, including the pericyclic wall and stomatal apparatus, was observed at different magnifications, and data were obtained and analyzed using image processing software.

The characteristics of stomata were from the fresh leaves of both species. Use a sharp blade to cut and harvest fresh tissue blocks quickly within 1–3 min. Leaf tissue was gently washed with PBS. The washed tissue blocks are immediately fixed by electron microscopy fixative for 2 h at room temperature, then transferred into 4 ℃ for preservation and transportation. Wash tissue blocks with 0.1 M PB (pH 7.4) for 3 times, 15 min each. Then transfer tissue blocks into 1% OsO4 in 0.1 M PB (pH 7.4) for 1–2 h at room temperature. After that, wash tissue blocks in 0.1 M PB (pH 7.4) for 3 times, 15 min each. Dry samples with Critical Point Dryer. Specimens are attached to metallic stubs using carbon stickers and sputter-coated with gold for 30 s. Observe and take images with scanning electron microscope.

Microscopic morphology of pollen

For *C. neriifolia* and *C. ilicifolia*, respectively, the pollen of five healthy and consistent degrees of openness of the plant was collected according to the acetic anhydride decomposition method to deal with the pollen (Ao and Liu 2001), and then the treated pollen was placed under the scanning electron microscope, which can be observed and measured to determine the shape of pollen grains, the size of pollen grains, pollen germination grooves, pollen grains outer surface ornamentation, the equatorial plane view, and the polar surface view, and at the same time, leave the image to be preserved. The pollen images from the SEM were processed using the Image Tool image processing software to obtain data for the two species. The pollen size was expressed as the average of the equatorial (E) and polar (P) axes, and the length of the pollen germination furrow was measured at the same time.

Chloroplast DNA extraction, assembly and annotation

The collected dried young leaves were used, and the modified CTAB method (Chen et al. 2004) was selected for the extraction of DNA from *C. neriifolia* and *C. ilicifolia* leaves. DNA integrity was detected by 1% agarose gel electrophoresis, and DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer. DNA was then subjected to random interruption, end repair, and junction ligation to construct sequencing libraries. Finally, the libraries that passed the quality test were sequenced using an Illumina high-throughput sequencing platform. Low-quality data were trimmed using the Trimmomatic software (Bolger et al. 2014). The filtered qualified CP sequences were compared with the sequences from sect. *Tuberculate* in the National Center for Biotechnology Information (NCBI) database and the filtered data were clipped from scratch using SOAPdenovo and NOVOPlasty software (Luo et al. 2012; Dierckxsens et al. 2017), to obtain the circular chloroplast genome sequence. Finally, the complete CP genome was obtained by online annotation, BLAST comparison, and manual correction, using *C. rubituberculata* Chang & Yu (MZ424202) as the reference sequence. The cp genome was mapped using the OrganellarGenomeDRAW (OGDRAW) software online tool (Lohse et al. 2007). ITS universal primers ITS1 and ITS4 (Xu et al. 2024), and PCR conditions were as follows: first 95 ℃ for 5 min, 1 cycle of 94 ℃ for 1 min, second 50 ℃ for 1 min, 72 ℃ for 50 s, 30 cycles of 95 ℃ for 1 min, then 50 ℃ for 1 min, 72 ℃ for 1 min, and 72 ℃ for 10 min. 72 ℃ for 1 min and 72 ℃ for 10 min. Finally, gel electrophoresis was performed, and the qualified samples were sent to Wuhan Prime Biotech for sequencing. The annotated

chloroplast genome and ITS were uploaded to the NCBI database to obtain GenBank accession numbers.

IR boundary expansion and contraction

The chloroplast genome sequences of *C. neriifolia* and *C. ilicifolia* were selected, and four chloroplast genome sequences were obtained from four sections. *Tuberculate* species data were downloaded from the NCBI for Biotechnology Information database. IR region boundary expansion and contraction analyses were performed, and comparative maps were plotted using IRscope (https://irscope.shinyapps.io/irapp/) (Amyiryousefi et al. 2018) online software.

Phylogenetic tree analysis

The chloroplast genome sequences of 18 species of the genus *Camellia* were downloaded from NCBI, and 21 chloroplast genomes were phylogenetically analyzed using the species *Apterosperma oblata* (GenBank accession number: NC035641) as an outgroup. Sequence comparisons were performed using MAFFT 7 (Katoh and Standley 2014) phylogenetic trees were reconstructed using the maximum likelihood (ML) method in IQ-TREE v1.6.12 (Nguyen et al. 2015), manually corrected with Mega X (Kumar et al. 2018), and the best tree building model was selected. The optimal model (GTR + I + G) was determined using MrModeltest v2.3, and finally, a Bayesian (BI) phylogenetic tree was reconstructed using MrBayes v3.2.7 (Huelsenbeck and Ronquist 2001). Phylogenetic trees were constructed based on protein-coding genes with ITS2 in the same way as for the whole chloroplast genome. Finally, a phylogenetic tree was constructed using iTOL (Interactive Tree Of Life) v4 (https://itol.embl.de/) (Letunic and Bork 2021) online tool.

Result

Morphological characteristics of *C. neriifolia* and *C. ilicifolia*

A detailed morphological comparison between the two species was performed. The results showed (Figs. 1, 2, Table 1) that there were some differences between the two species. For example, the differences in trunk (heavily debarked vs. slightly peeling), leaf type (smooth thin leathery, shiny vs. smooth leathery, obscure or slightly shiny), leaf margin (entire vs. serrate), flower type (subsessile vs. sessile), number of styles (3–4 vs. 3), and sepal (ovate vs. round) were more obvious between the *C. neriifolia* and *C. ilicifolia*.

Leaf epidermal characteristics of *C. neriifolia* and *C. ilicifolia*

Images obtained under the electron microscope showed (Fig. 3, Table 2) that the epidermis of the *C. neriifolia* leaves be distinctly deeply wavy in the anticlinal wall of the upper epidermis and wavy in the lower epidermis. However, the leaf hypodermis of *C. ilicifolia* leaves was shallowly undulating, and that of the upper epidermis was shallower compared to that of the leaf hypodermis. The upper epidermis of the *C. neriifolia* has a smaller number of wavy peaks on the anticlinal wall, and the leaf hypodermis has more wavy peaks. In contract, the number of peaks in the periplane of the upper and leaf hypodermis of *C. ilicifolia* is less, and the leaf hypodermis is slightly more than that of the upper epidermis.

The stomatal apparatus (annular type, with three secondary guard cells surrounding the guard cells) of both *C. neriifolia* and *C. ilicifolia* was irregularly arranged and scattered only in the leaf hypodermis of the leaf blade, with no distribution observed in the upper epidermis. The stomatal apparatus of *C. neriifolia* was subrounded and occasional stomatal clusters were observed under a microscope. Two to three rings of folds were observed when the individual stomatal apparatus was magnified; these rings were presumed to be piled-up keratin-like material. The length of the stomatal apparatus was counted to be 18–25 μm, the width of the stomatal apparatus was 19–24 μm, and the stomatal density was 94–113 number/mm^2. The stomatal apparatus of *C. ilicifolia* was broadly elliptic; no stomatal clusters were found under the microscope; no wrinkles were seen under magnification; the length of the stomatal apparatus was 26–35 μm, the width of the stomatal apparatus was 24–31 μm, and the stomatal density was 70–85 number/mm^2 (Table S2).

The leaf epidermis of both plants were observed under a scanning electron microscope. The results showed (Fig. 3, Table 2) that the morphology of the inner margin of outer rim was sinuolate in both *C. neriifolia* and *C. ilicifolia*. However, the cuticular ornamentation outside of the former is an ring edge, strip and ridge hunch, and that of the latter is an unconspicuous ring edge, a few ridge hunch.

Palynology characteristics of *C. neriifolia* and *C. ilicifolia*

The results of the study showed (Table 3, Fig. 4) that the size of *C. ilicifolia* pollen grains was 31–35 μm, the morphology of pollen grains was oblate spherical, trilobate orthotropic in polar view, pike-shaped in equatorial view, the polar-anodal ratio was 0.73–0.75, P × E = 24.74–25.15 × 33.56–33.92 μm, and pollen germination pores were of 3-pore furrow type; the pollen wall ornamentation was wrinkled wave to granular, with coarse reticulation ridges, shallow inter-ridge stripes, and deep and wide reticulation mesh. The size of *C. neriifolia* pollen grains was 32–36 μm; the morphology of the pollen grains was subglobular, trilobate deltoid in polar view and ellipsoid in equatorial view; the polar to equatorial ratio was 1.03–1.05, P × E = 36.14–36.72 × 35.06–35.64 μm;

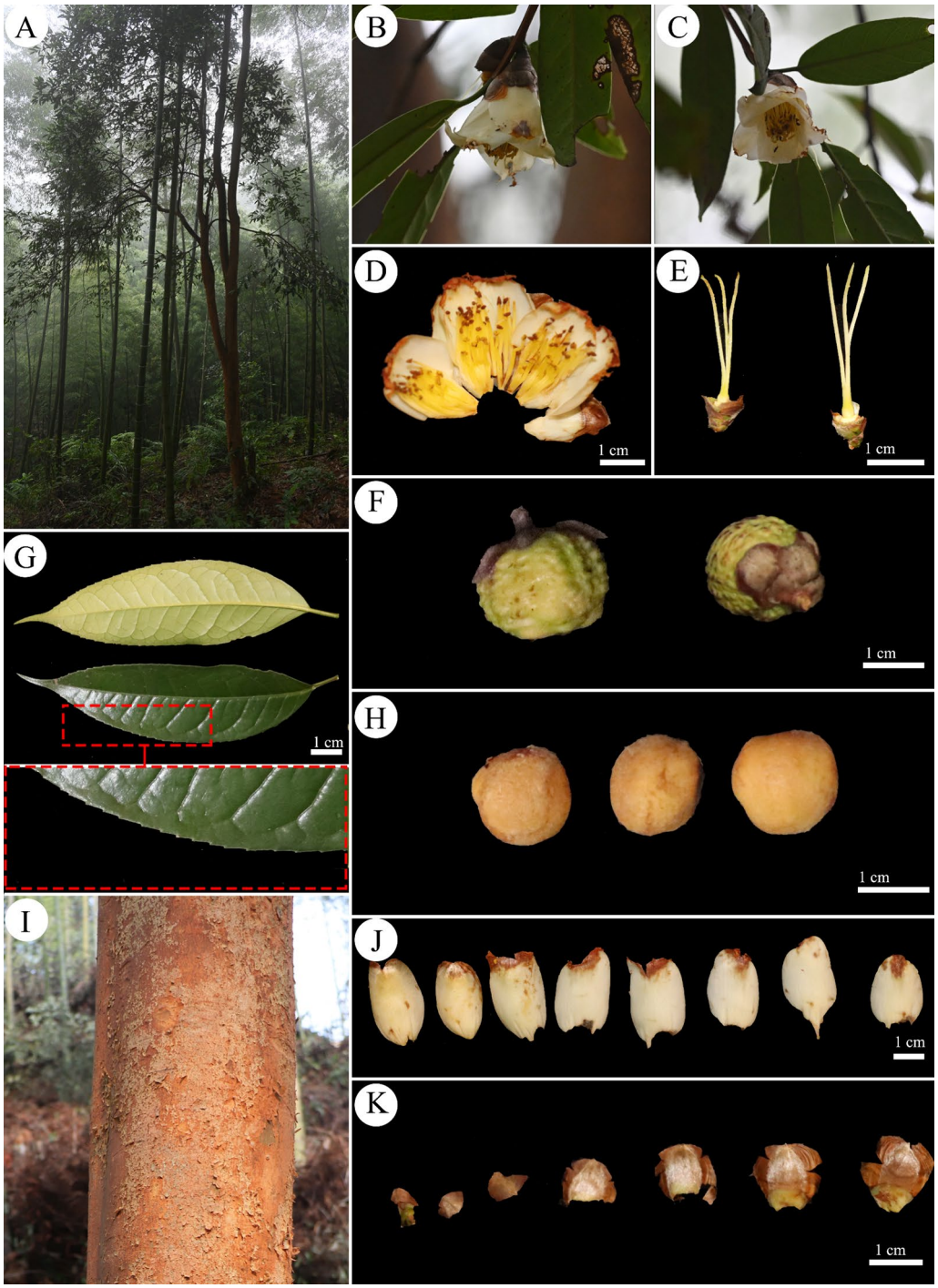

Fig. 1 Morphological characters of *C. ilicifolia* (**A** Habitat; **B** and **C** Flowers; **D** Flower anatomy; **E** Style; **F** Fruit; **G** Leaves; **H** Seeds; **I** Trunk; **J** Petals; **K** Calyces.)

the pollen germination pore was of 3-pore furrow type, with constriction in the middle of the furrow; the pollen wall ornamentation was crumpled wave to granular, with thicker and obvious ridges and warty grains; and the reticulum was obvious, with a deep and wide reticulum.

The pollen micromorphology of both species showed three germination furrows, but the length and type of the germination furrow varied. The length of the germination furrow of the *C. neriifolia* was about 28.04–28.47 μm; the germination furrow was deep and narrow, and the

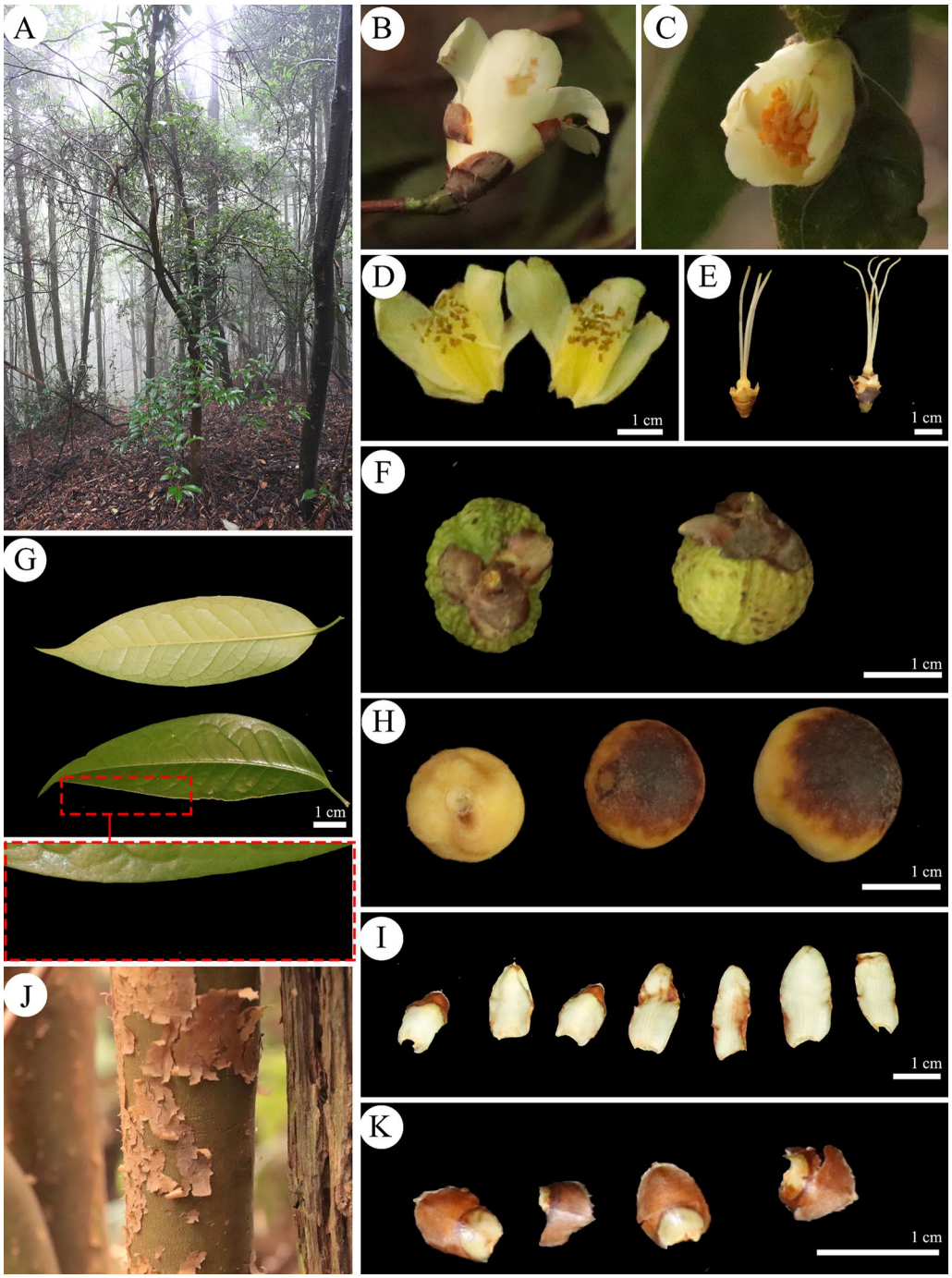

Fig. 2 Morphological characters of *C. neriifolia* (**A** Habitat; **B** and **C** Flowers; **D** Flower anatomy; **E** Style; **F** Fruit; **G** Leaves; **H** Seeds; **I** Trunk; **J** Petals; **K** Calyces.)

aperture furrow was long, thin, and narrow. The length of the *C. ilicifolia* germination pores is about 21.26–21.74 μm; the germination furrow is shallow, wide pike-type, and the aperture furrow gradually becomes wider and narrower from the two ends to the middle and stands out in the middle.

Characterization of *C. neriifolia* and *C. ilicifolia* chloroplast genomes

The whole chloroplast genomes of these two species were submitted to the NCBI GenBank, and GenBank accession numbers were obtained (Table 4). The cp genomes of both species were 157,067 bp in length (Table 4). Similar

Table 1 Comparison of morphological characters of *C. neriifolia* and *C. ilicifolia*

Species name	*C. neriifolia*	*C. ilicifolia*
Trunk	Reddish-brown, heavily debarked	Reddish brown, slightly peeling
Branches	Annual small-branched quadrangular, reddish brown	Annual small-branched quadrangular, reddish brown
Leaf type	Smooth thin leathery, shiny, narrowly lanceolate	Smooth leathery, obscure or slightly shiny, long oval or lanceolate
Leaf length (cm)	7.50–10.00	11.00–14.00
Leaf width (cm)	2.55–2.90	2.37–3.74
Leaf margin	Entire	Serrate
Petiole length (cm)	0.70–1.30	0.81–1.26
Flower type	Terminal, subsessile	Terminal, sessile
Flower colour	Faint yellow	White
Petal length (cm)	1.20–2.26	1.94–2.87
Petal width (cm)	0.80–1.20	1.30–1.80
Number of petals	6–8	8–12
Number of styles	3–4	3
Number of calyces	5–7	6–9
Sepal	Ovate	Round
Fruit shape	Capsule suborbicular, epidermis with verrucose projections	Capsule subglobose (lychee-like), epidermis with verrucose projections
Fruit diameter (mm)	14.0–19.8	13.0–18.5
Shell thickness (mm)	1.0–2.0	1.1–2.4
Ovaries	3–4	3–4
Habitat	Small trees, 1100–1200 m, under mixed montane and bamboo forests	Small trees or shrubs, forest edge, or bamboo forest around 950 m

to most angiosperms, both have a typical tetrameric structure (Fig. 5). It comprises an LSC region, an SSC region, and two IR regions. The total number of genes in both species was 132, including 87 CDS, 37 tRNAs, and 8 rRNAs genes (Table 4). The total GC content of the entire cp genome was 37.3%. However, there were differences in the GC content of each region, with the IR region having a manifestly higher GC content than that of the LSC or SSC regions (Table 4).

The IR boundary contraction and expansion analysis (Fig. 6) showed that the *rps19*, *rpl2* and *trnH* genes were relatively conserved, with the same gene positions and lengths in *C. neriifolia* and *C. ilicifolia*. However, interspecific differences were not obvious, with only slight differences in the lengths of the LSC regions. However, these two plants were compared with *C. lipingensis*, *C. pyxidiacea*, *C. leyeensis* and *C. anlungensis. C. anlungensis*, *C. neriifolia* and *C. ilicifolia* were located on the trnH gene, and *C. lipingensis*, *C. pyxidiacea*, and *C. leyeensis* were located on tRNA genes. The tendency of the six plants to contract and expand the IR boundaries was basically the same.

Phylogenetic tree analysis

Based on the results of the chloroplast whole-genome phylogenetic tree of 21 species (Fig. 7), *C. ilicifolia* clustered with seven *C. tuberculata* in Clade I and had a high support rate (ML = 100, BI = 1.00). *C. neriifolia* and *C. ilicifolia* are on the same branch of Clade I-2 but not clustered together (ML = 70, BI = 0.85). Meanwhile, the results of the phylogenetic tree constructed on the basis of protein-coding genes and ITS2 sequences showed that both *C. neriifolia* and *C. ilicifolia* were clustered in the tea group, and the two species were clustered in different branches with high support (Figs. 8, 9).

Taxonomic treatment

Combined the aforementioned evidence such as morphological, anatomy, and palynology fetures, as well as molecular phylogenetic results, it is demonstrated that *C. ilicifolia* and *C. neriifolia* act as independent species and both should be placed in sect. *Tuberculate*. Therefore, they are classified as two separate species:

Camellia neriifolia Chang

Camellia neriifolia Chang, in Acta Sci. Nat. Univ. Sunyat. (23)2: 79, 1984; *C. ilicifolia* var. *neriifolia* Ming, in Act. Bot. Yunania, 15(2): 125, 1993 et 21(2): 156, 1999.

Type: CHINA: Chishui, Jinsha, Shatian, Zeng Fanan 81,091 (SYS, GZFI) (Fig. 10A).

Description. Small macrophanerophytes, leaf blade thinly leathery, smooth and glabrous, dark green,

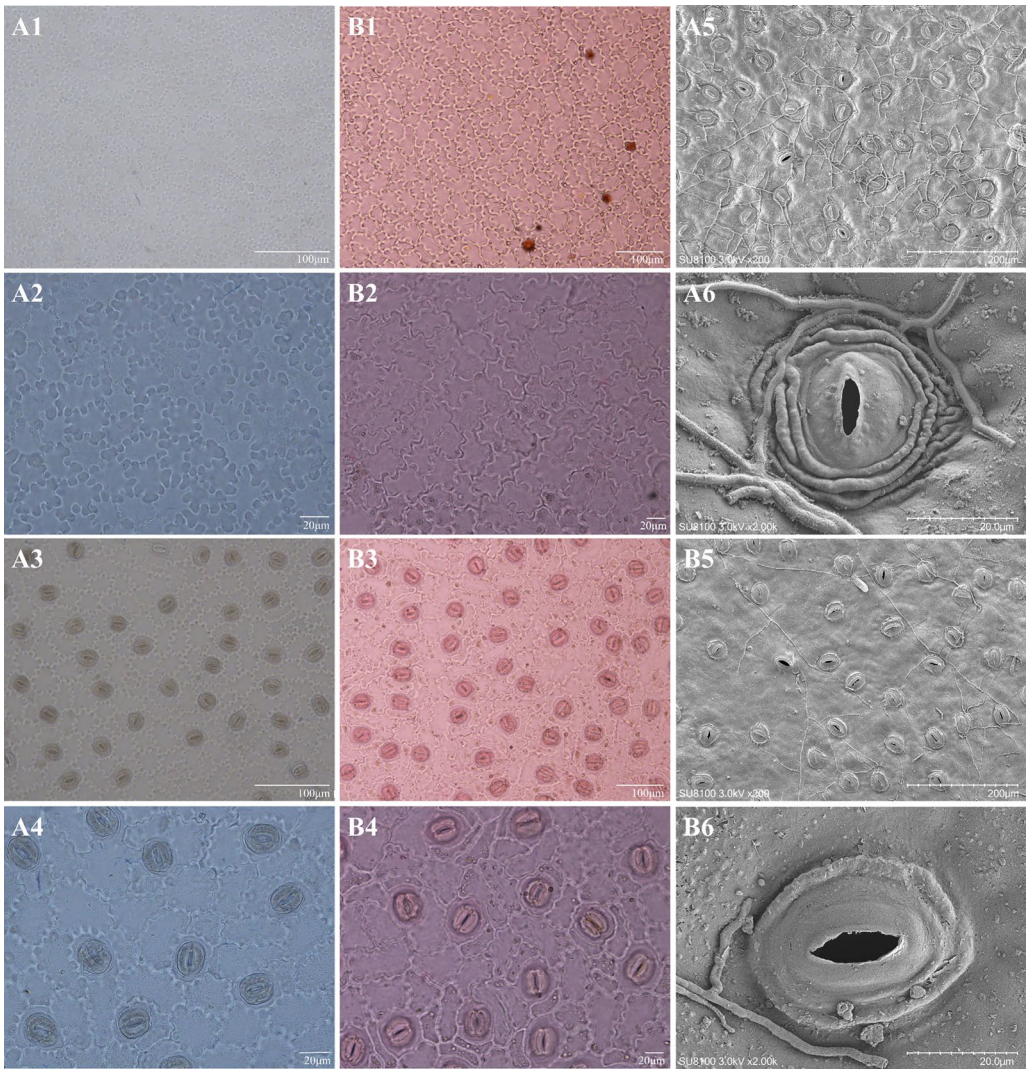

Fig. 3 Leaf epidermal micromorphological characters (**A** *C. neriifolia*; **B** *C. ilicifolia* 1,2: Leaf upper epidermis; 3,4: leaf hypodermis; 1,3: 20 times; 2,4: 40 times; 5,6: Characters of stomata)

obscure or slightly shiny adaxially, yellowish green abaxially; leaf blade symmetrical, narrowly lanceolate; length usually about 7.50–10.00 cm, width 2.55–2.90 cm; leaf apex caudate-acuminate, base broadly cuneate; leaf margins smooth, without any teeth or notches joining in a line; leaf parallel lateral veins 7–9 per side, visible above, more obvious below; reticulate veins not easily visible above, clearly visible below; petiole length 0.70–1.30 cm; flowers terminal, subsessile; flowers with 5–7 sepals, scarious, reddish-brown, 1.21–1.72 cm long, 0.44–0.81 cm wide, slightly ovate at the apex, similar to a fingernail cap, slightly hairy; petals 6–8 in number, faint yellow, 1.20–2.26 cm long, 0.80–1.20 cm wide; pistil with 3–4 styles, free, glabrous. Capsules are globose and glabrous, 14.0–19.8 mm in diameter, with a surface with verrucose projections. Fruit 3-loculed and 1-seeded per locule, seeds globose, approximately 0.68–1.64 cm in diameter, hairy. The fruiting pedicels are very short, with persistent sepals at the posterior end of the fruit. The branchlets are reddish-brown, and shoots are glabrous and glossy when dry. The trunk was yellowish-brown, with varying degrees of desiccation, and the trunk and branches were smooth with fine lines.

Distribution and habitat: Endemic to Guizhou, distributed in Chishui: Hutou Mountain in Yuanhou, Jinhe Village in Jinsha, Wild Boar Ping, and Stuffy Head Creek (Fig. 11). Most grow on steep slopes at 1100–1200 m above sea level, under mixed woods and bamboo forests in the mountains, sometimes forming small groups.

Table 2 Characteristics of leaf epidermis of *C. neriifolia* and *C. ilicifolia*

Species name	Pattern of anticlinal walls	Number of anticlinal wall crests	Sshape of stomata	Size of stomata (length/μm × width/μm)	Inner margin of outer rim	Cuticular ornamentation outside	Density of stomata (number/mm^2)	Type of stomatal apparatus
C. neriifolia	The upper epidermis of the leaves is distinctly deeply undulate, and the leaf hypodermis is undulate	Leaf upper epidermis less numerous, leaf hypodermis more crested	Subrounded	18.00–25.00 × 19.00–24.00	Sinuolate	Ring edge, strip and ridge hunch	Occasional	Circular type
C. ilicifolia	Leaf hypodermis is shallowly undulate, the upper leaf epidermis is shallowly undulate slightly paler than leaf hypodermis	Leaf upper surface and leaf hypodermis skins less numerous, but leaf hypodermis slightly more numerous	Broadly elliptic	26.00–35.00 × 24.00–31.00	Sinuolate	Unconspicuous ring edge, a few ridge hunch	Unprecedented	Circular type

Table 3 Morphological characteristics of the pollen of *C. neriifolia* and *C ilicifolia*

Species	Shape of pollen	Polar axis (P)/μm	Equatorial axis (E)/μm	P/E	Germination pores/μm			Pollen wall ornamentation
					Long	Wide	L/W	
C. ilicifolia	Oblate	27.74–25.15	33.56–33.92	0.73–0.75	21.26–21.74	5.11–5.43	3.91–4.25	Conspicuous
C. neriifolia	Subglobular	36.14–36.72	35.06–35.64	1.03–1.05	28.04–28.47	5.17–5.33	5.26–5.51	Conspicuous

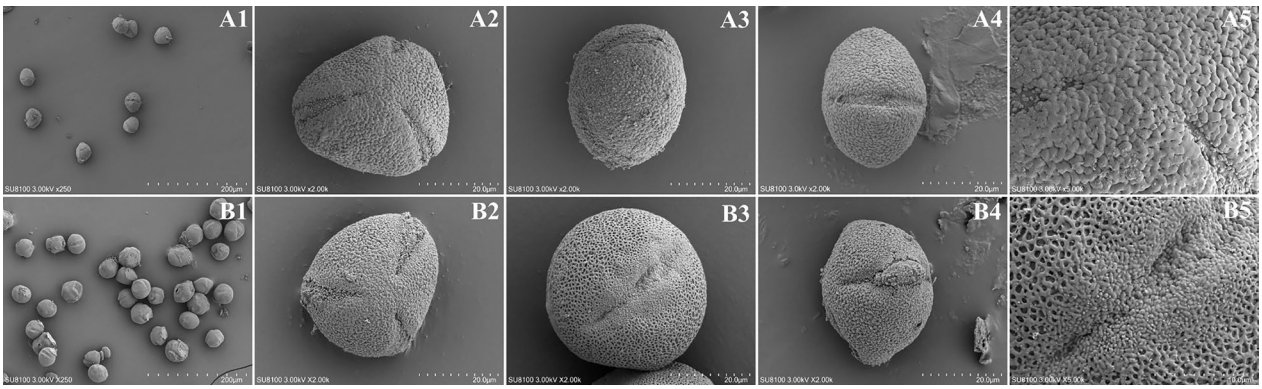

Fig. 4 Electron microscope scanning pollen morphology characteristics of *C. neriifolia* and *C. ilicifolia*. **A** *C. neriifolia* and **B** *C. ilicifolia*: 1. Ensemble view; 2. Polar view; 3. Equatorial view; 4. germination grooves; 5. Pollen wall ornamentation

Table 4 Genome-wide characterization of chloroplasts of *C. neriifolia* and *C. ilicifolia*

Species	*C. neriifolia*	*C. ilicifolia*
Genome size (bp)	157,067	157,067
GC (%)	37.3	37.3
LSC size (bp)	86,675	86,674
SSC size (bp)	18,282	18,283
IR size (bp)	52,110	52,110
GC in LSC (%)	35.31	35.31
GC in SSC (%)	30.61	30.61
GC in IR (%)	42.96	42.97
GC in CDS (%)	37.61	37.61
1st position GC (%)	45.37	45.37
2nd position GC (%)	38.04	38.04
3rd position GC (%)	29.46	29.43
Length of CDS	79,099	79,160
Number of genes	132	132
Number of CDS	87	87
Number of tRNA	37	37
Number of rRNA	8	8

Camellia ilicifolia Y. K. Li

Camellia ilicifolia Y. K. Li & H. T. Chang, Tax. *Camellia* 46, 1981; H. T. Chang and B. Bartholomew, Camellia 66, 1984; Flora of Guizhou, 5:9, 1988.

Type: CHINA: Chishui, Li YongKang 74,357 (SYS, GZAC) (Fig. 10B).

Description. Shrubs or small macrophanerophytes, leaf blade leathery, smooth and glabrous, adaxially dark green, glossy, abaxially yellow-green; leaf blade symmetrical, long elliptic or lanceolate; leaf blade length usually around 11.00–14.00 cm, width 2.37–3.74 cm; leaf apex acuminate or caudate-acuminate, base cuneate or rounded; leaf blade margins uniformly acute serrulate with regular inter-tooth intervals; proximal serrations sparser than distal ones; leaf margins glandular; parallel lateral veins 7–9 per side, visible on both sides; reticulate veins visible on both sides, clearer below; petiole length about 0.81–1.26 cm; flowers terminal, sessile; flowers with 6–9 sepals, scarious, reddish-brown, 1.46–1.88 cm long, 0.81–1.13 cm wide; apex slightly rounded, similar to fingernail shape; slightly hairy. Petals number 8–12, white, 1.30–1.80 cm wide, 1.95–2.87 cm long; pistil with 3 styles, free, glabrous. Capsules are subglobose and glabrous, about 13.0–18.5 mm in diameter, with a surface with verrucose projections. Fruit 3-loculed and 1-seeded per locule, seeds orbicular, approximately 0.84–2.13 cm in diameter, hairy. The fruiting pedicels are very short, with persistent sepals at the posterior end of the fruit. The branchlets are reddish-brown, and shoots are glabrous and glossy when dry. The trunk was yellowish-brown, with varying degrees of desiccation, and the trunk and branches were smooth with fine lines.

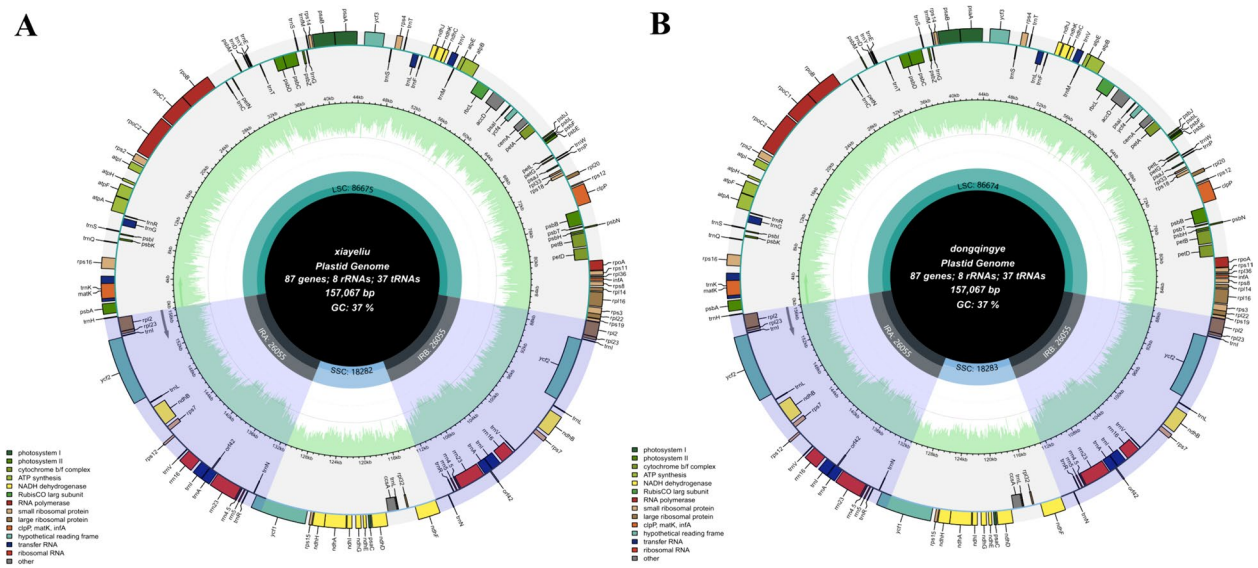

Fig. 5 Genome mapping of *C. neriifolia* (**A**) and *C. ilicifolia* (**B**)

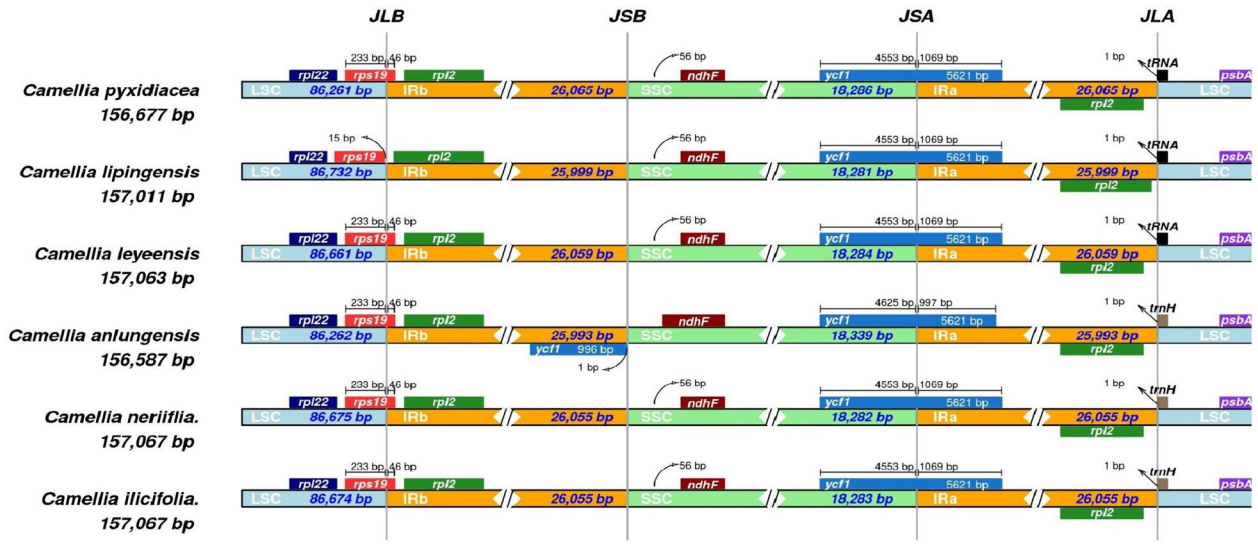

Fig. 6 Analysis of contraction and expansion of the IR boundaries of the *C. neriifolia and C. ilicifolia*

Distribution and habitat: Endemic to Guizhou, distributed in Taojiapo, Jindingshan, Zunyi, Jinshagou, and Chishui (Fig. 11). Most plants grow in steep forests and valleys, usually at an altitude of approximately 950 m, sometimes form small colonies. It grows at an altitude of 950 m.

Discussion

C. neriifolia and C. ilicifolia are controversial in traditional taxonomy in terms of ovary, leaves, and calyx (Min and Zhong 1993; Chang and Ren 1996). After reviewing a large number of specimens and field surveys, we found that leaf shape, petiole length, and fruit size were fairly stable among plant species populations, suggesting that these characteristics are key features of sect. *Tuberculata*. The leaves of seed plants evolved from a primitive branching system (Efroni et al. 2010). Fruit morphology is an important taxonomic tool (Song and Hong 2020). We found that fruit morphology was a very stable trait in both plants, making fruit difficult to use as key evidence. By observing the morphology, structure, characteristics, flowering period, and inflorescence of flowers, plants

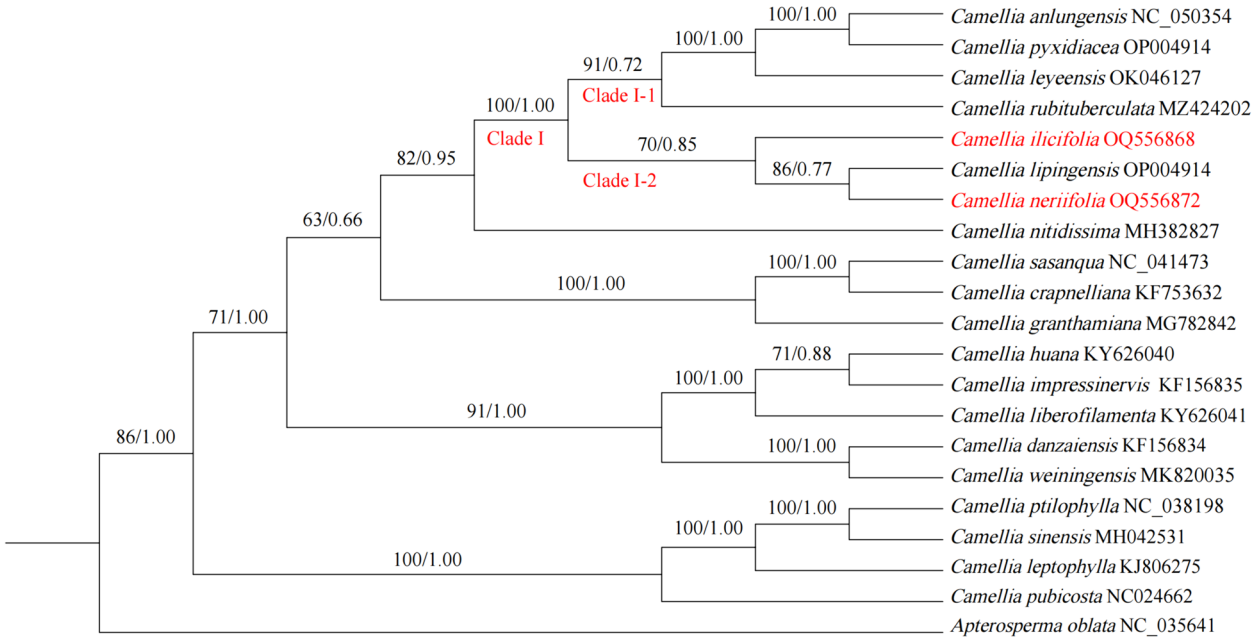

Fig. 7 Construction of a phylogenetic tree based on the chloroplast genome (Maximum Likelihood (ML) and Bayesian (BI) trees, BS ≥ 50% and PP ≥ 0.95 are indicated above branches as BS/PP)

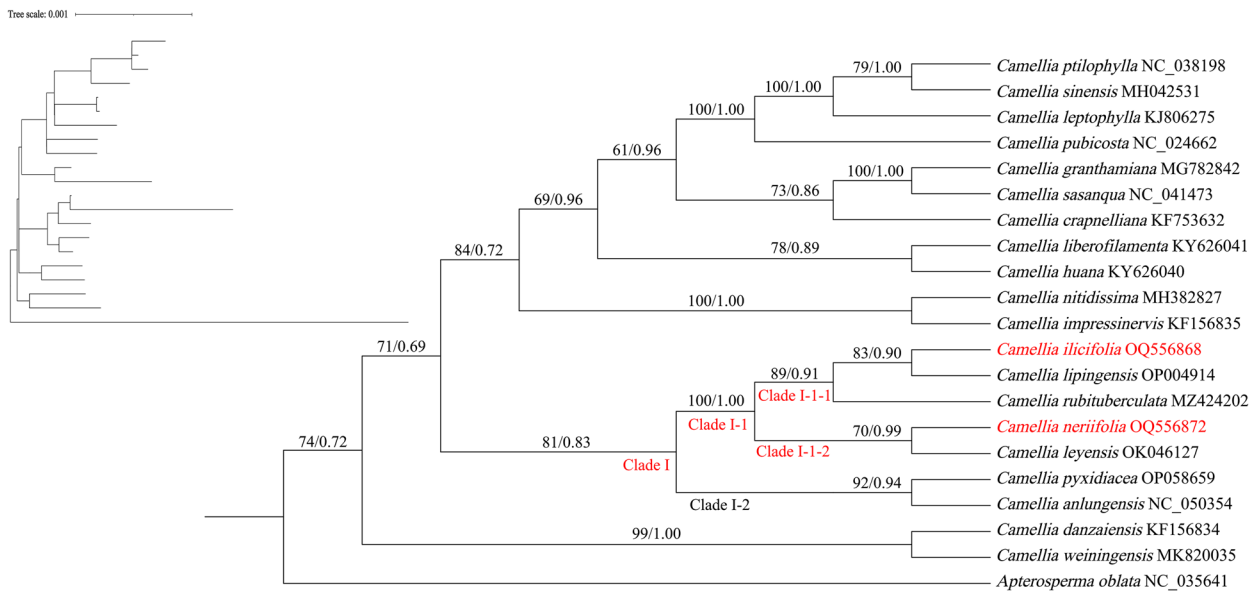

Fig. 8 Construction of a phylogenetic tree based on the PCGs (Maximum Likelihood (ML) and Bayesian (BI) trees, BS ≥ 50% and PP ≥ 0.95 are indicated above branches as BS/PP)

can be classified and identified, and their evolution and kinship can be studied (Decraene and Smets 1994). We found that these two species did not differ manifestly in calyx, flower type, or flower color. However, there were manifest differences between the two species in terms of trunks, leaf type, leaf margin, flower type, number of styles, and sepals. These characteristics provide an important taxonomic basis for the morphological delineation of the two species.

Leaf epidermal micromorphological features can be used as the basis for plant classification (Stace 1966; Brittan 1970; Kong 2001). In the taxonomic study of the

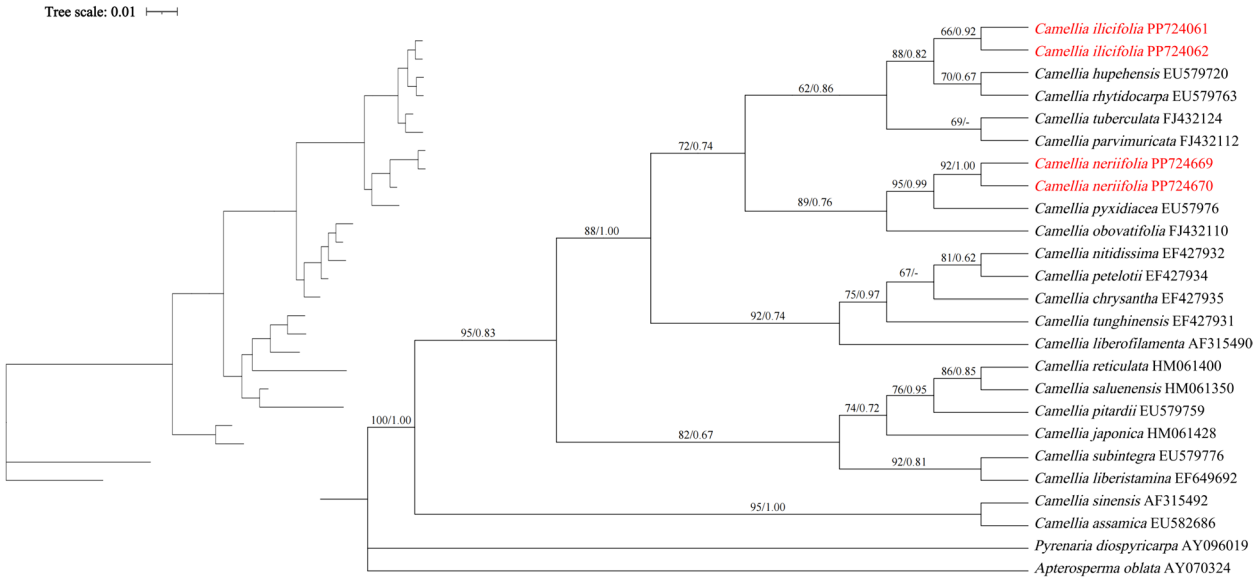

Fig. 9 Construction of a phylogenetic tree based on the ITS2 (Maximum Likelihood (ML) and Bayesian (BI) trees, BS ≥ 50% and PP ≥ 0.95 are indicated above branches as BS/PP)

Fig. 10 Information on type specimens of *C. neriifolia* and *C. ilicifolia* (**A** *C. neriifolia*, **B** *C. ilicifolia*)

genus *Camellia*, the difference in the number of upper and lower epidermal anticlinal wall crests can reflect interspecific differences to a certain extent, and the combination of the taxonomic indices of the anticlinal wall styles will be more conducive to the discovery of evolutionary relationships and developmental patterns between the two species. From these data, it is clear that the stomatal apparatus of *C. ilicifolia* is larger than that of *C. neriifolia*, and the species identity is obvious. This result is consistent with that of previous studies (Jiang

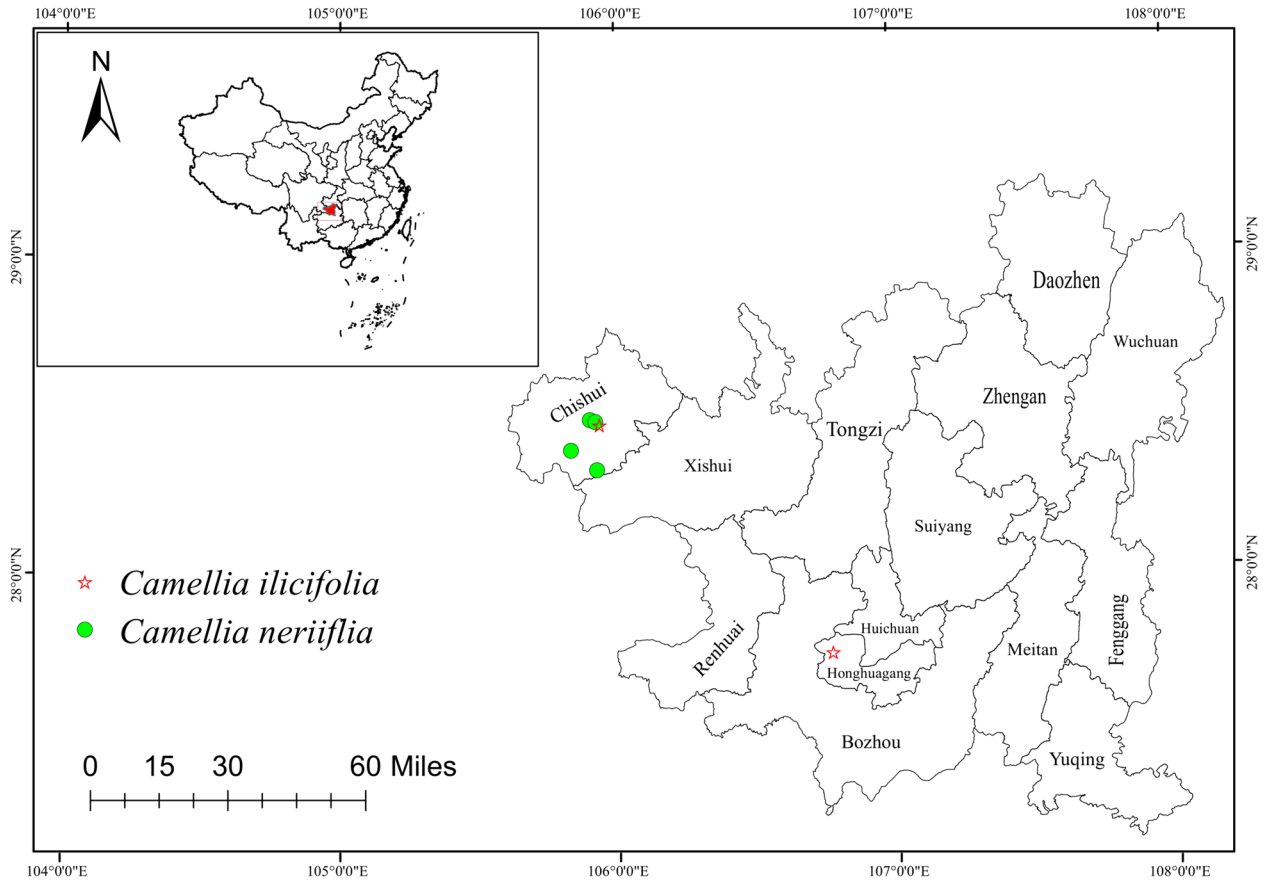

Fig. 11 Distribution map of the species of *C. neriifolia and C. ilicifolia*

2010). Thus, the leaf epidermal microstructures of both plants provided manifest evidence for the establishment of the two separate species. According to Erdtman's criteria for classifying pollen size, the pollen grains of both plants were small to medium-sized (Erdtman 1969). Morphological and data analyses of the pollen showed manifest differences in pollen shape, germination pores, and polar axes, supporting the independence of the two species. For example, the pollen morphology of *C. neriifolia* is sub-globose, whereas that *C. ilicifolia* is oblate. This is consistent with previous results (Wei et al. 1992; Hu et al. 2021).

Whole chloroplast genomes can provide powerful genetic resources for molecular phylogenetic studies of wild plant resources. Phylogenetic analyses have greatly deepened our understanding of the evolutionary relationships between *C. neriifolia* and *C. ilicifolia*. According to the molecular results of the present study, *C. neriifolia and C. ilicifolia* clustered on the same branch Clade I-2 with high support, which demonstrated the close affinity of the two species. Based on our fieldwork, Chishui Jinshagou was the most concentrated and dominant distribution area for both species. These sites highly overlap, and it is hypothesized that a high degree of natural hybridization may exist. The two species may have evolved over different periods.

Conclusion

In this study, we analyzed and compared for the first time the morphology, anatomy, palynology and molecular systematics traits of *C. neriifolia* and *C. ilicifolia*. The results showed that although not all traits were of systematic and taxonomic significance, some of them played a key role in the differentiation of the species, such as leaf margin type and sepal shape. Leaf epidermis, palynology and phylogeny also provide evidence for the independence of the two species. Therefore, both *C. neriifolia* and *C. ilicifolia* were treated as separate species in the sect. *Tuberculata* in this study. It provides an important reference value for the genetic diversity assessment, phylogeny, and population genetics of this species.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40529-024-00430-2.

Supplementary material 1.

Supplementary material 2.

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Not applicable.

Author contributions
Zhaohui Ran: Formal analysis (lead), investigation (lead), visualization (support), writing–review, and editing (equal). Xu Xiao: Formal analysis (supporting) and methodology (leading). Zhaohui Ran: Visualization (supporting); writing, review, and editing (equal). Xu Xiao: Investigation (Supporting Information). Zhi Li: Formal analysis (supporting); writing, review, and editing (supporting); funding acquisition (supporting); project administration (lead); writing, review, and editing (equal). Ming Tang: Formal analysis (supporting); writing–original draft (supporting); writing–review and editing (supporting).

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Availability of data and materials
GenBank accession numbers: OQ556872, OQ556868, PP724061-PP724062, and PP724669-PP724670. The Appendix Table for this article can be found online.

Declarations

Competing interests
There is no competing interests to declare.

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