

Short communication

Ceratopteris chunii and *Ceratopteris chingii* (Pteridaceae), two new diploid species from China, based on morphological, cytological, and molecular data

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ARTICLE INFO

Article history:

Received 13 October 2020

Received in revised form

16 October 2021

Accepted 20 October 2021

Available online 6 November 2021

Keywords:

Ceratopteris

Phylogeny

Chromosome

Taxonomy

Cryptic species

ABSTRACT

Understanding how natural hybridization and polyploidizations originate in plants requires identifying potential diploid ancestors. However, cryptic plant species are widespread, particularly in *Ceratopteris* (Pteridaceae). Identifying *Ceratopteris* cryptic species with different polyploidy levels is a challenge because *Ceratopteris* spp. exhibit high degrees of phenotypic plasticity. Here, two new cryptic species of *Ceratopteris*, *Ceratopteris chunii* and *Ceratopteris chingii*, are described and illustrated. Phylogenetic analyses reveal that each of the new species form a well-supported clade. *C. chunii* and *C. chingii* are similar to *Ceratopteris gaudichaudii* var. *vulgaris* and *C. pteridoides*, respectively, but distinct from their relatives in the stipe, basal pinna of the sterile leaf or subelliptical shape of the fertile leaf, as well as the spore surface. In addition, chromosome studies indicate that *C. chunii* and *C. chingii* are both diploid. These findings will help us further understand the origin of *Ceratopteris* polyploids in Asia.

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1. Introduction

The genus *Ceratopteris* is an aquatic or subaquatic annual plant, distributed in tropical and subtropical regions worldwide (Brongniart, 1821; Lloyd, 1974; Masuyama et al., 2002). However, because of the high degree of polymorphism within the genus, several reproductively isolated taxa are morphologically

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Peer review under responsibility of Editorial Office of Plant Diversity.

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indistinguishable (Grant, 1981; Paris et al., 1989; Masuyama et al., 2002; Masuyama, 2008). This phenomenon strongly suggests that many cryptic species exist in *Ceratopteris* (Liao et al., 2011; Kinoshita et al., 2020a). Consequently, the number of *Ceratopteris* species around the world remains unknown.

The genus *Ceratopteris* currently contains over 14 species names (IPNI 2021; Tropicos 2021), of which only four are widely accepted: *Ceratopteris cornuta* (P. Beauv.) Lepr., *Ceratopteris richardii* Brongn., *Ceratopteris thalictroides* (L.) Brongn., and *Ceratopteris pteridoides* (Hook.) Hieron (Brongniart, 1821, 1823; Leprieur, 1830; Hieronymus, 1905). *C. thalictroides* may be a cryptic species complex and the most polymorphic among the four species (Benedict, 1909; Lloyd, 1974). Masuyama and Watano (2010) demonstrated that *C. thalictroides* contains at least five taxa, including *Ceratopteris froesii* Brade., *C. thalictroides*, *C. gaudichaudii* var. *gaudichaudii* Brongn.,

C. gaudichaudii var. *vulgaris* Masuyama & Watano., and *C. oblongiloba* Masuyama & Watano (Brongniart, 1821; Brade, 1964; Masuyama and Watano, 2010). Liao et al. (2011) proposed two cryptic species of *C. thalictroides* (the south type and the north type species) in China. A new cryptic species of *Ceratopteris*, *Ceratopteris shingii* Y.H. Yan & R. Zhang, has been reported based on strong molecular and morphological evidence (Zhang et al., 2020b). Cytological results suggest that *C. cornuta*, *C. richardii* and *C. pteridoides* are diploids with $2n = 78$; other *Ceratopteris* species are tetraploid with $2n = 154$ or 156 (Smith et al., 1977; Hickok, 1979; Masuyama and Watano, 2005). The speciation of tetraploid *Ceratopteris* species is more complex than expected because of frequent hybridization. *Ceratopteris* is known to have cryptic allotetraploid taxa, but one of the diploid parents is likely extinct or has yet to be identified among known diploids (Hickok and Klekowski, 1974; Lloyd, 1974; Hickok, 1977, 1979; Adjie et al., 2007; Kinoshian et al., 2020b).

From 2017 to 2019, we collected and confirmed living individuals belonging to two species of *Ceratopteris*. Samples from Guangdong Province were sequenced using RNA-Seq (Shen et al., 2018) and have been reported as diploids, with a chromosome number $2n = 78$ (Zhang et al., 2019). However, the taxonomy of these samples has yet to be revised. Here we identified this specimen as *Ceratopteris chunii*. Another species collected from the Yangtze River area in central China and previously misidentified as *C. pteridoides* is here described as *Ceratopteris chingii*. We also confirm two new undescribed species through morphological observation, palynology, cytological study, and phylogenetic analysis.

2. Material and methods

2.1. Morphological description and scanning electron microscopy (SEM)

Morphological features were described based on specimens measured with Vernier calipers. Micromorphological features were examined under a JSZ6 dissecting microscope (Jiangnan Novel, China) (Tables 1 and 2).

Spores were picked from samples with an insect needle and fixed on a copper platform with conductive adhesive. The gold-coated spores were examined under a Quanta250 (FEI, Hillsboro, OR, USA) SEM. We observed 30 spores for each sample.

2.2. Chromosome counting

Root-tips were pretreated with P-dichlorobenzene at room temperature for 3 h and then fixed in ethanol: glacial acetic acid (3:1 v/v) for 1 h at 4 °C. The fixed root-tips were dissociated in 3% cellulase: 2.5% pectinase (1:1 v/v) for 15 min at 25 °C, and the chromosomes were stained with carbol fuchsin. The

photomicrographs were imaged on an Axio Scope A1 microscope (Carl Zeiss, Zena, Germany) (Zhang et al., 2019).

2.3. Phylogenetic analysis

Sequences for five plastid DNA regions (*rbcl*, *rpoC2*, *rbcl-atpB*, *trnL* (UAA)–*trnF* (GAA), and *trnW* (CCA)–*trnP* (UGG)) were used for the phylogenetic analysis according to Masuyama et al. (2002), Wei et al. (2017) and Zhang et al. (2020b). For primers and sequence amplification, we followed protocols described in Masuyama et al. (2002) and Abid et al. (2020). We sampled 25 species of *Ceratopteris* including the presumed new species. *Acrostichum speciosum* was chosen for the outgroup based on Zhang et al. (2020b). A list of samples, location information, GenBank accession numbers, and voucher information are provided in Table S1.

Phylogenetic trees were constructed based on maximum parsimony (MP) in MEGA X v.10.0.5 (Kumar et al., 2018), maximum likelihood (ML) in IQ-TREE v.1.6.8 (Nguyen et al., 2015), and Bayesian inference (BI) in MrBayes v.3.2.6 (Ronquist et al., 2012). Each DNA sequence matrix was aligned using MAFFT v.7 (Katoh and Standley, 2013) with manual adjustments using BioEdit v.4.0.6.2 (Hall, 1999).

For MP analysis, 1000 replicates were performed, with up to 10 tree-bisection-reconnection (TBR) searches per replicate and a maximum of 100 trees held per TBR search. In BI analysis, ModelFinder v.1.6.8 (Kalyaanamoorthy et al., 2017) was used to select the best-fit model of nucleotide substitution based on the Bayesian information criterion (BIC). The HKY + F model was selected for the cpDNA data set. Markov Chain Monte Carlo analyses were run for five million generations in BI analysis, with sampling every 500 generations, and the first 25% discarded as burn-in. We confirmed that the runs had converged by verifying that the average standard deviation of the split frequencies was below 0.01. For ML analysis, ModelFinder v.1.6.8 (Kalyaanamoorthy et al., 2017) was used to select the best-fit model of nucleotide substitution based on the BIC. The HKY + F model was selected for the cpDNA data set and 5000 replicates were performed for bootstrap analyses.

In addition to the MP phylogenetic trees, all phylogenetic analyses were performed in PhyloSuite 1.2.1 (Zhang et al., 2020a).

3. Results

3.1. Taxonomic treatment

3.1.1. *Ceratopteris chunii* Y.H. Yan, sp. nov. (Fig. 1)

Type: CHINA, Guangdong, Guangzhou, South China Botanical Garden, the Chinese Academy of Sciences (CAS), 23°11'N, 113°21'E, elev. 9 m, 20 August 2017. Yue-Hong Yan Fern09730 (holotype: CSH; isotypes: CSH, IBSC and PE).

Table 1
Morphometric data of *Ceratopteris chunii* and *C. gaudichaudii* var. *vulgaris* used for analysis of variance.

| Taxon | Stipe width of sterile leaf (cm) | Stipe length of sterile leaf (cm) | Blade length of sterile leaf (cm) | Ratio of stipe length to blade length (cm) | Blade length of 1st pinna (cm) | Ratio of 1st pinna of blade to stipe length (cm) | Voucher (Herbarium) |
|-------------------------------------------------------|----------------------------------|-----------------------------------|-----------------------------------|--------------------------------------------|--------------------------------|--------------------------------------------------|----------------------------|
| <i>Ceratopteris gaudichaudii</i> var. <i>vulgaris</i> | 0.122 | 0.731 | 1.580 | 0.463 | 1.225 | 1.676 | C. Gaudichaud, 1549 (MNHN) |
| <i>Ceratopteris gaudichaudii</i> var. <i>vulgaris</i> | 0.148 | 4.040 | 12.704 | 0.318 | 4.816 | 1.192 | B.C. Stone, 4321 (NL) |
| <i>Ceratopteris gaudichaudii</i> var. <i>vulgaris</i> | 0.155 | 2.505 | 7.220 | 0.347 | 2.738 | 1.093 | M. Evans, 628 (US) |
| <i>Ceratopteris gaudichaudii</i> var. <i>vulgaris</i> | 0.121 | 3.608 | 8.553 | 0.422 | 2.742 | 0.760 | L. Raulerson, 15,656 (US) |
| <i>Ceratopteris gaudichaudii</i> var. <i>vulgaris</i> | 0.142 | 3.147 | 6.221 | 0.506 | 2.631 | 0.836 | F.R. Fosberg, 35,454 (US) |
| <i>Ceratopteris chunii</i> | 0.100 | 7.000 | 14.000 | 0.500 | 2.702 | 0.386 | Y.H. Yan, Fern09730 (CSH) |
| <i>Ceratopteris chunii</i> | 0.150 | 9.500 | 12.500 | 0.760 | 3.572 | 0.376 | F.G. Wang, YYH15464 (CSH) |
| <i>Ceratopteris chunii</i> | 0.150 | 9.000 | 12.000 | 0.750 | 3.177 | 0.353 | F.G. Wang, YYH15463 (CSH) |
| <i>Ceratopteris chunii</i> | 0.200 | 9.000 | 11.300 | 0.796 | 3.708 | 0.412 | F.G. Wang, YYH15462 (CSH) |
| <i>Ceratopteris chunii</i> | 0.100 | 10.500 | 12.500 | 0.840 | 4.410 | 0.420 | F.G. Wang, YYH15461 (CSH) |

Table 2
Morphometric data of *Ceratopteris chingii* and *C. pteridoides* used for analysis of variance.

| Taxon | Stipe width of fertile leaf (cm) | Stipe length of fertile leaf (cm) | Blade length of fertile leaf (cm) | Width of fertile leaf (cm) | Ratio of stipe length to blade length (cm) | Aspect ratio of fertile leaf (cm) | Voucher (Herbarium) |
|---------------------------------|----------------------------------|-----------------------------------|-----------------------------------|----------------------------|--------------------------------------------|-----------------------------------|------------------------------------|
| <i>Ceratopteris pteridoides</i> | 1.925 | 13.000 | 29.500 | 23.475 | 0.441 | 0.703 | R.M. Lloyd, s.n. (GH) |
| <i>Ceratopteris pteridoides</i> | 0.836 | 14.545 | 36.364 | 14.545 | 0.400 | 1.500 | S.R. Hill & C.N. Horn, 27,110 (US) |
| <i>Ceratopteris pteridoides</i> | 0.182 | 18.182 | 34.545 | 26.727 | 0.526 | 0.612 | C.R. Sperlring, 6543 (US) |
| <i>Ceratopteris pteridoides</i> | 0.867 | 4.321 | 14.708 | 12.775 | 0.294 | 0.813 | A.H. Curtiss, 3690 (US) |
| <i>Ceratopteris pteridoides</i> | 1.727 | 7.273 | 17.309 | 18.182 | 0.420 | 0.552 | M.Y. Rimachi, 9213 (US) |
| <i>Ceratopteris pteridoides</i> | 1.455 | 7.273 | 20.000 | 21.818 | 0.364 | 0.583 | M.Y. Rimachi, 9213 (US) |
| <i>Ceratopteris pteridoides</i> | 2.909 | 23.636 | 30.909 | 9.091 | 0.765 | 0.800 | M.Y. Rimachi, 10,650 (US) |
| <i>Ceratopteris pteridoides</i> | 1.200 | 18.982 | 41.818 | 25.455 | 0.454 | 0.897 | M.Y. Rimachi, 10,650 (US) |
| <i>Ceratopteris pteridoides</i> | 1.436 | 11.091 | 23.636 | 14.545 | 0.469 | 0.863 | M.H. Grayum, 8030 (US) |
| <i>Ceratopteris pteridoides</i> | 2.782 | 9.091 | 21.818 | 25.455 | 0.417 | 0.500 | T.C. Plowman, 6380 (US) |
| <i>Ceratopteris pteridoides</i> | 1.018 | 16.364 | 30.909 | 33.273 | 0.529 | 0.437 | J. Zainúm, 19 Bo127 (US) |
| <i>Ceratopteris pteridoides</i> | 1.091 | 14.545 | 23.636 | 18.182 | 0.615 | 0.500 | N.C. Fassett, 28,557 (US) |
| <i>Ceratopteris pteridoides</i> | 0.727 | 8.891 | 20.000 | 19.200 | 0.445 | 0.579 | E.L. Ekman, H12109 (US) |
| <i>Ceratopteris pteridoides</i> | 0.545 | 14.545 | 21.818 | 21.818 | 0.667 | 0.333 | E.L. Ekman, H12109 (US) |
| <i>Ceratopteris pteridoides</i> | 0.836 | 10.909 | 29.091 | 20.000 | 0.375 | 0.909 | Gomez, 6766 (US) |
| <i>Ceratopteris pteridoides</i> | 2.878 | 12.887 | 26.632 | 18.782 | 0.484 | 0.732 | D.S. Conant, 948 (F) |
| <i>Ceratopteris pteridoides</i> | 1.261 | 13.068 | 34.014 | 23.678 | 0.384 | 0.885 | J. Popenoe, 1617 (NCU) |
| <i>Ceratopteris pteridoides</i> | 1.873 | 10.354 | 25.859 | 18.821 | 0.400 | 0.824 | S.G. Beck, 5523 (F) |
| <i>Ceratopteris pteridoides</i> | 1.506 | 16.202 | 31.452 | 25.592 | 0.515 | 0.596 | F.J. Roldán, 1686 (NY) |
| <i>Ceratopteris pteridoides</i> | 0.695 | 9.136 | 27.458 | 18.034 | 0.333 | 1.016 | T.M. Pedersen, 14,800 (RMNH) |
| <i>Ceratopteris pteridoides</i> | 2.776 | 6.343 | 19.949 | 19.581 | 0.318 | 0.695 | S. McDaniel, 17329 (F) |
| <i>Ceratopteris chingii</i> | 1.400 | 5.000 | 24.000 | 17.500 | 0.208 | 1.086 | H. Shang, SG2911 (CSH) |
| <i>Ceratopteris chingii</i> | 2.100 | 6.000 | 23.000 | 25.000 | 0.261 | 0.680 | H. Shang, SG2912 (CSH) |
| <i>Ceratopteris chingii</i> | 1.200 | 4.500 | 23.500 | 22.000 | 0.191 | 0.864 | H. Shang, SG2912 (CSH) |
| <i>Ceratopteris chingii</i> | 0.500 | 3.000 | 20.000 | 14.000 | 0.150 | 1.214 | H. Shang, SG2912 (CSH) |
| <i>Ceratopteris chingii</i> | 1.700 | 5.000 | 25.000 | 20.000 | 0.200 | 1.000 | H. Shang, SG2913 (CSH) |
| <i>Ceratopteris chingii</i> | 1.800 | 9.000 | 37.000 | 30.000 | 0.243 | 0.933 | H. Shang, SG2914 (CSH) |
| <i>Ceratopteris chingii</i> | 2.300 | 10.000 | 40.000 | 24.000 | 0.250 | 1.250 | H. Shang, SG2914 (CSH) |
| <i>Ceratopteris chingii</i> | 2.400 | 9.000 | 40.000 | 30.000 | 0.225 | 1.033 | H. Shang, SG2914 (CSH) |
| <i>Ceratopteris chingii</i> | 2.200 | 8.000 | 37.000 | 21.000 | 0.216 | 1.381 | H. Shang, SG2915 (CSH) |
| <i>Ceratopteris chingii</i> | 0.900 | 6.000 | 19.000 | 14.000 | 0.316 | 0.929 | H. Shang, SG2916 (CSH) |
| <i>Ceratopteris chingii</i> | 0.400 | 9.000 | 24.000 | 11.000 | 0.375 | 1.364 | H. Shang, SG2917 (CSH) |
| <i>Ceratopteris chingii</i> | 0.400 | 5.000 | 16.000 | 13.000 | 0.313 | 0.846 | H. Shang, SG2918 (CSH) |
| <i>Ceratopteris chingii</i> | 1.000 | 12.000 | 30.000 | 14.000 | 0.400 | 1.286 | H. Shang, SG2919 (CSH) |
| <i>Ceratopteris chingii</i> | 1.600 | 10.000 | 36.000 | 24.000 | 0.278 | 1.083 | H. Shang, SG2920 (CSH) |
| <i>Ceratopteris chingii</i> | 1.300 | 7.000 | 34.000 | 22.000 | 0.206 | 1.227 | H. Shang, SG2920 (CSH) |
| <i>Ceratopteris chingii</i> | 2.419 | 6.452 | 24.194 | 24.194 | 0.267 | 0.733 | J.M. Wang, 3886 (NAS) |
| <i>Ceratopteris chingii</i> | 2.581 | 4.839 | 20.968 | 16.129 | 0.231 | 1.000 | J.M. Wang, 3886 (NAS) |
| <i>Ceratopteris chingii</i> | 3.065 | 8.065 | 40.323 | 32.258 | 0.200 | 1.000 | J.M. Wang, 3886 (NAS) |
| <i>Ceratopteris chingii</i> | 1.129 | 5.484 | 17.742 | 16.129 | 0.309 | 0.760 | Y. Zou, 01679 (PE) |
| <i>Ceratopteris chingii</i> | 1.774 | 4.839 | 20.968 | 19.355 | 0.231 | 0.833 | Y. Zou, 01679 (PE) |
| <i>Ceratopteris chingii</i> | 0.600 | 7.000 | 15.000 | 14.000 | 0.467 | 0.571 | Y.H. Yan, YYH15457 (CSH) |
| <i>Ceratopteris chingii</i> | 0.600 | 6.500 | 19.000 | 15.000 | 0.342 | 0.833 | Y.H. Yan, YYH15458 (CSH) |
| <i>Ceratopteris chingii</i> | 0.300 | 7.000 | 16.800 | 7.000 | 0.417 | 1.400 | Y.H. Yan, YYH15459 (CSH) |

3.1.1.1. **Diagnosis.** Morphologically, *Ceratopteris chunii* and *C. gaudichaudii* var. *vulgaris* are similar, however, the sterile leaf of *C. chunii* has a longer stipe and shorter basal pinna than that of *C. gaudichaudii* var. *vulgaris*.

3.1.1.2. **Description.** Subaquatic, annual, 7–27 cm tall, soft and juicy, green when young and brownish when old. **Rhizomes:** erect, dense cover root. Scales sparse on stipe apex and dense on the base, brown to hyaline. **Leaves:** clustered and dimorphic. **Sterile leaves:** 13–23 cm long; stipes 4–11 cm long, 0.1–0.3 cm wide at base, green and semicylindrical; laminae 2–4 cm, 2–3-pinnate, broadly ovate or ovate-triangular, deeply divided, apex acute, base rounded-cuneate, pinna 1 × 0.5 cm, alternate, ovate to oblong, base rounded-cuneate. **Fertile leaves:** 8–27 cm long; stipes 4–12 cm long at base, 0.1–0.3 cm wide, stipe same as in sterile leaves; laminae 4–10 cm, 2–3-pinnate, oblong or ovate-triangular, base rounded-cuneate or rounded-truncate, apex acuminate; entire, terminal segments linear, acute to attenuate; pinna 1 × 4 cm, alternate, ovate or narrowly triangular. **Spores:** tetrahedral-globose, 120–131 μm in diameter,

granulate deposits, coarse ridges, and rodlets formed of coalescent particles on the surface. **Chromosomes:** 2n = 78, diploid.

Additional Specimens Examined —CHINA. Guangdong Province, Guangzhou City, South China Botanical Garden, CAS, 20 August 2017. *Yue-Hong Yan Fern09680* (CSH); 30 June 2020, *Fa-Guo Wang YYH15461–YYH15462* (PE and IBSC).

Habitat —*Ceratopteris chunii* is currently only found in Guangdong Province, China, where it grows in ponds, ditches, and rice fields at elevations of 9–21 m (Fig. 2A–C).

Chinese name —Huan Yong Shui Jue (焕镛水蕨).

Etymology —In honor of Professor Huan-Yong Chen for his contribution to plant investigations in China and the establishment of South China Botanical Garden, CAS.

Morphological evidence —Morphological comparisons show that the stipe width of the sterile leaf of *Ceratopteris chunii* and *C. gaudichaudii* var. *vulgaris* is similar ($P > 0.05$; Fig. 3). However, *C. chunii* has shorter basal pinnae ($P < 0.05$) and longer stipes ($P < 0.05$). These are important diagnostic characters for separating *C. chunii* from *C. gaudichaudii* var. *vulgaris*.

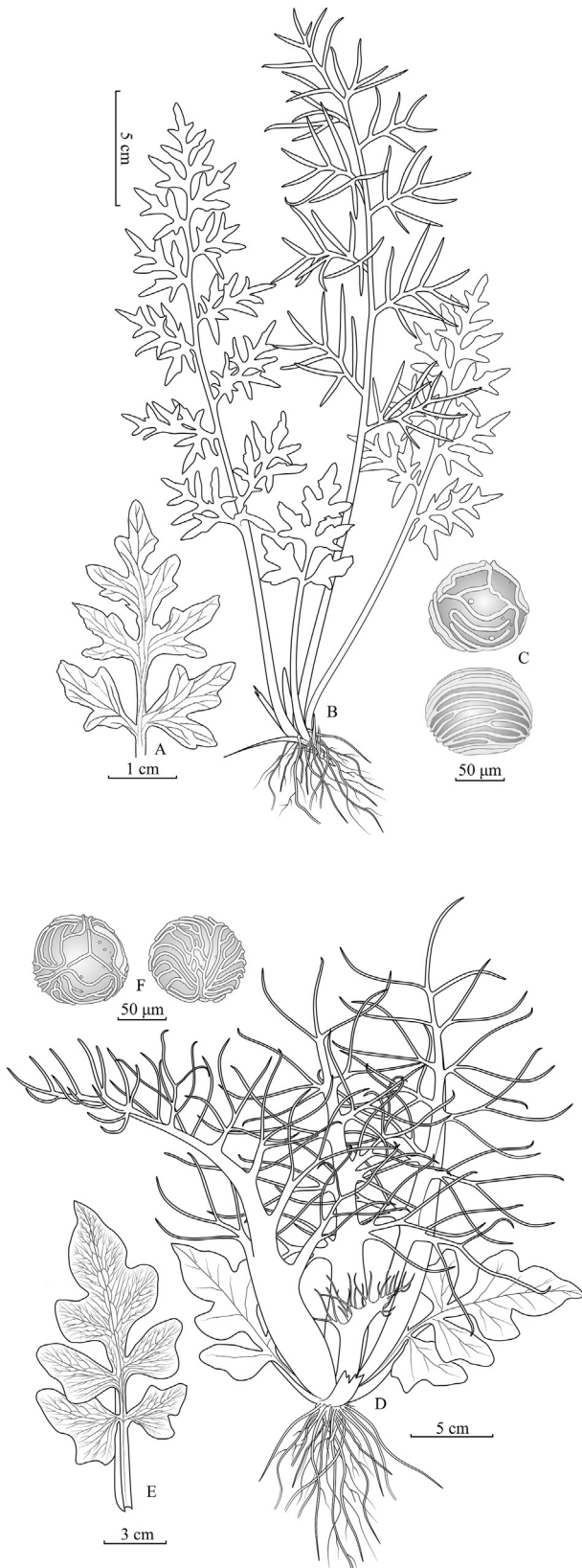


Fig. 1. Illustrations of *Ceratopteris chunii* Y.H. Yan, *sp. nov.* and *C. chingii* Y.H. Yan & Jun H. Yu, *sp. nov.* (A) Blade of sterile leaf of *C. chunii*; (B) Habit of *C. chunii*; (C) Spores of *C. chunii*; (D) Habit of *C. chingii*; (E) Blade of sterile leaf of *C. chingii*; (F) Spores of *C. chingii*.

Spore evidence —The spores of *Ceratopteris gaudichaudii* var. *vulgaris* and *C. thalictroides* are similar. Spores of both have dense granulate deposits and rodlets on the surface (Dettmann and Clifford, 1991; Tryon and Lugardon, 1991); however, *C. chunii* spores have comparatively fewer granulate deposits and rodlets (Fig. 4A).

Chromosome evidence —*Ceratopteris gaudichaudii* var. *vulgaris* is a tetraploid with $2n = 156$ (Masuyama and Watano, 2010), whereas *C. chunii* is considered diploid with a chromosome number of $2n = 78$ (Fig. S1A).

Phylogenetic evidence —The topologies of the phylogenetic trees from the MP, BI, and ML analyses were mostly identical, with support values shown in Fig. 5. The sampled populations of *Ceratopteris chunii* formed a monophyletic group with strong support ($MP_{BS} = 91\%$, $ML_{BS} = 94\%$, and $BI_{PP} = 1$), suggesting that *C. chunii* is an independent lineage from other previously reported *Ceratopteris* groups.

3.1.2. *Ceratopteris chingii* Y.H. Yan & Jun H. Yu, *sp. nov.* (Fig. 1)

Type: CHINA, Anhui, Chaohu, Chaohu wetland, in some reed marshes, $31^{\circ}38'N$, $117^{\circ}44'E$, elev. 5 m, 16 October 2018. Hui Shang SG2911 (holotype: CSH; isotypes: CSH, IBSC and PE).

3.1.2.1. **Diagnosis.** The morphological features of *Ceratopteris chingii* are similar to those of *C. pteridoides* except for the shorter stipe and the subelliptic shape of fertile leaf.

3.1.2.2. **Description.** Subaquatic, annual, 10–41 cm tall, soft and juicy, green when young and brownish when old. Stipe, rachis, and costa all obviously expanded toward base. **Rhizomes:** floating or penetrating deep into the mud, branched. Scales sparse on stipe apex, brownish hyaline. **Leaves:** clustered and dimorphic. **Sterile leaves:** 1–10 cm long; stipes 1–7 cm, 0.3–2 cm wide at base; green, smooth, and hemicylindrical; lamina ovate-triangular, 2–7 cm, 2-pinnate, terminal pinna blunt, lobes triangular to broadly loriform. **Fertile leaves:** subelliptic, 10–33 cm long; stipes 3–12 cm, 0.3–3 cm wide at base; stipe same as in sterile leaves; lamina 4–15 cm, 3-pinnate, base rounded-cuneate or rounded-truncate, apex acuminate; entire, terminal segments linear, acute to attenuate; pinna 3×6 cm, alternate, ovate or narrowly triangular. **Spores:** tetrahedral-globose, 95–113 μm in diameter, smooth, granulate deposits and coarse ridges on sporangium surface. **Chromosomes:** $2n = 78$, diploid.

Additional Specimens Examined —CHINA. Anhui and Hunan Provinces, Anhui Province Chaohu City, Chaohu wetland, in some reed marshes, 16 October 2018. Hui Shang SG2912–SG2920 (CSH); Hunan Province, Yueyang City, north of Dongting Lake National Nature Reserve at Caisang Lake, 12 October 2017, Xun Lin Yu YYH15457–YYH15458 (PE and IBSC).

Habitat —*Ceratopteris chingii* is currently found in Anhui and Hunan Provinces, China. It usually floats on the lakes or grows in wetlands, at elevations of 5–30 m (Fig. 2D–G).

Chinese name —Cu Geng Shui Jue (粗梗水蕨).

Etymology —In honor of Professor Ren-Chang Ching for his contribution to fern investigations in China and the establishment of Lushan Botanical Garden, CAS. The Chinese name preserves the name widely used in the past.

Morphological evidence —Morphological comparisons show that the stipe width of the fertile leaf of *Ceratopteris chingii* and *C. pteridoides* is similar ($P > 0.05$; Fig. 3). However, two important diagnostic characters separate *C. chingii* from *C. pteridoides*. The relative length of the stipe to blade is smaller in *C. chingii* ($P < 0.05$), indicating that the stipe is shorter. In addition, the aspect ratio



Fig. 2. Habitat of *Ceratopteris*. (A) Habitat of *C. chunii*; (B), (C) Young fertile leaf and sterile leaf of *C. chunii*. (D), (E) Habitat of *C. chingii*; (F) Young fertile leaf and sterile leaf of *C. chingii*; (G) Stipe of fertile leaf of *C. chingii*.

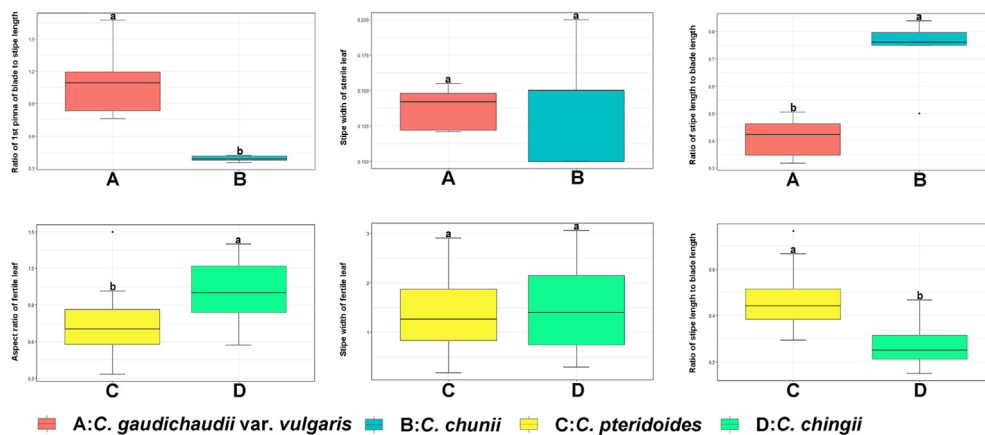


Fig. 3. Analysis of variance of the *Ceratopteris* morphometric data. (A) *C. gaudichaudii* var. *vulgaris*; (B) *C. chunii*; (C) *C. pteridoides*; (D) *C. chingii*.

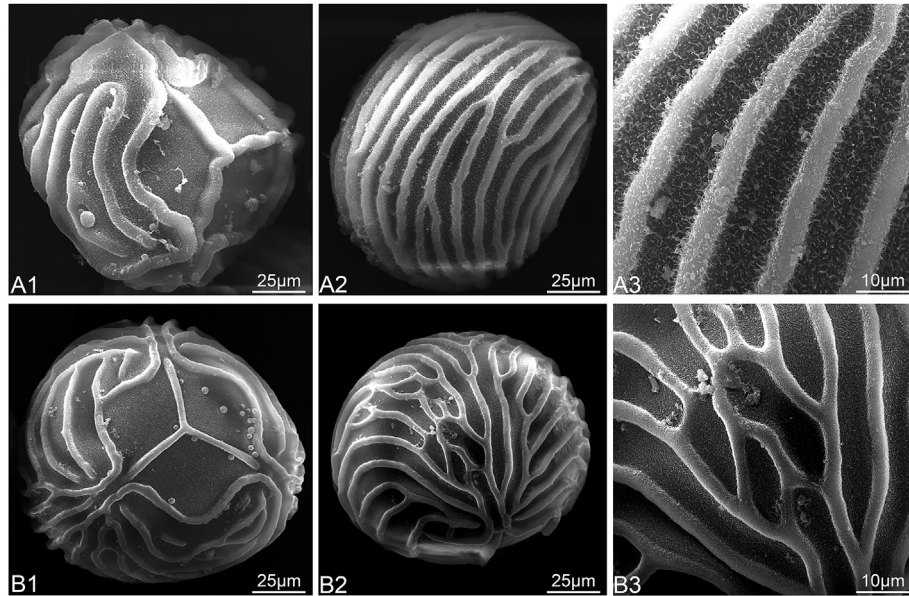


Fig. 4. Spore morphology of *Ceratopteris*. (A) *C. chunii*; (B) *C. chingii*.

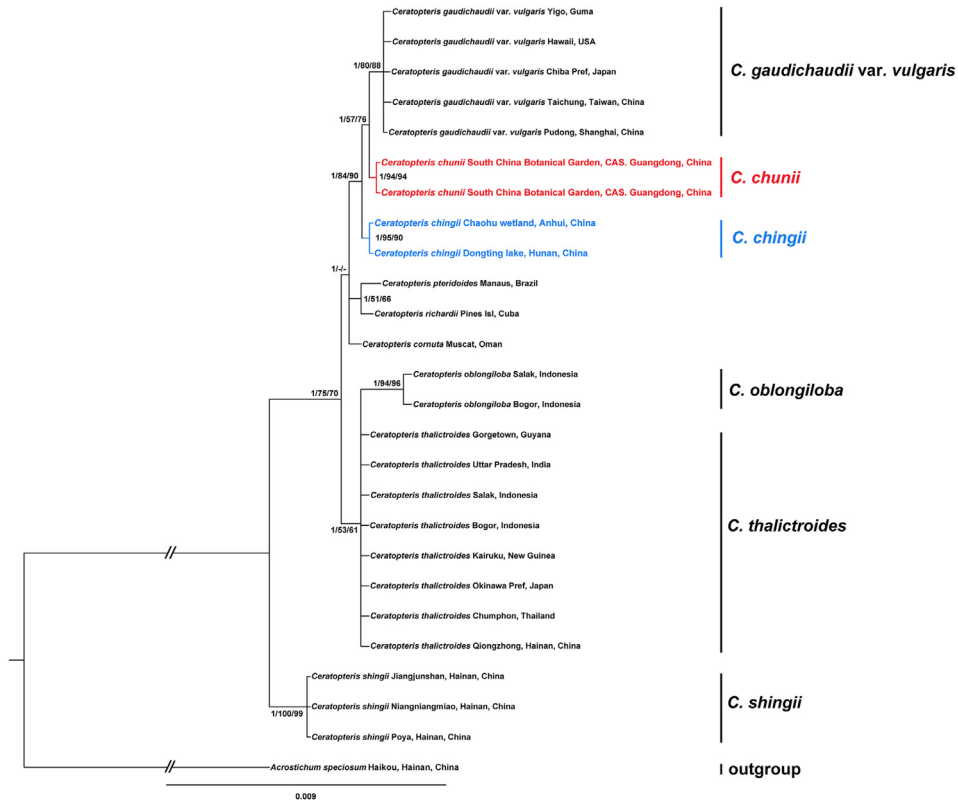


Fig. 5. Bayesian consensus tree of *Ceratopteris* based on the sequences of *rbcl*, *ropC2*, *rbcl-atpB*, *trnL* (UAA)–*trnF* (GAA) and *trnW* (CCA)–*trnP* (UGG) intergenic spacer regions. Numbers on the branches are support values (PPBI/BSML/BSMP). Dash (-) indicates nodes with BSMP or BSML < 50%. The branch length of the outgroups is shortened as indicated by "//".

indicates that the fertile leaf of *C. chingii* is subelliptical rather than triangular ($P < 0.05$).

Spores evidence—*Ceratopteris chingii* spores are relatively smooth with fewer granulate deposits on the surface (Fig. 4B) than those of *C. pteridoides* (Dettmann and Clifford, 1991; Tryon and Lugardon, 1991).

Chromosome evidence—*Ceratopteris chingii* chromosome number is $2n = 78$ (Fig. S1B), and this species was identified as diploid.

Phylogenetic evidence—MP, BI, and ML phylogenetic analyses all showed that *Ceratopteris chingii* formed a monophyletic clade with support of $MP_{BS} = 87\%$, $ML_{BS} = 95\%$, and $BI_{PP} = 1$, and was sister to the lineage including *C. gaudichaudii* var. *vulgaris* and *C. chunii* (Fig. 5).

4. Discussion

Generally, plants in relatively stable environments (e.g., aquatic plants) show little morphological variation due to convergent evolution (Schneider and Meyer, 2017). Consequently, identifying morphologically cryptic species such as those in *Ceratopteris* is challenging. Liao et al. (2011) noted that there are two cryptic species within *C. thalictroides* complex throughout China, which agreed with previous studies (Masuyama et al., 2002). However, only two species (*C. thalictroides* and *C. pteridoides*) are recorded in China (Lin and Masuyama, 2013). Zhang et al. (2020b) described a new endemic species *C. shingii* in Hainan, China. In addition, Kinoshita et al. (2020a) isolated a novel putative cryptic species from *C. thalictroides* using restriction-site associated DNA sequencing. Multiple lines of evidence indicate that there are many cryptic species in the genus *Ceratopteris*. Here, *C. chunii* and *C. chingii* are recognized as two new cryptic species of *Ceratopteris* in China.

Our phylogenetic analyses showed that *Ceratopteris chunii* from Guangdong Province formed a well-supported monophyletic group closely related to *C. gaudichaudii* var. *vulgaris* (Fig. 5). Masuyama and Watano (2010) separated *C. gaudichaudii* var. *vulgaris* (type from Japan) from *C. gaudichaudii* (type from Guam) at the variety level due to their morphological differences, although they have the same *rbcl* gene sequence and chromosome number ($2n = 156$, tetraploid) (type from Japan). Although morphologically similar to *C. gaudichaudii* var. *vulgaris*, *C. chunii* has a sterile leaf with a longer stipe and shorter basal pinna (Fig. 3). SEM showed that *C. chunii* spores have dense granulate deposits and rodlets on the surface (Fig. 4A). Most importantly, *C. chunii* is diploid ($2n = 78$) (Fig. S1A) rather than tetraploid. Based on these cytological analyses, we recommend that *C. chunii* be separated from these relatives. *C. chingii* has long been misidentified as *C. pteridoides* (type from South America) in China. Our cytological studies showed that *C. chingii* and *C. pteridoides* are both diploid with $2n = 78$ (Fig. S1B; Masuyama and Watano, 2005). The phylogenetic analyses indicated that *C. chingii* (type from Anhui and Hunan Provinces) and *C. pteridoides* were more distantly related; *C. chingii* formed a well-supported clade with $MP_{BS} = 87\%$, $ML_{BS} = 95\%$, and $BI_{PP} = 1$ (Fig. 5). Furthermore, the leaf-shape is nearly triangular, and the stipe of the fertile leaf is longer in *C. pteridoides*, as recorded by Hieronymus (1905), whereas those of *C. chingii* are subelliptical and short (Fig. 3). Most importantly, the spore surface of *C. chingii* is relatively smooth with fewer granulate deposits (Fig. 4B). Overall, our results indicate that *C. chingii* is a new cryptic species in China.

The origin of the tetraploid complex of *Ceratopteris thalictroides* is a mystery, and all cryptic species in *C. thalictroides* are tetraploid ($2n = 154, 156$; Masuyama and Watano, 2005). Various hypotheses have been proposed to explore the origin and speciation mechanism of the *C. thalictroides*

complex. Unfortunately, one of the diploid parents is likely extinct or has yet to be identified (Masuyama and Watano, 2005; Adjie et al., 2007; Kinoshita et al., 2020a). Here, the discovery of *C. chunii* and *C. chingii*, two diploid *Ceratopteris* in Asia, provides a framework for testing hypotheses about the origins of the tetraploid *C. thalictroides* complex. To unravel the origins of tetraploid *Ceratopteris*, future work should use nuclear gene sequencing or whole-genome sequencing.

Author contributions

Yue-Hong Yan, Yong-Bo Liu and Rui Zhang designed the experiments. Jun-Hao Yu drafted this paper; Field investigations and data analyses were performed by Jun-Hao Yu, Qiao-Ling Liu, Fa-Guo Wang, Xun-Lin Yu, and Xi-Ling Dai; Yue-Hong Yan, Rui Zhang, and Yongbo Liu revised the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The author declares no conflict of interest.

Acknowledgments

We would like to thank Hui Shang, Yu-Feng Gu, Li-Jun Chen and Wen Shao for their assistance in data analyses. The research was funded by the Biodiversity Survey and Assessment Project of the Ministry of Ecology and Environment, China (2019HJ2096001006), the Shanghai Municipal Administration of Forestation and City Appearance (grant number G192421), the Biological Resource Programme CAS (ZSZY-001-8), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020603) and the Basic Project of Ministry of Science and Technology of China under Grant (2015FY110200).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2021.10.002>.

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