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# Molecular phylogenetic identification of *Cyrtomium adenotrichum* based on *rbcL* and *psbA-trnH* barcoding

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## Abstract

Traditionally, new plant species identification relied primarily on morphological characteristics. However, molecular methods have now been demonstrated to provide more reliable identification. *Cyrtomium adenotrichum*, a fern species discovered in the Nandan limestone area of Guangxi, China, was initially described as a new species based on its distinct taxonomic morphology. To investigate the phylogenetic position of this newly described species, we conducted molecular phylogenetic analyses using the *rbcL* and *psbA-trnH* gene regions. Our study aimed to evaluate the effectiveness of these DNA barcodes in

distinguishing *C. adenotrichum* from closely related species. Maximum likelihood phylogenetic reconstruction revealed that both barcode regions clearly differentiated *C. adenotrichum* from its congeners, with significant topological divergence observed in the resulting trees. These findings demonstrate that *rbcL* and *psbA-trnH* serve as highly effective molecular markers for the accurate identification of *C. adenotrichum*.

**Key words:** Cyrtomium; *Cyrtomium adenotrichum*; *rbcl*; *psbA-trnH*; DNA barcoding

## Introduction

Cyrtomium Presl is a genus in the Dryopteris family, characterized by odd-pinnate leaves or leaf blades with pinnately lobed tips, reticulate venation, forming fairly large reticulate pores with veinlets, and having rounded cyst clusters and peltate endocysts. Since the genus was established in 1836, great attention has been paid to the division of species and parts within the genus (Motoji TAGAWA et al. 1934; Choi et al. 2022). There are about 50 species of Cyperus in the woeld, distributed in temperate regions of Asia, of which about 40 species were found in China (Lu et al. 2003). The rhizome of the same genus *C. fortumei* was once used as an anthelmintic and is now included in the Chinese Pharmacopoeia (2005 edition) as a legal drug (Yang et al. 2013). Pharmacological studies have shown that

imidacloprid in its rhizomes inhibits the growth of tumor cells by inducing tumor apoptosis (Yang et al. 2013), and the methanol extract of its roots can inhibit tyrosinase activity and melanin production in melan-α cells, inhibit melanin production (Choi et al. 2013). In addition, the ethanol extract of *C. macrophyllum* has an immunopotentiating effect on cyclophosphamide-induced immunosuppression in BALB/c mice (Ren et al. 2014; Ullah et al. 2022). Therefore, plants of the genus Cyrtomium have important medicinal value.

The DNA barcode is a short, standardized region of DNA that is often used to identify a specific species (Hebert et al. 2003; Coissac et al.2016). The application of different DNA barcoding technologies has greatly helped modern plant classification (Hebert et al. 2003; Kress et al. 2005; Hollingsworth et al. 2011; Hu et al. 2020) by revealing phylogenetic relationships between taxa and facilitating taxonomic decisions. This is particularly important for so-called “cryptic species”, i.e. species with low morphological differences but high genetic differences (Struck et al. 2018; Bryan et al. 2013; Heylen OCG et al. 2021; Beatriz et al. 2015). There are many coding and non-coding genes in the nuclear and plastid genomes, which are considered to be potential barcodes of plants (Mark et al. 2007; Lahaye et al. 2008; Hollingsworth et al. 2009; Rattray et al. 2024). The *rbcL* gene encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplasts, and its coding sequence is highly conserved (Blaxter et al. 2003;

Chase et al. 2005; Newmaster et al. 2006; Worthy et al. 2022). It has been widely used to resolve the phylogenetic relationships of ferns at the family or genus level (Pryer et al. 2011; Li et al. 2006; Geiger et al. 2005). In addition, the *psbA-trnH* gene is located between the *psbA* gene encoding the D1 protein of the photosynthetic system II reaction center and the *trnH* gene encoding tRNA histidine, and is one of the fastest evolving chloroplast spacers (Shaw et al. 2005a; Sang et al. 1997; Shaw et al. 2005b; Wang et al. 2023). Due to its high amplification efficiency in plants and its ability to well reflect the specificity of genes among populations (Shaw et al. 2005b), the Consortium Barcode of Life (CBOL) Plant Working Group recommends that *rbcL* and *psbA-trnH* sequences be used as one of the core barcodes for plant species identification (Wang et al. 2009).

In March 2024, our research team discovered a unique population of Cyrtomium during a field survey in the limestone of Nandan County, Guangxi. After field investigation and literature search, the species was found to be a new species and named *C. denotrichum* Y. Nong & R.H. Jiang, *sp. Nov*. (腺毛贯众) (Nong et al. 2024). Here, we provide a detailed molecular phylogenetic analysis of the new species *C. adenotrichum* and its species within the genus based on *rbcL* and *psbA-trnH* barcoding to clarify the group division of the species.

## Materials and methods

### Sample collection

Recently, in order to explore the phylogeny of *C. adenotrichum*, we obtained the specimen deposited in GXMI, specimen number Y Nong NY2024031701. The specimen was collected in China. Guangxi: Nandan, 24°48'47"N, 107°27'12"E, alt. 470 m, on the cliff at a gully; 17 March 2024.

### DNA extraction, PCR amplification and sequencing

The genomic DNA of all the samples extraction method and kit were referred to Zhao et al (2024). PCRs were performed in a volume of 30 μL, which consisted of 50 ng of template DNA (1 μL), 15 μL of 2×Taq PCR master mix (Vazyme, Nanjing, China), 2 μL of 10 μmol/L forward and reverse primers, and 11 μL of dd$H_2O$. The primers and programs were shown in Table 1. All the PCR products were detected via agarose gel electrophoresis, and the gel was photographed via a UV transilluminator. The product was purified via a Fast Pure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China), and the reaction mixture was sequenced on an ABI 3130xl automatic sequencer (Applied Biosystems, Foster City, California, USA). The detection, purification and sequencing methods of the PCR products were also referred to Zhao et al (2024).

Table. 1 PCR amplification primers and procedures.

| Barcode sequence | Primer name | Base sequence (5'→3') | Amplification program |
|---|---|---|---|
| *rbcL* | F | ATGTCACCACAAACAGAGACTAAAGC | 94 ℃ 5 min; 95 ℃ 30 s, 54.5 ℃ 30 s, 72 ℃ 45 s (35 cycles); 72 ℃ 10 min |
| | R | GCTAAATCAAGTCCACCACG | |
| *psbA-trnH* | psbA-F | GTTATGCATGAACGTAATGCTC | 94 ℃ 5 min; 94 ℃ 1 min, 55 ℃ 1 min, 72 ℃ 1.5 min (30 cycles); 72 ℃ 7 min |
| | trnH-R | CGCGCATGGTGGATTCACAATCC | |

**Sequence assembly and phylogenetic analysis**

The sequencing peak diagram was spliced and calibrated via Codon Code Aligner 8.0.2 software. The primers and low-quality regions of the sequenced *rbcL* and *psbA-trnH* sequences were removed and cut according to the annotation file to obtain the complete sequences (Zhao et al. 2024).

One hundred and ten (110) *rbcL* sequences from 25 species of the same genus were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 15 December 2024) and analysed together (Table S1). Seventy (70) *psbA-trnH* sequences from 8 species of the same genus were downloaded from

GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 15 December 2024) and analysed together (Table S2). All sequenced (*rbcL*s, *psbA-trnHs*) and reference sequences from Genbank were used to construct the phylogenetic tree. MAFFT (v7.505) was used for sequence alignment (Nakamura et al. 2018). ModelTest-NG (v0.1.7) was used for model validation of the phylogenetic tree (Darriba et al. 2020). According to the corrected Akaike information criterion (AICc), TIM2ef+I+G4 was selected for the *rbcL* barcode and TPM1uf was selected for the *psbA-trnH* barcode. The phylogenetic tree was constructed using RAxML-NG (v.1.1.0) (Kozlov et al. 2019). The support rate of each branch was checked using the bootstrap method (repeated 1000 times). The R package ggtree was used for visualization (Yu et al. 2017, Zhao et al. 2024).

## Results

### Sequences characterization

We compared the *rbcL* sequences of 112 samples by BLAST, and their identity was 100 %. A total of 110 sequences of the same genus species in GenBank were analyzed. *C. adenotrichum* had the shortest average sequence (479.50 bp), and *C. anomophyllum* had the lowest average GC content (46.69 %). 15 species had the longest average sequence (1154 bp), and *C. balansae* and *C. uniseriale* had the highest GC content (48.53 %) (Table S1). Afterwards, the *psbA-trnH* sequences of the 72 samples were compared by BLAST, and their identity was 100 %. A total of

70 sequences of the same genus species in GenBank were analyzed. *C. fortunei* had the shortest average sequence (342.43 bp), and *C. devexiscapulae* had the lowest average GC content (36.89 %) (Table S2). These results indicate that the rbcL and psbA-trnH sequences of C. adenotrichum differ from those of other species within the same genus.

**Phylogenetic analysis**

Phylogenetic analysis was performed based on the *rbcL* sequence level according to the ML method to better distinguish species. Distinct topological differences were observed in the rbcL sequences between *C. adenotrichum* and 25 congeneric species, demonstrating that this sequence can be used for identifying *C. adenotrichum* (Fig. 1). Using the same analysis method, there were also obvious topological differences between *C. adenotrichum* and 7 species of the same genus based on the *psbA-trnH* sequence level (Fig. 2), indicating that *psbA-trnH* can also distinguish and identify *C. adenotrichum* and its species within the genus Cyrtomium. Furthermore, in the phylogenetic trees constructed using *rbcL* and *psbA-trnH*, *C. adenotrichum* and *C. nephrolepioides* exhibited a closer genetic relationship, suggesting their sister-group affiliation within the genus *Cyrtomium*.

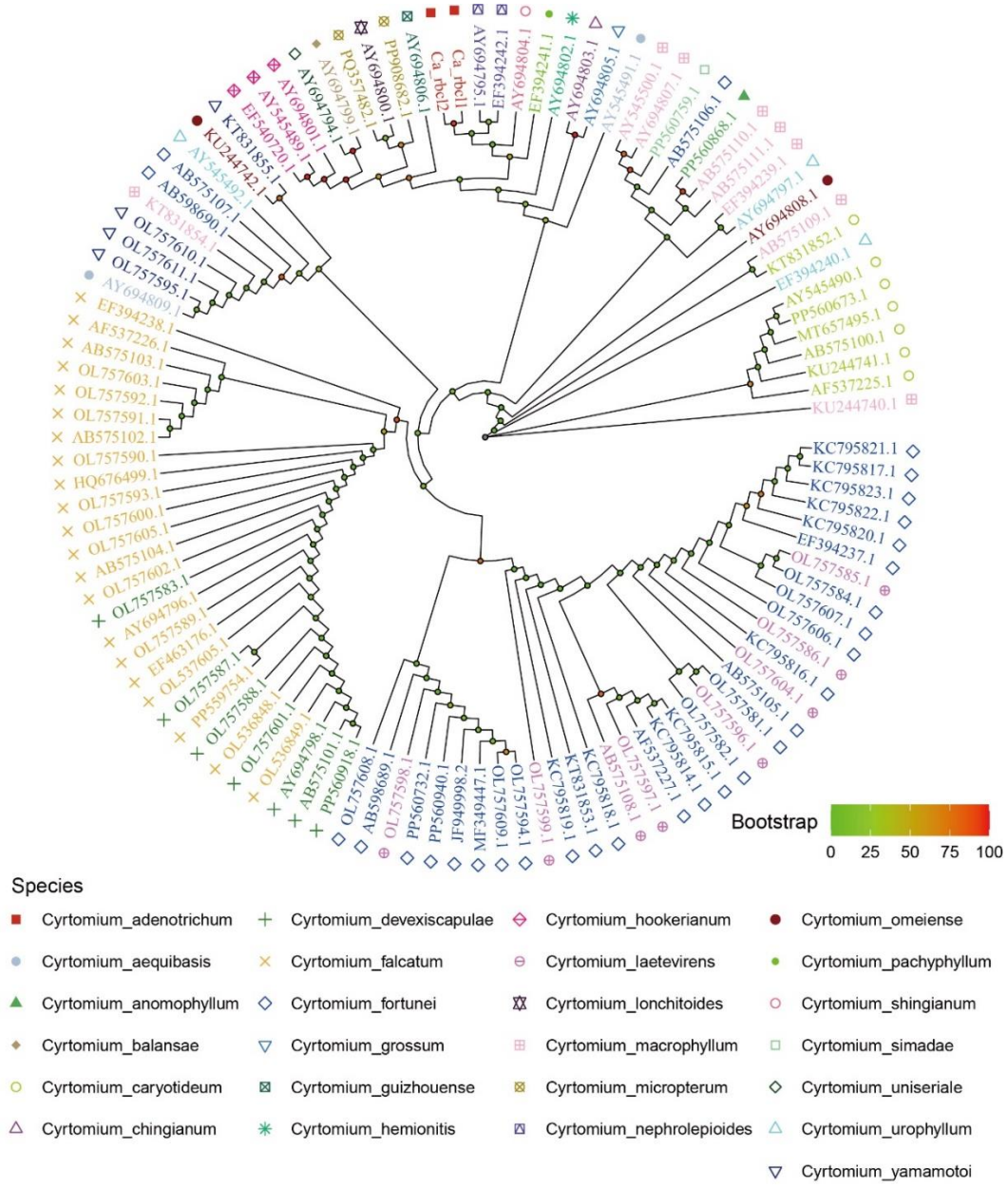


Fig. 1. *rbcL*-based phylogenetic analysis of *C. adenotrichum*s and other congeners. The source species of different sequences are marked with different colours and shapes, and the colour of the circles at the nodes changing from green to red represent an increase in bootstrap value.

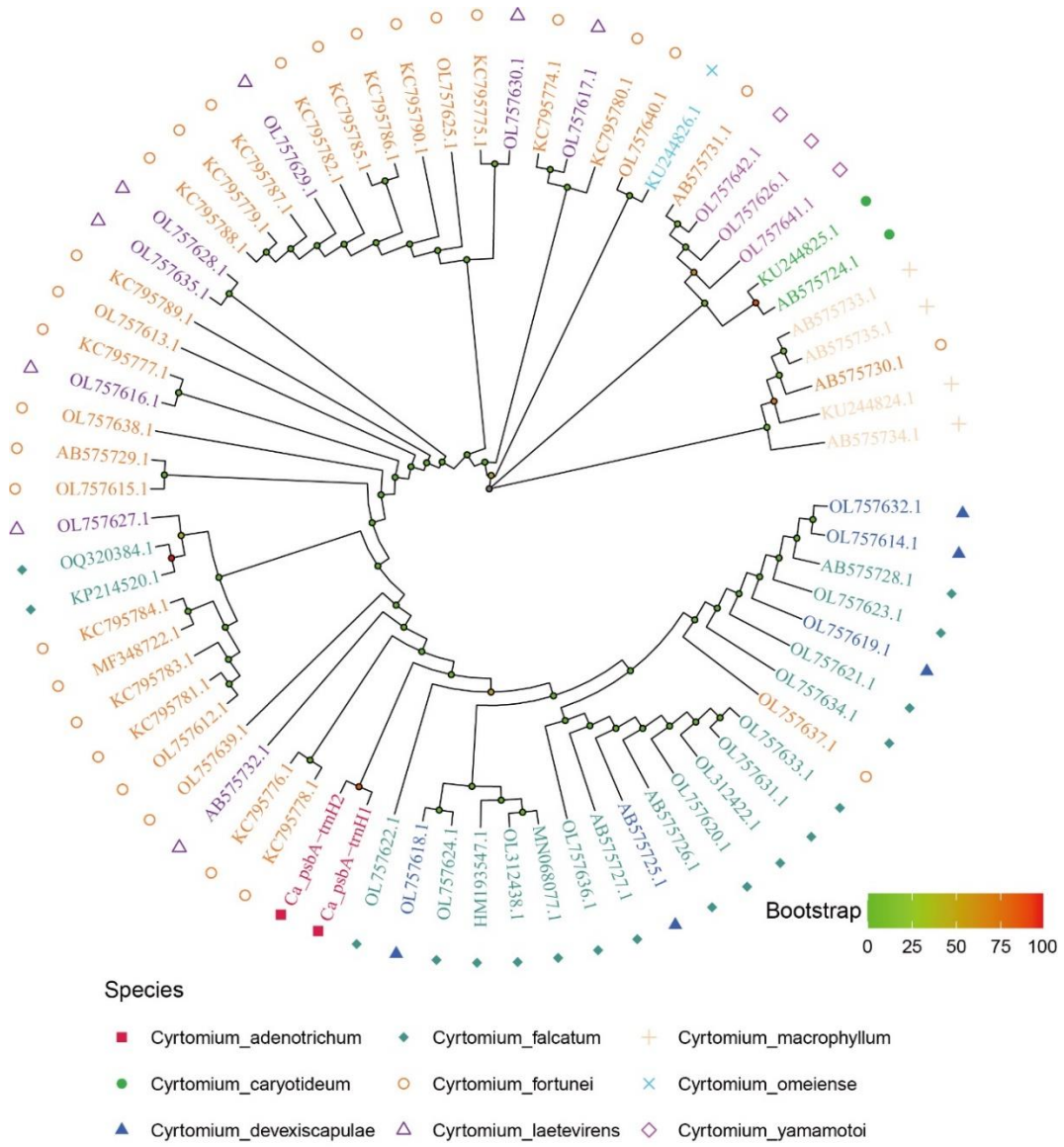


Fig. 2. *psbA-trnH* based phylogenetic analysis of *C. adenotrichum*s and other congeners. The source species of different sequences are marked with different colours and shapes, and the colour of the circles at the nodes changing from green to red represent an increase in bootstrap value.

## Discussion

Traditional classical taxonomy has gone through several centuries and has made important contributions to the systematic classification of the plant kingdom. The widespread application of scanning electron microscopes in plant taxonomy in the 1980s has enabled plant taxonomy to develop to a submicroscopic stage. With the development of science, the use of DNA genetic molecular level in plant taxonomy to identify species has been widely used, allowing plant taxonomy to enter a new stage from morphological research to the molecular level of internal genetic material (Hebert et al. 2003). Therefore, this paper used *rbcL* and *psbA-trnH* barcoding technology to explore the status of the new species *C. adenotrichum* and its congeners in plant taxonomy and their genetic relationships.

The main advantage of *rbcL* barcode is that it is easy to amplify and sequence. It has been used in Porphyra and its new species *P. aestivalis* sp. nov. (Hasebe et al. 1994), Aporosa and its new species *A. tetragona* Tagane & V. S. Dang (Tagane et al. 2015), Hemiboea and its new species *H. guangdongensis* comb. & stat. nov. (Li et al. 2019), Pterocladiella and its two new species *P. phangiae* sp. nov. and *P. megasporangia* sp. nov. (Sohrabipour et al. 2013), etc. In this study, a phylogenetic tree was constructed based on the *rbcL* barcode. *C. adenotrichum* and 25 species of the same genus were clearly clustered and identified (Fig. 1). The same species could be clustered into one category and could form independent branches at the

species level and at the family level, with a high support rate, so as to better distinguish species. This result shows that the *rbcL* barcode has a strong ability to identify and classify *C. adenotrichum* and its species of the same genus.

The chloroplast gene *psbA-trnH* sequence is a chloroplast intergenic gene fragment with a high amplification success rate and a short target fragment length (Chase et al. 2005; Lv et al. 2020). It has been successfully used to identify the genus Sideritis and its new species *S. elica* Aneva, Zhelev & Bonchev (Aneva et al. 2022), the genus Salvia and its new species *S. fimbriaticalyx* Mart.Gord. & Fragoso, sp. nov. (Fragoso-Martínez et al. 2021), the genus Euphorbia and its new species *E. tetrangularis* Hurbath & Cordeiro (Hurbath et al. 2018), etc. In this study, although the same species could not be grouped into one category based on the *psbA-trnH* barcode, *C. adenotrichum* and its species in the same genus could be clearly separated. Comparing the results of different studies, it was found that different primer fragments were selected, showing different results. The screening and identification of primers are crucial. Comprehensive analysis shows that the *psbA-trnH* primers used in this study need to be further optimized and need to be screened in multiple genera and even families.

## Conclusion

Both *rbcL* and *psbA-trnH* serve as effective DNA barcodes for accurate identification of *C. adenotrichum*, demonstrating high discriminative power at the

species level.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

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### Author contributions

Author Contributions: Conceptualization, Z.Z, J.W., and Y.H.; Data curation, Q.H.; Formal analysis, J.W.; Funding acquisition, Y.H.; Investigation, Y.H and K. L.; Methodology, Z.Z., J.W. and K.L.; Resources, Q.H. and K.L.; Software, J.W. and K. L.; Supervision, K.L. and Y.H.; Validation, J.W.; Visualization, J.W., Z.Z.; Writing–original draft, Z.Z. and K. L.; Writing–review & editing, Z.Z., J.W., and

Y.H. All authors have read and agreed to the published version of the manuscript.

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**Data availability**

All of the data that support the findings of this study are available in the main text.

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