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A new species of foliicolous lichenized fungi from southwest China: *Calopadia ruiliensis* sp. nov.

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Abstract: *Calopadia ruiliensis*, found on living leaves in southwest China, is described as a new species, based on morphology and phylogenetic analysis. It is characterized by a foliicolous thallus with yellow greenish to creamy yellow color, rounded apothecia with nonpruinose to thinly pruinose disc, a dark olive-green hypothecium and black-brown subhymenium, and 1-spored asci producing muriform ascospores. Phylogenetic analysis of LSU sequence data shows that the new species clusters in the genus *Calopadia* with high bootstrap support. The new species is compared with closely related and similar species of *Calopadia* and a comprehensive description and micrographs are provided.

Key words: Pilocarpaceae, new taxon, morphology, phylogeny, taxonomy

1. Introduction

The genus *Calopadia* was introduced by Vězda (1986), typified by *Calopadia fusca*. It is currently placed in the family Pilocarpaceae. *Calopadia* is characterized by a dispersed to centrally confluent, whitish to pale grey or pale green thallus, with or without UV fluorescence, apothecia with or without pruina, and transversely septate to muriform ascospores, as well as grey, ear-shaped campylidia producing filiform conidia (Vězda, 1986; Kalb and Vězda, 1987; Lücking et al., 2001; Lücking, 2008; Lumbsch et al., 2011; Seavey et al., 2011; Farkas and Flakus, 2012; Aptroot et al., 2014; Sanders et al., 2016). Twenty-eight species of *Calopadia* are listed in Index Fungorum.[1] However, molecular sequence data are available for only five species.[2]

Calopadia is a poorly studied genus in China, and only three species have been recorded (Santesson, 1952; Wei et al., 1991; Aptroot et al., 2003). During our surveys of the diversity of microfungi in Southwest China (Wu et al., 2014; Zeng et al., 2019), an interesting species of foliicolous lichenized fungi was collected from Ruili Botanical Garden in the Ruili region of Yunnan province. Morphological and phylogenetic analyses showed that it represents a new species, which is formally introduced here.

2. Materials and methods

2.1. Sample collection, morphological studies

Fresh living leaves of palms were collected from Ruili Botanical Garden in Ruili city, Yunnan Province, and brought to the laboratory in paper bags. Microscopic studies followed Wu et al. (2011, 2013). The samples were processed and examined using a stereomicroscope (Leica MZ16A and KEYENCEVHX-1000). Hand sections were mounted in water, Cotton Blue and Melzer's reagent. Photomicrographs of fungal structures were taken with a compound microscope (Nikon E800 and 80i). The holotype was deposited at the International Fungal Research and Development Centre, Research Institute of Resource Insects Herbarium (IFRD), and the isotype at the Herbarium of Cryptogams Kunming Institute of Botany, Academia Sinica (HKAS).

2.2. DNA extraction and PCR amplification

Genomic DNA extraction was performed directly using 10–15 fresh apothecia (fruit bodies). DNA was extracted

[1] Index Fungorum Partnership (2021). Index Fungorum [online]. Website http://www.indexfungorum.org/Names/Names.asp [accessed 00 Month Year].

[2] National Center for Biotechnology Information (2021). Taxonomy [online]. Website https://www.ncbi.nlm.nih.gov/taxonomy/?term=Calopadia [accessed 00 Month Year].

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Table 1. GenBank accession numbers of sequences used in the phylogenetic analysis.

Species	Strains	GenBank accession numbers (LSU)
Byssoloma leucoblepharum	BG3502	AY756317
Byssoloma sp.	F17228	EU601749
Byssoloma subdiscordans	AFTOL-ID4884	KJ766538
Byssolecania variabillis	AFTOL-ID2212	KJ766537
Calopadia ruiliensis	**IFRD9202**	**MF326268**
Calopadia lecanorella	F17252	EU601751
Calopadia sp.	F17098d	EU601752
Calopadia foliicola	BG16011	AY756318
Fellhaneral bouteillei	AFTOL-ID4917	KJ766559
Fellhaneral subtilis	BG28199	AY756321
*Lasioloma antillaru*m	AFTOL-ID4887	KJ766539
Lecidea floridensis	May3088	HQ660540
Lecidea cyrtidia	Lay07-0076	HQ660538
Lecidea sp.	FH7695	HQ660549
Leimonis erratica	AFTOL-ID4988	KJ766591
Micarea adnata	BG48	AY756326
Schadonia fecunda	GZU43170	AY756362
Sporopodium sp.	F12763	EU601750

LSU, 28s rDNA; newly generated sequence is in bold.

using a modified protocol of the EZNA Forensic DNA Kit (Omega Bio-Tek, Norcross, GA, USA). Partial large subunit (LSU) rDNA was amplified by using the LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') primer pair (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). PCR amplification was carried according to the following protocol: The final volume was 25μL, consisted of 12.5 μL of 2 × Power Taq PCR Master Mix, 1.5 μL of each primer (10 μM), 3 μL extract DNA and 6.5 μL deionised water. The PCR cycling parameters included: initial denaturation for 3 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 50 s at 55 °C, 1 min at 72 °C, and a final elongation for 10 min at 72 °C, The PCR amplification products were sequenced by BGI Ltd. (Beijing, China).

2.3. Phylogenetic analysis and K2P analysis

Phylogenetic analysis was performed based on LSU sequence data. Newly generated sequences were subjected to BLAST search to assess their affinities and aid in taxon sampling for the phylogeny (Table 1). Sequences were alignment using MUSCLE on the web server[3] using default settings (Edgar, 2004). The resulting alignment was further adjusted manually in BioEdit v.7.2.5[4] (Hall, 2013).

Maximum likelihood analysis was performed in RAxMLGUI v.1.3 (Silvestro and Michalak, 2012). The search strategy was set to rapid bootstrapping including 1000 bootstrap replicates and the analysis implemented the GTRGAMMA model of nucleotide substitution.

Bayesian analysis was performed in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) to evaluate posterior probabilities (PP) by Markov chain Monte Carlo sampling (MCMC) under the GTR+I+G model as suggested by MrModeltest 2.3 (Nylander, 2008). Six simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 100 generation, for a total of 100,000 trees. The first 20,000 trees were discarded as burn-in and the remaining 80,000 trees were used for calculating posterior probabilities (PP) in the majority rule consensus tree. The phylogenetic tree was visualized in TreeView v. 1.6.6[5] and annotated in Microsoft Power Point (2010).

The LSU sequences of the four sequenced species in *Calopadia* and related genera (*Lasioloma*, *Schadonia*,

[3] EMBL-EBI (2021). Multiple Sequence Alignment [online]. Website https://www.ebi.ac.uk/Tools/msa/muscle/ [accessed 00 Month Year].

[4] Hall T (2013). BioEdit version 7.2.5 [online]. Website http://www.mbio.ncsu.edu/bioedit/bioedit [accessed 00 Month Year].

[5] Page RDM (2001). TreeView: Tree drawing software for Apple Macintosh and Windows [online]. Website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html [accessed 00 Month Year].

Fellhanera, *Sporopodium*) were aligned using MEGA 7 (Kumar et al., 2016) and subjected to the calculation of pairwise distance method based on the K2P (Kimura's two parameter) model (Kimura, 1980).

3. Results

3.1. Molecular phylogenetic results

The phylogenetic analysis based on the LSU rDNA (comprising 1024 characters), including all available species of *Calopadia* in Genbank, and *Micarea adnata* as the outgroup, yielded a best scoring ML tree (Figure 1). The new species *Calopadia ruiliensis* (IFRD 9202) and *Calopadia lecanorella* grouped together with 87% ML bootstrap support and 0.99 Bayesian posterior probability, and the clade was close to *Calopadia* sp. and *Calopadia foliicola* (Figure 1). *Calopadia lecanorella* and *C. ruiliensis*showed a 95% similarity in the nuLSU sequences, with 27 bp differences (Table 2). Given that the morphological and anatomical data do not match with any other (nonsequenced species of *Calopadia*), *C. ruiliensis* is here introduced as a new species (Figure 2).

3.2. Taxonomy

Calopadia ruiliensis H.X. Wu, sp. nov. Figure 2.
Mycobank: MB 821712.; GenBank: MF326268.
Etymology: rui.li.en'sis. N.L. fem. adj. *ruiliensis*, pertaining to Ruili.

Thallus foliicolous, dispersed or continuous, yellow greenish to creamy yellow in color, indistinct, not fluorescent under UV (366 nm); isidia and soredia absent (Figures 2a–2c). Apothecia rounded, 0.3–0.55 mm diam and 200–300 µm high; disc convex, dark brown to black and thinly pruinose; margin distinct, slightly thickened, cream-colored (Figures 2d and 2e). Excipulum 30–70 µm broad (Figure 2f). Hypothecium 25–50 µm high, dark olive-green (Figures 2g and 2h, black arrows), subhymenium black-brown (Figures 2f and 2h, white arrows). Hymenium 80–130 µm high, hyaline (Figures 2f–2h). Paraphyses 1.5–2 µm wide, nonseptate (Figure 2i). Asci 70–120 × 20–25 µm ($\bar{x}$ = 93 × 20 µm; n = 20), 1-spored, clavate to cylindrical (Figures 2j–2m). Ascospores 55–110 × 11–15 µm ($\bar{x}$ = 81 × 12 µm; n = 25) and 4–7 times as long as broad, muriform, oblong-ellipsoid, hyaline (Figures 2n and 2o). Campylidia not observed. Secondary chemistry: no substances detected.

Material examined: CHINA. Yunnan Province, Ruili City, on the surface of living leaves ofPalms, 18 August 2014, H.X. Wu (IFRD 9202, holotype; HKAS 107495, isotype).

Notes: *Calopadia ruiliensis* can be identified by its nonpruinose to thinly pruinose apothecia with dark brown disc, dark olive-green hypothecium, black-brown subhymenium,1-spored, asci, and muriform ascospores

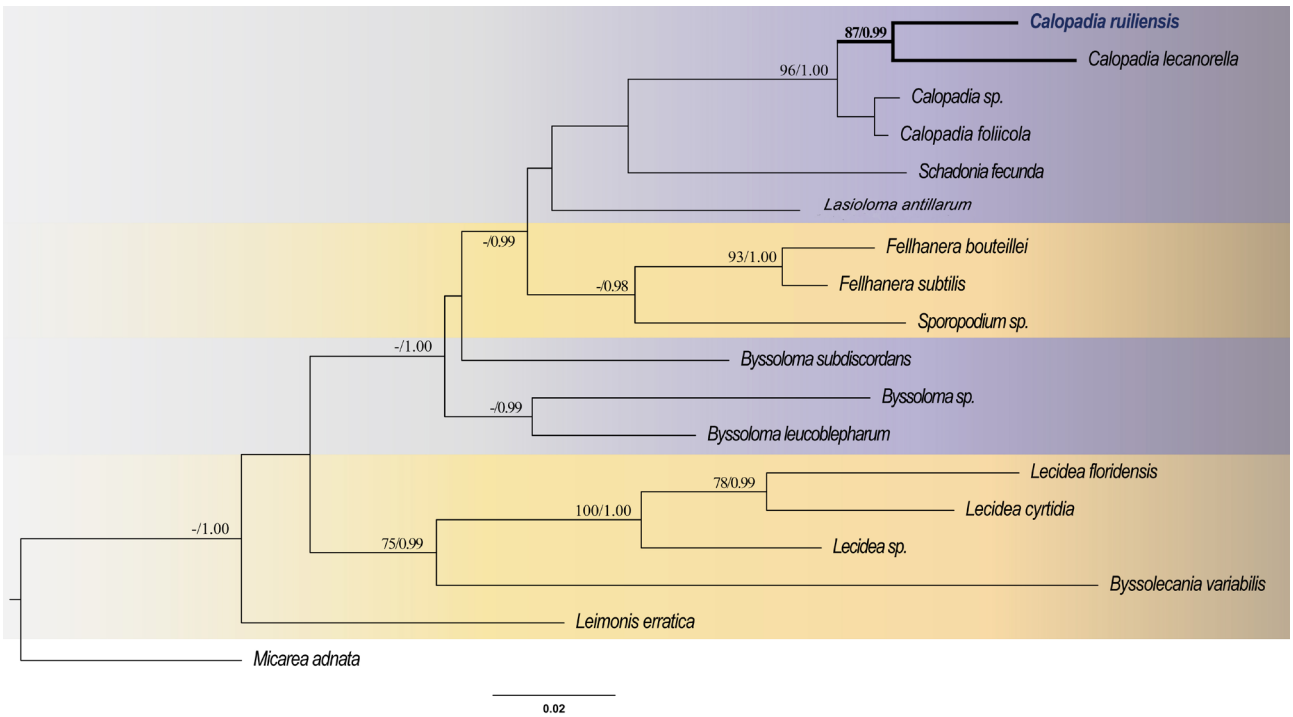

Figure 1. Phylogram generated from maximum likelihood analysis based on LSU sequence data, maximum likelihood bootstrap support values greater than 75 % and Bayesian posterior probabilities (PP) with values equal or greater than 0.99 are shown above nodes. The new is in bold. The outgroup is *Micarea adnata*.

Table 2. Intra- and intergeneric pairwise genetic divergence (%) based on Kimura's two parameter model for *Calopadia*.

	1	**2**	3	4	5	6	7	8	9
1. *L. antillarum*	0								
2. *C. ruiliensis*	9.4*	0							
3. *C. lecanorella*	9.9*	4.5	0						
4. *Calopadia* sp.	7.6*	3.7	3.9	0					
5. *C. foliicola*	7.8*	3.5	4.1	0.2	0				
6. *S. fecunda*	7.6	7.3	7.6	5.6	5.8	0			
7. *F. bouteillei*	7.9	7.3	7.1	5.4	5.6	6.4	0		
8. *F. subtilis*	6.9	7.5	7.1	6.0	6.2	5.6	2.2	0	
9. *Sporopodium sp.*	9.7	8.7	9.2	7.4	7.6	8.5	6.5	5.8	0

The number in the first row corresponds to the species name in the first column. The intargeneric pairwise genetic divergence of *Calopadia* are given in blue, new species in bold. The intargeneric pairwise genetic divergence between *Lasioloma antillarum* and other species in *Calopadia* are marked with asterisk.

55–110 × 11–15 µm in size ($\overline{x}$ = 81 × 12 µm; n = 25) (Figure 2). It is most similar to *Calopadia puiggarii* and *Calopadia subfusca*. In both species, the apothecia have dark brown disc and usually lack pruina, but can sometimes be thinly pruinose, resulting in an aspect similar to *C. ruiliensis*. In contrast, truly pruinose species such as *C. foliicola* and *C. perpallida* have a very thick pruina entirely covering the disc (Lücking 2008). Ascospore length largely agrees with that of *C. subfusca*, but the ascospores are much narrower (85–110 × 25–40 µm, about 2.5–3.5 as long as broad). *Calopadia puiggarii* typically has non-pruinose apothecia but with an aspect generally similar to *C. ruiliensis*; however, the ascospores are shorter and broader (55–85 × 15–30 µm, 3–4 times as long as broad; Lücking 2008; Sanders 2014). The ascospores of the morphologically similar *C. phyllogena* are similar in size and shape (60–110 × 10–15 µm, 5–7 times as long as broad), but the asci of the latter 2(–4)-spored. In our phylogenetic analysis, the new species clustered with *Calopadia lecanorella* (Figure 1). The latter species, however, has black apothecia and its ascospores are also shorter and broader (55–70 × 20–30 µm, 2.5–3 times as long as broad).

4. Discussion

Nearly 30 species are currently accepted in *Calopadia*[1] (Lücking, 2008). About one half are foliicolous and the other half occurs on various substrata (corticolous) (Kalb et al., 1987; Cáceres, 2007; Lumbsch et al., 2011; Aptroot and Cáceres, 2014). Most of the foliicolous species have been treated by Lücking (2008).

Calopadia ruiliensis sp. nov. belongs to a difficult complex of species with dark brown, nonpruinose to thinly pruinose apothecia and muriform ascospores (Lücking 2008).

Calopadia species are widespread in tropical regions (Aptroot et al., 2003; Kirk et al., 2008; Lücking, 2008; Farkas et al., 2012; Sanders, 2016). However, *Calopadia* is a poorly studied genus in China, as so far only *C.puiggarii* (Müll. Arg.) Vězda and *C. subcoerulescens* (Zahlbr.) Vězda have been recorded (Wei et al., 1991; Aptroot et al., 2003). The new species was collected in Ruili city, Yunnan province. Ruili city is situated at the northern border of the extension of the tropical rain forest in China, the natural habitat for species-rich assemblies of foliicolous lichens.

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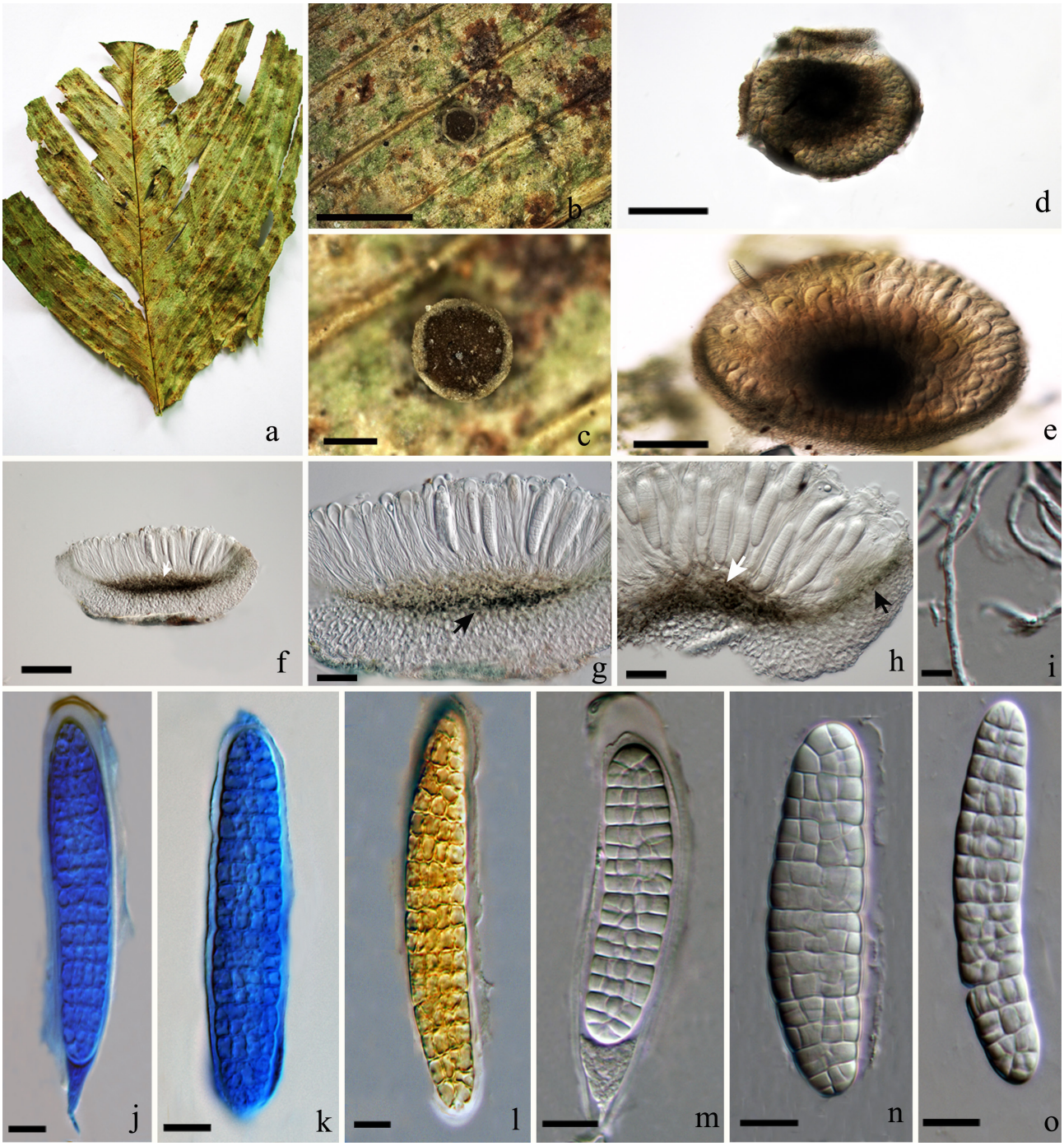

Figure 2. *Calopadia ruiliensis* a–c) Appearance of apothecia on lichen on leaves. d–e) Orbicular ascomata. f–h) Vertical section through apothecia with a developed basal layer. White arrows, black-brown pigment in subhymenium; black arrows, aeruginous pigmentation in hypothecium. i) Paraphyses. j–m) Bitunicate asci with ascospores. (j, k: in cotton blue; l: in Melzer's reagent; m: in water.) n, o) Ascospores. Scale bars: b = 1000 µm, c–d = 200 µm, e–f = 100 µm, g–h = 40 µm, j–o = 10µm.

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