



Standard Paper

Apothecia trump setae: *Paratricharia* belongs in the *Aulaxina* clade and is distant from *Tricharia* (lichenized Ascomycota: Gomphillaceae)

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Abstract

The phylogenetic relationships of the monospecific genus *Paratricharia*, with the single species *P. paradoxa*, within the family *Gomphillaceae* are resolved using newly generated sequences of the mtSSU and nuLSU markers for three specimens collected in Costa Rica. The results support placement as sister to the genus *Caleniopsis*, the two genera sister to a clade containing the genera *Aulaxina* and *Aulaxinella*. This placement confirms earlier studies based on cladistic analysis of phenotype characters and phenotype-based phylogenetic binning, suggesting that apothecial features are more informative for the phylogenetic placement of taxa within *Gomphillaceae* than thallus characteristics.

Keywords: *Batistomyces*; *Graphidales*; *Microxyphiomyces*; *Santricharia*; *Tricharia*

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Introduction

Gomphillaceae is the dominant element of foliicolous lichen communities in tropical rainforests, with well over 300 leaf-dwelling species known (Lücking *et al.* 2005, 2017; Lücking 2008; Xavier-Leite *et al.* 2022). The family is uniquely characterized by peculiar conidiomata, the so-called hyphophores, which are mostly setiform and produce hyphal drops that serve as asexual propagules (Vězda 1979; Sérusiaux & De Sloover 1986; Vězda & Poelt 1987; Lücking 1997, 2008; Lücking *et al.* 2005). Several lineages have also evolved sterile thallus setae, probably derived from setiform hyphophores; these were previously included in the collective genus *Tricharia* Fée s. lat. (Santesson 1952; Vězda & Poelt 1987; Lücking 1997).

The circumscription of the family, as well as the delimitation of genera contained within it, has undergone substantial changes since it was first described by Watson (1929) for the single genus, *Gomphillus* Nyl., and in its current sense by Vězda & Poelt (1987), who recognized ten genera. Given the sparse molecular data at the time (Lücking *et al.* 2004), Dennerlé & Péroni (1998) and Lücking *et al.* (2005) made an attempt to define genera based on a quantitative, cladistic analysis, and the latter authors increased the number of genera to 19. Until the year 2018, this number had increased to 27 (Lücking *et al.* 2017; Diederich *et al.* 2018). Recently, Xavier-Leite *et al.* (2022) provided the first broad molecular phylogenetic approach for the

Gomphillaceae, confirming most of the phenotypically defined genera but identifying an additional 19 genus-level clades which were subsequently formally instated (Xavier-Leite *et al.* 2023, 2024). The underlying phylogenetic study supported the notion that a combination of apothecial and hyphophore features characterizes genera in this family.

The genus *Paratricharia* Lücking (Lücking 1997) was established to accommodate a single species originally described in *Tricharia* Fée, *T. paradoxa* Lücking (Lücking 1991), which deviated from all other *Gomphillaceae* known at the time by forming apothecia with a carbonized zeorine margin, such as in the genus *Aulaxina* Fée, combined with robust, sterile black thallus setae, seen for example in the genus *Tricharia* as defined by Lücking *et al.* (2005). The new taxon further differed from other genera by producing a sterile columella in the centre of the apothecial disc, a feature otherwise known from the genus *Ocellularia* G. Mey. and its relatives in the sister family *Graphidaceae* (Kraichak *et al.* 2015).

Thus far, the phylogenetic relationships of *Paratricharia* have been unresolved. Phenotype-based cladistic analysis predicted the placement in a clade containing the genera *Aulaxina* and *Caleniopsis* Vězda & Poelt, as well as *Aplanocalenia* Lücking *et al.* and *Rolueckia* Papong *et al.* (the latter as *Caleniopsis*; Lücking *et al.* 2005). Phenotype-based phylogenetic binning predicted placement of the genus in the early diverging *Calenia tri-septata* Zahlbr. clade, now placed in the new genus *Caleniella* Xavier-Leite *et al.* (Xavier-Leite *et al.* 2024). These results ascribed a greater predictive power to apothecial features than to the presence of sterile, black setae, characteristic of the distantly placed genus *Tricharia* s. lat. in which the species was originally described (now divided into *Batistomyces* Xavier-Leite *et al.*,

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Microxyphiomyces Bat. et al., *Santricharia* Xavier-Leite et al. and *Tricharia*; Xavier-Leite et al. 2023), but not known in *Aulaxina* or *Caleniopsis*. Unfortunately, molecular data for *Paratracharia* had not been obtained for the study by Xavier-Leite et al. (2022).

Here, we resolve the phylogenetic placement of *Paratracharia* employing newly generated sequences of the mtSSU and nuLSU markers, obtained from material collected in Costa Rica.

Material and Methods

The newly sequenced specimens of *Paratracharia paradoxa* were collected in 1992 and 1997 at La Selva Biological Station in Costa Rica. Since then, the material had been kept in the freezer at -4 to -20 °C after drying and pressing, which apparently helped to preserve the DNA since we had no problems in amplifying the mtSSU and nuLSU markers after DNA extraction. We also included three specimens of *Gyalectidium*, representing *G. filicinum* Müll. Arg. and *G. imperfectum* Vězda, collected by Claudia Hartmann in Costa Rica.

Pieces of thalli from three different specimens were removed and placed in 1.5 ml Eppendorf tubes. Total genomic DNA was extracted at the laboratories of the Botanischer Garten und Botanisches Museum, Freie Universität Berlin, employing the Sigma-Aldrich REDEExtract-N-Amp Plant PCR Kit (St Louis, Missouri, USA), following the manufacturer's instructions but using lower amounts of reagents to obtain a lower amount of DNA extract.

Electrophoresis on a 1.5% agarose gel was used to assess the quality of the extracted DNA. In order to amplify the mitochondrial small subunit rRNA (mtSSU), the primer pair mrSSU1 and MSU7 (Zhou & Stanosz 2001) was used, with the following settings for the PCR: initial denaturation for 10 min at 94 °C, 45 s at 94 °C, 45 s at 50 °C, followed by 35 cycles of 45 s at 94 °C, then 10 min at 72 °C. Using the primer pair LR3 and LR0R (Vilgalys & Hester 1990), the nuclear large subunit rRNA (nuLSU) was amplified through PCR as follows: initial denaturation for 3 min at 95 °C, 45 s at 95 °C, 45 s at 54 °C, 1 min at 72 °C, then 35 cycles of 45 s at 95 °C, and a final elongation for 10 min at 72 °C. PCR products were purified with ExoSAP-IT™ (IT PCR Cleanup protocol) and sent for sequencing to Macrogen Europe (Amsterdam, the Netherlands; <https://dna.macrogen.com/en>).

The new sequences obtained were added to a subset of the concatenated mtSSU-nuLSU alignment provided by Xavier-Leite et al. (2022), for a total of 85 terminals representing the main clades of the *Gomphillaceae* (Table 1). We removed one mtSSU sequence previously identified as *Calenia triseptata* (MZ827298), since a recheck revealed it to represent *Aulaxina quadrangula*. Instead of using *Fissurina* (*Graphidaceae*) as an outgroup, we performed ingroup routing with the genus *Gyalidea*, an early diverging clade within the family (Xavier-Leite et al. 2022), to reduce alignment ambiguity. The resulting alignment, with a length of 949 bp for the mtSSU region and 660 bp for the nuLSU region, was manually inspected in BioEdit v. 7.2.0 (Hall 1999, 2011). Since only a small number of narrow, ambiguously aligned regions were identified using the HoT scores approach on the Guidance web server (<http://guidance.tau.ac.il>) (Penn et al. 2010a, b), we did not remove them prior to analysis.

The final concatenated alignment was analyzed with a maximum likelihood (ML) approach using RAxML v. 8.2.0 (Stamatakis 2014) locally, employing the reversible GTR model with 1000 non-parametric bootstrap replicates. To test for

potential monophyly of *Paratracharia* with *Tricharia* s. lat., we performed a Shimodaira-Hasegawa (SH) test as implemented in RAxML v. 8.2.0.

Results

The topology in our best-scoring ML tree matched the topology obtained in our previous study (Xavier-Leite et al. 2022), with the exception of the *Caleniella triseptata* clade, now positioned as an early diverging clade and no longer part of the *Aulaxina* clade; the previous result was caused by the erroneous mtSSU sequence now removed (MZ827298). Further early diverging clades included the genera *Rolueckia*, *Taitaia* Suija et al., and *Corticifraga* D. Hawksw. & R. Sant., the remaining genera forming a large, yet unsupported clade (Fig. 1). Within that clade, the topology largely matched that found in our previous analysis, with the difference that the *Calenia-Echinoplaca* complex formed a late-diverging clade; however, the backbone topology was overall not supported.

The three specimens of *Gyalectidium* clustered within their respective species, *G. filicinum* and *G. imperfectum* (Fig. 1). *Paratracharia paradoxa* was recovered with absolute support as sister to the genus *Caleniopsis*, this clade being strongly supported as sister to a clade containing the genera *Aulaxina* s. str. and *Aulaxinella* (Fig. 1). *Paratracharia* was not found to be closely related to either *Tricharia* s. str. or the *T. vainioi* R. Sant. and *T. santessonii* R. Sant. clades (*Microxyphiomyces*, *Santricharia*), the three groups that produce sterile, black setae. The SH test also rejected the monophyly of *Paratracharia* with *Microxyphiomyces*, *Santricharia* or *Tricharia* s. str. Since *Paratracharia* agrees with the latter three genera in the presence of robust, sterile, black setae, while its apothecial features are more in agreement with those of *Aulaxina* (Fig. 2), our results support the view that apothecial characters are more reliable in predicting the phylogenetic placement of this taxon. *Paratracharia* is now the fifth clade known to produce such large, sterile, black setae.

Discussion

Our study confirmed our previous assessment suggesting that apothecial features are more informative regarding the phylogenetic placement of *Paratracharia paradoxa* than the presence of sterile, black setae, based on which the species had originally been described in the genus *Tricharia* s. lat. (Lücking 1991). Instead, molecular data resolved this taxon close to *Caleniopsis* and *Aulaxina*, notably in the same area of the tree as a previous cladistic analysis based on phenotype data alone, as well as a phenotype-based binning approach, had suggested (Lücking et al. 2005; Xavier-Leite et al. 2024).

Given that phenotypic characters in *Paratracharia* are conflicting, with the apothecial features most similar to *Aulaxina*, the thallus features most similar to *Tricharia*, plus a columella as unique autapomorphy (Lücking 1997, 2008), the previous placement close to *Aulaxina* based on phenotype characters alone, using two different approaches, and its confirmation with molecular data, seems surprising. However, studies in other lichenized fungi have shown the predictive power of phenotype features when analyzed in the proper context, such as in the *Graphidaceae* or *Arthoniales* (Berger et al. 2011; Lücking & Kalb 2018; Perlmutter et al. 2020).

Table 1. GenBank Accession numbers of *Gomphillaceae* material used in this study, with voucher information, including collectors for the newly generated sequences (highlighted in bold)

Genus	Species	Country / Voucher	GB mtSSU	GB nuLSU
<i>Actinoplaca</i>	<i>strigulacea</i>	Guatemala	MZ827235	—
<i>Actinoplaca</i>	<i>strigulacea</i>	Guatemala / Brazil	MZ827235	MZ851583
<i>Asterothyrium</i>	<i>leucophthalmum</i>	Brazil	—	MZ851613
<i>Asterothyrium</i>	<i>leucophthalmum</i>	Brazil	—	MZ851619
<i>Asterothyrium</i>	<i>longisporum</i>	Costa Rica	AY341363	AY341349
<i>Asterothyrium</i>	<i>microsporum</i>	Brazil	—	MZ851549
<i>Asterothyrium</i>	<i>rotuliforme</i>	Brazil	—	MZ851615
<i>Asterothyrium</i>	<i>rotuliforme</i>	Guatemala	—	MZ851740
<i>Aulaxina</i>	<i>intermedia</i>	Brazil	MZ827227	MZ851657
<i>Aulaxina</i>	<i>opegraphina</i>	Brazil	MZ827294	MZ851672
<i>Aulaxina</i>	<i>quadrangula</i>	Brazil	MZ827291	MZ851678
<i>Aulaxina</i>	<i>quadrangula</i>	Brazil	MZ827295	MZ851673
<i>Aulaxina</i>	<i>submuralis</i>	Brazil	—	MZ851627
<i>Aulaxina</i>	<i>submuralis</i>	Brazil	MZ827224	MZ851618
<i>Aulaxinella</i>	<i>minuta</i>	Brazil	—	MZ851579
<i>Aulaxinella</i>	<i>minuta</i>	Brazil	MZ827230	MZ851578
<i>Calenia</i>	<i>depressa</i>	Costa Rica	MZ827256	MZ851711
<i>Calenia</i>	<i>lueckingii</i>	Costa Rica	—	MZ851716
<i>Calenia</i>	<i>lueckingii</i>	Mexico	—	MZ851741
<i>Calenia</i>	<i>phylogena</i>	Costa Rica	AY341366	AY341352
<i>Calenia</i>	<i>phylogena</i>	Mexico	—	MZ851744
<i>Caleniella</i>	<i>triseptata</i>	Brazil	—	MZ851691
<i>Caleniella</i>	<i>triseptata</i>	Brazil	—	MZ851663
<i>Caleniopsis</i>	<i>laevigata</i>	Brazil	—	MZ851667
<i>Caleniopsis</i>	<i>laevigata</i>	Brazil	—	MZ851675
<i>Corticifraga</i>	<i>peltigerae</i>	India	KY661684	KY661661
<i>Corticifraga</i>	<i>peltigerae</i>	Luxembourg	—	KY462801
<i>Echinoplaca</i>	<i>diffluens</i>	Brazil	—	MZ851538
<i>Echinoplaca</i>	<i>diffluens</i>	Mexico	AY341367	AY341353
<i>Echinoplaca</i>	<i>epiphylla</i>	Brazil	MZ827282	MZ851592
<i>Echinoplaca</i>	<i>epiphylla</i>	Mexico	AY341368	AY341354
<i>Echinoplaca</i>	<i>pellicula</i>	Brazil	—	MZ851484
<i>Echinoplaca</i>	<i>pellicula</i>	Guatemala	—	MZ851736
<i>Gomphillus</i>	<i>americanus</i>	USA	KY353115	KY381580
<i>Gomphillus</i>	<i>calycioides</i>	USA	MK318271	MH887485
<i>Gomphillus</i>	<i>ophiosporus</i>	Costa Rica	AY341371	AY341357
<i>Gyalectidium</i>	<i>areolatum</i>	Brazil	—	MZ851596
<i>Gyalectidium</i>	<i>areolatum</i>	Brazil	—	MZ851598
<i>Gyalectidium</i>	<i>filicinum</i>	Brazil	MZ827171	MZ851521
<i>Gyalectidium</i>	<i>filicinum</i>	Costa Rica, Hartmann s. n.	PP928950	—
<i>Gyalectidium</i>	<i>imperfectum</i>	Costa Rica, Hartmann s. n.	PP928951	—
<i>Gyalectidium</i>	<i>imperfectum</i>	Costa Rica, Hartmann s. n.	PP928952	—

(Continued)

Table 1. (Continued)

Genus	Species	Country / Voucher	GB mtSSU	GB nuLSU
<i>Gyalectidium</i>	<i>imperfectum</i>	Guatemala	MZ827236	MZ851731
<i>Gyalidea</i>	<i>fritzei</i>	Sweden	HM244744	HM244767
<i>Gyalidea</i>	<i>hyalinescens</i>	Costa Rica	DQ972996	DQ973046
<i>Microxyphiomyces</i>	<i>lancicarpus</i>	Brazil	MZ827225	MZ851629
<i>Microxyphiomyces</i>	<i>lancicarpus</i>	Brazil	MZ827274	MZ851628
<i>Microxyphiomyces</i>	<i>similis</i>	Brazil	MZ827214	—
<i>Microxyphiomyces</i>	<i>similis</i>	Brazil	MZ827218	—
<i>Microxyphiomyces</i>	<i>vainioi</i>	Brazil	MZ827199	MZ851527
<i>Microxyphiomyces</i>	<i>vainioi</i>	Brazil	MZ827280	MZ851585
<i>Monocalenia</i>	<i>monospora</i>	Costa Rica	KF833339	KF833325
<i>Monocalenia</i>	<i>monospora</i>	Cuba	MZ827264	MZ851728
<i>Paratracharia</i>	<i>paradoxa</i>	Costa Rica, Lücking s. n.	PP928953	PP928947
<i>Paratracharia</i>	<i>paradoxa</i>	Costa Rica, Lücking s. n.	PP928954	PP928948
<i>Paratracharia</i>	<i>paradoxa</i>	Costa Rica, Lücking s. n.	PP928955	PP928949
<i>Psathyromyces</i>	<i>heterellus</i>	Brazil	MZ827198	MZ851523
<i>Psathyromyces</i>	<i>heterellus</i>	Brazil	MZ827221	MZ851643
<i>Rolueckia</i>	<i>aggregata</i>	Brazil	—	MZ851690
<i>Rolueckia</i>	<i>aggregata</i>	Brazil	—	MZ851692
<i>Rolueckia</i>	<i>conspersa</i>	Brazil	—	MZ851644
<i>Roselvira</i>	<i>purulhensis</i>	Brazil	MZ827172	MZ851512
<i>Roselvira</i>	<i>purulhensis</i>	Brazil	MZ827283	MZ851595
<i>Rubrotricha</i>	<i>subhelminthospora</i>	Brazil	MZ827207	MZ851642
<i>Rubrotricha</i>	<i>subhelminthospora</i>	Brazil	MZ827220	MZ851639
<i>Santricharia</i>	<i>farinosa</i>	Brazil	—	MZ851515
<i>Santricharia</i>	<i>farinosa</i>	Brazil	—	MZ851516
<i>Spinomyces</i>	<i>aggregatus</i>	Brazil	—	MZ851588
<i>Spinomyces</i>	<i>albostrigosus</i>	Brazil	—	MZ851511
<i>Sporocybomyces</i>	<i>leucotrichoides</i>	Brazil	MZ827184	MZ851534
<i>Sporocybomyces</i>	<i>leucotrichoides</i>	Costa Rica	AY341369	AY341355
<i>Taitaia</i>	<i>aurea</i>	Kenya	—	MF372801
<i>Taitaia</i>	<i>aurea</i>	Kenya	MF372798	MF372796
<i>Tricharia</i>	<i>amazonum</i>	Brazil	—	MZ851482
<i>Tricharia</i>	<i>amazonum</i>	Guatemala	—	MZ851738
<i>Tricharia</i>	<i>longispora</i>	Brazil	MZ827219	MZ851638
<i>Tricharia</i>	<i>longispora</i>	Costa Rica	AY341374	AY341360
<i>Tricharia</i>	<i>paraguayensis</i>	Brazil	MZ827273	MZ851565
<i>Tricharia</i>	<i>paraguayensis</i>	Brazil	MZ827276	MZ851495
<i>Tricharia</i>	<i>urceolata</i>	Brazil	—	MZ851571
<i>Tricharia</i>	<i>urceolata</i>	Brazil	—	MZ851572
<i>Verruciplaca</i>	<i>verrucifera</i>	Brazil	MZ827169	MZ851517
<i>Verruciplaca</i>	<i>verrucifera</i>	Brazil	MZ827195	MZ851563
<i>Vezdamyces</i>	<i>vulgaris</i>	Brazil	—	MZ851481
<i>Vezdamyces</i>	<i>vulgaris</i>	Brazil	—	MZ851636

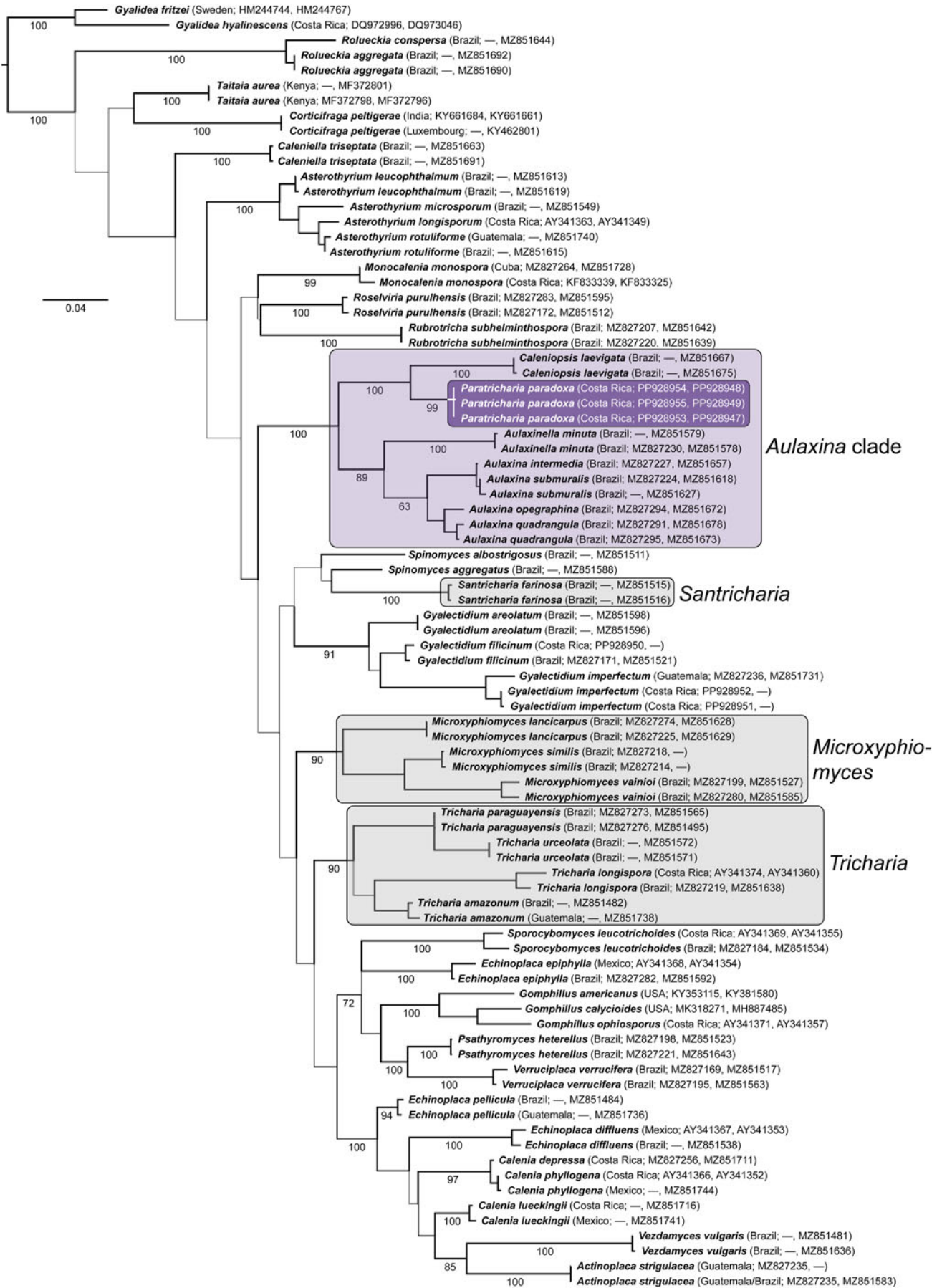


Figure 1. Best-scoring maximum likelihood tree of Gomphillaceae using the mtSSU and nuLSU markers, based on selected terminals representing most genus-level clades, showing placement of the genus Paratricharia. Bootstrap support values are shown below branches (to genus-level clades only). In colour online.

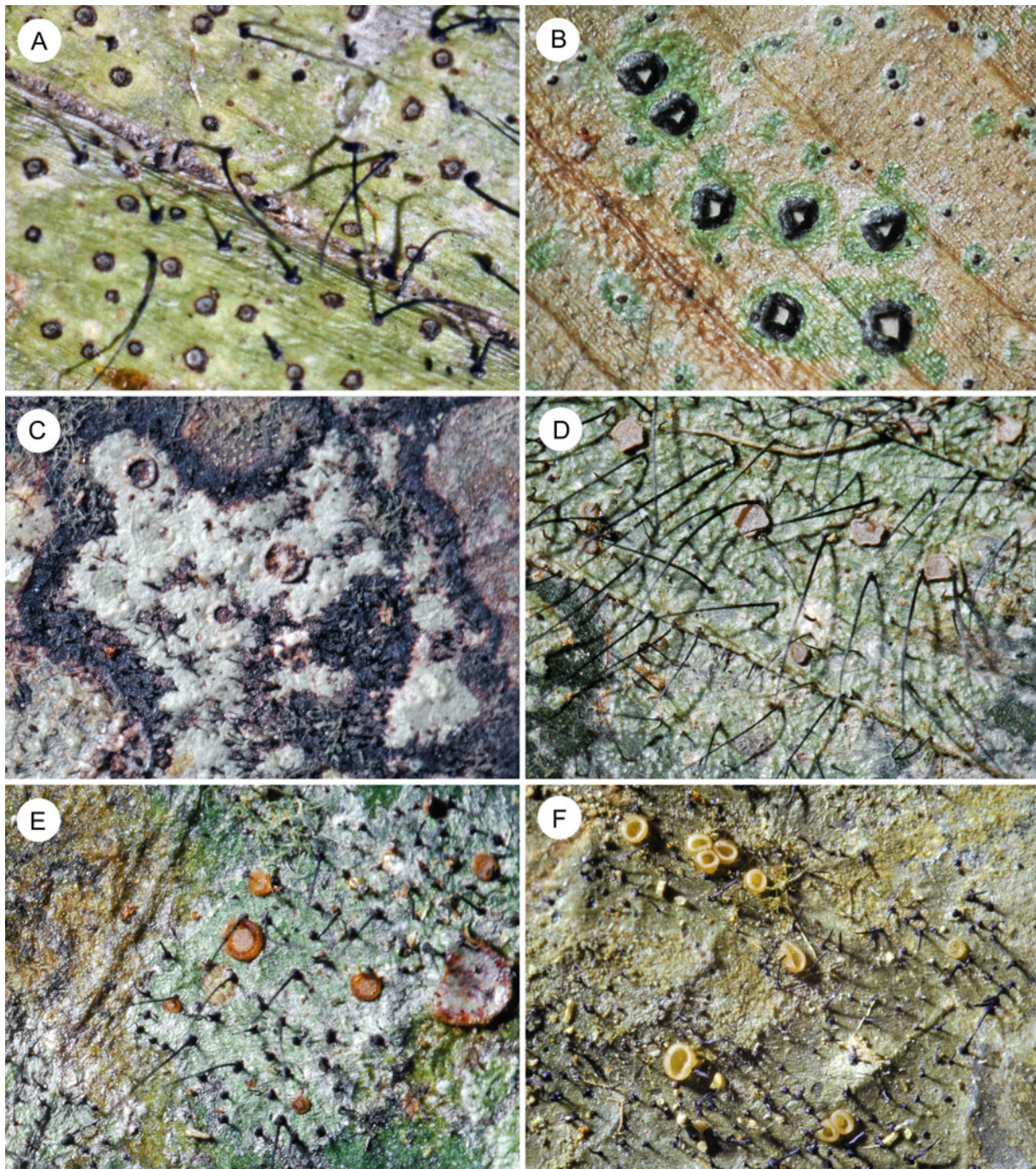


Figure 2. Morphology of selected genera and species of Gomphillaceae. A, *Paratracharia paradoxa*. B, *Aulaxina submuralis*. C, *Caleniopsis laevigata*. D, *Tricharia carnea*. E, *Microxyphiomyces vainioi*. F, *Batistomyces pallidus*. In colour online.


Our results support the findings by Xavier-Leite *et al.* (2022, 2024) that apothecial features are more conserved in the Gomphillaceae, thus having greater predictive power in the phylogenetic placement of taxa and showing a greater correlation with larger clades within the family. Hyphophore and thallus features are highly informative for genus-level clades, but similar phenotypes also evolved independently in different areas of the tree. Thus, in addition to *Paratracharia*, sterile, black setae are apomorphies for four genus-level clades in the family: *Tricharia* s. str., the *T. vainioi* clade (*Microxyphiomyces*), the *T. santessonii* clade (*Santricharia*) and the *T. pallida* Vězda clade (*Batistomyces*; see

Xavier-Leite *et al.* 2023). Similarly, genera with red-brown (*Rolueckia*, *Rubrotricha* Lücking *et al.*) or white setae (e.g. *Aderkomyces* Bat., *Arthotheliopsis* Vain., *Echinoplaca* Fée, *Psathyromyces* Bat. & Peres, *Roselviria* Xavier-Leite *et al.*) are not necessarily closely related (Xavier-Leite *et al.* 2022, 2023).

The function of these sterile setae is not known but for them to have evolved independently in so many clades, one would assume strong environmental pressure. Setiform hyphophores probably evolved to facilitate dispersal of the diahyphae in the semi-aquatic environment that leaf surfaces display during rainfall (Lücking 2001, 2008), but sterile setae do not bear diahyphae. Notably, in

almost all cases, the sterile setae are longer and more robust than the hyphophores in the same species (Vězda 1979; Sérusiaux & De Sloover 1986; Vězda & Poelt 1987; Lücking 1997, 2008).

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