Taxonomic and Phylogenetic Reassessment of *Pyrgidium* *(Mycocaliciale*) and Investigation of Ascospore Morphology

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Abstract: *Mycocaliciale* comprise non-lichenized either saprotrophic or lichenicolous fungi which occur in temperate and tropical regions. The mazaediate, saprotrophic and monospecific genus, *Pyrgidium*, is currently assigned to this order, yet the phylogenetic placement of the genus has remained uncertain due to the absence of molecular data. In order to investigate the systematic position of *Pyrgidium*, two specimens collected in Brazil and Thailand, respectively, were used to generate mtSSU, SSU, LSU and ITS sequences. However, given that most other representatives of this order only have LSU and ITS sequences available, the phylogenetic reconstruction was limited to these two markers. The phylogenetic analyses confirmed placement of the genus within *Mycocaliciale*, the genus possessing a sister group relationship with the lichenicolous genus *Sphinicrinita*. Detailed morphological descriptions and illustrations are provided, including those for type specimens of the various synonyms subsumed under the hitherto only accepted species, *Pyrgidium montellicum* (Beltr.) Tibell. The ascospore morphology was investigated using compound and scanning electronic microscopy (SEM). Principal component analysis (PCA) was performed for the ascospore size using PC-ORD 7. The molecular data and re-examination of the type specimens support the monospecific nature of this genus.

Keywords: Ascomycota; morphology; Mycocaliciale; PCA; saprotrophs; SEM

1. Introduction

Calicioid or mazaediate fungi are characterized by the production of ascospore masses accumulating on top of the ascomata after the disintegration of the asci [1,2]. Mazaediate fungi represent a heterogenous group of lichenized and non-lichenized lineages, traditionally assigned to the largely lichenized order *Caliciale* [3–7]. Vainio [8] pointed out the variable nutritional mode of the genera in this group and suggested excluding the non-lichenized genera from *Caliciale*, highlighting the absence of a photobiont when
establishing the genus *Mycocalicium*. Schmidt [9] introduced the family *Mycocaliciaceae* to accommodate the non-lichenized calicioid genera, including *Mycocalicium*, *Chaenothecopsis*, *Phaeocalicium*, *Stenocybe* and *Strongyleuma*. Nevertheless, *Caliciaceae*, *Mycocaliciaceae* and *Sphinctrinaceae* remained in the core group of *Calicium*, based on their shared morphological characteristics, such as stalked ascomata, dark, sclerotized hyphae and melanized ascospores [5,6,10,11]. Notably, *Mycocaliciaceae* have also independently evolved to have the trait of active spore dispersal without producing mazaedia [10].

Tibell [5,12] emphasized the heterogenous nature of *Caliciaceae*, and this was eventually resolved through phylogenetic analyses, which led to the placement of the calicioid lineages into different classes of *Ascomycota*, including *Arthoniomycetes*, *Eurotiomycetes*, *Lecanoromycetes* and *Leotiomyces*, and within two subclasses and several orders of *Lecanorales* [1,4,13]. Wedin and Tibell [4] showed that *Mycocaliciaceae* and *Sphinctrinaceae* form a monophyletic group within *Eurotiales*, whereas *Caliciaceae* are clustered close to *Lecanorales*. These placements were further supported by the nutritional biology and spore ornamentation [4]. Tibell and Wedin [6] then introduced *Mycocaliciaceae* to accommodate *Mycocaliciaceae* and *Sphinctrinaceae* in the *Eurotiales*, supporting findings of other studies [4–7,14]. Hibbett et al. [15] established the subclass *Mycocaliciomycetidae* for the single order *Mycocalicium*, and this classification was accepted in further works [16–24]. *Mycocaliciaceae* and *Sphinctrinaceae* share morphological characteristics, such as sessile to stalked ascomata, a sclerotized, blackish brown exciple, cylindrical asci, and dark brown ascospores with smooth or ornamented walls [6].

The *Mycocaliciaceae* family encompasses algicolous, lichenicolous and lignicolous species on bark, plant exudates, wood and lichens [25–29]. *Sphinctrinaceae* was introduced by Choisy [30] as a monogeneric family to accommodate *Sphinctrina*, and later, Tibell [12] added *Pyrgidium* to this family. Species of *Sphinctrina* are exclusively lichenicolous [5,31], while *Pyrgidium* includes one (presumably saprobic) bark-inhabiting fungus [5]. Given their shared morphological features and lack of clear phylogenetic separation, the *Sphinctrinaceae* family was treated as a synonym of *Mycocalicium* by Jaklitsch et al. [32], and this classification was followed in subsequent works [33,34].

The genus *Pyrgidium* was originally introduced by Nylander [35], with the type *P. bengaliense*. Nádvorník [36] combined *Trachylia leptocoria* Nyl. with *Pyrgidium*, while Tibell [37] transferred *Acolium montellicum* Beltr. to this genus. Tibell [5] considered *Pyrgidium* a monospecific genus, synonymizing *P. bengaliense* and *P. leptocoria* with *P. montellicum*. However, the genus was not studied in more detail afterwards, and the total number of species was defined as between one and three depending on the sources, including fungal databases and *Ascomycota* outlines [23,38,39]. The phylogenetic placement of *Pyrgidium* remained unresolved due to a lack of molecular data [6].

This study’s objective was to resolve the phylogenetic placement of *Pyrgidium* based on molecular analyses for the first time using LSU and ITS markers with the methods of maximum likelihood (ML) and Bayesian inference (BI) and, additionally, SSU and mtSSU to compare the sequence variation in materials from different tropical areas. Using both molecular and morphological data, we addressed the question of how many species can potentially be distinguished in this genus. Detailed morphological descriptions and illustrations are provided for both the freshly collected specimens and for the type specimens with the names previously assigned to *Pyrgidium*. The ascospore morphology of *Pyrgidium* was assessed with the aid of compound and SEM photographs, and PCA was performed to test the potential of specimens based on their ascospore sizes. We conclude that, at present, only one pantropical species, *P. montellicum*, should be recognized, agreeing with the previous findings of Tibell [5].

2. Materials and Methods
2.1. Sample Collection, Herbarium Examination and Morphological Studies

Fresh material was collected in Brazil and Thailand to ensure a broad geographic representation of this taxon. Type specimens of *Pyrgidium bengaliense*, *Trachyla leptocoria*, *Pyrgidium montellicum* Á. Nyl. and *Pyrgidium Á. Nyl.* are deposited at the Royal Botanic Gardens, Kew (K). The fresh material was preserved in 70% ethanol to ensure the preservation of the morphological traits. The specimens were identified based on their morphological characteristics and were compared with the type specimens to confirm their identity. The herbarium examination and morphological studies were conducted to assess the potential of specimens based on their ascospore sizes. We conclude that, at present, only one pantropical species, *P. montellicum*, should be recognized, agreeing with the previous findings of Tibell [5].
and *Acolium montellicum* were borrowed from the Uppsala University herbarium (UPS). Macro-morphological structures were observed with a dissecting microscope (MOTIC SMZ-168) and photographed with a ZEISS Discovery v8 stereomicroscope with an AxioCam ERC 5s camera (Carl Zeiss, Jena, Germany). Hand sections of the ascomata were mounted and examined in water and 5% KOH, and micro-morphological features were examined using a NIKON Eclipse 80i (Nikon Corporation, Tokyo, Japan) compound microscope fitted with a CANON 750D digital camera. For the scanning electron microscopy, ascospores from fresh and herbarium specimens of *Pyrgidium* were placed on a carbon-covered SEM mount, sputtered with palladium and examined under a scanning electron microscope (AI-FE-SEM/T) with 5 KV energy. All microscopic measurements were performed with Tarosoft Image Frame Work (0.9.0.7), and images of the photoplates were processed with Adobe Photoshop CS6 Extended 10.0 (Adobe Systems, San Jose, CA, USA). The freshly collected specimens were deposited in the ISE herbarium (Federal University of Sergipe, Brazil) and in the MFLU herbarium (Mae Fah Luang University, Chiang Rai, Thailand). Faces of the fungi numbers were registered following Jayasiri et al. [40].

2.2. DNA Extraction, PCR Amplification and Sequencing

The DNA isolation was carried out using hand-made sections of ascomata by the direct PCR method, using an E.Z.N.A.® Forensic DAT (D3591—01, Omega Bio-Tek, Norcross, GA, USA) DNA extraction kit and following the manufacturer’s instructions. DNA samples that were intended for use as a template for the PCR were stored at 4 °C to enable their use in regular work and duplicated at −20 °C for long-term storage. PCR was performed using specifications for each marker (Table 1). The purification and sequencing of the PCR products were performed by Tsingke Biotechnology Co., Ltd. (Kunming, China). The phylogenetic analyses were conducted following the recent protocol [41].

| Gene Region | Primers | PCR Condition | References |
|-------------|---------|---------------|------------|
| ITS         | ITS4 and ITS5 | 95 °C: 4 min, (94 °C: 1 min, 54 °C: 1 min, 72 °C: 45 s) × 35 cycles 72 °C: 5 min | [7,42] |
| LSU         | LROR and LR5  | 94 °C: 5 min, (94 °C: 40 s, 52 °C: 40 s, 72 °C: 40 s) × 35 cycles 72 °C: 10 min | [43,44] |
| SSU         | NS1 and NS4  | 95 °C: 15 min, (95 °C: 27 s, 54–56 °C: 30 s, 72 °C: 1 min) × 35 cycles 72 °C: 5 min | [42,45] |
| mtSSU       | mtSSU1 and mtSSU3R | 94 °C: 3 min, (94 °C: 3 min, 52 °C: 1 min, 72 °C: 1 min) × 35 cycles 72 °C: 10 min | [46] |

2.3. Phylogenetic Analyses

BLAST searches (NCBI) (https://www.ncbi.nlm.nih.gov; accessed on 15 January 2022) were performed for the newly generated sequences and, after the confirmation of their identity, the sequences were assembled in SeqMan [47] and deposited in GenBank (Table 2). For the phylogenetic analysis, we selected representative sequences of *Sphinctrinaceae* and *Mycocaliciaceae*, and for the outgroup taxa, we followed Tibell and Vinuesa [48]. The final combined LSU–ITS data set comprised 32 terminals, including five new sequences (Table 2). Given that most *Mycocalicites* are only represented by LSU and ITS sequences, we did not include the newly generated SSU and mtSSU sequences in the analysis but assessed and deposited them separately: MFLU 21-0135; SSU (ON979668), Cáceres and Aptroot 11449; mtSSU (ON979677). We followed Dissanayake et al. [41] for the phylogenetic analyses. Multiple alignments of the LSU and ITS were first performed separately with MAFFT 7 (http://mafft.cbrc.jp/alignment/server), using the default settings [49]. Ambiguous regions and introns were manually adjusted or trimmed, where necessary, using BioEdit7 [50]. The phylogenetic web tool “ALTER” [51] was used to convert the sequence alignments into the formats required for the ML and Bayesian analyses. The ML tree was generated
using RAxML-HPC2 8.2.8 on XSEDE [52] on the CIPRES Science Gateway platform [53], with 1000 bootstrap pseudoreplicates. MrBayes 3.1.2 was used to perform the Bayesian analysis [54]. We employed MrModeltest 2.3 [55] to select the best-fitting model using the Akaike information criterion (AIC), and GTR + I + G was selected as the best-fitting model for each marker. Markov Chain Monte Carlo sampling (MCMC) was run for 5,000,000 generations, and the trees were sampled every 100th generation. The first 10% of the trees that represented the burn-in phase were discarded, and the remaining 90% were used to calculate the posterior probabilities (PP) for the majority rule consensus tree. The resulting trees were visualized in FigTree 1.4.0 [56] and subsequently edited in Microsoft PowerPoint (2013) and Adobe Photoshop CS6 version 10.0.

Table 2. Taxa names, strain numbers and corresponding GenBank accession numbers of the LSU and ITS sequences used in the phylogenetic analyses. The newly generated sequences are shown in bold face.

| Taxa                      | Strain                | LSU Accessions          | ITS Accessions          | References |
|---------------------------|-----------------------|-------------------------|-------------------------|------------|
| Brunneocarpos banksiae    | CBS 141465            | NG_066277               | -                       | [29]       |
| Chaenothecopsis consociata| Tibell 22272          | DQ008999                | AY795851                | [48]       |
| Chaenothecopsis khayensis | H:JR 04G058           | -                       | NR_120165               | [57]       |
| Chaenothecopsis resinophila| H:JR 000424          | JX122782                | JX122780                | [58]       |
| Chaenothecopsis scheffleri| Rikkinen 13183       | KY499967                | KY499965                | [59]       |
| Chaenothecopsis subparoica| Tretiach (hb. Tretiach)| -                      | AY795869                | [48]       |
| Chaenothecopsis viridireagens| H:Tuovila 09-068    | JX119117                | JX119108                | [58]       |
| Chaenothecopsis pallida   | H:JR 010652           | JX122781                | JX122779                | [58]       |
| Chaenothecopsis pusiola   | Tibell 15884 (UPS)    | -                       | AY795865                | [48]       |
| Chaenothecopsis fennica   | Tibell 16024 (UPS)    | AY795995                | AY795857                | [48]       |
| Chaenothecopsis sitchensis| Tuovila 06-33 (TUR)  | KF157988                | -                       | [59]       |
| Chaenothecopsis golubkovae| Titov 6707 (UPS)     | AY795996                | AY795859                | [48]       |
| Chaenothecopsis viridireagens| Tibell 22803 (UPS)  | DQ013257                | AY795872                | [48]       |
| Fusichalara minuta        | CBS 709.88            | KX537758                | KX537754                | [60]       |
| Myccocalicium subtile     | Tibell 16744 (UPS)    | AY796004                | -                       | [48]       |
| Myccocalicium subtile     | Tibell 17164 (UPS)    | AY796005                | -                       | [48]       |
| Myccocalicium albignonum  | Tibell 19038          | AY796001                | AF223966                | [48]       |
| Phaeocalicium curtisi     | BIOUG24047-F02       | -                       | KT695401                | [61]       |
| Phaeocalicium populneum   | Tibell 19286 (UPS)    | AY796009                | AY795857                | [48]       |
| Phaeocalicium praeceiens  | Tuovila 09-240 (TUR) | KC904864                | KC904881                | [27]       |
| Pyrenula minatispora      | ABL AA11877           | -                       | KT820119                | [62]       |
| Pyrenula nitida           | F 9292               | DQ329023                | JQ927458                | [63,64]    |
| Pyrgidium montellicum     | MFLU 21-0135a        | ON979678                | ON979674                | This study |
| Pyrgidium montellicum     | MFLU 21-0135b        | ON979678                | ON979674                | This study |
| Pyrgidium montellicum     | Caceres and Aptroot 11449 | OP077215              | ON979667                | This study |
| Rhopalophora clavispora   | CBS 129.74           | MH872573                | KX537751                | [60]       |
| Rhopalophora clavispora   | CBS 281.75           | KX537756                | KX537752                | [50]       |
| Sphinctrina leucopoda     | Kalb 33829 (hb. Kalb)| AY796006                | AY795875                | [48]       |
| Sphinctrina turbinata     | AFITOL-ID 1721       | EF413632                | -                       | [14]       |
| Sphinctrina turbinata     | Tibell 22478 (UPS)   | -                       | AY795876                | [14]       |
| Stenocybe pullatula       | Tibell 17117 (UPS)   | AY796008                | AY795878                | [48]       |
| Verrucaria inverecundula  | FILIC650-13          | -                       | MK138796                | [65]       |

2.4. PCA

The PCA was performed in PC-ORD 7 to assess the size variation in the ascospores of Pyrgidium. The ascospore length and width, as well as the Q value (length:width ratio), were used as variables for the eight specimens of P. montellicum from various geographic regions, including Caceres and Aptroot 11449, Kurz 1866, L-008798, L-996762, Lindig 2865, MFLU 21-0135, Tibell 8232 and Tibell 8306. Measurements were taken from 50 ascospores of each specimen.

To test for significant differences in the ascospore size according to the geographic region by means of ANOVA with post hoc Tukey HSD, we grouped the measurements
into three categories: (1) the Neotropics (Costa Rica, Colombia, Brazil), (2) Europe (Italy), and Paleotropics (India, Thailand). The ANOVA and the post hoc Tukey HSD were performed online (https://www.socscistatistics.com/tests/anova/default2.aspx, accessed on 15 January 2022).

3. Results

3.1. Phylogenetic Analyses

The final LSU–ITS dataset comprised 32 taxa with 1532 aligned characters, including gaps (LSU: 894; ITS: 638). The best-scoring ML tree was selected to represent the relationships between the taxa, with the final ML optimization likelihood value of −10603.813268 (Figure 1). The parameters for the GTR + I + G model of the combined LSU and ITS data were as follows: the estimated base frequencies $A = 0.240252$, $C = 0.243881$, $G = 0.287821$, $T = 0.228046$, and the substitution rates $AC = 1.442589$, $AG = 2.659004$, $AT = 1.892966$, $CG = 1.070779$, $CT = 7.537779$ and $GT = 1.000000$. Bayesian posterior probabilities from the MCMC were evaluated with the final average standard deviation of split frequencies = 0.001450. The topologies of the ML and the Bayesian tree were manually compared and were largely congruent.

The genera of Mycocaliciales were resolved as monophyletic clades, except for Chaenothecopsis, which appears to be polyphyletic. The genus Pyrgidium formed a sister clade with Sphinctrina, and both clades were strongly supported. Pyrgidium itself formed two clades, one with a single specimen from Brazil and the other with two sequences from Thailand, the latter two clustering with a high level of statistical support.
Figure 1. Best-scoring ML tree based on the analysis of the combined LSU and ITS sequence data. Bootstrap support values equal to or greater than 70% and Bayesian posterior probabilities (BP) equal to or greater than 0.95 are given as ML/BP above the branches next to the nodes. Ex-type strains of genera other than *Pyrgidium* are displayed in bold, and the new sequences generated in this study are indicated in blue. The tree was rooted with *Fusichalara minuta* (CBS 709.88), *Pyrenula minutispora* (ABL AA11877), *P. nitida* (F 5929), *Rhopalophora clavispora* (CBS 281.75), *R. clavispora* (CBS 281.75), and *Verrucaria inverecundula* (FILIC650-13), following Tibell and Vinuesa [48].
3.2. PCA

The PCA indicated a homogeneous, unimodal distribution of the ascospore size measurements, with only one large cluster, although the data on the ascospore width included two outliers (Figure 2). There was some tendency of the samples to differentiate according to region, especially regarding the ascospore width; the samples from the Neotropics clustered more towards the left and those from the Paleotropics more towards the right of the first axis, which largely corresponded to the ascospore width. Mean values for the ascospore width were 6.30 μm (Neotropics), 6.42 μm (Europe) and 6.77 μm (Paleotropics). The mean values for the ascospore width were 3.45 μm (Neotropics), 3.52 μm (Europe) and 3.97 μm (Paleotropics). The mean values for the Q value (ratio) were 1.84 (Neotropics), 1.81 μm (Europe) and 1.75 μm (Paleotropics).

This tendency was significant in terms of the ascospore width and length (ascospore length ANOVA: f-ratio = 13.8751, p < 0.00001; ascospore width ANOVA: f-ratio = 51.2113, p < 0.00001) but not for the Q value or the length:width ratio (ANOVA: f-ratio = 2.6403, p = 0.0726). The length and width differed significantly between regions 1 and 2 (Neotropics, Europe), on one hand, and region 3 (Paleotropics), on the other, but not between regions 1 and 2 (length according to post hoc Tukey HSD: 1 vs. 2: Q = 1.77, p = 0.4230; 1 vs. 3: Q = 7.04, p = 0.0000; 2 vs. 3: Q = 5.27 (p = 0.0007; width according to post hoc Tukey HSD: 1 vs. 2: Q = 2.18, p = 0.2736; 1 vs. 3: Q = 13.36, p = 0.0000; 2 vs. 3: Q = 11.19, p = 0.0000).
Figure 2. PCA plot of the ascospore length and width. Measurements were taken for eight specimens, which included fresh and herbarium specimens from 50 ascospores of each specimen. Brazil: Cáceres and Aptroot 11449; Colombia: Lindig 2865; Costa Rica: Tibell 8232, Tibell 8306; India: Kurz 1866; Italy: Beltramiini s.n. (L-008798, L-996762); Thailand: MFLU 21-0135.

4. Taxonomy

4.1. Sphinctrinaceae M. Choisy, Bull. Mens. Soc. Linn. Soc. Bot. Lyon 19: 65 (1950)

Type genus: *Sphinctrina* Fr.

Syn.: *Mycocaliciaceae* A.F.W. Schmidt, Mitt. Staatsinst. Allg. Bot. Hamburg 13: 127 (1970).

Type genus: *Mycocalicium* Vain.

Notes: With the inclusion of *Mycocaliciaceae*, *Sphinctrinaceae* comprises seven genera, viz., *Brunneocarpos, Chaenothecopsis, Mycocalicum, Phaeocalicum, Pyrgidium, Sphinc-
4.2. Pyrgidium Nyl., Flora, Regensburg 50: 3 (1867)

Index Fungorum number: IF 4617; faces of fungi number: FoF 12620.

Type species: Pyrgidium montellicum (Beltr.) Tibell, lichenologist 14(3): 239 (1982).

Notes: Pyrgidium was previously assigned to Sphinctrinaceae, without molecular data, and the present molecular study supports this placement. According to Tibell [5], the genus comprises a single species, P. montellicum, found mainly in the neotropics but also known from other tropical regions. Pyrgidium is characterized by its sessile to stalked ascomata, blackish brown and sclerotized exciple, either simple or 1-septate, and broadly ellipsoid to oval, dark brown, ornamented ascospores [5].

4.3. Pyrgidium montellicum (Beltr.) Tibell, Lichenologist 14(3): 239 (1982) (Figures 3 and 4)

Index Fungorum number: IF 110065; faces of fungi number: FoF 10274.

Basionym: Acolium montellicum Beltr. 1858 [37].

Index Fungorum number: IF 110065; type: Italy. Bazzano, Beltramini s.n., ex Hb. Massalongo (UPS-Syntype).

Synonyms:
Pyrgidium leptoconium (Nyl.) Nádv., Stud. Bot. Čechoslov. 5: 125 (1942).

Index Fungorum number: IF 369854.

Trachylia leptoconia Nyl., Acta Soc. Sci. fenn. 7(2): 429 (1863) [37] (Figure 6).

Index Fungorum Number: IF 407801; type: Colombia (Colombia), Nova Granata, Fusagasuga, Lindig. A (Lindig 2865, UPS-Isotype).

Saprobic on bark. Thallus crustose, farinose or absent, and whitish. Prothallus is absent. Photobiont with loosely associated algae, sparsely present in the thallus, and mostly trentepohlioid or absent. Sexual morph: Ascomata are scattered, apothecial, rarely urn-shaped, 180–330 μm diam., 130–290 μm high (M = 255 × 210 μm), sessile to shortly stalked, almost sphaerical, mazaedioid, with a black disc. Excipulum of 10–55 μm thickened laterally, 27–90 μm thickened basally, mostly thickened basally and gradually becoming thinner towards the upper part, sometimes comprising sclerotized hyphae. Mazaedium filling the cavity of the ascoma and more or less projecting beyond the excipular edge. Paraphyses are 0.4–2 μm thick, septate, and unbranched. Asci of 18–56 × 3–9 μm (M = 37 × 6 μm, n = 30), cylindrical, 8-spored, unitunicate, tip-blunted, not narrowing towards the apex, with the ascus apex thickened in immaturity and reduced or inconspicuous in maturity, and short pedicellate. Ascospores of 4–10 × 2–5 μm (M = 7 × 3.5 μm, n = 40), broadly ellipsoid to oval, uniseriate to biseriate, and light to dark brown, (0-)1-septate, with a dark brown septum, small appendage present at one end in few ascospores, and with guttulates when immature, wall verrucose or with irregular, longitudinally arranged ridges. Asexual morph: unknown.

Notes: Pyrgidium montellicum has thus far been reported in Central and South America (Costa Rica, Colombia, Ecuador, Brazil, Argentina), Eurasia (Italy, Iran, Russia, China), the eastern Paleotropics (India, Sri Lanka, Thailand, Papua New Guinea) and Australasia [5,64,67–71] (https://www.gbif.org/species/3269679, accessed on 15 January 2022). The new collections of Pyrgidium montellicum studied here also confirm the previously reported presence of this taxon in Brazil, where it is known to be present in the Amazon [72], and the subtropical coast of Rio de Janeiro [5] and Thailand [69], which is now, for the first time, supported by molecular data. The Brazilian specimens were mostly over mature and the ascii were difficult to observe.
The collection of *Pyrgidium montellicum* from Thailand and Brazil clustered together in the phylogenetic analysis, providing strong support for the accurate placement of this lineage. No significant morphological differences were observed between the specimens representing the fresh collections and the types known by three names currently synonymized under the name *P. montellicum*, excepting the tendency of the paleotropical collections from India and Thailand to produce larger and especially broader ascospores. Base pair comparisons between the Brazilian and the Thai specimens revealed about a 4.7% difference in the ITS markers, correlating with the differences in the ascospore size. Even without evident morphological differences, such sequence divergences have been used to distinguish morphologically cryptic species in other cases [73,74]. Indeed, several studies have been conducted to assess the potentially cryptic nature of mycocalicioid species due to the limited number of taxonomically useful characteristics [75,76]. However, differences in a single molecular marker may not be seen as sufficient for establishing species boundaries in a cryptic lineage, especially if few specimens have been sequenced [74]. Here, a case could be made for the separation of the paleotropical populations into different species, but since only a few specimens have been examined molecularly and morphologically, for the time being, we agree with Tibell [5] that only a single sub-cosmopolitan species should be presently recognized. If the analysis of more material supports the encountered differences, *P. bengaliensis* could be resurrected for the paleotropical material. Apart from the size of the ascospores, several other characteristics, such as color (brown to dark brown), septation (0-1-septate), ornamentation ( verrucose, guttulates in immaturity and irregular ridges, longitudinally arranged), a thicker outer wall, and highly pigmented septa with constrictions at the septum, do not seem to provide any taxonomic value in this case (Table 3).

Material examined: Thailand, Chiang Mai, 128 Moo3, Bahn Pa Dheng, T. Pa Pae, A. Mae Taeng, on the bark of an unidentified tree, 10 September 2020, Vinodhini Thiagaraja (MFLU 21-0135); Brazil, Rondônia, Porto Velho, Parque Circuito, on the bark of *Hevea brasiliensis*, 11 March 2012, Andrê Aptroot and Marcela Eugenia da Silva Cáceres (Cáceres and Aptroot ISE 11449).

Description of the fresh material of *Pyrgidium montellicum*: Saprobic on bark. Thallus crustose, whitish and endoperidermal. Prothallus absent. Photobiont absent or loosely associated algal cells sparsely present in the thallus, and trentepohlioid. Sexual morph: Ascomata scattered, apothecial, 22–330 µm diam., 130–195 µm high M = 277 × 162 µm), sessile to shortly stalked, almost sphaerical, mazaedioid, with a black disc. Excipulum 25–55 µm thick laterally, 30–60 µm thick basally, brown to black, prosoplectenchymatous, with the edge comprised of sclerotized hyphae, the edge of the excipulum turned inward in the topmost part in immature ascomata and eventually turned outward in the topmost part in maturity, laterally and gradually becoming thinner. Mazaedium filling the cavity of the ascoma and more or less projecting beyond the excipular edge. Paraphyses of 1–2 µm thick, septate and unbranched. Asci of 25–40 × 4–7 µm (M = 32 × 5.5 µm, n = 30), cylindrical, shortly pedicellate, 8-spored, unitunicate, tip-blunted and not narrowing towards the apex, with the ascus apex thickened when immature and reduced or inconspicuous when mature. Ascospores of 5–9 × 2.5–4.5 µm (M = 5.5 × 3.5 µm, n = 40), broadly ellipsoid to oval, uniseriate to biseriate, light to dark brown and (0-)1-septate, with a dark brown septum, small appendage present at one end in a few ascospores, with guttulates when immature, wall verrucose or with irregular, longitudinally arranged ridges (Figure 7K–P). Asexual morph: unknown.
Figure 3. *Pyrgidium montellicum* (Thailand: MFLU 21-0135, Brazil: Cáceres and Aptroot 11449), (A–D). Ascomata on substrate (MFLU 21-0135), (E–G). Vertical section through the ascoma (MFLU 21-0135), (H). Vertical section through the exciple (MFLU 21-0135), (I). Loosely associated algae (MFLU 21-0135), (J). Paraphyses (MFLU 21-0135), (K₁–K₅). Asci (MFLU 21-0135), (L₁–L₁₀). Ascospores in water (MFLU 21-0135), (L₁₁–L₁₉). Ascospores in 5% KOH (MFLU 21-0135), (M–O). Ascomata on substrate (Cáceres & Aptroot 11449), (P). Vertical section through the ascoma (Cáceres and Aptroot 11449), (Q₁–Q₁₀). Ascospores in water (Cáceres and Aptroot 11449). Scale bars: (B–D), (M–O) = 500 µm, (C,D) = 200 µm, (E–G), (P) = 100 µm, (H) = 50 µm, (I) = 10 µm, (J) = 5 µm, (K₁–K₅) = 20 µm, (L₁) = 10 µm, (L₂–L₁₉), (Q₁–Q₁₀) = 5 µm.

The types of the three synonyms of *Pyrgidium montellicum* are characterized as follows:
**Figure 4.** *Pyrgidium montellicum* (type materials of *Acolium montellicum* (L-008798, L-996762) and non-type materials of *P. montellicum* (Tibell 8306, Tibell 8232)), (A–D). Details of herbarium specimens (A). Tibell 8306, (B). Tibell 8232, (C). L-008798, (D). L-996762. (E–I). Ascomata on substrate (E,H). Tibell 8306, (F). Tibell 8232, (G,I). L-008798. (J–L). Vertical section through the exciple, (J). Tibell 8306, (K). Tibell 8232, (L). L-996762. (M). Tibell 8232, (N). L-996762, L-008798. Asci, L-996762, L-008798. L-996762, 1981 L-996762, (P1–P22). Tibell 8306, (P1–P8). L-008798, (P12–P16). 1981 L-996762, (P17–P22). Tibell 8232). Scale bars: (E,F) = 1000 μm, (G,H) = 500 μm, (J–L) = 100 μm, (M) = 20 μm, (K) = 5 μm, (O1–O9) = 20 μm, (P1–P22) = 5 μm.

Description of the type *Acolium montellicum*: Saprobic on bark. Thallus crustose, farinose and whitish. Prothallus absent. Photobiont with loosely associated algae, sparsely
present in thallus, mostly trentepohlioid or absent. Sexual morph: Ascomata scattered, apothecial, rarely urn-shaped, 180–225 µm diam., 160–290 µm high (M = 202 × 225 µm), sessile to shortly stalked, almost sphaerical, mazaedioid, with a black disc. Excipulum 10–40 µm thickened laterally, 27–46 µm thickened basally, mostly thickened basally and gradually becoming thinner towards the upper part, sometimes comprising sclerotized hyphae. Mazaedium filling the cavity of the ascoma and more or less projecting beyond the excipular edge. Paraphyses 0.5–2 µm thick, septate and unbranched. Asci 25–40 × 2–6 µm (M = 32 × 4 µm, n = 30), cylindrical, 8-spored, unitunicate, tip-blunted and not narrowing towards the apex, with the ascus apex thickened in immaturity and reduced or inconspicuous in maturity, and short pedicellate. Ascospores 4–7 × 2–4 µm (M = 5.5 × 3 µm, n = 40), broadly ellipsoid to oval, uniseriate to biseriate, light to dark brown, (0-)1-septate, with a dark brown septum, small appendage present at one end in few ascospores, with guttulates when immature, wall verrucose or with irregular, longitudinally arranged ridges. Asexual morph: unknown.

Figure 5. Pyrgidium montellicum (type material of P. bengaliense) (Kurz 1866, Isotype) (A). Details of the herbarium specimen, (B–D). Ascomata on substrate, (E). Vertical section through the ascoma, (F). Vertical section through the exciple, (G). Paraphyses, (H1–H4). Asci, (I1–I4). Ascospores (in water), (I5–I14). Ascospores (in 5% KOH). Scale bars: (C) = 500 µm, (D) = 200 µm, (E) = 100 µm, (F) = 20 µm, (G) = 5 µm, (H1–H3) = 20 µm, (I1–I14) = 5 µm.

Description of the type Pyrgidium bengaliense: Saprobic on bark. Thallus inconspicuous, pruinose around the ascomata. Prothallus absent. Photobiont absent. Sexual morph: Ascomata

Description of the type Pyrgidium bengaliense: Saprobic on bark. Thallus inconspicuous, pruinose around the ascomata. Prothallus absent. Photobiont absent. Sexual morph: Ascomata
apothecial, 190–205 µm diam., 180–220 µm high (M = 197 × 200 µm, n = 10), not stalked, mazaedoid, with a black disc, sessile, scattered and almost spherical. *Excipulum* 30–55 µm thickened laterally, 60–90 µm thickened basally, brown to black, prosoplectenchymatous and comprising some sclerotized hyphae. *Mazaedium* filling the cavity of the fruit body and more or less projecting beyond the excipular edge. *Paraphyses* 1.2–1.8 µm thick, septate and simple. *Asci* 18–33 × 4–9 µm (M = 25.5 × 6.5 µm, n = 30), cylindrical, 8-spored, unitunicate, tip-blunted and not narrowing towards the apex, with the ascus apex thickened in immaturity and reduced or inconspicuous in maturity, and short pedicellate. *Ascospores* 5.5–10 × 3–5 µm (M = 7.75 × 4 µm, n = 40, ellipsoidal, overlapping bi-seriate, light brown to brown, 0-1-septate, with a slightly dark brown septum, sometimes constricted at the septum, with gattulates when immature, verrucose, irregular ridges that are longitudinally arranged (Figure 7G–J). Asexual morph: undetermined.
30–55 µm thickened laterally, 60–90 µm thickened basally, brown to black, prosoplectenchymatous and comprising some sclerotized hyphae. Mazaedium filling the cavity of the fruit body and more or less projecting beyond the excipular edge. Paraphyses 1.2–1.8 µm thick, septate and simple. Asci 18–33 × 4–9 µm (M = 25.5 × 6.5 µm, n = 30), cylindrical, 8-spored, unitunicate, tip-blunted and not narrowing towards the apex, with the ascus apex thickened in immaturity and reduced or inconspicuous in maturity, and short pedicellate. Ascospores 5.5–10 × 3–5 µm (M = 7.75 × 4 µm, n = 40, ellipsoidal, overlapping bi-seriate, light brown to brown, 0-1-septate, with a slightly dark brown septum, sometimes constricted at the septum, with gattulates when immature, verrucose, irregular ridges that are longitudinally arranged (Figure 7G–J). Asexual morph: undetermined.

Figure 6. Pyrgidium montellicum (type material of Trachylia leptoconia) (Lindig 2865, isotype) (A). Details of the herbarium specimen, (B–D). Ascomata on substrate, (E). Vertical section through the ascoma, (F). Vertical section through the exciple, (G). Paraphyses, (H₁–H₆). Asci, (I₁–I₁₃, I₁₅). Ascospores (in water), (I₁₄). Ascospore (in 5% KOH). Scale bars: (D) = 500 µm, (E) = 100 µm, (F) = 20 µm, (G) = 5 µm, (H₁–H₆) = 30 µm, (I₁–I₁₅) = 5 µm.

Description of the type Trachylia leptoconia: Saprobic on bark. Thallus crustose and farinose. Prothallus absent. Photobiont absent. Sexual morph: Ascomata apothecial, 220–240 µm diam., 220–235 µm high (M = 230 × 227 µm, n = 10), not stalked, mazaedioid, with a black disc, sessile, scattered and almost spherical. Mazaedium filling the cavity of the fruit body and more or less projecting beyond the excipular edge. Excipulum 22–28 µm thickened laterally, 35–55 µm thickened basally, brown to black, prosoplectenchymatous, hardly compromise any sclerotized hyphae, thickened basally and gradually becoming thinner towards the
upper part. Paraphyses 1.2–2 µm thick, septate and simple. Asci 34–56 × 4–5 µm (M = 45 × 4.5 µm, n = 30), cylindrical, 8-spored, unitunicate and tip-blunted, with the ascus apex thickened when immature and reduced or inconspicuous when mature, and short pedicellate. Ascospores 4.5–9 × 2–5 µm (M = 6.75 × 3.5 µm, n = 40), ellipsoidal, uniseriate, overlapping, light brown to brown, 0-1-septate, with a dark brown septum, small appendage present at one end in few ascospores, with gattulates when immature, and verrucose, irregular ridges that are longitudinally arranged (Figure 7D–F). Asexual morph: undetermined.

Table 3. Morphological comparison of the herbarium and fresh specimens based on this study.

|                         | P. bengaliense (Kurz 1866; Isotype UPS) | Trachylia leptoconia (Lindig 2865; Isotype UPS) | Acolium montellicum (L-008798; Syntype UPS) | Acolium montellicum (L-996762) | P. montellicum (Tibell 8306; Non-Type UPS) | P. montellicum (Cáceres and Aptroot 11449) | P. montellicum (MFLU 21-0135) |
|-------------------------|----------------------------------------|-----------------------------------------------|---------------------------------------------|---------------------------------|---------------------------------------------|---------------------------------------------|--------------------------------|
| Thallus                 | Absent                                 | Farinose                                      | Farinose                                    | Farinose                        | Absent                                      | Absent                                      | Farinose                      |
| Ascomata width (µm)     | 190–205                                | 220–240                                       | 215–225                                     | 185–190                         | 180–195                                     | 190–205                                     | 225–235                       | 180–330                        |
| Ascomata height (µm)    | 180–220                                | 220–235                                       | 160–170                                     | 175–190                         | 210–290                                     | 170–190                                     | 130–145                       | 135–195                        |
| Exciple (lateral) (µm)  | 30–55                                  | 22–28                                         | 26–31                                       | 25–40                           | 11–30                                       | 30–39                                       | 30–45                          | 25–55                          |
| Exciple (base) (µm)     | 60–90                                  | 35–55                                         | 33–46                                       | 28–40                           | 27–28                                       | 27–38                                       | 34–46                          | 30–60                          |
| Paraphyses width (µm)   | 0.9–2                                  | 1–2                                           | 0.8–1.6                                     | 1–2                            | 0.7–1.7                                     | 0.4–1.6                                     | –                             | 1–2                            |
| Asci length (µm)        | 18–33                                  | 34–56                                         | 31–36                                       | 29–39                           | 28–32                                       | 25–40                                       | –                             | 25–40                          |
| Asci width (µm)         | 4–9                                    | 4–5                                           | 3.5–5                                       | 3–6                            | 3–4                                         | 2–5                                         | –                             | 5–7                            |
| Ascospore length (µm)   | 5.5–10                                 | 4.5–9                                         | 4–5.5                                       | 5–7                            | 4.5–7                                       | 4.5–7                                       | 5–8                           | 6–9                            |
| Ascospore width (µm)    | 3–5                                    | 2–5                                           | 2.6–3.8                                     | 2–4                            | 2.5–4                                       | 2–4                                         | 2.7–4                          | 2.5–4.5                        |
| Q value                 | 1.94                                   | 1.82                                          | 1.76                                        | 1.85                           | 1.73                                        | 1.98                                        | 1.81                          | 1.55                           |
| No. of septates per ascospore | 0–1                                 | 0–1                                           | 0–1                                         | 0–1                            | 0–1                                         | 0–1                                         | 0–1                           | 0–1                            |
| Geographical occurrence | India                                  | Colombia                                      | Italy                                       | Italy                           | Costa Rica                                   | Costa Rica                                   | Brazil                         | Thailand                       |
5. Discussion

Although *Pyrgidium montellicum* is usually considered a saprotrophic taxon, several studies reported *Trebouxia* or allied cystococcaceous alga as photobionts, and some defined the taxon as commensal on lecanoralean lichens [6,37]. In addition, *P. montellicum* may serve as a host for lichenicolous fungi, such as *Chaenothecopsis rubina* Tibell [37]. In the material assessed in the present study, including both the fresh and historical specimens, no obligate and stable association with a particular photobiont was observed, but some specimens showed a weak association with trentepohlioid algae. Nádvorník [36] reported the presence of a *Trentepohlia* photobiont in the original material of *Pyrgidium leptoconium*, but this was not seen in the isotype material examined here. These findings support the notion that algal associations are accidental or facultative in the case of *P. montellicum* and that the taxon is primarily saprotrophic [37]. A similar situation can be observed with other borderline lichenized fungi, such as *Arthopyrenia salicis* A. Massal., *Cresporhaphis macrospora* (Eitner) M.B. Aguirre, *Requienella seminuda* (Pers.) Boise and *Splanchnonema lichenisatum* Aptroot and K.H. Moon, which are facultatively associated with various photobionts but were also recorded without any algal associations [77–79]. Notably, these lineages are found in predominantly non-lichenized clades, such as *Dothideomycetes* and *Sordariomycetes* [77–80].
suggesting initial evolutionary attempts at lichenization in these lineages. On the other hand, several bark-inhabiting fungi are known to have emerged from largely lichenized clades, and their saprotrophic mode evolved secondarily from their lichenized ancestors [81–83].

Eurotiomycetes comprise lichenized and lichenicolous lineages mainly in the orders of Pyrenulales and Verrucariales within the subclass of Chaetothyriomycetidae [14]. Among these, Pyrenula coryli A. Massal. has been recorded as non-lichenized [84]. Verrucariales largely encompass lichenized species or mycophycobioses [85,86]. In contrast, saprotrophic species are mainly found in Myccocalicioidae within the subclass Myccocaliciomycetidae, in the genera Brunncarpos, Chaenothecopsis, Myccalicium, Phaeocalicium, Stenocybe and Strongyleuma. Chaenothecopsis also comprise lichenicolous taxa, and one species, C. pusilla (Ach.) A.F.W. Schmidt, was found to be facultatively associated with algae [87,88].

Ascospore characteristics, such as color, size, septation and ornamentation, have been used for the generic and species delineation of myccocalicioid fungi [5,37]. Ascospores have been mostly recorded as 1-septate in Pyrgidium [5,37], although aseptate ascospores were also frequently observed in the material examined in this study. Sometimes, appendages were observed at one end of the ascospores (Tibell 8306; Tibell 8232; Lindig 2865; MFLU 21-0135). The ascospores of Pyrgidium have small warts or ridges that were visible under the scanning electron microscope. Nylander [35] defined the ascospore dimensions as 5–9 × 3–4 µm for Pyrgidium bengaliense, and the re-examination of the isotype material resulted in dimensions of 5.5–10 × 3–5 µm. The ascospore dimensions of the original material of P. leptoconia were given as 6–8 × 4–4.5 µm, whereas the isotype revealed measurements of 4.5–9 × 2–5 µm. Pyrgidium montellicum was originally described as having 5.5–7 × 3–4.5 µm large ascospores, while our measurements were 4–7 × 2–4 µm for the four nontype specimens. Variations in then ascospore size revealed limited clustering tendencies according to the geographic region, especially regarding the width, and both the length and width showed minor but significant differences between the groups from the Neotropics and Europe, on one hand, and the Paleotropics, on the other. However, due to the limited material examined, we refrained from dividing P. montellicum into more than one species at this point in time.

Tibell [37] described P. montellicum as having perithecial, urn-shaped ascomata, but later studies described them as apothecial [5,88]. The re-examination of several collections revealed both urn-shaped perithecial and apothecial ascomata, suggesting that these morphologies intergrade during ontogeny. The asci of all myccocalicioid fungi are known to arise from croziers [89,90], and careful microscopic observations revealed the presence of croziers in all the studied specimens of Pyrgidium. The ascus apex was almost reduced in maturity and could only be observed in the immature stage or after adding 5% KOH. Overall, considering all these characteristics, there are no clear-cut differences between the examined specimens, which, at present, supports the argument of Tibell [5] that we should consider Pyrgidium as a monospecific genus. However, the variation in the ITS associated with geography (Brazil vs. Thailand) warrants further attention and should be investigated using more material so as to assess the potential cryptic speciation through vicariance.

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