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A new hysteriform dothideomycete (Gloniaceae, Pleosporomycetidae *incertae sedis*), *Purpurepithecium murisporum* gen. et sp. nov. on pine cone scales

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Abstract – The family Gloniaceae is represented by the genera *Glonium* (plant saprobes) and *Cenococcum* (ectomycorrhizae). This work adds to the knowledge of the family, by introducing a new taxon from dead scales of pine cones collected on the ground in Chiang Mai Province, Thailand. Analysis of a combined LSU, SSU, RPB2 and TEF1 sequence dataset matrix placed it in Gloniaceae and *Purpurepithecium murisporum* gen. et sp. nov. is introduced to accommodate the new taxon. The genus is characterized by erumpent to superficial, navicular hysterothecia, with a prominent longitudinal slit, branched pseudoparaphyses in a gel matrix, with a purple pigmented epithecium, hyaline to dark brown muriform ascospores and a *Psiloglonium stygium*-like asexual morph which is produced in culture. The new taxon is illustrated and compared with similar genera.

Cenococcum* / *Glonium* / hysterothecia / *Purpurepithecium

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INTRODUCTION

Hysteriaceous ascomycetes are an interesting and important group of fungi and represented by few families and orders in the class Dothideomycetes (Hyde *et al.*, 2013, Wijayawardene *et al.*, 2014). Most species of this group are saprobic and mainly occur on twigs or bark of various woody plants and herbaceous plants in terrestrial and aquatic environments (Mugambi and Huhndorf 2009; Zhang *et al.*, 2009; Hyde *et al.*, 2013; Doilom *et al.* 2016) and worldwide in distribution. In addition, they can be endophytes (Xu *et al.*, 2015), ectomycorrhizae (Spatafora *et al.*, 2012) and are soft rot fungi (Yacharoen *et al.*, 2015). The majority of recent research on this group was carried out by Boehm *et al.* (2009a, b) and phylogenetic studies have shown that hysteriaceous taxa do not form a monophyletic group (Boehm *et al.*, 2009a, b; Mugambi and Huhndorf 2009; Voglmayr *et al.*, 2017). This has resulted in several changes to the classification of the group, such as the description of a new order, Mytilinidiales, introduction of two new families, Anteagloniaceae (Pleosporales) and Gloniaceae *incertae sedis*, along with several new genera and combinations (Boehm *et al.*, 2009a, b; Mugambi and Huhndorf 2009; Hyde *et al.*, 2013). Voglmayr *et al.* 2017 introduced *Stigmatodiscus pruni* with hysteriform ascomata and referred it to the Stigmatodiscales.

Corda (1842) originally proposed Gloniaceae Corda with a sub-familial taxonomic rank in the family Hysteriaceae, in which he placed both *Hysterographium* and *Glonium*. Boehm *et al.* (2009a) emended and restricted this sub-familial rank and elevated it to family rank using the monophyletic genus *Glonium*. The genus *Glonium* was retained as circumscribed first by von Höhnel (1918) and then by Petrak (1923). In addition, not enough taxa have been sequenced in Gloniaceae to establish whether they deserve ordinal status and therefore Boehm *et al.* (2009b) placed Gloniaceae in Pleosporomycetidae family, *incertae sedis*. *Glonium* produces darkly pigmented, carbonaceous ascomata on bark, wood or soil. The asci are clavate to cylindrical and fissitunicate, and the ascospores are hyaline to lightly pigmented, with a single conspicuous septum (Boehm *et al.*, 2009a). *Glonium* and *Psiloglonium* share the same asexual morph (ex. *Psiloglonium stygium*) (Lohman 1933, 1937). *Cenococcum* is a genus of ectomycorrhizal Dothideomycetes belonging to Gloniaceae based on a multigene phylogenetic analysis and there is no record of any sexual or asexual morph for this group (Spatafora *et al.*, 2012). It is the only genus of mycorrhizal fungi in Dothideomycetes and represents an independent origin of mycorrhizae among Ascomycota. Thus, the genome of *G. stellatum* is an important sampling point in understanding the genetic basis for transitions in fungal ecologies and the origin of mycorrhizae and plant-associated fungi (Peter *et al.*, 2016).

In this paper, we introduce the new genus *Purpurepithecium* in Gloniaceae based on morphological and multigene phylogenetic analysis. *Purpurepithecium* is typified by *Purpurepithecium murisporum* with distinctive morphological features, high statistical support in both maximum likelihood and bayesian analysis.

MATERIAL AND METHODS

Sample collection and specimen examination

Specimens were collected from Chiang Mai, Thailand in 2016. There were brought to laboratory and observed using a Motic SMZ 168 Series microscope.

Hand sections of fruiting structures were mounted in water for microscopic studies and photomicrography. The fungus was examined with a Nikon ECLIPSE 80i compound microscope and photographed with a Canon 450D digital camera connected to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for the figures were processed with Adobe Photoshop CS6 Extended version 10.0 software (Adobe Systems, USA). Isolations were carried from single ascospores, following a modified method of Chomnunti *et al.*, (2014).

Voucher specimens were deposited in the herbarium of Mae Fah Luang University (Herb. MFLU) and International Collection of Microorganisms from Plants (ICMP) Landcare Research, New Zealand (PDD). The living cultures were deposited in culture collection of Mae Fah Luang University (MFLUCC), Thailand with duplicates in BIOTEC Culture Collection (BCC), Bangkok, Thailand. [the latter under Material Transfer Agreement (MTA)]. Faces of fungi and IF numbers were obtained as in Jayasiri *et al.* (2015) and Index Fungorum (2017).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the growing mycelium after 30 days grown on MEA at 18ºC using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) following the manufacturer's protocol (Hangzhou, P.R. China). DNA amplifications were performed by Polymerase Chain Reaction (PCR). The partial large subunit nuclear rDNA (LSU) was amplified with primer pairs LROR and LR5 (Vilgalys and Hester, 1990). The small subunit nuclear rDNA (SSU) was amplified with primer pairs NS1 and NS4 (White *et al.*, 1990). The RNA polymerase II second largest subunit (RPB2) gene was amplified with primers fRPB2 and fRPB2-7cR (Sung *et al.*, 2007). The translation elongation factor 1-alpha gene (TEF1) was amplified by using primers EF1-983F and EF1-2218R (Rehner and Buckley 2005).

The amplification procedure was carried in a 50 µl reaction volume containing 2 µl DNA, 25 µl PCR mix, 19 µl distilled water 2 µl of each primer. The PCR reactions for amplification of LSU, SSU, RPB2 and TEF1 were performed under standard conditions (White *et al.*, 1990). Purification and sequencing of PCR products were carried at Shanghai Sangon Biological Engineering Technology and Services Co. (China).

Sequence alignment and phylogenetic analysis

Sequences generated from the LSU, SSU, RPB2 and TEF1 gene regions were carefully verified before further analyses. Multiple sequence alignments were produced with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html) and further improved manually where necessary and datasets analysed under different optimality criteria as outlined by Jeewon *et al.* (2013) All introns and exons were aligned individually. Ambiguously aligned regions with many leading or trailing gaps were excluded in alignments prior to tree building.

The final phylogenetic tree used to infer the taxonomic placement of our new taxon was generated based on DNA sequence analyses of a concatenated dataset of LSU, SSU, RPB2 and TEF1. A Maximum likelihood analysis was performed at CIPRES using RAxML v.7.2.8 as part of the "RAxMLHPC2 on TG" tool (Stamatakis *et al.*, 2008; Miller *et al.*, 2010). The general time reversible model (GTR) using proportion of invariable sites were applied with a discrete gamma distribution and

four rate classes. The best scoring tree was selected with a final likelihood value of -61728.844074. Maximum Likelihood bootstrap support (MLBS) equal or greater than 70% are given near to each node (Fig. 1).

The model of evolution was performed using jModeltest 2.1.7 (Guindon and Gascuel 2003; Darriba *et al.* 2012). Posterior probabilities (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.0b4 (Huelsenbeck and Ronquist 2001). Six simultaneous Markov chains were run for 5,000,000 generations and trees were sampled every 1000th generation. MCMC heated chain was set with a "temperature" value of 0.15. The distribution of log-likelihood scores was examined to determine stationary phase for each search and to decide if extra runs were required to attain convergence, using the program Tracer 1.5 (Rambaut and Drummond 2007). All sampled topologies beneath the asymptote (20%) were discarded as part of a burn-in procedure, the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. Bayesian Posterior Probabilities (BP) equal or greater than 0.90 is given near to each node (Fig. 1). Phylogenetic trees were drawn using FigTree v. 1.4 (Rambaut and Drummond 2008). The sequences of novel species are deposited in GenBank and the final matrices used for phylogenetic analyses were saved in TreeBASE (ID 21145).

RESULTS

Phylogenetic analyses

Multi genes (LSU, SSU, RPB2 and TEF1) were used for the phylogenetic analysis. The topologies of the obtained trees for each gene were compared manually, to verify that the overall tree topology of the individual datasets was congruent with the tree obtained from the combined alignment. The Bayesian analyses showed similar tree topologies and were congruent to those obtained in the ML analysis.

The combined gene analysis of LSU, SSU RPB2 and TEF1 sequence data representing the families and orders of hysteriform Dothideomycetes is shown in Fig. 1, which included 113 strains, representing 99 species and consisted of 4280 characters. *Schismatomma decolorans* (DUKE 0047570) is the outgroup taxon.

The Bayesian analysis resulted in 5000 trees after 5,000,000 generations. The first 1000 trees, representing the burn-in phase of the analyses were discarded, while the remaining tree was used for calculating posterior probabilities in the majority rule consensus tree and is shown in Fig. 1.

A best scoring RAxML tree resulted with the value of likelihood: -61728.844074. Phylogenetic trees obtained from ML and Bayesian analysis yielded trees with similar overall topology at the species level and in agreement with previous studies based on maximum likelihood and Bayesian analysis (Boehm *et al.*, 2009a; Schoch *et al.*, 2009; Hyde *et al.*, 2013).

The two strains of *Purpurepithecium murisporum* form a monophyletic clade with high statistical support and a sister group to *Glonium* spp. and *Cenococcum* spp. with separate branch length. Therefore, a new genus is introduced to accommodate this taxon in the family Gloniaceae.

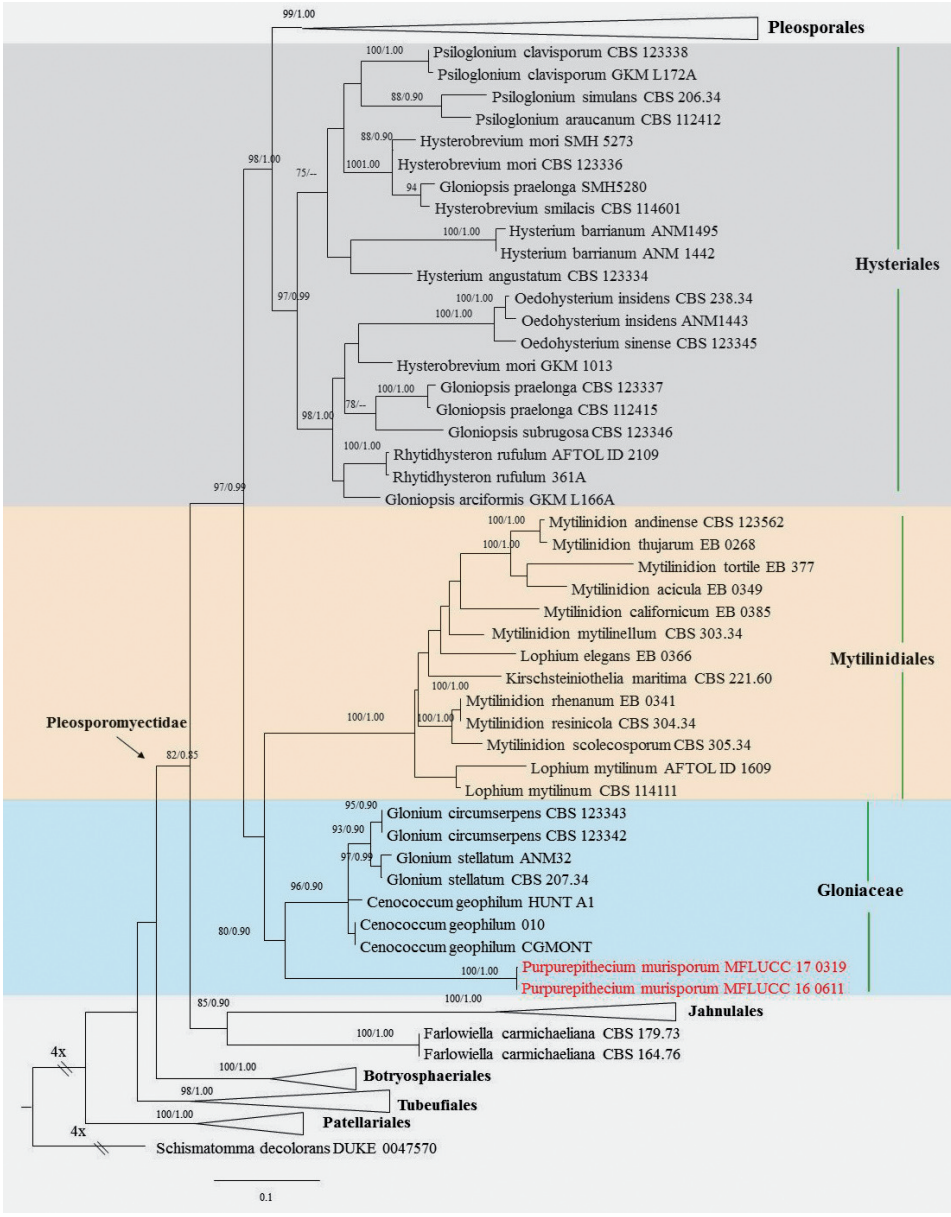

Fig. 1. Simplified phylogram showing the best RAxML maximum likelihood tree obtained from the combined multigene (LSU, SSU, RPB2 and TEF1) matrix of 113 taxa including major orders in Dothideomycetes, Arthoniomycetes (*Schismatomma decolorans*) selected as outgroup. MLBS above 70% and Bayesian posterior probabilities above 0.90 are given near to each branch. Newly generated strains in red.

TAXONOMY

Purpurepithecium Jayasiri & K.D. Hyde, ***gen. nov.***

Index Fungorum number: IF553000; *Facesoffungi number*: FoF 03109

Etymology: The generic epithet "Purpurepithecium" refers to the purple pigmented epithecium

Type species: *Purpurepithecium murisporum* Jayasiri & K.D. Hyde

Saprobic on scale of pine cones on ground. ***Sexual morph***: *Ascomata* hysterothecia, erumpent to superficial, scattered to gregarious, sometimes overlapping, navicular, flexuous, with a prominent longitudinal slit. *Epithecium* dense, swollen tip, branched, with purple pigmentation. *Peridium* wide, equally thick, narrower at base within the substrate, widest at mid-point, carbonaceous and brittle when dry. *Pseudoparaphyses* cellular, septate, persistent, wide, hyaline in mass, branched above. *Asci*, cylindrical to clavate, bitunicate, short-pedicellate. *Ascospores* asymmetric, when young, symmetric when mature, hyaline to dark brown, transversely septate or muriform, with curved ends, septation variable, usually 7-10 longitudinal and many vertical septa, constricted at the septa, gelatinous sheath absent. ***Asexual morph***: Hyphomycetous, punctiform, pulvinate, granular, black, shining. Mycelium immersed in the media, composed of branched, septate, smooth, subhyaline to pale brown, hyphae. *Conidiophores* pale brown, short, slender, articulated. *Conidiogenous cells*, hyaline, cells integrated, terminal, determinate. *Conidia* solitary, irregular, clavate, compact, two cells to muriform, 3-4 rows of transverse septa, constricted at the septa, dark and thickly banded at the septa, number of cells per conidium varies from 2 to 11, reddish-brown to brown, smooth. Basal cell subhyaline to pale brown, cuneiform, with thinner wall.

Purpurepithecium murisporum Jayasiri & K.D. Hyde ***sp. nov.*** **Figs 2-3**

Holotype: MFLU 16-2983

Index Fungorum number: IF553001; *Facesoffungi number*: FoF 03110

Etymology: The species epithet "murisporum" refers to the muriform ascospores

Saprobic on scale of pine cones on ground. ***Sexual morph***: *Ascomata* hysterothecia 228-320 × 260-293 × 700-1000 µm ($\bar{x}$ = 278 × 272 × 850 µm, n = 10), erumpent to superficial, with a prominent longitudinal slit, scattered to gregarious, sometime overlap each other, navicular, flexuous. *Epithecium* 2-2.5 µm wide, dense, cellular, swollen tip, branched, pigmented with purple. *Peridium* 28-62 µm ($\bar{x}$ = 41 µm) wide, narrower at base within the substrate, widest at mid-point, carbonaceous and brittle when dry. *Pseudoparaphyses* cellular, septate, persistent, 1-1.5 µm wide, hyaline in mass, branched above. *Asci* 107-157 × 23-30 µm ($\bar{x}$ = 124 × 25 µm, n = 30), cylindrical to clavate, bitunicate, short-stipitate. *Ascospores* 30-37 × 10-13 µm ($\bar{x}$ = 32 × 11 µm, n = 30), asymmetric, when young, mature symmetric, hyaline to dark brown, transversely septate or muriform, with curved ends, septation highly variable, usually 7-10 longitudinal and many vertical septa, prominently constricted at the median septum, gelatinous sheath absent. ***Asexual morph***: Hyphomycetes. punctiform, pulvinate, granular, black, shining. *Mycelium* 1.5-4.5 µm immersed in the media, composed of branched, septate, smooth, subhyaline to pale brown, hyphae. *Conidiophores* 25-30 × 3-4.5 µm, pale brown, short, slender, articulated. *Conidiogenous* 6-9 × 5-7 µm ($\bar{x}$ = 8 × 6 µm, n = 10), hyaline, cells integrated, terminal, determinate. *Conidia* 14-24 × 10-14 µm ($\bar{x}$ = 19 × 12 µm, n = 10), solitary, irregular, clavate, compact, two cell to muriform,

3-4 rows of transverse septa, constricted at the septa, dark and thickly banded at the septa. The number of cells per conidium varies from 2 to 11, reddish brown to brown, smooth, basal cell 4-6 × 2-4 µm, subhyaline to pale brown, cuneiform, with thinner wall.

Material examined: THAILAND, Chiang Mai Province, on decaying scales of a pine cone, 22 July 2015, Subashini C. Jayasiri C 095 (MFLU 16-2983, Holotype), PDD (Isotype), living culture MFLUCC 16-0611; BCC; *ibid.,* on decaying scales of pine cone, 23 December 2015, Subashini C. Jayasiri C 95-B (MFLU 17-0447, Paratype), living culture 17-0319. GenBank no: MFLUCC 16-0611- LSU KY799173, SSU KY887665, RPB2 KY799176 TEF1 KY887666; MFLUCC 17-0319 - LSU KY799174, SSU KY799175, RPB2 KY887667, TEF1 KY799177

Culture characteristics: Ascospores germinating on MEA within 24 h. Colonies growing on MEA 2 cm diam. after 30 days at 18 C, very slow growing, after this no more growth happen, chlamydospores formed, then asexual structures

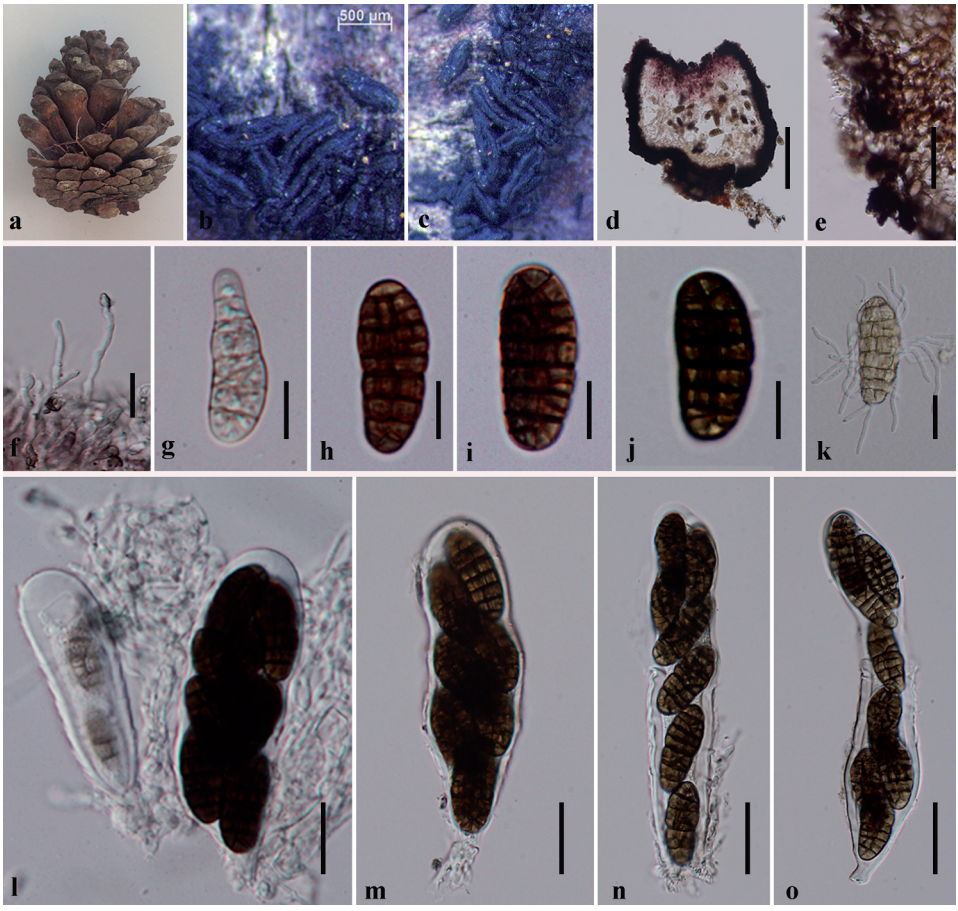

Fig. 2. *Purpurepithecium murisporum* (holotype). **a.** A pine cone. **b, c.** View of hysteriothecia on host surface. **d.** Section through hysteriothecium. **e.** Peridium. **f.** Pseudoparaphyses. **g-j.** Ascospores. **k.** Germinated ascospores. **l-o.** Asci. Scale bars: d = 100 µm, e = 20 µm, f = 10 µm, g-k = 10 µm, l-p = 30 µm.

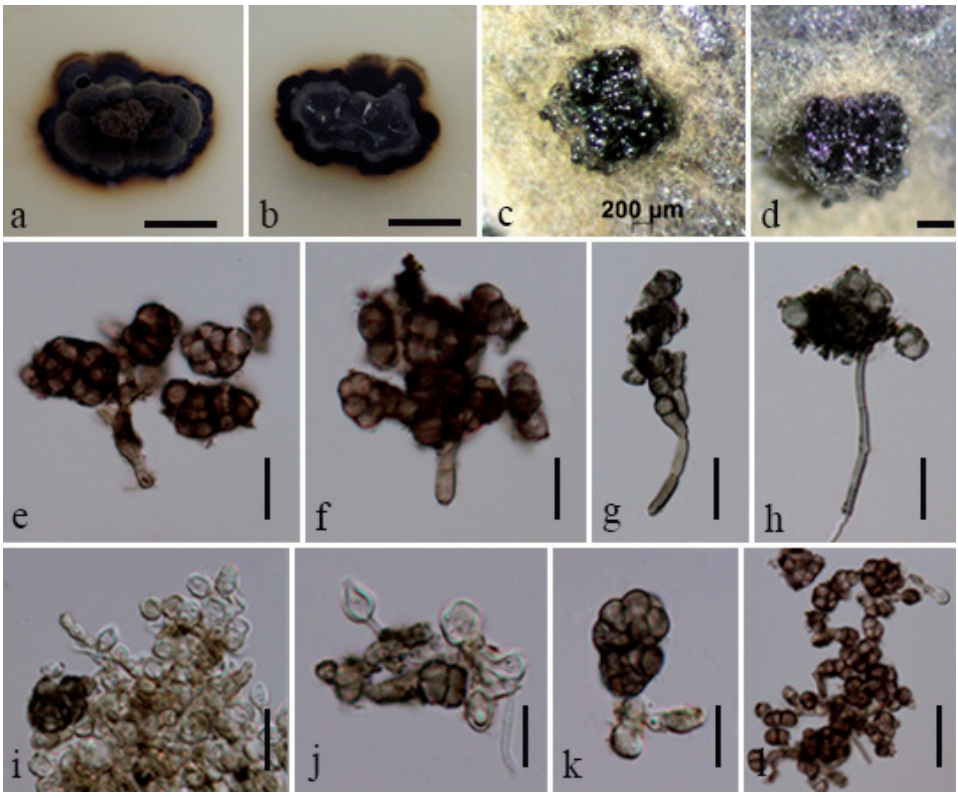

Fig. 3. *Purpurepithecium murisporum* asexual morph from culture. **a, b.** Forward and reverse view of the culture. **c, d.** Appearance of fruiting bodies in culture. **e-h.** Conidiophores with conidia. **i, j.** Conidiogenous cells. **k, l.** Conidia. Scale bar: a, b = 2 cm, c, d = 200 µm, e-k = 20 µm, l = 30 µm.

formed in culture. colonies circular, effuse, dense, dark brown, diffuse into media, many layers, smooth surface with entire to slightly undulate edge with brown yellow pigment (Fig. 3a, b).

DISCUSSION

In this study, we introduced a new genus in the family Gloniaceae based on morphological and multigene phylogenetic analysis. Gloniaceae comprises *Glonium* (type genus) and *Cenococcum*. However, *Cenococcum* is a genus of ectomycorrhizal Dothideomycetes, but no definitive sexual or asexual spore-producing structures are known, although it does produce vegetative hyphae and abundant sclerotia (Spatafora *et al.*, 2012). *Purpurepithecium murisporum* is similar to *Glonium* (e.g. *Glonium stellatum*) in producing hysterothecia, with persistent narrow cellular pseudoparaphyses in a gel matrix, branched with darkened apices and clavate to cylindric asci (Boehm *et al.*, 2009a). *Purpurepithecium murisporum*

differs from *Glonium stellatum* in that it has navicular hysterothecia, that are scattered to gregarious, with a prominent longitudinal slit, purple pigmented epithecium and hyaline to dark brown muriform ascospores.

In the phylogenetic analysis, *Purpurepithecium* strains separate from *Glonium* and *Cenococcum* spp. with high statistical support (80 MLBS, 0.90 PP) in a separate subclade. Therefore, *Purpurepithecium murisporum* provides sufficient data to be accommodated as the type for a new genus. *Glonium* and *Psiloglonium* share the same asexual morph (ex. *Psiloglonium stygium*) (Lohman 1933, 1937). *Psiloglonium stygium-like* (Basionym: *Sporidesmium stygium*) is characterized by small or widely spread effused patches of conidiomata and large, irregular, clavate, compact conidia supported at the base by a short, slender, articulated pedicel (Saccardo 1874). We also obtained the asexual morph for *Purpurepithecium murisporum* in culture which is somewhat similar to *Psiloglonium stygium* (Fig. 3).

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