

***Pustulomyces* gen. nov. accommodated
in *Diaporthaceae*, *Diaporthales*,
as revealed by morphology and molecular analyses**

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Abstract – A new coelomycetous genus, *Pustulomyces*, inhabiting decaying bamboo, is introduced based on morpho-molecular studies. *Pustulomyces* is characterized by immersed, pustule-like, acervular conidiomata, with dark ostiolar necks, enteroblastic, phialidic conidiogenous cells with a small collarette and fusiform, sigmoid, aseptate, guttulate conidia. In morphology it is similar with *Bambusicola* (*Bambusicolaceae*), but maximum-likelihood and Bayesian analyses of the combined LSU and TEF-1 alpha sequence data set shows *Pustulomyces* belongs in *Diaporthaceae*, *Diaporthales*. The new fungus is compared with other morphologically and phylogenetically similar genera.

***Pustulomyces* / asexual morph / coelomycetous fungi / molecular phylogeny / taxonomy**

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INTRODUCTION

Coelomycetes are asexual fungi which produce conidia in acervuli, pycnidia or stromata (Sutton, 1980; Wijayawardene *et al.*, 2012c). Approximately 1000 genera have so far been described but most of them are considered as ‘orphaned’ (Wijayawardene *et al.*, 2012a). A few new asexual morphs have been described on bamboo from Japan and Thailand (Dai *et al.*, 2012, 2014; Liu *et al.*, 2012; Tanaka *et al.*, 2009, 2010).

During our studies on diversity and taxonomy of microfungi inhabiting giant bamboo (*Poaceae*, *Bambusoideae*) in northern Thailand, several new taxa have been described (Dai *et al.*, 2012, 2014; Liu *et al.*, 2012). In this paper, we introduce a new coelomycetous genus which belongs to *Diaporthaceae*, *Diaporthales*, based on morpho-molecular and cultural studies.

MATERIALS AND METHODS

Collection and isolation of fungi. Fallen and decomposing bamboo culms and leaves were collected from various localities in Chiang Rai Province, Thailand. The samples were placed in plastic zip lock bags and brought to laboratory. The specimens were incubated in sterile moist chambers and examined at regular intervals until the resident fungi attained maturity and sporulated. The fungi were examined under stereoscope and compound microscope. Fungi were isolated by single spore isolation following the method of Chomnunti *et al.* (2011). The colonies were transferred to 1.5 ml microcentrifuge tube with 2% potato-dextrose agar (PDA) to deposit at 6°C and suspended in 2 ml screw cap microcentrifuge tube with 10% Glycerol to store at –20°C. Microscopic observations and photomicrographs were made as described in Boonmee *et al.* (2011). Type materials are deposited at herbarium of Mae Fah Luang University, Chiang Rai, Thailand (MFLU). The living cultures are deposited at Mae Fah Luang University Culture Collection (MFLUCC), the Research Institute of Resource Insects, Chinese Academy of Forestry (IFRD) and Landcare Research, New Zealand (ICPM) under Material Transfer Agreement (No.C29/2011) and IMCP 20065 under Material Transfer Agreement (No. 2013049115).

DNA extraction, PCR amplification and sequencing. Fungal isolates were grown on PDA for 30 d at 27°C and genomic DNA was extracted from fresh mycelia, following the specification of Biospin Fungus Genomic DNA Extraction Kit (BioFlux®). ITS5 and ITS4, NS1 and NS4 (White *et al.*, 1990) and LROR and LR5 (Vilgalys & Hester, 1990) primers were used for the amplification of internal transcribed spacers (ITS), small subunit rDNA (SSU) and large subunit rDNA (LSU) respectively. Translation elongation factor 1- α gene region (TEF 1-alpha) was amplified by using EF1-728F and EF1-986R primers (Carbone & Kohn, 1999). Polymerase chain reaction (PCR) amplification was carried out following the method of Phillips *et al.* (2008). Amplified PCR fragments were sequenced at Kunming Shuo Yang Technology Company, P.R. China. Generated new sequences of ITS, LSU, SSU and TEF1 regions are deposited in GenBank.

DNA sequence analysis. Blast searches at GenBank were carried out by both LSU and SSU rDNA sequences in order to reveal the closest taxa to our strain. Sequence data of closest taxa in *Diaporthaceae* were downloaded (Table 1) and it includes *Diaporthe*, *Mazzantia*, *Phaeocystroma*, *Stenocarpella* and

Table 1: The list of species used in this study. Abbreviations of isolates and culture collections: AR: Collection of A.Y. Rossman; ATCC: American Type Culture Collection, Virginia, USA; BCRC, Bioresource Collection and Research Center, Taiwan; BPI:USDA-ARS Systematic Mycology and Microbiology Laboratory U.S. National Fungus Collections, Baltimore Avenue, Beltsville, Maryland,U.S.A.; CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; MFLUCC: Mae Fah Luang University Culture Collection, ChiangRai, Thailand; STE-U: Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; UAMH: The University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden Edmonton, Alberta, Canada.

Species name	Source	GenBank accession numbers	
		LSU	TEFI
<i>Chapeckia nigrospora</i>	AR 3809	EU683068	
<i>Coniella australiensis</i>	IMI 261318	AF408336	
<i>Cryphonectria macrospora</i>	AR3444	AF408340	
<i>Cryphonectria nitschkei</i>	AR3433	AF408341	
<i>Diaporthe eres</i>	AR3538, CBS 694.94	AF408350	KC343821
<i>Diaporthe medusaea</i>	AR3422, CBS 109768	AF362560	JQ807376
<i>Diaporthe pardalota</i>	AR3478	AF408355	
<i>Gaeumannomyces graminis</i>	AR3400, CBS 235.32	AF362556	JX134695
<i>Gnomonia setacea</i>	AR3451, BPI 877645	AF362563	EU222009
<i>Harknessia eucalypti</i>	CBS342.97	AF408363	
<i>Harknessia gibbosa</i>	CBS 120033, CPC 17676	EF110615	
<i>Magnaporthe grisea</i>	Ina168, M82	AB026819	JX134696
<i>Mazzantia napelli</i>	AR3498, BPI 748443	AF408368	EU222017
<i>Melanconis alni</i>	AR3529, AR 3500	AF362566	EU221896
<i>Melanconis marginalis</i>	AR3442, CBS109744	AF408373	EU221991
<i>Melanconis stilbostoma</i>	AR3501, CBS 109778	AF408374	EU221886
<i>Ophiodiaporthe cyatheae</i>	BCRC 34961	JX570891	KC465406
<i>Phaeoacremonium sphinctrophorum</i>	CBS337.90	DQ173151	
<i>Phaeocytostroma megalosporum</i>	CBS:284.65	FR748103	FR748077
<i>Phaeocytostroma plurivorum</i>	CBS:113835	FR748104	FR748078
<i>Phomopsis vaccinii</i>	FAU475, CBS 160.32	AF439630	GQ250326
<i>Phragmoportha conformis</i>	AR3632, CBS 109783	AF408377	EU221993
<i>Pilidiella castaneicola</i>	CBS 143.97	AF408378	
<i>Pilidiella granati</i>	CBS152.33	AF408379	AY339362
<i>Pseudovalsa longipes</i>	AR 3541	EU683072	
<i>Pseudovalsa modonia</i>	AR 3558	EU683073	
<i>Pustulomyces bambusicola</i>	MFLUCC 11-0436	KF806753	KF806755
<i>Schizoparme straminea</i>	CBS 149.22, STE-U 3932	AF362569	AY339366
<i>Sillia ferruginea</i>	AR 3440, CBS 126567	EU683076	
<i>Stenocarpella macrospora</i>	CBS 117560	DQ377934	
<i>Stenocarpella maydis</i>	CBS 117559	DQ377937	FR748081
<i>Sydowiella fenestrans</i>	AR 3777, CBS 125530	EU683078	
<i>Togninia novae-zealandiae</i>	CBS 110156, UAMH9589	AY761081	AY179911
<i>Valsa ceratosperma</i>	AR3416, CBS116.21	AF408386	JX438577
<i>Valsa mali</i>	AR3417, ATCC 56632	AF362559	JX438571

Ophiidiaporthe (Fu *et al.*, 2013; Lamprecht *et al.*, 2011). Furthermore, we have included other families in *Diaporthales* i.e. *Cryphonectriaceae*, *Diaporthaceae*, *Gnomoniaceae*, *Harknessiaceae*, *Melanconidaceae*, *Pseudovalsaceae*, *Schizoparmaceae*, *Sydowiellaceae*, *Togniniaceae* and *Valsaceae*. (Crous *et al.*, 2012). The tree is rooted to *Gaeumannomyces graminis* (AR 3400) and *Magnaporthe grisea* (AB026819). Sequences were aligned using Bioedit (Hall, 2001) and ClustalX (Kohli & Bachhawat, 2003). Alignments were checked and manual adjustments were carried out when necessary. In the analysis, gaps were treated as missing data, and all characters were unordered and of equal weight (Begoude *et al.*, 2010; Liu *et al.*, 2011, 2012).

Maximum likelihood (ML) analysis was performed by RAxML (Stamatakis, 2006) implemented in raxmlGUI v.0.9b2 (Silvestro & Michalak, 2010). Fifty thorough maximum likelihood (ML) tree searches were done in RAxML under the general time reversible model (GTR), with each one starting from a separate randomised tree and the best scoring tree selected. One thousand non parametric bootstrap iterations were run with the GTR model and a discrete gamma distribution (Liu *et al.*, 2012).

Bayesian analyses were performed by using PAUP v. 4.0b10 (Swofford, 2002) and MrBayes v. 3.0b4 (Ronquist & Huelsenbeck, 2003). The model of evolution was estimated by using MrModeltest 2.2 (Nylander, 2004). Posterior probabilities (PP) (Rannala & Yang, 1996) were performed by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Liu *et al.*, 2012). Six simultaneous Markov chains were run for 1 m generations and trees were sampled every 100th generations (resulting 10 000 total trees) (Cai *et al.*, 2006). The first 2000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 8000 (post-burning) trees used for calculating posterior probabilities (PP) in the majority rule consensus tree (Cai *et al.*, 2006; Liu *et al.*, 2012).

Trees were visualized with TreeView (Page, 1996).

RESULTS

Phylogenetic analysis

Partial nucleotide combining sequences of LSU and TEF1 were used to determine the taxonomic placement of our strain. The LSU and TEF1 dataset was used in the phylogenetic analyses for generic placement (Fig. 1). ITS gene data was not included in the phylogenetic analyses as bootstrap of ITS analyses were low, but deposited in GenBank (accession number KF806752) as it is the preferred locus for use in fungal phylogenetics (Schoch *et al.*, 2011; Liu *et al.*, 2012). We have not included SSU sequence data in phylogenetic analysis but also deposited in GenBank (accession number KF806754), as most of the genera in this order lack SSU sequences in GenBank.

The dataset comprise one newly sequenced taxon and 32 taxa including two outgroup taxa. The final dataset consists of 1213 characters after alignment. The phylogenetic trees generated by Maximum likelihood (ML) and Bayesian analyses of combined LSU and TEF 1-alpha gene regions have shown that *Pustulomyces* is embedded within the family *Diaporthaceae* and close to *Phaeocytostroma* and *Stenocarpella* (Fig. 1). Bootstrap support (BS) values of ML and values of the Bayesian posterior probabilities (PP) from MCMC analyses are shown in Fig 1.

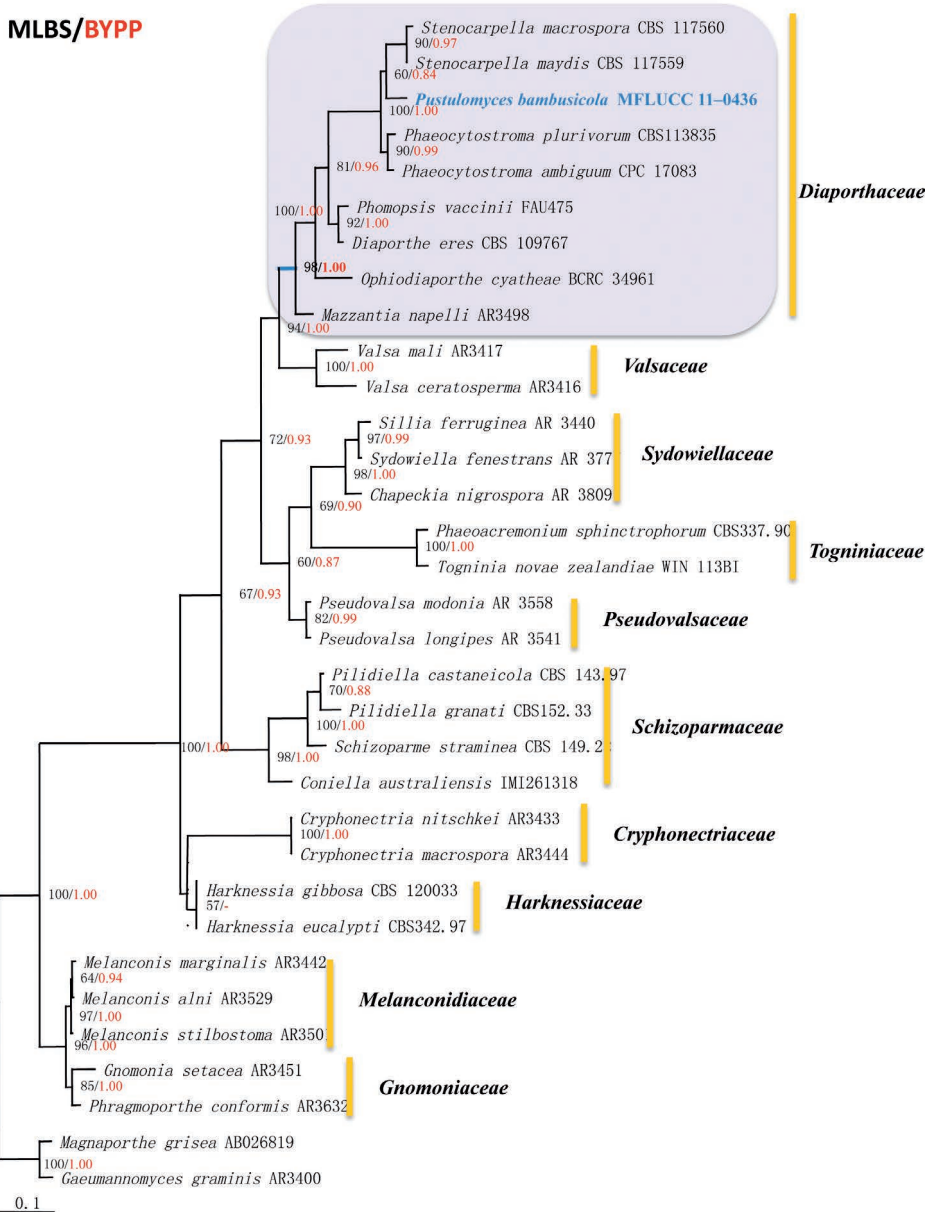


Fig. 1. Phylogenetic tree generated from maximum likelihood (ML) and Bayesian analysis based on combined LSU and TEF 1-alpha sequences. Data were analyzed with random addition sequence and treating gaps as missing data. Bootstrap support (BS) values above 50% are shown at nodes. Hyphen (“-”) indicates a value lower than 50% (BS) or 0.80 (PP). The original isolate numbers or GenBank codes are noted after the species names. Type species are highlighted with a reddish brown background. The tree is rooted with *Gaeumannomyces graminis* (AR3400) and *Magnaporthe grisea* (AB026819).

Taxonomy

Pustulomyces D.Q. Dai, D.J. Bhat & K.D. Hyde, **gen. nov.**

Mycobank: MB 806063

Etymology: With reference to its pustule-like conidiomata.

Saprobic on bamboo culms, forming slightly faded colonies on the host surface with conidiomata breaking through slightly to strongly raised areas. Sexual state: Unknown. Asexual state: *Conidiomata* acervular, immersed under the host epidermis, erumpent and pustule-like when mature, solitary, scattered, conical in section, coriaceous, with black, ostiolar necks. *Conidiomatal wall* comprising a few to several layers of cells of *textura angularis*, with dark brown outer layers and hyaline conidiogenous inner layer. *Conidiophores* cylindrical to irregular, hyaline, branched only at the base, septate, smooth, formed from the innermost layer cells of the conidiomata wall. *Conidiogenous cells* enteroblastic, phialidic, with a small collarette, determinate, integrated, cylindrical, slender, hyaline, smooth. *Conidia* fusiform, elongated, straight to curved, occasionally slightly sigmoid, pale brown, aseptate, smooth, with many guttules.

Notes: *Pustulomyces* can be compared with *Coleophoma*, *Diplozythiella*, *Discogloeum*, *Jahniella* and *Pseudothyrium* (Sutton, 1980). Amongst these genera, the conidiogenous cells and conidiogenesis in *Discogloeum* and *Pustulomyces* are similar, but *Discogloeum* has septate conidia (Nag Raj, 1971-1987). In *Jahniella*, the conidiogenous cells are holoblastic and distinct from *Pustulomyces*. *Coleophoma*, *Diplozythiella* and *Pseudothyrium* have phialidic, cylindrical to ampulliform conidiogenous cells. *Coleophoma* however, can be distinguished by the presence of cylindrical, hyaline and septate paraphyses. In *Diplozythiella*, conidia are hyaline and septate. In *Pseudothyrium*, conidiomata are subcuticular and conidia are cylindrical (Sutton, 1980).

Type species: *Pustulomyces bambusicola* D.Q. Dai, D.J. Bhat & K.D. Hyde.

Pustulomyces bambusicola D.Q. Dai, D.J. Bhat & K.D. Hyde, **sp. nov.** Fig. 2

Mycobank: MB 806064

Etymology: With reference to its occurrence on *Bambusa* sp.

Saprobic on bamboo culms, forming slightly faded colonies on the host surface with conidiomata breaking through slightly to strongly raised areas. Sexual state: Unknown. Asexual state: *Conidiomata* acervular, immersed under the host tissue, erumpent and pustule-like when mature, 150-200 µm high, 400-500 µm in diam., solitary, scattered, conical in section, coriaceous, with 40-60 µm long black, ostiolar necks. *Pycnidium wall* 5-15 µm wide in the upper region, with thin lower wall, comprising 1-2 layers of cells of *textura angularis*, with dark brown outer layers and hyaline conidiogenous inner layer. *Conidiophores* 2-4.5 × 1-2.5 µm (\bar{x} = 2.8 × 1.3 µm, n = 20), hyaline, branched only at the base, septate, cylindrical to irregular, smooth, formed from the inner cells of the conidiomata wall. *Conidiogenous cells* 5-8.5 × 1-2 µm (\bar{x} = 6.8 × 1.5 µm, n = 20), enteroblastic, phialidic, with a small collarette, determinate, integrated, cylindrical, slender, hyaline, smooth-walled. *Conidia* 10-15 × 2-3 µm (\bar{x} = 12.1 × 2.5 µm, n = 20), fusiform, straight to curved, occasionally slightly sigmoid, pointed at both ends, pale brown, aseptate, smooth-walled, with many guttules.

Culture on PDA: Colonies slow growing, 20 mm diam. after 15 days at 25-32°C, circular, with uneven margin, floccose at the center, drift white from front view and light green at the centre from back view after 5 days. Mature colonies in 15 d show dark green to yellow zones at the centre from front view.

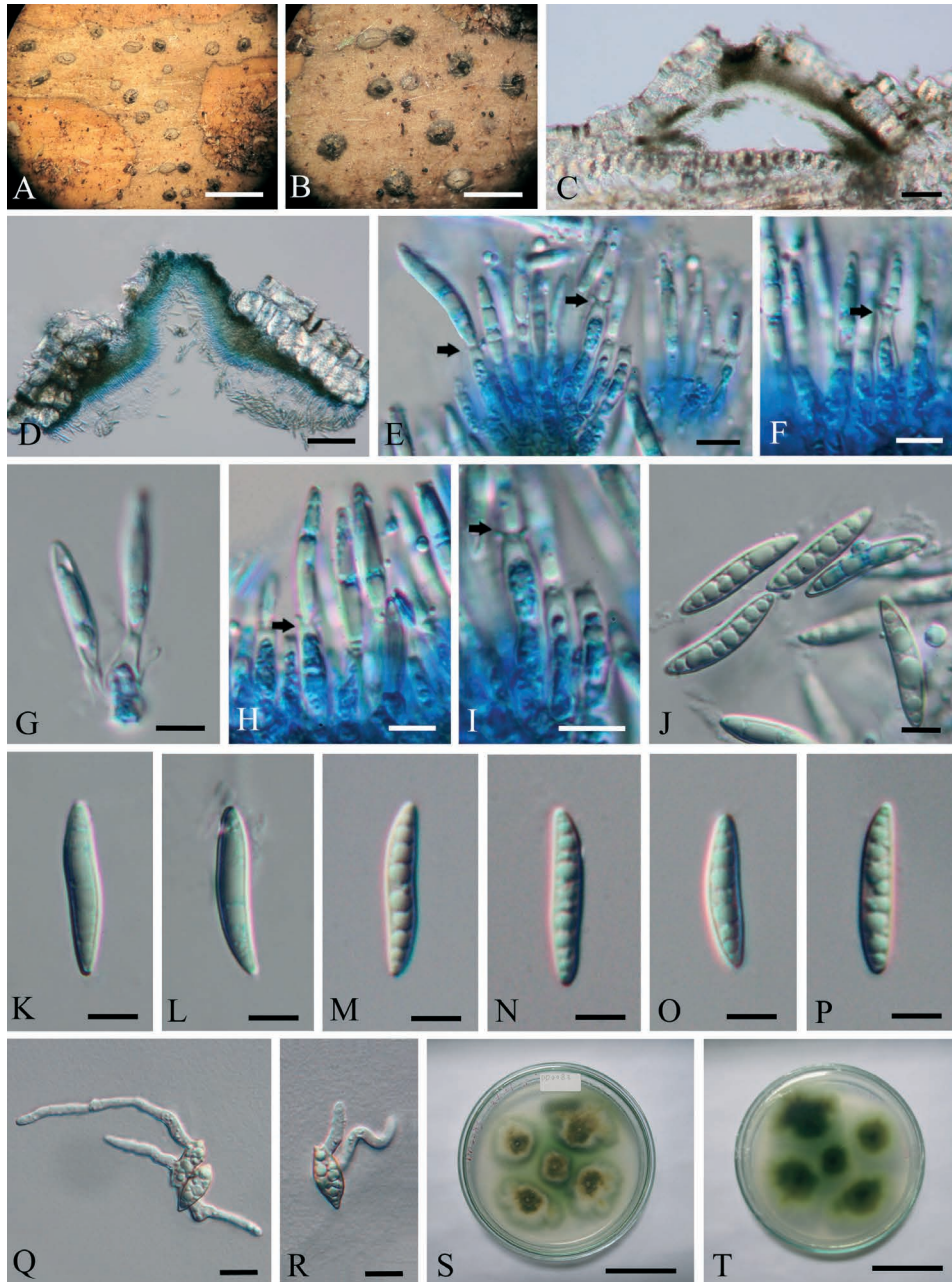


Fig. 2. *Pustulomyces bambusicola* (holotype) **A, B**. Conidiomata formed on host surface. **C**. Section of conidiomata. **D**. Section of ostioles. **E-H**. Conidiogenous cells and conidia. **I**. Conidiophores. **J-P**. Fusiform conidia. **Q, R**. Geminating conidia. **S**. Cultures on PDA from above after 15 days. **T**. Cultures on PDA from reverse after 15 days. **D-L**. Photograph in cotton blue. **C, M-R**. Photograph in water. Scale bars: **A** = 2 mm, **B** = 1 mm, **C, D** = 50 μ m, **E-P** = 5 μ m, **Q, R** = 10 μ m, **S, T** = 25 mm.

Material examined: THAILAND, Chiang Rai Province, Mae Fah Luang University, on dead culm of Bamboo, 19 July 2011, Dong-Qin Dai, DDQ0083 (MFLU13-0369, holotype), ex-type living culture = MFLUCC 11-0436 = IFRDCC 2536 = ICMP 20065.

Notes: *Pustulomyces bambusicola* is characterized by immersed, pustule-like, acervular conidiomata with dark ostiolar necks, enteroblastic, phialidic conidiogenous cells with a small collarette and fusiform, sigmoid, aseptate, guttulate conidia.

DISCUSSION

Diaporthales was introduced by Nannfeldt (1932) and is a distinct order within Sordariomycetes (Lumbsch & Huhndorf, 2010; Crous *et al.*, 2012). In recent studies (Crous *et al.*, 2012; Rossman *et al.*, 2007), ten families have been accepted in *Diaporthales*, viz. *Cryphonectriaceae*, *Diaporthaceae*, *Gnomoniaceae*, *Harknessiaceae*, *Melanconidaceae*, *Pseudovalsaceae*, *Schizoparmaceae*, *Sydowiellaceae*, *Togniniaceae* and *Valsaceae*. In our study, we have included all above mentioned families of *Diaporthales* to determine the placement of *Pustulomyces*.

Lumbsch & Huhndorf (2010) included four genera (i.e. *Apioporthella*, *Diaporthe*, *Leucodiaporthe* and *Mazzantia*) in the family *Diaporthaceae*. *Phaeocystostroma*, *Phomopsis* (= *Diaporthe*) and *Stenocarpella* are other accepted genera in this family (Lamprecht *et al.*, 2011; Udayanga *et al.*, 2011). Fu *et al.* (2013) introduced a new genus *Ophiodiaporthe* in *Diaporthaceae*. We have selected all the genera which have sequence data in the GenBank. However, we excluded *Apioporthella*, as no sequence data is lodged in GenBank.

The data set of combined LSU and TEF genes were used in the phylogenetic analyses to determine generic placement in *Diaporthaceae*. *Pustulomyces* is embedded within the *Diaporthaceae* (98%/1.00 MLBS/BYPP support (Fig. 1) and can be distinguished phylogenetically from *Phaeocystostroma* (100%/1.00 MLBS/BYPP support (Fig. 1). Morphological studies show *Pustulomyces* differs from the phylogenetically closest genera, *Phaeocystostroma* and *Stenocarpella*. *Phaeocystostroma* has long conidiophores, conidiogenous cells and paraphyses (longer than 15 μm) (Lamprecht *et al.*, 2011; Petrak, 1921; Sutton, 1980). Such characters are not observed in *Pustulomyces*. *Stenocarpella* has globose to elongated conidiomata, cylindrical to narrowly ellipsoid, 1-2-septate, conidia with tapered and truncate base (Sutton, 1980; Lamprecht *et al.*, 2011). *Pustulomyces* however, differs in having conical conidiomata, fusiform, aseptate conidia. Therefore, *Pustulomyces* is introduced as a new genus, with *P. bambusicola* as type species.

Pustulomyces is morphologically similar with *Bambusicola* (Dai *et al.*, 2012). Both of these genera have immersed conidiomata formed on the bamboo host and short, unobtrusive conidiophores. However, *Pustulomyces* has phialidic conidiogenous cells and aseptate conidia which distinguish it from *Bambusicola*, which has holoblastic, annelidic conidiogenous cells and 1-3-septate conidia. Molecular analyses also show *Bambusicola* to belong in *Bambusicolaceae*, *Pleosporales*, *Dothideomycetes* (Hyde *et al.*, 2013), while *Pustulomyces* belongs in *Diaporthaceae*, *Diaporthales*.

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