Integrating Different Lines of Evidence to Establish a Novel Ascomycete Genus and Family (*Anastomitrabeculia, Anastomitrabeculiaceae*) in Pleosporales

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Abstract: A novel genus, *Anastomitrabeculia*, is introduced herein for a distinct species, *Anastomitrabeculia didymospora*, collected as a saprobe on dead bamboo culms from a freshwater stream in Thailand. *Anastomitrabeculia* is distinct in its trabeculate pseudoparaphyses and ascospores with longitudinally striate wall ornamentation. A new family, *Anastomitrabeculiaceae*, is introduced to accommodate *Anastomitrabeculia*. *Anastomitrabeculiaceae* forms an independent lineage basal to *Halojulellaceae* in Pleosporales and it is closely related to *Neohendersoniaceae* based on phylogenetic analyses of a combined LSU, SSU and TEF1α dataset. In addition, divergence time estimates provide further support for the establishment of *Anastomitrabeculiaceae*. The family diverged around 84 million years ago (MYA) during the Cretaceous period, which supports the establishment of the new family. The crown and stem age of *Anastomitrabeculiaceae* was also compared to morphologically similar pleosporalean families.

Keywords: BEAST; Dothideomycetes; Pleosporales; Poaceae; taxonomy; three new taxa; trabeculate pseudoparaphyses

1. Introduction

*Pleosporales* is the largest order within *Dothideomycetes* (*Ascomycota*) [1]. The taxonomic and phylogenetic relationships of families and genera within this order are well documented [1–7]. *Pleosporales* comprises two suborders, *Massarineae* and *Pleosporineae* [1]. *Pleosporineae* includes economically important plant pathogens and *Massarineae* includes mainly saprobes from terrestrial or aquatic environments [1,3]. Zhang et al. [1] revised 174 genera and accepted 26 families in *Pleosporales*. The suborder *Massarineae* was resurrected to accommodate five families, the *Lentitheciaceae, Massarineae, Montagulaceae (Didynosphaeriaceae), Morosphaeriaceae* and *Trematosphaeriaceae* [1]. Hyde et al. [2] correlated morphology with phylogenetic evidence and accepted 41 families in this order. Tanaka et al. [3] introduced two new families, *Parabambusicolaceae* and *Sulcatisporaceae*, accepting 12 families in *Massarineae*. The family *Longipedicellataceae* was introduced, and the divergence time in *Pleosporales* was estimated with emphasis on *Massarineae* [4]. The crown age of *Pleosporales* was dated to 211 MYA and *Massarineae* was dated to 130 MYA [4]. Species boundaries in *Cucurbitariaceae* were revised [5] and the family, *Lentimurisporaceae*, was introduced in *Pleosporales* [6].
Species in this order are abundant and occur in terrestrial, marine and freshwater habitats [7–9]. The species can be epiphytes, endophytes or parasites of living leaves or stems, hyperparasites on fungi or insects, lichenized, or saprobes of dead plant stems, leaves or bark [7–9]. Currently, about 400 genera in 64 families are known in Pleosporales [1,2,7,10–13], with numerous coelomycetous and hyphomycetous taxa as their asexual morphs [1,13–15].

Several pleosporalean taxa are pathogens associated with a broad range of hosts including bamboo. Bamboo (Poaceae) comprises over 115 genera with around 1500 species [16–18], can be found in diverse climates [17], and are widely distributed in various forest types in Thailand [18,19]. It has been estimated that around 1100 fungal species belonging to over 200 genera have been described or recorded worldwide on bamboo and most of these bamboo-associated fungi are ascomycetes [20,21].

Divergence time estimates using molecular clock methodologies have been widely used in fungal taxonomy [4,11,22–27]. Several studies have applied molecular dating to provide additional evidence for higher taxa ranking in Pleosporales [4,6,7,11]. In this study, we introduce a novel bambusicolous species, Anastomitrabeculia didymospora within Anastomitrabeculia, which is accommodated in a new family, Anastomitrabeculiaceae, based on morphology, multi-loci phylogeny and divergence times estimates.

2. Materials and Methods

2.1. Sample Collection, Isolation and Identification

Dead bamboo culms were collected from a freshwater stream from Krabi province, Thailand, in 2015. The samples were incubated in plastic boxes with sterile and moist tissue at 25–30 °C for 3 days. Pure fungal colonies were obtained using single-spore isolation [28]. Germinating spores were transferred aseptically to potato dextrose agar (PDA) and malt extract agar (MEA) (Difco™). The cultures were incubated at 25 °C with frequent observations. Fungal characters were observed using a stereo microscope (Zeiss SteREO Discovery v. 8) fitted with an Axio Cam ERC5S and a Leica DM2500 compound microscope attached with a Leica MC190 HD camera. All microscopic measurements were carried out using Tarosoft (R) Image Frame Work program and the images were processed with Adobe Photoshop CS6 version 13.0 software (Adobe Systems, San Jose, CA, USA). The type specimens were deposited in the Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand, and pure cultures were deposited at the Mae Fah Luang University Culture Collection (MFLUCC). The new taxon was linked with Facesoffungi numbers (FoF) [29] and Index Fungorum (Index Fungorum 2020, http://www.indexfungorum.org/, accessed on 2 December 2020) and established based on guidelines recommended by Jeewon and Hyde [30].

2.2. DNA Extraction, PCR Amplification and DNA Sequencing

DNA extraction, PCR amplification, DNA sequencing and phylogenetic analysis were carried out as detailed in Dissanayake et al. [31]. Total genomic DNA was extracted from fresh mycelium with a Biospin Fungus Genomic DNA Extraction Kit (BioFlux®) (Hangzhou, PR. China) following the manufacturer’s protocol. The nuclear ribosomal large subunit 28S rRNA gene (LSU) [32], the nuclear ribosomal small subunit 18S rRNA gene (SSU) [33] and the translation elongation factor 1-alpha gene (TEF1α) [34] were amplified using primers (LSU: LROR/LR5, SSU: NS1/NS4 and TEF1α: 983F/2218R). Polymerase chain reaction (PCR) was performed using PCR mixtures containing 5–10 ng DNA, 1X PCR buffer, 0.8 units Taq polymerase, 0.3 µM of each primer, 0.2 mM dNTP and 1.5 mM MgCl2. PCR conditions were set at an initial denaturation for 3 min at 94 °C, followed by 40 cycles of 45 s of denaturation at 94 °C, annealing for 50 s at 56 °C for LSU, SSU and 52 °C for TEF1α and extension for 1 min at 72 °C, with a final extension of 10 min at 72 °C. All the PCR products were visualised on 1% Agarose gels with added 6 µL of 4S green dyes, per each 100 mL. Successful PCR products were purified and sequencing was performed by Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, PR. China). All sequences generated in this study were submitted to GenBank
Table 1. DNA sequences and GenBank numbers used for the phylogenetic analyses in this study. The ex-type strains are in bold and the new taxon introduced in this study is indicated in blue.

| Taxon                                | Strain Number   | GenBank Accession Numbers |
|---------------------------------------|-----------------|---------------------------|
|                                       |                 | LSU | SSU | TEFa |
| **Acrocalymma aquatica**              | MFLUCC 11-0208  | JX276952 | JX276953 | -    |
| **Acrocalymma fici**                  | CBS 317.76      | KP170712 | - | -    |
| **Acrocalymma medicaeignis**          | CPC 24340       | KP170713 | - | -    |
| **Acrocalymma medicaeignis**          | CPC 24341       | KP170714 | - | -    |
| **Acrocalymma medicaeignis**          | CPC 24345       | KP170718 | - | -    |
| **Acrocalymma pterocarpi**            | MFLUCC 17-0926  | MK347949 | MK347840 | -    |
|                                       |                 |     |     |      |
| **Aigialus grandis**                  | BCC 20000       | GU479775 | GU479739 | GU479837 |
| **Aigialus mangrovis**                | BCC 33563       | GU479776 | GU479741 | GU479840 |
| **Aigialus parvus**                   | BCC 18403       | GU479778 | GU479743 | GU479842 |
| **Aigialus rhizophorae**              | CBS 118232      | GU301796 | - | GU349048 |
| **Amniculicola immersa**              | CBS 123083      | FJ795498 | GU456295 | GU456273 |
| **Amniculicola lignicola**            | CBS 123094      | EF493861 | EF493863 | -    |
| **Amniculicola parva**                | CBS 123092      | GU301797 | GU296134 | GU349065 |
| **Amorosia littoralis**               | NN 6654         | AM292055 | AM292056 | -    |
| **Anastomitrabeculia didymospora**   | MFLUCC 16-0412  | JX276952 | JX276953 | -    |
| **Angustimassarina populi**           | MFLUCC 13-0034  | KP388642 | KP899128 | K075164 |
| **Anteaglonium abbreviatum**          | ANM 925a        | GQ221877 | - | -    |
| **Anteaglonium globosum**             | SMH 5283        | GQ221911 | - | GQ221919 |
| **Anteaglonium parasilenum**          | MFLUCC 14-0821  | KU922915 | KU922916 | -    |
| **Aquasubmersa japonica**             | HHUF 30468      | LC194340 | LC194298 | LC194382 |
| **Aquasubmersa japonica**             | HHUF 30469      | LC061856 | LC061581 | -    |
| **Arthonia dispersa**                 | UPSC 2583       | AY517381 | AY517379 | -    |
| **Ascochyta mangicola**               | BCC 09270       | GU479782 | GU479747 | GU479846 |
| **Ascochyta marina**                  | MD6011          | KT259205 | KT259207 | -    |
| **Ascochyta marina**                  | MD6012          | KT259206 | - | -    |
| **Ascochyta marina**                  | MF416           | MK007123 | MK007124 | -    |
| **Balnusandhika indica**              | GFUFC 18001     | KF460274 | - | -    |
| **Bambusicola massarinia**            | MFLUCC 11-0389  | JX442037 | JX442041 | -    |
| **Berkleasmium micronesicum**         | BCC 8141        | DQ280272 | DQ280268 | -    |
| **Berkleasmium nigroapicale**         | BCC 8220        | DQ280273 | DQ280269 | -    |
| **Bimuria novae-zelandiae**           | CBS 107.79      | AY016356 | AY016338 | DQ471087 |
| **Botryosphaeria dothidea**           | CBS 115476      | AY929047 | EU673173 | AY236898 |
| **Brevicollum hyalosporum**           | MAFN 243400     | LC271239 | LC271236 | LC271245 |
| **Brevicollum hyalosporum**           | MFLUCC 17-0007  | MG602200 | MG602202 | MG739516 |
| **Brevicollum hyalosporum**           | PUFN 17628      | MH198671 | - | -    |
| **Capnodium salicinum**               | CBS 131.34      | DQ676805 | DQ676997 | -    |
| **Cladosporium cladosporioides**      | CBS 170.54      | DQ676807 | DQ678004 | -    |
| **Clematidios palatina**              | MFLUCC 15-0084  | KU842381 | KU842382 | -    |
| **Corynespora cassicola**             | CBS 100822      | GU301808 | GU296144 | GU349052 |
| **Corynespora smithii**               | CAB 5649b       | GU323201 | - | GU349018 |
| **Craspiares quadrisporus**           | HHUF 30590      | LC271241 | LC271238 | LC271248 |
| **Crassiparipes quadrisporus**        | HHUF 30409      | LC100025 | LC100027 | -    |
| **Crassiparipes octosporum**          | KT 2144         | LC373108 | LC373084 | LC373120 |
| **Crassiparipes octosporum**          | KT 2894         | LC373109 | LC373085 | LC373121 |
| **Crassiparipes octosporum**          | KT 3008         | LC373110 | LC373086 | LC373122 |
| **Crassiparipes octosporum**          | KT 3029         | LC373111 | LC373087 | LC373123 |

(Table 1) and the ITS region of *Anastomitrabeculia didymospora* was deposited with the accession number MW413900 (MFLUCC 16-0412) and MW413897 (MFLUCC 16-0417).
| Taxon                              | Strain Number | GenBank Accession Numbers         |
|------------------------------------|---------------|----------------------------------|
|                                    |               | **LSU**  | **SSU**  | **TEF1α** |
| Crassiperidium octosporum          | KT 3046       | LC373112 | LC373088 | LC373124  |
| Crassiperidium octosporum          | KT 3188       | LC373113 | LC373089 | LC373125  |
| Crassiperidium octosporum          | KT 3468       | LC373114 | LC373090 | LC373126  |
| Crassiperidium octosporum          | KT 3604       | LC373115 | LC373091 | LC373127  |
| Crassiperidium octosporum          | KT 3605       | LC373116 | LC373092 | LC373128  |
| Crassiperidium octosporum          | MM 9          | LC373117 | LC373093 | LC373129  |
| Crassiperidium quadrisporum        | KT 27981      | LC373118 | LC373094 | LC373130  |
| Cryptoclypeus oxyphorus            | HHUF 30507    | LC373095 | LC373131 |
| Cryptocoryneum akitaense           | MAFF 245365   | LC373132 | LC373131 |
| Cryptocoryneum japonicum           | MAFF 245370   | LC373133 | LC373131 |
| Cryptocoryneum longicondensatum    | MAFF 245374   | LC373134 | LC373131 |
| Cyclothyriella rubronotata         | CBS 141486    | LX650544 | LX650507 | LX650519  |
| Cyclotrichia rubronotata           | CBS 121892    | LX650541 | -        | LX650516  |
| Cyclotrichia rubronotata           | CBS 385.39    | MH67543  | -        | -         |
| Cyclotrichia rubronotata           | CBS 419.85    | GU301875 | -        | GU349002  |
| Delitschia didyma                  | UME 31411     | DQ84090  | AF242264 |          |
| Delitschia winteri                 | CBS 225.62    | DQ678077 | DQ67922  | DQ67922  |
| Dendrographa decorans              | Ertz 5003     | AY548815 | AY548809 | -         |
| Dendrographa leucophana f. minor   |               | AF279382 | AF279381 | -         |
| Dendryphion europaeum              | CPC 22943     | KJ869203 | -        | -         |
| Dendryphion europaeum              | CPC 23231     | NG_059120| -        | -         |
| Didymosphaeria rubi-ulmifolii      | MFLUCC 14-0023| KJ436586 | KJ436588 | -         |
| Dissoconium aciculare              | CBS 204.89    | GU214419 | GU214523 | -         |
| Ernakulamia cochinensis            | PRC 3992      | LT964670 | -        | -         |
| Flavomyces fulophazii              | CBS 135761    | KP184040 | KP184082 | -         |
| Fuscostagonospora cytisii          | MFLUCC 16-0622| KY770978 | KY770977 | KY770977 |
| Fuscostagonospora sasae            | CBS 139687    | AB870548 | AB797258 | -         |
| Fusulina eucalyptorum              | CBS 145083    | MK047499 | -        | -         |
| Gordonomyces mucovaginatus         | CBS 127273    | JN712552 | -        | -         |
| Halotthia posidonae                | BBH 22481     | GU479786 | GU479752 | -         |
| Helminthosporium aquaticum         | MFLUCC 15-0357| KU697306 | KU697310 | -         |
| Helminthosporium velutinum         | MFLUCC 15-0423| KU697304 | KU697305 | -         |
| Helminthosporium velutinum         | MFLUCC 13-0243| KU697304 | KU697305 | -         |
| Heratomecyes thailandica           | MFLUCC 14-1140| KJ869203 | KJ869204 | -         |
| Hohos wagradensis                  | TI            | -        | -        | -         |
| Hysterium angustatum               | CBS 236.34    | FJ161106 | FJ161096 | -         |
| Hysterium angustatum               | MFLUCC 16-0623| MH535893 | MH535885 | MH535878 |
| Jahnula seychellensis              | SS2113        | ESI175665| ESI175643| -         |
| Latorua caligans                   | CBS 576.65    | KR873266 | -        | -         |
| Latorua grootfonteinensis          | CBS 369.72    | KR873267 | -        | -         |
| Lentimurispora urniformis          | MFLUCC 18-0497| MH179144 | MH179160 | MH188055 |
| Leoxyphium cacuminum               | CBS 505.75    | GQ387576 | GQ387515 | GU349069 |
| Lepidophyllum fissipora             | MFLUCC 11-0377| JP88646  | -        | -         |
| Lignosphaeria thailandica          | MFLUCC 11-0376| KPE88645 | -        | -         |
| Taxon                                      | Strain Number | GenBank Accession Numbers |
|--------------------------------------------|---------------|--------------------------|
| **Lindgomycetes ingoldianus**              | ATCC 200398   | AB521736 AB521719 -     |
| **Longistium tectonae**                    | MFLUCC 12 0562 | KU764700 KU712459 -     |
| **Lophiotaeba eburnoides**                 | HHUF 30079    | LC001707 LC001706 -     |
| **Lophiotaeba micional**                   | CBS 627.86    | GU301837 GU296167 GU349073 |
| **Macrodiplodopsis desmazeri**             | CBS 140062    | KR873372 - -            |
| **Magnicamarosporium dossypyrcola**        | MFLUCC 16-0419 | KY554212 KY554211 KY554209 |
| **Massarina eburnea**                      | CBS 473.64    | GU301843 GU296173 -     |
| **Massariosphaeria phacospora**            | CBS 611.86    | GU301843 GU296173 -     |
| **Mauritana rhizophorae**                  | BCC 28866     | GU371824 GU371832 GU371817 |
| **Medicopsis romeroi**                     | CBS 122784    | EU754207 EU754210 KF015678 |
| **Medicopsis romeroi**                     | CBS 132878    | KF015622 KF015648 KF015682 |
| **Murispora rubicunda**                    | IFRD 2017     | FJ795307 GU456308 GU456289 |
| **Neatoastrosphaeriella krabiensis**       | MFLUCC 11-0025 | JN846729 JN846739 -     |
| **Neohendersonia kickxii**                 | CBS 112403    | KX820266 - -            |
| **Neohendersonia kickxii**                 | CBS 122938    | KX820268 - -            |
| **Neohendersonia kickxii**                 | CBS 134276    | KX820267 - -            |
| **Neohendersonia kickxii**                 | CBS 122941    | KX820269 - -            |
| **Neohendersonia kickxii**                 | CBS 122941    | KX820269 - -            |
| **Neomassaria fabacearum**                 | MFLUCC 16-1875 | KX524145 KX524147 KX524149 |
| **Neomassaria formosana**                  | NTUCC 17-007  | MH174756 MH174759 MH174762 |
| **Neomassarina chromolaenae**              | MFLUCC 17-1480 | MT214466 MT214419 MT235785 |
| **Neomassarina pandanicolana**             | MFLUCC 16-0270 | MG298945 - MG298947 -   |
| **Neomassarina thailandica**               | MFLUCC 10-0552 | KX672157 KX672160 KX672163 |
| **Neotorula aquatica**                     | MFLUCC 150342 | KU500576 KU500583 -     |
| **Neotorula submersa**                     | KUMCC 15-0280 | KX789217 - -            |
| **Ocellitambusa bambusae**                 | MFLUCC 13-0855 | KU863112 KU872116 -     |
| **Ocellitambusa postula**                  | MFLUCC 11-0502 | KU863115 KU872118 -     |
| **Ohleria modesta**                        | MGC           | KX650562 KX650563 KX650533 |
| **Paradictyoarthrinium diffractum**        | MFLUCC 13-0466 | KP744498 KP753960 -     |
| **Paradictyoarthrinium diffractum**        | MFLUCC 12-0557 | KP744497 - -            |
| **Paradictyoarthrinium hydei**             | MFLUCC 13-0465 | MG747497 - -            |
| **Paradictyoarthrinium tectonicola**       | MFLUCC 13-0465 | KP744500 KP753961 -     |
| **Periconia thailandica**                  | MFLUCC 17-0065 | KU753888 KU753889 -     |
| **Phaeoseptum aquaticum**                  | CBS 123113    | JN640072 - -            |
| **Phaeoseptum terricola**                  | MFLUCC 10-0102 | MH105779 MH105780 MH105781 |
| **Phyllosticta capitansis**                | CBS 226.77    | KF206289 KF766300 -     |
| **Piedraia hortae**                        | CBS 480.64    | GU214466 - -            |
| **Polyphosphaeria fusca**                  | CBS 125425    | AB524607 AB524666 AB524822 |
| **Preussia nigricans**                     | CBS 363.69    | DQ384098 DQ384087 -     |
| **Preussia nigricans**                     | CBS 264.69    | GU301872 GU296197 GU349027 |
| **Pseudoastrosphaeriella bambusae**        | MFLUCC 11-0205 | KT955475 KT955455 KT955437 |
| **Pseudoastrosphaeriella longicolla**      | MFLUCC 11-0171 | KT955476 KT955456 KT955438 |
| **Pseudoastrosphaeriella thailandensis**   | MFLUCC 10-0553 | KT955477 KT955456 KT955439 |
| **Pseudoastrosphateria eylonicola**        | HHUF 28984    | LC194381 LC194339 LC194418 |
| **Pseudomassariosphaeria broonicola**      | MFLUCC 15-0031 | KT305994 KT305996 KT305999 |
| **Pseudotetraploa curvippendiculata**      | CBS 125426    | AB524610 AB524669 AB524825 |
| **Quadritcruva septentrionalis**           | CBS 125428    | AB524617 AB524476 AB524832 |
| **Racodium rupestre**                      | L424          | EU048582 EU048577 -     |
| **Racodium rupestre**                      | L424          | EU048582 EU048577 -     |
| **Ramusculica thailandica**                | MFLUCC 13-0284 | KP888647 KP899131 KP305767 |
| **Rimora mangrovi**                        | JK 5246A      | GU301868 GU296193 -     |
| **Roccella fusciformis**                   | Teher 8171    | FJ638979 - -            |
| **Rostriconidium aquaticum**               | KUMCC 15-0297 | MG208144 - MG207995 -   |
Table 1. Cont.

| Taxon                              | Strain Number | GenBank Accession Numbers  |
|------------------------------------|---------------|----------------------------|
|                                    |               | LSU | SSU | TEF1α          |
| Rostriconidium aquaticum           | MFLUCC 16-1113| MG208143 | -     | MG207994       |
| Salsuginea ramicola                | KT 2597.1     | GU479800 | GU479767 | GU479861       |
| Salsuginea ramicola                | CBS 125781    | MH877872 | -     | -              |
| Scoris spongiosa                   | CBS 325.33    | MH866910 | GU214696 | -              |
| Seriascoma didymospora             | MFLUCC 11-0179| KU863116 | KU872119 | -              |
| Sigarisporea arundinis              | JCM 13550     | AB618998 | AB618679 | LC001737       |
| Sigarisporea ravennica             | MFLUCC 14-0005| KP698414 | KP698415 | -              |
| Splanchnonema platani              | CBS 222.37    | KR909316 | KR909318 | KR909319       |
| Sporodesmioides thailandica        | KUMCC 16-0012 | KX437758 | KX437760 | KX437767       |
| Sporormia fimetaria                | UPS:Dissing Gr.81.194 | GQ203729 | -     | -              |
| Sporormiella minima                | CBS 52450     | DQ468046 | -     | DQ468003       |
| Stagonospora pseudocaricis         | CBS 135132    | KF251762 | KF251259 | KF252741       |
| Stemblygium vesicarium             | CBS 191.86    | DQ247804 | DQ247812 | DQ471090       |
| Stemblygium vesicarium             | CBS 714.68    | DQ678049 | DQ767648 | DQ677888       |
| Sulcattispora acerina              | KT2982        | LC014610 | LC014605 | LC014615       |
| Sulcosporium thailandicum          | MFLUCC 12-0004| KT426563 | KT426564 | -              |
| Tetraphiala quercus                | CBS 143396    | MH107966 | -     | MH108030       |
| Tetralsapheria sascolia            | KT 563        | AB524631 | AB524490 | AB524838       |
| Torula gaodangeris                 | MFLUCC 17-0234| NG_059827 | NG_063641 | -              |
| Torulha herbarum                   | CBS 111855    | KF443386 | KF443391 | KF443403       |
| Triplosapheria maxima              | MAF 239682    | AB524637 | AB524496 | -              |
| Tubufa chiangmaiensis              | MFLUCC 11-0514| KF301538 | KF301543 | KF301557       |
| Tubufa japonica                    | MFLUCC 12-0545| KJ880036 | KJ880035 | KJ880037       |
| Vargamycetes aquaticus             | CBS 639.63    | KY853539 | -     | -              |
| Vargamycetes aquaticus             | HKUCC 10830   | DQ408575 | -     | -              |
| Versicloripsis triplexatum          | HHUF 28815    | AB330081 | AB524501 | -              |
| Westerdykella dispersa             | CBS 297.56    | MH869191 | -     | -              |
| Westerdykella ornata               | CBS 379.55    | GU301880 | GU296208 | GU349021       |
| Xenomassariosphaeria rosae         | MFLUCC 15-0179| MG829092 | MG829192 | -              |

2.3. Phylogenetic Analysis

The sequence data were assembled using BioEdit v. 7.2.5 [35] and subjected to a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest matches with taxa in Pleosporales. Reference sequence data of this order and some representatives of other orders of Dothideomycetes were downloaded from previously published studies [1,6,36–39]. The sequences were automatically aligned using default settings in MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) [40]. A combined dataset of three gene regions (LSU, SSU and TEF1α) was prepared and manually adjusted using BioEdit and AliView [41]. Phylogenetic analyses of the combined dataset were performed using maximum likelihood, maximum parsimony and Bayesian inference method. Maximum likelihood analyses (ML), including 1000 bootstrap pseudoreplicates, were performed at the CIPRES web portal [42] using RAxML v. 8.2.12 [43]. Maximum parsimony analysis was conducted using PAUP v.4.0b 10 [44] with the heuristic search option and number of replications 1000 each. The Tree Length (TL), Consistency Indices (CI), Retention Indices (RI), Rescaled Consistency Indices (RC) and Homoplasy Index (HI) were documented.

The best model for different genes partition was determined in JModelTest version 2.1.10 [45] for posterior probability (PP). The general time reversible (GTR) model with a discrete gamma distribution plus invariant site (GTR+I+G) substitution model was used for the combined dataset. Posterior probabilities [46] were estimated by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.2.6 [47]. Four simultaneous Markov chains were run for 10 million generations and trees were sampled every 1000th generation, thus resulting in 10,000 trees. The suitable burn-in phase was determined by inspecting traces
in Tracer version 1.7 [48]. The first 10% of generated trees representing the burn-in phase of the analyses were discarded, while the remaining trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree. The phylogenograms were visualized with FigTree v1.4.0 program [49] and edited using Adobe Illustrator CS6 v15.0 (Adobe Systems, USA).

2.4. Fossil Calibration and Divergence Time Estimates

Divergence times were estimated with BEAST 2.6.2 [50] based on the methodology described in Phukhamsakda et al. [4]. The aligned sequence dataset (LSU, SSU and TEF1α) used for the phylogenetic analyses were loaded into BEAUTI 2.6.2 to prepare the XML file. Nucleotide substitution models were determined using JModelTest version 2.1.10. The GTR+I+G nucleotide substitution model was applied to LSU and TEF1α partitions. The symmetrical (SYM) model with a discrete gamma distribution plus invariant site (SYM+I+G) substitution model was applied to the SSU partition. The data partitions were set with unlinked substitution, linked clock model and linked tree. An uncorrelated relaxed clock model with lognormal distribution was used. The Yule speciation process, which assumes a constant rate of speciation divergence, was used as the tree prior [51]. The analysis was performed in BEAST 2.6.2 for 100 million generations, sampling every 1000 generations. The effective sample size (ESS) was analysed with Tracer version 1.7 to check that the values were greater than 200, as recommended by Drummond et al. [52]. The first 20% trees were discarded as the burn-in phase and the remaining trees were combined in LogCombiner 2.6.2. The maximum clade credibility was calculated in TreeAnnotator v 2.6.2. The phylograms were visualized with FigTree v.1.4.0 program.

To estimate the divergence time for Anastomitrabeculiaceae, the fossil Metacapnodium succinum (Metacapnodiacae) was used to set the crown age of Capnodiales using a normal distribution, mean of 100 MYA, SD of 150 MYA, giving 95% credibility interval of 346 MYA [4,23,53,54]. The fossil Margaretbarromyces dictyosporus was used to calibrate the crown age of Aigialus (Aigialaceae) using a gamma distribution, with an offset of 35 MYA, a shape of 1.0, scale of 25, providing 95% credibility interval of 110 MYA [4,55–57]. The split between Arthoniomycetes (outgroup) and Dothideomycetes was used as the secondary calibration using a normal distribution, mean of 300 MYA, SD of 50 MYA, giving 95% credibility interval of 382 MYA [22,36,53,54].

3. Results

3.1. Phylogenetic Analyses

The combined gene alignment comprised 196 strains and 2800 characters (LSU: 860 characters, SSU: 1039 characters and TEF1α: 901 characters). Among the 2800 characters, there were 1492 conserved sites (53%), 364 variable sites (13%) and 944 parsimony informative sites (34%). The parsimony analysis of the data matrix yielded one most parsimonious tree out of 1000 (CI = 0.265, RI = 0.659, RC = 0.175, HI = 0.735, Tree Length = 7606). Based on BLAST search in the NCBI GenBank of the LSU gene, the newly generated taxon MFLUCC 16-0412 and MFLUCC 16-0417 show 95% similarity to Crassiperidium quadrisporum (KT 27981 and KT 27982). The topology of the phylogenetic tree based on the LSU gene was generally congruent with the overall topology of the tree based on the combined dataset. Phylogenetic trees generated from maximum likelihood, maximum parsimony and Bayesian analysis of the combined dataset resulted in similar topologies with some exception. The position of Cyclothryiellaceae and Longiostiolaceae differed between the three methods. The best scoring RAxML tree had a final likelihood value of −40,523,297855 (Figure 1). The new taxon formed an independent lineage basal to the Halojulellaceae with strong Bayesian inference support and moderate support from maximum likelihood (0.99 PP/65% MLBT). A new genus Anastomitrabeculia is therefore introduced within Anastomitrabeculiaceae to accommodate the new species.
Figure 1. The best scoring RAxML tree based on a combined LSU, SSU and TEF1α dataset. RAxML bootstrap support and maximum parsimony values ≥60% (BT), as well as Bayesian posterior probabilities ≥0.90 (BYPP) are shown, respectively, near the nodes. The ex-type strains are in bold and the scale bar indicates 0.06 changes per site. The tree is rooted with species of Arthoniomycetes and the new taxon is indicated in blue.
3.2. Fossil Calibration and Divergence Time Estimates

The topology of the maximum clade credibility (MCC) tree (Figure 2) was congruent with the tree obtained from the Bayesian inference analysis and the maximum likelihood analysis. The divergence times of the dating analysis are listed in Table 2. The crown age of *Dothideomycetes* is estimated at 263 MYA during the Permian period based on the MCC tree. The split of *Arthoniomycetes* and *Dothideomycetes* occurred around 323 MYA during the Carboniferous period. The crown age of *Pleosporales* is estimated at 206 MYA, and *Hysteriales* diverged from *Pleosporales* approximately 236 MYA during the Triassic period. The crown age of *Anastomitrabeculiaceae* is estimated at around 2.6 MYA, and it diverged from *Halojulellaceae* at around 84 (52–116) MYA. *Anastomitrabeculiaceae* formed an independent lineage with close relationship to *Halojulellaceae* with strong posterior probability in the MCC tree (0.99 BYPP). The divergence time of *Anastomitrabeculiaceae* was compared to Pleosporalean families with trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath (Table 3). The divergence time of *Anastomitrabeculiaceae* was also compared to *Didymosphaeriaceae* as they are morphologically similar by having trabeculate pseudoparaphyses and cylindrical asci.

Table 2. Divergence time estimates obtained from BEAST analysis. The median and the 95% Highest Posterior Density are provided in million years ago (MYA). The geological time scales are given based on the median node age.

| Nodes                                | Node Age      | Geological Time Period |
|--------------------------------------|---------------|------------------------|
| *Arthoniomycetes–Dothideomycetes*    | 323 (310–349) | Carboniferous          |
| *Dothideomycetes* crown group        | 263 (216–313) | Permian                |
| *Hysteriales–Pleosporales*           | 236 (188–300) | Triassic               |
| *Pleosporales* crown group           | 206 (171–254) | Triassic               |
| *Capnodiales* crown group            | 147 (99–200)  | Jurassic               |
| *Anastomitrabeculiaceae* stem group  | 84 (52–116)   | Cretaceous             |
| *Aigialaceae–Aigialus* sp.           | 37 (18–56)    | Eocene                 |
| *Anastomitrabeculiaceae* crown group | 2.6 (0.19–6.61)| Neogene               |

Table 3. Divergence time estimates obtained from BEAST analysis for families with similar morphology to *Anastomitrabeculiaceae*. The crown age and the stem age are provided in million years ago (MYA).

| Families                     | Crown Age | Stem Age |
|------------------------------|-----------|----------|
| *Aigialaceae*                | 102       | 141      |
| *Amniculicolaceae*           | 90        | 177      |
| *Anastomitrabeculiaceae*     | 2.6       | 84       |
| *Anteagloniaceae*            | 52        | 98       |
| *Bambusicolaceae*            | 29        | 57       |
| *Cyclothyriellaceae*         | 66        | 95       |
| *Delitschiaceae*             | 78        | 131      |
| *Didymosphaeriaceae*         | 47        | 81       |
| *Fuscostagonosporaceae*       | 26        | 63       |
| *Lindgomycetaceae*           | 31        | 92       |
| *Neomassariaceae*            | 82        | 131      |
| *Pseudoastrosphaeriellaceae*  | 56        | 147      |
| *Tetraplosphaeriaceae*        | 91        | 189      |
Figure 2. Maximum clade credibility (MCC) tree of families in Dothideomycetes using BEAST. Numbers at nodes indicate posterior probabilities (PP) for node support. Bars correspond to the 95% highest posterior density (HPD) intervals. Posterior probabilities greater than 0.95 are given near the nodes. The new taxon is indicated in blue. Geological time scales are given at the base together with scale in million years ago (MYA) [58].
3.3. Taxonomy

_Anastomitrabeculiaceae_ Bhunjun, Phukhams and K.D. Hyde, _fam. nov._

Index Fungorum number: IF556817, Facesoffungi number: FoF 09521.

Etymology: Referring to the name of the type genus.

Saprobic on dead bamboo culms submerged in freshwater. **Sexual morph:** Ascomata immersed under a clypeus to semi-immersed, gregarious, uniloculate, globose to subglobose, carbonaceous, black. **Ostiole** central, apex well developed. **Peridium** multi-layered, sub-carbonaceous or coriaceous, with dark brown to hyaline cells arranged in a *textura angularis*. **Hamathecium** composed of numerous, filamentous, trabeculate pseudoparaphyses, septate, anastomosing between the asci and at the apex. **Ascii** bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, bulbous pedicel, with an ocular chamber. **Ascospores** biseriate, broadly fusiform, septate, smooth-walled, with wall ornamentation, surrounded by mucilaginous sheath.

Note: *Anastomitrabeculiaceae* is introduced to include _Anastomitrabeculia_, which is reported as a saprobe on bamboo culms. *Anastomitrabeculiaceae* is characterised by semi-immersed, coriaceous or carbonaceous ascomata with septate, trabeculate pseudoparaphyses and hyaline ascospores with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. _Anastomitrabeculiaceae_ formed a well-supported independent lineage closely related to *Halojulellaceae*, but *Halojulellaceae* differs by its cellular pseudoparaphyses and golden-brown ascospores.

**Type genus:** _Anastomitrabeculia_ Bhunjun, Phukhams and K.D. Hyde.

_Anastomitrabeculia_ Bhunjun, Phukhams. and K.D. Hyde, _gen. nov._

Index Fungorum number: IF556680, Facesoffungi number: FoF 09522.

Etymology: Referring to the trabeculate pseudoparaphyses anastomosing between the asci and at the apex.

Colonies on natural substrate umbonate at the centre, circular, black shiny dots are visible on the host surface. **Ascomata** on surface of the host, immersed under a clypeus, gregarious, uniloculate, subglobose, carbonaceous. **Ostiole** orange pigment near ostiole. **Peridium** comprising multilayers of brown to hyaline cells of *textura angularis*, inner layers composed of thin, hyaline cells. **Ascii** 8–spored, bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, with a bulbous pedicellate, rounded at the apex, with an ocular chamber. **Ascospores** biseriate, broadly fusiform, tapering towards the ends, hyaline, with guttules in each cell, constricted at the septa, with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath.

Note: *Anastomitrabeculia* is established as a monotypic genus. It is characterised by the presence of carbonaceous ascomata, with orange pigment near ostiole and ascospores with longitudinally striate wall ornamentation. *Anastomitrabeculiaceae* is morphologically similar to members of *Pleosporales* in having perithecioid ascomata, bitunicate asci and hyaline ascospores.

**Type species:** _Anastomitrabeculia didymospora_ Bhunjun, Phukhams and K.D. Hyde.

_Anastomitrabeculia didymospora_ Bhunjun, Phukhams and K.D. Hyde, _sp. nov._

Index Fungorum number: IF556559; Facesoffungi number: FoF 09523 Figure 3.

Etymology: Referring to the didymosporous ascospores.

Holotype–MFLU 20-0694.

Saprobic on dead bamboo culms submerged in freshwater. **Sexual morph:** Ascomata 430–460 μm high, 435–575 μm diam., immersed under a clypeus to semi-immersed, gregarious, uniloculate, globose to subglobose, carbonaceous, rough, black, ostiolate. **Ostiole** 160 μm high, 270 μm diam., central, apex well developed, papillate, with pore-like opening, with periphyses filling the ostiolar canal, dark brown to black, orange pigment near ostiole. **Peridium** 6–18 μm wide, comprising 3–5 layers of brown to hyaline cells of *textura angularis*, inner layers composed of thin, hyaline cells. **Hamathecium** of dense, long, 0.8–1.25 μm wide (*X = 1 μm, n = 50*), filiform, filamentous, trabeculate pseudoparaphyses, septate, branched,
embedded in a gelatinous matrix, anastomosing between the asci and at the apex. *Asci* 125–160 × 15–20 µm (x = 145 × 17 µm, n = 20), 8-spored, bitunicate, fissitunicate, broad cylindrical to cylindrical-clavate, with bulbous pedicellate, rounded at the apex, with an ocular chamber. *Ascospores* 18–28 × 7–10 µm (x = 22.5 × 9 µm, n = 20), biseriate, broadly fusiform, tapering towards the ends, hyaline, 1-septate at the centre, constricted at the septum, cell above septate enlarged, straight, smooth-walled, with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. **Asexual morph:** Undetermined.

Figure 3. *Anastomitrabeculia didymospora* (MFLU 20-0694, holotype). (a) Ascomata on bamboo. (b) Close-up of ascomata. (c) Vertical section of ascoma. (d) Ostiolar canal. (e) Peridium layer. (f) Trabeculate pseudoparaphyses. (g–i) Asci. (j) Pedicel. (k–o) Ascospores showing mucilaginous sheath. (p) Culture characteristics on PDA from above and below (9 cm diameter petri dish). Scale bar: (b) = 500 µm, (c) = 200 µm, (d–i) = 50 µm, (j–o) = 10 µm.

Culture characters: Ascospores germinating on MEA and PDA within 24 h with germ tubes developing from basal cells. Colonies on MEA and PDA umbonate at the centre, circular, friable, reaching 20 mm diameter after four weeks of incubation at 25 °C. Culture on MEA with white aerial mycelium, dark brown at the centre and paler towards the edge from above and below. Culture on PDA dark brown from above and below.

Material examined: THAILAND, Krabi province (8.1° N, 98.9° E), on dead bamboo culms, 15 December 2015, C. Phukhamsakda, KR001 (MFLU 20-0694, holotype), ibid,
18 December 2015 (MFLU 20-0695, paratype); ex-type living culture MFLUCC 16-0412; ex-paratype living culture, MFLUCC 16-0417.

4. Discussion

In this study, we introduce a new species, genus and family for a collection of Pleosporales found on bamboo. The introduction of new taxa, even at the family level, is not surprising, considering that about 93% of fungi remain unknown to science despite ca. 2000 species described every year [59,60]. Pleosporalean species can occur in terrestrial, marine and freshwater habitats [7–9]. Several studies have reported new pleosporalean taxa from freshwater or marine habitats or from bambusicolous hosts [1,3]. Pleosporales have unique characters such as perithecioid ascomata typically with a papilla and bitunicate, generally fissitunicate asci, bearing mostly septate ascospores of different colours and shapes, with or without a gelatinous sheath [7]. The morphology of Anastomitrabeculaceae is similar to members of the Pleosporales based on the presence of pseudoparaphyses, perithecioid ascomata, bitunicate asci and hyaline ascospores. Anastomitrabeculaceae is characterised by semi-immersed to superficial ascomata, trabeculate pseudoparaphyses, cylindrical asci and ascospores with longitudinally striate wall ornamentation, surrounded by mucilaginous sheath. The newly discovered species formed a well-supported independent lineage basal to the Halojulellaceae based on phylogenetic analyses of the combined dataset (0.99 PP/65% MLBT). Halojulellaceae differs by its cellular pseudoparaphyses and golden brown ascospores [2]. The new taxon is also phylogenetically closely related to Neohendersonsoniaceae, which differs by its cellular pseudoparaphyses and smooth-walled ascospore [61]. A novel genus Anastomitrabeculia is therefore introduced to accommodate one new species, Anastomitrabeculia didymospora. A new family, Anastomitrabeculaceae, is also introduced to accommodate this independent lineage.

Several pleosporalean families such as Aigialaceae, Amniculicolaceae, Anteagloniaceae, Astrosphaeriellaceae, Bambusicolaceae, Biastrisoraceae, Caryosporaceae, Cyclothyriellaceae, Delitschiaceae, Didymosphaeriaceae, Fuscostagonosporaceae, Lindgomycetaceae, Melanommataceae, Neomassariaceae, Pseudoastrosphaeriellaceae, Striagutulaceae and Tetraplosphaeriaceae share similar characters to Anastomitrabeculaceae in having trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath [7]. The nature of pseudoparaphyses is often overlooked, but they have taxonomic relevance at the genus and possibly family levels [7], but not at the ordinal level [62]. These families differ from Anastomitrabeculaceae mainly by their ascospores, for example, Aigialaceae and Amniculicolaceae have brown and muriform ascospores [7]. Anteagloniaceae differs by having a peridium composed of dark brown cells of textura epidermoidea, cellular or trabeculate pseudoparaphyses and small, uniseriate ascospores [2]. Astrosphaeriellaceae differs by its brown, sub-fusiform to fusiform, dark brown ascospores [2]. Caryosporaceae differs by its broad-fusiform, ovoid or ellipsoid, brown ascospores [64]. Bambusicolaceae species have also been isolated from dead bamboo culms, but they differ from Anastomitrabeculaceae by their cellular pseudoparaphyses and multi-seriate, smooth-walled ascospores [2]. Cyclothyriellaceae differs by its uniseriate, ellipsoid to fusiform, brown ascospores with several eusepta [65]. Fuscostagonosporaceae differs in having globose to subglobose ascomata, fissitunicate ascospores with long stipes and narrowly fusiform ascospores [66]. Anastomitrabeculaceae shares several characters with Didymosphaeriaceae in having immersed ascomata formed under a clypeus, trabeculate pseudoparaphyses and cylindrical asci. Didymosphaeriaceae and Melanommataceae differ in having cellular or trabeculate pseudoparaphyses and brown, multi-septate, muriform ascospores [7]. Lindgomycetaceae differs by the presence of cellular or trabeculate pseudoparaphyses and brown, multi-septate ascospores with bipolar mucilaginous appendages [7]. Neomassariaceae differs by its immersed ascomata and ellipsoid ascospores. Pseudoastrosphaeriellaceae differs by its brown to reddish-brown ascospores with longitudinal ridges towards the ends and Striagutulaceae differs in having brown, ellipsoid...
ascospores with paler end cells. Tetraplosphaeriaceae differs by its immersed ascomata and slightly curved, pale brown ascospores [7].

Divergence time estimate has been widely used as supporting evidence to clarify taxonomic status of extant or novel families in fungal taxonomy [4,6,23,24,26,27,67]. In this study, the MCC tree was congruent with the topology of the trees generated from Bayesian inference analysis and maximum likelihood analyses. The divergence time estimates for the crown age of Dothideomycetes (263 MYA), the split of Dothideomycetes and Arthoniomycetes (323 MYA), the crown age of Pleosporales (206 MYA) and the divergence of Hysteriales from Pleosporales (236 MYA) are similar to previous studies [4,7,11]. Hyde et al. [27] recommended that the divergence times of families should be between 50 and 150 MYA. The stem age is usually preferred to the crown age in taxa ranking as it is not affected by the sample size of the clade [27]. Based on the MCC tree, Anastomitrabeculiaceae and Halojulellaceae share the stem age of 84 MYA which supports the establishment of Anastomitrabeculiaceae.

The divergence time of Anastomitrabeculiaceae was also compared to Pleosporalean families with trabeculate pseudoparaphyses, cylindrical asci and ascospores with a sheath (Table 3). Cyclothyriellaceae has an estimated crown age of 66 MYA and it diverged at 95 MYA. Fuscostagonosporaceae has a crown age of approximately 26 MYA and it diverged around 63 MYA. Bambusicolaceae, which was also isolated from dead bamboo culms, has a crown age of 29 MYA and a stem age of about 57 MYA. The stem age of Anastomitrabeculiaceae lies within the range of divergence times of those with similar morphology, but the crown age of Anastomitrabeculiaceae (2.6 MYA) is much earlier compared to these families. Bambusicolaceae was introduced by Hyde at al. [2] to accommodate three bambusicolous taxa, and it currently has 15 species [7]. Fuscostagonosporaceae was introduced by Hyde at al. [66] to accommodate one bambusicolous taxon and it currently has four species [7]. Ariyawansa et al. [64] introduced the pleosporalean family, Caryosporaceae, which is morphologically similar to Astrosphaeriellaceae and Trematosphaeriaceae [7]. Based on Liu et al. [11], the stem age of Caryosporaceae (85 MYA) is similar to Trematosphaeriaceae (88 MYA) compared to Astrosphaeriellaceae (113 MYA), but the crown age of Caryosporaceae (2 MYA) is much earlier compared to Astrosphaeriellaceae (55 MYA) and Trematosphaeriaceae (65 MYA). Astrosphaeriellaceae currently has 111 species, and Trematosphaeriaceae has 103 species, whereas Caryosporaceae has ten species [7]. Compared to their morphologically similar families, the early crown of Anastomitrabeculiaceae and Caryosporaceae could be due to their smaller sample size. Therefore, further collections are needed for an accurate estimation of the crown age as it is affected by the sample size of the clade [27]. This could also be due to rapid speciation of pleosporalean fungal species given their high adaptation capabilities.

The estimated crown age of Pleosporales (206 MYA) lies within the early Triassic period. The origin of monocotyledons is estimated within the late Cretaceous period (around 145 MYA) [68]. This period is associated with the diversification of pleosporalean families, which continued during the early Cretaceous period when there was a major diversification and radiation of angiosperms, which favoured further diversification of Pleosporalean families to adapt to various hosts [69].

Hosts and their symbionts can speciate in parallel, which relates to a high level of congruence between the phylogeny of the hosts and their symbionts [70,71]. Therefore, studies focusing on divergence time is important for a better understanding of host–pathogen interaction as well as co-evolutionary interactions [72]. This study uses a polyphasic approach based on morphology, multi-locus phylogenetic analyses and divergence time estimates. By implementing a polyphasic approach, we provide strong evidence for introducing the new family based on congruent results supporting the establishment of a new family.

**Author Contributions:** Conceptualization, C.S.B., C.P.; methodology, C.S.B., C.P.; resources, K.D.H.; writing—original draft preparation, C.S.B.; writing—review and editing, C.S.B., C.P., R.J., I.P. and K.D.H.; supervision, K.D.H.; funding acquisition, K.D.H. All authors have read and agreed to the published version of the manuscript.
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