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Splanchnonema-like species in Pleosporales: introducing *Pseudosplanchnonema* gen. nov. in Massariaceae

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Abstract

In this paper we introduce a new genus *Pseudosplanchnonema* with *P. phorcioides* comb. nov., isolated from dead branches of *Acer campestre* and *Morus* species. The new genus is confirmed based on morphology and phylogenetic analyses of sequence data. Phylogenetic analyses based on combined LSU and SSU sequence data showed that *P. phorcioides* formed a distinct clade within the family Massariaceae and is sister to *Massarina eburnea*, the type species of *Massarina*. The new genus *Pseudosplanchnonema* differs from *Massarina* in having ascomata without clypei, a thick peridium and larger, 1-septate, guttulate, dark brown ascospores. The new genus is compared with genera in the family Massariaceae and a detailed description and illustrations of the species *P. phorcioides*, including its asexual morph, is provided.

Key words: morphology, *Morus* sp., multi-gene analysis, Pleomassariaceae, SSU

Introduction

The family Massariaceae was introduced by Munk (1956) to accommodate species with immersed, flattened or spherical ascomata, cellular pseudoparaphyses, clavate to cylindro-clavate asci, and hyaline, fusiform to narrowly fusiform, 1 to 3-septate ascospores with or without a mucilaginous sheath. Previously these species had been placed under the genus *Massaria* (Munk 1956). The family is typified by the genus *Massarina*, which was established to segregate taxa with hyaline ascospores based on *Massarina*, *Keissleriella*, *Metasphaeria*, *Pseudotrachia* and *Trichometasphaeria* (Munk 1956, Hyde *et al.* 2013). Following its introduction, many studies have been conducted on the above mentioned genera, with the exception of *Massarina*, have been transferred to other families (Suetrong *et al.* 2009, Zhang *et al.* 2012, Hyde *et al.* 2013, Wijayawardene *et al.* 2014). *Massarina* has also been placed within Lophiostomataceae in Pleosporales (Bose 1961, Barr 1992, Aptroot 1998, Thambugala *et al.* 2015). Massariaceae was considered as a synonym of Lophiostomataceae in some subsequent studies (Barr 1987). However, recent morphological and molecular studies provide evidence that these two families evolved separately (Thambugala *et al.* 2015). Hence, Massariaceae and Lophiostomataceae were treated as separate families in the order Pleosporales (Liew *et al.* 2002, Zhang *et al.* 2009a, 2012, Hyde *et al.* 2013, Wijayawardene *et al.* 2014).

Members of the family Massariaceae are found in terrestrial habitats, and are saprobic on wood or twigs (Hyde *et al.* 2013). This narrow generic concept, which has been described above, was also accepted for *Massarina*, which comprised *M. eburnea* (Tul. & C. Tul.) Sacc. and *M. cisti* S.K. Bose (Zhang *et al.* 2009b, 2012); molecular data is lacking for many other *Massarina* species. *Massarina eburnea*, *M. cisti* and *M. igniaria* (C. Booth) Aptroot together with *Byssothecium circinans* Fuckel forms a distinct monophyletic clade which can be considered as *Massarina*

sensu stricto (Liew *et al.* 2002, Zhang *et al.* 2012, Hyde *et al.* 2013). The polyphyletic *Massarinaceae sensu lato* clade consists of morphologically diverse asexual species such as *Aquaticheirospora lignicola* Kodsueb & W.H. Ho, *Corynespora olivacea* (Wallr.) M.B. Ellis, *Helminthosporium chlorophorae* M.B. Ellis, *H. solani* Durieu & Mont., *H. velutinum* Link, *Neottiosporina paspali* (G.F. Atk.) B. Sutton & Alcorn and *Stagonospora sensu stricto* (Kodsueb *et al.* 2007, Shearer *et al.* 2009, Suetrong *et al.* 2009, Zhang *et al.* 2012, Hyde *et al.* 2013, Quaedvlieg *et al.* 2013).

Splanchnonema is typified by *S. pustulatum* Corda, and presently thought to belong in the family Pleomassariaceae (Barr 1979, Zhang *et al.* 2012, Hyde *et al.* 2013, Liu *et al.* 2015). It was established by Corda (1829) to accommodate species with immersed, depressed, subglobose ascomata with a thin peridium, clavate to broadly cylindrical, short, furcate pedicellate asci and obovoid, asymmetrical, reddish brown ascospores (Zhang *et al.* 2012, Hyde *et al.* 2013). Currently 44 species has been described as *Splanchnonema* (Index Fungorum 2015). Barr (1993) suggested that this genus was polyphyletic, since it comprises species with different ascospore and ascomata types. Many studies have stated that *Splanchnonema* needs further attention, since they suspected that this genus may include morphologically similar, but phylogenetically distinct *Pleomassaria* species (Tanaka *et al.* 2005, Zhang *et al.* 2012).

This study presents a morpho-molecular study conducted of splanchnonema-like species collected in Italy and Russia. We introduce a new genus *Pseudosplanchnonema* and a new combination *P. phorcioides*. Phylogenetic species recognition based on combined multilocus alignment of SSU and LSU sequence data, using Maximum-parsimony (MP), Maximum Likelihood (ML) and Bayesian analyses showed the phylogenetic position of the new genus in the family Massarinaceae.

Materials and Methods

Sample collection, specimen examination and isolation

Decaying plant materials were collected in Italy and Russia, and returned to the laboratory in paper bags for further study. Materials lacking fruiting bodies were incubated in moist chambers to promote their development and were examined under a Motic SMZ 168 dissecting microscope. Hand sections of the fruiting structures were mounted in water, agitated gently and observed under a Nikon Eclipse 80i compound microscope and photographed by a Canon 450D digital camera fitted to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work, version 0.9.7 software and images were processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems Inc., United States). Single ascospores were isolated following the method described in Chomnunti *et al.* (2014). Single germinating ascospores were transferred to potato dextrose agar and incubated at 25 °C for two days. Specimens are deposited in the Mae Fah Luang University herbarium (MFLU), Chiang Rai, Thailand and BIOTEC Bangkok Herbarium, Thailand. Cultures are deposited in the Culture Collection at Mae Fah Luang University (MFLUCC), Chiang Rai, Thailand and China General Microbiological Culture Collection Center (CGMCC), Beijing, China. Facesoffungi numbers were obtained as in Jayasiri *et al.* (2015).

DNA Extraction, DNA Amplification and Sequencing

Total genomic DNA was extracted from fresh cultures using a modified protocol of Armaleo and Clerc (1995). DNA amplification was performed by Polymerase Chain Reaction (PCR). Partial sequences from large sub unit of nuclear ribosomal RNA (LSU) and small sub unit of nuclear ribosomal RNA (SSU) were amplified. NS1 and NS4 primer pairs were used to amplify a region spanning the SSU gene region (White *et al.* 1990) and LSU gene region was amplified by primer pairs LROR and LR5 (Vilgalys & Hester 1990).

PCR was performed as follows by modifying the thermal cycling program outlined in White *et al.* (1990): initial denaturing step of 95 °C for 3 min, followed by 34 amplification cycles of denaturation at 95 °C for 30 s, annealing for 30 s and elongation at 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The annealing temperatures differed for the two gene regions were 52 °C for LSU and 54 °C for SSU. The PCR products, spanning approximately 1200 nucleotides and 950 nucleotides (respectively of LSU and SSU regions), were checked on 1% agarose electrophoresis gel and stained with ethidium bromide. DNA sequencing was performed at Sunbiotech Company, Beijing, China.

Phylogenetic analyses

SSU and LSU gene data were used in the analysis. DNASTar V.5.1 was used to obtain consensus sequences from data generated from forward and reverse primers. The generated sequences were analyzed using the GenBank BLAST search engine of the National Center for Biotechnology Information to establish possible sister groups of the newly

sequenced taxa. In addition, fungal members from different families of the Pleosporales were also included in the analyses. All reference sequences were obtained from GenBank based on previously published studies (Zhang *et al.* 2009a, 2009b, 2012, Ariyawansa *et al.* 2014, Hyde *et al.* 2013, Liu *et al.* 2015) as listed in Table 1. Combined datasets of SSU and LSU genes were aligned using Clustal X1.81 (Thompson *et al.* 1997) and using default settings of MAFFT v.7 (Kato & Toh 2008, <http://mafft.cbrc.jp/alignment/server/>). The alignment was checked and improved manually where necessary using BioEdit (Hall 1999).

TABLE 1. Accession details of Massarinaceae and representative taxa of Pleosporales available in GenBank and newly generated sequences used in the phylogenetic study.

Taxon Name	Culture Collection ¹	GenBank Accessions ²	
		SSU	LSU
<i>Aigialus grandis</i>	BCC 18419	GU479738	GU479774
<i>Bambusicola bambusae</i>	MFLUCC 11-0614	JX442039	JX442035
<i>Bambusicola irregulispora</i>	MFLUCC 11-0437	JX442040	JX442036
<i>Bambusicola massarinia</i>	MFLUCC 11-0389	JX442041	JX442037
<i>Bambusicola splendida</i>	MFLUCC 11-0439	JX442042	JX442038
<i>Bimuria novae-zelandiae</i>	CBS 107.79	AY016338	AY016356
<i>Byssosphaeria rhodomphala</i>	GKM L153N	-	GU385157
<i>Byssosphaeria salebrosa</i>	SMH 2387	-	GU385162
<i>Byssosphaeria schiedermayeriana</i>	GKM 1197	-	GU385161
<i>Byssosphaeria villosa</i>	GKM 204N	-	GU385151
<i>Byssothecium circinans</i>	CBS 675.92	GU205235	GU205217
<i>Cochliobolus heterostrophus</i>	CBS 134.39	AY544727	AY544645
<i>Corynespora cassiicola</i>	CBS 100822	GU296144	GU301808
<i>Corynespora leucadendri</i>	CBS 135133	-	KF251654
<i>Corynespora olivacea</i>	CBS 114450	-	GU301809
<i>Corynespora smithii</i>	CABI 5649b	-	GU323201
<i>Curcubitaria berberidis</i>	CBS 394.84	GQ387544	GQ387605
<i>Didymosphaeria rubi ulmifoli</i>	MFLUCC 14-0023	KJ436588	KJ436586
<i>Dothidotthia aspera</i>	CPC 12933	EU673228	EU673276
<i>Dothidotthia symphoricarpi</i>	CBS 119687	EU673224	EU673273
<i>Entodesmium rude</i>	CBS 650.86	-	GU301812
<i>Helicascus nypae</i>	BCC 36752	GU479755	GU479789
<i>Herpotrichia diffusa</i>	CBS 250.62	GU205239	-
<i>Herpotrichia juniperi</i>	CBS 200.31	DQ678029	DQ678080
<i>Herpotrichia macrotrichia</i>	GKM 196N	-	GU385176
<i>Kalmusia ebuli</i>	CBS 123120	JN851818	JN644073
<i>Karstenula rhodostoma</i>	CBS 690.94	GU296154	AY787933
<i>Katumotoa bambusicola</i>	MAFF 239641	AB524454	AB524595
<i>Keissleriella cladophila</i>	CBS 104.55	GU205241	GU205221
<i>Lentithecium aquaticum</i>	CBS 123099	GU296156	GU301823
<i>Lentithecium arundinaceum</i>	CBS 123131	GU456298	GU456320
<i>Lentithecium fluviatile</i>	CBS 122367	GU296158	GU301825
<i>Leptosphaeria biglobosa</i>	CBS 303.51	-	GU301826
<i>Leptosphaeria maculans</i>	DAOM 229267	DQ470993	DQ470946
<i>Leptosphaerulina australis</i>	CBS 317.83	EU754067	EU754166
<i>Letendraea helminthicola</i>	CBS 884.85	AY016345	AY016362
<i>Letendraea padouk</i>	CBS 485.70	GU296162	GU849951
<i>Massarina cisti</i>	CBS 266.62	FJ795490	FJ795447
<i>Massarina eburnea</i>	CBS 473.64	GU296170	GU301840
<i>Massarina igniaria</i>	CBS 845.96	GU296171	GU301841
<i>Massarina</i> sp.	CBS 183.58	GU205250	GU205225
<i>Melanomma pulvis-pyrius</i>	CBS 124080	GU456302	GU456323
<i>Monotosporella tuberculata</i>	CBS 256.84	-	GU301851
<i>Montagnula opulenta</i>	CBS 168.34	NG_013127	NG_027581
<i>Morosphaeria ramunculicola</i>	BCC 18405	GU925839	GU925854
<i>Morosphaeria velataspora</i>	BCC 17059	GU925841	GU925852
<i>Neokalmusia brevispora</i>	NBRC 106240	AB524460	AB524601

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TABLE 1. (Continued)

Taxon Name	Culture Collection ¹	GenBank Accessions ²	
		SSU	LSU
<i>Neokalmusia scabriscpora</i>	NBRC 106237	AB524453	AB524594
<i>Neottiosporina paspali</i>	CBS 331.37	EU754073	EU754172
<i>Ophiosphaerella sasicola</i>	MAFF 239644	AB524458	AB524599
<i>Paraconiothyrium fuckelii</i>	CBS 508.94	-	JX496209
<i>Paraconiothyrium fungicola</i>	CBS 113269	AY642527	JX496133
<i>Paraconiothyrium hawaiiense</i>	CBS 120025	EU295655	JX496140
<i>Paraconiothyrium minitans</i>	CBS 122788	EU754074	EU754173
<i>Paraphaeosphaeria michotii</i>	CBS 591.73	GU456305	GU456326
<i>Phaeosphaeria oryzae</i>	CBS 110110	GQ387530	GQ387591
<i>Phoma exigua</i>	CBS 431.74	EU754084	EU754183
<i>Pleomassaria siparia</i>	CBS 279.64	DQ678027	DQ678078
<i>Pleospora herbarum</i>	CBS 191.86	GU238232	GU238160
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC 14-0618	KP683374	KP683373
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC 13-0533	KM875455	KM875454
<i>Pseudosplanchnonema phorcioides</i>	MFLUCC 13-0611	KP683377	KP683376
<i>Prosthemia betulinum</i>	CBS 127468	AB553644	AB553754
<i>Prosthemia canba</i>	JCM 16966	AB553646	AB553760
<i>Prosthemia intermedium</i>	MAFF 242292	AB553647	AB553771
<i>Prosthemia neobetulinum</i>	CBS 121.51	AB553640	AB553747
<i>Prosthemia orientale</i>	JCM 16965	AB553642	AB553750
<i>Prosthemia stellare</i>	CBS 126964	AB553650	AB553781
<i>Pyrenochaeta nobilis</i>	CBS 407.76	EU754107	EU754206
<i>Pyrenophora phaeomes</i>	DAOM 222769	JN940960	JN940093
<i>Setomelanomma holmi</i>	CBS 110217	GQ387572	GQ387633
<i>Splanchnonema platani</i>	CBS 221.37	DQ678013	DQ678065
<i>Splanchnonema pupula</i>	MFU 14-0807	-	KP659197
<i>Stagonospora duoseptata</i>	CBS 135093	-	KF251758
<i>Stagonospora macropycnidia</i>	CBS 114202	GU296198	GU301873
<i>Stagonospora cf. paludosa</i>	CBS 130005	-	KF251757
<i>Stagonospora paludosa</i>	CBS 135088	-	KF251760
<i>Stagonospora perfecta</i>	CBS 135099	-	KF251761
<i>Stagonospora pseudocaricis</i>	CBS 135132	-	KF251762
<i>Stagonospora pseudovitensis</i>	CBS 135094	-	KF251764
<i>Stagonospora uniseptata</i>	CBS 135090	-	KF251767

¹BCC: BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand; CABI: Commonwealth Agricultural Bureaux International, UK; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Center for Plant Conservation, US; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; GKM: G.K. Mugambi; JCM: Japanese Collection of Microorganisms, RIKEN Institute of Physical & Chemical Research, Japan; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MFU: Mae Fah Luang University Herbarium, Chiang Rai, Thailand; NBRC: NITE Biological Resource Center Culture Collection; SMH: S.M. Huhndorf.

²SSU: Small (18S) Sub Unit rRNA gene; LSU: Large (28S) Sub Unit rRNA gene. Taxonomic novelties are shown in bold face.

Maximum-parsimony (MP) analysis was performed using PAUP v. 4.0b10 (Swofford 2002) to obtain the most parsimonious tree. Ambiguously aligned regions were excluded and gaps were treated as missing data. Trees were inferred using the heuristic search option with Tree Bisection Reconnection (TBR) branch swapping and 1000 random sequence additions. Maxtrees were set up to 5000 and branches of zero length were collapsed and all multiple parsimonious trees were saved. Descriptive tree statistics for parsimony such as Tree Length (TL), Consistency Index (CI), Retention Index (RI), Relative Consistency Index (RC) and Homoplasy Index (HI) were calculated under different optimality criteria. Clade stability was evaluated by 1000 bootstrap replications resulting from maximum parsimony analysis (Hillis & Bull 1993). The Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed to determine whether the trees inferred under different optimality criteria, were significantly different.

The best model of evolution for each gene region was determined by using MrModeltest 2.2 (Nylander 2004). A maximum likelihood analysis was performed in raxmlGUIv.0.9b2 (Silvestro & Michalak 2010). The search strategy was set to rapid bootstrapping with one thousand non parametric bootstrapping iterations using the general time

reversible model (GTR) with a discrete gamma distribution. The best scoring tree was selected with a final likelihood value of -9735.27 . The resulting replicates were plotted on to the best scoring tree obtained previously.

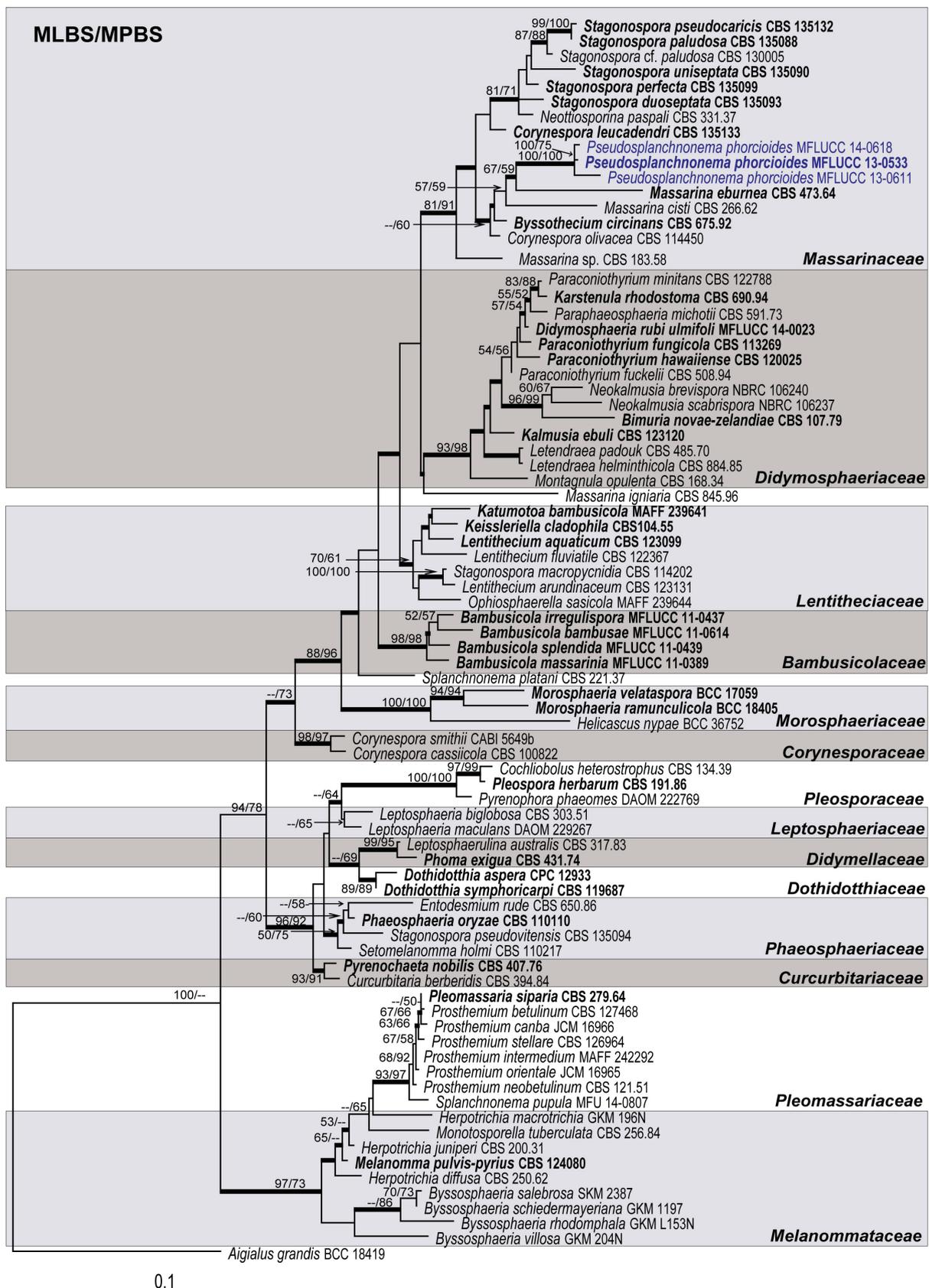


FIGURE 1. Phylogram generated from Maximum Likelihood analysis based on combined SSU and LSU gene dataset. Bootstrap support values for maximum parsimony (MP) and maximum likelihood (ML) greater than 50 % are indicated near the nodes and branches with Bayesian posterior probabilities above 0.90 are indicated in bold. The tree is rooted with *Aigialus grandis* (BCC 18419). The ex-types strains are in bold and the species introduced in the study are indicated in blue.

Posterior probabilities (PP) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). Kimura 2-parameter model coupled with discrete gamma distribution and a proportion of invariant site (K80+I+G) was applied for SSU gene region and symmetrical model with discrete gamma distribution coupled with a proportion of invariant sites (SYM+I+G) was applied for LSU gene region. Four simultaneous Markov chains were run for 1000000 generations and trees were sampled every 100th generation and 10000 trees were obtained. The first 2000 trees, representing the burn-in phase of the analyses, were discarded while remaining 8000 trees were used for calculating posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01) (Crous *et al.* 2006). Phylogenetic trees were drawn using Treeview v. 1.6.6 (Page 1996). The sequences of novel species in this study are deposited in GenBank.

Results and Discussion

Phylogenetic Analyses

The combined dataset of SSU and LSU alignment composed of 81 strains representing 81 taxa with *Aigialus grandis* (BCC 18419) as the outgroup taxon. Individual trees resulted from the maximum parsimony analysis, maximum likelihood and Bayesian analyses were similar in topology and not significantly different from the combined tree, and comprised 1875 total characters (1017 characters in the SSU region and 858 in the LSU region) including gaps. Among them, 1446 were constant, 150 were variable and parsimony uninformative and the remaining 279 were parsimony informative. Kishino-Hasegawa (KH) test showed length= 1275 steps, CI= 0.435, RI= 0.772, RC= 0.336 and HI= 0.565. Maximum Likelihood analysis used 1000 bootstrap replicates and yielded a tree with the following model parameters: alpha= 0.1651, $\Pi(A)$ = 0.257288, $\Pi(C)$ = 0.208787, $\Pi(G)$ = 0.285140 and $\Pi(T)$ = 0.248785. The first of 1,000 equally most parsimonious trees is shown (Fig. 1).

The 81 taxa analyzed in the phylograms formed 14 familial clades, i.e. Bambusicolaceae, Corynesporaceae, Curcurbitariaceae, Didymosphaeriaceae, Didymellaceae, Dothidotthiaceae, Lentitheciaceae, Leptosphaeriaceae, Massarinaceae, Melanommataceae, Morosphaeriaceae, Pleomassariaceae, Pleosporaceae and Phaeosphaeriaceae. The genus *Massarina* together with *Byssothecium circinans*, *Corynespora olivacea*, *C. leucadendri*, *Neottiosporina paspali* and *Stagonospora sensu stricto* clustered in Massarinaceae and formed a distinct clade with strong support, similar to previous studies (Liew *et al.* 2002, Kodseub *et al.* 2007). The phylogenetic analyses provides good evidence that the clade comprising of splanchnonema-like species herein, described as a new genus, *Pseudosplanchnonema*, belongs in Massarinaceae, where it forms a distinct clade separated from other genera of the family with a relatively high bootstrap value (100%) and high Bayesian posterior probability (1.00) (Fig. 1). *Pseudosplanchnonema phorcioides* separated from *Massarina eburnea* (CBS 473.64), the type species of the genus *Massarina* with a ML bootstrap value (67%) and a significant support of 0.90 Bayesian posterior probability. *Pseudosplanchnonema phorcioides* also forms a sister clade with *M. cisti* (CBS 266.62) with MP and ML bootstrap supports of 57% and 59% respectively and Bayesian posterior probability of 0.66. In accordance with this result, LSU (27.37 %) and SSU (24.90 %) gene regions also shows very low similarity between *P. phorcioides* and *M. cisti*. LSU region showed 36 base differences with 5 indels and SSU region showed 13 base pair differences with 4 indels. *Pseudosplanchnonema phorcioides* differs morphologically from other *Massarina* species such as *M. beaurivagea*. Morphology coupled with molecular data suggests it is better to accommodate these species in a new genus.

Taxonomy

The genus *Pseudosplanchnonema*, typified by *P. phorcioides*, is introduced in the family Massarinaceae. It is well-differentiated from the other genera in the family based on morphology and molecular phylogeny.

Pseudosplanchnonema Chethana & K.D. Hyde, *gen. nov.*

Index Fungorum number: IF551021, Facesoffunginumber: FoF 00568.

Etymology:—The specific generic epithet *Pseudosplanchnonema* is given based on its morphological resemblance to the genus *Splanchnonema*.

Type species:—*Pseudosplanchnonema phorcioides* (I. Miyake) Chethana, Camporesi & K.D. Hyde.

Saprobic or parasitic on dead branches or wood. **Sexual morph:** *Pseudostromata* with immersed, perithecial ascomata solitary, scattered, gregarious, subglobose to globose, verruculose, dark brown to black, ostiolate. *Ostirole* short, papillate, opening to exterior through bark. *Peridium* comprising several-layers, outer layer composed of dark brown to reddish brown, heavily pigmented, thick-walled cells of *textura angularis*, inner layer comprising hyaline to pale brown, thin-walled cells. *Hamathecium* comprising filiform, broad, pseudoparaphyses, anastomosing above the asci, embedded in a gelatinous matrix. *Asci* 8-spored, bitunicate, fissitunicate, cylindrical to clavate, with a short pedicel and an ocular chamber best seen in immature asci. *Ascospores* overlapping, uni to biseriate, fusiform to ellipsoidal, widest near the centre, with acute rounded ends, sometimes slightly curved, hyaline to pale brown when young, dark brown at maturity, 1-septate, constricted at the septum, sometimes with pseudosepta, smooth-walled, surrounded by a mucilaginous sheath. **Asexual morph:** Coelomycetous, phoma-like. *Conidiomata* pycnidial, solitary or aggregated, slightly erumpent, oval to globose, with a verruculose wall, initially brown and becoming black at maturity. *Pycnidial wall* comprising multi-layered, brown, outer cells of *textura angularis* and thin, hyaline, inner cells. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* enteroblastic, phialidic, hyaline, smooth-walled, formed from the inner layer of the pycnidial wall. *Conidia* subglobose to ellipsoidal, hyaline, aseptate, straight to curved, smooth and thin-walled, with rounded ends.

Pseudosplanchnonema phorcioides (I. Miyake) Chethana, Camporesi & K.D. Hyde, *comb. nov.*

Basionym: *Massaria phorcioides* (I. Miyake), Techn. Rep. Imper. Sericult. Exp. Stat. Tokyo 1:316 (1916).

≡ *Splanchnonema phorcioides* (I. Miyake) P. Leroy, L. Gauthier & M.E. Barr, Bull. Soc. mycol. Fr. 116(3): 209 (2001).

Index Fungorum number: IF550800, *Facesoffunginumber:* FoF: 00199; Figs. 2, 3.

Saprobic on dead branch of *Morus* sp. **Sexual morph:** *Pseudostromata* with immersed, perithecial ascomata, 159–483 µm diam. (\bar{x} =307.53 µm, n=20), solitary, gregarious, globose, black, short ostiolate. *Ostirole* 60–90 µm high, 80–120 µm diam. (\bar{x} =78.6 × 108.6 µm, n=10), short, papillate, opening to exterior through bark. *Peridium* 30.5–82 µm (\bar{x} =47.14 µm) wide at side walls, up to 18.9 µm wide near the apex and 60.48 µm wide at the base, comprising 6 to 7 layers of cells, outer 3–4 layers composed of dark brown, thick-walled cells of *textura angularis*, inner 2–3 layers comprising hyaline to pale brown, thin-walled cells. *Hamathecium* comprising broad, filiform 2–4 (\bar{x} =3.4) µm wide, septate pseudoparaphyses, embedded in a gelatinous matrix. *Asci* 161–286 × 35–51 µm (\bar{x} =193.2×41.9 µm, n=10), 8-spored, bitunicate, fissitunicate, cylindrical to clavate, with a short pedicel and an ocular chamber best seen in immature asci. *Ascospores* 50–66 × 14–20 µm (\bar{x} =56.6 × 17.1 µm, n=15), overlapping biseriate, hyaline to pale brown when young, dark brown at maturity, fusiform to ellipsoidal, widest near the centre, with acute rounded ends, sometimes slightly curved, 1-sub-median septate, constricted at the septum, 5-guttulate, sometimes with pseudosepta between the guttules, smooth-walled, surrounded by a mucilaginous sheath. **Asexual morph:** Coelomycetous, phoma-like. *Conidiomata* 382–805 µm high, 270–480 µm diam. (\bar{x} =556.8 × 425.8 µm, n=10), pycnidial, solitary or aggregated, slightly erumpent, oval to globose, with verruculose wall, initially brown and becoming black at maturity. *Pycnidial wall* comprising multi-layered, brown, outer cells of *textura angularis* and thin, hyaline, inner cells. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* 3.2–6.9 µm × 1.5–2.5 µm (\bar{x} =4.5 × 1.7 µm, n=10), enteroblastic, hyaline, smooth-walled, formed from inner layer of pycnidial wall. *Conidia* 3.4–8.6 µm × 1.2–3.5 µm (\bar{x} =5.9 × 2.1 µm, n=40), subglobose to oblong, hyaline, aseptate, straight or occasionally slightly curved, 0–2-guttulate, smooth-walled, with rounded ends.

Cultural Characteristics:—Ascospores germinating on water agar within 24 h and the germ tubes of 3–4 µm diam. produced near the septum. Colonies slow growing on PDA, attaining 6 mm diam. after 14 days at 28 °C, edge entire, greenish black in the centre, greenish grey towards rim, white at the margin with a circular cottony mycelium on the surface and reverse black in the centre and grey towards the ends of the mycelium. *Conidia* in mass, white.

Material examined:—ITALY. Province of Forli-Cesena: Modigliana, Montebello (Ibola Valley), on dead branches of *Morus* sp. (Moraceae), 13 May 2013, E. Camporesi IT-1220 tris (MFLU 14-0929, **holotype**), (**isotype** in BBH, under the code of BBH 39847); ex-type living culture, MFLUCC 13-0533, CGMCC 3.17583; ITALY. Province of Forli-Cesena: Santa Sofia, Camposonardo, dead and hanging branches of *Acer campestre* L. (Aceraceae), 3 May 2013, E. Camporesi IT-1254 (MFLU 14-0752), living culture, MFLUCC 13-0611; RUSSIA. Rostov region: Shakhty city, Central Park, on dead branch of *Morus alba* L. (Moraceae), 5 May 2014, Timur Bulgakov (MFLU 15-0004), living culture, MFLUCC 14-0618.

Notes:—Morphologically *Pseudosplanchnonema* shows a resemblance to genus *Splanchnonema*, as typified by *Splanchnonema pustulatum*. *Splanchnonema pustulatum* differs from *Pseudosplanchnonema phorcioides* in

possessing larger, depressed, subglobose ascomata and clavate, reddish brown, 2-septate ascospores (Zhang *et al.* 2012, Liu *et al.* 2015). Our new collection of *Pseudosplanchnonema phorcioides* is identical to *Splanchnonema phorcioides*. Both collections have immersed, ostiolate ascomata, 8-spored, clavate, biseriata asci and fusiform, dark brown, ascospores surrounded by a gelatinous sheath (Tanaka *et al.* 2005). Hence we transfer this species to *Pseudosplanchnonema* as a new combination. According to the molecular data, *P. phorcioides* clusters in a well-separated clade in Massarinaceae. Leroy *et al.* (2000) has described *Splanchnonema phorcioides* as parasitic. Hence we have described the *Pseudosplanchnonema phorcioides* as also parasitic. *Pseudosplanchnonema* differs from *M. eburnea* in having smaller ascomata lacking a clypeus, a thicker peridium composed of two layers the inner of hyaline and outer of dark brown cells, anastomosing pseudoparaphyses, and larger, dark brown, 1-septate, guttulate ascospores which sometimes have pseudosepta.

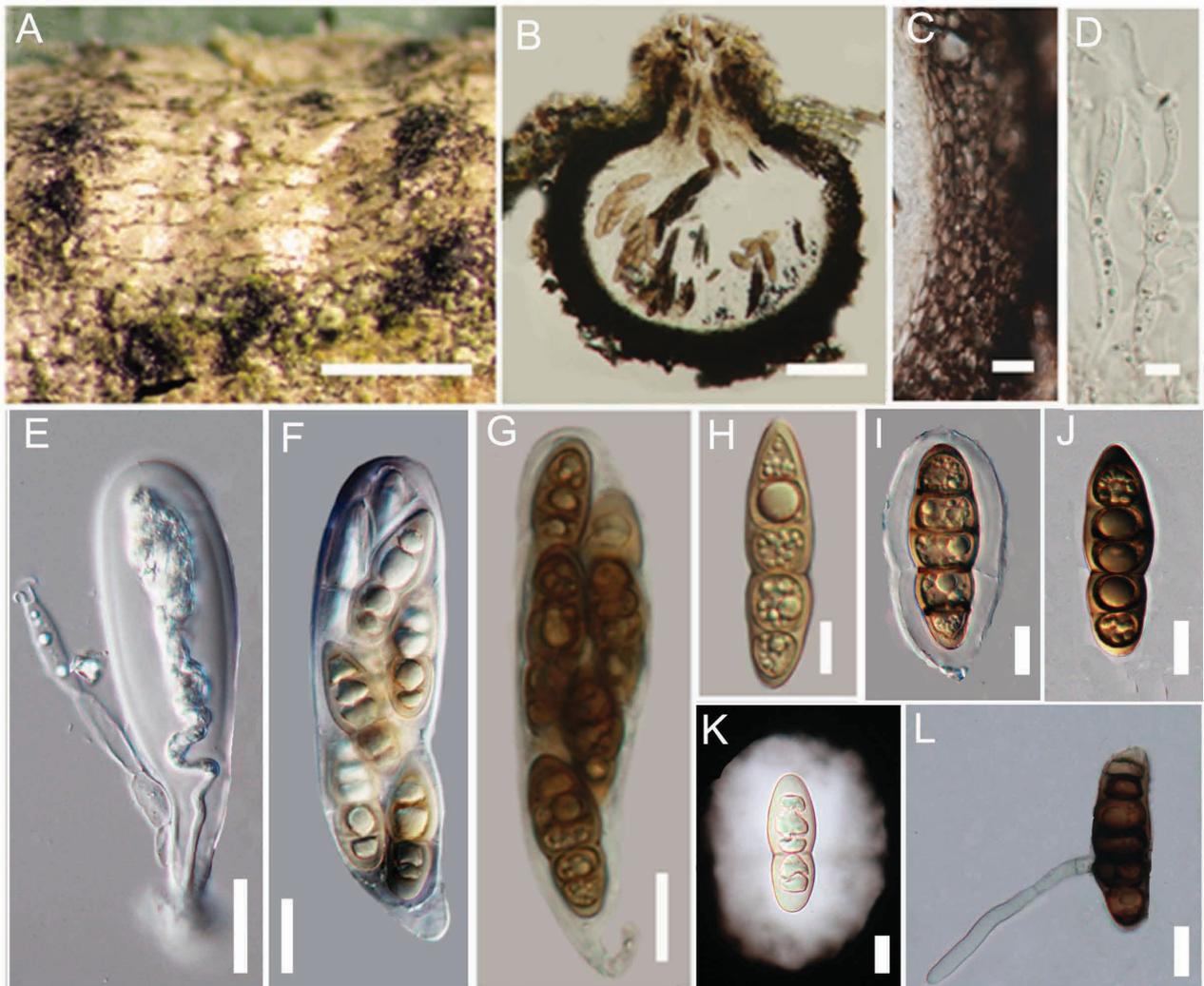


FIGURE 2. *Pseudosplanchnonema phorcioides* (MFLUCC 13-0533). **A.** Pseudostroma immersed in the host tissue, **B.** Section of the ascoma (TS), **C.** Section of the peridium, **D.** Hyaline, pseudoparaphyses, **E.** Immature ascus, **F–G.** Mature asci, **H–J.** Brown ascospores, **K.** Ascospore with a gelatinous sheath stained in Indian ink, **L.** Germinating ascospore. Scale bars: **A=** 1 mm, **B=** 500 μ m, **C=** 20 μ m, **D=** 10 μ m, **E–G=** 20 μ m, **H=** 20 μ m, **I–L=** 10 μ m.

Several asexual genera have been shown to be associated with Massarinaceae by molecular phylogenetic studies (Kodsueb *et al.* 2007, Shearer *et al.* 2009, Suestrong *et al.* 2009, Zhang *et al.* 2012). *Byssothecium*, represented by *B. circinans* differs from *Pseudosplanchnonema* in having minute, thin-walled conidiomata and subglobose, broadly papillate pseudothecia with smaller asci and ascospores in its sexual morph (Boise 1983). *Aquaticheiospora* represented by *A. lignicola* differs from *Pseudosplanchnonema* in possessing synnematosus conidiomata, larger, hyaline to pale brown, cheiroid conidia (Kodsueb *et al.* 2007). *Cheiosporium* represented by *C. triseriale* differs by having sporodochial conidiomata, macronematous, septate conidiophores, monoblastic, doliiform or broad-cylindrical conidiogenous cells, larger, olivaceous to brown, 0–1-septate, cheiroid conidia truncated at the base (Cai *et al.* 2008,

Kodsueb *et al.* 2007). *Corynespora* represented by *C. olivacea*, *Helminthosporium* represented by *H. chlorophorae*, *H. solani*, *H. velutinum* and *Periconia* represented by *P. igniaria* differed from the asexual morph of *Pseudosplanchnonema* by possessing macronematous, mononematous conidiophores, *Corynespora* and *Helminthosporium* differed by possessing brown, elongate, multi-cellular, tetric conidia, whereas *Periconia* differed by globose to cylindrical conidia (Ellis 1971, Hyde *et al.* 2013). *Neottiosporina* represented by *N. paspali* and *Saccharicola* represented by *S. bicolor* differed from *Pseudosplanchnonema* in having smaller conidiomata and transversely septate conidia (Sutton 1980).

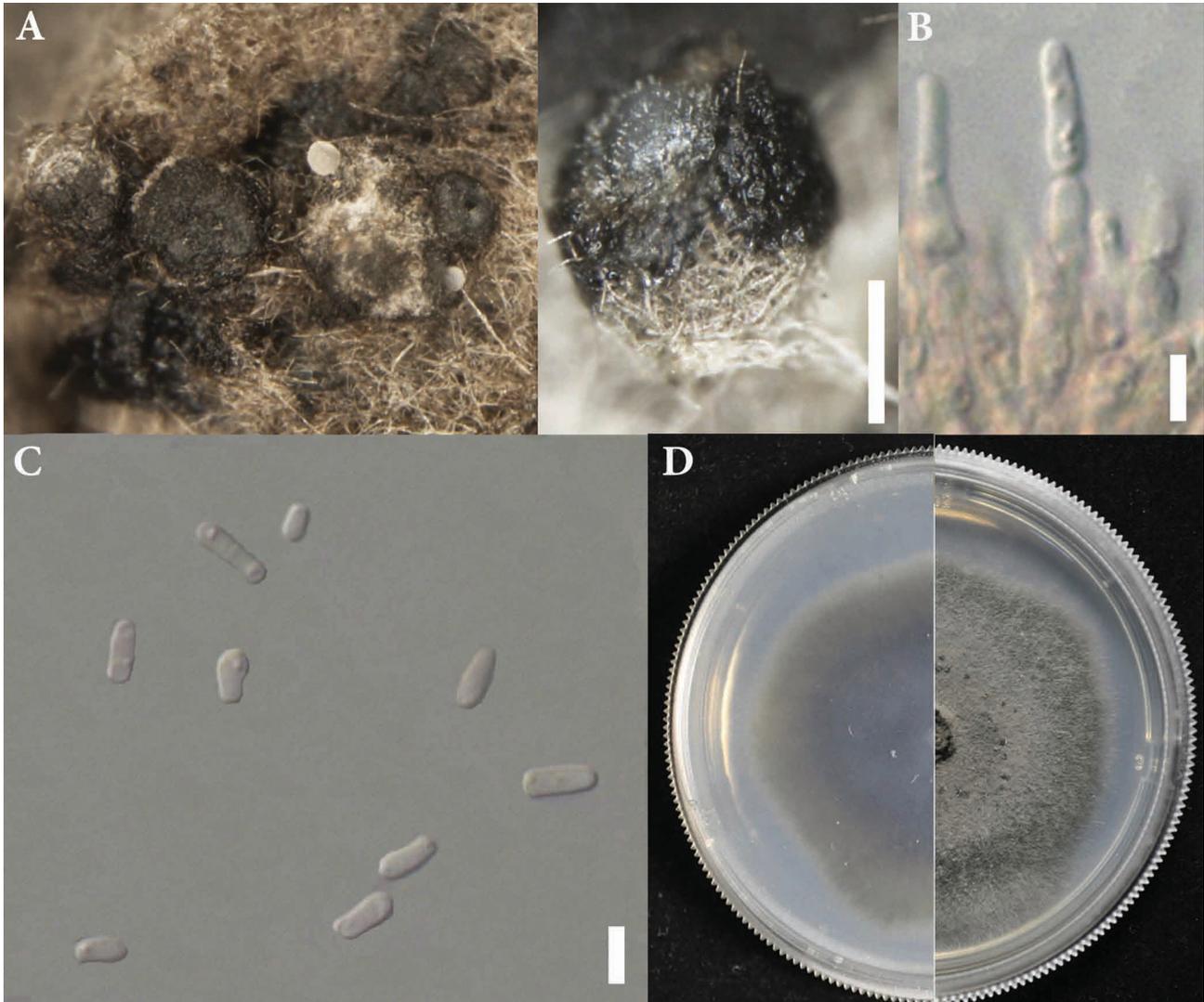


FIGURE 3. Asexual morph of *Pseudosplanchnonema phorcioides* (MFLUCC 13-0533). **A.** Conidiomata on the PDA, **B.** Conidiogenous cells, **C.** Conidia, **D.** Upper-view (right) and the reverse view (left) of the colony on PDA. Scale bars: **A**= 200 μm , **B**= 3 μm , **C**= 5 μm .

Many studies have been conducted on splanchnonema-like species isolated from the *Morus* species, with distinct phylogeny and morphological characters similar to *Pseudosplanchnonema* (Tanaka & Harada 2004, Tanaka *et al.* 2005, Liu *et al.* 2015). Our study shows the importance of studying these splanchnonema-like taxa since they are scattered across Pleosporales with distinct phylogenetic lineages. Therefore, further studies with molecular data are essential to interpret correct generic concepts for splanchnonema-like taxa.

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