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The genus *Pseudodidymosphaeria*

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Abstract

The genus *Pseudodidymosphaeria* is revisited with an overview of its history, a generic description with amendments and notes and illustrations of the genus. Molecular data from two species of the genus are analyzed using single and combined ITS and LSU gene datasets and the workflow of phylogenetic analysis is provided in an appendix. The genus *Pseudodidymosphaeria* formed a well-supported clade in the family Massarinaceae.

Key words – ARB – ITS – LSU – Massarinaceae – Poaceae

Introduction

The family Massarinaceae was established to accommodate four genera, *Keissleriella*, *Massarina*, *Metasphaeria*, *Pseudotrichia*, and *Trichometasphaeria* (Munk 1956). Barr (1987) treated Massarinaceae as a synonym of Lophiostomataceae. However, these two families are now recognized as separate lineages in Pleosporales, Dothideomycetes (Zhang et al. 2012, Hyde et al. 2013, Thambugala et al. 2015b, Wijayawardene et al. 2018). During the past 60 years many genera were included or removed from Massarinaceae depending on the various taxonomic treatments and accessibility of sequence data (Zhang et al. 2012, Hyde et al. 2013, Wijayawardene et al. 2014, 2017, Tanaka et al. 2015, Thambugala et al. 2015a). Wijayawardene et al. (2017) accepted nine genera in Massarinaceae including *Bambusistroma* D.Q. Dai & K.D. Hyde, *Helminthosporium* Link, *Longiostiolum* Doilom et al., *Massarina* Sacc., *Pseudodidymosphaeria* Thambug. & K.D. Hyde, *Pseudosplanchnonema* Chethana & K.D. Hyde, *Semifissispora* H.J. Swart, *Stagonospora* (Sacc.) Sacc. and *Suttonomyces* Wijayaw. et al. However, the phylogenetic placements of *Bambusistroma* and *Longiostiolum* in the order Pleosporales are doubtful.

Thambugala et al. (2015a) established *Pseudodidymosphaeria* Thambugala & K.D. Hyde in Massarinaceae, with *P. spartii* (Fabre) Thambugala et al., which occurred on *Spartium junceum* L. (Fabaceae), as the type species. Subsequently, Li et al. (2016) introduced a second species, *P. phlei* Phukhamsakda, Camporesi & K.D. Hyde, found on a dead stem of *Phleum pretense* L. (Poaceae). *Pseudodidymosphaeria phlei* has also been reported on *Arundo donax* L. (Poaceae) (Tibpromma et al. 2017). *Pseudodidymosphaeria* species have only been recorded from three different hosts in Italy.

Materials and Methods

Morphological study

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A reference specimen of *Pseudodidymosphaeria spartii* was obtained from the Herbarium of Mae Fah Luang University (MFLU), Thailand. Morphological observations and photomicrographs were made following the method of Thambugala et al. (2015b).

Phylogenetic analyses

All sequences used in this study were downloaded from GenBank (Table 1), following previous publications (Tanaka et al. 2015, Thambugala et al. 2015a, Li et al. 2016, Voglmayr & Jaklitsch 2017). The sequence data were imported into the ARB v. 6.0.6 software package and aligned using MAFFT v.7.055b (using the E-INS-i alignment strategy, Katoh & Standley 2013) as implemented in ARB. The alignments were checked visually and improved manually where necessary using ARB_EDIT4 (Ludwig et al. 2004, Westram et al. 2011). Maximum likelihood analyses for single and combined gene alignments of ITS and LSU sequences were performed using RAxML (v.7.7.2, Stamatakis 2006) calculating 1,000 bootstrap replicates and applying the GTRGAMMAI model of nucleotide substitution (ARB workflow and single gene trees are provided in Appendices A and B). *Periconia digitata* was selected as the outgroup taxon in each analysis. The most likely trees were viewed with Xfig v.3.2 patchlevel 5c (Protocol 3.2), and finalized using Adobe Illustrator CS3.

Table 1 Culture collection and GenBank accession numbers of sequences used in the phylogenetic analyses.

Name	Culture Collection no. *	LSU	ITS
Helminthosporium solani	CBS H-13302	KY984341	KY984341
Helminthosporium tiliae	CBS 136907	KY984345	KY984345
Helminthosporium velutinum	CBS 139923	KY984352	KY984352
Helminthosporium velutinum	WU 38886	KY984359	KY984359
Massarina eburnea	CBS 473.64	GU301840	AF383959
Massarina eburnea	CBS 139697	AB521735	LC014569
Periconia digitata	CBS 510.77	AB807561	LC014584
Pseudodidymosphaeria phlei	MFLU 15–1360	KY264748	KY264744
Pseudodidymosphaeria phlei	MFLUCC 14–1061	KU754541	KU764780
Pseudodidymosphaeria spartii	MFLUCC 13–0273	KP325436	KP325434
Pseudodidymosphaeria spartii	MFLUCC 14–1212	KP325437	KP325435
Pseudosplanchnonema phorcioides	MFLUCC 14–0618	KP683373	KP683372
Pseudosplanchnonema phorcioides	MFLUCC 13–0611	KP683376	KP683375
Stagonospora cf. paludosa	CBS 130005	KF251757	KF251254
Stagonospora paludosa	CBS 135088	KF251760	F251257
Stagonospora pseudocaricis	CBS 135132	KF251762	KF251259

*CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. WU: Fungarium of the Department of Botany and Biodiversity Research, University of Vienna

Results and Discussion

Phylogeny of single and combined ITS and LSU gene datasets

The concatenated and single ITS and LSU datasets comprised 15 taxa in Massarinaceae, representing five genera, with *Periconia digitata* as the outgroup taxon. The resulting single gene trees show almost identical underlying phylogenies, just differing in unsupported internal nodes. Therefore, only the combined gene tree is discussed here (Fig. 1). Analysis of the combined ITS and LSU dataset for Massarinaceae resulted in five clades corresponding to five genera. *Pseudodidymosphaeria* forms a well-defined and well-supported monophyletic genus in the family

Massarinaceae in the single and concatenated gene trees (Fig. 1). Both *Pseudodidymosphaeria* species, *P. phlei* and *P. spartii*, received high bootstrap support values.

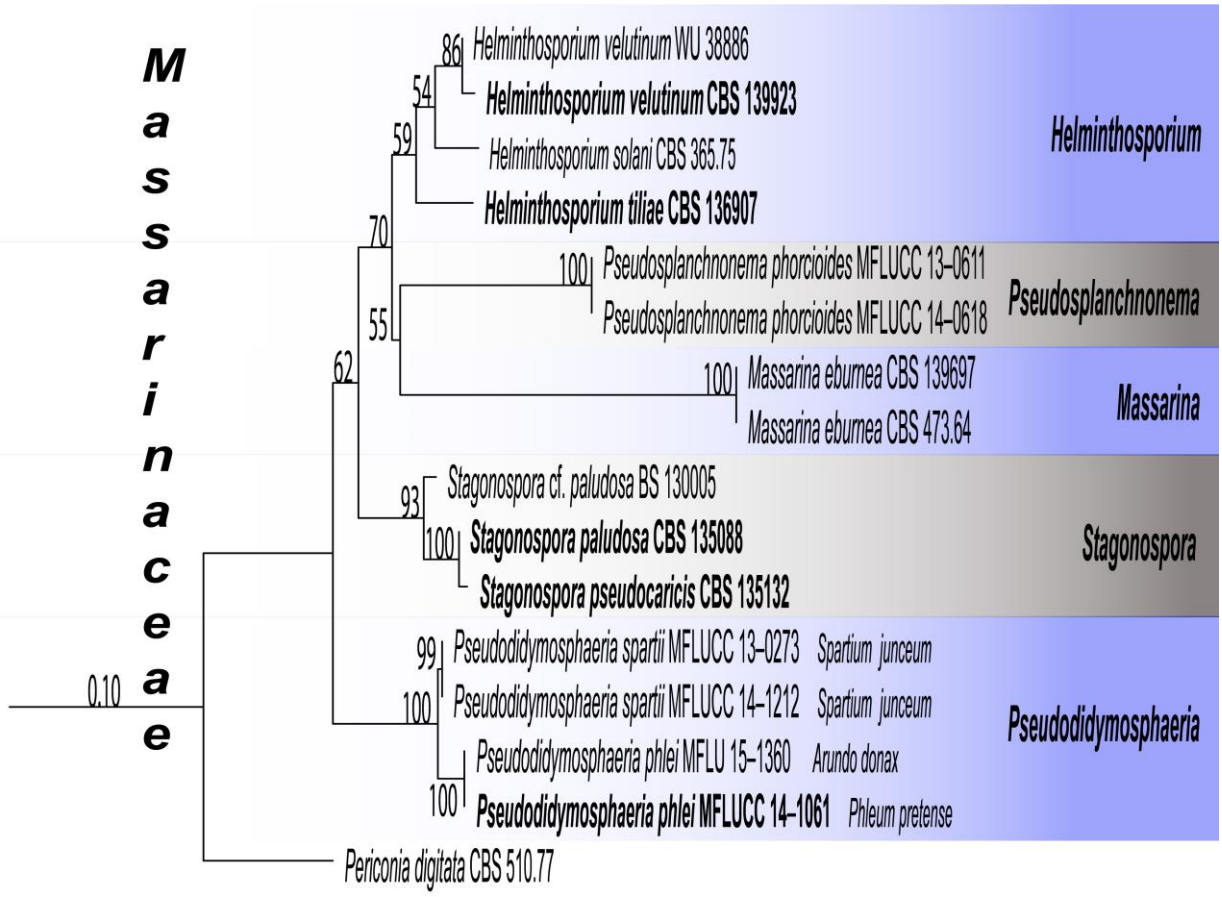

Fig. 1 – Phylogenetic relationships among Massariaceae. Maximum likelihood RAxML analysis of combined LSU and ITS sequence data of species of Massarinaceae. Bootstrap support values exceeding 50% are given above the nodes. Culture accession numbers are mentioned along with the species name, while hosts for *Pseudodidymosphaeria* species are given after the culture accession numbers. The tree is rooted to *Periconia digitata* and type strains are highlighted in boldface.

Taxonomy

Massarinaceae Munk, Friesia 5: 305 (1956)

The family is characterized by immersed or superficial, globose, conical globose to lenticular, ostiolate ascomata, bitunicate, clavate to cylindrical asci and ellipsoid to fusoid, hyaline to pigmented, 1–3-septate ascospores and coelomycetous or hyphomycetous asexual morphs (Hyde et al. 2013, Chethana et al. 2015, Tanaka et al. 2015, Thambugala et al. 2015a, Voglmayr & Jaklitsch 2017).

Pseudodidymosphaeria Thambug. & K.D. Hyde, Phytotaxa 231 (3): 273 (2015)

Saprobic on *Spartium junceum* L. and grasses (Poaceae) in terrestrial habitats. Sexual morph: *Ascomata* scattered, or in small groups, immersed or semi-immersed to erumpent, globose to subglobose, with or lacking ostioles. *Peridium* 1–2-layered, composed of hyaline to brown compressed cells of *textura angularis and textura prismatica*, cells towards the inside lighter and somewhat flattened, at the outside, darker. *Hamathecium* of dense, long, branched, septate, cellular pseudoparaphyses. *Asci* 8-spored, bitunicate, fissitunicate, cylindro-clavate, pedicellate, rounded at the apex, with an ocular chamber. *Ascospores* uniseriate to obliquely uniseriate or bi-seriate, ellipsoid with broadly obtuse ends, brown to reddish brown, 1-septate, verrucose, surrounded by a

mucilaginous sheath. Asexual morph: *Conidiomata* solitary or in groups, scattered, globose to subglobose, dark brown to black, pulvinate, unilocular. *Conidiomatal wall* comprising several cell layers; outer layers composed of brown to lightly pigmented cells of *textura angularis* to *textura globosa*, becoming thin-walled and hyaline towards the inner region. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* formed from the cells lining the inner walls of the conidiomata, phialidic, fusiform to cylindrical, determinate, hyaline. *Conidia* solitary, ovoid, straight, oval to ellipsoidal, producing conidia at their tips, smooth, hyaline, aseptate.

Type species – *Pseudodidymosphaeria spartii* Thambugala, E. Camporesi & K.D. Hyde, Fig. 2

Notes – The unique combinations of morphological features of *Pseudodidymosphaeria* are different from the other recognized genera in Massarinaceae (Chethana et al. 2015, Tanaka et al. 2015, Thambugala et al. 2015a, Li et al. 2016, Voglmayr & Jaklitsch 2017). *Pseudodidymosphaeria phlei* is distinct from the type species *P. spartii* in having semi-immersed to erumpent ascomata, larger peridium cell walls, with 2–3 wall layers, and smaller ascospores, with less distinct, rounded ends (Li et al. 2016). However, these species morphologically resemble *Didymosphaeria* species and epitypification and molecular analyses of *Didymosphaeria* species will certainly reveal additional taxa belonging to *Pseudodidymosphaeria*.

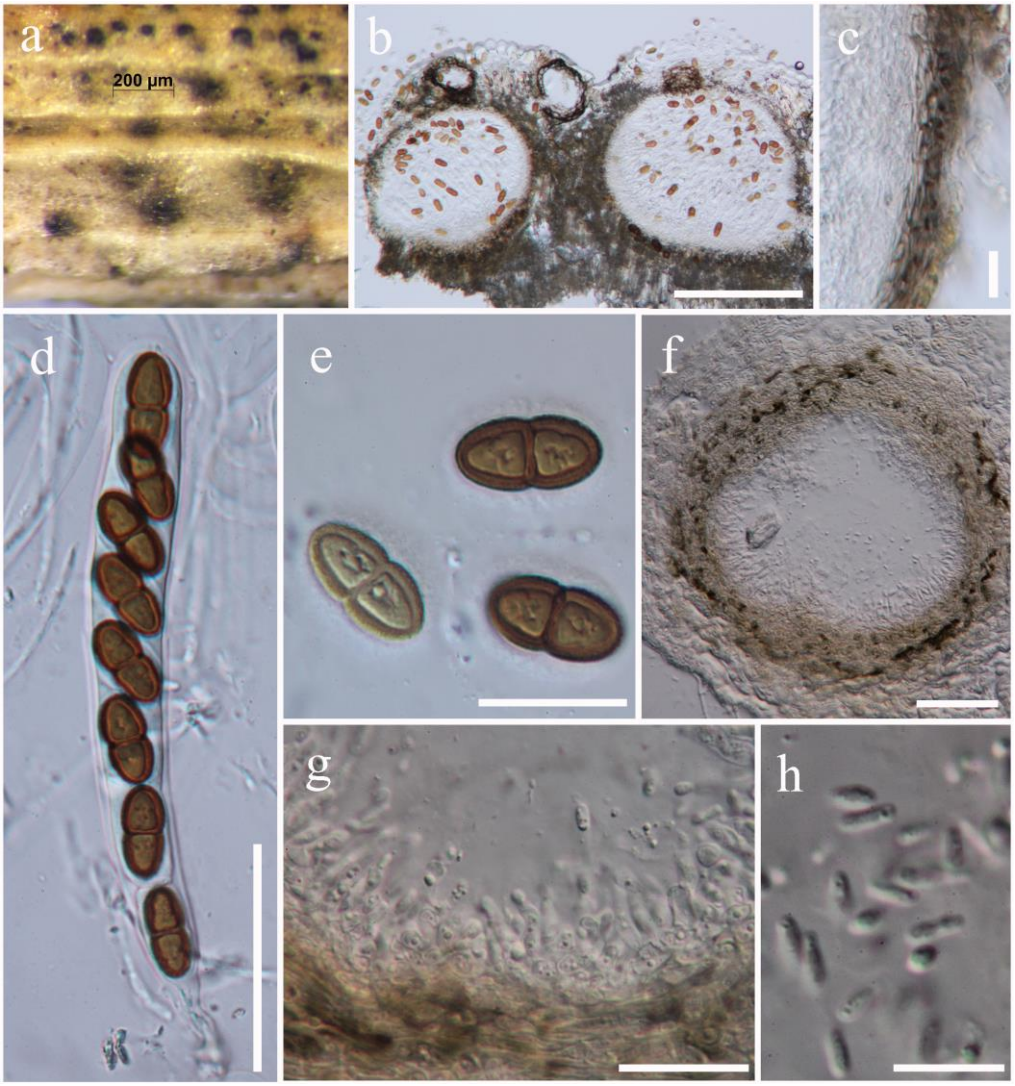

Fig. 2 – *Pseudodidymosphaeria spartii* (MFLU 14–0578) a. Appearance of ascomata on host tissue. b. Vertical sections through ascomata. c. Peridium. d. Ascus. e. Ascospores. f. Conidiomata. g. Conidiogenous cells and developing conidia. h. Conidia. Scale bars: b = 200 µm, c = 15 µm, d, f = 50 µm, e, g = 20 µm, h = 10 µm.

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Appendix A

Multi-locus phylogenetic analyses of genus *Pseudodidymosphaeria* in Massarinaceae using ARB

1) ARB (http://www.arb-home.de/) was installed on a QIIME 2 Core VirtualBox Image (v 2017.12, https://qiime2.org/), on which libxm4 and Xfig had been installed previously.
2) A new ARB database was created using sixteen LSU sequences (Table 1) downloaded from GenBank (https://www.ncbi.nlm.nih.gov) in GenBank format.
    a. Sequences were imported into the alignment "ali_LSU"
    b. the newly created import filter ("GB_MFU.ift", https://www.arb-silva.de/fileadmin/silva_databases/imp_exp_filters/GB_MFU.ift) was applied to import a maximum of sequence associated information
3) The sequence accession number was preserved.
    a. The accession was copied to new field called "Acc_LSU"
        i. Sequences with entries in the ali_LSU/data field were searched (Species > Search and query) and the accession numbers were copied using "More functions > Modify Fields of Listed Species" in the "SEARCH and QUERY" window.
4) Imported LSU sequences were aligned using MAFFT (Sequences > Align Sequences > Mafft).
5) A selected sequence was copied to a new 'species' called 'filter' and used as a filter sequence for phylogenetic analyses.
    a. Positions in the newly created filter sequence, which correspond to ambigiously aligned regions were replaced by Gap symbols ("-").
6) Successive import of sequences from other genes
    a. A new alignment was created (Sequence > Sequenmce/Alignment Admin) for ITS sequences (ali_ITS), Reference sequences were imported (File > Import > Import from external format) in GenBank format and using the filter "GB_silva_modified_Persoh_v3.ift".
    b. Sequence Accession numbers were copied to the corresponding field, i.e. 'Acc_ITS'.
    c. A filter sequence, always called 'filter', was created and modified appropriately.
7) Merging of sequences
    a. A new field ("individual") was created (Species > Database fields admin > create fields…)
    b. Strain or specimen Ids were copied (using "More functions > Modify Fields of Listed Species" in the "SEARCH and QUERY" window) to the field "individual" and curated.
    c. Expert mode was enabled (Properties > Toggle expert mode)
    d. Sequence of the same individual were merged (Species > Merge Species > Create merged species from similar species) using entries in the database field "individual" as identifier.
    e. Database entries with single sequences were deleted; i.e. species having no entry in the "merged_species" field were searched (Species > Search and query) and deleted (Delete Listed).
8) Calculating phylogenetic trees using RAxML.
    a. Only positions in which the filter sequence has no Gap ("-") were considered for phylogenetic reconstructions
    b. The resulting trees were renamed.
    c. To assure traceability of the analyses, the alignment (including the filter sequence) underlying the phylogenetic tree was copied to a new alignment, which was renamed including the name of the corresponding tree.

9) Calculating multi-gene phylogenetic tree
    a. Single gene alignments (including the filter sequences) were concatenated (Sequence > Concatenate Sequences/Alignments)
    b. Phylogenetic tree was calculated as detailed above based on the positions specified by the filter sequence.
    c. Tree was renamed and the underlying alignment copied to a correspondingly named alignment for documentation.

Appendix B

Single gene trees generated in this study

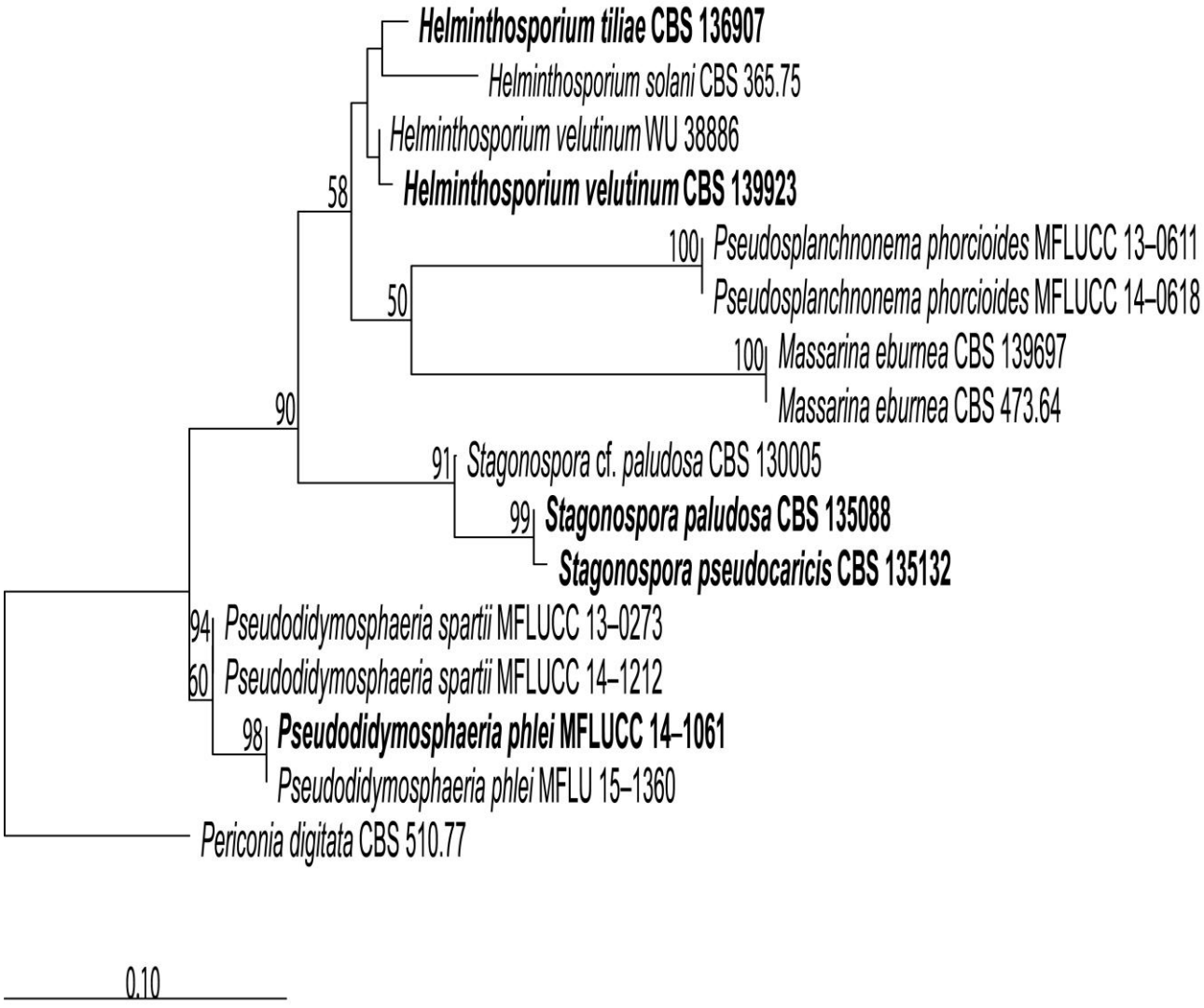

Fig. 3 – Maximum likelihood tree from analysis of LSU sequence data of species in Massarinaceae. Bootstrap support values greater than 50% are given above and below the nodes. Culture accession numbers are mentioned along with the species name. The tree is rooted to *Periconia digitata* and type strains are in black bold.

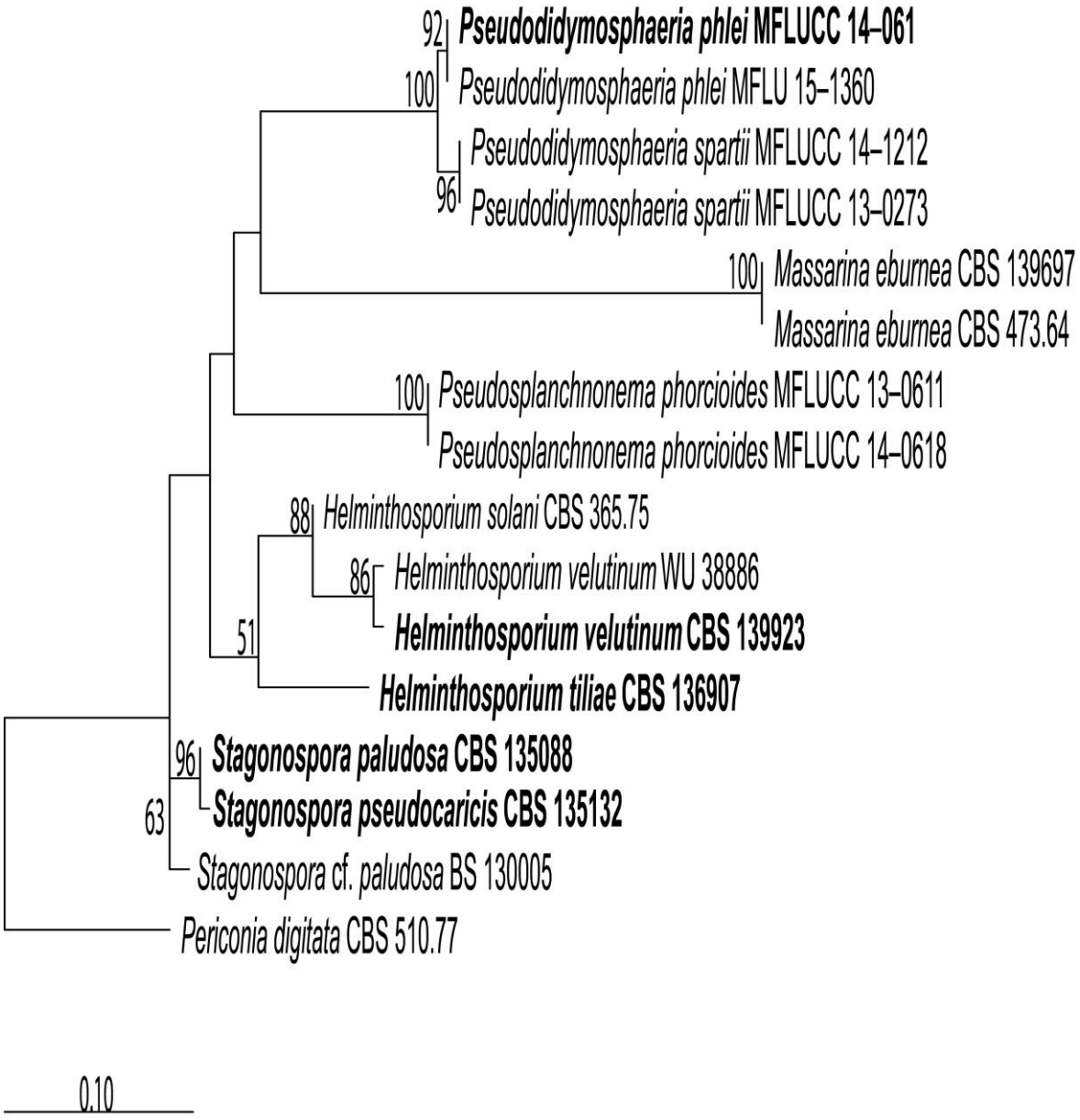

Fig. 4 – Maximum Likelihood tree from analysis of ITS sequence data of species in Massarinaceae. Bootstrap support values greater than 50% are given above and below the nodes. Culture accession numbers are mentioned along with the species name. The tree is rooted to *Periconia digitata* and type strains are in black bold.