The genus *Pseudodidymosphaeria*

**Thambugala KM**, **Peršoh D**, **Perera RH** and **Hyde KD**

1. Center of Excellence in Fungal Research, Mae Fah Luang University, Chiang Rai 57100, Thailand
2. Geobotany, Ruhr-Universität Bochum, Universitätsstraße 150, 44780 Bochum, Germany


**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**


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**
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.

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

*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.

![Phylogenetic relationships among Massariaceae](image)

**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).


*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.
Acknowledgments

We are grateful to the Directors and Curators of MFLU for making specimens available for examination. Kasun M. Thambugala thanks Milan C. Samarakoon for helpful comments and advice on the manuscript.

References

Barr ME. 1987 – Prodomus to class Loculoascomycetes. Publ. by the author, Amherst
Thambugala KM, Hyde KD, Tanaka K, Tian Q et al. 2015b – Towards a natural classification and backbone tree for Lophiostomataceae, Floricolaceae, and Amorosiaceae fam. nov. Fungal Diversity 74, 199–266.
Appendix A

Multi-locus phylogenetic analyses of genus *Pseudodidymosphaeria* in Massarinaeae 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 libxml4 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 ambiguously aligned regions were replaced by Gap symbols (“-“).

6) Successive import of sequences from other genes
   a. A new alignment was created (Sequence > Sequencmce/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

![Phylogenetic Tree]

**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.