Sulcispora supratumida sp. nov. (Phaeosphaeriaceae, Pleosporales) on Anthoxanthum odoratum from Italy

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Abstract

Sulcispora is typified by S. pleurospora. We collected a sulcispora-like taxon on leaves of Anthoxanthum odoratum L. in Italy and obtained single ascospore isolates. Combined ITS, LSU, SSU and tef1 sequence analyses suggested that Sulcispora is placed in the family Phaeosphaeriaceae and a newly collected Sulcispora species is introduced here as S. supratumida sp. nov. Detailed descriptions and illustrations are provided for Sulcispora supratumida and it is compared with the type species, S. pleurospora.

Keywords

Combined gene analysis, Dothideomycetes, graminicolous fungi, new species, spore septation
Introduction

Phaeosphaeriaceae is a highly diverse and large family in the order Pleosporales (Hyde et al. 2013) with more than 42 accepted genera (Hyde et al. 2017; Karunarathna et al. 2017; Wanasinghe et al. 2018). Members of Phaeosphaeriaceae are pathogens or hyper-parasites on living plants and humans and sapropes of decaying plant matter (Tennakoon et al. 2016; Ahmed et al. 2017).

*Sulcispora* was proposed by Shoemaker and Babcock (1989) as a monotypic genus to accommodate *Sulcispora pleurospora* (= *Phaeosphaeria pleurospora* Niessl). Some morphological characters of *Phaeosphaeria pleurospora* did not fit within species concepts of *Phaeosphaeria* and Shoemaker and Babcock (1989), therefore, introduced the genus *Sulcispora*. The genus name refers to the numerous furrows on the ascospore wall (Shoemaker and Babcock 1989). *Sulcispora pleurospora* has been reported on monocotyledonous hosts in genera such as *Anthoxanthum*, *Carex*, *Deschampsia*, *Sesleria* and *Tofieldia* (Leuchtmann 1984; Shoemaker and Babcock 1989).

In this study, we collected sulcispora-like species associated with leaf spots of *Anthoxanthum odoratum* in Italy. We compared the morphological characters of our collection with the isotype of *Sulcispora pleurospora*. Morphologically, our collection differs from the type species of *Sulcispora*, *S. pleurospora*. Therefore, we introduce our collection as a new species. Combined ITS, LSU, SSU and tef1 sequence analysis including taxa in Phaeosphaeriaceae indicates that the here-studied fungus grouped with “*Phaeosphaeria pleurospora*” (CBS 460.84) with high support value.

Methods

Sample collection, specimen examination and single spore isolation

Specimens were collected from *Anthoxanthum odoratum* L. from Italy in 2013. They were examined and photographed using a Carl Zeiss Discovery V8 stereo-microscope fitted with Axiocam. Sections of ascomata were taken by hand under a stereo-microscope. Sections and other micro-morphological characters were photographed using a Nikon Eclipse 80i compound microscope fitted with a Canon 450D digital camera. All microscopic measurements were made with Tarosoft image framework (v. 0.9.0.7). Colony characteristics were recorded from cultures grown on Malt Extract Agar (MEA).

Single spore isolation was carried out following the method described by Chomnunti et al. (2014). Germinated ascospores were aseptically transferred into fresh MEA plates and incubated at 20 °C to obtain pure cultures and later transferred to MEA slants and stored at 4 °C for further study. The holotype and paratype specimens were deposited at the Mae Fah Luang University (MFLU) fungaria and the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS), respectively. Living cultures were deposited at the Mae Fah Luang Culture Collection (MFLUCC).
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MycoBank (http://www.mycobank.org/) and Facesoffungi (Jayasiri et al. 2015) numbers were obtained for the new strain. The new species was established based on recommendations outlined by Jeewon and Hyde (2016).

**DNA extraction, PCR amplification and DNA sequencing**

Fresh fungal mycelium grown on MEA for four weeks at 20°C was used for DNA extraction (Jeewon et al. 2002). Genomic DNA extraction and PCR reactions were carried out using ITS4/ITS5 for internal transcribed spacer nrDNA (ITS), LR5/LROR for large subunit nrDNA (LSU), NS1/NS4 for large subunit nrDNA (SSU) and 983F/2218R for translation elongation factor 1 (tef1) genes according to the same protocol of Maharachchikumbura et al. (2012). The PCR products were observed on 1% agarose electrophoresis gel stained with ethidium bromide. Purification and sequencing of PCR products were carried out at the Kunming Institute of Botany, Chinese Academy of Science, Kunming, China. Sequence quality was checked and sequences were condensed with DNASTAR Lasergene v.7.1. Sequences derived in this study were deposited in GenBank (Table 1).

**Sequence alignment and phylogenetic analysis**

BLASTn searches were made using the newly generated sequences to assist in taxon sampling for phylogenetic analyses. In addition, representatives of the Phaeosphaeriaceae were selected following Tennakoon et al. (2016) and Wanasinghe et al. (2018) (Table 1). Combined multi-locus sequence data of ITS, LSU, SSU and tef1 regions were aligned using default settings of MAFFT v.7 (Katoh et al. 2017) and manually adjusted using BioEdit 7.1.3 (Hall 1999) to allow maximum alignment and minimum gaps. Maximum likelihood analysis was performed by RAxML (Stamatakis and Alachiotis 2010) implemented in raxmlGUIv.1.3 (Silvestro and Michalak 2012). The search strategy was set to rapid bootstrapping and the analysis carried out using the GTRGAMMAI model of nucleotide substitution with 1000 replicates. The model of evolution was estimated by using MrModeltest 2.2 (Nylander 2004).

For the Bayesian inference (BI) analyses of the individual loci and concatenated ITS, LSU, SSU and tef-1 alignment, the above mentioned model test was used to determine the best fitting nucleotide substitution model settings for MrBayes v. 3.0b4. A dirichlet state frequency was predicted for all three data partitions and GTR+I+G as the best model for all single gene and combined datasets. The heating parameter was set to 0.2 and trees were saved every 1000 generations (Ronquist and Huelsenbeck 2003). The Markov Chain Monte Carlo (MCMC) analysis of four chains started in parallel from a random tree topology. The Bayesian analysis lasted 10,000,000 generations (average standard deviation of split frequencies value = 0.0098) and the consensus trees and posterior probabilities were calculated from the 9,998,000 trees sampled.
Table 1. Isolates used in this study and their GenBank and culture accession numbers. The strain of *Sulcispora supratumida* sp. nov. is set in bold font and all ex-type strains are annotated with “T”.

| Taxon                              | Culture accession no | ITS            | LSU            | SSU            | tef-1          |
|------------------------------------|----------------------|----------------|----------------|----------------|----------------|
| *Allophaeosphaeria muriformia*     | MFLUCC 13-0349T      | KP765680       | KP765681       | KP765682       | –              |
| A. subcilindrospora                 | MFLUCC 13-0380T      | T           | T             | T             | –              |
| *Amarengraphium anumphiadile*      | MFLUCC 16-0296T      | T             | T             | T             | –              |
| *Ampleomycys quisqualis*           | CBS 129.79T          | T             | T             | T             | –              |
| *Bhatiellae roseae*                | MFLUCC 17-0664T      | MG828873       | MG828989       | MG829101       | –              |
| *Chaetosphaeronema hispidulum*     | CBS 216.75           | T             | T             | T             | –              |
| *Dactyldina dactylidis*            | MFLUCC 14-0963T      | MG828887       | MG829003       | MG829114       | MG829199       |
| *D. shoemakeri*                    | MFLUCC 14-0966T      | MG828886       | MG829002       | MG829113       | MG829200       |
| *Dematiopleospora mariae*          | MFLUCC 13-0612T      | T             | T             | T             | –              |
| *Didymella exigua*                 | CBS 183.55T          | GU377794       | EU754155       | EU754056       | –              |
| *Didymocyrtis caloplacae*          | CBS 129338           | T             | T             | T             | –              |
| *D. ficuzzae*                      | CBS 128019           | T             | T             | T             | –              |
| *D. cladoniicola*                  | CBS 128026           | T             | T             | T             | –              |
| *Embarria clematidis*              | MFLUCC 14-0976T      | MG828871       | MG828987       | MG829099       | MG829194       |
| *Entodesmium rude*                 | CBS 650.86           | –             | GU301812       | GU349012       | –              |
| *Equiseticoila fusispora*           | MFLUCC 14-0522T      | KU987668       | KU987669       | KU987670       | MG520895       |
| *Galliicola pseudophaeosphaeria*    | MFLUCC 14-0527T      | T             | T             | T             | –              |
| *Hawksworthiana clematidicola*     | MFLUCC 14-0910T      | MG828901       | MG829011       | MG829120       | MG829202       |
| *H. lonicerae*                     | MFLUCC 14-0955T      | MG828902       | MG829012       | MG829121       | MG829203       |
| *Italica achilleae*                | MFLUCC 14-0959T      | MG828903       | MG829013       | MG829122       | MG829204       |
| *Junaceicola alpine*               | CBS 456.84           | T             | T             | T             | –              |
| *J. luzulae*                       | MFLUCC 16-0780       | KX449529       | KX449530       | KX449531       | MG520898       |
| *Leptospora rubella*               | CPC 11006            | DQ195780       | DQ195792       | DQ195803       | –              |
| *Loratospora aestuarii*            | JK 5535B             | –             | GU301838       | GU296168       | –              |
| *L. luzulae*                       | MFLUCC 14-0826       | KT328497       | KT328495       | KT328496       | –              |
| *Melnikia anthoxanthii*            | MFLUCC 14-1010T      | KU842805       | KU842804       | –              | –              |
| *Murphiaphaeria galatellae*        | MFLUCC 14-0614T      | KT338333       | KT338329       | KT338331       | MG520900       |
| *Neosetophoma italica*             | MFLUC14-0826T        | KT711356       | KT711361       | KT711366       | –              |
| *N. samarorum*                     | CBS 138.96T          | FJ427061       | KF251664       | GQ387517       | –              |
| *Neostagonospora caricis*          | CBS 135092/S616T     | KF251163       | KF251667       | –              | –              |
| *N. elegiae*                       | CBS 135101T          | KF251164       | KF251668       | –              | –              |
| *Nodulosphaeria hirta*             | MFLUCC 13-0867       | KU708849       | KU708845       | KU708854       | KU708853       |
| *N. senecionis*                    | MFLUCC 15-1297       | KT290257       | KT290258       | KT290259       | –              |
| *Ophiobolus cirsi*                 | MFLUCC 13-0218T      | KM014664       | KM014662       | KM014663       | –              |
| *O. diseminaris*                   | AS2L14-6             | –             | –             | –             | KP117305       |
| *Ophiophaeralia agrostidi*         | MFLUCC 11-0152T      | KM434271       | KM434281       | KM434290       | KM434299       |
| *Panaleptophthaeria dryadis*       | CBS 643.86           | JF740213       | GU301828       | KC584632       | GU349009       |
| *Paraphalmona chrysanthemicoila*   | CBS 522.66           | FJ421665       | KF251670       | GQ387521       | –              |
| *P. radicina*                      | CBS 111.79T          | KF251172       | KF251676       | EU754092       | –              |
| *Parastagonospora nodorum*         | CBS 110109T          | KF251177       | KF251681       | EU754076       | –              |
| *P. poagena*                       | CBS 136776T          | KJ869116       | KJ869174       | –              | –              |
| *Phaeosphaeria chiangrainai*       | MFLUCC 13-0231T      | KM434270       | KM434280       | KM434289       | KM434298       |
| *P. oryzae*                        | CBS 110110T          | KF251186       | KF251689       | GQ387530       | –              |
| *P. papaya*                        | S528                 | KF251187       | KF251690       | –              | –              |
Sulcispora supratumida sp. nov. (Phaeosphaeriaceae, Pleosporales)...

Results

Phylogenetic inferences

The combined ITS, LSU, SSU and tef-1 sequence data set comprised 69 strains of Phaeosphaeriaceae with Didymella exigua as the outgroup taxon. All individual trees generated under different criteria and from single gene datasets were essentially similar in topology and not significantly different from the tree generated from the concat-
ated dataset. Maximum likelihood analysis with 1000 bootstrap replicates yielded a tree with the likelihood value of ln: -13019.593920 and the following model parameters: alpha: 0.144187; \( \Pi(A) \): 0.245356, \( \Pi(C) \): 0.229408, \( \Pi(G) \): 0.267562 and \( \Pi(T) \): 0.257674. The best scoring RAxML tree is shown in Figure 1. Maximum likelihood bootstrap values \( \geq 50\% \) and Bayesian inference (BI) \( \geq 0.9 \) are given at each node.

The phylogenetic trees obtained from maximum likelihood were topologically congruent to previous studies on Phaeosphaeriaceae (Phookamsak et al. 2014; Thambugala et al. 2014; Tennakoon et al. 2016; Karunarathna et al. 2017; Wanasinghe et al. 2018). This phylogenetic analysis showed the placement of 45 genera within Phaeosphaeriaceae. The here-studied strain clustered with CBS 460.84 (one of Leuchtmann’s Swiss strains of *S. pleuropora* from *Carex firma*) with 100% bootstrap support value. The ITS sequence of the CBS 460.84 is almost identical to our strain (MFLUCC 14–0995). However no LSU, SSU and tef-1 sequences were obtained from CBS 460.84 in GenBank. The herbarium specimen of CBS 460.84 is in Westerdijk Fungal Biodiversity Institute (CBS) under accession number CBS H-15991 (SWITZERLAND, Kr. Graubünden, Zügenschlucht near Davos, *Carex firma*, A. Leuchtmann). However, CBS has presently stopped sending specimens on loan, hence we could not compare morphological characters of the here studied strain with CBS 460.84. Additionally *Sulcispora* sisterly clustered with the type species of *Loratospora*, *L. aestuarii* with low support and the second species of *Loratospora*, *L. luzulae*. was distantly clustered.

**Taxonomy**

*Sulcispora supratumida* Senan., Camporesi & K.D. Hyde, sp. nov.

MycoBank No: MB826887

Facesoffungi No: FoF 04782

Figure 2

**Etymology.** The species epithet is based on the two Latin words “supra” meaning upper and “tumidus” meaning swollen, referring to the position of swollen cells of ascospores.

**Type.** ITALY. Province of Forli-Cesena, Premilcuore, Passodella Valbura, on dead leaves of *Anthoxanthum odoratum* L. (Poaceae), 25 May 2013, Erio Camporesi, IT 1306 (MFLU 15–0038, holotype; HKAS 83865, paratype): living cultures, MFLUCC 14–0995.

**Description.** Saprobic on leaves of *Anthoxanthum odoratum* L., visible as black spots, occurring on the upper surface of entire leaf. **Sexual morph.** Ascomata 110–150 \( \times \) 90–140 \( \mu m \) (\( \bar{x} = 140–125 \mu m, n = 10 \)), scattered, solitary, immersed, uniloculate, globose, black. **Ostiole** 35–40 \( \mu m \) (\( \bar{x} = 39 \mu m, n = 10 \)) wide, papillate, central, periphysate. **Periphyses** 15–20 \( \mu m \) long, hyaline. **Peridium** comprising 2–4 layers of brown to dark brown, thick-walled, cells of *textura angularis* to *textura globularis*. **Hamathecium** comprising
Figure 1. Maximum likelihood majority rule consensus tree based on a combined dataset of ITS, LSU, SSU and tef-1 sequences. Bootstrap support values ≥50% and Bayesian inference (BI) ≥0.9 are given at the nodes. The tree is rooted to *Didymella exigua* (CBS 183.55). The culture accession numbers are given after the species names. All ex-type strains are in bold. The newly introduced species from this study is in bold red.
Figure 2. Sulcispora supratumida (MFLU 15–0038). a Leaves of Anthoxanthum odoratum b Appearance of ascomata on host surface c Cross section of ascoma d Peridium e Pseudoparaphyses f–i Asci j–n Ascospores o Upper surface of the culture p Lower surface of the culture. Scale bars: 200 µm (b), 50 µm (c), 20 µm (d–i), 10 µm (j–n).

2–4 µm wide, cellular, hyaline, branched, septate, pseudoparaphyses, constricted at the septa, anastomosing mostly above the asci and embedded in a mucilaginous matrix. Asci 85–125 × 20–35 µm (x = 100 × 30 µm, n = 20), 8-spored, few, bitunicate, fissitunicate, subglobose to clavate, short pedicellate, apically rounded, with an ocular chamber, arising from the base of the ascoma and attached to parenchymatous cell matrix at base. Ascospores 30–35 × 6–9 µm (x = 35 × 7 µm, n = 25), bi-seriate to tri-seriate, narrowly fusiform, narrowing towards the end cells, reddish to dark brown, 6-septate, second septum supra-median, slightly constricted, not constricted at other septa, second segment swollen, straight, with 12–16 longitudinal furrows on surface, lacking a mucilaginous sheath. Asexual morph. Undetermined.
**Table 2.** Ascospore morphology comparison of *Sulcispora* species

| Species name                 | Herbarium type data     | Host                      | No of septa | Swollen cell | Reference                  |
|------------------------------|-------------------------|---------------------------|-------------|--------------|----------------------------|
| *Sulcispora pleurospora*     | FH 196419 (isotype)     | *Deschampsia cespitosa*   | 5–6         | 3<sup>rd</sup> | Shoemaker and Babcock 1989 |
|                              | F6952, F6949, F6951     | *Deschampsia cespitosa*   | 6           | 3<sup>rd</sup> | In this study               |
|                              | (isotype)               | (Poaceae)                 |             |              |                            |
|                              | M (1 collection),       | 6 monocotyledous hosts,   | 6–8         | 3<sup>rd</sup> or 4<sup>th</sup> | Leuchtmann 1984            |
|                              | ZT (8 collections)      | 1 dicotyledous host        |             |              |                            |
| *Sulcispora supratumida*     | ZT (6 collections)      | *Seleria caerulea*        | 6           | 2<sup>nd</sup> | Leuchtmann 1984            |
|                              |                         | (Poaceae)                 |             |              |                            |
|                              | MFLU 15-0038 (holotype) | *Anthoxanthum odoratum*   | 6           | 2<sup>nd</sup> | In this study               |
|                              |                         | (Poaceae)                 |             |              |                            |

**Culture characteristics.** 2 cm diameter after 4 weeks incubated in dark at 25 °C on MEA, pinkish-white, circular, slightly woolly, margin lobate, effuse, lacking aerial mycelium, tightly attached to the media.

**Discussion**

Shoemaker and Babcock (1989) observed type specimens of *Phaeosphaeria pleurospora* and found that the ascospores of *P. pleurospora* with striated ornamented walls are different to those of other genera in Phaeosphaeriaceae. Hence, they introduced the genus *Sulcispora* to accommodate *P. pleurospora* and placed it in Phaeosphaeriaceae. *Sulcispora pleurospora* has some similarities with *Phaeosphaeria exarata* Shoemaker & C.E. Babc., in having very large cells in the peridium, ascospores with a continuous sheath and ornamented wall of ascospores with coarse, longitudinal ridges (Shoemaker and Babcock 1989).

In this study, a combined gene sequence analysis of taxa amongst the Phaeosphaeriaceae provides substantial evidence to support *Sulcispora* as a distinct genus in Phaeosphaeriaceae. *Sulcispora pleurospora* differs from other genera in having immersed ascomata with a relatively thin wall, cellular pseudoparaphyses, short pedicellate asci and brown ascospores (Phookamsak et al. 2014).

Leuchtmann (1984) reported variation of ascospore septation amongst several collections of *Phaeosphaeria pleurospora* from different host plants. *Phaeosphaeria pleurospora*, collected from *Seleria caerulea* (L.) Ard. and *Carex firma* Mygind ex Host, usually formed 6-septate ascospores and the second segment was swollen. Our collection is morphologically identical to Leuchtmann’s collection. However, the isotype and some of Leuchtmann’s collections from other host plants had 5–8-septate ascospores and the third or fourth segment was swollen (Table 2). Therefore Leuchtmann (1984) characterised *Phaeosphaeria pleurospora* as a species with 5–8 septate ascospores. However, Leuchtmann’s collection of *Sulcispora pleurospora* is likely to comprise more than a single species and possibly constitutes a species complex.
Based on the morphology, we identified our collection as different from the isotype of *Sulcispora pleurospora*. Hence, we introduced a new species as *Sulcispora supratumida* sp. nov. However, the ITS sequence of our strain clustered with that of CBS 460.84 (one of Leuchtmann’s Swiss strain of *S. pleurospora* from *Carex firma*) with 100% bootstrap support value. There are only two base pair differences between the ITS regions of both strains. Since there are no sequence data of other DNA regions of *Sulcispora pleurospora* deposited in GenBank, we could not confirm whether or not CBS 460.84 is *Sulcispora supratumida*. However, it would eventually be practical to obtain the living strain of CBS 460.84 and generate further sequence data.

### Keys for species in *Sulcispora*

1. Ascomata erumpent, long papillate, 5–8-septated, ascospores with 3rd swollen cell
   - Ascomata immersed, short papillate, 6-septated, ascospores with 2nd swollen cell

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