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Stemphylium revisited

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Abstract: In 2007 a new *Stemphylium* leaf spot disease of *Beta vulgaris* (sugar beet) spread through the Netherlands. Attempts to identify this destructive *Stemphylium* sp. in sugar beet led to a phylogenetic revision of the genus. The name *Stemphylium* has been recommended for use over that of its sexual morph, *Pleospora*, which is polyphyletic. *Stemphylium* forms a well-defined monophyletic genus in the *Pleosporaceae, Pleosporales* (*Dothideomycetes*), but lacks an up-to-date phylogeny. To address this issue, the internal transcribed spacer 1 and 2 and intervening 5.8S nr DNA (ITS) of all available *Stemphylium* and *Pleospora* isolates from the CBS culture collection of the Westerdijk Institute (N = 418), and from 23 freshly collected isolates obtained from sugar beet and related hosts, were sequenced to construct an overview phylogeny (N = 350). Based on their phylogenetic informativeness, parts of the protein-coding genes calmodulin and glyceraldehyde-3-phosphate dehydrogenase were also sequenced for a subset of isolates (N = 149). This resulted in a multi-gene phylogeny of the genus *Stemphylium* containing 28 species-clades, of which five were found to represent new species. The majority of the sugar beet isolates, including isolates from the Netherlands, Germany and the UK, clustered together in a species clade for which the name *S. beticola* was recently proposed. Morphological studies were performed to describe the new species. Twenty-two names were reduced to synonymy, and two new combinations proposed. Three epitypes, one lectotype and two neotypes were also designated in order to create a uniform taxonomy for *Stemphylium*.

Key words: Morphology, Multi-gene phylogeny, *Pleospora*.
Taxonomic novelties: New combinations: *Stemphylium armeriae* (Corda) Woudenb. & Crous, *S. halophilum* (J. Webster) Woudenb. & Crous; **New species:** *S. canadense* Woudenb. & Crous, *S. chrysanthemicola* Woudenb. & Crous, *S. lucomagnoense* Woudenb. & Crous, *S. novae-zelandiae* Woudenb. & Crous, *S. simmonsii* Woudenb. & Crous; **Typification (Basionyms): Epitypifications:** *Alternaria lancipes* Ellis & Everh., *Stemphylium solani* G.F. Weber, *Thyrospora astragali* Yoshii; **Lectotypification:** *Thyrospora astragali* Yoshii; **Neotypifications:** *Pleospora pomorum* A.S. Horne, *Thyrospora lycopersici* Enjoji.

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INTRODUCTION

In 2007 a new leaf spot disease associated with a *Stemphylium* sp. was first discovered on sugar beet (*Beta vulgaris*) in the Netherlands, which subsequently spread rapidly throughout the country in the following years (Hanse 2013). The causal agent was recently formally named as *Stemphylium beticola* (Crous *et al.* 2016), but the genus itself was not treated in that study.

Stemphylium is a dematiaceous hyphomycete, which can be distinguished from other hyphomycetes forming phaeodictyospores based on the percurrent rejuvenation of its conidiophores, and apically swollen conidiogenous cells. Other closely related genera mostly display a geniculate, sympodial proliferation, e.g. *Alternaria* (Simmons 2007). *Stemphylium*, with *S. botryosum* as type species, forms a well-defined monophyletic genus in the family *Pleosporaceae, Pleosporales* (Câmara *et al.* 2002; Inderbitzin *et al.* 2009). However, the sexual morph to which *Stemphylium* is linked, *Pleospora*, is known to be polyphyletic. The type species of *Pleospora*, *Pleospora herbarum*, has *Stemphylium herbarum* as asexual morph (Simmons 1985), but several *Pleospora* spp. have been linked to a range of different asexual genera (e.g. Inderbitzin *et al.* 2006; De Gruyter *et al.* 2013; Ariyawansa *et al.* 2015; Crous & Groenewald 2017). The latest comprehensive phylogenetic study on *Pleospora* species with *Stemphylium* asexual morphs was published in 2009 (Inderbitzin *et al.* 2009), which left many unnamed and potentially new *Stemphylium* species. The *Pleospora herbarum* clade *sensu* Inderbitzin *et al.* (2009) illustrated the problems with identification in the genus. Based on a multi-gene phylogeny five species should be synonymised, but RAPD fingerprints (Chaisrisook *et al.* 1995), morphology and ecology studies supported them to be separate species. Some researchers therefore chose to retain all the species names (e.g. Inderbitzin *et al.* 2009), while others again chose to synonymise them (e.g. Köhl *et al.* 2009). With the uptake of the one fungus-one name initiative in the International Code of Nomenclature for algae, fungi and plants (ICN, McNeill et al., 2012), name changes in these genera became necessary. The use of *Stemphylium* over *Pleospora* has subsequently been recommended by the Working Group on *Dothideomycetes* of the International Committee on the Taxonomy of Fungi (Rossman *et al.* 2015).

The aim of the present study was to construct a phylogenetic overview of the genus *Stemphylium*. All available *Stemphylium* and *Pleospora* isolates from the CBS collection, together with *Stemphylium* isolates collected from sugar beet from different parts of the Netherlands as well as from the UK and Germany, were included in the study. The internal transcribed spacer 1 and 2 and intervening 5.8S nr DNA (ITS) were sequenced to construct a draft overview phylogeny. Using a reduced set of isolates, the phylogenetic informativeness of six commonly used protein-coding genes, namely partial actin (*actA*), beta-tubulin (*tub2*), calmodulin (*cmdA*), translation elongation factor 1-alpha

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(*tef1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and DNA-directed RNA polymerase second largest subunit (*rpb2*) were also evaluated. Based on these results, the two most promising genes were additionally sequenced for the genus *Stemphylium*, and used to construct a multi-gene phylogeny.

MATERIALS AND METHODS

Isolates

Four-hundred-and-forty-one isolates were included in this study, comprising of 418 *Pleospora* and *Stemphylium* isolates from the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands (Supplementary Table 1) and 23 isolates received from the IRS (the research and knowledge centre for sugar beet cultivation in The Netherlands), Bergen op Zoom, the Netherlands (Supplementary Table 2). The dataset includes 48 (ex-)type strains. Freeze-dried strains from the CBS culture collection were revived in 2 mL malt/peptone (50 %/50 %) and subsequently transferred to oatmeal agar (OA) (Crous *et al.* 2009). Strains stored in liquid nitrogen were transferred to OA directly from the −185 °C storage. For the isolation methods of the IRS isolates see Hanse *et al.* (2015).

Morphology

Isolates were grown on potato carrot agar (PCA, Crous et al., 2009) and synthetic nutrient-poor agar (SNA, Nirenberg, 1976) at moderate temperatures under CoolWhite fluorescent light with an 8 h photoperiod. After 7 and 14 d the growth rates were measured and the colony characters noted. Colony colours were rated according to Rayner (1970). Morphological descriptions of asexual structures were made for isolates grown on SNA for 7 d. Slides were prepared with the cellotape technique (Schubert *et al.* 2007) using Titan Ultra Clear Tape (Conglom Inc., Toronto, Canada) and Shear's medium as mounting fluid. Morphological descriptions of sexual structures were made for isolates grown on PCA for 14 d, with 85 % lactic acid as mounting fluid. The mean plus/minus standard deviation values were derived from measurements of 30 structures, with extremes given in parentheses. Photographs of characteristic structures were made with a Zeiss Axio Imager A2 microscope equipped with a Nikon DS-Ri2 high-definition colour camera using differential interference contrast (DIC) optics and the Nikon software NIS-elements D v. 4.50. Adobe Bridge CS5.1 and Adobe Photoshop CS5 Extended, v. 12.1, were used for the final editing and photographic preparation. Nomenclatural data were deposited in MycoBank (Crous *et al.* 2004).

DNA isolation, PCR and sequencing

DNA extraction was performed using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. The ITS region, *gapdh*, *tef1* and *rpb2* gene regions were amplified and sequenced with respectively the primers V9G (De Hoog and Gerrits van den Ende, 1998)/ITS4 (White *et al.* 1990), gpd1/gpd2 (Berbee *et al.* 1999), EF1-728F/EF1-986R (Carbone & Kohn 1999), and RPB2-5F2 (Sung *et al.* 2007)/fRPB2-7cR (Liu *et al.* 1999) as described in Woudenberg *et al.* (2013). The *actA* gene region was amplified and sequenced with ACT-512F/ACT-783R (Carbone & Kohn 1999) as described in De Gruyter *et al.* (2009). For the *tub2* gene region several primer combinations and PCR programs were tested, but no PCR product could be obtained. The *cmdA* gene region was amplified and sequenced with the primers CALDF1/CALDR2 (Lawrence *et al.* 2013). The PCR mixture consisted of 1 µl 50× diluted genomic DNA, 1× NH4+ reaction buffer (Bioline, Luckenwalde, Germany), 2 mM $MgCl_2$, 20 µM of each dNTP, 0.2 µM of each primer and 0.25 U *Taq* DNA polymerase (Bioline). The PCR conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 59 °C and 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. The PCR products were sequenced in both directions using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Bleiswijk, the Netherlands) and analysed with an ABI Prism 3730xl DNA Analyser (Thermo Fisher Scientific) according to the manufacturer's instructions. Consensus sequences were computed from forward and reverse sequences using the Bionumerics v. 4.61 software package (Applied Maths, St-Marthens-Latem, Belgium). Generated sequences were deposited in GenBank (Table 1, Supplementary Table 1).

Identification of best loci

Based on the ITS sequence results and former sequence data (Inderbitzin *et al.* 2009), seven isolates representing clade 10 (Fig. 1), namely CBS 378.54, CBS 116598, CBS 116599, CBS 134496, CBS 136590, GV11-196-a1-3 and IFZ2013-024, were selected to determine which gene would be the most informative in distinguishing species within this clade. In addition to ITS, the *actA*, *cmdA*, *gapdh*, *rpb2* and *tef1* gene regions were amplified and sequenced as described above. Unfortunately the beta-tubulin PCRs did not give any results, even when following previously published PCR primers and methods (Bt2a/Bt2b, Glass & Donaldson 1995) which are supposed to work on *Stemphylium* species (Lawrence *et al.* 2013). A sequence comparison from the five additional gene regions of the seven selected isolates was made in Bionumerics v. 4.61 (Applied Maths) and by eye (Table 2).

Phylogenetic analyses

In Bionumerics v. 4.61 (Applied Maths), a quick UPGMA phylogeny was constructed from the ITS sequences of the 441 included isolates to assign them to clusters of closely related or identical isolates. For those isolates belonging to the *Stemphylium* clade, a multiple sequence alignment of the ITS sequences was generated with MAFFT v. 7.271 (http://mafft.cbrc.jp/alignment/server/index.html) using the FFT-NS-i method. With Findmodel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) the best nucleotide substitution model was determined. Bayesian analyses were performed with MrBayes v. 3.2.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The sample frequency was set at 1 000 and the temperature value of the heated chain was set at 0.1. The run stopped when the average standard deviation of split frequencies reached below 0.01. Burn-in was set to 25 % after which the likelihood values were

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