Is morphology in Cercospora a reliable reflection of generic affinity?

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Abstract

Cercospora (Mycosphaerellaceae) is a large genus of fungi comprising many important plant pathogens. In recent years DNA-based studies have revealed multiple genera of cercosporoid fungi being poly- and paraphyletic. Among these genera, the genus Cercospora has always been perceived as monophyletic. In the present study, phylogenetic inferences based on partial gene sequences of the LSU, ITS, ACT, TEF1-α and HIS loci, elucidated a cercospora-like taxon from Ammi majus to cluster in a clade apart from Cercospora s. str. In spite of numerous Cercospora spp. presently known from their DNA sequence data, this collection represents the first concrete evidence to the fact that the morphological characters previously attributed to Cercospora s. str. evolved more than once in the Mycosphaerellaceae. The genus Neocercospora is subsequently introduced to accommodate the Iranian taxon occurring on A. majus. Further collections on other hosts and from different continents are now required to establish the prevalence and relative importance of species of Neocercospora.

Key words: biodiversity, cercosporoid hyphomycetes, Mycosphaerellaceae, Neocercospora, phylogeny

Introduction

Cercosporoid fungi or Cercospora s. lat. belonging to Mycosphaerellaceae (Capnodiales), include numerous economically significant plant pathogens causing leaf spots on a wide variety of woody and herbaceous plants, but also can cause necrotic lesions on flowers, fruits, bracts, seeds and stems (Goodwin et al. 2001, Crous & Braun 2003, Agrios 2005). They are found in different geographical and climatic zones across the world, and are especially abundant and diverse in tropical and subtropical areas (Braun et al. 2013, 2014). The frequent association of cercosporoid fungi with plant diseases has stimulated substantial interest in this group, and much of this attention has been focused on the systematics of species and genera in this complex (Deighton 1976, Pretorius et al. 2003, Braun & Crous 2005, Crous et al. 2006, Arzanlou et al. 2008, Nakashima et al. 2011, Braun et al. 2013).

The first genus of cercosporoid hyphomycetes, Passalora, was introduced by Fries (1849), followed by Cercospora introduced by Fresenius (in Fuckel 1863). Since then, the taxonomy of this group has proven highly problematic. Chupp (1954) published the first monograph of cercosporoid hyphomycetes in which he followed a very broad generic concept and reduced many of the cercosporoid genera to synonymy with the genus Cercospora. Contrary to this approach, Deighton (1967, 1973, 1976, 1979, 1987, 1990) and Ellis (1971, 1976) in their treatments of cercosporoid fungi narrowed the generic concept of Cercospora s. lat. and divided it into smaller morphological units. Later, Crous & Braun (2003) reviewed the genera of cercosporoid fungi and, due to numerous morphologically intermediate taxa and the first phylogenetic results based on DNA sequence data being available at the time (Crous et al. 2000), rearranged them into four genera viz. Cercospora, Passalora, Pseudocercospora and Stenella. These cercosporoid genera are mainly separated based on a combination of characters, of which the structure of the conidiogenous loci (scars) and hila, and the presence or absence of pigmentation in conidiophores and conidia are considered to be the most important (Crous & Braun 2003).

With progress towards a stable phylogeny for the Mycosphaerellaceae (Arzanlou et al. 2007, Crous et al. 2007,
2009a, 2009b, Braun et al. 2013, Crous et al. 2013a, Groenewald et al. 2013), most of the assumptions made by Crous & Braun (2003) regarding generic circumscriptions have been confirmed. However, several newly segregated cercosporoid genera have been also introduced, or old genera resurrected to reflect monophyletic, morphologically separated clades, e.g. Pallidocercospora, Paracercospora (Crous et al. 2013a), Phaeocercospora (Crous et al. 2012), Neopseudocercospora (Crous et al. 2013b) and Porocercospora (Amaradasa et al. 2014). In this regard, many cercosporoid genera have been revealed to represent polyphyletic taxa. Among these genera, the genus Cercospora which is recognised by having pigmented conidiophores with conspicuous (thickened and darkened) conidiogenous loci and hyaline conidia with conspicuous hila, has until now been supposed to be monophyletic (Groenewald et al. 2013, Bakhshi et al. 2015), at least as far as included phylogenetically-proven species are concerned. This monophyly is assumed based on the phylogenetic association of taxa to the type species of Cercospora, C. apii (see Crous & Braun (2003), and Braun et al. (2013) for a discussion on the identity of the type species).

Members of cercosporoid fungi are known to be widely distributed, occurring on a broad range of plant hosts in many countries, including Iran. The biodiversity of cercosporoid fungi in Iran has recently received much attention (Bakhshi et al. 2014, 2015). Bakhshi et al. (2015) revised the taxonomy of the genus Cercospora in Iran by applying the Consolidated Species Concept (Quaedvlieg et al. 2014). These results indicated a rich diversity of Cercospora spp. in the north and north-west of Iran, including six novel species and several new host records (Bakhshi et al. 2015).

During the course of the present study, two isolates of cercosporoid fungi morphologically resembling species of the genus Cercospora were recovered from Bishop’s flower (Ammi majus L.). A subsequent phylogenetic study based on different gene regions revealed these isolates to represent an undescribed genus. The aim of this study was thus to describe this novel cercospora-like genus and also elucidate the phylogenetic relationship of this genus to Cercospora and allied genera in Mycosphaerellaceae.

Material and Methods

Isolates

Symptomatic Bishop’s flower (Ammi majus) leaves were collected in the field from Firouragh in the Khoy region, West Azerbaijan province, Iran, and taken to the laboratory. Leaves were examined directly under a Nikon SMZ 1500 stereo-microscope to observe sporulation. Single spore isolates derived from conidia, directly lifted from conidiophores on Bishop’s flower leaves, were grown on 2% malt extract agar (MEA; Fluka, Hamburg, Germany) using a previously described procedure (Bakhshi et al. 2011). Dried specimens are maintained in the Fungarium of the Iranian Research Institute of Plant Protection, Tehran, Iran (IRAN). Representative cultures were deposited in the Culture Collection of Tabriz University (CCTU) and the Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands).

DNA extraction, amplification and sequencing

Fungal isolates were grown on MEA for 10 d at 25°C in the dark. The mycelia were harvested with a sterile scalpel and genomic DNA isolated using the protocol of Möller et al. (1992). DNA samples were diluted 50–100 times in preparation for further DNA amplification reactions. Parts of the following loci were amplified and sequenced: 28S nrRNA gene (LSU) with the primer pairs LROR (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990), the internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) of the nrDNA operon with the primer pairs V9G (de Hoog & Gerrits van den Ende 1998) and ITS4 (White et al. 1990), a fragment of the actin gene (ACT) with the primer set ACT-512F and ACT-783R (Carbone & Kohn 1999), part of the translation elongation factor 1-alpha (TEF1-α) using the primer set EF1-728F (Carbone & Kohn 1999) and EF-2 (O’Donnell et al. 1998) and a fragment of the histone H3 gene (HIS) with the primer set CvH3F and CvH3R (Crous et al. 2004b). All PCR reaction mixtures and conditions were performed in a total volume of 12.5 μl as described by Bakhshi et al. (2015).

Both strands of the PCR fragments were sequenced with the BigDye® Terminator Cycle Sequencing reaction Kit v. 3.1 (Applied Biosystems™, Foster City, CA, USA), following the manufacturer’s instructions, using the same primer pairs used for amplification. Sequencing amplicons were purified through Sephadex G-50 Superfine columns (Sigma Aldrich, St. Louis, MO) in a 96-well MultiScreen HV plate (Millipore, Billerica, MA) and analysed with an ABI Prism 3730xl DNA Analyzer (Life Technologies Europe BV, Applied Biosystems™, Bleiswijk, The Netherlands) according to manufacturer’s recommendation.
### TABLE 1

A list of isolates and their GenBank accessions used in phylogenetic analyses. Bold accession numbers were generated in this study.

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Phylogenetic analyses

The DNA sequences generated with forward and reverse primers were edited using MEGA v. 6 (Tamura et al. 2013) and a consensus sequence was generated manually for each set of forward and reverse sequences. Megablast searches of the NCBI’s GenBank nucleotide database were used to supplement the sequence data obtained in this study. Sequences were aligned with the MAFFT online interface using default settings (http://mafft.cbrc.jp/alignment/server/) (Katoh

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Morphology

Morphological characterisations were based on both structures from herbarium material (in planta) and structures from culture (in vitro). For in planta descriptions, diseased leaf tissues were observed under a stereo-microscope and relevant morphological structures (stromata, conidiophores and conidia) were picked up from lesions with a sterile inoculation needle and mounted on glass slides in clear lactic acid. For in vitro descriptions, cultures were plated on fresh MEA and subsequently incubated at 20°C under continuous near-ultraviolet light, and examined after 2–4 weeks for sporulation. Observations were made at × 1 000 magnification for microscopic structures using a Nikon Eclipse 80i light microscope, and 95% confidence intervals were derived for the 30 measurements with extreme values given in parentheses. High-resolution photographs of microscopic fungal structures mounted in clear lactic acid were captured with a Nikon digital sight DS-fi1 high definition colour camera mounted on the Nikon Eclipse 80i light microscope. Adobe Photoshop CS3 was used for the final editing of acquired images and photographic preparations.

Colony macro-morphology was assessed on MEA at 25°C in the dark in triplicate. After 20 days, the colony diameter was measured and the colony colour was assessed according to the mycological colour charts of Rayner (1970). Descriptions, nomenclature and illustrations were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004a).

Results

DNA sequencing and phylogenetic analyses

For two cercospora-like strains obtained from *Ammi majus* in this study, amplification of the five loci (LSU, ITS, ACT, TEF1-α, HIS), yielded fragments of approximately 900, 700, 200, 300 and 400 bp, respectively. Two BI phylogenetic analyses were performed: 1) an initial analysis of the LSU region to determine the generic position of the obtained isolates in the Mycosphaerellaceae; 2) a combined analysis of the ITS, ACT, TEF1-α and HIS to fully resolve the phylogenetic relation of the isolates in this study with the different species of the *Cercospora s. str.* clade (Groenewald et al. 2013, Bakhshi et al. 2015).

**LSU dataset**

During phylogenetic analyses, the obtained LSU sequences in this study were aligned with LSU sequence data of 33 Mycosphaerellaceae taxa in order to establish how these isolates are related to other well-established genera within Mycosphaerellaceae. The final aligned LSU dataset contained 35 ingroup taxa with a total of 735 characters, containing 114 unique site patterns and *Cladosporium herbarum* (GenBank accession DQ678074) served as the outgroup taxon. The results of MrModeltest recommended a general time reversible (GTR) substitution model with inverse gamma rates and dirichlet base frequencies. During the generation of the tree, a total of 512 trees were saved, and the 50% majority rule consensus tree (Fig. 1) and posterior probabilities (PP) were calculated from the remaining 384 (75%) trees. The phylogenetic analysis of the Mycosphaerellaceae LSU dataset showed isolates from *Ammi majus* clustering in a distinct monophyletic clade that is sister to the *Phloeospora*, *Ramulispora* and *Pseudocercosporella capsellae* clades (Fig. 1).
FIGURE 1. Consensus phylogram (50% majority rule) of 512 trees resulting from a Bayesian analysis of the LSU sequence alignment using MrBayes v. 3.2.2. The scale bar represents the average number of substitutions per site, and posterior probability values are shown at the nodes. GenBank accession numbers are shown in brown text and bold accession numbers were generated in this study. Clades of different genera are indicated in coloured blocks and names of the genera are shown in purple text. The tree is rooted to *Cladosporium herbarum* (GenBank accession DQ678074).
IS MORPHOLOGY A REFLECTION OF GENERIC AFFINITY?  Phytotaxa 213 (1) © 2015 Magnolia Press • 27

FIGURE 2. Consensus phylogram (50% majority rule) of 622 trees resulting from a Bayesian analysis of the combined 4-gene (ITS, TEF1-α, ACT and HIS) sequence alignment using MrBayes v. 3.2.2. The scale bar represents the average number of substitutions per site, and posterior probability values are shown at the nodes. Clades of different genera are indicated in coloured blocks and names of the genera are shown to the right of the block. The tree is rooted to Cladosporium herbarum (strain CBS 121621).
Multi-locus dataset

The combined ITS/TEF1-α/ACT/HIS alignment contained 45 taxa including *Cladosporium herbarum* (isolate CBS 121621) as outgroup taxon, and 1659 characters including alignment gaps were used. The gene regions in the alignment were 1–514 for ITS, 519–1092 for TEF1-α, 1097–1293 for ACT and 1298–1655 for HIS. The alignment contained a total of 720 unique site patterns: 155 (ITS), 357 (TEF1-α), 122 (ACT), 86 (HIS). The results of the MrModeltest analyses recommended a HKY+I+G for TEF1-α and HIS, while a GTR+G for ITS and ACT. All partitions had dirichlet base frequencies. The Bayesian analysis generated 622 trees from which 154 trees were discarded (25% burn in). The 50% majority rule consensus tree (Fig. 2) and posterior probabilities were calculated from the remaining 468 trees. Based on the results of combined gene tree, the isolates from *Ammi majus* cluster in a distinct well-supported clade sister to the clade including *Pseudocercosporella capsellae* strains (Fig. 2).

Taxonomy

Based on the LSU (Fig. 1) and multi-locus (Fig. 2) DNA datasets, cercospora-like isolates occurring on *Ammi majus* clustered in a separate clade, distinct from *Cercospora s. str.*, suggesting that they represented a distinct genus in the Mycosphaerellaceae. Due to their distinct phylogenetic placement, a new genus, *Neocercospora*, is hereby introduced for the isolates occurring on *Ammi majus*.

**Neocercospora** M. Bakhshi, Arzanlou, Babai-ahari & Crous, gen. nov. MycoBank MB 812284

Foliicolous and caulicolous, phytopathogenic. *Mycelium* internal. *Stromata* substomatal, weakly to moderately developed, brown. *Caespituli* amphigenous, punctiform, brown. *Conidiophores* aggregated in loose to moderately dense fascicles, arising from the upper cells of substomatal to intraepidermal brown stromata; conidiophores aseptate, reduced to conidiogenous cells. *Conidiogenous cells* unbranched, pale brown to brown, smooth, subcylindrical to cone-shaped, wider at the base, unilocular, sympodial, subdenticulate; loci conspicuous, thickened, darkened, somewhat refractive, apical or formed on shoulders caused by geniculation. *Conidia* solitary or catenate, in unbranched chains, hyaline, smooth, guttulate or not, cylindrical, subcylindrical to obclavate-cylindrical, straight to slightly curved, septate; hilum flattened, moderately thickened, darkened and somewhat refractive.

**Type species:**—*Neocercospora ammicola* M. Bakhshi, Arzanlou, Babai-ahari & Crous.  
**Etymology:**—New genus resembling *Cercospora* in morphology.

**Neocercospora ammicola** M. Bakhshi, Arzanlou, Babai-ahari & Crous, sp. nov. (Fig. 3, 4.) MycoBank MB 812288

Type:—IRAN. West Azerbaijan Province: Khoy, Firouragh, on leaves and stems of *Ammi majus* L. (Apiaceae), Sept. 2012, M. Arzanlou (holotype IRAN 16461 F, culture ex-type CCTU 1186 = CBS 136450).

**Description in planta:**—Foliicolous and caulicolous, phytopathogenic. *Leaf spots* amphigenous, circular to subcircular, 1–4 mm diam., brown, with raised, dark brown border. *Mycelium* internal. *Stromata* substomatal, weakly to moderately developed, brown, 5–18 μm diam. *Caespituli* amphigenous, punctiform, brown. *Conidiophores* aggregated in loose to moderately dense fascicles, arising from the upper cells of substomatal to intraepidermal brown stromata; conidiophores aseptate, reduced to conidiogenous cells. *Conidiogenous cells* unbranched, pale brown to brown, smooth, subcylindrical to cone-shaped, wider at the base, unilocular and multilocular, sympodial, subdenticulate; loci conspicuous, thickened, darkened, somewhat refractive, apical or formed on shoulders caused by geniculation, 1–2.5 μm diam. *Conidia* solitary or catenate, in unbranched chains, hyaline, smooth, guttulate or not, cylindrical, subcylindrical to obclavate-cylindrical, straight to slightly curved, septate; hilum flattened, moderately thickened, darkened and somewhat refractive.

**Description in vitro on MEA:**—*Mycelia* consisting of hyaline, branched, septate, smooth hyphae, 2–6 μm diam, guttulate, gradually becoming pale to medium brown and somewhat verruculose at fertile regions. *Conidiophores* solitary or in loose fascicles, unbranched, pale brown, becoming darker towards the apex, semi-macronematous to
macronematous, up to 85 μm tall, (3–)3.5–4(–5) μm wide, 0–6-septate, septa 10–20 μm apart (but not observed in planta), often reduced to solitary conidiogenous cells. Conidiogenous cells integrated, terminal or lateral or terminal on hyphae when 1-celled, medium brown to brown, (15–)20–25(–35) × 3–3.5(–4.5) μm, uni- and multilocular, sympodial, subdenticulate; loci moderately conspicuous, slightly thickened and darkened, somewhat refractive, apical or formed on shoulders caused by geniculation, 1–2.5 μm diam. Conidia solitary or catenate, in unbranched chains, hyaline, smooth, guttulate or not, cylindrical to subcylindrical, straight to gently curved, indistinctly 1–9-septate, (25–)45–60(–95) × (2–)2.5–3(–4) μm; apex obtuse or subobtuse, base obconically truncate or truncate with slight basal taper to hilum; hila flattened, with marginal thickening along the rim, somewhat refractive, 1–2 μm diam.

**FIGURE 3.** Neocercospora amnicola (CBS 136450) (in vivo). a. Leaf spots on Ammi majus. b–f. Fasciculate conidiophores reduced to conidiogenous cells. g–m. Solitary and catenate conidia. Scale bars = 10 μm.
**Cultural characteristics:**—Colonies on MEA after 20 days at 25ºC in the dark up to 35 mm diam., erumpent with smooth, uneven margins and moderate aerial mycelium; surface olivaceous black, reverse iron-grey.

**Habitat/Distribution:**—Known to inhabit *Ammi majus*, West Azerbaijan Province, Iran.

**Etymology:**—Named after the host genus from which it was isolated, *Ammi*.

**Other material examined:**—IRAN. West Azerbaijan Province: Khoy, Firouragh, on *Ammi majus*, Sept. 2012, M. Arzanlou (CCTU 1187).

**FIGURE 4.** *Neocercospora amnicola* (CBS 136450) (*in vitro*). a. Colony on MEA. b–d. Conidiophores and conidiogenous cells. e. Terminal conidiophore on hypha. f. Conidiophore reduced to a conidiogenous cell. g–l. Solitary and catenate conidia. Scale bars = 10 μm.
Discussion

Since the application of molecular techniques to delineate genera of cercosporoid fungi, several genera, e.g. Pseudocercospora (Frank et al. 2010, Crous et al. 2013a), Stenella (Arzanlou et al. 2007) and Passalora (Braun et al. 2013, Hyde et al. 2013) have been revealed as being polyphyletic within the Mycosphaerellaceae. For many years the genus Cercospora has been treated as a general concept to accommodate a wide range of cercosporoid hypomycetes that have pigmented conidiophores with conspicuously thickened and darkened conidiogenous loci (scars) and hyaline conidia formed singly with thickened and darkened conidial hila (Crous & Braun 2003). By using this concept combined with a multi-locus molecular phylogenetic approach, the genus Cercospora was assumed to be monophyletic (Groenewald et al. 2013, Bakhshi et al. 2015). However a comprehensive phylogenetic examination of all known Cercospora species is required to confirm this assumption.

In the present study, we introduce the novel genus Neocercospora to accommodate the isolates occurring on Bishop’s flower, which are cercospora-like in morphology, but cluster apart from Cercospora s.str. Based on the LSU phylogeny generated here (Fig. 1), Neocercospora resides in the Mycosphaerellaceae, with close neighbours being Phloeospora, Ramulispora and Pseudocercosporella capsellae (Fig. 1). In the combined gene tree, Neocercospora is a sister taxon to Pseudocercosporella capsellae, and clearly distinct from Cercospora, forming a well-supported clade with high Bayesian posterior probability (Fig. 2). Morphologically Neocercospora appears cercospora-like in morphology, but has conidiophores that are reduced to conidiogenous cells, and conidia that can occur in chains. Although both characteristics have been recorded among species of Cercospora s. str., further collections of additional species would have to reveal if it is the combination of these two features that in fact separates Neocercospora from Cercospora. In any case, non-congeneric, phylogenetically differentiated species should in future not be assigned to Cercospora s. str. to avoid further polyphyly.

In this study, as in numerous other recent studies (Crous et al. 2012, 2013a, 2013b, Hyde et al. 2013, Quaedvlieg et al. 2014), the limits of sole morphology-based classification for genera as well as species within the Mycosphaerellaceae were confirmed. There are several genera that are readily distinguishable based on even a single locus, but are visually impossible to identify based on solely morphological characteristics. For example, the genus Phaeocercospora is morphologically similar to and indistinguishable from Pseudocercospora species with consistently percurrently proliferating conidiogenous cells, but phylogenetically it is distinct (Crous et al. 2012). Microcyclosporella (Frank et al. 2010) is another genus that was introduced on the basis of phylogenetic data, showing that it clusters within the Mycosphaerellaceae. However, it is morphologically close to, and easily confused with, species of Microcyclospora (Frank et al. 2010) in Teratosphaeriaceae and Pseudocercosporella in Mycosphaerellaceae (Frank et al. 2010). The phylogenetic placement of such genera demonstrates that previous generic concepts and the sole reliance on particular morphological features are not always congruent with molecular phylogenies.

Our results also show that a single species of Pseudocercosporella, namely P. oxalidis, resides in the Cercospora s. str. clade. Pseudocercosporella was established based on P. ipomoeae by Deighton (1973) to accommodate cercosporella-like asexual morphs of Mycosphaerella, having unthickened and inconspicuous conidial scars. Recent phylogenetic studies have indicated Pseudocercosporella as being polyphyletic and comprising a genetically heterogeneous assemblage of fungi (Crous et al. 2009a, 2013a, Frank et al. 2010). Furthermore, Frank et al. (2010) revealed fungi with a pseudocercosporella-like morphology to reside in at least five clades distinct from the type species P. bakeri (= P. ipomoeae, see Braun 1995). The genus Pseudocercosporella is therefore in need of taxonomic revision pending the recollection of additional species thus far not known from culture. Based on our multi-gene phylogenetic data, P. oxalidis must be placed in the genus Cercospora, even though it has unthickened conidial hila and conidiogenous scars. Presently we are of the opinion that such a decision is premature, and that more species of Pseudocercosporella still need to be recollected and cultured to resolve the genera involved in this complex.

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