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 CylH3F and CylH3R (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.

Species	Culture accession number(s)[1]	GenBank accession numbers			
		ITS	TEF1-α	ACT	HIS
Caryophylloseptoria lychnidis	CBS 109102	KF251289	KF253237	KF253598	–
Caryophylloseptoria silenes	CBS 109103	KF251293	KF253241	KF253602	–
Cercospora althaeina	CBS 248.67; CPC 5117	JX143530	JX143284	JX143038	JX142546
Cercospora apii	CBS 114418; CPC 10924	AY840517	AY840484	AY840448	AY840382
Cercospora apii	CBS 116455; CPC 11556	AY840519	AY840486	AY840450	AY840384
Cercospora apii	CBS 553.71; IMI 161116; CPC 5083	DQ233320	DQ233344	DQ233370	DQ233422
Cercospora armoraciae	CBS 136132; CCTU 1117	KJ886418	KJ886257	KJ885935	KJ886096
Cercospora armoraciae	CBS 136134; CCTU 1190	KJ886422	KJ886261	KJ885939	KJ886100
Cercospora armoraciae	CBS 250.67; CPC 5088	JX143545	JX143299	JX143053	JX142561
Cercospora beticola	CBS 124.31; CPC 5070	AY840523	AY840490	AY840454	AY840388
Cercospora beticola	CBS 116456; CPC 11557	AY840527	AY840494	AY840458	AY840392
Cercospora campi-silii	CBS 132625; CPC 14585	JX143561	JX143315	JX143069	JX142577
Cercospora canescens complex	CBS 111133; CPC 1137	AY260065	DQ835084	DQ835103	DQ835157
Cercospora capsici	CBS 118712	GU214653	JX143322	JX143076	JX142584
Cercospora celosiae	CBS 132600; CPC 10660	JX143570	JX143326	JX143080	JX142588
Cercospora conyzae-canadensis	CBS 135978; CCTU 1119	KJ886445	KJ886284	KJ885962	KJ886123
Cercospora cylindracea	CCTU 1114	KJ886450	KJ886289	KJ885967	KJ886128
Cercospora cylindracea	CCTU 1189	KJ886452	KJ886291	KJ885969	KJ886130
Cercospora pseudochenopodii	CCTU 1176	KJ886518	KJ886357	KJ886035	KJ886196
Cercospora senecionis-walkeri	CBS 132636; CPC 19196	JX143649	JX143408	JX143162	JX142670
Cercospora sojina	CBS 132018; CPC 12322	GU214655	JX143418	JX143172	–
Cercospora sorghicola	CBS 136448; CCTU 1173	KJ886525	KJ886364	KJ886042	KJ886203
Cercospora sp. G	CBS 136023; CCTU 1020	KJ886529	KJ886368	KJ886046	KJ886207
Cercospora sp. G	CCTU 1116	KJ886537	KJ886376	KJ886054	KJ886215
Cercospora violae	CBS 251.67; CPC 5079	JX143737	JX143496	JX143250	JX142758
Cercospora zeae-maydis	CBS 117757	DQ185074	DQ185086	DQ185098	DQ185122
Cercospora zeae-maydis	CBS 132678; CPC 15602	JX143743	JX143502	JX143256	JX142764
Cercospora zebrina	CCTU 1110	KJ886546	KJ886385	KJ886063	KJ886224
Cercospora zebrina	CBS 118790	JX143748	JX143510	JX143264	JX142772
Cercospora zeina	CBS 118820; CPC 11995	DQ185081	DQ185093	DQ185105	DQ185129
Cladosporium herbarum	CBS 121621	EF679363	EF679440	EF679516	EF679670
Neocercospora ammicola	CBS 136450; CCTU 1186	**KR232407**	**KR232409**	**KR232411**	**KR232413**
Neocercospora ammicola	CCTU 1187	**KR232408**	**KR232410**	**KR232412**	**KR232414**
Passalora eucalypti	CBS 111318	GU269845	GU384558	GU320548	–
Phloeospora ulmi	CBS 344.97	JQ324974	JQ324986	GU320528	–
Phloeospora ulmi	CBS 613.81	GU269825	JQ324987	GU320529	–
Pseudocercospora abelmoschi	CPC 14478; CBS 132103	GU269647	GU384365	GU320355	–
Pseudocercospora eucalyptorum	CPC 12568; CBS 132309	GU269796	GU384506	GU320497	–
Pseudocercospora humulicola	CPC 11358; CBS 131585	GU269723	GU384438	GU320427	–
Pseudocercospora norchiensis	CBS 114641	GU269772	GU384484	GU320475	–
Pseudocercospora vitis	CPC 11595; CBS 132012	GU269829	GU384541	GU320533	–
Pseudocercosporella arcuata	CPC 10050	GU269850	JQ325006	GU320554	–
Pseudocercosporella capsellae	CBS 112033	KF251306	KF253254	KF253615	–
Pseudocercosporella capsellae	CBS 127.29	KF251326	KF253273	KF253632	–
Pseudocercosporella oxalidis	CBS 118758	GU269756	GU384467	GU320458	–

1 CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; CCTU: Culture Collection of Tabriz University, Tabriz, Iran; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.

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

& Standley 2013). The alignments were manually checked and improved where necessary using MEGA v. 6 and were concatenated with Mesquite v. 2.75 (Maddison & Maddison 2011).

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate Bayesian Inference (BI) phylogenetic trees with Bayesian probabilities using MrBayes v. 3.2.2 (Ronquist *et al.* 2012). Models of nucleotide substitution were selected independently for each locus under the Akaike Information Criterion (AIC) using MrModeltest v. 2.3 (Nylander 2004). The analyses of two parallel MCMC algorithms, each consisting of four chains, were run from random trees for 100 000 000 generations until the average standard deviation of split frequencies reached a value of 0.01, with trees saved every 1 000 generations and the heating parameter was set to 0.15. The first 25% of saved trees were discarded as the 'burn-in' phase and the posterior probabilities (Rannala & Yang 1996) were calculated from the remaining trees. The resulting phylogenetic tree was printed with Geneious v. 5.6.7 (Drummond *et al.* 2012). All new sequences generated during this study were deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov) and the accession numbers of the sequences used for the phylogenetic analyses are listed in Table 1. The alignment and phylogenetic trees were deposited in TreeBASE (www.TreeBASE.org).

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-f1 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).

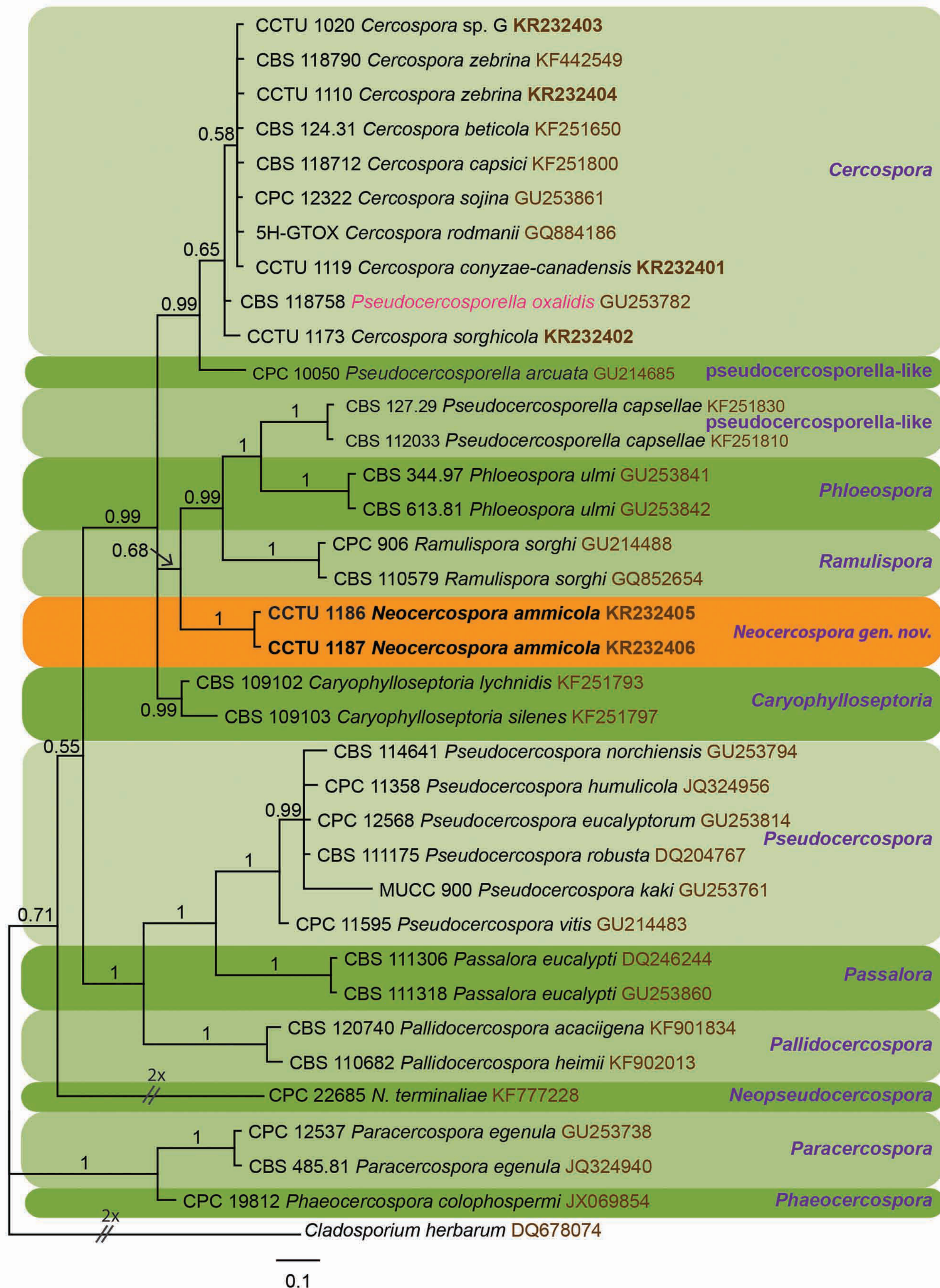

FIGURE 1. Consensus phylogram (50% majority rule) of 512 trees resulting from a Bayesian analysis of the LSU sequence alignment using MrBayes v. 3.2.2. The scale bar represents the average number of substitutions per site, and posterior probability values are shown at the nodes. GenBank accession numbers 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).

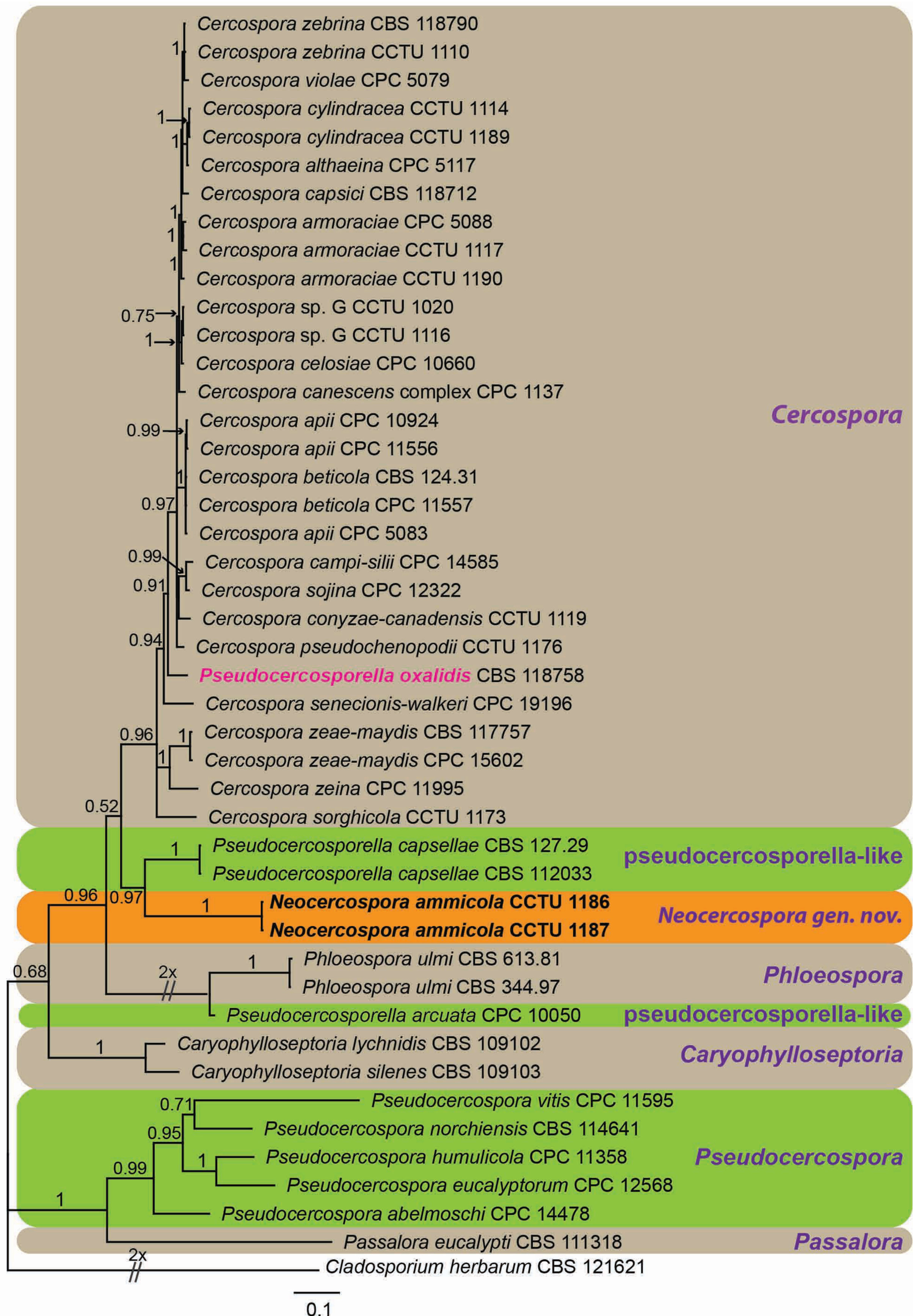

FIGURE 2. Consensus phylogram (50% majority rule) of 622 trees resulting from a Bayesian analysis of the combined 4-gene (ITS, TEF1-α, ACT and HIS) sequence alignment using MrBayes v. 3.2.2. The scale bar represents the average number of substitutions per site, and posterior probability values are shown 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, uni- to multilocal, 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 (1–12), arising from the upper cells of substomatal to intraepidermal brown stromata, up to 18 µm wide and 27 µm high, emerging through stomata or erumpent; conidiophores aseptate, reduced to conidiogenous cells. *Conidiogenous cells* unbranched, pale brown to brown, smooth, subcylindrical to cone-shaped, wider at the base, gradually becoming narrower towards the apex, occasionally geniculate-sinuous, (10–)14.5–17(–25) × 2.5–3.5(–4) µm, unilocal and multilocal, 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, 1–10-septate, (15–)35–50(–110) × (2.5–)3–3.5(–4) µm, apex obtuse, base obconically truncate or truncate with slight basal taper to hilum, 1–2 µm diam., 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 multilocal, 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 ammicola* (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).

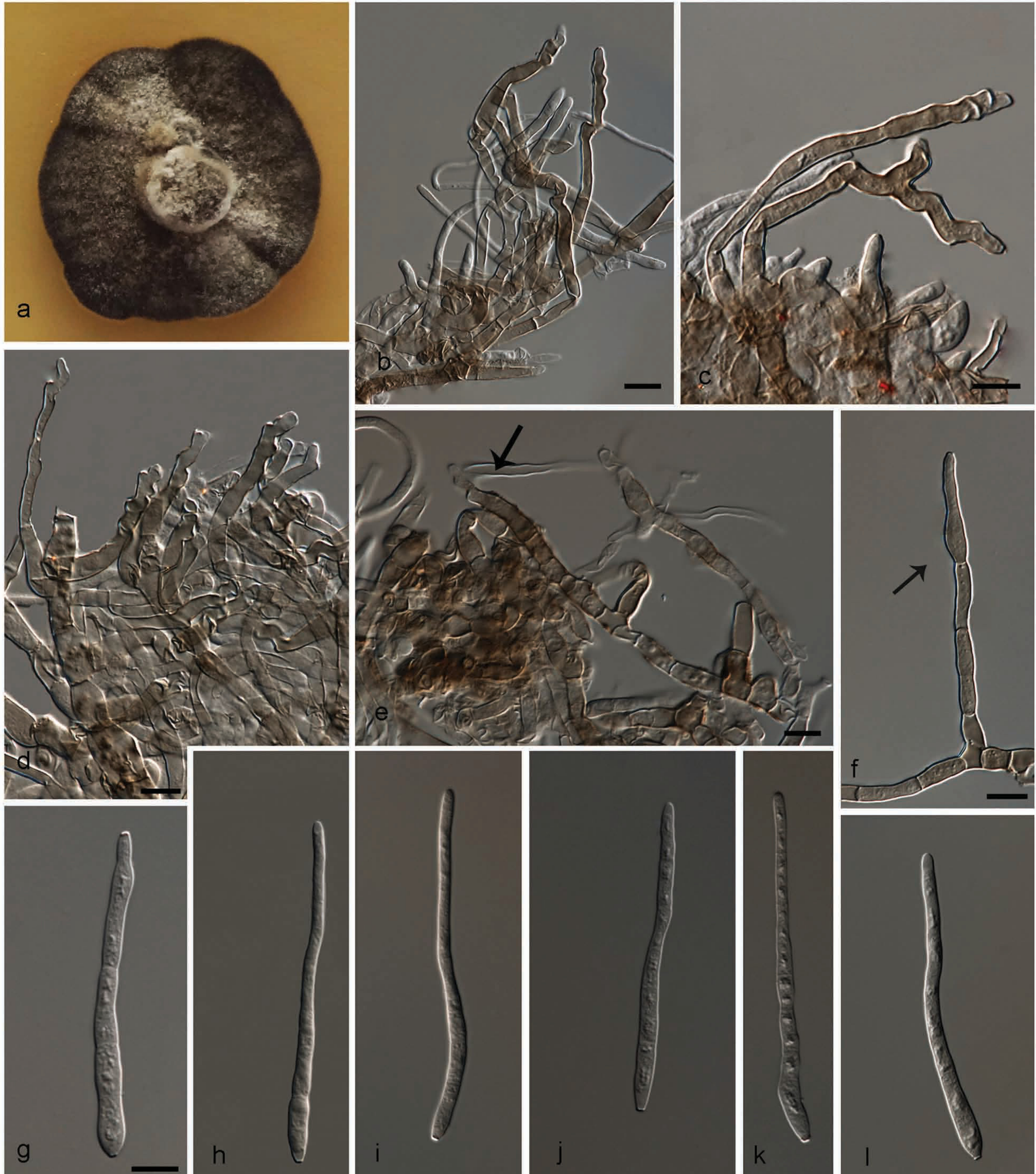

FIGURE 4. *Neocercospora ammicola* (CBS 136450) (*in vitro*). a. Colony on MEA. b–d. Conidiophores and conidiogenous cells. e. Terminal conidiophore on hypha. f. Conidiophore reduced to a conidiogenous cell. g–l. Solitary and catenate conidia. Scale bars = 10 µm.

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 hyphomycetes 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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