Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis

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Partial rDNA sequences were obtained from 26 isolates representing species of *Cercospora*, *Passalora*, *Paracercospora*, *Pseudocercospora*, *Ramulispora*, *Pseudocercosporella* and *Mycocentrospora*. The combined internal transcribed spacers (ITS) including the 5·8S rRNA gene and 5′ end of the 25S gene (primer pairs F63/R635) on rDNA were amplified using PCR and sequenced directly. The ITS regions including the 5·8S varied in length from 502 to 595 bp. The F63/R635 region varied from 508 to 519 bp among isolates sequenced. Reconstructed phylogenies inferred from both regions had highly similar topologies for the taxa examined. Phylogenetic analysis of the sequences resulted in four well-supported clades corresponding to *Cercospora*, *Paracercospora*/*Pseudocercospora*, *Passalora* and *Ramulispora*, with bootstrap values greater than 92% for each clade. Based on the results of the analysis, a new combination for *Pseudocercosporella aestiva* is proposed in *Ramulispora*, and *Paracercospora* is reduced to synonymy with *Pseudocercospora*.

Mycosphaerella (Dothideales, Mycosphaerellaceae) is one of the largest genera of ascomycetes (Corlett, 1991, 1995), and has been linked to at least 27 different coelomycete or hyphomycete anamorph genera (Kendrick & DiCosmo, 1979). Sutton & Hennebert (1994) reviewed this list of anamorph genera, many of which were incorrectly cited by von Arx (1983) as synonyms of cercosporoid genera that are presently recognized by Braun (1995*a*), or that have been shown to have teleomorph affinities elsewhere. Some of these include *Marssonina* with *Diplocarpon* (Sutton, 1980), *Polythrincium* with *Cymadothea* (Sivanesan, 1984) and *Phyllosticta* with *Guignardia* teleomorphs (van der Aa, 1973). Other genera were, however, accepted as synonyms, namely *Ramularia* (= *Ovularia*) (Hughes, 1949; Braun, 1995*a*), *Pseudocercospora* (= *Cercoseptoria*) (Deighton, 1987) and *Cladosporium* (= *Heterosporium*) (David, 1997). Twenty-three genera were eventually accepted as anamorphs of *Mycosphaerella* (Sutton & Hennebert, 1994). *Asteromella* is, however, now commonly accepted as the spermatial state that occurs with most species of *Mycosphaerella* (Crous & Wingfield, 1996). *Stigmina* Sacc. *sensu* Sutton & Pasco (1989) and *Lecanosticta* have been shown to not have teleomorphs in *Mycosphaerella* (Barr, 1996; Crous, 1998; Crous & Corlett, 1998; Crous, 1999), while new genera such as *Cercostigmina*, and *Xenostigmina* have been erected for *Stigmina*-like species with *Mycosphaerella* teleomorphs (Braun, 1993; Crous, 1998; Crous & Corlett, 1998). In addition, *Mycovellosiella* and *Thedgonia* have recently been linked to *Mycosphaerella* (Crous *et al*., 1998; Kaiser & Crous, 1998), as well as *Phaeophleospora* (= *Kirramyces*) (Crous & Wingfield, 1997*b*; Crous, Ferreira & Sutton, 1997; Crous, 1998), *Colletogloeopsis* (*Crous* & *Wingfield*, 1997*a*), *Uwebraunia* (Crous & Wingfield, 1996) and *Sonderhenia* (Park & Keane, 1984; Swart & Walker, 1988). Some of the genera reported as possible anamorphs of *Mycosphaerella* by Sutton & Hennebert (1994) are based on older literature citations reporting a species of *Mycosphaerella* as occurring in close proximity to a certain anamorph [i.e. *Toxosporium camptospermum* and *Mycosphaerella abietis* (Rostr.) Lindau *fide* Grove, 1937 (= *Delphinella abietis* (Rostr.) E. Müll.) *fide* Müller & Von Arx, 1962], and still have to be confirmed in culture.

To place this large number of anamorph genera into perspective, the various characters used to separate them need to be considered in more detail. In a treatment of cercosporoid fungi (generally accepted as genera having teleomorph affinities with *Mycosphaerella*), Chupp (1954) placed all species in *Cercospora*. Subsequent to this monographic study, Deighton's taxonomic treatment of this complex (1967, 1974, 1976, 1979) delimited species into smaller, more natural units that have subsequently been recognized in treatments of these fungi from Taiwan (Hsieh & Goh, 1990), China (Guo & Hsieh, 1995), South Africa (Crous & Braun, 1996), Russia (Braun & Melnik, 1997) and elsewhere. Features that are presently acknowledged as important in separating these genera include mycelium (presence or absence of superficial mycelium, and texture thereof), conidiophores (arrangement, branching, pigmentation), conidiogenous cells (placement, proliferation, scar type) and conidia (formation, shape, septation and pigmentation). Considerable attention has been given to conidiomatal structure and mode of conidiogenesis (von Arx, 1983; Sutton & Hennebert, 1994), while David (1993) used

scanning electron microscopy to show differences in the type of conidial scar structure between *Mycosphaerella* anamorphs such as *Cladosporium*, *Cercospora* and *Stenella*.

The proliferation of cercosporoid genera from the concept of Chupp (1954), to the nearly 50 recognized by Braun (1995*a*), may be explained in part, by the 43 different conidiogenous events and different conidiomatal structures recognized by Sutton & Hennebert (1994). Taking the plasticity of conidiogenesis and conidiomatal structures into consideration, however, many of these genera are in fact not so dissimilar. Species of *Pseudocercospora* can have conidiophores occurring separately on superficial mycelium, or aggregated in fascicles or sporodochia that appear almost acervular once subepidermal (Crous, 1998). The separation of coelomycete genera with acervuli and hyphomycetes with sporodochia is but one aspect that needs further study via molecular systematics.

With the advent of molecular techniques, nucleotide sequences sampled from genomes have been commonly employed in recent years by systematists to investigate the phylogeny of various groups of fungi. Genes coding for ribosomal RNAs and non-coding regions lying between these genes (rDNA) have commonly been used in phylogenetic studies (Bruns, White & Taylor, 1991; Lee & Taylor, 1991; Berbee & Taylor, 1992; Swann & Taylor, 1993; O'Donnell & Gray, 1995; O'Donnell *et al*., 1997). Ribosomal DNAs are composed of variously conserved and variable regions which have proven to be useful for inferring fungal relationships at different taxonomic levels (Bruns, White & Taylor, 1991). The internal transcribed spacers (ITS) and the adjacent 5′ end of the 25S rRNA gene (F63/R635), which is the most variable region of the gene (Peterson & Kurtzman, 1991), are often variable both within and between species (Lee & Taylor, 1991; Peterson & Kurtzman, 1991).

The present study presents the first in a series of molecular systematic studies towards the establishment of a more natural taxonomy for *Mycosphaerella* and its anamorph form genera. The aim of this study was first to establish if *Cercospora*, *Pseudocercospora*, *Paracercospora* and *Passalora* represent monophyletic taxa, and secondly to determine their relationship to non-*Mycosphaerella* cercosporoid genera such as *Ramulispora* (teleom. *Tapesia*) and *Mycocentrospora* (teleom. unknown).

MATERIALS AND METHODS

Fungal isolates and cultural growth. In total, 26 isolates representing seven species of *Cercospora*, two species of *Passalora*, two varieties of one species of *Paracercospora*, two species of *Pseudocercospora*, three species of *Ramulispora*, one species of *Pseudocercosporella*, and one species of *Mycocentrospora* were studied (Table 1). All culture isolates obtained for this study were initially grown on 20 ml V-8 juice agar (V-8 juice, 200 ml; $CaCO_3$, 3 g; agar, 20 g; distilled water, volume to 1000 ml) in 100 mm plastic Petri dishes. Working in a biological safety hood, inoculum from each isolate was aseptically ground in a 10-brock grinder to generate multiple hyphal fragments that were inoculated into 100 ml V-8 juice liquid growth medium (filtered V-8 juice, 100 ml; dextrose, 10 g; malt extract, 5 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; brought to 1000 ml with distilled water and adjusted to pH 4·5 using 1 N HCl) in a 500 ml Erlenmeyer flask. The flasks were incubated at 21 °C on a flat bed shaker for 5–7 d. At harvest mycelium was cleaned and separated from the growth medium by vacuum filtration through miracloth, followed by multiple washings in sterile, double distilled water. Harvested mycelium was labelled, immediately wrapped in aluminium foil, frozen using liquid nitrogen and then lyophilized for 24 h. The lyophilized mycelium was stored at −80° until needed for DNA extraction. Prior to harvest, growth medium and hyphae were examined microscopically to ensure cultural purity.

DNA Extraction. Genomic DNAs were isolated from lyophilised hyphae based on the method of O'Donnell (1992). The DNA was further purified by digestion with RNaseA to remove RNAs (Sambrook, Fritsch & Maniatis, 1989), followed by extraction with phenol/chloroform and ethanol precipitation. The final pellet was resuspended in 50–100 µl of TE buffer. Approximately 1 µg of DNA was usually obtained from 20 mg of lyophilized mycelium.

PCR amplification. The ITS and 5′ end of the 25S rDNA were amplified with primer pairs ITS4/ITS5 (White, Bruns & Taylor, 1990) and F63/R635 (Georgiev *et al*., 1981; Gueho, Kurtzman & Peterson, 1989), respectively. Double stranded products were obtained with Amplitaq® DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) in a 100 µl volume using the supplied buffer. Each PCR reaction contained 0·2 mM dNTPs and 0·1 µM of each primer. Fourty PCR cycles were performed on an MJ Research PTC-100-60 programmable Thermal Controller (MJ Research, Watertown, MA) using the following parameters: 94° (30 s), 50° (1 min), and 72° (2 min), followed by a final 10-min extension period at 72°. Amplified PCR products were purified by preparative gel electrophoresis in low melting point agarose (Gibco, BRL, Grand Island, NY). Single-stranded templates for DNA sequencing were generated by asymmetric amplification of purified double stranded PCR products using primer ratios of 1:50 or 1:25 with the aforementioned PCR programme. The asymmetric products were then purified using Millipore Ultrafree Microconcentrators 30000 NMWL membrane filter units (Millipore Bedford, MA).

DNA sequencing. Sequencing of single-stranded DNA templates was performed using Sequenase version 2.0 as described by the manufacturer (U.S. Biochemical, Cleveland, OH), except the initial labelling reactions were run for 3 min. Both strands of ITS and F63/R635 regions were sequenced using forward and reverse primers. Additional accuracy within the ITS region was achieved by using the two internal primers ITS2 and ITS3 (White *et al*., 1990). Sequencing reactions with both dGTP and dITP were run in parallel for each single-stranded template to resolve compressions due to secondary structure problems. Single-stranded PCR products for *Cercospora apii* (CA2), *Cercospora hayi* (CH6) and *Paracercospora fijiensis* (PF7) could not be amplified efficiently when ITS5 and R635 were used as excess primers. These templates were

Table 1. Cercosporoid isolates included in the analyses, abbreviations, taxa, teleomorph state if known, and origin for ITS and F63/R635 sequences. (ATCC = American Type Culture Collections; MPPD = St Paul, Univ. Minnesota Plant Pathology Dept)

		Teleomorph State	Origin
CA1	*Cercospora apii* Fresen.	—	ATCC 12246
CA2	*C. apii*	—	MPPD 12119 (Celery, Wisconsin)
CA29	*C. apii*	—	ATCC 26667 (Celery, Florida)
CB4	*C. beticola* Sacc.	—	MPPD 12120 (Sugar beet, Minnesota)
CCA19	*C. canescens* Ellis & G. Martin	—	ATCC 32779 (Mungbean, Taiwan)
CH5	*C. hayi* Calp.	—	ATCC 12234 (Banana, Cuba)
CH6	*C. hayi*	—	ATCC 28246 (Banana, Honduras)
CK35	*C. kikuchii* (T. Matsumoto & Tomoy.) M. W. Gardner	—	Soybean, Illinois
CK39	*C. kikuchii*	—	Soybean, Illinois
CN17	*C. nicotianae* Ellis & Everh.	—	ATCC 18366 (Tobacco, Tennessee)
CS42	*C. sojina* Hara	—	Race 6 (Soybean, North Carolina)
CS43	*C. sojina*	—	Race 2 (Soybean, North Carolina)
CS13	*C. sojina*	—	ATCC 44531 (Soybean, Illinois)
PP15	*Passalora personata* (Berk. & M. A. Curtis) S. H. Khan & M. Kamal	*Mycosphaerella berkeleyi* W. A. Jenkins	MPPD 12121 (Peanut, Oklahoma)
PA16	*P. arachidicola* (Hori) U. Braun	—	MPPD 12122 (Peanut, Oklahoma)
MA12	*Mycocentrospora acerina* (Hartig) Deighton	—	ATCC 34539 (Carrot, Norway)
PF7	*Paracercospora fijiensis* (M. Morelet) Deighton	*M. fijiensis* M. Morelet	ATCC 22116 (Banana, Philippines)
PF8	*P. fijiensis*	*M. fijiensis*	ATCC 22117 (Banana, Hawaii)
PFD9	*P. fijiensis* var. *difformis* (J. L. Mulder & R. H. Stover) Deighton	*M. fijiensis* var. *difformis* J. L. Mulder & R. H. Stover	ATCC 36054 (*Musa* AAA, Honduras)
PCR18	*Pseudocercospora cruenta* (Sacc.) Deighton	*M. cruenta* Latham	ATCC 262271 (Cowpea, Puerto Rico)
PM10	*Ps. musae* (Zimm.) Deighton	*M. musicola* J. L. Mulder	ATCC 22115 (Banana, Philippines)
PM11	*Ps. musae*	*M. musicola*	ATCC 36143 (*Musa* AAA, Honduras)
RAE22	*Ramulispora aestiva* (Nirenberg) E. L. Stewart & Crous	—	Wheat, Washington
RAN45	*R. anguioides* (Nirenberg) Crous	*Tapesia* state unknown	ATCC 60971 (Wheat, Germany)
RH26	*R. herpotrichoides* (Fron) Arx	*Tapesia yallundae* Wallwork & Spooner	Ph90-19-3 (Wheat, Washington)
RAC44	*R. acuformis* (Nirenberg) Crous	*T. acuformis* (Boerema, Pieters & Hamers) Crous	ATCC 60973 (Rye, Germany)

sequenced using purified double-stranded PCR products that were denatured either by alkali according to the manufacturer's instruction included in Sequenase version 2.0, or by boiling prior to sequencing (Winship, 1989). Electrophoreses of sequencing reactions were conducted on 5% acrylamide/8·3 M urea or 5% Long Ranger™ Acrylamine (AT Biochemicals, Malvern, PA). The gels were washed for 10 min with 1000 ml of 20% ethanol prior to drying to exposing to Kodak SB film. DNA sequences were read by an electronic digitizer (Bio-Rad Laboratories, Hercules, CA), aligned by Megalign module from the LaserGene software package version (1·60 dz (DNASTAR Inc. Madison, Wisconsin) using the CLUSTAL V method (Higgins, Bleasby, & Fuchs, 1991) and hand-adjusted by Program Editor of WordPerfect Library version 1.1 (WordPerfect Corporation, Orem, UT). Only slight visual adjustment was needed in a few obvious mismatch positions.

Phylogenetic analysis. *Mycocentrospora acerina* (MA12) was chosen as outgroup based on morphology and lack of a known teleomorph. Phylogenetic relationships among cercosporoid genera were estimated by using software packages MEGA version 1.01 (Kumar, Tamura & Nei, 1993) and PAUP version 3.1.1 (Swofford, 1993). Data analysis by the MEGA programme was run on a Dell Dimension TM P133V computer, whereas the PAUP analysis was run on a Power MacIntosh 63500/250 computer.

The Kimura 2-parameter, Jukes–Cantor and other algorithms were used to generate distance matrices using pre-aligned DNA sequences from all 26 isolates listed in Table 1. Sites containing alignment gaps were excluded either entirely or only during the pair-wise comparisons in the calculation. The phylogenies based on estimated distance matrices were then produced by the neighbour-joining (NJ) method (Saitou & Nei, 1987). Support for the internal branches was obtained by bootstrap analysis (Felsenstein, 1985) with 1000 replications.

When performing maximum parsimony (MP) analysis with PAUP, select, representative taxa having identical DNA sequences were excluded in the data set in order to run the data analysis efficiently. Consequently, the data sets for the F63/R635 region (5′ end of 25S rDNA), ITS region and their combination were reduced to 11, 17 and 17 taxa, respectively. Phylogenetic trees were then constructed using the heuristic search with 1000 random-addition sequences or the branch and bound search. Sequence gaps were treated as missing data or scored as additional character-states conveying phylogenetic information in separate analyses. A strict consensus tree would be determined if more than one maximally parsimonious tree was obtained. The validity of the resulting trees was tested by the bootstrap method with 1000 replications, and decay index analysis (Bremer, 1988) performed by saving and swapping on all trees up to 10 steps longer than the most parsimonious ones.

RESULTS

Nucleotide sequences of ITS and F63/R635 regions. The combined ITS regions including the 5·8S rRNA gene among cercosporoid taxa examined in this study varied in length from 502 bp in *Paracercospora fijiensis* to 595 bp in *Pseudocercospora* species. The ITS size in most taxa was, however, comparable to that of *P. fijiensis*. The 5·8S rDNA was highly conserved in all isolates sequenced. The sequence alignment of the combined ITS region showed that *Ramulispora* species each had several insertion sites that ranged from 7 to 31 bp in the ITS 1 spacer region that accounted for approximately 75 bp of the 595 bp sequence. The aligned ITS sequences, therefore, yielded 616 sites of which 235 positions were

Fig. 1

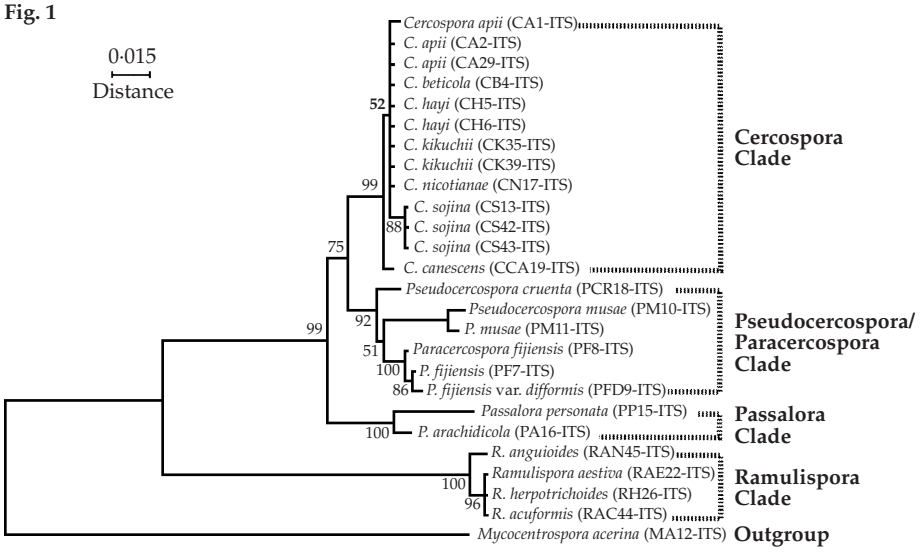
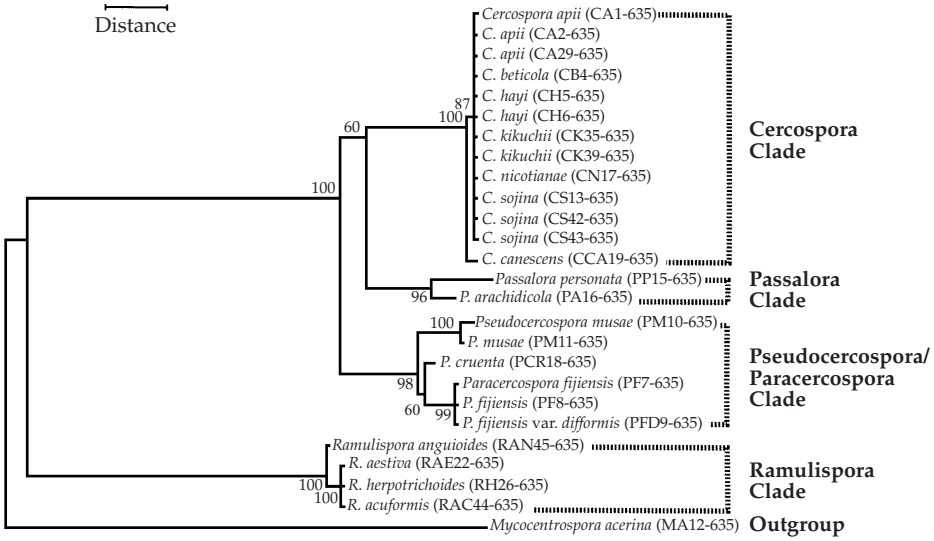


Fig. 2



Figs 1–2. For legend see facing page.

variable. Three isolates of *Cercospora apii* (CA1, CA2 and CA29) shared identical ITS 1 and 2 regions with selected isolates from *C. beticola* (CB4), *C. hayi* (CH6), and *C. nicotianae* (CN17). Similarly, isolates from *C. sojina* (CS13, CS42, CS43) and isolates of *Ramulispora aestiva* (RAE22) and *R. herpotrichoides* (RH26, RAC44), respectively, had identical sequences in the ITS region. There were 131 parsimony-informative sites among these aligned sequences.

The 5′ end of 25S rDNA sequenced with primer pair F63/R635 was 508–519 bp long. The introduction of alignment gaps extended the region to 522 bp in length of which 179 sites were variable. Unlike the ITS sequence, this region possessed only a few insertion/deletion sites and variable positions were more evenly distributed. Three isolates of *Paracercospora fijiensis* (PF7, PF8, PFD9) had identical F63/R635 sequences, as was the case with *Ramulispora aestiva* (≡ *Pseudocercosporella aestiva*) (RAE22) and *R. herpotrichoides* (RH26, RAC44). The same was also true for *C. apii* (CA1, CA2, CA29), *C. beticola*, *C. hayi* (CH5 and CH6), *C. kikuchii* (CK35, CK39), *C. nicotianae* (CN17), and *C. sojina* (CS13, CS42, CS43). There were 104 parsimony-informative sites among these sequences. Overall, as expected, the F63/R635 region exhibited less sequence variability than the ITS region. The average distances between taxa were 4·3% for F63/R635 and 10·1% for ITS respectively, calculated after removing gap sites from the data sets. These values were estimated from 17 taxa with distinct sequences. Mean percentage of nucleotide frequencies for A, T, C, and G on the ITS and F63/R635 regions were 21·5, 25·4, 26·1 and 27·1, and 24·2, 22·8, 22·7 and 30·3, respectively.

Phylogenetic analysis. Phylogenetic trees obtained using the Kimura 2-parameter model and NJ tree-building method from two DNA sequence data sets alone and in combination are shown in Figs 1–3. Sites consisting of the alignment gaps were removed before the distance calculation. Analyses were

Fig. 3

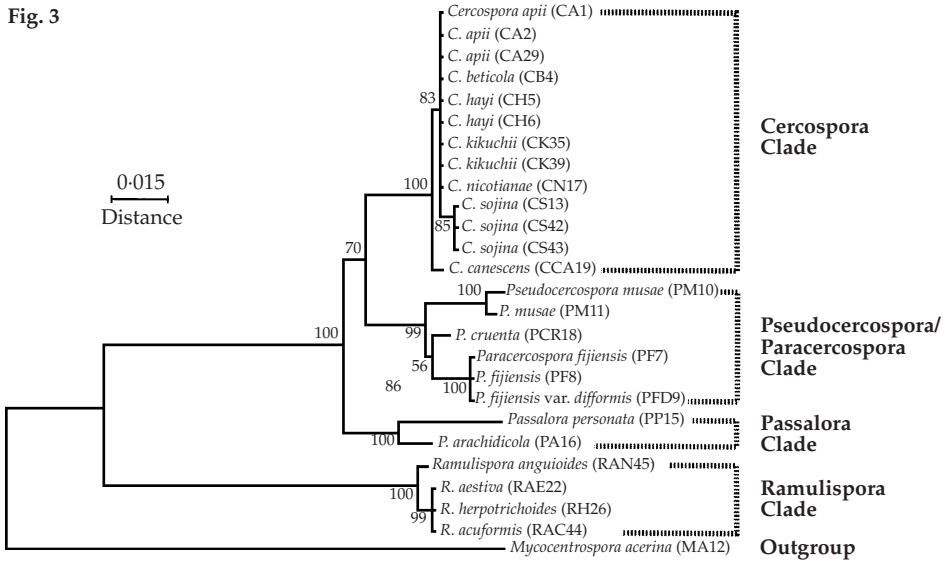
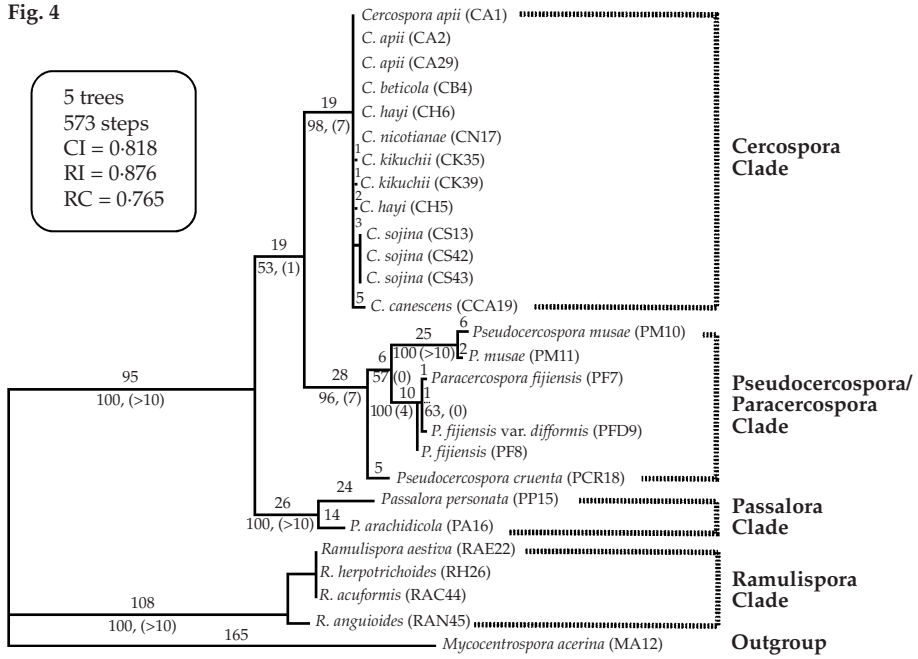


Fig. 4



Figs 1–4. Phylogenetic relationships among species of *Cercospora*, *Passalora*, *Paracercospora*, *Pseudocercospora*, *Ramulispora*, *Pseudocercosporella* and *Mycocentrospora*. The phylogeny for Figs 1–3 was constructed with the Kimura 2-parameter model and NJ tree-building method using MEGA version 1.01. Numbers along the nodes are bootstrap values > 50%. **Fig. 1.** Determined from ITS DNA sequences. **Fig. 2.** Determined from F63/R635 DNA sequences. **Fig. 3.** Determined from combined ITS and F63/R635 DNA sequences. **Fig. 4.** Determined from combined ITS and F63/R635 DNA sequences. The phylogeny was constructed by the branch and bound search option with 1000 bootstrap replication using PAUP 3.1.1. This consensus of five trees has a total length of 573 steps. Numbers above the nodes and branches are branch lengths. Numbers preceding parentheses are percentages determined from bootstrap analyses. Numbers in parentheses are decay values.

also conducted such that gaps were only excluded during the pair-wise comparisons. Similar tree topologies and branch lengths as well as bootstrap values were, however, obtained as shown in Figs 1–3 indicating that these gaps did not introduce complications in distance estimation. Phylogenetic inference based on distance analysis can be affected by the A, T, C and G frequencies, transition/transversion substitution ratios and the rate of nucleotide substitution at each site (Kumar *et al*., 1993). Various distance-estimating models addressing these issues such as Jukes-Cantor, Tajima-Nei, Tamura, Tamura-Nei and Gamma distances included in the MEGA package were also used to construct phylogenetic trees in this study. They all gave nearly identical topographies and similar bootstrap values of nodes, as indicated in Figs 1–3 for individual and combined data sets.

Phylogenetic analysis by the MP method was performed

using a subset of taxa representing those with identical DNA sequences. This strategy reduced the number of parsimony-informative sites from 133, 116 and 249 to 131, 104 and 235 respectively, for the original ITS, F63/R635 and combined ITS, F63/R635 sequence data for 26 isolates. For all data sets, including the combined data with 20 sequence gaps scored as phylogenetic informative sites, trees with highly similar topologies to NJ trees were derived using either the heuristic or the branch and bound search option. A parsimony analysis of 17 taxa with combined ITS and F63/R635 sequences based on the branch and bound search yielded five most parsimonious trees with 573 steps. The strict consensus of the five trees is shown in Fig. 4, on which the taxa with the same sequences as their representatives were added to their respective terminal nodes. The consistency (CI), retention (RI) and rescaled consistency (RC) indices for the trees were 0·818, 0·876 and 0·765, respectively. Similar analyses produced a strict consensus (358 steps, CI = 0·853, RI = 0·835, RC = 0·712) of 12 trees for the ITS region, a strict consensus (218 steps, CI = 0·881, RI = 0·877, RC = 0·773) of two trees for the F63/R635 region and a consensus (604 steps, CI = 0·858, RI = 0·874, RC = 0·750) of five trees for the combined data with some gaps scored as informative sites (data not shown). Based on three NJ trees (Figs 1–3) and a MP tree (Fig. 4), four well-supported clades [*Cercorspora* (CE) clade, *Paracercospora*/*Pseudocercospora* (PP) clade, *Passalora* (PA) clade, and *Ramulispora* (RA) clade] were consistently observed with bootstrap values greater than 92% for each clade. The results show that the ITS and F63/R635 regions (Figs 1, 2) both can be used to infer phylogenies among cercosporoid genera. The placement of the PA clade was different in trees generated from ITS and F63/R635 regions (Figs 1, 2). The PA clade appeared as a sister group to the CE clade in the NJ analysis of the F63/R635 region (Fig. 2). When the ITS alone or combined ITS and F63/R635 data were analysed, however, the PA clade consistently appeared as a sister group to the CE and PP clades in both NJ and MP analyses (Figs 1, 3, 4). The difference may be caused by short branch lengths connecting these groups to their sister clades. More characters are needed to thoroughly resolve the relationships among these clades. Similarly, the combined data could not resolve a decisive position for *Pseudocercospora cruenta* within the PP clade (Figs 1–4). The support for the interior nodes by decay analysis in the MP tree (Fig. 4) was generally in agreement with the bootstrap analysis. The four clades were also strongly supported by decay analysis (Fig. 4).

DISCUSSION

In this study, analysis of aligned sequences of the ITS region and 5′ end of the 25S gene on rDNA has allowed phylogenetic relationships to be estimated among selected cercosporoid taxa. The results showed that the phylogenetic trees generated from both regions had mostly similar topologies for the taxa examined, even though the ITS region revealed more overall variability than the F63/R635 region, but only slightly more in terms of phylogenetically informative sites. The combined data appear to enhance the phylogenetic resolution as reflected in a slight increase in bootstrap support. Phylogenetic reconstruction with these data has consistently identified well-supported monophyletic clades using various estimating models. Most clades are, at least to some degree, supportive of the traditional taxonomies.

Cercospora clade (*teleomorph*, Mycosphaerella)

Cercospora is presently confined to species that have fasciculate, pigmented conidiophores, usually situated on a weakly developed stroma, and sympodial proliferating conidiogenous cells with darkened, thickened, refractive loci. Conidia are hyaline (rarely subhyaline), acicular to narrowly cylindrical-filiform, multiseptate, with darkened, thickened, refractive conidial hila. Conidial scars in *Cercospora sensu stricto* are darkened throughout with the exception of a minute central pore (Braun, 1995*a*). The fact that all the *Cercospora* spp. compared in this study formed a single cluster supports the concept of *Cercospora sensu* Braun (1994).

Pseudocercospora/Paracercospora *clade* (*teleomorph*, Mycosphaerella)

Pseudocercospora Speg., *An. Mus. Nac. Hist. B. Aires* **20**: 438 (1910); *emend* Deighton (1976).

= *Helicomina* L. S. Olive, *Mycologia* **40**: 17 (1948).

= *Ancylospora* Sawada, *Rep. Govt. Res. Inst. Formosa* **87**: 78 (1944) (*nom. non rite publ.*).

= *Cercocladospora* G. P. Agarwal & S. M. Singh, *Proc. Natn. Acad. Sci. India, B*, **42**: 439, 1972 (1974), *fide* Deighton (1976).

= *Cercosporiopsis* Miura, *Flora of Manchuria and East Mongolia, 3, Cryptogams* 527 (1928) (*nom. illegit.*).

= *Cercoseptoria* Petr., *Annls. Mycol.* **23**: 69 (1925), *fide* Deighton (1987).

= *Paracercospora* Deighton, *Mycol. Pap.* **144**, 47–48 (1979).

With several recent large treatments of cercosporoid fungi (Hsieh & Goh, 1990; Goh & Hsieh, 1995; Crous & Braun, 1996), it has become obvious that *Pseudocercospora* contains an extraordinarily large number of species. Cercosporoid taxa with pigmented conidiophores (on superficial mycelium, in fascicles or sporodochia), with unthickened conidial loci, and pigmented scolecospores, multiseptate conidia with unthickened conidial hila are accommodated in *Pseudocercospora*. Conidiogenous cells usually proliferate sympodially, although fine, inconspicuous percurrent proliferations are found to be present on conidiogenous cells of most species. In species where percurrent proliferation is the norm, *Cercostigmina* is more suitable, which forms an intermediate between *Pseudocercospora* and *Stigmina*, which again has wide, flaring, irregular, percurrent proliferations and distoseptate conidia. Several species not fitting the concept of *Pseudocercospora sensu stricto* by having some conidial catenulation, hyaline conidia or conidia with oblique and longitudinal septa have, however, also been included in *Pseudocercospora*, making the distinction between this and similar genera more difficult (Braun, 1995*a*). The species compared in the present study are representative of *Pseudocercospora sensu stricto*, and form one cluster as was expected. The fact that *Paracercospora* isolates also cluster in this clade, however, raise serious doubts about the validity of the latter genus.

Deighton (1979) separated *Paracercospora* from *Pseudocercospora* based on the narrow thickening along the rim of the scar on the conidiogenous cell and the conidial hilum. Crous (1998) recently described *Pseudocercospora basiramifera* Crous from *EucHalyptus*. *In vivo* the conidiogenous cells and conidia clearly exhibited a narrow thickening along the rim as defined for *Paracercospora*. Once cultured, however, this thickening was no longer visible on either the conidial hila or conidiogenous loci. Based on the unreliability of this feature in culture, the latter species was therefore described in *Pseudocercospora*. The findings from the present study clearly showed isolates of these two genera to represent one group, and further support reducing *Paracercospora* to synonymy with *Pseudocercospora*.

Passalora *clade* (*teleomorph*, Mycosphaerella)

Passalora (= *Cercosporidium*) is primarily distinguished from *Phaeoisariopsis* by not having synnematal conidiomata (Braun, 1995*b*). Essentially *Passalora* contains *Cercospora*-like species with slightly thickened conidial scars and subglobose, ellipsoid-ovoid or broadly obclavate-fusiform, sparsely septate conidia, as well as species with obclavate-fusiform, pigmented, multiseptate conidia. From this description, it thus becomes clear that the generic circumscription of *Passalora* is such that it accommodates species not easily placed in its segregate genera. Further research comparing more species of the *Passalora*/*Phaeoisariopsis*/*Cercospora*-complex would be required to address this issue. *Passalora personata*, which is regarded as a species of *Passalora sensu stricto*, *grouped separately from the Cercospora* and *Pseudocercospora* clades in this study, thus supporting the traditional morphological scheme.

Mycocentrospora (teleomorph unknown)

Mycocentrospora represents a heterogeneous assemblage of species ranging from saprotrophys to aquatic hyphomycetes. It was recently treated by Braun (1993), who confined it to species with rostrate *Mycocentrospora*-like conidia with pedicellate bases. *M. acerina*, the type species of the genus, was used as an outgroup in this study. Presently no teleomorph is known, and results of this study found this isolate to group quite distinct from those clades with *Mycosphaerella* teleomorphs, suggesting that its teleomorph affinities would reside elsewhere.

Ramulispora *clade* (*teleomorph*, Tapesia)

Ramulispora aestiva (Nirenberg) E. L. Stewart & Crous, comb. nov.

≡ *Pseudocercosporella aestiva* Nirenberg, *Z. Pflanzenkr. Pflanzenschutz* **88**: 246 (1981).

Four *Pseudocercosporella*-like species have been associated with eyespot disease of wheat, namely *R. herpotrichoides*, *R. acuformis*, *R. anguioides* and *Ps. aestiva*. Based on RAPD polymorphisms and distinct mating types, *R. herpotrichoides* and *R. acuformis*, which were formerly considered as varieties of *R. herpotrichoides*, were distinguished from each other, and their teleomorphs recognized as species of *Tapesia* (Robbertse, Campbell & Crous, 1995; Dyer *et al*., 1996). The anamorph genus *Ramulispora*, which is confined to species occurring in grasses and cereals (von Arx, 1983), and has *Tapesia* teleomorphs, is morphologically similar to species of *Pseudocercosporella*, which have *Mycosphaerella* teleomorphs. In their treatment of this group of cereal pathogens, Robbertse *et al*. (1995) were uncertain of the taxonomic status of *Ps. aestiva* Nirenberg, and retained it in *Pseudocercosporella*. Braun (1995*a*) also stated that its status remains unclear. In the present study, however, all the species of *Ramulispora* formed one clade with *Ps. aestiva*, which suggests that it has affinities to *Tapesia*, and not *Mycosphaerella*. Based on these results, a new combination for *P. aestiva* is thus proposed in *Ramulispora*.

Is Mycosphaerella *heterogeneous?*

Crous (1998) suggested that *Mycosphaerella* is polyphyletic. This concept is not altogether novel to mycologists and plant pathologists, and various divisions have been proposed by Klebahn (1918), von Arx (1949, 1983) and Barr (1972). Klebahn (1918) proposed *Septorisphaerella* for species with *Septoria* anamorphs, *Ramulisphaerella* for those with *Ramularia* anamorphs, and *Cercosphaerella* for those with *Cercospora* anamorphs. A comparison of the conidiogenous events and conidiomatal structures also led Sutton & Hennebert (1994) to conclude that although *Mycosphaerella* is heterogeneous, certain series of anamorph genera could be recognized based on these features. In a subsequent study comparing 18 different *Mycosphaerella* anamorphs occurring on *Eucalyptus* (Crous, 1998), 27 different anamorph and teleomorph features were used to group species using multiple correspondence analysis (Greenacre, 1992). Several groups were recognized. Coelomycete anamorph species in *Sonderhenia* formed a group separate from those in *Colletogloeopsis* and *Phaeophleospora*. Among the cercosporoid taxa, species of *Uwebraunia* clustered separate from *Mycovellosiella*, while the *Pseudocercospora* spp. formed another group, including a species of *Stenella*, which was unexpected. Although correspondence analysis has its limitations, it clearly illustrated the heterogeneous nature of *Mycosphaerella*. The rDNA sequence analysis of the present study also separated cercosporoid genera into groups corresponding to *Cercospora*, *Pseudocercospora* and *Passalora*. The cercosporoid genera compared in this study still represented a monophyletic group, however, distinct from *Ramulispora* and *Mycocentrospora*, which lack *Mycosphaerella* teleomorphs. These results further suggest that the concepts of Klebahn (1918), proposing the segregation of genera from *Mycosphaerella* on the basis of their anamorphs should be explored further. Future research comparing various coelomycete and hyphomycete anamorphs of *Mycosphaerella* is presently underway to further clarify the generic bounries of the anamorph form genera and teleomorphs, and to test the hypothesis that *Mycosphaerella* is polyphyletic.

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