Phylogenetic Analysis of *Cercospora* and *Mycosphaerella* Based on the Internal Transcribed Spacer Region of Ribosomal DNA

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Accepted for publication 26 March 2001.

ABSTRACT

Goodwin, S. B., Dunkle, L. D., and Zismann, V. L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the internal transcribed spacer region of ribosomal DNA. Phytopathology 91:648-658.

Most of the 3,000 named species in the genus *Cercospora* have no known sexual stage, although a *Mycosphaerella* teleomorph has been identified for a few. *Mycosphaerella* is an extremely large and important genus of plant pathogens, with more than 1,800 named species and at least 43 associated anamorph genera. The goal of this research was to perform a large-scale phylogenetic analysis to test hypotheses about the past evolutionary history of *Cercospora* and *Mycosphaerella.* Based on the phylogenetic analysis of internal transcribed spacer (ITS) sequence data (ITS1, 5.8S rRNA gene, ITS2), the genus *Mycosphaerella* is monophyletic. In contrast, many anamorph genera within *Mycosphaerella* were polyphyletic and were not useful for grouping species. One exception was *Cercospora*, which formed a highly supported monophyletic group. Most *Cercospora* species from cereal crops formed a subgroup within the main *Cercospora* cluster. Only species within the *Cercospora* cluster produced the toxin cercosporin, suggesting that the ability to produce this compound had a single evolutionary origin. Intraspecific variation for 25 taxa in the *Mycosphaerella* clade averaged 1.7 nucleotides (nts) in the ITS region. Thus, isolates with ITS sequences that differ by two or more nucleotides may be distinct species. ITS sequences of groups I and II of the gray leaf spot pathogen *Cercospora zeae-maydis* differed by 7 nts and clearly represent different species. There were 6.5 nt differences on average between the ITS sequences of the sorghum pathogen *Cercospora sorghi* and the maize pathogen *Cercospora sorghi* var. *maydis*, indicating that the latter is a separate species and not simply a variety of *Cercospora sorghi*. The large monophyletic *Mycosphaerella* cluster contained a number of anamorph genera with no known teleomorph associations. Therefore, the number of anamorph genera related to *Mycosphaerella* may be much larger than suspected previously.

Additional keywords: *Dothistroma*, *Lecanosticta*, mating type, *Mycocentrospora*.

Fungi in the genus *Cercospora* are among the most prevalent and destructive plant pathogens. As a group, they are nearly universally pathogenic, occurring on a wide range of hosts in almost all major families of dicots, most monocot families, and even some gymnosperms and ferns (26). Chupp (6) listed over 1,800 species names in his monograph of the genus in 1954, and the list had grown to over 3,000 by 1987 (26). In a major effort to clarify the taxonomy of the genus, Deighton (12–15) segregated and reclassified many *Cercospora* species into other genera, including *Cercosporella*, *Cercosporidium*, *Paracercospora*, *Pseudocercospora*, *Pseudocercosporella*, and *Pseudocercosporidium*, among others. This broad assemblage is referred to as the *Cercospora* complex, with members of *Cercospora* proper having conidia that are acicular, hyaline, and septate with a conspicuous hilum produced on pigmented, unbranched, septate, smooth conidiophores (17,27).

Many species of *Cercospora* are characterized by the production of a phytotoxic metabolite of polyketide origin called cercosporin (3). Although this compound may enhance virulence (33), it is not a universal pathogenicity factor because it is not produced by all species (3,16,18,21). Fajola (18) concluded that cercosporin production is associated with "true" *Cercospora* species and suggested that those species that do not produce cercosporin may belong to other, related genera. However, the ability to produce cercosporin is often specific to strains or isolates (16,21,35), and is influenced by various environmental and nutritional conditions (21). These inconsistencies preclude definitive application of cercosporin production to taxonomy.

Due to the paucity of useful morphological and physiological characters, taxonomy of the *Cercospora* complex remains confusing and depends heavily on the host. This is further complicated because most species have no known sexual stage. For those few species in which a sexual stage has been identified, the teleomorph is in the genus *Mycosphaerella* (5–7,29,34). Examples include the banana pathogens *Mycosphaerella fijiensis* (*Cercospora fijiensis* = *Paracercospora fijiensis*) and *Mycosphaerella musicola* (*Cercospora musae* = *Pseudocercospora musae*) and the peanut pathogen *Mycosphaerella arachidis* (*Cercospora arachidicola*) (7). Many other associations between *Cercospora* species and *Mycosphaerella* teleomorphs have been reported but not confirmed.

Mycosphaerella also is a very large genus, with over 1,800 names and at least 500 species associated with more than 40 anamorph genera (7). Similar to *Cercospora*, the taxonomy of *Mycosphaerella* is complicated, and several competing classification systems have been proposed (5,29,34). Due to the large number of associated anamorphs, Crous and Wingfield (10) concluded that *Mycosphaerella* was a polyphyletic assemblage of presumably monophyletic anamorph genera. Barr (5) agreed, and separated

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Publication no. P-2001-0511-01R

species with *Dothistroma* and *Lecanosticta* anamorphs into a new genus, *Eruptio*. There clearly is a great need for increased understanding of the phylogenetic relationships within *Mycosphaerella*.

Recent molecular analyses have begun to clarify the taxonomic confusion surrounding *Mycosphaerella* and a few of its associated anamorph genera. Stewart et al. (30) used ribosomal DNA sequence analyses to divide species with cercosporoid anamorphs into three clusters. One group corresponded to the genus *Cercospora sensu stricto*, the second included *Paracercospora* and *Pseudocercospora*, and the third was composed of species of *Passalora*. Because no other species with *Mycosphaerella* teleomorphs were included, it was not possible to determine the phylogenetic relationships of the cercosporoid species to other anamorph genera. Goodwin and Zismann (20) identified a monophyletic group that included six out of seven species of *Mycosphaerella* tested. The *Mycosphaerella* cluster included seven anamorph genera, two of which had no known teleomorph associations. The only exception was *Mycosphaerella pini* (anamorph *Dothistroma septospora*), which clustered outside the main *Mycosphaerella* group. No species of *Cercospora* were included in that study. Therefore, the relationships between *Cercospora* and the other anamorphs tested could not be determined.

Neither of the previously described studies included species of *Cercospora* infecting cereal crops. One *Cercospora* of recent importance to grain production is the gray leaf spot pathogen of maize, *Cercospora zeae-maydis* (23). Although epidemics of gray leaf spot have caused substantial economic losses in the midwestern and eastern U.S. corn belts during the past several years, nothing is known about the phylogenetic relationships of the causal organisms. Analyses of amplified fragment length polymorphisms (AFLPs) and internal transcribed spacer (ITS) sequence data revealed that gray leaf spot is caused by two sibling species of *Cercospora*, designated group I and group II (16,35). ITS sequences of the two groups differ by 7 nucleotides (nts); based on AFLP data, the groups are as different from each other as they are from the sorghum pathogen *Cercospora sorghi* or the soybean pathogen *Cercospora kikuchii* (35). The gray leaf spot sibling species also differed in their production of cercosporin; isolates of *Cercospora zeae-maydis* group I produce cercosporin, whereas those of group II do not (16,35). *Cercospora* may contain other cryptic species in addition to those within *Cercospora zeae-maydis*. For example, due to their different host specificities, Chupp (6) suggested that the sorghum pathogen *Cercospora sorghi* and the corn pathogen *Cercospora sorghi* var. *maydis* might be different species, even though they are identical morphologically. However, the evolutionary relationships of these two taxa to each other, to the gray leaf spot pathogens, and to other species of *Cercospora* are not known.

The purpose of this research was to perform a large-scale phylogenetic analysis of the genus *Mycosphaerella* and associated anamorphs to test hypotheses about the evolutionary history of the genus *Cercospora*. The first goal was to test the hypothesis that the *Cercospora* species from cereal crops form a monophyletic group with the true *Cercospora* clade as defined by Stewart et al. (30). The second goal was to develop empirical data on the number of nucleotide differences within and between species to determine whether groups I and II of *Cercospora zeae-maydis* represent different species. The third goal was to test Chupp's (6) hypothesis that *Cercospora sorghi* and *Cercospora sorghi* var. *maydis* are different species. The fourth goal was to test the hypothesis that *Cercospora* species that produce cercosporin form a monophyletic group. Within *Mycosphaerella*, the primary goal was to test the hypothesis of Crous and Wingfield (10) and Barr (5) that the genus *Mycosphaerella* is polyphyletic. A secondary goal within *Mycosphaerella* was to determine whether anamorph genera are monophyletic.

MATERIALS AND METHODS

Sources of isolates and culture methods. The ITS region (ITS1, 5.8S rRNA gene, ITS2) was sequenced from 15 isolates representing five species each of *Cercospora* and *Mycosphaerella* (Table 1). Most isolates were received as axenic cultures from collaborators or were purchased from the American Type Culture Collection (*Mycosphaerella citrullina* and *Mycosphaerella fragariae*). Cultures of *Cercospora kalmiae* and *Mycosphaerella macrospora* were isolated from infected leaves of mountain laurel (*Kalmia latifolia*) and iris (*Iris germanica*), respectively, showing symptoms of leaf spot disease. ITS sequences of *Mycosphaerella brassicicola* were obtained from DNA samples provided by G. Kema (Wageningen, the Netherlands). For DNA extraction, cul-

TABLE 1. Summary information for isolates of six anamorph genera analyzed for the internal transcribed spacer sequence database

Anamorph	Teleomorph	Isolate	Host	Location	GenBank no.
Ascochyta cucumis	*Mycosphaerella citrullina*[a]	ATCC 16241[b]	*Cucumis melo*	Florida	AF297228
Asteromella brassicae	*Mycosphaerella brassicicola*	IPO99156	*Brassica oleracea*[c]	France	AF297227
Asteromella brassicae	*Mycosphaerella brassicicola*	IPO99157	*Brassica oleracea*[c]	France	AF297236
Asteromella brassicae	*Mycosphaerella brassicicola*	IPO99510	*Brassica oleracea*[d]	The Netherlands	AF297223
Cercospora arachidicola	*Mycosphaerella arachidis*	–[e]	*Arachis hypogaea*	–	AF297224
Cercospora asparagi	–	–	*Asparagus officinalis*	–	AF297229
Cercospora beticola	–	–	*Beta vulgaris*	–	AF297222
Cercospora kalmiae	–	Ceka 1	*Kalmia latifolia*	Virginia	AF297226
Cercospora kikuchii	–	C4RI99	*Glycine max*	Indiana	AF291708
Cercospora nicotianae	–	ATCC 18366	*Nicotiana tabacum*	Tennessee	AF297230
Cercospora sorghi[f]	–	TX3	*Sorghum bicolor*	Texas	AF291707
Cercospora sorghi var. *maydis*	–	NC	*Zea mays*	North Carolina	AF297233
Cercospora sorghi var. *maydis*	–	Kenya 1	*Zea mays*	Kenya	AF297232
Cercospora zeae-maydis group I[f]	–	GBIN11	*Zea mays*	Indiana	AF291709
Cercospora zeae-maydis group II[f]	–	LSNCX1	*Zea mays*	North Carolina	AF291710
Cladosporium iridis	*Mycosphaerella macrospora*	Myma 1	*Iris germanica*	Indiana	AF297231
Paracercospora fijiensis	*Mycosphaerella fijiensis*	rCRB2	*Musa* sp.	–	AF297234
Paracercospora fijiensis	*Mycosphaerella fijiensis*	8837	*Musa* sp.	–	AF297225
Ramularia brunnea	*Mycosphaerella fragariae*	ATCC 24113	*Fragaria*	Illinois	AF297235

[a] This culture was listed as *Mycosphaerella citrullina* by ATCC, but is considered *Didymella bryoniae* by Corlett (7). Cluster analysis confirmed that it is not related to *Mycosphaerella*.
[b] Accession number, American Type Culture Collection.
[c] Cauliflower.
[d] Brussels sprouts.
[e] Not known.
[f] From Wang et al. (35).

tures of *Cercospora arachidicola*, *Cercospora kalmiae*, and *Mycosphaerella macrospora* were grown in complete medium (CM) (10 ml of solution A [10 g of $Ca(NO_3)_2 \cdot 4H_2O$ in 100 ml of H_2O], 10 ml of solution B [2 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, and 1.5 g of NaCl in 100 ml of H_2O, pH 5.3], 10 g of glucose, 1 g of yeast extract, and 1 g of casein hydrolysate in 1 liter of total volume), that of *Mycosphaerella fragariae* in malt medium (15 g of malt extract, 3 g of peptone, and 30 g of glucose per liter), and those of *Mycosphaerella fijiensis* in potato dextrose broth (Difco Laboratories, Detroit). The isolate of *Mycosphaerella citrullina* was grown in rabbit-food medium (25 g of commercial rabbit-food pellets per liter) and those of *Cercospora sorghi* var. *maydis* in V8 medium as described in Wang et al. (35). The remaining isolates were grown in both CM and malt media. Cultures were grown at room temperature on a shaking platform at 150 rpm, harvested by vacuum filtration, lyophilized overnight, and stored at –80°C. All isolates were maintained on solid media (the same as described previously for each species but with 1.5% agar) at room temperature. Long-term storage of cultures was on lyophilized filter paper disks at –80°C or as agar disks containing mycelia in water at 4°C. Kanamycin (50 μg/ml) was added to all media to prevent bacterial contamination.

DNA extraction, polymerase chain reaction amplification, and sequencing. DNA was extracted according to the method of Ossanna and Mischke (25) with minor modifications (20) and was quantified with a fluorometer (DyNAQuant 2000; Hoefer Scientific Instruments, San Francisco). The complete ITS region of each species was amplified with primers ITS4 and ITS5 of White et al. (36). Amplification was completed in a thermalcycler (9600; Perkin-Elmer, Foster City, CA) as described by Nakasone (24) with the following cycling parameters: 94°C for 2 min, 30 cycles of 93°C for 30 s, 53°C for 2 min, 72°C for 2 min, and a final extension of 10 min at 72°C. Amplification of products of the correct size was verified on 1% agarose gels. The remaining amplified product was purified with a polymerase chain reaction (PCR) prep kit (Wizard; Promega Corp., Madison, WI) according to the manufacturer's instructions, except the DNA was eluted in sterile water rather than Tris-EDTA. Purified products were cloned with the TA cloning kit (Invitrogen Corp., Carlsbad, CA), and the presence of inserts was confirmed by digestion with *Eco*RI and agarose electrophoresis. Plasmid DNA was prepared with a miniprep kit (Promega), as described previously, and the DNA samples were quantified with a fluorometer. DNA samples were sequenced with the ThermoSequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ) by mixing 8 pmol of CY-5-labeled primer with approximately 500 ng of plasmid DNA in a total volume of 26 μl. Six microliters of the DNA solution was added to each of four tubes containing 2 μl of A, C, G, or T termination mix and mixed by pipetting up and down. DNA was amplified in a thermalcycler at 94°C for 3 min, followed by 25 cycles of 55°C for 30 s, 72°C for 2 min, and 94°C for 30 s. After adding 6 μl of stop dye, 6 μl of each reaction was analyzed on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech). Each clone was sequenced in both directions with the M13 reverse and M13-40 primers. Three to six clones per fungal isolate were sequenced to minimize the impact of errors caused by PCR amplification.

Assembling the ITS database. To identify additional species closely related to *Cercospora*, a BLAST (1) search was performed on the ITS sequence of the *Cercospora sorghi* var. *maydis* isolate from North Carolina. Sequences of 52 species with high similarity to the ITS sequence of *Cercospora sorghi* var. *maydis* were downloaded from GenBank and added to the database (Table 2). The ITS sequences for *Cercospora sorghi* and groups I and II of *Cercospora zeae-maydis* were taken from Wang et al. (35) (Table 1). In addition, the entire data set for a recent paper by Stewart et al. (30) was downloaded from TreeBASE (available on-line from the Harvard University Herbaria), converted into FASTA format, and added to the database. The ITS sequence for *Phaeosphaeria nodorum*, shown to cluster outside the *Mycosphaerella* group in a previous analysis (20), was included as an outgroup. Multiple sequences of the same species were retained if they differed or were listed originally as separate species in the database. The final database contained sequences of 94 isolates representing 77 species and varieties in 24 anamorph and eight teleomorph genera (Tables 1 and 2). The anamorphs of species of *Mycosphaerella* listed in Tables 1 and 2 are as indicated in Corlett (7).

DNA sequence alignment and analysis. All sequences were trimmed to include the complete ITS1, 5.8S ribosomal RNA gene, and ITS2 sequences. Seven bases, each of the 18S and 26S gene sequences, were included at the beginning and end of most sequences, respectively, to aid alignment. The aligned region corresponds to bases 48 to 508 of the *Cercospora sorghi* var. *maydis* North Carolina isolate. The DNA sequences were aligned by a three-step process with the profile mode of ClustalX (32). First, a simultaneous multiple alignment of all sequences was performed to identify groups of closely related taxa. Then a separate alignment was performed for each group and saved as a different profile. Finally, the profiles were aligned to each other using the original dendrogram as a guide. Sequences that did not cluster with any of the others in the initial step were aligned as separate profiles. Each profile was checked by eye and edited manually if necessary before proceeding to the next step. Following alignment, genetic distances among all isolates were calculated, and a neighbor-joining tree was prepared with the Draw N-J Tree option of ClustalX. This option uses Kimura's two-parameter method for estimating evolutionary distances (22) and implements the neighbor-joining algorithm of Saitou and Nei (28). Bootstrap analysis (1,000 replications) was performed on the resulting tree with the Bootstrap N-J Tree option of ClustalX, and the final tree was visualized and printed with Njplot.

Analysis of cercosporin production. Cultures of *Cercospora* species were grown for 5 to 10 days on dilute (0.2×) potato dextrose agar at 25°C under a 12-h photoperiod provided by two fluorescent bulbs (Phillips, Somerset, NJ). Cylinders of agar medium with mycelium were removed, and the reddish-purple pigment was extracted into 5 N KOH as described by Jenns et al. (21). Compounds showing a green color in alkali and having a characteristic absorption spectrum with A_{max} at 480, 595, and 640 nm were assumed to represent cercosporin (4).

Intraspecific sequence differences. For 25 taxa, multiple ITS sequences were available in GenBank or in our database. For each species with two or more sequences, a separate alignment was made with ClustalX, and the number of differences among isolates within species was tabulated. To determine which type of mutation occurred most commonly, a separate count was made for transitions, transversions, and insertions/deletions (indels) within the entire ITS region. To test the hypothesis that ITS1 is more variable than ITS2, counts were made for each region separately. For many taxa, this analysis used sequences in addition to those that were included in the phylogenetic trees.

PCR amplification with mating-type primers. In an attempt to determine the mating type of *Cercospora* and *Mycosphaerella* isolates, Loculoascomycete primers ChHMG1 and ChHMG2 of Arie et al. (2) were synthesized commercially (Operon Technologies Inc., Alameda, CA) and used in PCR analysis. These primers amplify the high mobility group (HMG) mating-type gene (MAT-2) in *Cochliobolus* and *Mycosphaerella zeae-maydis*. DNA of *Cercospora sorghi*, *Cercospora zeae-maydis* groups I and II, *Mycosphaerella citri*, and *Mycosphaerella graminicola* was extracted as described previously. DNA of *Cochliobolus heterostrophus* (MAT-1 and MAT-2) and *Bipolaris sorghicola* (MAT-2) were included as positive and negative controls. PCR conditions were as described in Arie et al. (2). Amplification products were separated on agarose gels, stained with ethidium bromide, and photographed under ultraviolet illumination.

TABLE 2. Additional DNA sequences for the internal transcribed spacer database that were obtained from GenBank, TreeBASE, or other published sources[a]

Anamorph	Teleomorph	Isolate	GenBank no.
–	*Dothidea hippophaes*[b]	CBS 186.58	AF027763
–	*Dothiora cannabinae*	CBS 737.71	AJ244243
–	*Dothiora rhamni-alpinae*	CBS 745.71	AJ244245
–	*Elsinoë banksiae*	–	AF097572
–	*Elsinoë proteae*	–	AF097578
–	*Mycosphaerella africana*	STE-U 794	AF173314
–	*Mycosphaerella keniensis*	STE-U 1084	AF173300
–	*Mycosphaerella marksii*	STE-U 935	AF173316
Asteromellopsis insculpta	*Dothidea insculpta*	CBS 189.58	AF027764
Capnobotryella renispora	–	CBS 214.90	AJ244238
Cercospora apii	–	CA29	TreeBASE
Cercospora beticola	–	CB4	TreeBASE
Cercospora canescens	–	CCA19	TreeBASE
Cercospora hayi	–	CH5	TreeBASE
Cercospora hayi	–	CH6	TreeBASE
Cercospora kikuchii	–	CK35	TreeBASE
Cercospora kikuchii	–	CK39	TreeBASE
Cercospora nicotianae	–	CN17	TreeBASE
Cercospora sojina	–	CS43	TreeBASE
Cladosporium allii-cepae	*Mycosphaerella allii-cepae*	96-1	AB026160
Cladosporium cladosporioides	–	CBS 170.54	AJ244241
Cladosporium fulvum	–	–	L25430
Cladosporium herbarum	*Mycosphaerella tassiana*	CBS 111.82	AJ238469
Cladosporium herbarum	*Mycosphaerella tassiana*	CBS 399.80	AJ244227
Cladosporium macrocarpum	–	CBS 175.62	AJ244229
Cladosporium oxysporum	–	–	L25432
Cladosporium sphaerospermum	–	CBS 122.47	AJ244228
Cladosporium tenuissium	–	P196	AF132797
Colletogloeopsis molleriana	*Mycosphaerella molleriana*	STE-U 1214	AF173301
Dothistroma septospora	*Mycosphaerella pini*	–	AF013227
Dothistroma septospora	*Mycosphaerella pini*	MP002	AF211197
Hormonema dematioides	*Sydowia polyspora*	–	AF013228
Hormonema dematioides	*Sydowia polyspora*	CBS 128.64	AJ244262
Hormonema macrosporum	–	CBS 536.94	AJ244247
Hortaea werneckii	–	CBS 359.66	AJ244249
Hortaea werneckii	–	CBS 373.92	AJ238474
Lacazia loboi	–	–	AF035674
Lecanosticta acicola	*Mycosphaerella dearnessii*	–	AF260818
Lecanosticta acicola	*Mycosphaerella dearnessii*	MDUS1	AF211196
Mycocentrospora acerina	–	MA12	TreeBASE
Mycovellosiella tasmaniensis	*Mycosphaerella tasmaniensis*	STE-U 1457	AF173307
Paracercospora fijiensis	*Mycosphaerella fijiensis*	ATCC 22116	AF181705
Paracercospora fijiensis	*Mycosphaerella fijiensis*	PF7	TreeBASE
Paracercospora fijiensis	*Mycosphaerella fijiensis*	PF8	TreeBASE
Paracercospora fijiensis var. *difformis*	*Mycosphaerella fijiensis* var. *difformis*	PFD9	TreeBASE
Passalora arachidicola	–	PA16	TreeBASE
Passalora personata[c]	*Mycosphaerella berkeleyi*	PP15	TreeBASE
Phaeotheca triangularis	–	CBS 471.90	AJ244256
Pseudocercospora cruenta	*Mycosphaerella cruenta*	PCR18	TreeBASE
Pseudocercospora musae	*Mycosphaerella musicola*	ATCC 22115	AF181706
Pseudocercospora musae	*Mycosphaerella musicola*	PM10	TreeBASE
Pseudocercospora musae	*Mycosphaerella musicola*	PM11	TreeBASE
Ramichloridium cerophilum	–	CBS 103.59	AF050286
Ramularia brunnea	*Mycosphaerella fragariae*	STE-U 656	AF173312
Ramularia collo-cygni	–	STE-U 2045	AF173310
Ramulispora acuformis	*Tapesia acuformis*	RAC44	TreeBASE
Ramulispora aestiva	–	RAE22	TreeBASE
Ramulispora anguioides	–	RAN45	TreeBASE
Ramulispora herpotrichoides	*Tapesia yallundae*	RH26	TreeBASE
Septoria passerinii	–	ATCC 26515	AF181696
Septoria passerinii	–	ATCC 26516	AF181697
Septoria tritici	*Mycosphaerella graminicola*	T48	AF181694
Sphaceloma australis	*Elsinoë australis*	Val-2, Bat0	U28057
Sphaceloma fawcettii	*Elsinoë fawcettii*	S36954, Marc3	U28058
Sphaceloma sp.	*Elsinoë leucospermi*	STE-U 2042	AF131089
Stagonospora nodorum	*Phaeosphaeria nodorum*	N2	AF181710
Stenella araguata	–	CBS 486.80	AJ244261
Stenella citri-grisea	*Mycosphaerella citri*	Fellsmere	AF181703
Stenella parkii	*Mycosphaerella parkii*	STE-U 353	AF173311
Trimmatostroma abietina	–	CBS 290.90	AJ244267
Trimmatostroma abietina	–	CBS 618.84	AJ244266
Trimmatostroma salicis	–	CBS 300.81	AJ244264
Trimmatostroma salinum	–	MZKI B-962	AJ238676
Uwebraunia ellipsoidea	*Mycosphaerella ellipsoidea*	STE-U 1224	AF173302
Uwebraunia juvenis	*Mycosphaerella juvenis*	STE-U 1005	AF173299

[a] – Indicates not known. CBS = Centraalbureau voor Schimmelcultures accession number; ATCC = American Type Culture Collection accession number.
[b] Listed as *Dothidea berberidis* at CBS.
[c] The anamorph for this species is listed as *Phaeoisariopsis personata* by Corlett (7), but was named a *Passalora* by Stewart el al. (30).

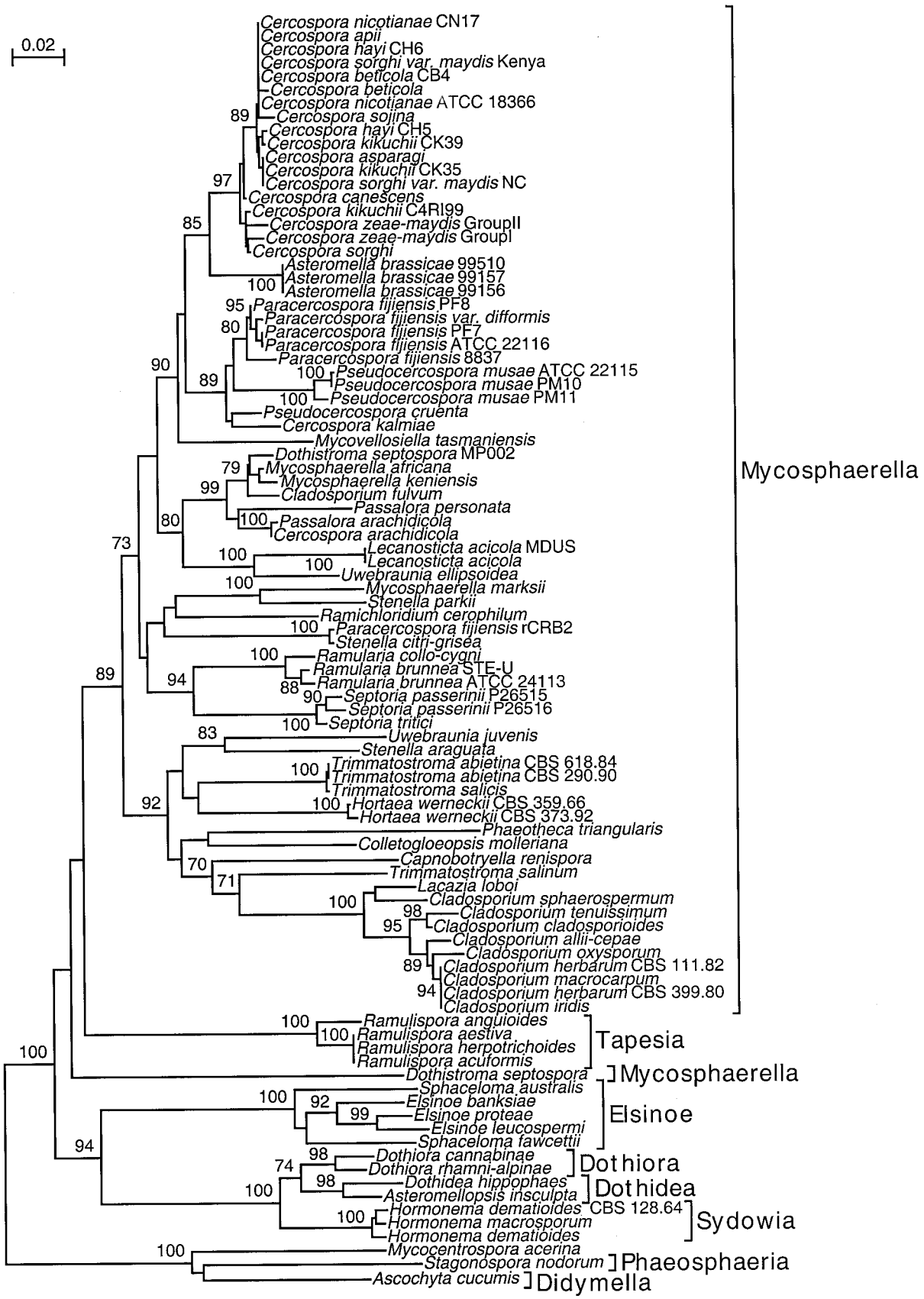

Fig. 1. Unrooted neighbor-joining tree of 94 sequences of the internal transcribed spacer (ITS) region of ribosomal DNA from species of *Mycosphaerella* and related anamorphs and teleomorphs. All bootstrap values of 70 or greater (percentage of 1,000 replications) are indicated, rounded to the nearest integer. The ITS sequence of *Phaeosphaeria nodorum* was used as an outgroup. All species are indicated by anamorph name, if known, otherwise by teleomorph. If more than one isolate of a species was analyzed, isolate designations are provided after the species name. The probable teleomorph genus for each major group, if known, is indicated by brackets. Branch lengths are proportional to genetic distance, which is indicated by a bar at the upper left.

RESULTS

ITS sequencing and alignment. The length of the ITS region, including the primer region, for the 15 isolates sequenced ranged from 548 nts for *Mycosphaerella fijiensis* isolate 8837 to 574 for *Mycosphaerella macrospora*. The extensive length variation commonly detected among fungi (20) was not found in the species sequenced in this study. GenBank accession numbers for the 15 sequences are indicated in Table 1.

A BLAST (1) search of the *Cercospora sorghi* var. *maydis* North Carolina isolate on the GenBank database identified strong matches with many species of *Mycosphaerella*, as well as the anamorph genera *Trimmatostroma*, *Ramularia*, and *Cladosporium*. The highest BLAST score was obtained to an isolate of *Guignardia bidwellii*, followed by *Mycosphaerella tasmaniensis* and *Mycosphaerella africana*. All sequences downloaded from GenBank had expected values of 4×10^{-89} or lower in the BLAST results.

Alignment of the 94 sequences required 45 profile steps, with the original simultaneous multiple alignment as a guide. Minor manual editing was required on approximately half of the profiles. Use of the Profile mode of ClustalX to build the alignment ensured that accurate relationships among species within each group were maintained at each step. This yielded a better result with generally higher bootstrap support compared with the original simultaneous multiple alignment (data not shown).

For most of the profile alignments, the gap opening and extension penalties were left at the default values of 15.00 and 6.66, respectively. A few of the sequences downloaded from GenBank contained large insertions or deletions. These usually occurred at or near the 5′ end of ITS1. Aligning these sequences was more difficult and required lowering the gap opening and extension penalties until an accurate alignment could be obtained.

Cercosporin production. For each species that produced a reddish-purple pigment in the agar medium, cercosporin was confirmed by spectrophotometric analysis. Confirmed cercosporin producers were *Cercospora asparagi*, *Cercospora beticola*, *Cercospora nicotianae*, and *Cercospora sorghi* var. *maydis*. The isolates of *Cercospora kikuchii*, *Cercospora sorghi*, and *Cercospora zeae-maydis* group I produced cercosporin in a previous study (35). Isolates of *Cercospora arachidicola*, *Cercospora sojina*, *Cercospora zeae-maydis* group II, *Mycosphaerella brassicicola*, *Mycosphaerella macrospora*, *Mycosphaerella fijiensis*, and *Mycosphaerella fragariae* were tested and did not produce cercosporin.

Phylogenetic analyses. Most of the *Cercospora* species tested formed a single, monophyletic group with high (97%) bootstrap support (Fig. 1). The only exceptions were *Cercospora kalmiae* and *Cercospora arachidicola*, which clustered with species of *Pseudocercospora* and *Passalora*, respectively. Interestingly, *Asteromella brassicae* (teleomorph *Mycosphaerella brassicicola*) was a sister group to the main *Cercospora* cluster and separated most of the *Cercospora* species from a *Paracercospora*/*Pseudocercospora*/*Cercospora kalmiae* cluster. Two species of *Mycosphaerella* with no known anamorphs, *Mycosphaerella africana* and *Mycosphaerella keniensis*, clustered with *Cladosporium fulvum* and *Dothistroma septospora* (teleomorph *Mycosphaerella pini*), which was a sister group to a *Passalora*/*Cercospora arachidicola* cluster (Fig. 1).

In addition to *Cercospora*, three other anamorph genera clearly were polyphyletic. Species of *Stenella* were in three widely separated clusters (Fig. 1). The two species with *Uwebraunia* anamorphs were phylogenetically unrelated, and *Trimmatostroma salinum* was in a different cluster from *T. abietina* and *T. salicis*.

Anamorph genera that formed monophyletic clusters included *Ramularia* and *Septoria* (Fig. 1). All species of *Cladosporium* except *Cladosporium fulvum* formed a monophyletic group that included *Lacazia loboi*, a fungus with previously unknown phylogenetic affinities that was isolated from the skin of bottlenose dolphin (31). The *Cladosporium*, *Ramularia*, and *Septoria* clusters each had 100% bootstrap support (Fig. 1).

Most species with *Mycosphaerella* teleomorphs formed a monophyletic group with high (89%) bootstrap support. The only exception was one isolate of *Mycosphaerella pini* (anamorph *Dothistroma septospora*), which did not cluster with any other species (Fig. 1). A second isolate of *Mycosphaerella pini* clustered within the large *Mycosphaerella* group together with *Mycosphaerella africana*, *Mycosphaerella keniensis*, and *Cladosporium fulvum*. The only species within the *Mycosphaerella* cluster with a different teleomorph was one isolate of *Guignardia bidwellii* (GenBank Accession No. AF216533), which clustered as a sister taxon to *Mycosphaerella brassicicola* near the large *Cercospora* cluster (data not shown). However, because this sequence appeared unrelated to those from other species in the genus *Botryosphaeria* (sometimes considered a synonym for *Guignardia* [19]), it was assumed to have been identified incorrectly and was excluded from further analysis.

Two species in this analysis clustered with the outgroup taxon *Phaeosphaeria nodorum*. One of these was labeled as *Mycosphaerella citrullina* when it was received from the ATCC. However, the correct name for this species is *Didymella bryoniae* (7) (anamorph *Ascochyta cucumis*). The other species was *Mycocentrospora acerina*, which has no known teleomorph (19).

In addition to the species tested in this study, reports of cercosporin production or nonproduction for other species were taken from the literature (3,18) and added on to a second analysis of a reduced data set with the *Septoria* cluster as an outgroup (Fig. 2). All of the cercosporin-producing species were within the monophyletic *Cercospora* cluster that had 97% bootstrap support. The only taxa within this cluster that did not produce cercosporin were *Cercospora sojina* and *Cercospora zeae-maydis* group II. All species outside this cluster for which data were available did not produce cercosporin, including *Mycosphaerella brassicicola* (anamorph *Asteromella brassicae*), the most closely related species with a confirmed *Mycosphaerella* teleomorph (Fig. 2).

Nucleotide differences between and within species. The number of nucleotide differences between species for the 12 taxa in the monophyletic *Cercospora* cluster ranged from 0 to 14 (Table 3). Five taxa (*Cercospora apii*, *Cercospora beticola*, *Cercospora hayi*, *Cercospora nicotianae*, and *Cercospora sorghi* var. *maydis*) had isolates with identical ITS sequences, although single isolates of *Cercospora beticola*, *Cercospora hayi*, and *Cercospora sorghi* var. *maydis* differed from the others by 5, 2, and 1 nts, respectively. The largest nucleotide difference was between *Cercospora zeae-maydis* group II and one isolate of *Cercospora beticola* (Table 3). There were 7 nt differences between the sequences of *Cercospora zeae-maydis* groups I and II, and an average of 6.5 nts between *Cercospora sorghi* and the two isolates of *Cercospora sorghi* var. *maydis*. The overall mean number of differences between taxa within the main *Cercospora* cluster was 5.28 nts over all 66 pairwise comparisons.

Within the large, monophyletic *Mycosphaerella* cluster, 25 taxa were represented by two or more sequences in the databases. The number of sequences available per species ranged from 2 to 8 with a mean of 3.28 (Table 4). The numbers of transitions, transversions, and insertions/deletions (indels) within species ranged from 0 to 6, 0 to 7, and 0 to 9, respectively. Over all 25 taxa, transitions and indels occurred at approximately the same frequency, with means of 1.08 and 0.96 of each per taxon, respectively. Transversions only occurred about one half as often, with a mean of 0.56 transversions per taxon.

There was little difference in the number of changes between ITS1 and ITS2. The total number of differences between sequences within species ranged from 0 to 12 for ITS1 compared with 0 to 10 for ITS2 (Table 4). Intraspecific variation among all 25 taxa averaged 1.36 differences between ITS1 sequences and

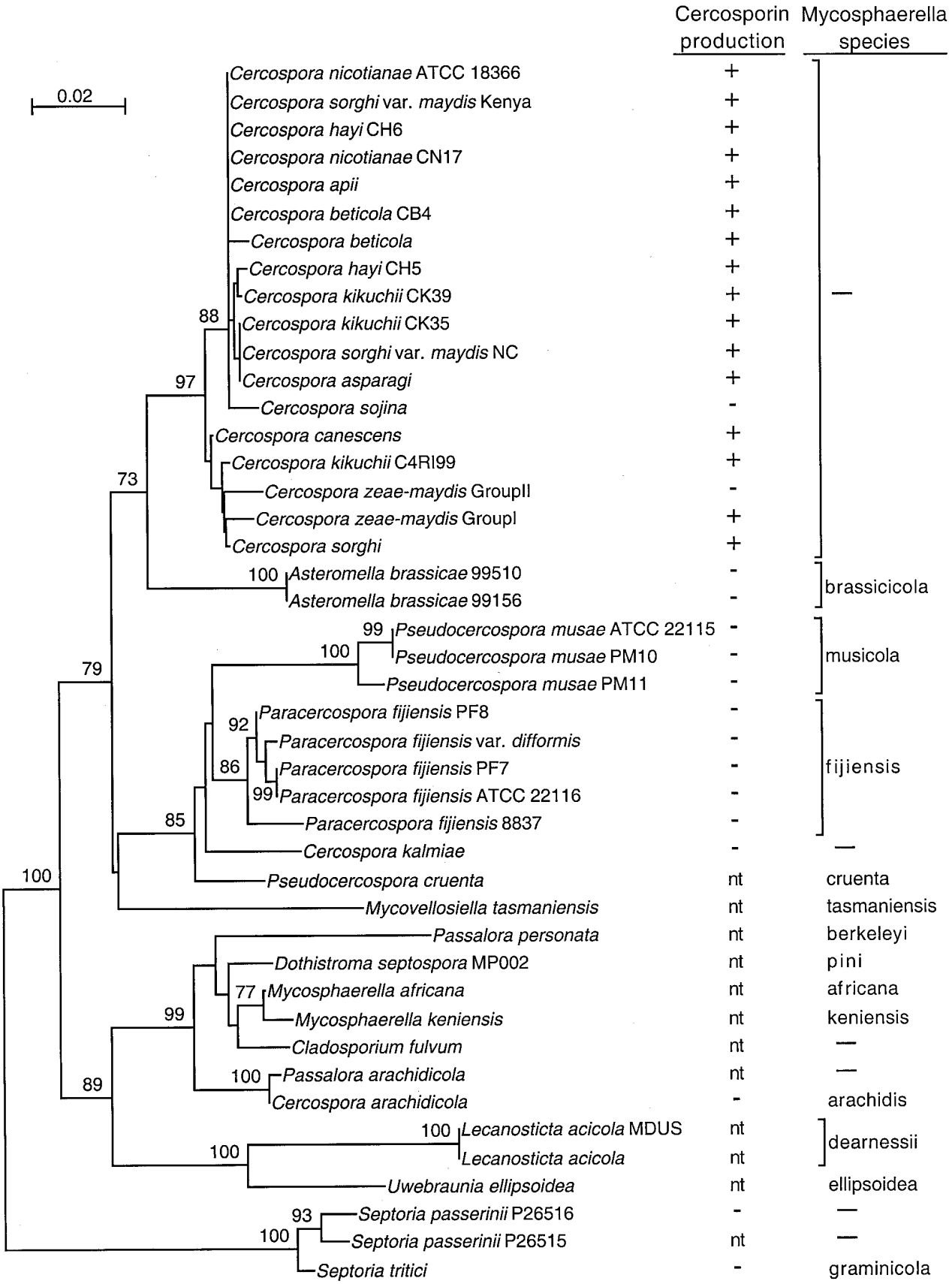

Fig. 2. Relationship between cercosporin production and phylogeny in the genera *Cercospora* and *Mycosphaerella*. Unrooted neighbor-joining tree from a reduced data set of 44 internal transcribed spacer sequences. All bootstrap values above 70 (percentage of 1,000 replications) are indicated and rounded to the nearest integer. The *Septoria* cluster was used as an outgroup. If more than one isolate of a species was analyzed, isolate designations are provided after the species name. Species that produce cercosporin are indicated by +, those that do not produce cercosporin are indicated by –, and those that were not tested are indicated by nt. All species are listed by anamorph name, if known, otherwise by teleomorph. For those with a known teleomorph, the species is indicated to the right. Branch lengths are proportional to genetic distance, which is indicated by a bar at the upper left.

1.24 differences between those of ITS2. The mean number of total differences among sequences within species ranged from 0 to 8.5, with a mean over all 25 taxa of 1.67 (Table 4). The corresponding numbers for taxa in the *Cercospora* cluster ranged from 0 to 5 with an overall mean of 1.27. There were no differences in the 5.8S gene sequences within any species.

The above numbers were inflated greatly by the inclusion of *Hortaea werneckii*, which had sequences with more than twice the number of nucleotide differences of any other taxon. Other species with much higher variation compared with the mean value were *Cercospora beticola*, *Cercospora kikuchii*, *Mycosphaerella fragariae*, and *Mycosphaerella musicola*, with 5, 4.7, 7, and 4.7 differences, respectively. The multiple isolates of *Cercospora beticola*, *Mycosphaerella fragariae*, and *Mycosphaerella musicola* clustered together with their respective species. However, the three isolates of *Cercospora kikuchii* were in slightly different clusters within the main *Cercospora* group.

Single isolates of *Mycosphaerella fijiensis* and *Septoria passerinii* were excluded from the previous analysis. One isolate of *Mycosphaerella fijiensis* was very different from the others of this species and instead clustered with *Mycosphaerella citri* (anamorph *Stenella citri-grisea*). This isolate was assumed to be mislabeled or misidentified. The ITS sequence for one isolate of *Septoria passerinii* differed from that of six other isolates by 7 nts. The isolate with a different sequence did not come from the same host as the other six isolates and was assumed to be a different species by Goodwin and Zismann (20).

PCR amplification with mating-type primers. Primers ChHMG1 and ChHMG2 amplified the expected band of approximately 300 bp from the MAT-2 isolates of *Cochliobolus heterostrophus* and *Bipolaris sorghicola*, but not from the *Cochliobolus heterostrophus* negative (MAT-1) control. There was no amplification at all with DNA of *Mycosphaerella citri*. Multiple bands were obtained for the other species but all were much larger than 300 bp and did not appear to be the MAT-2 (HMG mating type) gene (data not shown).

DISCUSSION

The large-scale analysis presented in this study provides unprecedented resolution of phylogenetic relationships within *Cercospora* and *Mycosphaerella*. The three main groups of cercosporoid species identified by Stewart et al. (30) and Crous et al. (9) were confirmed and extended. There is a strongly supported monophyletic group that includes all of the true *Cercospora* species, including those from cereal crops. However, the cereal pathogens *Cercospora zeae-maydis* (both groups) and *Cercospora sorghi*, as well as one isolate of *Cercospora kikuchii* were in a separate subcluster within *Cercospora*, indicating some degree of evolutionary divergence from the other *Cercospora* species.

The short branch lengths among species within the *Cercospora* cluster indicate that they all shared a common ancestor relatively recently. Because virtually all of these species produce cercosporin, the common ancestor probably was a producer. Lack of cercosporin production by species outside this cluster indicates that this trait may have evolved only once, and supports Fajola's (18) hypothesis that noncercosporin producers are in different anamorph genera. One possible explanation for these results is that all *Cercospora* species share a common ancestor that acquired the ability to produce cercosporin. Ability to produce cercosporin allowed the ancestral *Cercospora* species to expand its host range, leading to a rapid, recent adaptive radiation. This would explain the occurrence of a large number of closely related species, some with identical ITS sequences, on widely divergent hosts. *Cercospora* species that do not produce cercosporin presumably lost this ability following the species radiation. Testing additional species for cercosporin production and sequencing additional genes are required to test this hypothesis thoroughly.

Our data show conclusively that groups I and II of *Cercospora zeae-maydis* represent different species. One problem with ITS data is that it is not clear how many differences there are between closely related species compared with the amount of variation that exists within species. For taxa within *Mycosphaerella*, on average there were 1.7 nt differences in ITS sequences within species, with slightly more differences within ITS1 than ITS2. This number was biased upward by the inclusion of *Hortaea werneckii*, a highly variable species (11). Without *Hortaea werneckii*, the mean was 1.38, which was close to the 1.27 for species in the *Cercospora* cluster. From this analysis, taxa with ITS sequences that differ by two or more nucleotides may be distinct species. ITS sequences from the two groups of *Cercospora zeae-maydis* differ by 7 nts, which is greater than the mean of 5.3 nts between species within the *Cercospora* cluster. These findings corroborate the conclusions based on amplified fragment length polymorphism (AFLP) analyses (35) and suggest that AFLP data are, in fact, valid indicators of genetic similarity among closely related species.

The phylogenetic analysis also strongly supports Chupp's (6) suggestion that *Cercospora sorghi* and *Cercospora sorghi* var. *maydis* are different species. The ITS sequences of these taxa differed on average by 6.5 nts. The two isolates of *Cercospora sorghi* var. *maydis* tested had ITS sequences that were virtually identical to those of *Cercospora apii*, *Cercospora asparagi*, *Cercospora beticola*, *Cercospora hayi*, *Cercospora kikuchii*, and *Cercospora nicotianae*, and clearly are much more closely related to those species than they are to *Cercospora sorghi*. Interestingly,

TABLE 3. The number of differences between sequences of the internal transcribed spacer region of the ribosomal DNA in pairwise comparisons among 12 closely related species, varieties, and groups of *Cercospora*[a]

Cercospora	*apii*	*asparagi*	*beticola*	*canescens*	*hayi*	*kikuchii*	*nicotianae*	*sojina*	*sorghi*	*sorghi* var. *maydis*	*zeae-maydis* group I	*zeae-maydis* group II
C. apii	…	2	0–5	3	0–2	1–6	0	3	6	0–1	8	9
C. asparagi	–	…	2–5	5	2	1–8	2	5	8	1–2	10	11
C. beticola	2.5	3.5	…	3–8	0–7	1–11	0–5	3–8	6–11	0–6	8–13	9–14
C. canescens	–	–	5.5	…	3–5	3–4	3	6	3	3–4	5	6
C. hayi	1.0	2.0	3.5	4.0	…	1–6	0–2	3–5	6–8	0–2	8–10	9–11
C. kikuchii	2.67	4.0	5.17	3.67	2.67	…	1–6	4–9	2–7	0–7	4–9	5–10
C. nicotianae	–	–	2.5	–	1.0	2.67	…	3	6	0–1	8	9
C. sojina	–	–	5.5	–	4.0	5.67	–	…	9	3–4	11	12
C. sorghi	–	–	8.5	–	7.0	5.33	–	–	…	6–7	4	5
C. sorghi var. *maydis*	0.5	1.0	3.0	3.5	1.0	2.83	0.5	3.5	6.5	…	8–9	9–10
C. zeae-maydis group I	–	–	10.5	–	9.0	7.33	–	–	–	8.5	…	7
C. zeae-maydis group II	–	–	11.5	–	10.0	8.33	–	–	–	9.5	–	…

[a] Above diagonal indicates number of nucleotide differences between species. For species with two or more sequences available, the range of differences is indicated. Below diagonal indicates mean number of differences between species for those with two or more sequences available. – Indicates that only one sequence was available for both species in this comparison so a mean could not be calculated.

no other species from cereal hosts were in the *Cercospora* subgroup that contained *Cercospora sorghi* var. *maydis*.

All of the *Cercospora* species tested grouped within a much larger cluster of species that have *Mycosphaerella* teleomorphs. Thus, the genus *Cercospora* must have evolved within the *Mycosphaerella* lineage. The teleomorphs for these *Cercospora* species, if they exist, most likely will be in *Mycosphaerella*. This agrees with the unconfirmed report of a *Mycosphaerella* teleomorph for *Cercospora zeae-maydis* (23).

The large-scale phylogenetic analysis provided evidence that *Mycosphaerella* is monophyletic and contains numerous polyphyletic anamorph genera. This is in contrast to the hypothesis of Crous and Wingfield (10) who suggested that *Mycosphaerella* is a polyphyletic assemblage of monophyletic anamorphs. The only ITS sequence of a *Mycosphaerella* species that did not cluster with this genus in our analysis was one isolate of *Mycosphaerella pini*. However, the sequence of this isolate was very different from that of a second isolate of this species that clustered well within *Mycosphaerella*. Therefore, the aberrant isolate most likely was misidentified. Within the *Mycosphaerella* cluster, the only isolate with a different teleomorph was one of *Guignardia bidwellii*. The sequence for this isolate was very different from those for *Botryosphaeria* species that also were present in GenBank (data not shown). Because *Guignardia* is considered a synonym of *Botryosphaeria* (19), the GenBank sequence for *Guignardia bidwellii* probably came from an isolate that was misidentified or mislabeled. Overall, the data provide very strong support for the hypothesis that the genus *Mycosphaerella* is monophyletic, which confirms the results of Crous et al. (9) and Goodwin and Zismann (20) from analyses of much smaller data sets.

Although *Mycosphaerella* clearly appears to be monophyletic, branch lengths among groups within *Mycosphaerella* are quite long. Genetic distances between some clusters within *Mycosphaerella* are larger than those between the teleomorph genera *Dothiora*, *Dothidea*, and *Sydowia*. Therefore, the *Mycosphaerella* teleomorph probably is of ancient origin and has been maintained through a long period of evolutionary history by selection. The long branch lengths lead others to conclude incorrectly that the genus is polyphyletic. This issue could only be resolved by a large-scale phylogenetic analysis.

In contrast to the teleomorph, certain anamorph genera associated with *Mycosphaerella* clearly are polyphyletic. This was particularly evident for *Stenella* and the new genus *Uwebraunia*, which had representatives in very different clusters. A monophyletic origin for *Uwebraunia* was already in question by morphological analysis of the teleomorphs. Crous (8) noted that the two species of *Uwebraunia* included in the phylogenetic analysis have teleomorphs with different shaped ascospores. Therefore, it is not surprising that *Uwebraunia* is polyphyletic. Evidently, many anamorph characters are highly mutable; the same anamorph probably arose multiple times by convergent evolution. Thus, anamorphs in *Mycosphaerella* in general may not be useful for resolution of phylogenetic relationships. This supports the conclusion of von Arx (34) that anamorphs should not be used to separate groups within *Mycosphaerella.*

Based on these results, some recent changes in the taxonomy of *Mycosphaerella* should be revisited. For example, Barr (5) erected the new teleomorph genus *Eruptio* to include species with anamorphs in *Dothistroma* and *Lecanosticta* on the assumptions that: (i) these anamorphs are closely related; and (ii) they are different from other species within *Mycosphaerella*. Our large-scale phylogenetic analysis contradicted both of these assumptions. These two anamorphs are not particularly closely related and both are located well within the *Mycosphaerella* cluster. Therefore, the teleomorph names for *Dothistroma septospora* and *Lecanosticta acicola* should remain within *Mycosphaerella*.

Not all anamorphs were polyphyletic. Anamorphs that were clearly monophyletic included *Cercospora sensu* Stewart et al. (30), *Ramularia*, *Septoria*, and all of the *Cladosporium* species except *Cladosporium fulvum*. For *Ramularia* and *Septoria*, the number of species tested was too small for firm conclusions. However, *Cercospora* and *Cladosporium* formed well-supported

TABLE 4. Number of nucleotide differences in the internal transcribed spacer (ITS) region among isolates within species for taxa in the *Mycosphaerella* cluster

Species	No. of sequences	Type of change			No. of differences				
		Transitions	Transversions	Indels[a]	ITS1	ITS2	Total	Range[b]	Mean
Cercospora apii	3	0	0	0	0	0	0	0	0.0
Cercospora beticola	2	0	2	3	4	1	5	–	5
Cercospora hayi	2	2	0	0	0	2	2	–	2
Cercospora kikuchii	3	5	1	1	1	6	7	2–7	4.7
Cercospora nicotianae	2	0	0	0	0	0	0	–	0
Cercospora sojina	3	0	0	0	0	0	0	0	0.0
Cercospora sorghi	4	0	0	0	0	0	0	0	0.0
Cercospora sorghi var. *maydis*	2	1	0	0	0	1	1	–	1
Cercospora zeae-maydis group I	4	0	0	0	0	0	0	0	0.0
Cercospora zeae-maydis group II	4	0	0	0	0	0	0	0	0.0
Cladosporium allii-cepae	3	0	0	0	0	0	0	0	0.0
Cladosporium herbarum	3	0	0	0	0	0	0	0	0.0
Cladosporium sphaerospermum	2	0	0	0	0	0	0	–	0
Hortaea werneckii	8	6	7	9	12	10	22	1–16	8.5
Mycosphaerella brassicicola	3	0	0	1	1	0	1	0–1	0.7
Mycosphaerella citri	2	0	0	1	0	1	1	–	1
Mycosphaerella dearnessii	5	0	0	2	2	0	2	0–2	1.0
Mycosphaerella ellipsoidea	2	1	1	0	1	1	2	–	2
Mycosphaerella fijiensis	5[c]	3	1	2	2	4	6	0–5	2.8
Mycosphaerella fragariae	2	2	2	3	7	0	7	–	7
Mycosphaerella graminicola	4	0	0	0	0	0	0	0	0.0
Mycosphaerella musicola	3	6	0	1	3	4	7	1–7	4.7
Septoria passerinii	6[d]	0	0	0	0	0	0	0	0.0
Trimmatostroma abietina	2	0	0	0	0	0	0	–	0
Trimmatostroma salinum	3	1	0	1	1	1	2	1–2	1.3
Overall mean	3.28	1.08	0.56	1.96	1.36	1.24	2.60	–	1.67

[a] Insertions, deletions, or both.
[b] The range was only calculated when three or more sequences were available.
[c] One isolate of *Mycosphaerella fijiensis* that probably was misidentified was excluded.
[d] Isolate P26515 of *Septoria passerinii* was considered a separate species by Goodwin and Zismann (20) so was excluded from this analysis.

monophyletic groups. Interestingly, the *Cladosporium* cluster included *Lacazia loboi*, the cause of lobomycosis in humans and bottlenose dolphins (31).

In addition to addressing phylogenetic questions, the large-scale analysis identified a number of sequences from isolates that probably were misidentified, mislabeled, or misclassified. Two of these were *Mycosphaerella pini* and *Guignardia bidwellii* as discussed previously. The others were *Mycosphaerella fijiensis* and *Mycosphaerella citrullina*. Five isolates of *Mycosphaerella fijiensis* clustered together, but the sixth isolate had an ITS sequence that was almost identical to that of *Stenella citri-grisea* (*Mycosphaerella citri*). The most likely explanations for this are that the isolate was misidentified or mislabeled, or that there was contamination during PCR amplification. The isolate received as *Mycosphaerella citrullina* from the ATCC clustered with *Phaeosphaeria nodorum* in the *Pleosporales*, not within *Mycosphaerella*. This isolate was simply misclassified; the correct name for *Mycosphaerella citrullina* is *Didymella bryoniae* (7), which is supported by the phylogenetic analysis.

The *Mycosphaerella* cluster included a number of species with no known teleomorphs. These included *Capnobotryella*, *Hortaea*, *Lacazia*, *Phaeotheca*, and *Trimmatostroma*. Many of these are black yeasts that are found on a variety of substrates and on humans (11), but are evolutionarily related to the large group of plant pathogens within *Mycosphaerella*. Because these anamorphs have not been associated with *Mycosphaerella* previously, the true number of anamorphs within *Mycosphaerella* may be much larger than the 43 listed by Corlett (7).

In addition to *Cercospora* and *Mycosphaerella*, phylogenetic analysis indicated the probable teleomorph association for *Mycocentrospora acerina*. This species was used as an outgroup by Stewart et al. (30), but did not cluster with any other species. Our analysis revealed that it clustered with *Phaeosphaeria nodorum* and *Didymella bryoniae* in the *Pleosporales*. An expanded analysis (data not shown) confirmed that it clustered within the *Phaeosphaeria*/*Leptosphaeria* clade identified by Goodwin and Zismann (20). Thus, *Mycocentrospora acerina* probably has a teleomorph related to those genera.

The Loculoascomycete HMG mating-type primers described by Arie et al. (2) may not be useful for species of *Mycosphaerella*. In our preliminary analyses, we were unable to amplify a MAT-2 HMG homologue from species of *Cercospora* and *Mycosphaerella*. The only species of *Mycosphaerella* tested by Arie et al. (2) was *Mycosphaerella zeae-maydis*, which did contain a homologous MAT-2 idiomorph. However, *Mycosphaerella zeae-maydis* is a synonym for *Didymella zeae-maydis* (7) and, therefore, it is not a species of *Mycosphaerella*. The species of *Didymella* tested in our analysis (*Didymella bryoniae*, listed as *Mycosphaerella citrullina* in the collection of the ATCC) clustered with *Stagonospora nodorum*, the anamorph of *Phaeosphaeria nodorum*. The ChHMG1 and ChHMG2 primers (2) did amplify the HMG sequence from isolates of *Phaeosphaeria nodorum* (S. B. Goodwin and V. L. Zismann, *unpublished data*). The most likely explanation for lack of amplification with species of *Cercospora* and *Mycosphaerella* is that *Mycosphaerella zeae-maydis* is classified incorrectly and is not really a *Mycosphaerella*. Therefore, the primers developed by Arie et al. (2) may be useful for some Loculoascomycetes, but not *Mycosphaerella* species. Inclusion of *Mycosphaerella zeae-maydis* in a phylogenetic analysis and cloning of the mating-type genes from a *Mycosphaerella* species are needed to test this hypothesis thoroughly.

ACKNOWLEDGMENTS

This work was supported by USDA CRIS project 3602-22000-009-00D. Published as paper 16389, Purdue University Agricultural Experiment Station. We thank M. Daub for providing cultures of several *Cercospora* species, G. Kema for providing DNA and cultures for isolates of *Mycosphaerella brassicicola*, J. Cavaletto and B. Roberts for generating some of the sequence data and submitting the sequences to GenBank, respectively, M. McClenning for providing general technical support, and M. Scholler for providing helpful comments on a previous draft of the manuscript.

LITERATURE CITED

1. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
2. Arie, T., Christiansen, S. K., Yoder, O. C., and Turgeon, B. G. 1997. Efficient cloning of ascomycete mating type genes by PCR amplification of the conserved MAT HMG box. Fungal Genet. Biol. 21:118-130.
3. Assante, G., Locci, R., Camarda, L., Merlini, L., and Nasini, G. 1977. Screening of the genus *Cercospora* for secondary metabolites. Phytochemistry 16:243-247.
4. Balis, C., and Payne, M. G. 1971. Triglycerides and cercosporin from *Cercospora beticola*: Fungal growth and cercosporin production. Phytopathology 61:1477-1484.
5. Barr, M. E. 1996. Planistromellaceae, a new family in the Dothideales. Mycotaxon 60:433-442.
6. Chupp, C. 1954. A Monograph of the Fungus Genus *Cercospora*. Ronald Press Co., New York.
7. Corlett, M. 1991. An annotated list of the published names in *Mycosphaerella* and *Sphaerella*. Mycol. Mem. 18:1-328.
8. Crous, P. W. 1998. *Mycosphaerella* spp. and Their Anamorphs Associated with Leaf Spot Diseases of *Eucalyptus.* The American Phytopathological Society, St. Paul, MN.
9. Crous, P. W., Aptroot, A., Kang, J.-C., Braun, U., and Wingfield, M. J. 2000. The genus *Mycosphaerella* and its anamorphs. Stud. Mycol. 45: 107-121.
10. Crous, P. W., and Wingfield, M. J. 1996. Species of *Mycosphaerella* and their anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. Mycologia 88:441-458.
11. de Hoog, G. S., Zalar, P., Urzì, C., de Leo, F., Yurlova, N. A., and Sterflinger, K. 1999. Relationships of dothideaceous black yeasts and meristematic fungi based on 5.8S and ITS2 rDNA sequence comparison. Stud. Mycol. 43:31-37.
12. Deighton, F. C. 1967. Studies on *Cercospora* and allied genera. II. *Passalora*, *Cercosporidium*, and some species of *Fusicladium* on *Euphorbia*. Mycol. Pap. 112:1-80.
13. Deighton, F. C. 1973. Studies on *Cercospora* and allied genera. IV. *Cercosporella* Sacc., *Pseudocercosporella* gen. nov. and *Pseudocercosporidium* gen. nov. Mycol. Pap. 133:1-62.
14. Deighton, F. C. 1976. Studies on *Cercospora* and allied genera. VI. *Pseudocercospora* Speg., *Pantospora* Cif. and *Cercoseptoria*. Mycol. Pap. 140:1-168.
15. Deighton, F. C. 1979. Studies on *Cercospora* and allied genera. VII. New species and redispositions. Mycol. Pap. 144:1-56.
16. Dunkle, L. D., and Levy, M. 2000. Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90:486-490.
17. Fajola, A. O. 1978. Cultural studies in Cercospora taxonomy: I. Interrelationships between some species from Nigeria. Nova Hedwigia 29:912-921.
18. Fajola, A. O. 1978. Cercosporin, a phytotoxin from *Cercospora* spp. Physiol. Plant Pathol. 13:157-164.
19. Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. Fungi on Plants and Plant Products in the United States. The American Phytopathological Society, St. Paul, MN.
20. Goodwin, S. B., and Zismann, V. L. 2001. Phylogenetic analyses of the ITS region of ribosomal DNA reveal that *Septoria passerinii* from barley is closely related to the wheat pathogen *Mycosphaerella graminicola*. Mycologia (In press.)
21. Jenns, A. E., Daub, M. E., and Upchurch, R. G. 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79:213-219.
22. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111-120.
23. Latterell, F. M., and Rossi, A. E. 1983. Gray leaf spot of corn: A disease on the move. Plant Dis. 67:842-847.
24. Nakasone, K. K. 1996. Morphological and molecular studies on *Auriculariopsis albomellea* and *Phlebia albida* and a reassessment of *A. ampla*. Mycologia 88:762-775.
25. Ossanna, N., and Mischke, S. 1990. Genetic transformation of the biocontrol fungus Gliocladium virens to benomyl resistance. Appl. Environ.

Microbiol. 56:3052-3056.
26. Pollack, F. G. 1987. An annotated compilation of *Cercospora* names. Mycol. Mem. 12:1-212.
27. Pons, N., and Sutton, B. C. 1988. *Cercospora* and similar fungi on yams (*Dioscorea* species). Mycol. Pap. 160:1-78.
28. Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
29. Sivanesan, A. 1984. The Bitunicate Ascomycetes and their Anamorphs. J. Cramer, Vaduz, Liechtenstein.
30. Stewart, E. L., Liu, Z., Crous, P. W., and Szabo, L. J. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. Mycol. Res. 103:1491-1499.
31. Taborda, P. R., Taborda, V. A., and McGinnis, M. R. 1999. *Lacazia loboi* gen. nov., comb. nov., the etiologic agent of lobomycosis. J. Clin. Microbiol. 37:2031-2033.
32. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.
33. Upchurch, R. G., Walker, D. C., Rollins, J. A., Ehrenshaft, M., and Daub, M. E. 1991. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. Appl. Environ. Microbiol. 57:2940-2945.
34. von Arx, J. A. 1983. *Mycosphaerella* and it anamorphs. Proc. K. Ned. Akad. Wet., Series C 86:15-54.
35. Wang, J., Levy, M., and Dunkle, L. D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269-1275.
36. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols, A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.