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Phylogenetic analyses of *Septoria* species based on the ITS and LSU-D2 regions of nuclear ribosomal DNA

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Abstract: The phylogenetic relationships of 17 selected *Septoria* spp. (eight with a known *Mycosphaerella* teleomorph), *Phloeospora ulmi* (teleomorph *M. ulmi*) and 18 additional taxa (10 with a *Mycosphaerella* teleomorph) were inferred from ITS and D2-LSU nrDNA sequences. In total, 10 anamorph genera associated with *Mycosphaerella* were represented. Intraspecific variation in ITS was limited in *Septoria,* with the exception of three strains that all were identified as *S. rubi* but originated from different *Rubus* spp. and probably belong to different species of *Septoria*. The results of D2 region sequencing confirmed *Mycosphaerella sense lato* (including *Davidiella* and *Eruptio*) as monophyletic. The cereal pathogen *Septoria tritici,* which is closely related to *S. passerinii* as found in ITS analysis, clusters with *Ramularia* spp. in the D2 analyses, distant from the other *Septoria* spp. The pathogens *S. apiicola, S. linicola* and *S. populicola* cluster in a major clade containing *Phl. ulmi,* and other *Septoria* spp. and *Cercospora* spp. Short branch lengths in this clade suggest a very recent evolution. *Septoria castaneicola* and *S. pyricola* also might represent relatively distant lineages. Both analyses of the regions indicated that *Septoria* is not monophyletic within *Mycosphaerella* and that conidiomatal structure (acervulus, pycnidium) has little value for predicting phylogenetic relatedness. As a consequence, the separation between the acervular *Phloeospora* and pycnidial *Septoria* is untenable. The loss of the teleomorph most likely has occurred several times in the evolutionary history of *Mycosphaerella*.

Key words: *Cercospora, Didymella,* morphology, *Mycosphaerella, Phloeospora, Ramularia, Stagonospora*

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INTRODUCTION

Septoria Sacc. nom. cons. is an anamorph genus accommodating many plant-pathogenic coelomycetes. Most taxa occur on leaves, causing leaf spot diseases. Species such as *Septoria apiicola* Speg. of celery and *S. passerini* Sacc. of barley are of considerable economic importance. The type species of *Septoria* is *S. cytisi* Desm., a fungus occurring on *Cytisus laburnum* (= *Laburnum anagyroides*), and several other species in the Fabaceae (Farr 1992, Muthumary 1999). To date, more than 2000 names have been described in *Septoria* and, although groups that are associated with certain hosts families or geographical regions recently have been revised (Jørstad 1965, 1967, Constantinescu 1984, Sutton and Pascoe 1987, 1989, Farr 1991, 1992, McPartland 1995, Wu et al 1996, Shin 1999), most taxa never have been re-examined since their introduction. In the late 19th and early 20th centuries numerous *Septoria* collections on previously unrecorded host plants were described as new species.

Septoria currently is characterized by pycnidial conidiomata, holoblastic, hyaline, smooth-walled conidiogenous cells with sympodial and/or percurrent proliferation, and filiform, hyaline, smooth-walled multiseptate conidia (Sutton 1980, Constantinescu 1984, Farr 1992). However, it also includes taxa with apparently nonproliferating, phialidic conidiogenous cells (sensu Sutton 1980), e.g., *S. tritici,* and species that vary in their conidiomatal structure from acervuloid to pycnidial. Due to the limited number of useful morphological characters and the paucity of physiological and other data in vitro, the taxonomy of the *Septoria*-like species still remains confusing and largely dependant on the host. As pointed out by Verkley and Priest (2002), *Septoria*-like anamorphs of *Mycosphaerella* are not always easy to distinguish morphologically from certain coelomycetous anamorphs of the Pleosporales (viz. *Stagonospora* anamorphs of *Leptosphaeria* and *Phaeosphaeria*).

Knowledge of the phylogenetic relationships among *Septoria* is still fragmentary. The sexual state of the type species is unknown, and only a relatively small number of other species have been linked unequivocally to teleomorphs, all of which now are classified in *Mycosphaerella* Johanson (Dothideales). *Mycosphaerella* is a species-rich ascomycete genus that has been associated with 27 anamorph genera, mostly

hyphomycetous (von Arx 1983, Sutton and Hennebert 1994), 23 of which were accepted by Crous et al (2000). Recent molecular studies have shown that *Mycosphaerella* is monophyletic (Stewart et al 1999, Crous et al 2000, 2001b, Goodwin et al 2001), and that the taxa with anamorphs in *Cladosporium* s. str. that are associated with *Mycosphaerella*-like teleomorphs, such as *M. tassiana* (De Not.) Johanson, form a sister group of the *Mycosphaerella* clade (Braun et al 2003). In a study focussing primarily on cercosporoid anamorphs of *Mycosphaerella,* Crous et al (2001b) concluded that morphological characters such as conidiomatal structure (conidiophores solitary, fasciculate to synnematous, sporodochia, pycnidia, or acervuli) and conidial shape, size and septation are uninformative phylogenetically and are not useful for generic separation of *Mycosphaerella* anamorphs. In studies of other genera of ascomycetes, anamorphs similarly have been found to vary in conidiomatal structure from acervular through pycnidial to complex eustromatic (Samuels and Seifert 1987, Verkley 1999). In the foliicolous *Septoria*-like fungi, this raises the question whether *Septoria* with pycnidial conidiomata can be meaningfully distinguished from *Phloeospora* Wallr. (type species *Phl. ulmi,* teleomorph *Mycosphaerella ulmi*) with acervular conidiomata. Based on similarities of conidiomatal development, von Arx (1983) and Braun (1995) adopted a wider concept of *Septoria* that included *Phloeospora.* Another question is whether *Septoria* forms a monophyletic group within *Mycosphaerella.*

The aim of the present study is to investigate the phylogenetic relationships of *Septoria* species and other anamorphic genera linked to *Mycosphaerella.* The D2 region of the 28S ribosomal RNA gene is relatively conserved and informative at the generic level and above. The ITS region was chosen to assess the phylogenetic relationships of a large set of taxa within the main *Mycosphaerella* clade, using *Cladosporium* as outgroup. In total we selected 17 taxa with *Septoria* anamorphs, eight of which have known *Mycosphaerella* teleomorphs. Other *Mycosphaerella* species and anamorph taxa were added, so that in total 10 anamorphic genera that have been associated with *Mycosphaerella* were included. To further investigate the value of the conidiomatal structure, we also included a strain of *Phl. ulmi.*

MATERIAL AND METHODS

The 77 strains used in this study are listed in TABLE I. Most are preserved in the CBS culture collection under the teleomorph name. Names of confirmed alternate states also are listed in TABLE I. Morphology in vitro was studied as described by Verkley (1998b).

DNA isolation.—Strains were transferred from agar cultures to 2 mL liquid medium (2% malt extract) and incubated on a rotary shaker (300 rpm) for 2–3 wk at room temperature. Liquid cultures were transferred to 2 mL tubes, centrifuged and washed twice with sterile water. Subsequently, 0.1 g of silica (Merck, Darmstadt, Germany) and 300 μL of CTAB extraction buffer was added (200 mm tris [hydroxynethyl]aminomethane [Tris]-HCL, pH 7.5, 1.5 M NaCl, 20 mM EDTA, 2% hexadecyltrimethylammoniumbromide [CTAB]). Tissue was ground with a micropestle for 1 min, another 200 μL of CTAB extraction buffer was added, and the lysate was incubated 10 min at 65 C. A chloroform/isoamylalcohol (24:1) extraction was performed and 2 volumes of ethanol were added, followed by incubation at 20 C for 30 min. The precipitate was centrifuged 5 min and the pellet was washed with 70% ethanol. The pellet was allowed to dry and dissolved in 100 μL TE. Finally, 2.5 μL RNase (10 mg/mL) was added and the solution was incubated 5 min at 37 C. For DNA isolation, the FastDNAkit (Omnilabo 6050073, BIO 101) also was used.

Sequence analysis.—For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9D (de Hoog and Gerrits van den Ende 1998) and LS266 (Masclaux et al 1995), or V9G (de Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990). PCR was performed in 50 μL reaction volumes and each reaction contained 10–100 ng of genomic DNA, 25 pM of each primer, 40 μM dNTP, 1.0 unit Supertaq DNA polymerase and 5 μL 10× PCR buffer (SphaeroQ, Leiden, The Netherlands). The amplification was performed in a thermocycler (Applied Biosystems Foster City, California), with this program: 1 min 95 C, 30× (1 min 95 C, 1 min 55 C, 2 min 72 C) followed by a final extension of 5 min at 72 C. PCR product was cleaned with GFx columns (Amersham Pharmacia 27-9602-01) and analyzed on a 2% agarose gel to estimate the concentration. The PCR product was sequenced using internal primers ITS5 and ITS4 or ITS1 and ITS4 (White et al 1990). Sequencing was performed with the BigDye terminator chemistry (Part number 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex colums (Amerham Pharmacia 17-0041-01) and separated and analyzed on an automated sequencer (ABI Prism 3700 DNA Analyzer; Applied Biosystems). Forward and reversed sequences were matched using SeqMan from the Lasergene package (DNAstar Inc., Madison, Wisconsin). For D2 sequence analyses the MicroSeq; TM D2 LSU rDNA Fungal Sequencing Kit (Applied Biosystems) was used according to manufacturer's instructions.

Phylogenetic analyses.—Pairwise and global alignment of consensus sequences were performed in Bionumerics 2.5 (Applied Maths, Kortrijk, Belgium). Manual adjustments were made in the global alignment where necessary. Maximum-parsimony and neighbor-joining distance methods were used to infer phylogenetic hypotheses. Parsimony analyses were done using PAUP version 4.0b10 (Swofford 2002). The heuristic searches were performed with these parameters: characters were unordered with equal weight, and random taxon addition. The tree bisection-reconnection

TABLE I. Fungal isolates included for ITS and LSU-D2 sequence analyses in alphabetical order of the anamorph name

ITS GenBank	D2 GenBank	Anamorph	Teleomorph	Origin
	AY152608	*Ascochyta lycopersici* (Plowr.) Brun.	*Didymella lycopersici* Kleb.	CBS 735.74; *Lycopersicon esculentum,* Netherlands
	AY152611	*A. pinodes* L. K. Jones	*D. pinodes* (Berk. & Bloxam) Petr.	CBS 235.55; *Pisum* sp.
	AY152610	*A. rabiei* (Pass.) Labr.	*D. rabiei* (Kovachevski) Arx**	CBS 237.37 (ex type); *Cicer arietinum,* Bulgaria
	AY152629	*Cercospora apii* Fres.	*Mycosphaerella* state unknown	CBS 536.71; *Apium graveolens,* Rumania
AY152576	AY152632	*C. beticola* Sacc.	*Mycosphaerella* state unknown	CBS 539.71; *Beta vulgaris,* Rumania
	AY152628	*C. carotae* (Pass.) Kaznowski & Siemaszko	*Mycosphaerella* state unknown	CBS 101.65; *Daucus carota,* Norway
AY152577	AY152630	*C. kukuchii* (Matsumoto & Tomoyasu) Gardner	*Mycosphaerella* state unknown	CBS 128.27 (ex type); *Glycine max,* Japan
	AY152631	*C. nicotianae* Ellis & Everh.	*Mycosphaerella* state unknown	CBS 570.69; *Nicotiana tabacum*
	AY152642	*C. zonata* Winter	*Mycosphaerella* state unknown	CBS 557.71; *Vicia narbonensis,* Rumania
AY152598	AY152627	*Cercosporidium magnoliae* (J. B. Ellis & Harkn.) Sivan.	*Mycosphaerella milleri* Hodges & Haasis	CBS 541.63; *Magnolia grandiflora,* North Carolina, USA
AY152552	AY152617	*Cladosporium herbarum* (Pers. : Fr.) Link	*Davidiella tassiana* (De Not.) Crous & U. Braun	CBS 289.49; *Allium schoenoprasum,* Switzerland
AY302597		*Cl. herbarum* (Pers. : Fr.) Link	*D. tassiana* (De Not.) Crous & U. Braun	CBS 223.31; ex *Mycosphaerella tulasnei,* F. D. Heald
	AY152615	*Dendryphion penicillatum* (Corda) Fr.	*Pleospora papaveracea* (De Not.) Sacc.	CBS 432.50; *Papaver somniferum,* Germany
	AY152618	*Lecanosticta acicola* (Thüm.) Syd.	*Eruptio acicola* (Dearn.) M. E. Barr***	CBS 322.33; *Pinus* sp., USA
	AY152604	*Naemospora* sp.	*Lachnellula willkommii* (Hartig) Dennis	CBS 170.35; *Larix decidua,* Massachusetts, USA
	AY152605	*Naemospora* sp.	*L. occidentalis* (Hahn & Ayers) Dharne	CBS 160.35; *Larix decidua,* Massachusetts, USA
AY152575		*Phloeospora ulmi* (Fr. : Fr.) Wallr.	*Mycosphaerella ulmi* Kleb.	CBS 344.97; *Ulmus glabra,* Austria
	AY152644	*Ph. ulmi* (Fr. : Fr.) Wallr.	*M. ulmi* Kleb.	CBS 101564; *Ulmus* sp., Netherlands
	AY152609	*Phoma* sp.	*Didymella cannabis* (Winter) Arx	CBS 234.37; *Cannabis sativa*
AY152590	AY152621	*Pseudocercospora* sp.	*Mycosphaerella laricina* R. Hartig	CBS 326.52; *Larix decidua,* Switzerland
AY152595		*Ramularia grevilleana* (Tul. & C. Tul.) Jørst.	*M. fragariae* (Tul.) Lind.	CBS 259.36; *Fragaria* sp., Netherlands
AY152597		*R. grevilleana* (Tul. & C. Tul.) Jørst.	*M. fragariae* (Tul.) Lind.	CBS 719.84; *Fragaria* sp., Netherlands
AY152596	AY152625	*R. grevilleana* (Tul. & C. Tul.) Jørst.	*M. fragariae* (Tul.) Lind.	CBS 298.34; *Fragaria* sp., Netherlands
AY152593		*Ramularia* sp.	*M. punctiformis* (Pers. : Fr.) Starb.	CBS 943.97; *Quercus* sp., Netherlands
AY152594		*Ramularia* sp.	*M. punctiformis* (Pers. : Fr.) Starb.	CBS 184.97; *Acer pseudoplatanus,* Netherlands
	AY152624	*Ramularia vallisumrosae* Cavara	*Mycosphaerella* state unknown	CBS 271.38; *Narcissus* sp., UK
	AY152616	*Sclerophoma pythiophila* (Corda) Höhn.; *Hormonemamatioides* Lagerberg & Melin	*Sydowia polyspora* (Bref. & von Tav.) E. Müller	CBS 544.95; *Larix decidua,* Netherlands
	AY152638	*Septoria aceris* (Lib.) Berk. & Br.	*Mycosphaerella latebrosa* (Cooke) Schröt.	CBS 183.97; *Acer pseudoplatanus,* Netherlands
AY152553		*S. aceris* (Lib.) Berk. & Br.	*M. latebrosa* (Cooke) Schröt.	CBS 687.94; *Acer pseudoplatanus,* Netherlands
AY152571	AY152634	*S. aciculosa* Ellis & Everh.	*Mycosphaerella* state unknown	CBS 177.77; *Fragaria* sp., New Zealand
AY152572		*S. apiicola* Speg.	*Mycosphaerella* state unknown	CBS 395.52, IMI 092627; *Apium* sp., Netherlands
AY152573	AY152645	*S. apiicola* Speg.	*Mycosphaerella* state unknown	CBS 389.59; *Apium graveolens,* Italy

TABLE I. Continued

ITS GenBank	D2 GenBank	Anamorph	Teleomorph	Origin
AY152574		*S. apiicola* Speg.	*Mycosphaerella* state unknown	CBS 400.54, IMI 092628; *Apium graveolens,* Netherlands
AY152579	AY152641	*S. berberidis* Niessl	*Mycosphaerella berberidis* (Auerswald) Lind.	CBS 324.52; *Berberis vulgaris,* Switzerland
AY152566	AY152648	*S. calendulae* Bernaux	*Mycosphaerella* state unknown	CBS 349.58; *Calendula arvensis,* Italy
AY152588		*S. castaneicola* Desm.	*Mycosphaerella* state unknown	CBS 102377; *Castanea sativa,* Netherlands
AY152589		*S. castaneicola* Desm.	*Mycosphaerella* state unknown	CBS 102323; *Castanea sativa,* Netherlands
AY152569	AY152635	*S. gerberae* Syd. & P. Syd.	*Mycosphaerella* state unknown	CBS 410.61; *Gerbera jamesonii,* Italy
	AY152647	*S. glycines* Hemmi	*Mycosphaerella* state unknown	DAOM 226224
AY152563		*S. lamiicola* Sacc.	*Mycosphaerella* state unknown	CBS 109113; *Lamium album,* Austria
AY152564		*S. lamiicola* Sacc.	*Mycosphaerella* state unknown	CBS 102328; *Lamium album,* Netherlands
AY152570	AY152636	*S. linicola* (Speg.) Garovaglio	*Mycosphaerella linicola* Naumov	CBS 316.37; *Linum usitatissimum,* Argentina
	AY152633	*S. linicola* (Speg.) Garovaglio	*M. linicola* Naumov	DAOM 226246
AY152583		*S. populicola* Peck	*M. populicola* G. Thompson	CBS 100045; *Populus trichocarpa,* Washington, USA
AY152584		*S. populicola* Peck	*M. populicola* G. Thompson	CBS 100052; *Populus trichocarpa,* Washington, USA
AY152585		*S. populicola* Peck	*M. populicola* G. Thompson	CBS 100044; *Populus trichocarpa,* Washington, USA
AY152586	AY152637	*S. populicola* Peck	*M. populicola* G. Thompson	CBS 100051; *Populus trichocarpa,* Washington, USA
AY152587		*S. populicola* Peck	*M. populicola* G. Thompson *M. pyri* (Auerswald) Boerema	CBS 100047; *Populus trichocarpa,* Washington, USA
AY152591	AY152626	*S. pyricola* (Desm.) Desm.	*M. pyri* (Auerswald) Boerema	CBS 222.31; *Pyrus communis*
AY152592		*S. pyricola* (Desm.) Desm.	*M. grossulariae* (Fr.) Lind.	CBS 640.72; *Pyrus communis,* Netherlands
AY152581	AY152640	*S. ribis* (Lib.) Desm.		CBS 235.37; *Ribes nigrum,* Netherlands
AY152565	AY152646	*S. rubi* West.*	*M. rubi* Roark	CBS 238.37; *Rubus strigosus,* Illinois, USA
AY152578		*S. rubi* West.*	*M. rubi* Roark	CBS 102327; *Rubus fruticosus* s.l., Netherlands
AY152580		*S. rubi* West.*	*M. rubi* Roark	CBS 109017; *Rubus idaeus,* Austria
AY152558		*S. scabiosicola* Desm.	*Mycosphaerella* state unknown	CBS 108981; *Knautia arvensis,* Austria
AY152559		*S. scabiosicola* Desm.	*Mycosphaerella* state unknown	CBS 102336; *Knautia arvensis,* Netherlands
AY152560		*S. scabiosicola* Desm.	*Mycosphaerella* state unknown	CBS 317.37
AY152561		*S. scabiosicola* Desm.	*Mycosphaerella* state unknown	CBS 182.93; *Succissa pratensis,* France
AY152562		*S. scabiosicola* Desm.	*Mycosphaerella* state unknown	CBS 102335; *Knautia arvensis,* Netherlands
AY152567		*S. sii* Rob. & Desm.	*Mycosphaerella* state unknown	CBS 102369; *Berula erecta,* Netherlands
AY152568		*S. sii* Rob. & Desm.	*Mycosphaerella* state unknown	CBS 118.96; *Berula erecta,* Netherlands
AY152601	AY152622	*S. tritici* Rob.	*Mycosphaerella graminicola* (Fuckel) Schröt.	CBS 100330 (IPO 6566.1); *Triticum aestivum*
	AY152623	*S. tritici* Rob.	*M. graminicola* (Fuckel) Schröt.	CBS 100329 (IPO 6569.81); *Triticum aestivum*
AY152602		*S. tritici* Rob.	*M. graminicola* (Fuckel) Schröt.	CBS 100335; *Triticum aestivum*
AY152603		*S. tritici* Rob.	*M. graminicola* (Fuckel) Schröt.	CBS 392.59; *Triticum aestivum*

TABLE I. Continued

ITS GenBank	D2 GenBank	Anamorph	Teleomorph	Origin
	AY152606	*Spilocaea pomi* Fr.	*Venturia inaequalis* (Cooke) Winter	CBS 595.70; *Malus sylvestris,* Netherlands
	AY152612	*Stagonospora avenae* (Frank) Bisset	*Phaeosphaeria avenaria* f. sp. *avenaria* (Weber) O. E. Eriksson	DAOM 226215
	AY152613	*Stagonospora* sp.	*Ph. avenaria* f. sp. *triticea* (T. Johnson) Shoemaker & C. E. Babc.	DAOM 226222
	AY152607	*Stemphylium botryosum* Wallr.	*Pleospora herbarum* (Pers. : Fr.) Rabenh. var. *herbarum*	CBS 191.86; *Medicago sativa,* India
	AY152614	*St. botryosum* Wallr.	*Pl. herbarum* (Pers. : Fr.) Rabenh. var. *herbarum*	DAOM 195299; *Medicago sativa,* Ontario
AY152599	AY152619	*Stenella parkii* Crous & Alfenas	*Mycosphaerella parkii* Crous et al.	CBS 387.92 (=''STE-U 353''; ex type); *Eucalyptus grandis,* Brazil
	AY152639	Unknown	*M. harthensis* (Auersw.) Migula	CBS 325.52; *Betula* sp., Switzerland
AY152600	AY152620	Unknown	*M. marksii* Carnegie & Keane	CBS 682.95 (''STE-U 842''); *Eucalyptus grandis,* South Africa
AY152554	AY152643	Unknown****	*M. brassicicola* (Fr.) Lind.	CBS 267.53; *Brassica oleracea,* Netherlands
AY152555		Unknown****	*M. brassicicola* (Fr.) Lind.	CBS 228.32; *Brassica oleracea,* Denmark
AY152556		Unknown****	*M. brassicicola* (Fr.) Lind.	CBS 174.88; *Brassica oleracea,* Germany
AY152557		Unknown****	*M. brassicicola* (Fr.) Lind.	CBS 173.88; *Brassica oleracea,* Germany

* = unconfirmed.

** = nomen inval. (no latin diagnosis).

*** = *Mycosphaerella dearnessi* M. E. Barr.

**** = *Asteromella brassicae* (F. Chevallier) Boerema & van Kesteren spermatial state.

(TBR) algorithm was used in branch swapping, with branches collapsing if the maximum branch length was zero. The maximum number of trees was set at 10 000. Alignment gaps were treated as fifth base in the D2 analyses, where they were confined to highly conserved regions. In the ITS analyses, gaps were treated as missing data because they also had to be introduced in less conserved regions. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition and 1000 replicates, with maxtrees set at 100. Forty-six strains were included in the analysis of D2, with two species of the order Helotiales, (viz. *Lachnellula willkommii* [CBS 170.35] and *L. occidentalis* [CBS160.35]) as multitaxon outgroup. Several pleosporalean taxa were included in the selection for this region but not for the more variable ITS region, because the sequences could not be unambiguously aligned. Seventy-three strains were included in the analysis of ITS, with three strains of *Cladosporium herbarum* as multitaxon outgroup. Neighbor-joining analyses were performed in Bionumerics and PAUP, in both cases without pairwise corrections. Stability of clades was tested using 1000 neighbor-joining bootstrap replications.

The following additional sequences were retrieved from GenBank (teleomorph names in TABLE I or here in brackets): *Septoria tritici,* AF181692, AF181693; *Cercospora apii,* CA1; *C. beticola,* CB4; *C. kikuchii,* CK35, CK39; *Paracercospora fijiensis* var. *fijiensis* (Morelet) Deighton [*M. fijiensis* Meredith & Lawrence], PF7; *Pa. fijiensis* var. *difformis* (J. L. Mulder & R. H. Stover) Deighton [*M. fijiensis* var. *difformis* J. L. Mulder & R. H. Stover], PFD9; *Pseudocercospora musae* (Zimm.) Deighton [*M. musicola* J. L. Mulder], PM10, PM11; *Ps. cruenta* (Sacc.) Deighton [*M. cruenta* Latham], PCR18; *Mycosphaerella latebrosa,* AF362051 (CBS 183.97), AF362067 (CBS 652.85); *Septoria passerini,* AF181697, AF181699; *Dothidea sambuci* Pers. : Fr., AF382387; and *Pleospora herbarum,* AF382386.

RESULTS

D2 LSU.—The alignment for the phylogenetic analyses of the D2 region of the LSU comprised 334 characters, of which 111 (33%) were parsimony informative. The remaining 223 characters were uninformative and excluded from the parsimony analysis. In the neighbor-joining analyses, 135 informative and autapomorphic characters were included to obtain accurate branch lengths in the phylograms, while all constant characters were excluded. The heuristic search involving 5000 random sequence input orders yielded 24 MPTs of 361 steps, with consistency index (CI) 0.526, retention index (RI) 0.850, rescaled consistency index (RCI) 0.448 and homoplasy index (HI) 0.474. The strict-consensus tree with results of the bootstrap analysis is shown in FIG. 1. Maximum bootstrap support (100%) was found for the major *Mycosphaerella* clade and its direct sister, a strain of *Cladosporium herbarum*. Within *Mycosphaerella,* the two species of *Ramularia* clustered with two strains of *S. tritici* (*M. graminicola*) (95%). These clusters showed 100% internal homology in D2 sequences: (i) *Septoria glycines* and *S. calendulae*; (ii) *S. gerberae* and *S. aciculosa,* and the two strains of *S. linicola*; and (iii) *Cercospora apii, C. kikuchii, C. nicotianae* and *C. beticola. Cercospora zonata* and *C. carotae* had divergent D2 sequences. *Dothidea sambuci* and *Sydowia polyspora,* the only other Dothideales represented in this selection, clustered in a well-supported sister group (96%) of *Mycosphaerella,* but for all represented Dothideales there was less support (72%). Additional well-supported clusters were the *Didymella* spp., the two formae speciales of *Phaeosphaeria avenaria,* and two strains representing *Pleospora herbarum* and *Pl. papaveracea.* CBS 191.86 is most likely misidentified as *Pl. herbarum.* These results agree with those of the neighbor-joining analyses (FIG. 2). Again, in neighbor joining there was high support for the *Ramularia* spp. and *M. graminicola,* which clustered with *M. laricina* (anamorph *Pseudocercospora* sp.). Within *Mycosphaerella,* a large cluster with 93% bootstrap support comprised *Phl. ulmi, M. harthensis, M. brassisicola,* all *Cercospora* and *Septoria* spp. except *S. tritici* and *S. pyricola.* In parsimony analysis the support for this cluster was 84%.

ITS.—Of the 513 characters in the alignment of the ITS region, 196 (38%) parsimony-informative characters were used for the phylogenetic analyses. Five small regions comprising 25 characters with insertions/deletions or ambiguous position homology were excluded. For the parsimony analysis, the remaining uninformative 292 characters also were excluded from the analyses. In the neighbor-joining analyses constant characters were excluded but autapomorphic characters included to obtain accurate branch lengths in the phylograms, so that in total 203 characters were used. The heuristic search involving 5000 random sequence input orders yielded 530 MPTs of 514 steps (CI 0.591, RI 0.866, RCI 0.512 and HI 0.409). The strict-consensus tree is shown in FIG. 3. Bootstrap supports of more than 50% are indicated. The results of the neighbor-joining analysis obtained with Bionumerics and PAUP were the same (FIG. 4) and largely agreed with those of the parsimony analysis in PAUP (FIG. 3). As expected, most strains of the same species clustered in highly supported clades in the bootstrap analysis and showed 100% homology or differed by a single position. A difference on 2–4 positions was observed within the strain pairs of *Pseudocercospora musae, M. punctiformis, S. castaneicola* and outgroup strains of *Cladosporium herbarum.* The three strains of *S. rubi* showed a

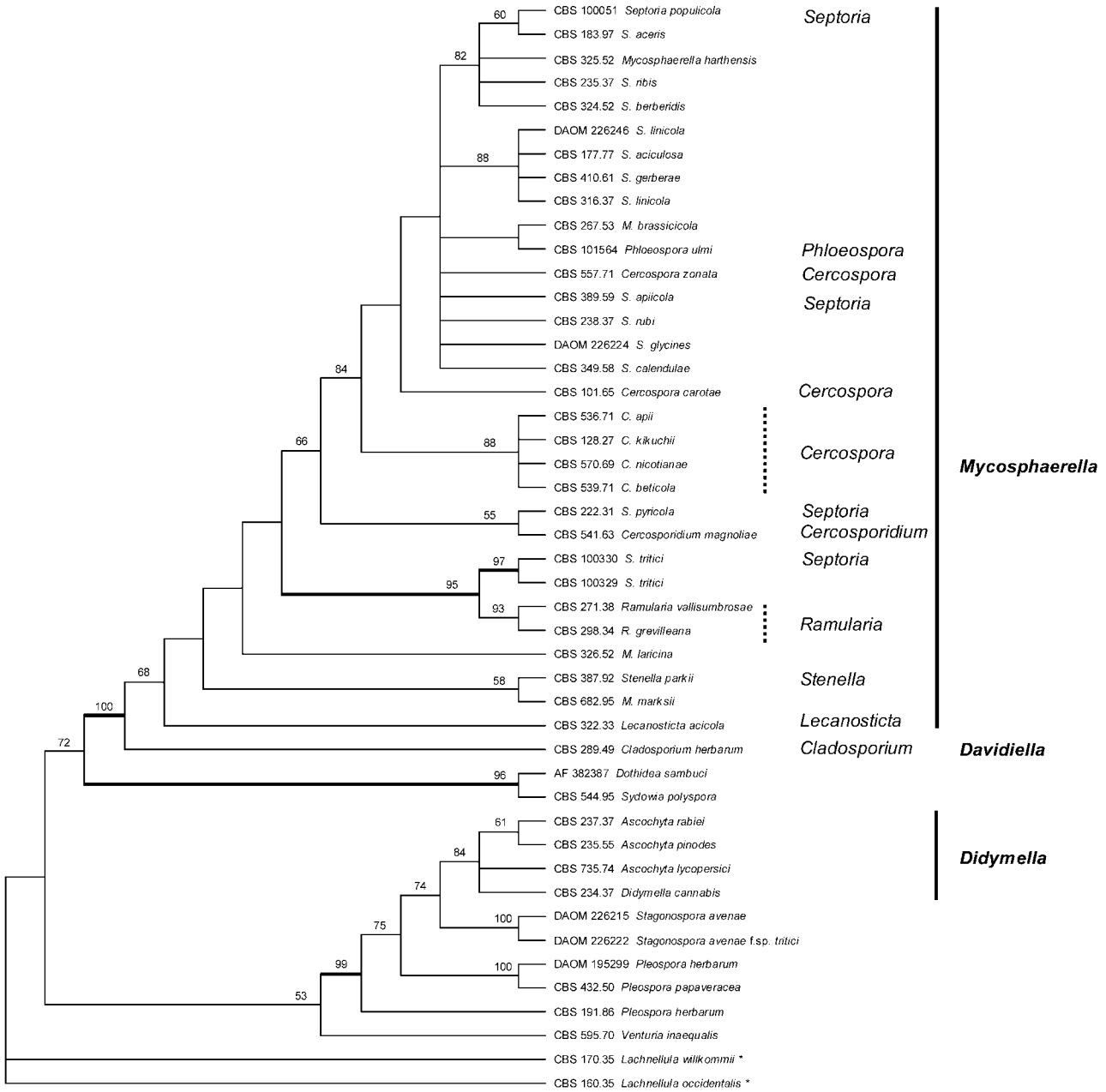

FIG. 1. Strict-consensus tree of 24 MPTs of 361 steps (CI = 0.526, RI = 0.850, RCI = 0.448, HI = 0.474), obtained in PAUP using a heuristic search of the D2 region of the LSU rRNA gene, using 111 parsimony-informative characters. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 95% or higher values are in bold. Species are presented by anamorph name, if known (teleomorph names are given in TABLE I). Two presumed outgroup taxa are marked with asterisk (*Lachnellula willkommii* and *L. occidentalis*).

higher diversity, with CBS 109017 (isolated from *Rubus idaeus,* Austria) differing from CBS 238.37 (from *R. strigosus,* Illinois) by 5 base positions in ITS 1, 2 positions in the 5.8 S gene, and 6 in ITS 2. It differed from CBS 102327 (from *R. fruticosus* s.l., Netherlands) by 11 positions in ITS 1, also 2 positions in the 5.8S gene and 5 in ITS 2. CBS 238.37 differed from 102327 by 10 positions in ITS 1 and 3 in ITS 2 (5.8 S gene identical).

High bootstrap percentages were found for several clades, including one comprising the three species of *Cercospora* (parsimony 96/neighbor joining 98), a *Ra-*

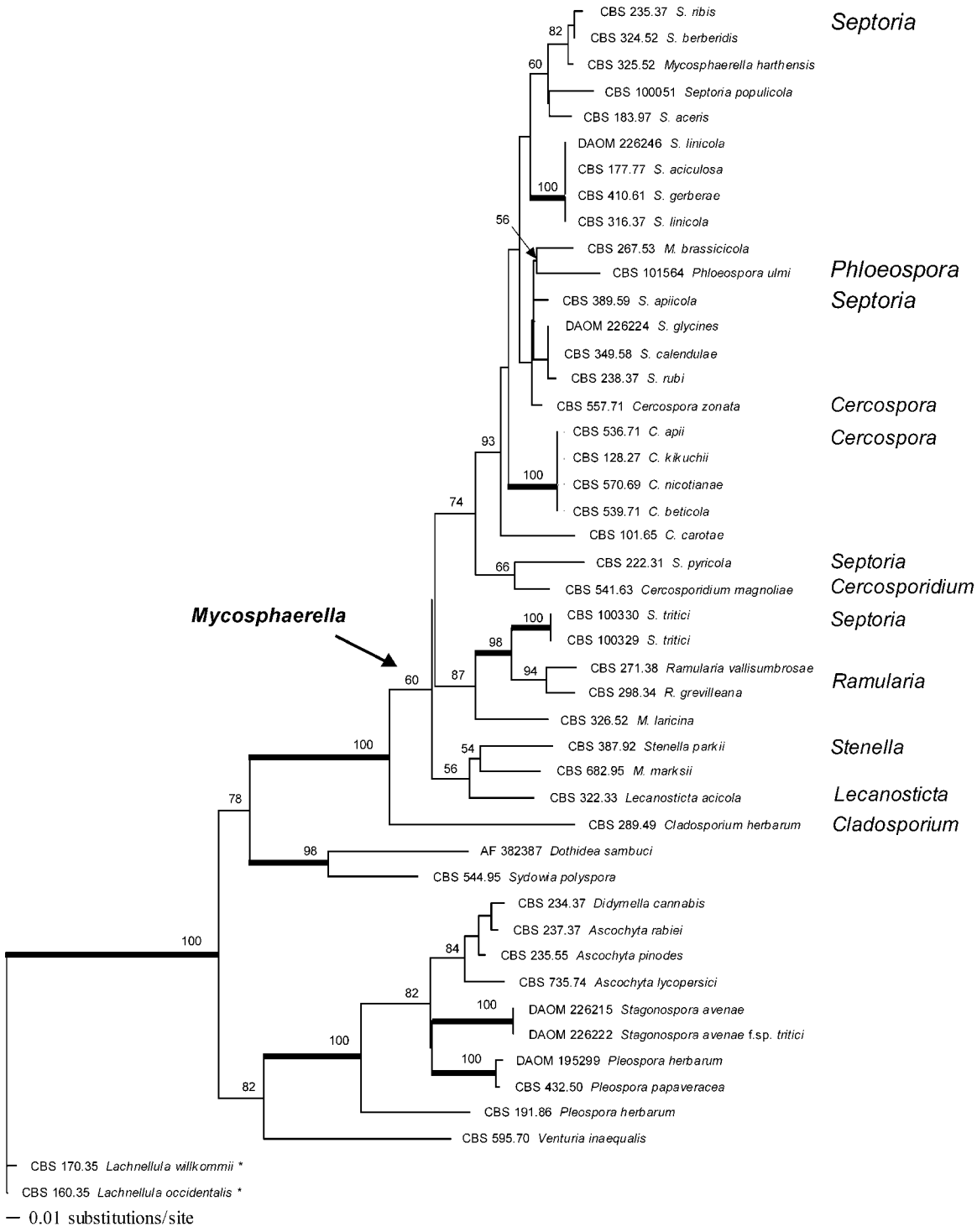

FIG. 2. Neighbor-joining tree derived from 135 parsimony-informative and autapomorphic characters of the D2 region of the LSU rRNA gene, calculated in PAUP without pairwise corrections. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 95% or higher values are in bold. Length of branches is proportional to number of changes. Species are presented by anamorph name, if known (teleomorph names are given in TABLE I). Sequences of taxa marked with asterisk were used as outgroup to root the tree (*Lachnellula willkommii* and *L. occidentalis*).

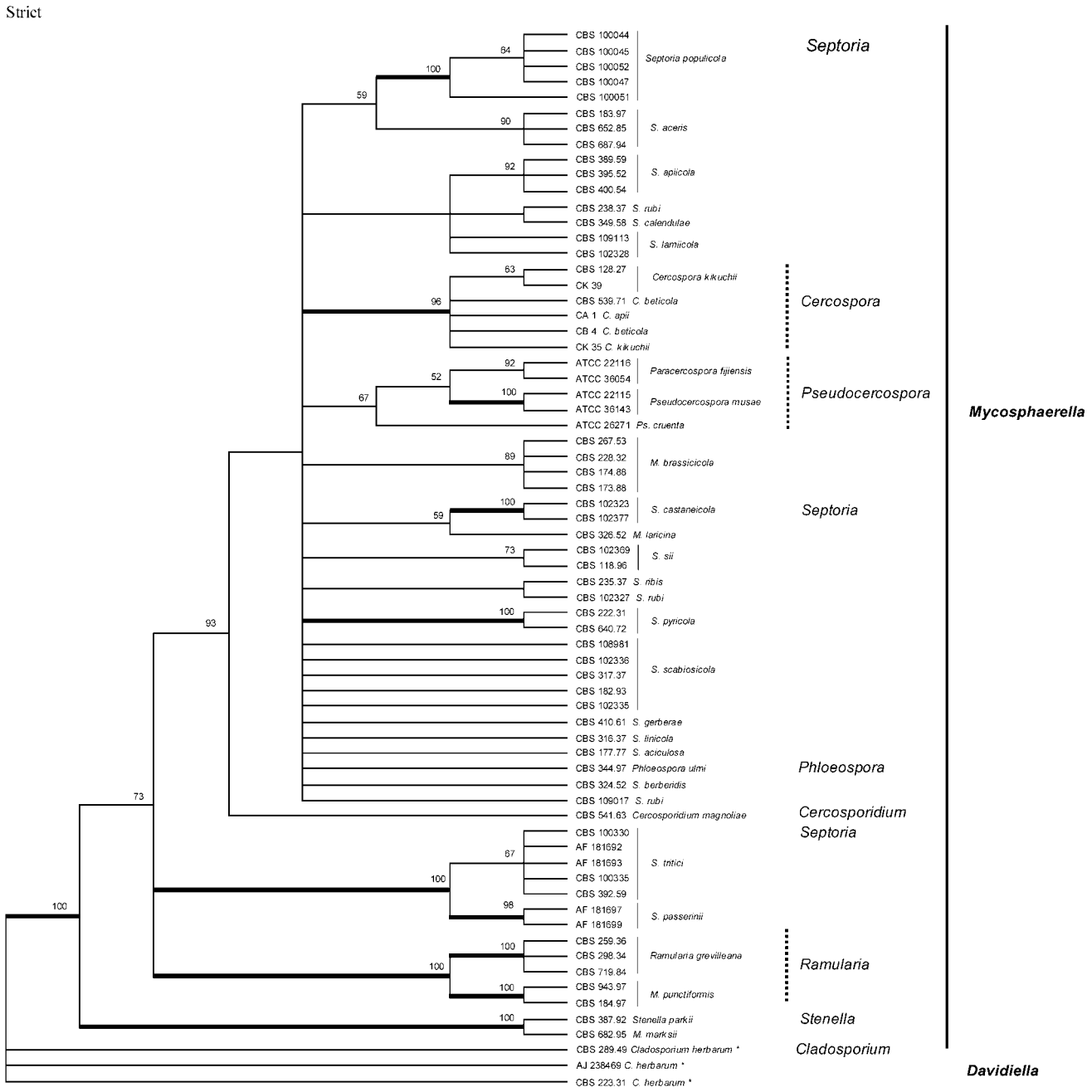

FIG. 3. Strict-consensus tree of 530 MPT's of 514 steps (CI = 0.591, RI = 0.866, RCI = 0.512, HI = 0.409), obtained in PAUP using a heuristic search of the ITS1-5.8SrDNA-ITS2 region using 196 parsimony-informative characters. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 95% or higher values are in bold. Species are presented by anamorph name, if known (teleomorph names are given in TABLE I). Two strains of *Cladosporium herbarum* were used as outgroup and are marked with asterisks.'

mularia-clade (100/100), a clade including the cereal pathogens *Septoria tritici* and *S. passerini* (100/100) and a clade including *M. marksii* and *Stenella parkii* (100/98). A large subclade including *Cercosporidium magnoliae, Cercospora* and *Paracercospora* spp., *Phloeospora ulmi* and most *Septoria* spp. obtained 93% bootstrap support in the parsimony analysis, in the strict consensus tree in a polytomy with the clades of *S. tritici* and *S. passerini* and *Ramularia*. Maximum support also was found for all ingroup taxa of the main *Mycosphaerella* clade. A subcluster with 80% bootstrap support in the neighbor-joining analysis contained 40

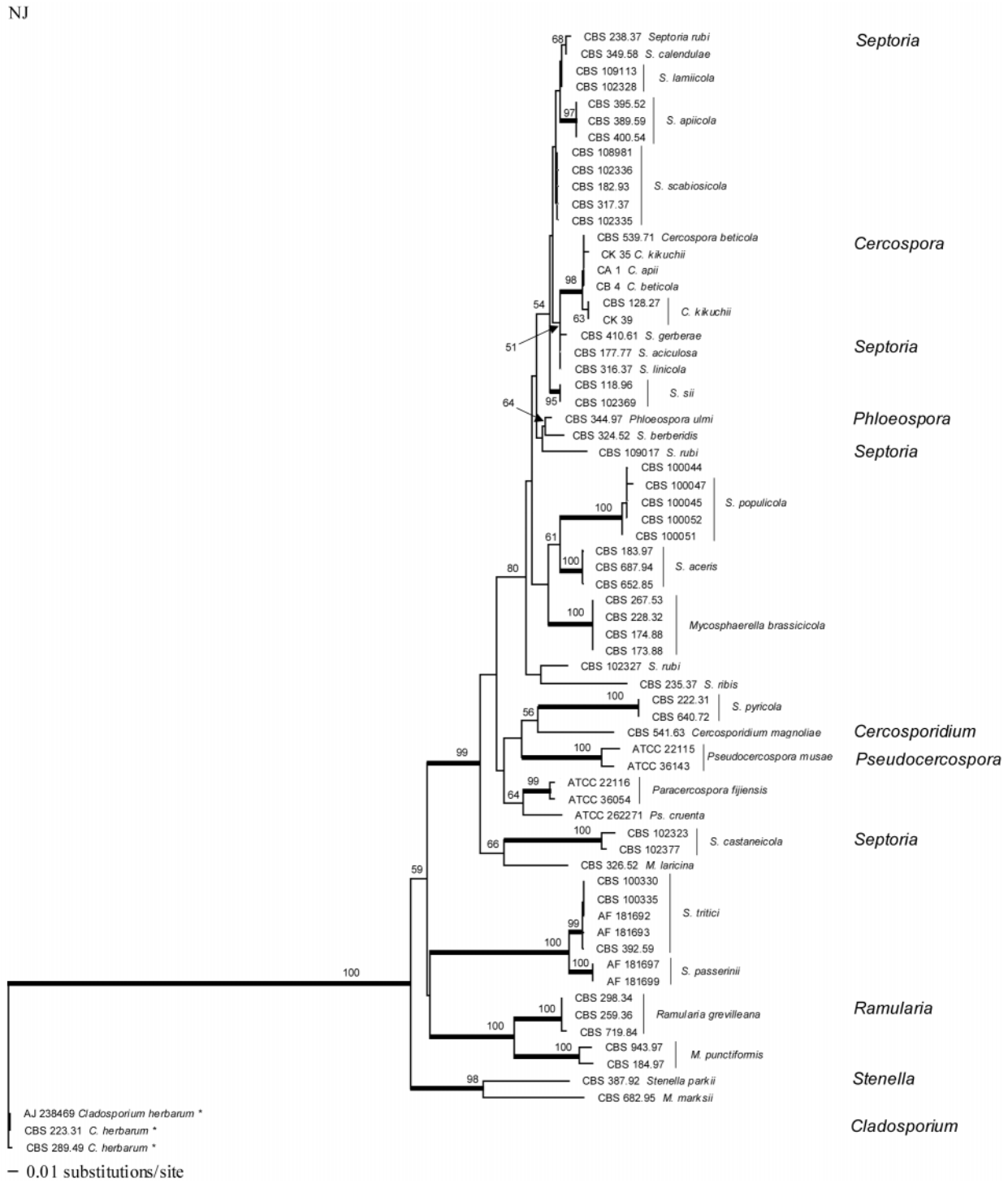

FIG. 4. Neighbor-joining tree derived from 203 parsimony-informative and autapomorphic characters of the ITS1-5.8SrDNA-ITS2 region calculated in PAUP without pairwise corrections. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 95% or higher values are in bold. Length of branches is proportional to number of changes. Species are presented by anamorph name, if known (teleomorph names are given in TABLE I).

strains, *M. brassicicola,* the *Cercospora* spp., *Phl. ulmi* and all *Septoria* spp. analysed, except *S. castaneicola, S. pyricola, S. tritici* and *S. passerini*.

DISCUSSION

Septoria species are among the most common and widespread leaf-spotting fungi worldwide. The taxonomy is complicated, and life cycles and ecology are still poorly understood. Previous molecular studies of *Mycosphaerella* mostly focussed on the hyphomycetous anamorphs. The present study is the first large-scale molecular phylogenetic study of *Septoria,* and it provides important new insights into the complex evolution of coelomycetous anamorphs in *Mycosphaerella*.

No more than four *Septoria* species previously have been included in molecular phylogenetic studies. Goodwin and Zismann (2001) sequenced the 5.8 S ribosomal RNA gene and flanking internal-transcribed spacers (ITS 1 and ITS 2) for a dozen strains of *S. tritici* and *S. passerini*. These authors demonstrated that *S. passerini* (teleomorph unknown) belongs in *Mycosphaerella* and is closely related to *S. tritici* (teleomorph *M. graminicola*). Based on ITS sequence analysis, Crous et al (2001b) found that two strains of *S. aceris* (teleomorph *M. latebrosa*) clustered with *Cercospora* spp., distant from a third strain, which belonged to *S. tritici*. They suspected that several possible lineages within *Mycosphaerella* were associated with *Septoria* anamorphs.

All *Septoria* species clustered within *Mycosphaerella*. *Septoria* has been regarded a heterogeneous assemblage due to the considerable variation in its conidial morphology, conidiogenesis and conidiomatal structures (Sutton 1980, Constantinescu 1984, Sutton and Hennebert 1994, Verkley 1998a, b, Muthumary 1999). We were unable to obtain material of *S. cytisi,* the type species of *Septoria*. This species has been studied extensively in planta, and several new varieties have been described recently (Farr 1992, Muthumary 1999). Morphologically this species certainly can be regarded as a typical *Septoria,* with well-developed pycnidia, sympodial as well as percurrent conidiogenous cell proliferation and relatively long, pluriseptate conidia. Transmission electron microscopy (TEM) of *S. chrysanthemella* Sacc., *S. quercicola* Sacc. and *S. aceris* has shown that these species also exhibit percurrent and sympodial proliferation, even in a single conidiogenous cell (Verkley 1998a, b). According to Sutton (1980), *S. tritici* and *S. apiicola* have phialidic conidiogenous cells. We observed percurrent and sympodial proliferation in all sporulating *Septoria* cultures used in the present study, including those of *S. apiicola* and also *S. passerini,* which is closely related to *S. tritici* (Goodwin and Zismann 2001). Our cultures of *S. tritici* seemed to confirm that the species is phialidic, but the conidiogenous cells are small and should be studied by TEM. Sutton's observations on conidiogenesis in *S. apiicola* probably are based on cryptically proliferating cells, which under the light microscope easily can be mistaken for phialides, as reported for *S. chrysanthemella* (Verkley 1998a).

Representatives of the order Pleosporales were included in this study to detect possible affinities with *Septoria* species, but none were found. *Leptosphaeria* Ces. & De Not. and *Phaeosphaeria* I. Miyake species have anamorphs in *Stagonospora* (Sacc.) Sacc., that sometimes have been placed in *Septoria*. For example, *Stagonospora nodorum* (Berk.) Castell. & Germano, the anamorph of the cosmopolitan wheat pathogen *Phaeosphaeria nodorum* (E. Müll.) Hedjar. (syn. *Leptosphaeria nodorum* E. Müll.), still often is referred to as *Septoria nodorum* (Berk.) Berk. Verkley and Priest (2000) pointed out that it may be impossible to delimit *Septoria* from *Stagonospora* by strict morphological criteria alone.

Our results support the conclusions of Crous et al (2001b), that conidiomatal structure has little value for predicting phylogenetic relatedness among taxa in *Mycosphaerella*. *Phloeospora ulmi,* the type species of the anamorph genus *Phloeospora,* is characterized by acervuli on the host. It clustered in our phylogenetic analyses with *Cercospora* spp. forming caespituli and most *Septoria* spp. forming pycnidia. *Septoria aceris,* which also clusters among these fungi, often forms conidiomata that are similar to the acervuli of *Phl. ulmi,* for which it has been treated under *Phloeospora* by Jørstad (1965). *Phloeospora ulmi* and *S. aceris* also agree in conidiogenesis. It seems unlikely that a separation between *Phloeospora* and *Septoria* is tenable.

The results also indicate that *Septoria* is not monophyletic within *Mycosphaerella* and that the development of pycnidial forms with multiseptate hyaline conidia might have occurred more than once. Three lineages roughly are indicated here (viz. a major clade of *Septoria* spp. closely related to *Cercospora* spp., including several notorious pathogens such as *S. apiicola, S. linicola* and *S. populicola,* a second lineage containing the cereal pathogens *Septoria tritici* and *S. passerini* and possibly separate from these two lineages, one represented by *S. castaneicola,* and another by *S. pyricola*). A clade including *S. tritici* and species with *Ramularia* anamorphs was supported by high bootstrap values in the D2 analyses only. The ITS analyses of Crous et al (2001b) and Goodwin et al (2001) also suggest a close relationship of *S. tritici* and *S. passerinii* with *Ramularia,* one which contrasts

with many morphological differences distinguishing *Ramularia* and *Septoria* anamorphs. The addition of *S. pyricola, S. castaneicola* and *M. laricina* to our ITS dataset may explain why this relationship is not confirmed in the analyses of that region. *Ramularia* spp. clustered in a single group within *Mycosphaerella* in earlier studies (Crous et al 2001b, Goodwin et al 2001), although the numbers of included strains were small.

Crous et al (2001b) already reported the clustering of *Cercospora* species with two strains of *S. aceris* (*M. latebrosa*), as well as *M. brassicicola*. Three out of four *Cercospora* spp. with identical D2 sequences (*C. apii, C. kikuchii, C. beticola*) also clustered in the analyses of ITS with high bootstrap support (parsimony 96%, neighbor joining 98%). *Cercospora zonata* and *C. carotae,* which were included here only in the analysis of the D2 region, did not cluster with these species. Stewart et al (1999) analyzed ITS and partial LSU rDNA sequences of selected cercosporoid anamorphs and identified a robust *Cercospora* cluster including *C. apii, C. beticola, C. kikuchii, C. nicotianae* and *C. hayi* Calp. Goodwin et al (2001) found some divergence within *C. kikuchii*. We included the ex-type strain of *C. kikuchii* (CBS 128.27), and it clustered with another isolate of that species, CK 39, which fell within *Cercospora sensu* Stewart et al (1999). The short branch lengths among the species of *Cercospora* suggest a relatively recent development from a shared common ancestor (Goodwin et al 2001).

The variation seen in the D2 region was lower than in the ITS region. The D2 sequences of *S. aciculosa, S. gerberae* and *S. linicola* were identical, indicating that this region is too conserved for species recognition, and the ITS sequences of these species also were similar. For example, *S. gerberae* CBS 410.61, isolated from *Gerbera* in Italy, differed from *S. linicola* CBS 316.37, from *Linum* in Argentina by only a single base position in ITS 2 (A-T). The isolates of *Septoria scabiosicola* originating from *Knautia* and *Succissa* (*Dipsacaceae*) differed from the two strains of *S. lamiicola* from *Lamium album* (*Lamiaceae*) by one position in ITS 1 and one in ITS 2. These species are also similar in their morphology in planta and in vitro and most likely are related closely although they occur on plants of different families.

S. rubi strains were isolated from three different *Rubus* species and most likely represent three distinct species. CBS 238.37, isolated from *R. strigosus* from Illinois and preserved in CBS culture collection as *M. rubi,* unfortunately is now degenerated and sterile. However, we were able to compare the morphology of the two European isolates in culture. On oatmeal agar (OA), CBS 102327 (*R. fruticosus,* Netherlands) formed 1–5-septate conidia 30−80 × 1.2–2.0 μm, similar to those seen in planta. According to Jørstad (1965), the conidia of *S. rubi* measure 24–64 × 1.5–2 μm on *R. idaeus,* while Teterevnikova-Babayan (1987) gives 20–90 × 1.5–2 μm as conidial measurements for this species. CBS 109017 (*R. idaeus,* Austria) produced 3–8-septate conidia 58–108 × 2.8–3.2 μm on OA, slightly longer than those formed in planta. These conidia clearly are wider than those of *S. rubi* and agree more with those of *S. rosae* Desm. (40–72 × 2.5–3.5 μm cf. Jørstad 1965; 48–95 × 2.5–4.5 μm, cf. Teterevnikova-Babayan 1987). That species, however, is only known from *Rosa*. A dozen *Septoria* names have been described from the host genus *Rubus*. As noted by Jørstad (1965), the taxonomy and host specificity of *Septoria* spp. and possibly related *Mycosphaerella* spp. described on *Rubus* and other *Rosaceae* still are unclear.

In the ITS analyses, *S. castaneicola* is closer to *M. laricina* and species with *Pseudocercospora* anamorphs than are other *Septoria* spp. We were unable to obtain D2 sequences for these species. Compared to most *Septoria* spp., *S. castaneicola* has much darker colonies on OA and is slow growing. The conidia are relatively wide and clearly constricted at the septa, but those features also are observed in other taxa, e.g., *Phl. ulmi*. A *Pseudocercospora/Paracercospora* clade was identified by Stewart et al (1999), based on ITS and partial LSU sequence data, and was confirmed in later studies (Crous et al 2001b, Goodwin et al 2001). As a result, *Paracercospora* recently was placed in the synonymy of *Pseudocercospora* by Crous et al (2001b).

Septoria pyricola (teleomorph *M. pyri*) also seems distant from other *Septoria* species, and its conidia are aberrant because of their pale greenish pigmentation. The ITS and D2 analyses indicate a possible relationship to *M. milleri,* but the anamorph of that species, *Cercosporidium magnoliae* (*Phaeoisariopsis magnoliae* [Ell. & Harkn.] Jong & Morris), is vastly divergent from *S. pyricola*. It is cercosporoid, with synnema-like caespituli and sympodial conidiogenous cells producing hyaline to pale olivaceous, smooth to slightly verruculose, (1–)2(–3)-septate conidia. It would fit *Passalora* Fr. or *Pseudocercospora* in the sense of Crous et al (2001b). In this case, only the conidial pigmentation supports the molecular results, which is interesting in light of the suggestion of Crous et al (2001b) that the presence or absence of pigmentation in conidiophores and conidia is a character of value at generic level in anamorphs of *Mycosphaerella*.

Mycosphaerella marksii and *Stenella parkii* (teleomorph *M. parkii*) cluster in a clade that is well supported by ITS analyses. Crous et al (2000) found a clustering of two other strains representing these species, based on ITS sequence data. The anamorph of

M. marksii is unknown, but Crous et al (2000) found verruculose hyphae in culture and suggested that the species might form an anamorph similar to *Stenella* Syd., which is characterized by pigmented verruculose superficial hyphae and mononematous scattered conidiophores. However, as shown by Goodwin et al (2001), some *Mycosphaerella* species with *Stenella* anamorphs may not be closely related (viz. *St. citri-grisea* [Fisher] Sivan. [teleomorph *M. citri* J. O. Whiteside] and also the type species of *Stenella, St. araguata* Syd). A strain of *St. araguata* (CBS 486.80) was sequenced by these authors and found to cluster with *Uwebraunia juvenis* Crous & M. J. Wingf. (teleomorph *M. juvenis* Crous & M. J. Wingf.).

In addition to *Septoria, Mycosphaerella* also produces two other pycnidial anamorphs, *Phaeophleospora* Rangel and *Sonderhenia* H. Swart & J. Walker. These genera differ considerably in morphology from *Septoria*. *Phaeophleospora* and *Sonderhenia* both have brown and percurrent conidiogenous cells, but in the former genus these cells are verruculose, while in the latter they are smooth-walled. Conidia are brown and smooth to verruculose in both genera, yet are distoseptate in *Sonderhenia* and eu-septate in *Phaeophleospora*. In earlier ITS and LSU sequence studies, *Sonderhenia* was found to cluster with *Pseudocercospora* Speg. and *Mycovellosiella* Rangel (Crous et al 2001a). *Phaeophleospora* clustered in ITS studies with the acervular *Colletogloeopsis* Crous & M. J. Wingf. and the mononematous *Scolecostigmina* U. Braun, distant from the included *Septoria* spp. (Crous et al 2001b). Because of the affinities among taxa with pycnidial and nonpycnidial anamorphs, Crous et al (2001b) suggested that conidiomatal structure is less reliable as a phylogenetic marker in *Mycosphaerella* than are other characters such as conidiogenesis and conidial pigmentation (Crous et al 2001b). Our results confirm this, but they also suggest that conidiogenous cell proliferation may be poorly informative in these fungi.

Species with unknown teleomorph affinities group with pleomorphic species in well-supported clades (see TABLE I). Some taxa that have been extensively studied have likely lost the teleomorph, e.g., *Septoria passerini* and *S. apiicola*. In some species, there would seem to be a sound ecological reason for failure to produce a teleomorph. For example, *Septoria lamiicola* and *S. scabiosicola* are found only in summer on living leaves and these leaves are degraded in the fall before teleomorph development can occur. We conclude that the loss of the teleomorph most likely has occurred several times in the evolutionary history of *Mycosphaerella* and that it also involved taxa with *Septoria* anamorphs.

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