Mycosphaerella punctiformis revisited: morphology, phylogeny, and epitypification of the type species of the genus *Mycosphaerella* (*Dothideales, Ascomycota*)

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Mycosphaerella punctiformis, the type species of the genus *Mycosphaerella*, is epitypified by material collected on *Quercus robur* in The Netherlands. The teleomorph is described *in planta*, and the *Ramularia* anamorph, for which the new name *R. endophylla* is proposed, and the *Asteromella* spermatial state are characterized *in vitro*. Sequence data of the nuclear ribosomal DNA are presented and analyzed together with other *Mycosphaerella* spp. with *Ramularia* and several other anamorphs. Several strains originating from *Quercus*, *Acer* and *Tilia* showed diverging ITS sequences, indicating that the *M. punctiformis* complex may comprise more than a single phylogenetic species, but this could not be confirmed by the analysis of our dataset. An endophytic phase is established for the first time in the life-cycle of *M. punctiformis*, as the species was repeatedly isolated from surface sterilized green healthy leaves of *Quercus robur* in summer at the epitype locality.

INTRODUCTION

The genus *Mycosphaerella* is one of the largest genera of ascomycetes, comprising many plant pathogens of economically important crops, but also saprobic species. Teleomorph morphology is relatively simple and uniform in *Mycosphaerella*, but the genus is unequalled in the diversity of the associated anamorphs. Indeed, 27 anamorphic genera have been associated with *Mycosphaerella* (von Arx 1983, Sutton & Hennebert 1994), 23 of which were accepted by Crous *et al.* (2000). Klebahn (1918) and Laibach (1922) suggested segregating groups of species from *Mycosphaerella* based on their association with a particular anamorph, but genera they proposed did not become widely used. Recent molecular studies indicate that characters used to define the anamorph genera, such as conidiomatal structure, and conidial shape, size, and septation, are not always phylogenetically informative, and that some generic concepts for the anamorphs need to be revised (Crous *et al.* 2000, Crous, Kang & Braun 2001, Verkley *et al.* 2004). However, a group of species with *Cladosporium* anamorphs was recently segregated under the name *Davidiella* (Braun *et al.* 2003); it is a close sister group of other *Mycosphaerella*.

Mycosphaerella punctiformis, the type species of *Mycosphaerella*, was originally described as *Sphaeria punctiformis* from fallen leaves of *Quercus robur*. Microscopical examination of the lectotype material of *M. punctiformis* deposited in L, confirmed the identity. However, the over 200 yr old herbarium specimen does not provide an unambiguous application of the name, because recent molecular work has shown that *M. punctiformis* as currently circumscribed comprises cryptic species that are morphologically indistinguishable. Several strains in the CBS collection that had been morphologically identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia*, were found to be heterogeneous in their sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene array. As no ex-type strain is available, we tried to obtain ribosomal DNA from the type material of *M. punctiformis*, but failed. In accordance with Art. 9.7 of the *Code*, we sought to settle the application of the name by selecting an epitype for *M. punctiformis*. The main purpose of this paper is to epitypify *M. punctiformis* with material recently collected from the type host *Quercus robur* in The Netherlands, and to give a full phenotypic characterization of the teleomorph, and (syn)anamorphs in culture. Because the anamorph will

be the only sporulation observed in most ecological and endophyte studies, we consider it useful to also formally name this conidial state. Fresh ascomata of *M. punctiformis* were collected on dead fallen leaves of the type host *Quercus robur*, checked for agreement with the lectotype material, and ascospore isolates were made. We also obtained ecological data from a biodiversity study of foliar ascomycetous endophytes of *Quercus* in the epitype locality. We sequenced the ITS region of rDNA of the available strains of *M. punctiformis*, and also included a number of additional taxa in the sequence analyses to investigate the phylogenetic relationships of *M. punctiformis* with other *Mycosphaerella* species with *Ramularia* and several other anamorphs. Furthermore, partial small subunit (SSU) sequences of the ex-epitype strain of *M. punctiformis* were analysed with other data available in order to obtain further support for the phylogenetic position within the *Mycosphaerella* clade.

MATERIALS AND METHODS

Isolation from fruit bodies on decaying leaves and endophytic mycelia from green leaves

Strains used in this study are listed in Table 1. Dead fallen leaves with ascomata were collected in March to May of 2002 and 2003 in the forested area 'De Stompert' in The Netherlands, from three mature trees of *Quercus robur*. Leaves were incubated in a moist chamber for several hours in the laboratory at *ca* 20 °C. They were then cut into square pieces and glued to the inside of Petri dish lids to allow ascospores to be discharged on to 2% malt extract agar (MEA). Germinating ascospores were examined after 24 h, illustrated and transferred to MEA. Fresh green leaves from the same trees were collected monthly between May and November, put in plastic bags and transported to the lab. On the same day, leaves were sterilized in domestic bleach water (5% chlorine) for 5 min, followed by three rinses in sterile water. Small squares of about 0.5 cm² were placed onto MEA with 50 ppm streptomycin, aureomycin and penicillin to inhibit bacterial growth, placed on the laboratory bench in diffuse daylight, and regularly checked for fungal growth. Mycelia growing out of the margin were transferred to 2% MEA and oatmeal agar (OA; Centraalbureau voor Schimmelcultures 2001) and preliminarily identified morphologically.

Phenotypic characterization

For microscopic examination, fruiting structures were mounted in tap water. Line drawings were made with a drawing tube, and photographic images with a Nikon Coolpix 995 digital camera. For the description of colony features and sporulating structures, isolates were transferred onto OA and 3% MEA plates and placed in an incubator at 15 ° under n-UV (12 h rhythm). Colours are described according to Rayner (1970).

DNA extraction and sequencing

Strains were transferred from agar cultures to 2 ml liquid medium (2% malt extract) and incubated on a rotary shaker (300 rpm) for 3 wk at room temperature. Liquid cultures were transferred to 2-ml tubes, centrifuged and washed twice with sterile water. DNA was extracted using the FastDNAkit (Omnilabo 6050073, BIO 101, CA) according to the manufacturer's instructions. For ITS sequence analysis a part of the ribosomal RNA gene cluster was amplified by PCR using primer pairs V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990). Part of the 18S rRNA gene (SSU) was amplified using primers NS1 and NS4 (White *et al.* 1990). PCR was performed in 50 μl reaction volumes, each reaction containing 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). PCR was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following program: 1 min at 95 °, 30 cycles (1 min 95 °, 1 min 55 °, 2 min 72 °) followed by a final extension of 5 min at 72 °. PCR products were cleaned with GFX columns (Amersham Pharmacia, NJ) and analyzed on a 2% agarose gel to estimate concentrations. ITS1 and ITS4 (White *et al.* 1990) were used as internal sequencing primers for the ITS region. The SSU region was sequenced using the PCR primers. Sequencing was performed with the BigDye terminator chemistry (Part no. 403049, Applied Biosystems) following the manufacturer's instructions. The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia 17-0041-01), and separated and analyzed in an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were matched using SeqMan (DNAstar, Madison, WI).

Phylogenetic analyses

Pairwise and global alignment of consensus sequences were performed in Bionumerics 3.0 (Applied Maths, Kortrijk, Belgium), and manually adjusted where necessary. Parsimony analysis was done using PAUP v. 4.0b10 (Swofford 2003). The heuristic search was performed with the following parameters: characters unordered with equal weight, random taxon addition, branch swapping with tree bisection-reconnection (TBR) algorithm, branches collapsing if the maximum branch length was zero. Maxtrees was set at 10 000. Alignment gaps were treated as missing characters. Parsimony bootstrap analyses were performed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 100.

Neighbour-joining analyses was performed using PAUP, with GTR (Gamma = 0.5, and rates for variable

sites equal), and 1000 neighbour-joining bootstrap replications to test the stability of clades. BLAST searches in GenBank revealed highest similarity to species of *Mycosphaerella*. GenBank accession numbers, taxon names and other information about the sequences from GenBank used in this study are given in Table 1. Gen-Bank accession numbers (marked with *) of sequences generated in this study are also given in Table 1. A strain of *Davidiella tassiana* (sub *Mycosphaerella tassiana*) was defined as outgroup for the ITS dataset and sequences of *Botryosphaeria* species were used as outgroup for the SSU dataset. The alignments and trees were lodged in TreeBASE (study accession S1126).

RESULTS

Phylogenetic analyses

The alignment of the ITS sequences comprised 513 characters, of which 168 (36%) were parsimony-informative. 23 of these characters were excluded from the analysis because they were positioned in small insertions/deletions or regions with ambiguous position homology. Furthermore, 322 uninformative characters were also excluded, so that 145 characters were used in the parsimony analysis. In the neighbour joining analysis in total 213 characters were included, as constant characters were excluded, but autapomorphic characters were included to obtain accurate branch lengths in the phylogram. The heuristic search yielded 580 most parsimonious trees (MPT) of 535 steps (C.I. = 0.505, R.I. = 0.878, R.C.I. = 0.443, and homoplasy index = 0.495). The strict consensus tree is shown in Fig. 1. Several highly supported multi-taxon clades were the same in the parsimony and neighbour joining analyses (neighbour joining trees not shown). Among these was a clade comprising all included strains with *Ramularia* anamorphs (parsimony 99%/neighbour joining minimum 100%), which in the parsimony analysis formed a sister group to the clade with the cereal pathogens *Mycosphaerella graminicola* and *Septoria passerini* (100/92). The support for the two clades together was, however, lower (61/<50). Further highly supported clades were the one with *Cercospora* spp. (90/97), a clade with *M. crystallina*, *M. heimii*, *M. heimioides* and *M. colombiensis* (99/95), and a clade with *M. africana*, *M. keniensis*, *M. aurantia*, *M. hedericola*, *Mycosphaerella* sp. (from *Coprosma* sp.), *M. confusa*, and *Passalora fulva* (91/81). The *Ramularia* clade was rather unresolved in parsimony and neighbor joining analyses. In the parsimony analysis, only a clade comprising four strains identified as *M. punctiformis* from *Quercus*, *Acer* and *Tilia* was well-supported (100/95). With their closest sister *M. phacae-frigidae*, these strains also obtained good support in both analyses (91/77).

BLAST results of the SSU sequence of *M. punctiformis* (AY490775) supported the close association of *M. punctiformis* with other *Mycosphaerella* species. The alignment of the SSU sequences included 1067 characters, of which 1006 were constant, 21 were parsimony uninformative and 40 were parsimony informative. The heuristic search yielded eleven most parsimonious trees of 81 steps (C.I. 0.852, R.I. 0.919, R.C. index 0.783, and H.I. 0.148). The strict consensus tree is shown in Fig. 2. The topology of the eleven trees only differed in the internal ordering of groups in the *Mycosphaerella* clade. Two main clades are delimited in the SSU tree, the first clade contains isolates of *Mycosphaerella* (98% bootstrap support) and the other isolates of *Davidiella* (100% bootstrap support). The sequence of *M. puncti-formis* groups closest to the sequences of a *Mycosphaerella* sp. isolated from *Acacia* (AY251116) and a sequence of *Septoria tritici* (AY251117). However, this association does not have significant bootstrap support.

Phenotypic characterization (Figs 3–10)

A description of the teleomorph *in planta*: *Leaf spots* not observed. *Ascomata* developing on fallen dead leaves, predominantly hypophyllous, black, subepidermal, erumpent to superficial, globose, 70–110 μm diam; apical ostiole 5–10 μm wide; wall consisting of 2–3 layers of medium brown textura angularis. *Asci* aparaphysate, fasciculate, bitunicate, subsessile, cylindrical, straight, 8-spored, 30–45×5–$7(-9)$ μm. *Ascospores* multiseriate, overlapping, hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, medianly 1-septate, constricted at the septum, tapering towards both ends, but more prominently towards the lower end, $(6-)8$–$9(-10) \times (2-)3$ μm (av. 9×3 μm). Germinating ascospores hyaline, distorting, forming germ tubes 4–6 μm diam apically, parallel to the long axis from both ascospore cells, and simultaneously also laterally, from one or both ascospore cells, at an angle of 90° or less to the long axis (Germination pattern D; Crous 1998).

Free conidia possibly belonging to *M. punctiformis* were occasionally observed in late summer on older leaf lesions caused by pathogens such as *Septoria quercicola* and *Discula* sp.

Colony description (diffuse daylight, 15°): *Colonies* on OA reaching 28–31 mm diam in 27 d, spreading (low), sometimes in the centre with some elevated mycelium, margin even or slightly lobed, glabrous, pale Honey to Olivaceous Buff or Rosy Vinaceous to Rosy Buff, colony surface glabrous or with appressed pure white aerial hyphae or conidiophores; in the centre submerged and superficial mycelium Rosy Buff to Salmon and concolourous on reverse, or becoming Dark Violet to dark Purple due to the deposition of violet pigment on the outer surface of vegetative hyphae, surrounding medium then often becoming Coral to red by diffusing pigments, and Coral to Flesh on reverse. In a few isolates, the colony was dominated by olivaceous colours (underneath a white covering of

Table 1. Fungal isolates included for ITS and SSU sequence analyses (in alphabetical order of the teleomorph names).

GenBank accession no.				
ITS	SSU	Teleomorph	Anamorph	Origin
	U42476	*Botryosphaeria rhodina*	*Lasiodiplodia theobromae*	No data available
	U42477	*B. ribis*	*Fusicoccum* sp.	No data available
AY251078	AY251096	*Davidiella tassiana*	*Cladosporium herbarum*	ATCC 66670 (='STE-U 5101'); CCA-treated Douglas-fir pole, New York, USA
	AY251094	*Davidiella* state unknown	*Cl. cladosporioides*	ATCC 66669 (='STE-U 5100'); Creosote-treated southern pine pole, New York, USA
	AY251092	*Davidiella* state unknown	*Cl. colocasiae*	STE-U 4323; *Colocasia esculenta*, Fiji Islands
	AY251098	*Davidiella* state unknown	*Cl. sphaerospermum*	CBS 188.54 (='STE-U 3686', ATCC 11290)
	AY251097	*Davidiella* state unknown	*Cl. uredinicola*	ATCC 46649 (='STE-U 5390'); Fungicolous on *Cronartium fusiforme* f. sp. *quercum* on *Quercina nigra* leaves, Alabama, USA
AF173314		*Mycosphaerella africana*	Unknown	STE-U 794 (ex-type); *Eucalyptus viminalis*, South Africa
AY490773		*M. africana*	Unknown	CBS 680.95 (=STE-U 796; ex type); *Eucalyptus viminalis*, South Africa
AY150331		*M. aurantia*	Unknown	CBS 110500; *Eucalyptus globulus*, Australia
AF222838		*M. colombiensis*	*Pseudocercospora colombiensis*	STE-U 1106; *Eucalyptus*, Colombia
AF362058		?*M. confusa*	*Ps. rubi*	CBS 256.35
AF222839		*M. crystallina*	*Ps. crystallina*	STE-U 801 (ex type); *Eucalyptus bicostata*, South Africa
AY490757		*M. crystallina*	*Ps. crystallina*	CBS 681.95, STE-U 802 (ex type); *Eucalyptus bicostata*, South Africa
AY266152		*M. fijiensis*	*Ps. fijiensis*	ATCC 22116, PF7; *Musa* sp., Philippines
AY266150		*M. fijiensis*	*Ps. fijiensis*	ATCC 36054, PFD9; *Musa* sp., Honduras
AF181706		*M. musicola*	*Ps. musae*	ATCC 22115; *Musa* sp., Philippines
AY288148		*M. musicola*	*Ps. musae*	PM11, ATCC 36143; *Musa*, Honduras
AY152590		*M. laricina*	*Pseudocercospora* sp.	CBS 326.52; *Larix decidua*, Switzerland
AY152595		*M. fragariae*	*Ramularia grevilleana*	CBS 259.36; *Fragaria* sp., The Netherlands
AY152597		*M. fragariae*	*R. grevilleana*	CBS 719.84; *Fragaria* sp., The Netherlands
AY152596		*M. fragariae*	*R. grevilleana*	CBS 298.34; *Fragaria* sp., The Netherlands
AF297235		*M. fragariae*	*R. grevilleana*	ATCC 24113; *Fragaria* sp., Illinois, USA
AF173312		*M. fragariae*	*R. grevilleana*	STE-U 656; *Fragaria* sp., South Africa
AY152601		*M. graminicola*	*Septoria tritici*	CBS 100330 (=IPO 6566.1); *Triticum aestivum*, The Netherlands
AY152602		*M. graminicola*	*S. tritici*	CBS 100335; *Triticum aestivum*, The Netherlands
AY152603		*M. graminicola*	*S. tritici*	CBS 392.59; *Triticum aestivum*
AF181692		*M. graminicola*	*S. tritici*	IPO 323; *Triticum aestivum*, The Netherlands
AF181693		*M. graminicola*	*S. tritici*	T1; *Triticum aestivum*, Minnesota, USA
AF362068	AY251117	*M. graminicola*	*S. tritici*	STE-U 658; *Triticum* sp., South Africa
AY152581		*M. grossulariae*	*S. ribis*	CBS 235.37; *Ribes nigrum*, The Netherlands
AY490772		*M. hedericola*	Unknown	CBS 441.86; *Hedera helix*, France
AF452508		*M. heimii*	*Pseudocercospora heimii*	CMW5705
AF452509		*M. heimii*	*Ps. heimii*	CMW5707
AF222841		*M. heimii*	*Ps. heimii*	No data available
AF452512		*M. heimii*	*Ps. heimii*	CMW5713
AF222842		*M. heimioides*	*Ps. heimioides*	STE-U 1312; *Eucalyptus*, Indonesia
AF173300		*M. keniensis*	Unknown	STE-U 1084; *Eucalyptus grandis*, Kenya
AY490768		*M. latebrosa*	*Septoria aceris*	CBS 183.97; *Acer pseudoplatanus*, The Netherlands
AY152553		*M. latebrosa*	*S. aceris*	CBS 687.94; *Acer pseudoplatanus*, The Netherlands
AY490769	AY251114	*M. latebrosa*	*S. aceris*	CBS 652.85; *Acer pseudoplatanus*, The Netherlands
AY152600		*M. marksii*	Unknown	CBS 682.95 (='STE-U 842'); *Eucalyptus grandis*, South Africa

AY152599		*M. parkii*	*Stenella parkii*	CBS 387.92 (= 'STE-U 353; ex type); *Eucalyptus grandis*, Brazil
AY490758		M. phacae-frigidae	*Ramularia* sp.?	CBS 234.55; *Phaca frigida*, Switzerland
AY152583		*M. populicola*	*Septoria populicola*	CBS 100045; *Populus trichocarpa*, Washington, USA
AY152584		*M. populicola*	*S. populicola*	CBS 100052; *Populus trichocarpa*, Washington, USA
AY152585		*M. populicola*	*S. populicola*	CBS 100044; *Populus trichocarpa*, Washington, USA
AY152586		*M. populicola*	*S. populicola*	CBS 100051; *Populus trichocarpa*, Washington, USA
AY152587		*M. populicola*	*S. populicola*	CBS 100047; *Populus trichocarpa*, Washington, USA
AY490759		'*M. punctiformis*'	*Ramularia* sp.	CBS 515.69; *Acer pseudoplatanus*, The Netherlands
AY490760		'*M. punctiformis*'	*Ramularia* sp.	CBS 742.79; *Tilia* sp., Germany
AY152593		'*M. punctiformis*'	*Ramularia* sp.	CBS 943.97; *Quercus* sp., The Netherlands
AY152594		'*M. punctiformis*'	*Ramularia* sp.	CBS 184.97; *Acer pseudoplatanus*, The Netherlands
AY490761		*M. punctiformis*	*R. endophylla*	CBS 942.97; *Quercus* sp., Belgium
AY490762		*M. punctiformis*	*R. endophylla*	CBS 113871 (SS); *Quercus robur*, The Netherlands
AY490763	AY490775*	*M. punctiformis*	*R. endophylla*	CBS 113265 (SS; ex-epitype); *Quercus robur*, The Netherlands
AY490764		*M. punctiformis*	*R. endophylla*	CBS 113868; leaf endophyte *Quercus robur*, The Netherlands
AY490765		*M. punctiformis*	*R. endophylla*	CBS 113869; leaf endophyte *Quercus robur*, The Netherlands
AY490766		*M. punctiformis*	*R. endophylla*	CBS 113870; leaf endophyte *Quercus robur*, The Netherlands
AF222848		*M. punctiformis*	*R. endophylla*	KC1
AY152591		*M. pyri*	*Septoria pyricola*	CBS 222.31; *Pyrus communis*
AY152592		*M. pyri*	*S. pyricola*	CBS 640.72; *Pyrus communis*, The Netherlands
AY490767		*M. rubella*	*Ramularia* sp.?	CBS 288.49; *Angelica sylvestris*
AY152575		*M. ulmi*	*Phloeospora ulmi*	CBS 344.97; *Ulmus glabra*, Austria
AY490774		*Mycosphaerella* sp.	*Septoria* sp. (in culture)	CBS 113113; *Coprosma* sp., New Zealand
AY490771		*Mycosphaerella* sp.	*S. quercicola*	CBS 663.94; *Quercus robur*, The Netherlands
	AY251115	*Mycosphaerella stromatosa*	*Pseudocercospora stromatosa*	STE-U 1731; *Protea* sp., South Africa
	AY251116	*Mycosphaerella* sp.		STE-U 3837; *Acacia* sp., Venezuela
AF173310		*Mycosphaerella* state unknown	*Ramularia collo-cygni*	STE-U 2045; *Hordeum* sp., Germany
AJ417496		*Mycosphaerella* state unknown	*Ramularia* sp.	'ascomycete 2'; *Quercus robur*, Germany
AY259131	AY251110	*Mycosphaerella* state unknown	*Ramulispora sorghi*	STE-U 905; *Sorghum* sp., South Africa
AY259132	AY251111	*Mycosphaerella* state unknown	*R. sorghi*	STE-U 906; *Sorghum* sp., South Africa
AY166268		*Mycosphaerella* state unknown	*Cercospora apii*	CA1, ATCC 12246
AY152576		*Mycosphaerella* state unknown	*C. beticola*	CBS 539.71; *Beta vulgaris*, Romania
AY266165		*Mycosphaerella* state unknown	*C. beticola*	MPPD12120, CB4; *Beta vulgaris*, Minnesota, USA
AY152577		*Mycosphaerella* state unknown	*C. kikuchii*	CBS 128.27 (ex type); *Glycine max*, Japan
AY166260		*Mycosphaerella* state unknown	*C. kikuchii*	CK 39; *Glycine max*, Illinois, USA
AY266161		*Mycosphaerella* state unknown	*C. kikuchii*	CK 35; *Glycine max*, Illinois, USA
AY260078	AY251104	*Mycosphaerella* state unknown	*C. zebrina*	STE-U 3955; *Trifolium pratense*, Canada
AY152572		*Mycosphaerella* state unknown	*Septoria apiicola*	CBS 395.52 (=IMI 092627); *Apium* sp., The Netherlands
AY152573		*Mycosphaerella* state unknown	*S. apiicola*	CBS 389.59; *Apium graveolens*, Italy
AY152574		*Mycosphaerella* state unknown	*S. apiicola*	CBS 400.54 (=IMI 092628); *Apium graveolens*, The Netherlands
AY152588		*Mycosphaerella* state unknown	*S. castaneicola*	CBS 102377; *Castanea sativa*, The Netherlands
AF279582	AF279583	*Mycosphaerella* state unknown	*S. epambrosiae*	*Ambrosia artemisiifolia*
AY490770		*Mycosphaerella* state unknown	*S. hippocastani*	CBS 411.61; *Aesculus hippocastanum*, Germany
AY152563		*Mycosphaerella* state unknown	*S. lamiicola*	CBS 109113; *Lamium album*, Austria
AY152564		*Mycosphaerella* state unknown	*S. lamiicola*	CBS 102328; *Lamium album*, The Netherlands
AF181697		*Mycosphaerella* state unknown	*S. passerinii*	ATCC 26516; *Hordeum vulgare*, Minnesota, USA
AF181699		*Mycosphaerella* state unknown	*S. passerinii*	P78; *Hordeum vulgare*, Minnesota, USA
	AY251108	*Mycosphaerella* state unknown	*Passalora dodonaeae*	STE-U 1223; *Dodonaea* sp., South Africa

Table 1. (*Cont.*)

ITS	SSU	Teleomorph	Anamorph	Origin
				GenBank accession no.
AY251069	AY251109	*Mycosphaerella* state unknown	*Pas. fulva*	CBS 119.46 (='STE-U 3688'); *Lycopersicon esculentum*, The Netherlands
	AY251103	*Mycosphaerella* state unknown	*Pas. janseana*	CBS 145.37 ('STE-U 4303'); *Oryza sativa*, Arkansas, USA
AY152559		*Mycosphaerella* state unknown	*Septoria scabiosicola*	CBS 102336; *Knautia arvensis*, The Netherlands
AY152561		*Mycosphaerella* state unknown	*S. scabiosicola*	CBS 182.93; *Succissa pratensis*, France
	AY251095	*Sphaerulina polyspora*	Unknown	CBS 354.29 (='STE-U 4301')
	AY251113	? *Sphaerulina rhemiana*	*S. rosae*	CBS 355.58 (='STE-U 4302'); leaf of *Rosa* sp.
	AY251106	Unknown	*Pseudocercospora angolensis*	CBS 149.53 (='ATCC 11669); leaf of *Citrus sinensis*, Angola
	AY251107	Unknown	*Ps. protearum* var. *leucadendri*	STE-U 1869; *Leucadendron* sp., South Africa

* GenBank accession no. of LSU sequence = AY490776.

conidiophores) and greyish Sepia to Hazel or Olivaceous on reverse.

Colonies on MEA reaching 21–30 mm diam in 27 d, restricted and up to 5 mm high in the centre, margin weakly to distinctly lobed, glabrous or finely felty of pure white aerial hyphea, Buff, pale Olivaceous or Rosy Buff, colony surface Pale Vinaceous or Pale Violet, and then often the surrounding medium becoming Coral to red by diffusing pigments, or greyish, but largely covered by pure white aerial hyphae or conidiophores; reverse Dark Purple to Blood Colour, or Fawn to Vinaceous Buff with Dark Brick, Brick and Cinnamon areas.

TAXONOMY

Ramularia endophylla Verkley & U. Braun, sp. nov.
(Figs 11–16)

Conidiophora unicellulares (=cellulae conidiogenae), simplicia, subcylindrica vel cylindrica, (6–)10–30 × 2.5–4(–5) μm, recta vel geniculata-sinuosa, hyalina, levia; cicatrices conidiales leniter incrassatae et fuscae, circa 1 μm latae; *conidia* hyalina, levia vel sublevia, hila incrassata, fusca, refractiva, 0.5–1(–1.5) μm lata; conidia primaria solitaria, ovoidea, ellipsoidea vel subcylindrica, continua, apice rotundato, basin versus leniter attenuata, 6–15 × 2–4 μm; conidia secundaria catenata, saepe ramicatenata, in OA praecipue ellipsoidea vel cylindrica, in MEA ovoidea vel ellipsoidea-fusiformia, recta vel curvata, 0–1-septata, in OA 7–29 × 3–4(–5) μm, in MEA (4–)7–10(–15) × (3–)4–5 μm.

Typus: **The Netherlands:** *Utrecht*: Soesterberg, 'De Stompert', on dead leaf of *Quercus robur* ('B3'), April 2003, *G. Verkley s.n.* [ex-epitypus *Mycosphaerella punctiformis*] (CBS 113265–holotypus; culture kept metabolically inactive, in liquid nitrogen and lyophilized).

Conidiophores simple, subcylindrical or cylindrical, (6–)10–30 × 2.5–4(–5) μm, straight or geniculate-sinuous, hyaline, smooth-walled, arising from terminal or intermediary hyphal cells at the colony surface, often without a basal septum; conidial scars somewhat thickened and darkened, about 1 μm wide; *conidia* formed holoblastically, hyaline, walls smooth to minutely roughened, hila conspicuous, thickened, darkened, refractive, 0.5–1(–1.5) μm wide; *primary conidia* solitary, ovoid, or ellipsoid to subcylindrical, aseptate, rounded at the top and somewhat attenuated towards the base, 6–15 × 2–4 μm; *secondary conidia* catenate, often in branched, acropetal chains, on OA predominantly ellipsoid to cylindrical, on MEA ovoid to ellipsoid-fusiform, straight to curved, 0–1-septate, ends with a single hilum rounded to attenuated, branching ends often with hila on short projections, on OA 7–29 × 3–4(–5) μm, on MEA (4–)7–10(–15) × (3–)4–5 μm.

Asteromella *spermatial state*

Description *in vitro*: *Spermogonia* submerged or on the agar surface, pycnidial, globose, mostly aggregated in larger complexes containing several merging cavities and one or several rather undifferentiated ostioles, black to dark brown; *conidiomatal walls* composed of
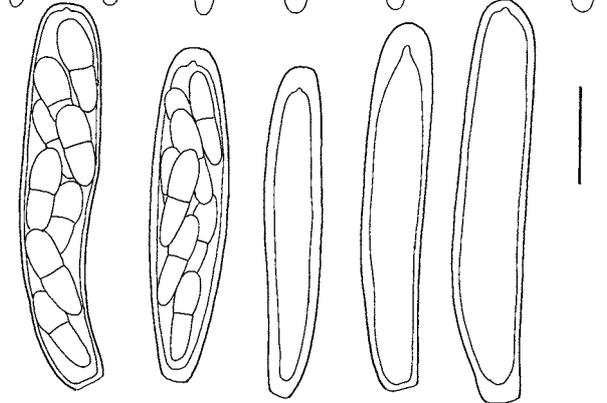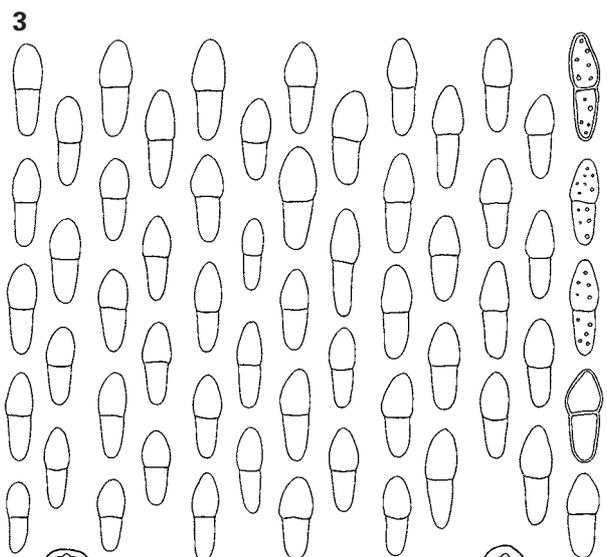
Fig. 1. Strict consensus tree of 580 most parsimonious trees of 535 steps obtained in a heuristic search of 168 parsimony-informative characters of the ITS1-5.8SrDNA-ITS2 region calculated in PAUP. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Species are labelled by teleomorph name, if known (anamorph names are given in Table 1).
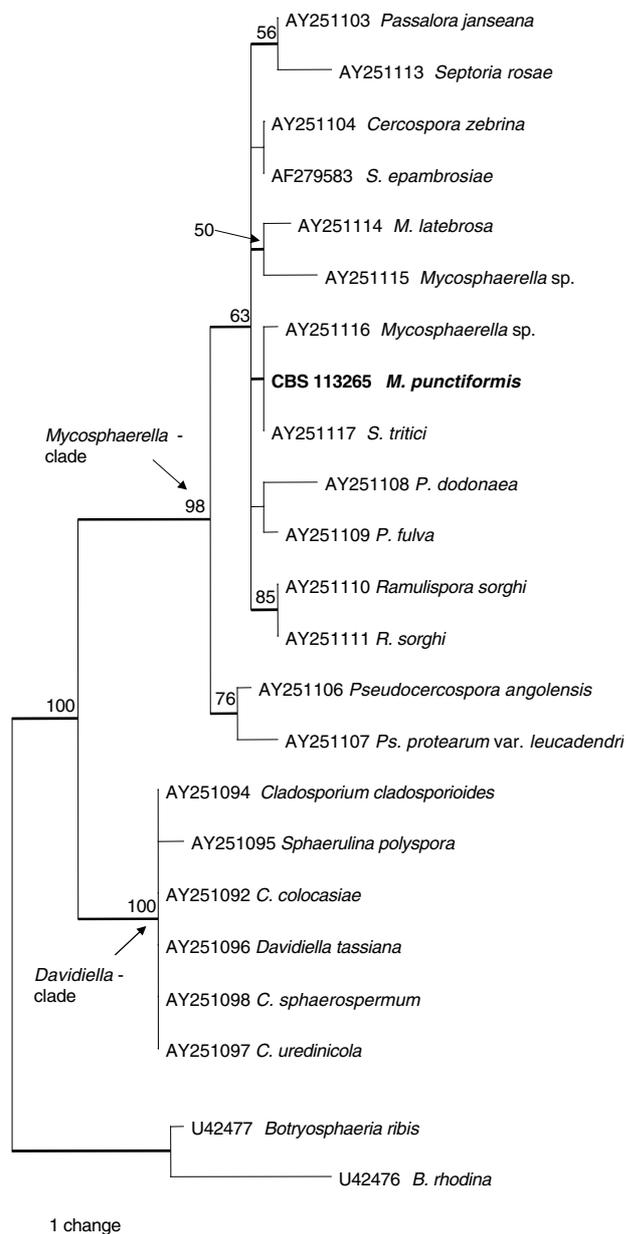
Fig. 2. One of eleven most parsimonious trees obtained from a heuristic search of the SSU sequence alignment. Bootstrap support values from 1000 replicates are shown at the nodes and the scale bar represents a single change. Branches that were maintained in the Strict consensus tree are thickened and the tree is rooted to *Botryosphaeria ribis* and *Botryosphaeria rhodina*.
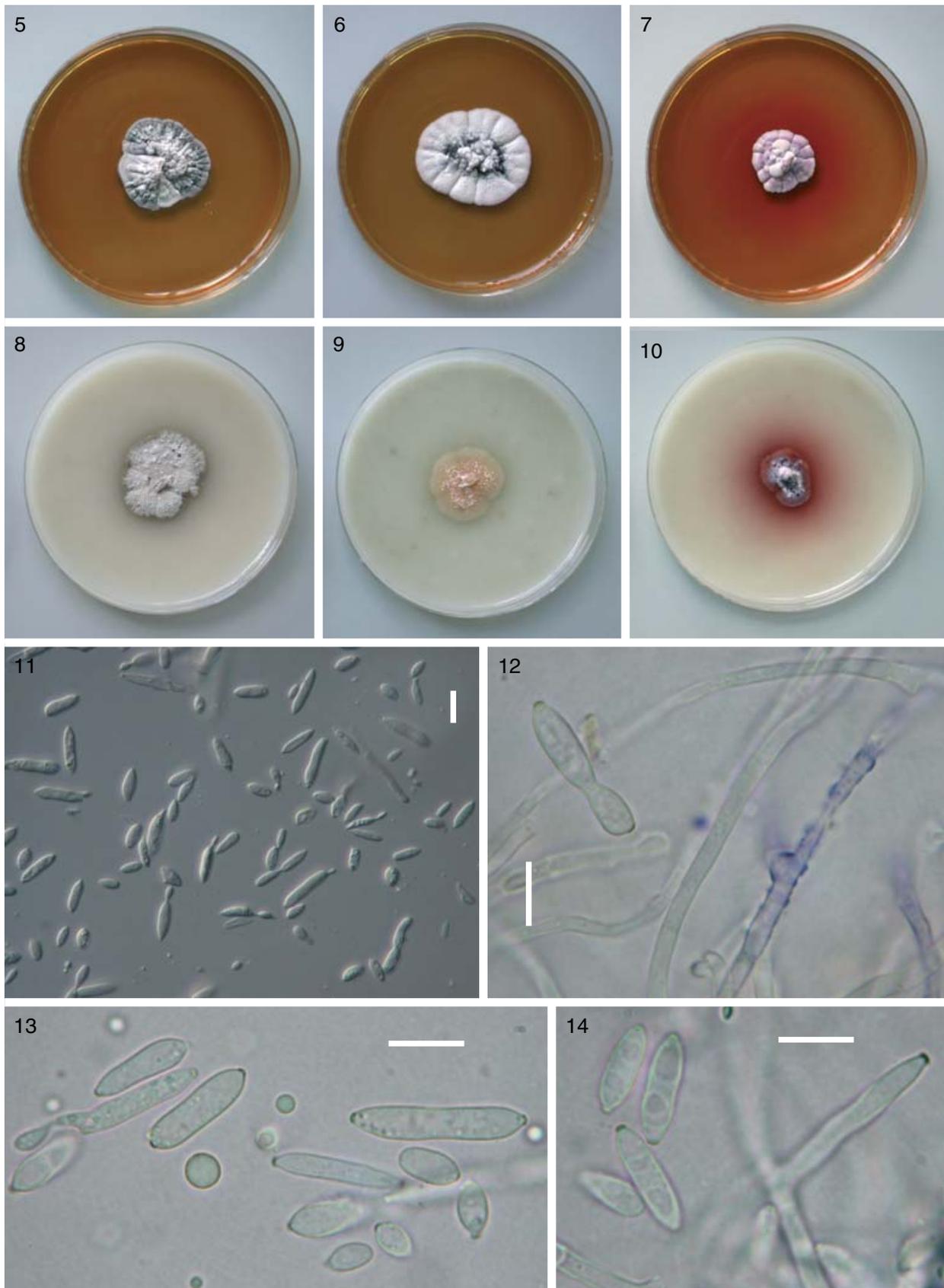

Figs 3–4. *Mycosphaerella punctiformis*, epitype (CBS herb. 7949). **Fig. 3.** Ascospores and asci *in planta*. **Fig. 4.** Germinating ascospores on MEA. Bars = 10 μm.

an outer layer of thick-walled, brown textura angularis, and an inner layer of hyaline, irregular to isodiametric cells; *spermatogenous cells* phialidic, determinate, hyaline, discrete or integrated in simple, septate, more rarely branched, hyaline spermatiophores with acropleurogenous openings; *spermatia* ellipsoid to sub-cylindrical, with rounded ends, hyaline, smooth-walled, aseptate, 3–4(–5) × 1–1.5 μm, whitish in mass.

Mycosphaerella punctiformis (Pers.: Fr.) Starbäck, *Bih. Kongl. Svenska Vetensk.-Akad. Handl.* **15**(3, 2): 9 (1889).

Sphaeria punctiformis Pers., *Ann. Bot.* (*Usteri*) **11**: 26. 1794 : Fr., *Syst. Mycol.* **2**: 525 (1823).
Sphaerella punctiformis (Pers.: Fr.) Rabenh., *Herb. Vivum Mycol., ed. nov., cent.* 3, no. 264 (1856).

Typus: **The Netherlands**: On lower surface of dead leaves of *Quercus* (*Fagaceae*), *Persoon s.n.* (L-Persoon – **lectotypus hic designatus**); *Utrecht*: Soesterberg, 'De Stompert', *G. Verkley*

Figs 5–14. *Mycosphaerella punctiformis in vitro* (diffuse daylight, 18 °C). **Figs 5–7.** Isolates on MEA, after 27 d. **Fig. 5.** CBS 113870. **Fig. 6.** CBS 113868. **Fig. 7.** CBS 113869. **Figs 8–10.** Isolates on OA, after 27 d. **Fig. 8.** CBS 113870. **Fig. 9.** CBS 113869. **Fig. 10.** CBS 113868. **Figs 11–14.** Conidia and conidiogenous cells on OA. Bars = 10 μm.
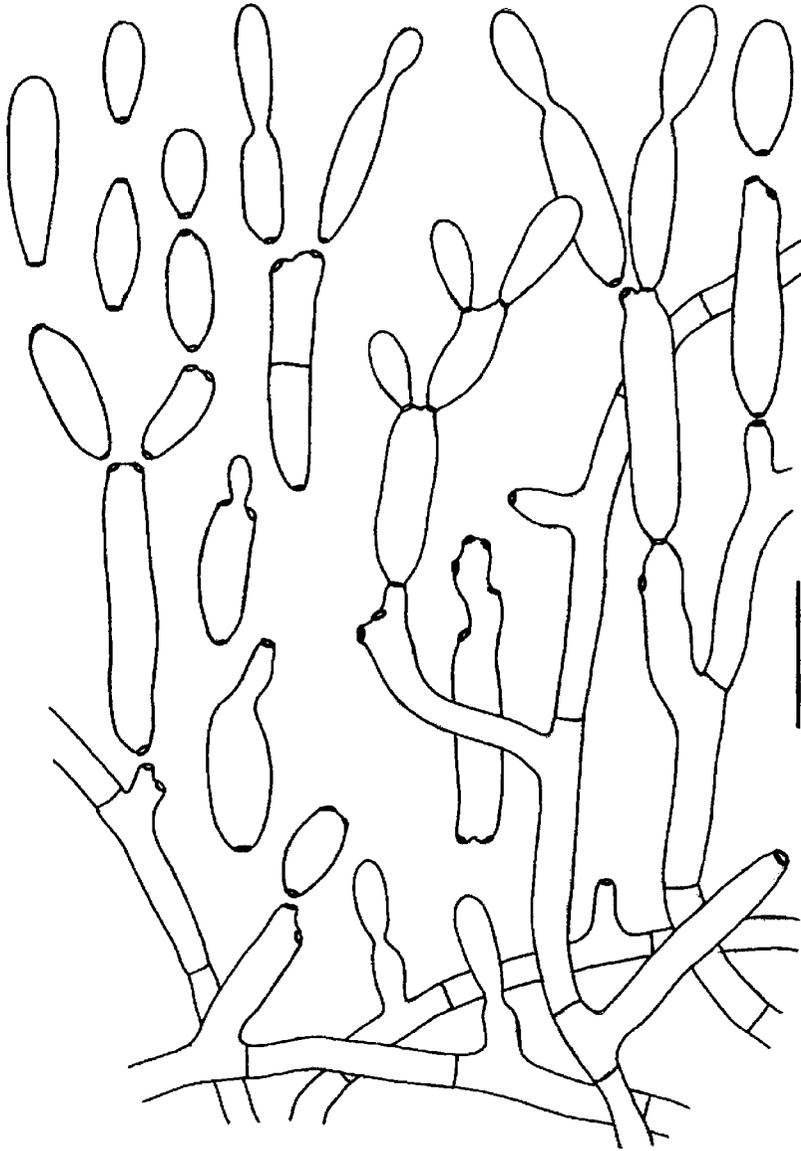
Fig. 15. *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on OA. Bar = 10 μm.
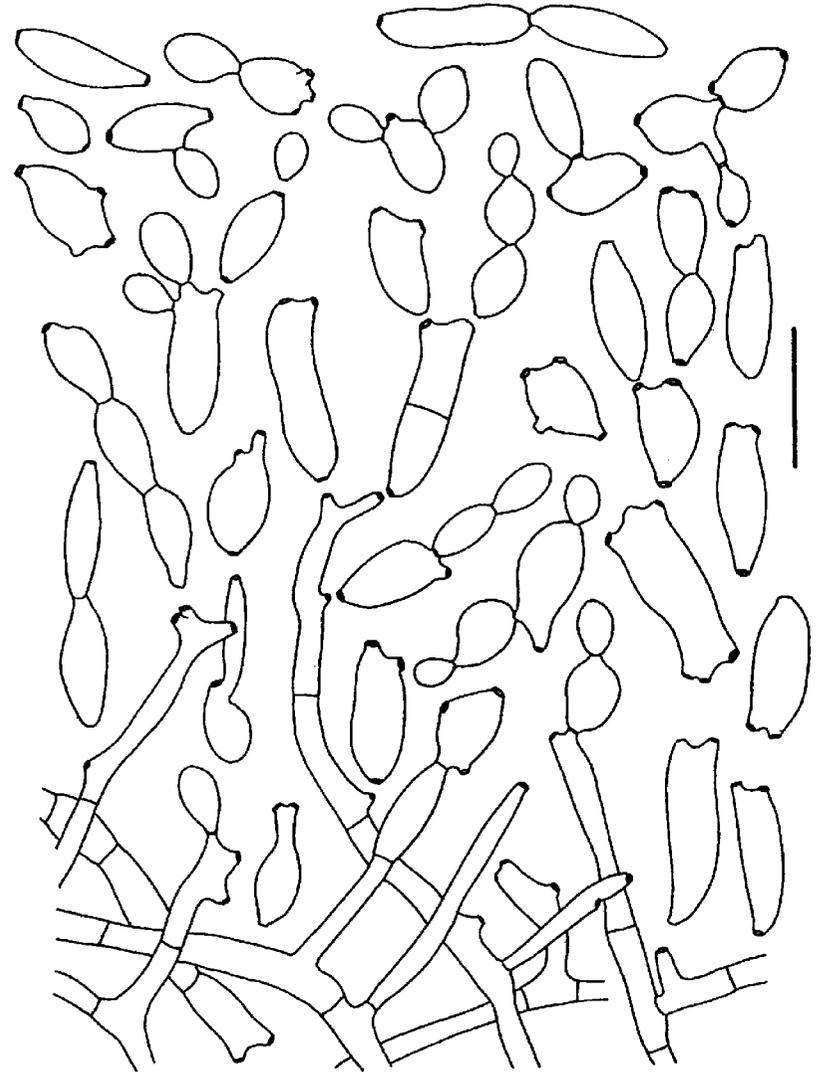
Fig. 16. *Mycosphaerella punctiformis* (CBS 113265 – ex-epitype). Conidiogenous cells and conidia on MEA. Bar = 10 μm.

s.n., on dead leaf of *Quercus robur* ('B3'), April 2003 (CBS herb. Nr 7949 – **epitypus hic designatus**); living single asco-spore (SS) culture CBS 113265 – (ex-epitype; also with the holotype of *Ramularia endophylla*).

The lectotype is the only material under this name in the Persoon herbarium that was not classified in another (often invalid) variety by himself. It is typical for the species, with cylindrical asci, and ascospores 8–10 × 2–3 µm.

Endophytic isolates examined: **The Netherlands**: *Utrecht*: Soesterberg, 'De Stompert', ex living leaf of *Quercus robur*, 'AugB3H8', Aug. 2002 (CBS 113868); *loc. cit.*, substr., 'AugB2L12', Aug. 2002 (CBS 113869), and 'AugB3H7' (CBS 113870).

DISCUSSION

Previous work showed that ITS sequences are fairly constant within most species of *Mycosphaerella*, and that some species may not even be discriminated by ITS sequences (Verkley *et al.* 2004). ITS sequence diver-gences among *Mycosphaerella* states which are identified as *M. punctiformis* found on dead leaves of *Quercus*, *Tilia*, and *Acer*, indicate that this morphospecies could in fact represent a species complex. *M. phacae-frigidae*, which grouped with four *M. punctiformis* strains, can be distinguished morphologically from *M. punctiformis* by the larger ascospores (11–13 × 3–3.5 µm in the holotype of *M. phacae-frigidae* in ZT; A. Aptroot, un-publ.). *M. punctiformis*, as we epitypify it here, has been fully characterized phenotypically on the basis of iso-lates from *Quercus*. Future work including morpho-logical analysis of strains from other hosts, and also sequence analysis of additional genes, may provide evi-dence to delimit *M. punctiformis s. str.* from other cryptic species. The host range of *M. punctiformis* in this re-stricted sense is therefore still unknown. The characters of the teleomorph from which CBS 113265 was isolated comply with the original material of *M. punctiformis* in Persoon's herbarium in L. The main aim of the work presented here, is to link the name *M. punctiformis* to this material, and in accordance with Art. 9 of the *Code*, to epitypify *M. punctiformis* with herbarium specimen CBS 7949 (teleomorph on leaves), and an ex-epitype strain CBS 113265. Other *M. punctiformis* strains which originated from *Tilia*, *Acer*, and *Quercus* differ in ITS sequence by more than 20 positions from the epitype strain and other strains of *M. punctiformis s. str.* However, the ITS data proved insufficient to re-solve possible cryptic species within the *M. punctiformis* complex. Therefore, all isolates studied here are for the moment considered as *M. punctiformis s. lat.*

We repeatedly isolated endophytic *Ramularia* strains from surface-sterilized, fresh, green leaves of *Quercus robur* trees collected between June and September. Because they were morphologically and genetically identical to the epitype strain, we were able to prove that *M. punctiformis* can asymptomatically colonize living *Quercus* leaves. Its presence becomes evident by the spermogonia, which develop in large numbers when oak leaves or parts hereof go into senescence naturally or due to activities of fungi or other invaders. Although *R. endophylla* conidia were occasionally seen near leaf lesions, we were unable to confirm that conidial sporulation of *M. punctiformis* does occur *in planta* or on dead leaves in nature. This is in accordance with Braun (1998), who listed the *Ramularia* anamorph of *M. punctiformis* as an insufficiently known taxon, formed in culture only. The life-cycle of *M. puncti-formis* seems to be similar to that described in *M. buna*, a fungus with a *Pseudocercospora* anamorph which endophytically colonizes *Fagus crenata* foliage in Japan (Kaneko & Kakishima 2001, Kaneko, Kakishima & Tokumasu 2003).

On oaks in The Netherlands, *M. punctiformis* is commonly accompanied by the weakly pathogenic *Septoria quercicola*, which forms pycnidia within small leaf spots. We recently also discovered its teleomorph in small numbers on dead leaves, including those of the epitype specimen. The teleomorph of *S. quercicola* differs from *M. punctiformis* in the wider asci (35–50 × 9–12 µm) and longer ascospores (13–20 × 3.5–5 µm, av. 17 × 4.5 µm), which are not constricted at the septum and taper about equally towards both ends. Our ITS sequence analyses indicate that this *Mycosphaerella* species, which is probably different from all published species on oaks (Gilman & Wadley 1952) and for which an applicable name has not yet been found, is relatively distant from taxa of the *Ramularia* clade, as well as other taxa with *Septoria* anamorphs.

Host specificity in the *M. punctiformis* complex is still insufficiently known. Brefeld & Tavel (1891) regarded *M. punctiformis* as a plurivorous species. They noted that it was less abundant on oak than *M. maculiformis*, a species originally described from *Corylus*. According to Brefeld & Tavel, *M. maculiformis* can be dis-tinguished from *M. punctiformis* by the more densely arranged ascomata, cylindrical asci and larger asco-spores. However, they have been seen as synonymous for a long time, and the type specimens of both species were recently re-examined and found to contain (at least) morphologically indistinguishable fungi. Klebahn (1918) studied the ascomata of *M. puncti-formis* on *Tilia*, *Corylus*, and *Quercus* and briefly described and illustrated the *Ramularia* anamorphs in culture. Klebahn noted that there were only minor differences between the teleomorphs from the various tree species, and that the isolates showed only some differences in pigmentation but were otherwise indis-tinguishable. He tentatively classified these fungi as host-specific forms of *M. punctiformis*. Von Arx (1949, Müller & von Arx 1962) considered *M. maculiformis* as a synonym of *M. punctiformis*, which he regarded as plurivorous. Later authors followed this concept (Barr 1972, Sivanesan 1984), but as is shown here, the situation is more complex and may involve more than one species.

All *Mycosphaerella* species with *Ramularia* anamorphs grouped in a single, monophyletic group which obtained high bootstrap support particularly in the parsimony analysis. This was also the case in earlier molecular studies, in which fewer taxa had been included (Crous *et al.* 2001, Goodwin, Dunkle & Zismann 2001, Verkley *et al.* 2004). As in those studies, *M. graminicola* and *Septoria passerinii* form the closest sister group, but support for the joined clades remains limited. The epitypification of the type species of *Mycosphaerella* will enable the unambiguous application of the name *M. punctiformis*, and facilitate the naming of possible future segregates from *Mycosphaerella*.

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