CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Wageningen University & Research Publications

Mycol. Res. **107** (6): 689–698 (June 2003). © The British Mycological Society
DOI: 10.1017/S0953756203007883 *Printed in the United Kingdom.*

Phylogeny and taxonomy of root-inhabiting *Cryptosporiopsis* species, and *C. rhizophila* sp. nov., a fungus inhabiting roots of several *Ericaceae*

Gerard J. M. VERKLEY[1], Jantineke D. ZIJLSTRA[2], Richard C. SUMMERBELL[1] and Frank BERENDSE[2]

[1] *Centraalbureau voor Schimmelcultures, P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands.*
[2] *Nature Conservation and Plant Ecology Group, Wageningen University, Bornesteeg 69, 6708 PD Wageningen, The Netherlands.*
E-mail: *verkley@cbs.knaw.nl*

Received 8 December 2002; accepted 28 March 2003.

Three *Cryptosporiopsis* species have thus far been isolated from roots of woody plants. A fourth species, which was recently isolated from roots of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus* in The Netherlands, is described here as new. Sporulation on the natural substratum has not been observed and the morphological description of this fungus is therefore based on characters expressed on oatmeal and malt extract agars. The phenotypic characters indicated a close relationship with the other root-inhabiting species of *Cryptosporiopsis* and species of the associated teleomorph genus *Pezicula*. This relationship was confirmed by phylogenetic analyses using sequence data of the 5.8S nuclear rDNA and flanking internal transcribed spacers. In order to facilitate recognition of this possibly under-recognized category of root inhabitants, a key to the root-inhabiting *Cryptosporiopsis* species based on characters *in vitro* is given.

INTRODUCTION

Root inhabiting ascomycetes have been implicated to play an important role in functioning of ecosystems. For example, as a result of their symbiosis with ericoid mycorrhizal fungi, ericaceous plants are capable of growing in nutrient-stressed and even in highly polluted environments (Smith & Read 1997). Inoculation with specific endophytic or mycorrhizal ascomycetes can increase resistance against certain root-pathogenic fungi (Sylvia & Chemelli 2001). The biodiversity of root-inhabiting fungi is becoming a major topic in soil ecology, rhizosphere and mycorrhizal research (Sylvia & Chemelli 2001, Perotto, Girlanda & Martino 2002, Vandenkoornhuyse *et al*. 2002). Molecular approaches are revealing an unexpected taxonomic and genetic diversity among the ascomycetes that are isolated from healthy roots, particularly of members of the *Ericaceae*. Ever more sterile morphotypes are tentatively identified as members of the discomycete order Helotiales by comparisons of ITS sequences with those in GenBank and EMBL databases. Some have already been recognized as ericoid mycorrhizal fungi (Monreal, Berch & Berbee 1999, Berch, Allen & Berbee 2002, Perotto *et al*. 2002). Three species of *Cryptosporiopsis* have thus far been isolated from roots of woody plants. *Cryptosporiopsis* species are the anamorphs of *Pezicula* and *Neofabraea*, two genera of the *Helotiales* which are mainly known as endophytes or pathogens of above ground parts of woody plants (Verkley 1999, Abeln, de Pagter & Verkley 2000, de Jong *et al*. 2001). Some species are producers of secondary metabolites with antibacterial, fungicidal and herbicidal activity (Noble *et al*. 1991, Schulz *et al*. 1995, 2002). According to Kowalski (1983), the wood and bark endophyte *P. cinnamomea*, with its anamorph *Cryptosporiopsis grisea*, can also spread into the roots of dying trees. Thus far, only two *Cryptosporiopsis* species that were isolated exclusively from roots have been formally described based on morphological characters *in vitro*, *viz C. radicicola* from roots of *Quercus robur* (Kowalski & Bartnik 1995), and *C. melanogena* from roots of *Q. robur* and *Q. petraea* (Kowalski, Halmschlager & Schrader 1998). The teleomorphs of these species are unknown, but on the basis of partial 18S rDNA and ITS sequence analyses, Abeln *et al*. (2000) concluded that they belong to the monophyletic genus *Pezicula sensu* Verkley (1999), a concept including the genus *Ocellaria*.

As part of an ecophysiological study of ericaceous plant communities in The Netherlands, we repeatedly isolated a fungus from healthy, surface-sterilized roots of several *Ericaceae*. No sporulation was observed in

Table 1. Isolates of *Cryptosporiopsis rhizophila* used in this study. All strains were isolated from roots of plants collected in The Netherlands.

GenBank accession no.	CBS accession no.	Host	Geographic origin
AY176753	109839	*Erica tetralix*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176754	110602	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176755	110603	*Calluna vulgaris*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176756	110604	*Calluna vulgaris*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176757	110606	*Erica tetralix*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176758	110609	*Erica tetralix*	Prov. Drenthe, Dwingeloo, Nat. Park Dwingelderveld
AY176759	110612	*Vaccinium vitis-idaea*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176760	110616	*Vaccinium myrtillus*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide
AY176761	110617	*Vaccinium myrtillus*	Prov. Gelderland, Hoog Buurlo, Hoog Buurlosche heide

nature, but the morphological features expressed by some isolates on oatmeal agar indicated that it was a species of *Cryptosporiopsis* resembling *C. radicicola* and *C. melanogena* of oak roots. To test our hypothesis that the fungus isolated from the roots of *Ericaceae* is a genetically distinct entity within the genus *Pezicula*, we performed ITS sequence analyses comparing data derived from earlier work (Abeln *et al.* 2000) and additional data from GenBank. Because the teleomorph is as yet unknown and only an anamorph name can be applied, we describe this fungus as a new species of *Cryptosporiopsis* based on morphological characters *in vitro*.

MATERIALS AND METHODS

Isolation and phenotypic characterization of root-inhabiting fungi

New strains used in this study are listed in Table 1. Whole plants of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus* were collected in heather and vicinal forest vegetations in The Netherlands, and placed with intact root system and surrounding soil in plastic bags. Plants were regularly moistened and within 14 d treated in the laboratory as follows. Soil and superficial debris were removed from the roots by rinsing in tap water. Roots tips were cut off 1 cm behind the apex and attached soil particles were removed with forceps under a stereomicroscope. Tips were surface sterilized in 4 times diluted domestic bleach water (4% chlorine, final concentration 1%) for 3 min, followed by three rinses in sterile water. Three tips were placed in each Petri dish on 2% malt extraction agar (MEA) or potato dextrose agar (PDA) with 20 mg l^{-1} streptomycin to inhibit bacterial growth. Mycelia growing out of the root tips were transferred after about 7 d to 2% MEA and PDA. Pure cultures were regularly checked for sporulation. For morphological description, strains were incubated on oatmeal agar (OA) and 3% MEA (Centraalbureau voor Schimmelcultures 2001). Petri dishes were placed in an incubator at 15 °C in the dark, and at the same temperature with n-UV (12 h rhythm). The colours were 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 2–3 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, Carlsbad, 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). 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 amplification was performed in an Applied Biosystems (Foster City, CA) thermocycler with the following program: 1 min 95 °, 30× (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, Piscataway, NJ) and analysed on a 2% agarose gel to estimate the concentration. The PCR products were sequenced using internal primers 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 columns (Amerham Pharmacia), and separated and analyzed on an automated sequencer (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 2.5 (Applied Maths, Kortrijk, Belgium). Manual adjustments were made in the global alignment where necessary. Maximum parsimony methods and neighbour-joining distance methods were used to infer phylogenetic hypotheses. Parsimony analyses were done using PAUP v. 4.0b10 (Swofford 2002). The heuristic searches were performed

Table 2. Sequences included in this study with GenBank accession nos., species name, strain, geographic origin and source publication.

GenBank	Taxon	Strain	Host	Geographic origin	Source
AF335454	*Bisporella citrina*	–	Unknown	Unknown	F 140146 (UBC); Berbee *et al*. (unpubl.)
AF141196	*Cryptosporiopsis melanogena*	CBS 898.97 (ex type)	*Quercus petraea* (root)	Austria	Abeln *et al*. (2000)
AF141193	*C. radicicola*	CBS 640.94 (ex type)	*Quercus robur* (root)	Poland	Abeln *et al*. (2000)
AF141164	*Dermea acerina*	CBS 161.38	*Acer rubrum*	Ontario, Canada	Abeln *et al*. (2000)
AF141158	*D. ariae*	CBS 134.46	*Sorbus* sp.	Nova Scotia, Canada	Abeln *et al*. (2000)
AF141159	*D. cerasi*	CBS 136.46	*Prunus* sp.	Maryland, USA	Abeln *et al*. (2000)
AF141157	*D. hamamelidis*	CBS 137.46	*Hamamelis virginiana*	Ontaria, Canada	Abeln *et al*. (2000)
AF141160	*D. padi*	CBS 140.46	*Prunus* sp.	New York, USA	Abeln *et al*. (2000)
AF141162	*D. prunastri*	CBS 143.46	*Prunus* sp.	Quebec, Canada	Abeln *et al*. (2000)
AF141163	*D. viburni*	CBS 145.46	*Viburnum* sp.	Ontario, Canada	Abeln *et al*. (2000)
AF149067	*Hymenoscyphus ericae*	UBCtra 141	*Gaultheria shallon*	Canada	Berch *et al*. (2002)
AF149068	*H. ericae*	UBCtra 241	*Gaultheria shallon*	Canada	Berch *et al*. (2002)
AF149069	*H. ericae*	UBCtra 274	*Gaultheria shallon*	Canada	Berch *et al*. (2002)
AF141190	*Neofabraea alba*	CBS 452.64	*Malus sylvestris*	England	Abeln *et al*. (2000)
AF141189	*N. malicorticis*	CBS 355.72	*Malus sylvestris*	Portugal	Abeln *et al*. (2000)
AF141175	*Pezicula acericola*	CBS 245.97	*Acer spicatum*	Ontario, Canada	Abeln *et al*. (2000)
AF141170	*P. aurantiaca*	CBS 201.46	*Alnus crispa* var. *mollis*	Nova Scotia, Canada	Abeln *et al*. (2000)
AF141191	*P. carpinae*	CBS 923.96	*Carpinus betulus*	Germany	Abeln *et al*. (2000)
AF141186	*P. cinnamomea*	CBS 625.96	*Quercus robur*	Germany	Abeln *et al*. (2000)
AF141182	*P. corni*	CBS 285.39	*Cornus circinata*	Ontario, Canada	Abeln *et al*. (2000)
AF141179	*P. corticola*	CBS 259.31 (ex type?)	*Malus sylvestris?*	Denmark	Abeln *et al*. (2000)
AF141176	*P. corylina*	CBS 249.97	*Corylus cornuta*	Ontario, Canada	Abeln *et al*. (2000)
AF141194	*P. eucrita*	CBS 662.96	*Pseudotsuga menziesii*	Netherlands	Abeln *et al*. (2000)
AF141183	*P. frangulae* subsp. *frangulae*	CBS 286.39	*Rhamnus* sp.	Germany	Abeln *et al*. (2000)
AF141181	*P. ocellata*	CBS 949.97	*Salix* sp.?	Luxemburg	Abeln *et al*. (2000)
AF141188	*P. pruinosa*	CBS 292.39	*Amelanchier* sp.	Ontario, Canada	Abeln *et al*. (2000)
AF141178	*P. rubi*	CBS 253.97	*Rubus* sp.	New York, USA	Abeln *et al*. (2000)
AF141172	*P. sporulosa*	CBS 224.96 (ex type)	*Larix decidua*	Netherlands	Abeln *et al*. (2000)
AF141171	*P. subcarnea*	CBS 203.46	*Acer pennsylvanicum*	Ontario, Canada	Abeln *et al*. (2000)
AF141168	*Scleropezicula alnicola*	CBS 200.46	*Alnus incana*	Ontario, Canada	Abeln *et al*. (2000)
AF149074	'Salal root associated fungus'	'UBCtra 288'	*Gaultheria shallon*	Canada	Berch *et al*. (2002)
AF149087	'Salal root associated fungus'	'UBCtra 29'	*Gaultheria shallon*	Canada	Berch *et al*. (2002)
AJ301960	'Ascomycete sp.'	BBA 71218	*Erica* sp.	Germany	GenBank

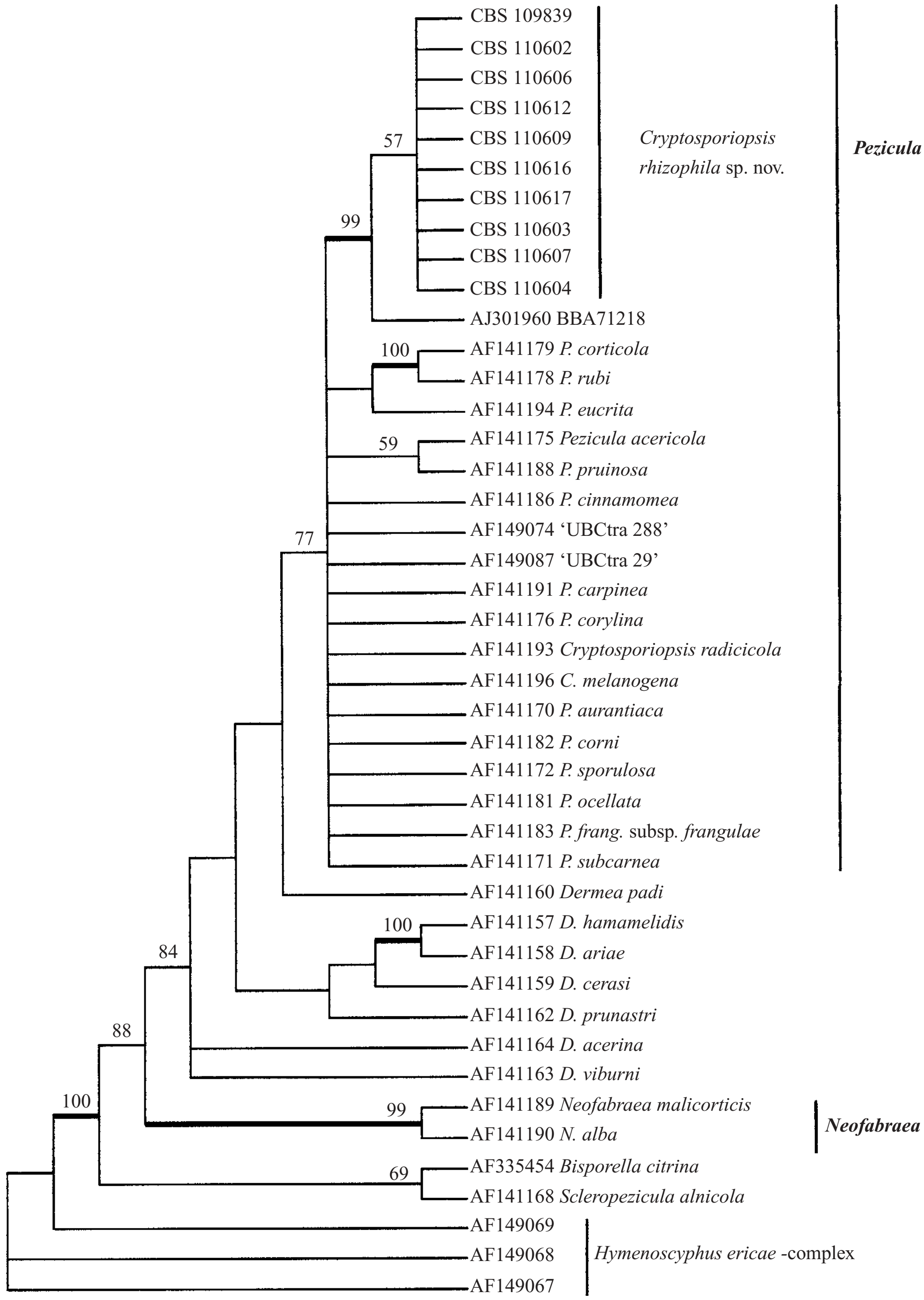


Fig. 1. Strict consensus tree of 95 MPTs of 409 steps using 135 parsimony-informative characters of the ITS region. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50%. Branches supported by 90% or higher values are in bold. Accession numbers of sequences taken from GenBank are indicated before the taxon name. GenBank numbers of sequences of *C. rhizophila* strains are given in Table 1. Species are presented by teleomorph name, if known. Sequences of the *Hymenoscyphus ericae*-complex were used as outgroup to root the tree.

with the following parameters: characters were unordered with equal weight, and random taxon addition. The tree bisection-reconnection (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 10000. 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 were performed in Bionumerics and PAUP, in both cases without pairwise corrections. Stability of clades was tested with 1000 neighbour-joining bootstrap replications. BLAST searches in GenBank revealed highest similarity to species of *Pezicula*, *Dermea*, and *Cryptosporiopsis*, of

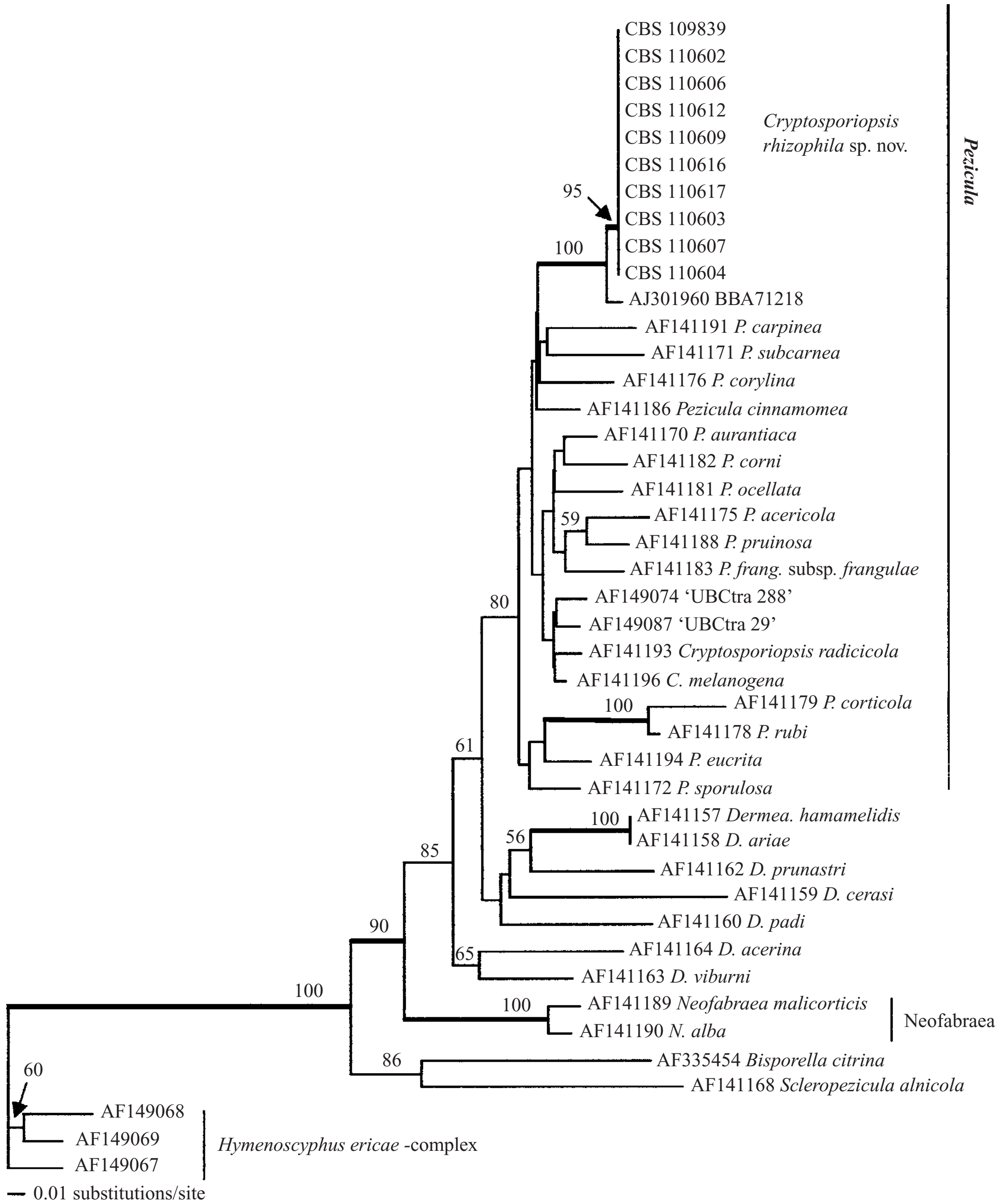


Fig. 2. Neighbour-joining tree derived from 135 parsimony-informative and 42 autapomorphic characters of the ITS region, calculated in PAUP without pairwise corrections. Numbers at the branches are bootstrap values obtained from 1000 replications and rounded to the nearest integer, shown only for branches supported by more than 50 %. Branches supported by 90 % or higher values are in bold. Length of branches is proportional to number of changes. Species are presented by teleomorph name, if known. Sequences of the *Hymenoscyphus ericae*-complex were used as outgroup to root the tree.

which a part of the 18S, ITS 1, 5.8S rDNA, and ITS 2 had been sequenced by Abeln *et al.* (2000). In our analyses only the ITS region of these sequences was included. Three additional sequences from GenBank were also included: one highly similar sequence of an unidentified ascomycete (BBA71218), and two of isolates from roots of *Gaultheria shallon*. GenBank accession numbers, taxon names and other information about these sequences are given in Table 2. GenBank accession numbers of the strains of *C. rhizophila* are given in Table 1. Two species classified in the family *Helotiaceae* of the same order were also included: *Bisporella citrina*, AF335454, and *Hymenoscyphus ericae*, AF149067, 149068, 149069. The last three sequences were defined as outgroup.

RESULTS

Phylogenetic analyses

The investigated strains of *Cryptosporiopsis rhizophila* showed 100 % identity in ITS 1-5.8S rDNA-ITS 2. The alignment of all 43 taxa comprised 499 characters, 135 (27 %) of which were parsimony-informative. The

Key to the species of Cryptosporiopsis *isolated from roots*

The key is based on characters expressed on OA and MEA in the dark at 15 °. It is followed by a formal description of the new species from *Ericaceae*. Previously described *Cryptosporiopsis* species from roots were treated by Kowalski & Bartnik (1995), Kowalski *et al.* (1998), and Verkley (1999).

1 Colonies on OA intially colourless, but later becoming greyish, Buff or brownish, or almost black with age; aerial mycelium may be well-developed, but without elevated surface structures; conidiogenous cells borne directly on vegetative hyphae, or in sporodochial conidiomata provided with seta-like brown-walled hyphae; macroconidia to 37 µm long and 9 µm wide, containing oil droplets up to 3 µm diam. 2

Colonies on OA soon becoming brownish, cinnamon or olivaceous, with distinct globular to columnar surface structures which are composed of entangling hyphae and rise well above the aerial mycelium; sporulating only in simple to complex, intially closed, eustromatic conidiomata; macroconidia often very large, 29–58 × 9.5–16 µm, with oil droplets up to 2 µm diam. . . . . . **C. grisea** (teleomorph *Pezicula cinnamomea*)

2(1) Chlamydospores present, basal cell of the seta-like hyphae swollen, macroconidia 22–37 µm long 3

Chlamydospores absent, basal cell of seta-like hyphae not swollen, macroconidia 16.2–25 µm long . . . . **C. rhizophila**

3(2) Colonies becoming homogenously grey to black on MEA, forming scattered, very dark brown areas on OA; macroconidia 25–37 × 5.5–9 µm, usually formed within a few weeks . . . . **C. melanogena**

Colonies beige, pale or dark brown on MEA; macroconidia 22–35 × 6–7.5 µm, usually formed sparsely and later . **C. radicicola**

remaining 364 characters were all uninformative and were excluded from the parsimony analyses. The heuristic search using 5000 random sequence input orders yielded 95 most-parsimonious-trees (MPT) of 409 steps, with consistency index (CI) 0.494, retention index (RI) 0.697, rescaled consistency index (RCI) 0.344, and homoplasy index (HI) 0.506. The strict consensus tree is shown in Fig. 1. Bootstrap supports over 50% are indicated. *C. rhizophila* and the strain BBA71218 formed a highly supported clade (99%), which was nested within the *Pezicula* clade, comprising the oak root endophytes *C. radicicola* and *C. melanogena*, the two strains isolated from the roots of *Gaultheria shallon* (UBCtra 288 and 29), and all included *Pezicula* species. This clade was supported by 77% of the bootstrap replications. The two species of *Neofabraea* grouped in a well-supported clade, but the species of *Dermea* showed a paraphyletic arrangement. *Scleropezicula alnicola* grouped with *Bisporella citrina* (69% bootstrap support). In addition to the 135 informative characters, 42 autapomorphic characters were also included the neighbour-joining analysis (see Fig. 2). The results of this analysis were similar to those of the parsimony analysis, showing 80% bootstrap support for the *Pezicula* cluster, which included the root-inhabiting species as well as *C. rhizophila* (Fig. 7). *C. radicicola* and *C. melanogena* clustered with the two strains isolated from the roots of *Gaultheria shallon* (UBCtra 288 and 29), but bootstrap support was low. As in the parsimony analysis, the cluster of *C. rhizophila* strains and BBA71218 obtained very high bootstrap support. The cluster comprising only the *C. rhizophila* strains was much higher suppported in the neighbour-joining analysis (95%) than in the parsimony analysis (57%).

TAXONOMY

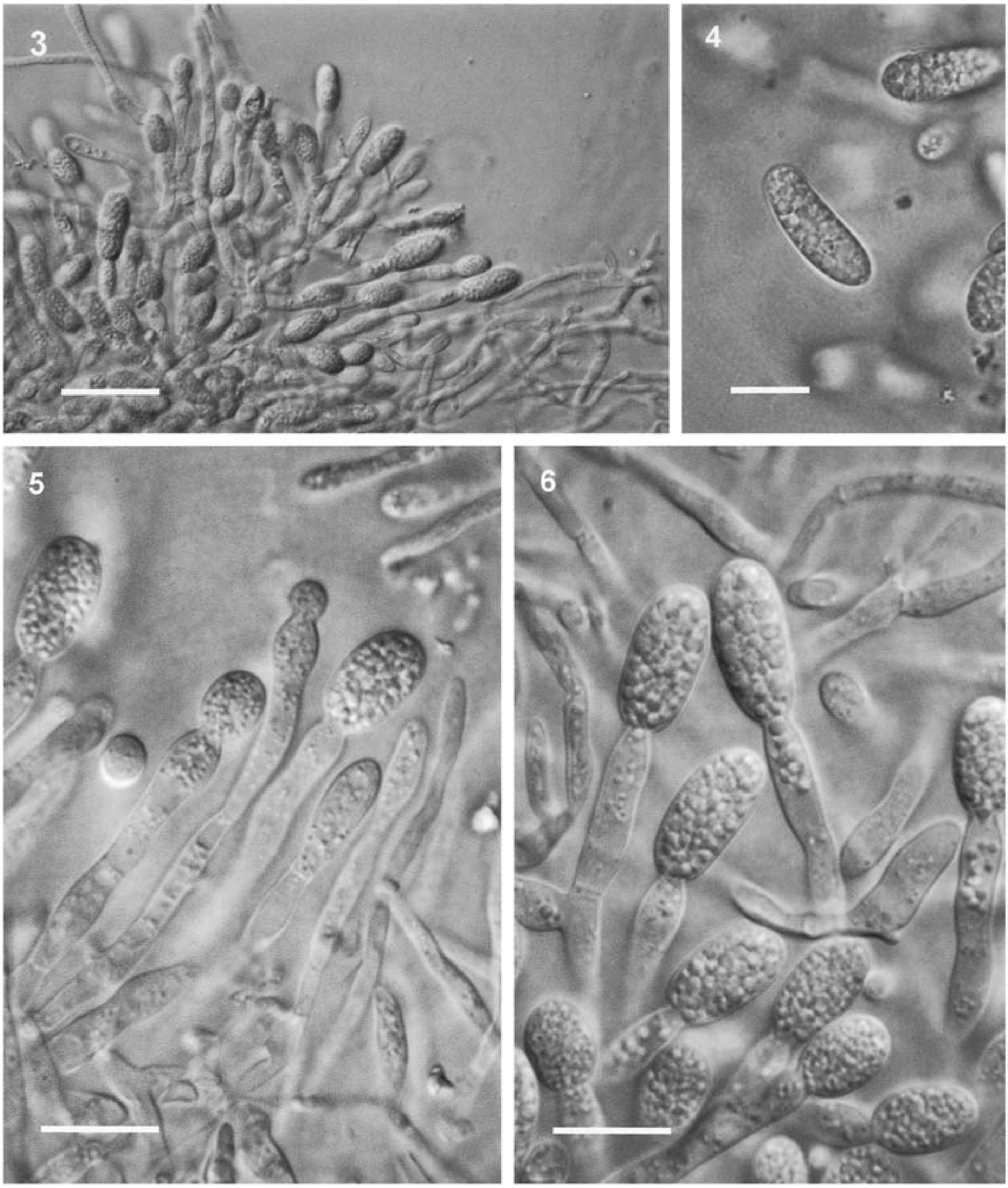
Cryptosporiopsis rhizophila Verkley & Zijlstra, **sp. nov.** (Figs 3–7)

Etym.: rhizophilus, root-loving.

Conidiomata *in vitro* typice sporodochia et plerumque setis fuscis septatis praedita. *Cellulae macroconidiogenae* plerumque in conidiophoris simplicae vel interdum ramosae, septatae, acrogenae vel acropleurogenae integratae, phialidicae, cylindricae vel clavatae, 7–13(–18) × 3–5 µm. Macroconidia ellipsoidea vel breve cylindrica, vulgo curvata, hyalina, continua, interdum 1-septata, guttas numerosas 1–2.5(–3) µm diametro continentia, 16–25 × 6–7.5 µm. Microconidia ellipsoidea, apice rotundato et basi late truncato vel leviter attenuato, hyalina, continua, 4–5.5 × 1–2 µm.

Typus: **The Netherlands**: *Prov. Drenthe*: Nationaal Park Dwingelderveld, near 'schaapskooi', isol. ex root of *Erica tetralix*, Sept. 2000, *J. D. Zijlstra 335* (**CBS** – holotypus [dried culture on OA]; CBS 109839 – cultura viva).

Sporulation ocurring directly on immersed or superficial vegetative hyphae, or in superficial, hemispherical sporodochia which may become surrounded by tufts of 70–140 µm long, septate seta-like hyphae with somewhat thickened, smooth, pale to dark brown walls, ending in a hyaline blunt tip, 4 µm wide at the base, and often rising above the surface in sticky bundels. In addition, erect synnema-like columns are also formed which are composed of entangled hyphae bearing conidiogenous cells in the lower part. *Conidiogenous cells* mostly integrated in simple, rarely branched, septate, acrogenous or acropleurogenous conidiophores, more rarely discrete and borne on rather undifferentiated sterile tissue consisting of hyphal to isodiametric cells, determinate, phialidic, older ones with a well-visible periclinal thickening, cylindrical to clavate, widest just below the apex, 7–13(–18) × 3–5 µm. *Macroconidia* ellipsoid to short-cylindrical, mostly curved, aseptate, hyaline, with age occasionally medianly 1-septate and golden yellow, containing numerous oil droplets 1–2.5(–3) µm diam. Measurements in water (18–)20–24(–25) × (6–)6.5–7(–7.5) µm (dark; on average 22.5 × 7 µm; $N=20$); (16–)18–21.5(–23) × (6–)6.5–7(–7.5) µm (n-UV; on average 20 × 7 µm; $N=20$). Conidial masses whitish, with age yellow or Cinnamon. *Microconidiogenous cells* integrated in separate cylindrical, acrogenous or acropleurogenous conidiophores, phialidic, with a periclinal thickening at the apex and often a minute collarette. *Microconidia* ellipsoid, with a rounded apex and a broadly truncate



Figs 3–6. *Cryptosporiopsis rhizophila* (CBS 109839 on OA). **Fig. 3.** Sporodochium, Bar = 25 µm. **Fig. 4.** Conidia. Bar = 10 µm. **Figs 5–6.** Conidiogenous cells. Bars = 10 µm.

or slightly attenuated base, hyaline, aseptate, with granular contents, 4–5.5 × 1–2 µm (dark and n-UV).

Colony morphology: on OA reaching a diam of 44 mm in 10 d (80 mm in 21 d), with an even or slightly ruffled margin; first glabrous and colourless, but after 3–4 weeks immersed mycelium becoming Beige to Buff, and in the centre and some scattered areas Umber, slightly Olivaceous or Sepia, reverse in these areas becoming dull Hazel; aerial mycelium variable, remaining very scanty or becoming well-developed within a few weeks, whitish to Buff, woolly-floccose, covering most of the colony surface; sporulation starting at about 10–14 d, at first only in the centre from small clusters of macro- and microconidiogenous cells arising from undifferentiated hyphae, but later also in sporodochia which are scattered over the colony surface. On MEA reaching a diam of 10–12 mm in 10 d (20 mm in 21 d), with an even to irregular, later often distinctly lobed, glabrous, Saffron to Ochreous margin; surface mostly covered by dense, pure white, woolly aerial mycelium,

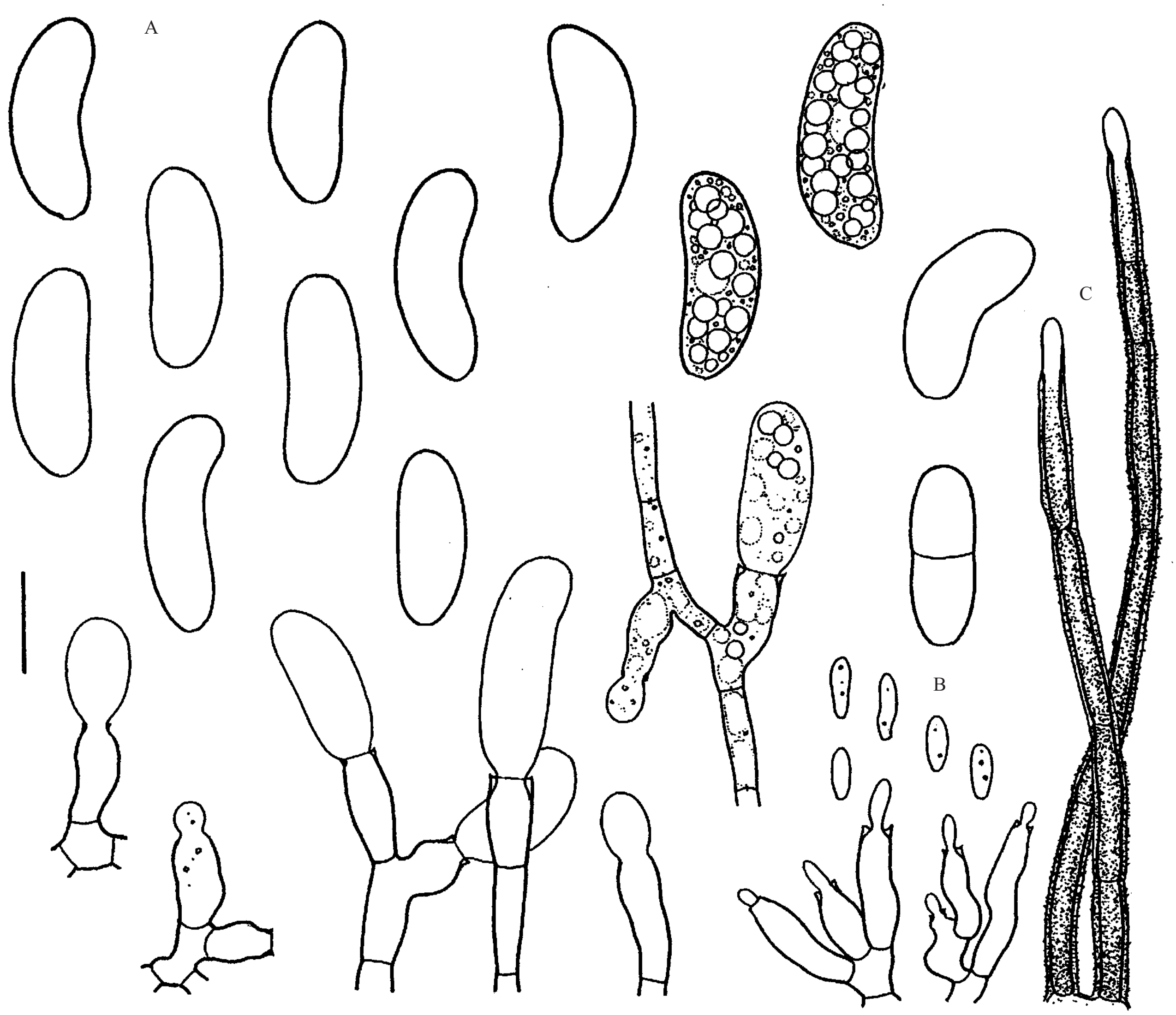


Fig. 7. *Cryptosporiopsis rhizophila* (CBS 109839 on OA). (A) Macroconidia and macroconidiogenous cells (B) Microconidia and microconidiogenous cells (C) Setae. Bar = 10 μm.

which near the margin becomes Salmon with some yellow after several weeks; reverse homogeneously Cinnamon or Bay, later Chestnut in the centre. A red diffusible pigment is clearly visible in the medium surrounding 3-week-old colonies. Isolates examined are listed in Table 1. Sporulation was observed on OA in type strain CBS 109839, and also in CBS 110604, 110606 and 110612.

Hosts: isolated from root tips of *Calluna vulgaris*, *Erica tetralix*, *Vaccinium vitis-idaea*, and *V. myrtillus*.

Distribution: Known only from two localities in The Netherlands.

DISCUSSION

In the extended body of literature on fungi isolated from stringently washed or surface-disinfected roots of woody plants, reports of *Cryptosporiopsis* species are very rare. Previously, however, we have noted that unidentified *Cryptosporiopsis* species could regularly be obtained from serially washed roots of apparently healthy *Cornus canadensis* (*Cornaceae*) plants in Ontario (Summerbell 1989). Since *Cryptosporiopsis* species are often slow to sporulate in culture and also may be difficult to recognize as coelomycetes – a step that is necessary in order to access useful morphological identification literature – it is possible that such isolates have been seen in other studies but not identified.

C. rhizophila can be placed in *Cryptosporiopsis* on the grounds of the macro- and microconidiogenous cells which are integrated, phialidic, determinate, and hyaline. The presence of ellipsoid, pluriguttulate, 0-septate macroconidia and much smaller microconidia are also characteristic of this anamorphic genus (Verkley 1999). The oak-root-inhabiting *C. radicicola* and *C. melanogena* have several features in common with *C. rhizophila*. The macroconidia of these fungi are more or less distinctly curved, and contain oil droplets that are larger than those found in other *Cryptosporiopsis* species. The phialides are frequently directly borne on vegetative hyphae. Conidiomata are sporodochial and often provided with seta-like hyphae that are not found in other *Cryptosporiopsis* species. There are, however, also differences between the three root fungi. The two species from oak roots form chlamydospores, but these are not found in *C. rhizophila*. The basal cells of the setae of *C. radicicola* and *C. melanogena* are swollen, whilst those of *C. rhizophila* are not. In *C. melanogena* macroconidia are 25–37 × 5.5–9.0 μm (Kowalski *et al.* 1998), and in *C. radicicola* they are 22–35 × 6–7.5 μm, while in *C. rhizophila* they are on average shorter, and never over 25 μm long. Some isolates of *C. rhizophila* produced a red diffusible pigment on MEA, and this has not been observed in the other two species.

In congruence with morphological data, the ITS sequence analyses also indicate that *C. rhizophila* is a member of the genus *Pezicula*, and that it is congeneric with *C. radicicola* and *C. melanogena*. The internal topology of the *Pezicula* clade is, however, largely unresolved. Thus far, ITS sequences show no variation within species of *Pezicula*, and also the strains of *C. rhizophila* all had identical ITS sequences. The ITS sequence of isolate BBA 71218 differs by three base position from that of *C. rhizophila*, indicating that it is most likely specifically distinct. Unfortunately, we have so far been unable to obtain any information about the phenotype of this fungus which was isolated from roots of *Erica* sp. (H. I. Nirenberg, pers. comm.).

The neighbour-joining analysis indicates that *C. rhizophila* could also be closely related to *Pezicula carpinea*, *P. subcarnea*, *P. cinnamomea* and *P. corylina*, rather than to the other root-isolates including *C. radicicola*, *C. melanogena*, and the unidentified strains from *Gaultheria*. However, sequencing of more loci will be necessary to clarify whether the root-inhabiting species indeed represent multiple lineages within *Pezicula*, or a single lineage. The conidiomata of the anamorph of *P. carpinea* (*C. fasciculata*), are relatively similar to those of *C. rhizophila*, because they are also sporodochial or 'acervuloid' in culture (Verkley 1999), while *in planta* they develop as true acervuli. *P. carpinea*, which is the type species of the genus *Pezicula*, occurs mainly on *Carpinus betulus* in Eurasia and on *C. caroliniana* in North America, but also on *Fagus sylvatica*. It has also, however, been isolated from living bark of other trees in a study by Kowalski & Kehr (1992) on endophytes in forest tree species. As far as is known, it has not been isolated from roots. *P. corylina* is so far only known from North America, where it is confined to *Corylus* spp. In culture, this species forms eustromatic, initially closed conidiomata resembling those formed *in planta* (Groves 1941, Verkley 1999). Such conidiomata are also formed by the ubiquitous *P. cinnamomea*.

Cryptosporiopsis rhizophila is to our knowledge the first morphologically described species of *Cryptosporiopsis* from roots of *Ericaceae*. Because it has been repeatedly isolated from surface-sterilized, healthy roots of several *Ericaceae*, it can be regarded as an endophytic fungus. The association of *C. rhizophila* with the plants has been confirmed by microscopical observations of hyphae in living, healthy rootlets of sterile *Calluna* seedlings grown *in vitro*, that were successfully infected after inoculation with this fungus (Zijlstra *et al.*, unpubl.). Some Helotiales are experimentally confirmed ericoid mycorrhizal symbionts, *viz*. *Hymenoscyphus ericae*, and several probably closely related, unnamed *mycelia sterilia* (Read 1974, Monreal *et al.* 1999, Vrålstad, Schumacher & Taylor 2002, Vrålstad, Myre & Schumacher 2002). Berch *et al.* (2002) reported that resynthesis experiments conducted with the salal root isolates UBCtra 288 and 29 had been unsuccessful. Little is known about the role of these apparently root-associated members of *Pezicula*. The resynthesis experiments recently initiated in Wageningen are expected to shed more light on the possible role of *C. rhizophila* as a mycorrhizal partner.

Endophytic fungi that were reported as isolated from twigs and branches of ericaceous plants and identified as *Cryptosporiopsis* sp. (Fischer *et al.* 1984), may have been *P. myrtillina* or *P. acericola*, both of which have been found on aboveground parts of ericaceous hosts. *P. myrtillina* occurs in Europe and North America on recently dead twigs and branches of several *Ericaceae*, *viz*. *Calluna vulgaris*, *Vaccinium myrtillus* and *V. uliginosum*, and also on *Rhododendron ferrugineum* and *R. maximum*. No cultures are available of this species, and the anamorph is unknown (Verkley 1999). Morphologically, the apothecia of *P. myrtillina* resemble those of *P. rubi* and *P. eucrita*, species which in our sequence analyses are more distantly related to *C. rhizophila* than are the oak fungi (31 and 20 base positions difference in ITS1 and ITS2, respectively, with *C. rhizophila*). *Pezicula acericola* normally occurs on *Acer*, *Cornus* and *Quercus* spp., but also on *Rhododendron ferrugineum*. This species is different from *C. rhizophila* in morphology and ITS sequence.

ACKNOWLEDGEMENTS

Mieke Starink-Willemse is gratefully acknowledged for sequencing of the new strains. Pedro W. Crous is kindly thanked for a critical reading of the manuscript, and Helgard I. Nirenberg for providing information about the strain BBA71218.

REFERENCES

Abeln, E. C., de Pagter, M. A. & Verkley, G. J. M. (2000) Phylogeny of *Pezicula*, *Dermea* and *Neofabraea* inferred from partial sequences of the nuclear ribosomal RNA gene cluster. *Mycologia* **92**: 685–693.

Berch, S. M., Allen, T. R. & Berbee, M. L. (2002) Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. *Plant and Soil* **244**: 55–66.

Centraalbureau voor Schimmelcultures (2001) *List of Cultures, Fungi and Yeasts*. 35th edn. Centraalbureau voor Schimmelcultures, Utrecht.

de Hoog, G. S. & Gerrits van den Ende, A. H. G. (1998) Molecular diagnostics of clinical strains of filamentous basidiomycetes. *Mycoses* **41**: 183–189.

de Jong, S. N., Levesque, C. A., Verkley, G. J. M., Abeln, E. C., Rahe, J. E. & Brown, P. G. (2001) Phylogenetic relationships among *Neofabraea* species causing tree cankers and bull's-eye rot of apple based on DNA sequencing of ITS nuclear rDNA, mitochondrial rDNA, and the β-tubulin gene. *Mycological Research* **105**: 658–669.

Fischer, P. J., Anson, A. E. & Petrini, O. (1984) Novel antibiotic activity of an endophytic *Cryptosporiopsis* sp. isolated from *Vaccinium myrtillus*. *Transactions of the British Mycological Society* **83**: 145–148.

Groves, J. W. (1941) *Pezicula carnea and Pezicula subcarnea*. *Mycologia* **33**: 510–522.

Kowalski, T. (1983) Vorkommen von Pilzen in durch Luftverunreinigung geschädigten Wäldern im Oberschlesischen und Krakauer Industriegebiet IX. Mykoflora von *Quercus robur* L. und *Q. rubra* L. an einem Standort mit mittlerer Immisionsbelastung. *European Journal of Forest Pathology* **13**: 46–59.

Kowalski, T. & Bartnik, C. (1995) *Cryptosporiopsis radicicola* sp. nov. from roots of *Quercus robur*. *Mycological Research* **99**: 663–666.

Kowalski, T., Halmschlager, E. & Schrader, K. (1998) *Cryptosporiopsis melanigena* sp. nov., a root-inhabiting fungus of *Quercus robur* and *Q. petraea*. *Mycological Research* **102**: 347–354.

Kowalski, T. & Kehr, R. D. (1992) Endophytic fungal colonization of branch bases in several forest tree species. *Sydowia* **44**: 137–168.

Monreal, M., Berch, S. M. & Berbee, M. (1999) Molecular diversity of ericoid mycorrhizal fungi. *Canadian Journal of Botany* **77**: 1580–1594.

Noble, H., Mary, H., Langley, D., Sidebottom, P. J., Lane, S. J. & Fisher, P. J. (1991) An echinocandin from an endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in *Pinus sylvestris* and *Fagus*. *Mycological Research* **95**: 1439–1440.

Perotto, S., Girlanda, M. & Martino, E. (2002) Ericoid mycorrhizal fungi: some new perspectives on old acquaintances. *Plant and Soil* **244**: 41–53.

Rayner, R. W. (1970) *A Mycological Colour Chart*. Commonwealth Mycological Institute, Kew.

Read, D. J. (1974) *Pezizella ericae* sp. nov., the perfect state of a typical mycorrhizal endophyte of *Ericaceae*. *Transactions of the British Mycological Society* **63**: 381–382.

Schulz, B., Sucker, J., Aust, H. J., Krohn, K., Ludewig, K., Jones, P. G. & Döring, D. (1995) Biologically active secondary metabolites of endophytic *Pezicula* species. *Mycological Research* **99**: 1007–1015.

Schulz, B., Boyle, C., Draeger, S., Römmert, A.-K. & Krohn, K. (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* **106**: 996–1004.

Smith, S. E. & Read, D. J. (1997) *Mycorrizal Symbiosis*. Academic Press, San Diego.

Summerbell, R. C. (1989) Microfungi associated with the mycorrhizal mantle and adjacent habitats within the rhizosphere of black spruce. *Canadian Journal of Botany* **67**: 1085–1095.

Swofford, D. L. (2002) *PAUP: phylogenetic analysis using parsimony*. Sinauer Associates, Sunderland, MA.

Sylvia, D. M. & Chemelli, D. O. (2001) Interactions among root-inhabiting fungi and their implications for biological control of root pathogens. *Advances in Agronomy* **73**: 1–33.

Vandenkoornhuyse, P., Baldauf, S. L., Leyval, C., Straczek, J. & Young, J. P. W. (2002) Extensive fungal diversity in plant roots. *Science* **295**: 2051.

Verkley, G. J. M. (1999) A monograph of the genus *Pezicula* and its anamorphs. *Studies in Mycology* **44**: 1–180.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Vrålstad, T., Schumacher, T. & Taylor, A. F. S. (2002) Mycorrhizal synthesis between fungal strains of *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. *New Phytologist* **153**: 143–152.

Vrålstad, T., Myre, E. & Schumacher, T. (2002) Molecular diversity and phylogenetic affinities of root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. *New Phytologist* **155**: 131–148.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Corresponding Editor: R. S. Currah