Cryptosporiopsis melanigena sp. nov., a root-inhabiting fungus of *Quercus robur* and *Q. petraea*

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A new species of *Cryptosporiopsis* is described. It was isolated frequently from roots of healthy-looking and declining oaks (*Quercus robur* and *Q. petraea*). The relationship to *C. radicicola*, a similar species also occurring on oak roots, is discussed. The new species is separated from *C. radicicola* by means of morphological and physiological characters, genomic analysis by the PCR-based RAPD technique as well as by the production of metabolites.

Cryptosporiopsis species are known as anamorphs of *Pezicula* (Sutton, 1980). Most are found on the lignified above ground portions of plants (Groves, 1939; Wollenweber, 1939; Sutton, 1980) and a few species have been reported sporadically from assimilation organs (Sankaran, Sutton & Balasundran, 1995). Recent surveys on the mycoflora of tree roots indicate that *Cryptosporiopsis* species are also very common on tree roots (Kowalski, 1983; Hennon, Shaw & Hansen, 1990; Krzan, 1991; Holdenrieder & Sieber, 1992; Neumüller, 1996) and can also be found in the rhizosphere (Summerbell, 1989), although in most cases it was not possible to determine the species.

On oak the most common species is *Cryptosporiopsis quercina* Petr. [teleomorph: *Pezicula cinnamomea* (DC.) Sacc.]. It occurs predominantly on twigs and trunks (Sutton, 1980; Butin & Kowalski, 1983; Kowalski, 1983) but can spread into the roots of dying trees (Kowalski, 1983). *Cryptosporiopsis radicicola* Kowalski & Bartnik was described from oak roots as a new species; it is confined to plant parts below the soil surface (Kowalski & Bartnik, 1995). It was also the most frequently isolated species from fine roots of declining oaks in Poland (Bartnik, 1996). Similar results were obtained during investigations on the fungal community of roots from healthy and declining oaks in Austria (Halmschlager & Kowalski, 1996). In this study another *Cryptosporiopsis* was obtained which could not be attributed to a known species. It is described here formally and compared and contrasted with species having similar characteristics, especially *C. radicicola*, using morphological, physiological, genetical and biochemical

Table 1. List of *Cryptosporiopsis* isolates used for physiological, genetical and biochemical studies (strains chosen for growth test, RAPD analysis or hplc are indicated with +, nos in parentheses refer to lanes given in Fig. 19)

	Isolate no.	Source	Geographic origin	Growth test	RAPD	hplc
C. melanigena	IFBW 14	*Quercus robur*	Niederweiden, Austria	+	−	
	IFBW 15	*Q. robur*	Niederweiden, Austria	+	+ (9)	+
	IFBW 16	*Q. robur*	Niederweiden, Austria	+	+ (10)	
	IFBW 13	*Q. robur*	Niederweiden, Austria	+	+ (11)	
	IFBW 18	*Q. robur*	Niederweiden, Austria	+	+ (12)	
C. radicicola	IFBW 2	*Q. petraea*	Patzmannsdorf, Austria	+	+ (3)	+
	IFBW 3	*Q. petraea*	Patzmannsdorf, Austria	+	+ (4)	
	IFBW 4	*Q. petraea*	Patzmannsdorf, Austria	+	+ (5)	
	IFBW 5	*Q. petraea*	Patzmannsdorf, Austria	+	+ (6)	
	IFBW 6	*Q. petraea*	Patzmannsdorf, Austria	+	+ (7)	
C. quercina	Ke/T 102	*Abies alba*	Regensburg, Germany	−	+ (19)	
	Ke/Es 1	*Fraxinus excelsior*	Essehof, Germany	−	+ (18)	
C. abietina	Ke/K 28	*Pinus sylvestris*	Essehof, Germany	−	+ (20)	
C. fasciculata	Ke/Ca 70	*Carpinus betulus*	Essehof, Germany	−	+ (21)	

Strains were derived as follows: IFBW, sampled by E. Halmschlager; Ke, Culture Collection of the Institute for Plant Protection in Forestry BBA Braunschweig, Germany; sampled by T. Kowalski.

criteria. Characteristics that support the attribution of the new species to *Cryptosporiopsis* were the same as discussed by Kowalski & Bartnik (1995) for *C. radicicola*.

MATERIALS AND METHODS

The fungus described in this paper was isolated frequently from roots (diam. < 2–30 mm) of healthy and declining 80–120-yr-old oak trees (*Quercus robur* L. and *Q. petraea* (Matt.) Lieb.) from Patzmannsdorf and Niederweiden (60 km north, and 50 km west of Vienna, respectively). Because no conidiomata could be found on natural substrata its description is based on morphology *in vitro*. Isolation, sterilization and incubation of root samples were performed according to Kowalski & Halmschlager (1996). For morphological investigations cultures were grown on 2% malt extract agar (MEA, 20 g l^{-1} malt extract Difco, 15 g l^{-1} agar Difco) supplemented with 100 mg l^{-1} streptomycin sulphate. Culture colours were classified using the German industrial standard colour swatch (RAL). Photomicrographs were taken using a Zeiss Axiophot microscope in bright field or interference contrast. Single spore isolates were prepared from macroconidia according to Kreisel & Schauer (1987). Size of macroconidia was assessed separately for young (6-wk-old) and older (4-6-mo-old) cultures by measuring 60 conidia of each type in deionized water. Extension of colonies in the range from 5° to 30° C (steps of 5°) was measured after 21 d by taking the average of two colony diameters at right angles for each plate. Mean radial growth was then calculated for each strain. Two Petri dishes per strain were used and the experiment was conducted twice. Growth tests were also carried out for the morphologically similar *C. radicicola*, using the same method. The isolates tested are listed in Table 1.

Fungal isolates used for RAPD analysis (Table 1) were grown in shake flasks in 50 ml of 2% (w/v) malt extract medium at 20° for 10 d, using a rotary shaker (110 rpm). The mycelium was harvested by vacuum filtration, washed with sterile water, frozen quickly at −70° and lyophilized. The freeze-dried mycelium was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The total DNA from freeze-dried mycelium was isolated by QIAmp Tissue Kit (Qiagen GmbH, Hilden, Germany).

The DNA was amplified in a 25 µl reaction mixture containing template DNA (2 ng), 10 mM TRIS-HCl pH 8·3, 10 mM KCl, 4 mM $MgCl_2$, 0·2 µM Random Primer OPY-18 (Operon Technologies, Alameda, CA), 5 units of Taq DNA polymerase (Perkin Elmer, Norwalk, CT), and 100 µM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim). Amplifications were performed in a Perkin Elmer Cetus Gene Amp PCR-System 9600 (Norwalk, CT) with the following conditions (Pastrick, Rumpenhorst & Bürgermeister, 1995): 150 s at 94° followed by 40 cycles of 20 s at 92°, 15 s at 38° and 1 min at 72°. The program ended with a final extension at 72° for 420 s. The amplification products were separated by electrophoresis in 1·4% agarose gel, stained with ethidium bromide and photographed under uv light.

Banding patterns from RAPD markers were compiled in a two-discrete-character matrix by scoring 1 for the presence of major bands and 0 for their absence. Generation of cladograms with estimation of branch lengths was performed by the Jukes–Cantor option in DNADIST and application of the FITCH program to the computed distance matrix (PHYLIP package; Felsenstein, 1989).

Hplc profiles of secondary metabolites were determined for one selected isolate of *C. melanigena* and *C. radicicola* each within a routine screening at Bender/Boehringer Ingelheim Vienna (Table 1). Isolates were inoculated onto a rice medium (Bender/Boehringer Ingelheim Vienna, unpubl.) and cultivated at 28° for 12 d. Extraction of mycelium was performed using ethyl acetate–isopropanole and butanol. Hplc analysis was performed by Waters Alliance DAD–hplc equipment using a RP-18 Waters Symmetry column 2 × 150 mm ('Microbore') with a gradient of 0·1% phosphoric acid–acetonenitril: 5–100% ACN in 16 min and a flow rate of 0·5 ml min^{-1} at a wavelength of 225 nm.

RESULTS

Morphological investigations

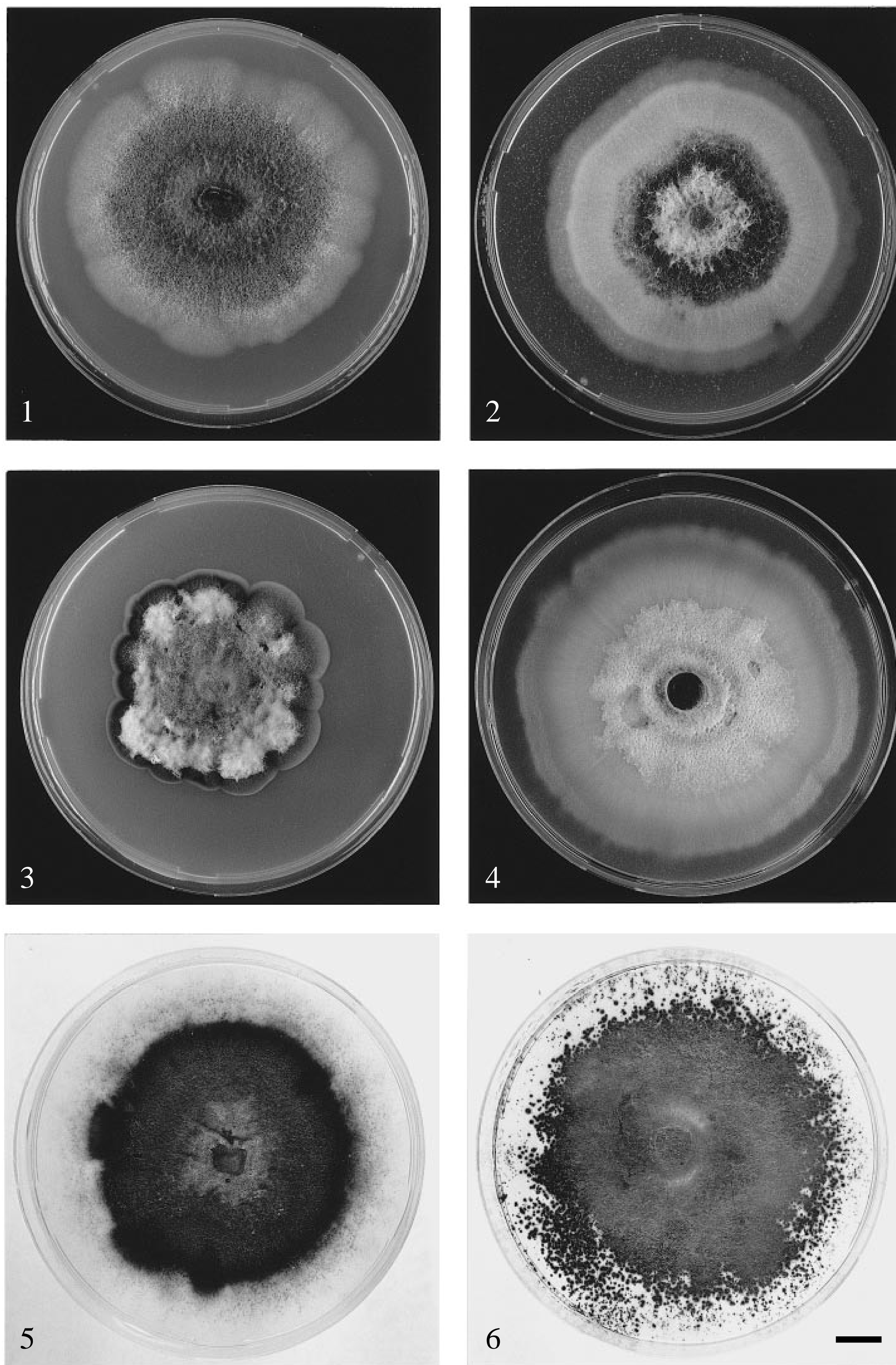
Cryptosporioposis melanigena Kowalski & Halmschlager sp. nov. (Figs 1–16)

Etym.: melas, melanos (Gr.), black

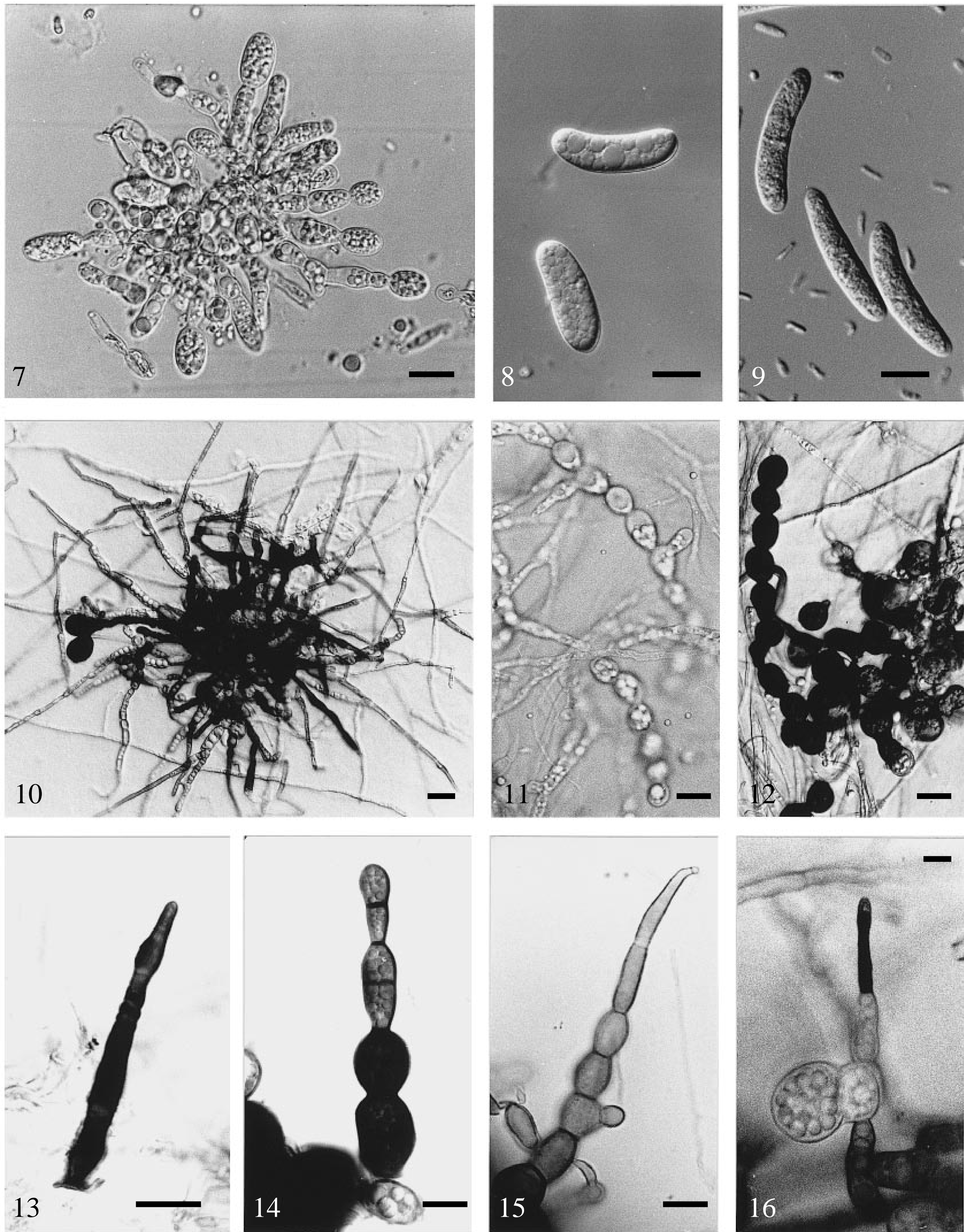
Coloniae in agaro maltoso primum albo-griseae, paulo a centro griseo-atrae aut atrae, ad marginem applanatae, reverso griseo-atrae. *Mycelium* aerium floccosum aut lanatum, hyphis vegetativis hyalinis aut nigro-brunneis, 2–8 µm latis, levibus vel raro verrucosis compositum. Chlamydosporae numerosae, 1–2(–5) cellulas (12–20 × 12–25 µm) habentes, hyalinae aut atrae. Setae 50–170 µm longae, in basi 3·5–11 µm latae, rectae, septatae, nigro-brunneae, in apice obtusae. *Conidiomata* acervularia, 300–600 µm lata, strato conidiophororum a textura globulosa nascentium composita. *Conidiophora* hyalina, singularia vel ad basim ramificata, septata, 9–40 × 4–5 µm. *Cellulae conidiogenae* discretae, hyalinae, leves, cylindricae, rectae vel leniter curvatae 7–15 × 2·5–3·5 µm. *Macroconidia* holoblastica, hyalina aut virentia-brunnea, levia, guttulata, cylindrica, leniter curvata, non septata, in coloniis vetustis raro 1–4 septata, 25–37 × 5·5–9·0 µm, in apice obtusa, in basi abrupte deminuta, cicatrice complanata et protusa, pariete 1–1·5 µm crassa. *Microconidia* holoblastica, hyalina aut brunnescentia, bacilliformia aut claviformia, 4·5–6·9 × 1·2–1·5 µm. Medium solidum non discoloratum. Status teleomorphicus ignotus.

Ex radicibus *Quercus petraeae* (Matt.) Lieb., Patzmannsdorf/Austria, legit Halmschlager, 24 Jun. 1993. Holotypus (colonia exsiccata) in herbario ZT, cultura viva in CBS Baarn (CBS 898.97) et MUCL Louvain-la-Neuve (MUCL 40435).

Colonies on MEA initially whitish-grey, becoming black from the centre with floccose to lanose aerial *mycelium* becoming downy towards the margin. Reverse greyish-black to black, light grey at the margin. Optimum of growth *in vitro* was at 20°. No discoloration of medium was observed. Vegetative hyphae in aerial and immersed mycelium hyaline to brownish-black, 2–5(–8) µm wide, smooth, occasionally verrucose. Chlamydospores abundant, consisting of 1–2(–5) cells (12–20 × 12–25 µm), hyaline to black. Setae-like structures 50–170 µm long and 3·5–11 µm wide at the base, straight, septate, brownish-black, blunt-ended, arising predominantly from chlamydospores but also from vegetative hyphae and conidiomata. *Conidiomata* acervular of 300–600 µm diam., with a row of 'phialides' (*sensu* Sutton, 1980) borne on a



Figs 1–6. Colonial morphologies of *Cryptosporiopsis melanigena* (bar, 1 cm). **Fig. 1.** Greyish colony after 6 wk on MEA at 20°. **Fig. 2.** Whitish colony becoming black from the centre after 6 wk on MEA at 20°. **Fig. 3.** Slow growing colony after 9 wk on MEA at 20°. **Fig. 4.** Whitish-grey colony after 2 mo on MEA at 20° followed by 2 mo at 6° in the dark. **Fig. 5.** Dark pigmented colony surrounded by loosely running hyphae after 4 mo on MEA at 20° followed by 2 mo at 6° in the dark. **Fig. 6.** Dark pigmented colony with dense patches of mycelium after 4 mo on MEA at 20° followed by 2 mo at 6° in the dark.



Figs 7–16. *Cryptosporiopsis melanigena.* **Fig. 7.** Fragment of sporodochium with conidiogenous cells and developing macroconidia (bar, 10 µm). **Fig. 8.** Macroconidia from young colonies (bar, 10 µm). **Fig. 9.** Macroconidia and microconidia from old colonies (bar, 10 µm). **Fig. 10.** Hyphal clump with developing chlamydospores found in old colonies (bar, 20 µm). **Fig. 11.** Hyaline chlamydospores (bar, 20 µm). **Fig. 12.** Dark pigmented chlamydospores (bar, 20 µm). **Figs 13–16.** Different forms of setae are found on MEA (bar, 10 µm).

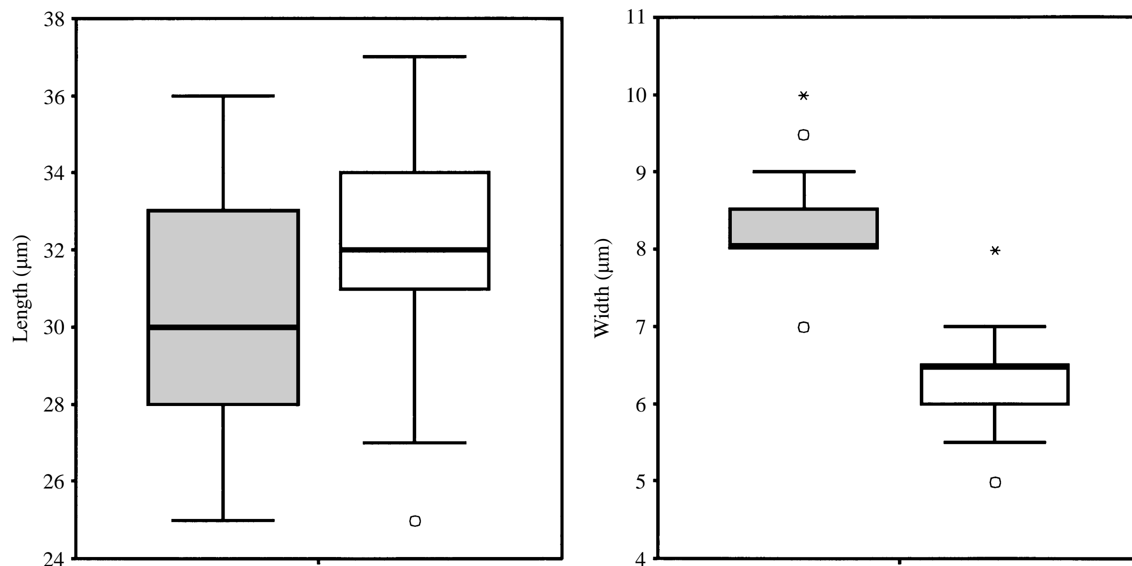


Fig. 17. Boxplots of conidial size of *C. melanigena* depending on age of macroconidia. ▨, young; □, old. (0 = outliers, cases with values between 1·5 and 3 box lengths from upper or lower edge of box; * = extremes, cases with values more than 3 box lengths from the upper or lower edge of box.)

stromatic layer of textura globulosa, which is up to 25 μm thick, consisting of cells with a diam. of 4·0–8·0 μm. Development of conidiomata *in vitro* was sparse and did not occur earlier than 6 wk after the start of incubation at 20°. *Conidiophores* hyaline, becoming golden-brown at the base, tapering towards the apex, unbranched or branched at base, septate, 9–40 × 4–5 μm (Fig. 7). *Conidiogenous* cells discrete, hyaline, smooth, cylindrical, straight or slightly curved, 7–15 × 2·5–3·5 μm. *Macroconidia* holoblastic, hyaline to greenish-brown, smooth, guttulate, cylindrical, slightly curved, aseptate, in old colonies occasionally 1–4 septate and distinctly curved; 25–37 × 5·5–9 μm, with obtuse apex and base tapered to a more or less distinct 1–1·5 μm wide scar; mass of macroconidia forming off-beige to greenish-beige (RAL 1000) droplets. Sporadically macroconidia formation was also found on solitary phialides (12–27 × 3–3·5 μm). *Microconidiophores* hyaline, septate, branched, 15–30 × 2·5–3·1 μm found in cushion-like aggregations with diam. up to 50 μm. Microconidiogenous cells hyaline, cylindrical 6–15 × 2–3 μm. *Microconidia* holoblastic, hyaline to brownish, thin-walled, straight or slightly curved, bacilliform to clavate, 4·5–6·9 × 1·2–1·5 μm (Fig. 9). Teleomorph unknown.

Having had access to more than 90 isolates of *C. melanigena* from oak roots, as well as 20 single spore isolates, it was possible to obtain further information on the morphological variability of this fungus. Isolates differ in intensity of culture pigmentation, growth rate, size of macroconidia, colour of chlamydospores and shape of setae.

Different pigmentation was observed within cultures of the same age (Figs 1–2) but also after subculturing. Some subcultures of isolates with dark pigmentation remain whitish-grey even after 4 months (Fig. 4). On the other hand cultures grown from single spore isolates always maintained a greyish-black to black colour (Figs 5–6). Fast growing cultures reached 68 mm diam. at 20° after 6 wk (Fig. 2), whereas slow growing isolates only reached 47 mm after 9 wk (Fig. 3). Outlines appeared circular (Fig. 2) or irregular (Fig. 3). In old colonies the compact mycelium either was surrounded by loosely running hyphae (Fig. 5) or by dense patches of mycelium consisting of dark pigmented and swollen hyphal cells (Fig. 6).

Macroconidia were found to be different in shape, size, colour and structure of cytoplasm depending on the age of cultures. Young conidia predominantly were hyaline, ellipsoid, straight or slightly curved and 28–33 × 8–8·6 μm (Figs 8, 17), whereas in old cultures macroconidia were more cylindrical and distinctly curved, greenish-brown and mostly 31–34 × 6–6·5 μm (Figs 9, 17). The old conidia were either filled with granular cytoplasm like the young ones or were eguttulate.

Chlamydospores were subglobose with a diameter of up to 15 μm to elliptical and up to 20(–40) × 12(25) μm, hyaline (Fig. 11) to dark-pigmented (Fig. 12), filled with granular cytoplasm, one- to five-celled with transverse or occasionally longitudinal septa, developing apically or intercalary from mycelium, single, in chains or forming clusters (Figs 10–12). Setae-like hyphae either were aculeate to acuminate or torulose (Figs 13–16).

Growth rates of five *C. melanigena* isolates in comparison with the same number of *C. radicicola* isolates were examined in a range 5–30°. Although growth of both species was observed at a wide range of temperatures, *ca* 20° was optimum for *C. melanigena*, whereas that of *C. radicicola* was *ca* 25°. Between 25° and 30° isolates of *C. radicicola* grew two to three times faster than the *C. melanigena* isolates, with no overlap in the growth rates of individual *C. radicicola* or *C. melanigena* isolates (Fig. 18). This suggests that a growth test at 25° might be used as a supplementary aid for separation of the two species.

RAPD- and hplc-analysis

Four *C. melanigena* isolates, five *C. radicicola* isolates and four reference strains were subjected to RAPD analysis. Since

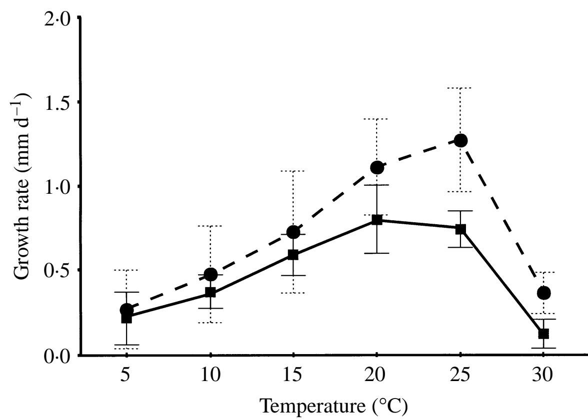


Fig. 18. Mean radial growth curves of five *C. melanigena* (——) isolates in comparison with five isolates of *C. radicicola*. (– –). Vertical bars indicate 95% confidence interval for mean.

RAPD patterns suffer from reproducibility between different experiments, it is essential that all strains to be compared are processed in one batch.

In this study one decamer was applied as primer (OPY-18: 5′-dGTGGAGTCAG-3′). With this primer, amplification products ranging in size from 200 to over 1500 bases were generated. From these products, reproducible RAPD markers were generated (Fig. 19). All isolates of *C. melanigena* had the same RAPD pattern, which was clearly distinct from the RAPD pattern obtained from *C. radicicola*. RAPD patterns of both species were different from those of the reference strains. The patterns of RAPD fragments from isolates of *C. melanigena*, *C. radicicola* and the reference strains were scored by hand and converted to a matrix for presence (1) or absence (0) of bands. This matrix of 19 characters was subjected to computer analysis with the programs DNADIST and FITCH for

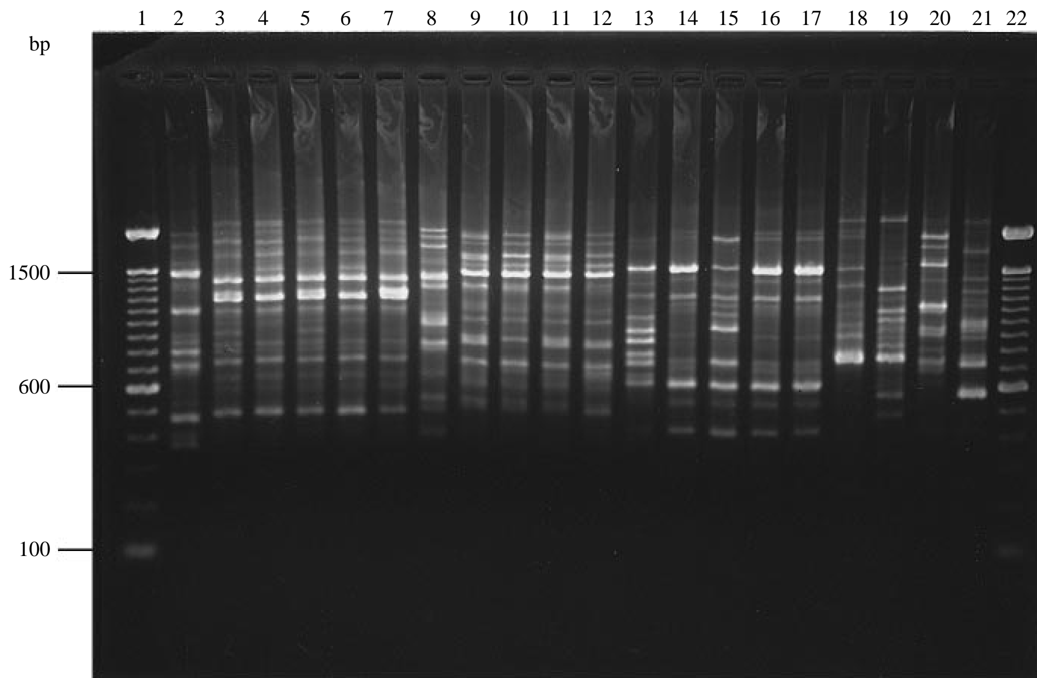


Fig. 19. Pattern of fragments from RAPD analysis of *Cryptosporiopsis* spp. primed by the OPY-18: 5′-dGTGGAGTCAG-3′. The order of strains reflects the one for RAPD+ strains in Table 1: *C. melanigena* (lanes 9–12), *C. radicicola* (lanes 3–7), *C. quercina* (lanes 18, 19), *C. abietina* (lane 21), *C. fasciculata* (lane 20) and Lambda DNA digested by *Pst* I (lane 22). (In order to demonstrate comparability, patterns of all strains being processed in the batch are given, although some lanes are not relevant for this paper.)

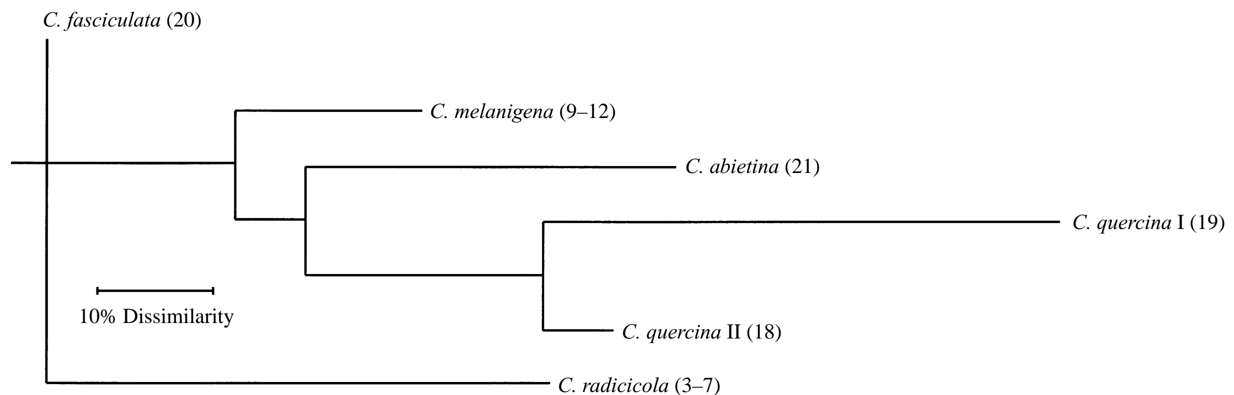


Fig. 20. Cladogram of 13 *Cryptosporiopsis* isolates based on data from RAPD analysis. The information derived from primer was joined in a matrix of 19 characters. A distance matrix according to Jukes & Cantor (1969) was created (DNADIST, PHYLIP package) and fed to the program FITCH. The bar is sized to 10% dissimilarity.

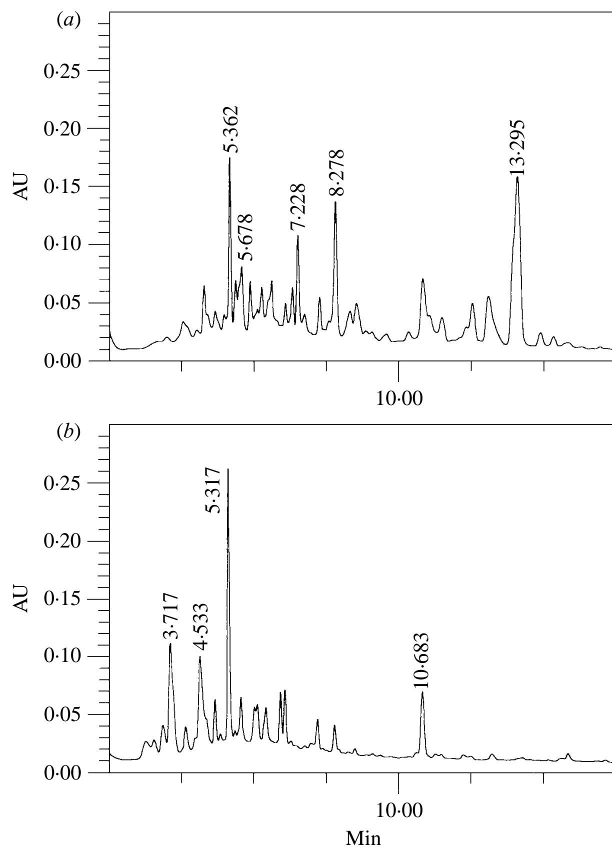


Fig. 21. Hplc profile of secondary metabolites of (*a*) *C. melanigena* in comparison with (*b*) *C. radicicola*.

estimation of branch lengths [set to Jukes & Cantor (1969) distance methods within DNADIST, and global rearrangements within FITCH]. The resulting cladogram is shown in Fig. 20. All isolates of *C. melanigena* (9–12) and *C. radicicola* (3–7) were clustered within one branch each and were separated by branch length, which is also found between well-differentiated species such as *Cryptosporioposis abietina* Petr., *C. quercina* Petr. and *C. fasciculata* (Tode ex Tul.) Petr. The two reference strains of *C. quercina* grouped together in one cluster.

The hplc profile of secondary metabolites of *C. melanigena* in comparison with morphologically similar *C. radicicola* is shown in Fig. 21. Although both profiles show similarities there are some specific substances only found in *C. melanigena* but not in *C. radicicola*.

DISCUSSION

Sutton (1980) pointed out that there are few morphological differences between *Cryptosporiopsis* species, and characters used to distinguish taxa are unreliable. For delimitation of *Cryptosporiopsis* species derived from above ground portions of plants, apart from differences in conidiomatal structure, conidial morphology and size, host specificity is an essential criterion. This character was not suitable for species delimitation in soil-borne *Cryptosporiopsis* spp. because the newly discovered fungus, as well as being morphologically similar to *C. radicicola*, is also found on *Q. robur*.

Morphological similarities between *C. radicicola* and *C. melanigena* concern characteristics of macroconidia and the presence of chlamydospores and setae-like vegetative hyphae. However, some other culture characteristics such as the structure and grey to black colour of colony as well as the late and sparse formation of sporodochia and solitary phialides *in vitro* easily allow *C. melanigena* to be distinguished from *C. radicicola* morphologically. From all other species *C. melanigena* is easily distinguished by its more distinctly curved macroconidia. Conidia of similar shape are only found in *C. perennans* (Zeller & Childs) Wollenw. but are 7·2–20 × 3–6·4 µm (Arx, 1958; Dugan, Grove & Rogers, 1993).

Furthermore, physiological, genetic and biochemical features have been shown to be useful in differentiating *C. melanigena* from *C. radicicola*. The two species could be clearly differentiated in culture on the basis of a simple growth test at 25°. These temperature-growth differences may indicate that *C. melanigena* is a species requiring cooler conditions than *C. radicicola*. Such growth tests had been found to be useful if species were not easily distinguished from each other by microscopic morphology [e.g. *Ophiostoma piceae* (Münch) Syd. & P. Syd. and *Ophiostoma quercus* (Georgév.) Nannf.] (Halmschlager *et al.*, 1994; Wulf & Kowalski, 1994).

Based on RAPD analysis, genetic differences between the two *Cryptosporiopsis* species from roots of *Quercus* sp. were also obtained. All isolates of *C. melanigena* and of *C. radicicola* had the same RAPD patterns, each forming one branch of the cladogram. The lack of variation between the isolates of *C. melanigena* and *C. radicicola* could reflect the fact that each species was derived from a single site. Dissimilarity between the two groups of *Cryptosporiopsis* isolates from oak roots was computed at 75% and groups were separated by branch lengths which are also found between well-differentiated species such as *C. quercina* and *C. abietina*. The results obtained from genomic analysis by the PCR-based RAPD technique therefore strongly support the morphological and physiological data.

Comparing the hplc profiles of *C. melanigena* and *C. radicicola* it becomes evident that specific compounds are synthesized by *C. melanigena* but not by *C. radicicola*. Due to the fact that fungal pigments are often metabolites or part of secondary metabolite pathways (Fox, 1993) it is tempting to speculate that the differences observed may reflect the different pigmentation of the two species in culture. The production of specific pigments like melanins has been used as a taxonomic character (Hermanides-Nijhof, 1977; de Hoog & Yurlova, 1994; Schulz *et al.*, 1995). On the other hand, synthesis of melanin often is not a constant character (Horvath, Brent & Cropper, 1976; Cernáková *et al.*, 1980). This was also true for hyaline isolates of *C. melanigena*, which became black-pigmented after some period of incubation in the dark. Strains of *C. radicicola*, however, always remain beige to brown. Further investigations should clarify if secondary metabolites of *C. melanigena* also show strongly fungicidal, herbicidal and to a lesser extent algicidal and antibacterial activity as found by Schulz *et al.* (1995) for other endophytic *Pezicula* strains.

Distinguishing *C. melanigena* is not only important taxonomically but also from a phytopathological point of

view: *Cryptosporiopsis* yields several important plant pathogens (Butin, 1983; Kowalski, 1983; Taylor, 1983; Kehr, 1991) as well as endophytes (Kowalski & Kehr, 1996) showing antibiotic activity (Stillwell, 1966; Fisher, Anson & Petrini, 1984; Schulz *et al*., 1995). Further investigations in progress will deal with the ecology of the fungus and its role in the 'oak decline' syndrome of *Quercus robur* and *Q. petraea*.

We thank the Austrian Ministry of Science for financial support of the international cooperation with the senior author. Dr H. Haag (Bender/Boehringer Ingelheim Vienna) is acknowledged for providing the hplc patterns. Special thanks are due to S. Krumböck for her excellent technical assistance.

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(*Accepted 27 May 1997*)