Mycorrhiza (2009) 19:481–492
DOI 10.1007/s00572-009-0239-0

ORIGINAL PAPER

The ectomycorrhizal morphotype *Pinirhiza sclerotia* is formed by *Acephala macrosclerotiorum* sp. nov., a close relative of *Phialocephala fortinii*

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Received: 20 November 2008 / Accepted: 17 March 2009 / Published online: 5 May 2009

Abstract Relatively few ectomycorrhizal fungal species are known to form sclerotia. Usually, sclerotia are initiated at the extraradical mycelium. In this study, we present anatomical and ultrastructural evidence for the formation of sclerotia directly in the hyphal mantle of the mycorrhizal morphotype *Pinirhiza sclerotia*. A dark-pigmented fungal strain was isolated from *Pinirhiza sclerotia* and identified by molecular tools as *Acephala macrosclerotiorum* sp. nov., a close relative of *Phialocephala fortinii* s.l. As dark septate fungi are known to be mostly endophytic, resyntheses with *Pinus sylvestris* and *A. macrosclerotiorum* as well as *Populus tremula*×*Populus tremuloides* and *A. macrosclerotiorum* or *P. fortinii* s.l. were performed under axenic conditions. No mycorrhizas were found when hybrid aspen was inoculated with *A. macrosclerotiorum* or *P. fortinii*. However, *A. macrosclerotiorum* formed true ectomycorrhizas in vitro with *P. sylvestris*. Anatomical and ultrastructural features of this ectomycorrhiza are presented. The natural and synthesized ectomycorrhizal morphotypes were identical and characterized by a thin hyphal mantle that bore sclerotia in a later ontogenetic stage. The Hartig net was well-developed and grew up to the endodermis. To our knowledge, this is the first evidence at the anatomical and ultrastructural level that a close relative of *P. fortinii* s.l. forms true ectomycorrhizas with a coniferous host.

Keywords *Acephala macrosclerotiorum* sp. nov. · Anatomy · Axenic culture · Ectomycorrhiza · Sclerotia

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Introduction

Relatively few ectomycorrhizal fungal species form sclerotia. Sclerotia survive for long periods in soil and resist environmental conditions that would usually damage mycelium (Bullock et al. 1980; Shaw and Sidle 1983). The development and structure of sclerotia have been studied in detail for *Pisolithus tinctorius* (Grenville et al. 1985a), *Cenococcum geophilum* (Massicotte et al. 1992; Peterson et al. 2004), *Hebeloma sacchariolens* (Fox 1986a), and *Paxillus involutus* (Grenville et al. 1985b; Fox 1986b; Moore et al. 1991). Other genera of mycorrhizal fungi such as *Gyrodon*, *Boletus*, *Austropaxillus*, and *Cortinarius* are also known to form these structures (Peterson et al. 2004).

Sclerotia are normally initiated at the extraradical mycelium and are sometimes associated with rhizomorphs (Peterson et al. 2004). At maturity, each sclerotium usually develops a melanized outer covering, the rind that surrounds a cortex of compact hyphae, and a medulla of loosely or isodiametric organized hyphae (Grenville et al. 1985a, b). The cortical region of the sclerotia stores proteins, lipids, polysaccharides, and polyphosphates (Moore et al. 1991), making these structures ideal propagules to provide infective hyphae for reinfection of roots following environmental stresses (Grenville et al. 1985c).

The mycorrhizal morphotype *Pinirhiza sclerotia* was first collected and described by Wöllecke (2001). This morphotype is easy to recognize in the field due to the dark-pigmented sclerotia formed directly on the surface of the ectomycorrhizal mantle. Therefore, it is amazing that this morphotype was previously overlooked. Fox (1986a) studied the ultrastructure of sclerotium-like bodies on the mantle of the ectomycorrhizal fungus *H. sacchariolens* on birch.

Over 600 species of vascular plants harbor dark septate (DS) fungi (Jumpponen and Trappe 1998). DS fungi are a miscellaneous group of ascomycetous anamorphic fungi that colonize living root tissues intracellularly and intercellularly (Jumpponen 2001, Sieber 2002). However, decayed coarse roots, decomposing stumps, and living stems of different tree species are also colonized (Menkis et al. 2004; Menkis et al. 2006). Hyphae change from being thick-walled and melanized to thin-walled and hyaline after root contact and often produce pigmented exudates on their surface (Currah and Tsuneda 1993). Yu et al. (2001) suggested that the hyaline hyphae produced by melanized DS fungi were often unnoticed in microscope studies and may have resulted in underestimation of DS fungi abundance. Mandyam and Jumpponen (2005) observed that melanized and hyaline structures cooccurred and often occupied the same tissues when DS fungal colonization was high. In addition, DS fungi develop sclerotium-like structures, the so-called microsclerotia, which act as dispersal units when host cells are sloughed (Haselwandter and Read 1980; Addy et al. 2005).

DS fungi are known from harsh environments such as arid, alpine, arctic, antarctic, and subantarctic zones (Christie and Nicolson 1983; Blaschke 1991; Hambleton and Currah 1997; Laursen et al. 1997; Schadt et al. 2001; Frenot et al. 2005). However, some data indicates that DS fungi are also common in roots of *Picea abies* from the temperate zone (Ahlich et al. 1998; Grünig et al. 2001, 2002a, b). The most frequent root endophyte is *Phialocephala fortinii* (Ahlich and Sieber 1996; Ahlich et al. 1998), the anamorph of an inoperculate discomycete that belongs to the Helotiales (Gernandt et al. 2001; Jacobs et al. 2003; Piercey et al. 2004; Zijlstra et al. 2005). *P. fortinii* has a global distribution, colonizes a wide variety of plants (Currah and van Dyk 1986; Jumpponen 1999), and lacks host specificity (Stoyke et al. 1992; Jumpponen and Trappe 1998; Addy et al. 2000; Yu et al. 2001; and citations therein).

Mandyam and Jumpponen (2005) suggested that DS fungi are as abundant as mycorrhizal fungi; indeed, they often coexist (Wagg et al. 2008). In ectomycorrhizal root tips, any DS colonization may be difficult to find since many intercellular and intracellular structures may be hidden in the hyphal mantle or the Hartig net (Mandyam and Jumpponen 2005).

DS fungi may function as pathogens, saprophytes, or in mutualistic associations (Jumpponen 2001; Grünig et al. 2008b). A mycorrhiza-like relationship has been postulated for *P. fortinii* (Haselwandter and Read 1980; Ahlich and Sieber 1996; Jumpponen et al. 1998). Fernando and Currah (1996) were the first to present anatomical data showing that *P. fortinii* forms ectomycorrhiza with *Salix glauca* in an axenic resynthesis experiment. Although several authors suggested mycorrhiza formation for *P. fortinii* and Scots pine (*Pinus sylvestris*) from molecular field data (Jonsson et al. 1999a, b; Heinonsalo and Sen 2007), axenic resynthesis experiments for final proof are lacking.

P. fortinii-like dematiaceous fungi are thought to be a species complex (Harney et al. 1997; Grünig et al. 2008b). Using intersimple sequence repeat–polymerase chain reaction (ISSR-PCR), Grünig et al. (2001) found high intraspecific genetic variability in *P. fortinii* and explained this phenomenon by the presence of a species complex. Six different ISSR types were identified as *P. fortinii* based on the morphology of conidiophores and/or ITS1 and ITS2 sequence comparisons (Grünig et al. 2002a). In a consecutive study, Grünig et al. (2002b) compared the two methods, ISSR-PCR and sequence analysis of ITS1 and ITS2, and found the genus *Phialocephala* to be polyphyletic. *P. fortinii* s. l. is composed of at least eight cryptic species, all detected as endophytes in tree roots (Grünig et al. 2004; Piercey et al. 2004; Queloz et al. 2005; Grünig et al. 2009). Another DS

root endophyte, *Acephala applanata*, is closely related to *P. fortinii* s.l. but differs from it by a lower growth rate, absence of aerial mycelium, and has never been observed to sporulate (Grünig and Sieber 2005). *A. applanata* showed a host preference for *P. abies*, although it was also isolated from *P. sylvestris* and *Pinus mugo* (Grünig et al. 2006).

The aims of the present investigation were to collect and describe the ectomycorrhizal morphotype *Pinirhiza sclerotia* from the field and isolate and identify the fungal symbiont. Axenic resyntheses with *P. sylvestris* or *Populus tremula*× *Populus tremuloides* were performed with *A. macrosclerotiorum* sp. nov. and a *P. fortinii* s.l. strain of the *P. fortinii* s.l.–*A. applanata* species complex (PAC) to test the ectomycorrhizal status and host specificity. Different ontogenetical states of *P. sylvestris–A. macrosclerotiorum* sp. nov. from axenic culture were demonstrated.

Materials and methods

Isolation and cultivation of the fungal strains

Soil cores containing roots were taken with a soil corer (3 cm in diameter) in spring 2004 from an 81-year-old *P. sylvestris* plot, named "Hubertusstock" (N 52°56′38″, E 14° 13′22″), located in the Biosphere Reserve Schorfheide-Chorin near Eberswalde, Brandenburg, Germany. Soil debris was carefully removed and ectomycorrhizas of *Pinirhiza sclerotia* (Wöllecke 2001) were separated. Twenty healthy-looking ectomycorrhizas were surface sterilized in vials (50 mL) containing 20 mL of 30% hydrogen peroxide (H_2O_2) for 1 min under constant agitation. Mycorrhizal tips were then transferred to new vials and rinsed with sterile distilled water. Thereafter, tips were placed on MMN 1/10 strength agar (medium a; Kottke et al. 1987) that contained (per liter): 0.025 g NaCl, 0.05 g $CaCl_2$, 0.05 g KH_2PO_4, 0.025 g $(NH_4)_2HPO_4$, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.005 mg $FeCl_3{\cdot}6H_2O$, 100 µg thiamine hydrochloride, 10 mL stock solution of trace elements, and 20 g agar–agar. Stock solution of trace elements contained (per liter): 3.728 g KCl, 1.546 g H_3BO_3, 0.845 g $MnSO_4{\cdot}H_2O$, 0.125 g $CuSO_4{\cdot}5H_2O$, 0.018 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.575 g $ZnSO_4{\cdot}7H_2O$. Agar plates were controlled daily to cut off fast-growing contaminations by soil fungi. Outgrowing hyphae of a single DS fungus were easily recognized by their dark pigmentation. This strain was isolated and named strain EW 76. EW 76 is described as the new species *Acephala macrosclerotiorum* B. Münzenberger & B. Bubner in this paper and was used to perform the resynthesis experiments. In addition, a culture of *P. fortinii* s.l. (strain 75), which was isolated from a dark brown ectomycorrhizal morphotype on the hybrid aspen clone Esch5 (*P. tremula* L.×*P. tremuloides* Michx.) (Kaldorf et al. 2004), was kindly provided by F. Buscot, Helmholtz Centre for Environmental Research (UFZ), Halle. Both fungal strains were cultivated on MMNC agar (medium b; Kottke et al. 1987) that contained (per liter): 0.025 g NaCl, 0.05 g $CaCl_2$, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.005 mg $FeCl_3{\cdot}6H_2O$, 100 µg thiamine hydrochloride, 10 g glucose monohydrate, 10 g malt extract, 1 g casein hydrolysate, 10 mL stock solution of trace elements, and 20 g agar–agar. For comparison with existing culture collections at the Institute of Integrative Biology, ETH Zürich, both strains were also cultivated on malt agar (20 g L^{-1} malt extract (DIFCO) and 20 g L^{-1} agar–agar).

Plant culture

Seeds of *P. sylvestris* (Federal Forestry Office Annaburg, Annaburg, Germany) were surface sterilized for 20 min in 30% H_2O_2 and rinsed with sterile distilled water. Seeds were germinated on modified Benecke agar (Münzenberger et al. 1997) that contained (per liter): 2 g PIPES ($C_8H_{18}N_2O_6S_2$), 0.1 g KH_2PO_4, 0.2 g NH_4NO_3, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.005 mg $FeCl_3{\cdot}6H_2O$, 10 mL stock solution of trace elements (see above), and 10 g agar–agar. The pH was adjusted to 5 with H_2SO_4. The light and temperature settings in the growth chamber were: 20°C day for 16 h and 18°C night for 8 h (lamps OSRAM daylight white and Flora violet 1:1, 170 µE m^{-2} s^{-1}). Plants of the clone Esch5 (*P. tremula*× *P. tremuloides*) were grown in vitro on "woody plant medium" (Duchefa, Haarlem, The Netherlands) containing 20 mg L^{-1} sucrose and 8 g L^{-1} agar–agar but without hormones in Magenta vessels (Sigma-Aldrich, Munich, Germany). Temperature was kept at 24°C with 24 h continuous light (OSRAM daylight white). Plants approximately 10–12 cm high were subcultivated by cutting the whole shoot tip of 1–2 cm in length and transferring it to a fresh Magenta vessel containing the same medium. Rooting started 7–10 days after subcultivation.

Mycorrhizal synthesis

Seedlings of Scots pine with well-developed cotyledons and rooted hybrid aspen cuttings were transferred into 500 and 1,000 mL sterile Erlenmeyer flasks containing 250 and 350 mL of a perlite–*Sphagnum* mixture (90:10, *w*/*w*), respectively. There were ten flasks planted with Scots pine and 20 with hybrid aspen. The perlite–*Sphagnum* mixture in flasks with pine was moistened with 140 mL of MMN nutrient solution (medium a) containing 0.1% glucose monohydrate, and flasks with hybrid aspen were moistened with 200 mL. Each flask contained one seedling or cutting. Each Scots pine seedling was inoculated with five and each hybrid aspen with 15 small discs (5 mm in diameter) of

agar colonized by vigorously growing mycelium of *A. macrosclerotiorum* (strain EW76) and *P. fortinii* s.l. (strain 75), respectively. Thus, the following combinations were carried out: *P. sylvestris–A. macrosclerotiorum*; *P. tremula*×*P. tremuloides–A. macrosclerotiorum*; and *P. tremula*×*P. tremuloides–P. fortinii*. Flasks were sealed with a cotton plug and incubated in the same growth chamber. A total of 30 mycorrhizal root tips were harvested after 8, 16, 21, and 28 weeks from the Erlenmeyer flasks containing Scots pine seedlings. Mycorrhizas did not form on any of the aspen cuttings inoculated with *A. macrosclerotiorum* or *P. fortinii* s.l. (strain 75) even after 28 weeks.

Light and transmission electron microscopy

The 30 *P. sylvestris–A. macrosclerotiorum* mycorrhizas were fixed overnight with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.2) at room temperature. Following six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer for 1 h in the dark at room temperature. Thereafter, mycorrhizas were washed in distilled water and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five washes in distilled water, samples were dehydrated by immersion for 10 min each in 25%, 50%, 70%, and 95% acetone and three times for 1 h in 100% acetone. Samples were embedded in Spurr's plastic (Spurr 1969) and 26 mycorrhizas were sectioned with a diamond knife on an Ultracut Reichert Ultramicrotome (W. Reichert-LABTEC, Wolfratshausen, Germany). Sections (0.5 µm thick) were stained with crystal violet for light microscopy. For ultrastructure, serial sections about 80 nm thick were mounted on formvar-coated, single-slot copper grids, stained with lead citrate at room temperature for 5 min, and washed with distilled water. The ultrastructure of *P. sylvestris–A. macrosclerotiorum* was studied with a ZEISS 109 transmission electron microscope (Zeiss, Oberkochen, Germany) at 80 kV.

Amplification and sequencing

DNA was extracted from fresh mycelium of an agar plate using DNA extraction kit (Quiagen, Hilden, Germany). PCR was performed with the Accuprime® *Taq* Polymerase System (Invitrogen, Karlsruhe, Germany) using the supplied buffer II with following final concentrations: 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP and 500 nM of each of the primers ITS1F (Gardes and Bruns 1993) and NL4 (O'Donnell 1993). The total volume of the reaction was 25 µL, comprising 5 µL of template DNA. The thermocycler, a GeneAmp® PCR system 9700 (ABI, Darmstadt, Germany), was programmed as follows: 3 min denaturation at 94°C, ten cycles with decreasing annealing temperature (94°C for 30 s, 60–50°C for 45 s, and 68°C for 60 s), 35 cycles with constant annealing temperature (94°C for 30 s, 50°C for 45 s, and 68°C for 60 s), and 7 min strand completion at 68°C. After purification of the PCR product with QIAquick® PCR Purification Kit (Quiagen), 20 µL was sent to GATC Biotech AG (Konstanz, Germany) for sequencing. The PCR products were sequenced with the primers NL1 (O'Donnell 1993), ITS4 (White et al. 1990), ITS1F, and NL4. Sequences were assembled with the Lasergene® Software Package (DNASTAR, Madison, USA).

Phylogenetic analysis of sequence data

Sequences closely matching the ITS sequence of the newly described mycorrhizal fungus were sought using the BLAST algorithm (Zhang et al. 2000; http://www.ncbi.nlm.nih.gov). These sequences and the sequences of the ITS regions of additional species known to be closely related were aligned and subjected to phylogenetic analysis using parsimony as the optimality criterion (PAUP 4.0.0b10; Swofford 2001) (Fig. 7). The first 60 bp of the alignment, i.e., the beginning of ITS1, was excluded from the analysis due to alignment uncertainties. The bootstrap 50% majority-rule consensus tree (100 replicates) was presented as a phylogram using *Hyphodiscus hymeniophilus* (P. Karst.) Baral as an outgroup.

Results

Mycorrhiza synthesis of hybrid aspen with *A. macrosclerotiorum* or *Phialocephala fortinii*

No mycorrhizas were formed when hybrid aspen was inoculated with *A. macrosclerotiorum* or *P. fortinii*. To confirm the lack of Hartig net hyphae, squash mounts were made from fine roots of hybrid aspen.

Morphology of *Pinus sylvestris–Acephala macrosclerotiorum* mycorrhizas

The mycorrhizal systems were rarely ramified. Few mycorrhizas (<10%) had a single dichotomous ramification. A mycorrhiza from the field, formerly named *Pinirhiza sclerotia*, is shown in Fig. 1a. The thin to nearly absent hyphal mantle and the black to dark brown emanating hyphae are characteristic of this morphotype (Wöllecke 2001). The best feature for morphological identification is the black hemispherical sclerotia (100–300 µm in diameter), which are attached directly to the hyphal mantle surface (Fig. 1a). Rhizomorphs were not observed.

Several ontogenetic stages were observed in axenic culture. Eight weeks after inoculation, mycorrhizas were

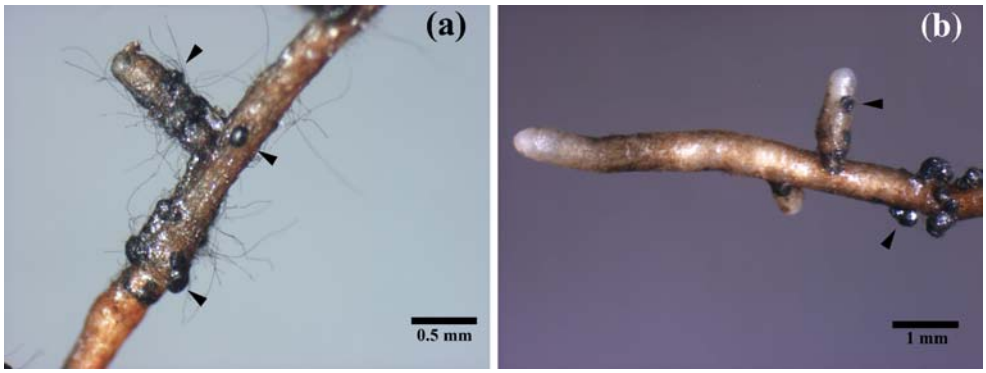

Fig. 1 Morphology of *P. sylvestris–A. macrosclerotiorum* mycorrhizas. **a** Mycorrhiza from the field. **b** Mycorrhiza from axenic culture after 21 weeks. Numerous sclerotia are visible in **a** and **b** (*arrowheads*)

characterized by a thin hyphal mantle and a Hartig net. Sclerotia were not yet formed and root hairs were still present. Ten weeks after inoculation, the first sclerotia were observed on the most proximal parts of the mycorrhizas and at the secondarily formed mycorrhizas. A ramified mycorrhiza of *P. sylvestris–A. macrosclerotiorum* sp. nov. harvested after 21 weeks is shown in Fig. 1b. Numerous sclerotia can be seen.

Light microscopy

Field material of *P. sylvestris–A. macrosclerotiorum* sp. nov. was characterized by a thin plectenchymatous mantle consisting only of a loose network of single hyphae arranged in zero to four hyphal layers (mantle thickness 0–12 µm). A mantle covering the complete root surface was not developed. The hyphae of the mantle and the emanating hyphae were membranously dark brown to blackish with a smooth surface. The fungal cell walls were slightly thicker (up to 0.5 µm) and a black matrix appeared in contact zones between hyphae (Fig. 2a). The surface of the hyphal mantle was formed by curved DS hyphae (Fig. 2a). The septa were not constricted. There were no color reactions of hyphae in KOH (15%), ethanol (70%), formol (40%), iron(II)sulfate, Melzer's reagent, cotton blue, toluidine blue, lactic acid, or potassium iodide. Hyphae did not autofluoresce (365 nm). The Hartig net was a well-developed palmetti-type. Outer cortical cells were surrounded by two hyphal layers and the remaining cortical cell layers were surrounded with one hyphal layer. A young sclerotium is shown in Fig. 2b. Cells of the sclerotium were even-shaped and surrounded by the hyphae of the hyphal mantle. A mature sclerotium on a mycorrhiza is shown in Fig. 2c. Occasionally, sclerotia bore different cell types. Walls of the outer cells were highly melanized, whereas the more central hyphae were thick-walled and partly not melanized. As ectomycorrhizas were only detected with *P. sylvestris–A. macrosclerotiorum* sp. nov. in axenic flask cultures, this morphotype is the only one described in detail in the following sections. Different ontogenetical stages could be harvested after 21 weeks of axenic culture. A longitudinal median section through a mycorrhiza is shown in Fig. 3. The hyphal mantle consisted of only three to four cell layers. The hyphae of the hyphal mantle formed a loose prosenchyma changing to a pseudoparenchyma near the root surface. Sometimes, emanating hyphae were visible. No sclerotia were developed during this ontogenetical stage.

Sclerotium initials were observed as a part of the hyphal mantle in another ontogenetical stage (Fig. 4). Cells of sclerotia were clearly differentiated by their melanized cell

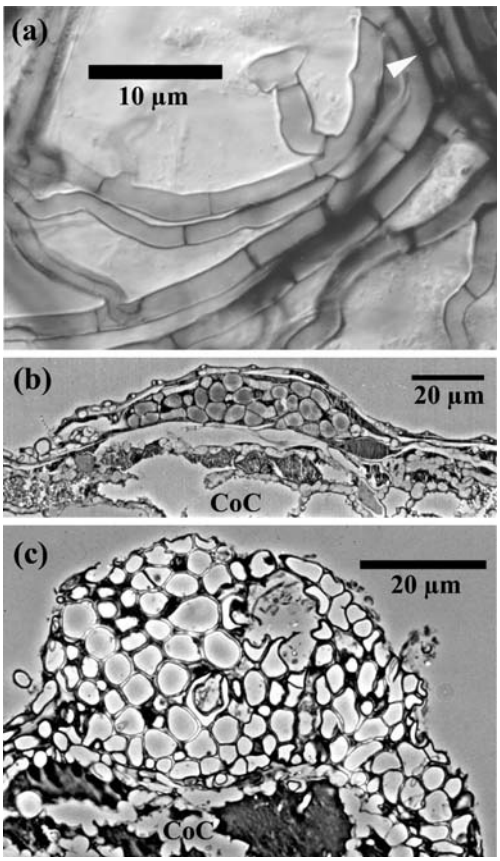

Fig. 2 Anatomy of the *P. sylvestris–A. macrosclerotiorum* mycorrhiza from the field. **a** Curved DS hyphae on the surface of the hyphal mantle. Dark matrix between hyphae is visible (*arrowhead*). **b** A young sclerotium of even-shaped cells surrounded by a hyphal mantle; *CoC* cortical cell. **c** Sclerotium bearing different cell types

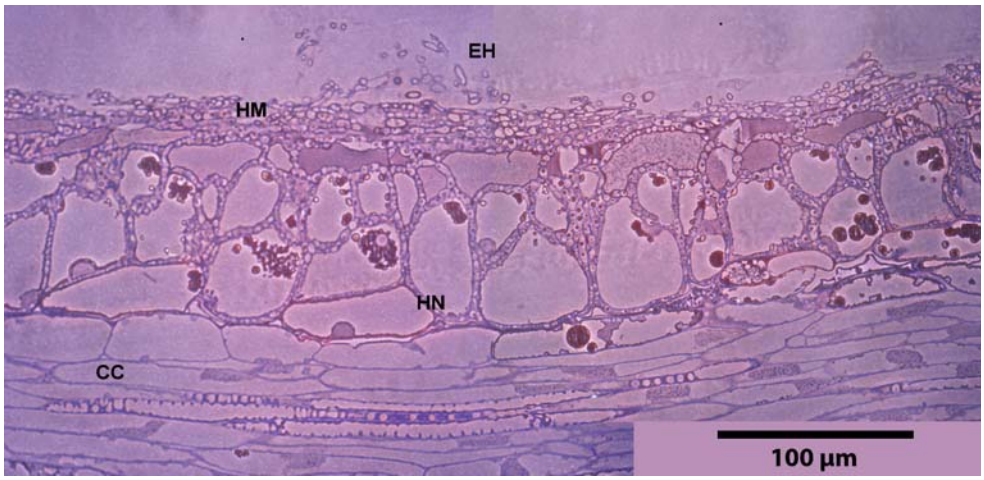

Fig. 3 Anatomy of *P. sylvestris–A. macrosclerotiorum* mycorrhiza in axenic culture. This ontogenetical stage showed no sclerotia. *EH* emanating hyphae, *HM* hyphal mantle, *HN* Hartig net, *CC* central cylinder

walls. The sclerotia shape varied from roundish to oval (Fig. 5). In this stage, the sclerotia were easily detached from the ectomycorrhizal mantle because they break through the hyphal mantle in mature mycorrhizas.

Ultrastructure

Occasionally, sclerotia were characterized by different cell types. The framed sclerotium of Fig. 5 was further studied for ultrastructure (Fig. 6). The sclerotium consisted of two different cell types: the cell walls of the outer cells were thick-walled and highly melanized, whereas those of the inner cells were less thick-walled and not melanized (Fig. 6a). A mantle of thin-walled hyphae surrounded the sclerotium, and the Hartig net in the root cortex was well-developed. The nucleus inside the cortical cell showed that the host cells were viable. The inner cells of the sclerotium bore many lipid droplets (Fig. 6b). In this stage, the fungal cytoplasm was well-organized; in contrast, the cytoplasm of the outer cells was disorganized (Fig. 6c). The septal pores were simple and associated with a Woronin body, confirming that the newly described fungus belongs to the Ascomycetes (Fig. 6d). The pore was plugged with electron-dense material, and cytoplasm was well-organized with visible mitochondria and endoplasmic reticulum. No glycogen was found.

Molecular identification and phylogenetic analysis

We propose to describe the mycorrhiza-forming strain EW 76 as a new species, namely, as *A. macrosclerotiorum* sp. nov. The epithet *macrosclerotiorum* was chosen to distinguish the new fungus from "microsclerotia" typically formed by many other DS fungi. *A. macrosclerotiorum* is closely related to

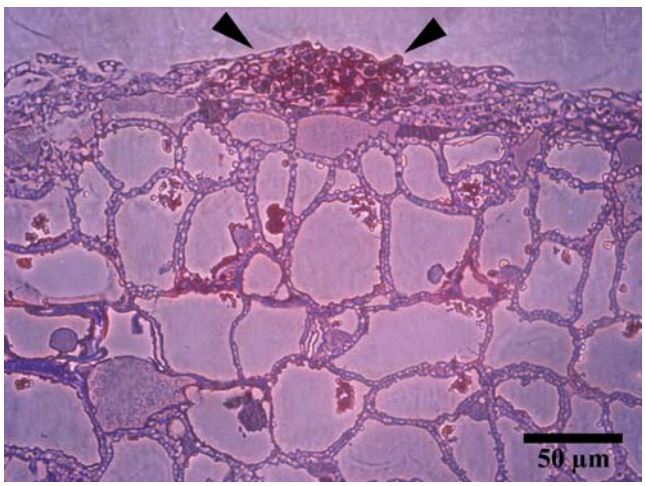

Fig. 4 Sclerotium initial (*arrowheads*) after 21 weeks of axenic culture. This tangential section is close to the root apex. Note the melanized cell walls of the sclerotium

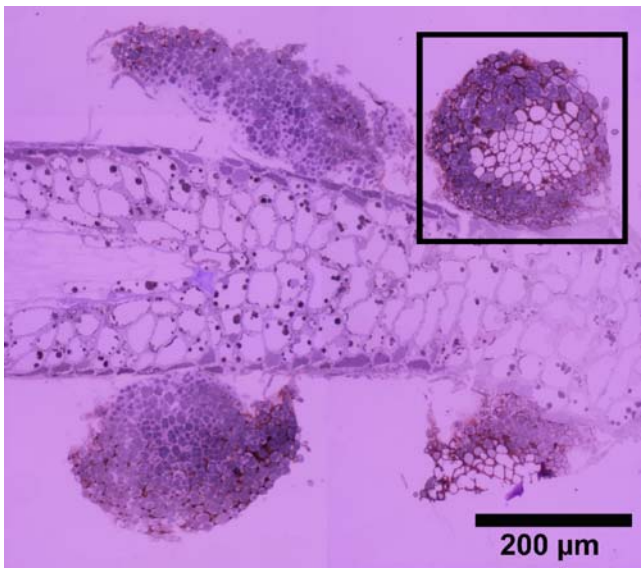

Fig. 5 Semithin section (0.5 µm) through a mature mycorrhiza with loosely attached sclerotia after 21 weeks of axenic culture. For orientation, root apex follows on the *left margin*. *Framed picture* shows the area that was cut ultrathin (approximately 80 nm; Fig. 6)

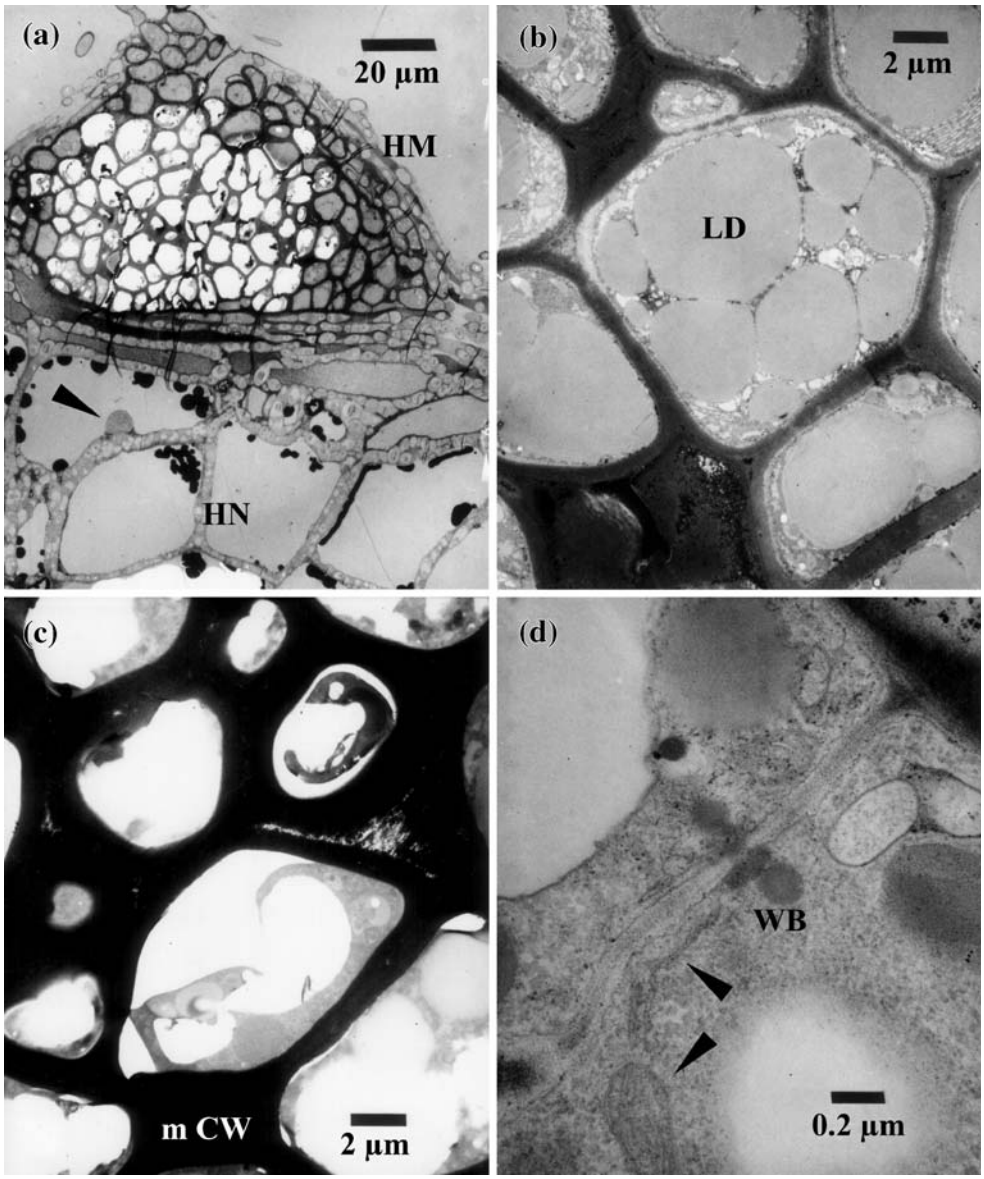

Fig. 6 Ultrastructure of framed sclerotium shown in Fig. 5. **a** Outer cells of the sclerotium are highly melanized, inner ones have thick cell walls but without melanin. *Dark lines* are artefacts due to hardness of specimen (*HM* hyphal mantle, *HN* Hartig net, *arrowhead* nucleus inside a cortical cell). **b** Many lipid droplets (*LD*) can be seen in the inner cells. **c** Outer cells are highly melanized and cytoplasm is disorganized (*mCW* melanized cell wall). **d** Simple septal pore shows fungus is an Ascomycete (*WB* Woronin body, *arrowheads* endoplasmic reticulum and mitochondrion)

species of the PAC and other DS fungi (Fig. 7) (Grünig et al. 2008a, 2009). The rDNA sequence of the ITS regions (including the 5.8S RNA gene) was identical to the sequence of a fungus isolated previously from a root tip of *P. sylvestris* in Lithuania and deposited as *Acephala* sp. 6 at the GenBank (Menkis et al. 2004). Another *Acephala* sp. 6 isolate from fine roots of *P. abies* had a nearly identical sequence. *Acephala* sp. 6 and *A. macrosclerotiorum* were, thus, considered conspecific. All other relationships fall into a basal polytomy. The hitherto undescribed *Acephala* sp. 1, endophytic in roots of *Cassiope mertensiana*, is the closest known relative of the new species with a total of 19 nucleotide differences (4.75%; eight transitions, nine transversions, and two insertions in *A. macrosclerotiorum*) in the ITS regions (including the 5.8S RNA gene). The helotialean ascomycete *Phaeomollisia piceae* is the closest validly described relative (Grünig et al. 2009). Its ITS regions differ by 13 transitions, 12 transversions, and six insertions/deletions (6.4%), respectively.

The sequences of the 5.8S RNA gene of *A. macrosclerotiorum*, *P. piceae*, and species of the PAC were identical, whereas that of *Phialocephala compacta*, another closely related species, has a transition from adenine to cytosine at position 90 of the 5.8S RNA gene in the midst of a *Hin*fI restriction site, transforming it into a *Drd*II restriction site.

Taxonomy

A. macrosclerotiorum sp. nov. B. Münzenberger & B. Bubner MycoBank, no. MB 513098

Coloniae in agaro maltoso 40–44 mm diametro post 20 dies temperatura 20°C crescentes; mycelium aereum confer-

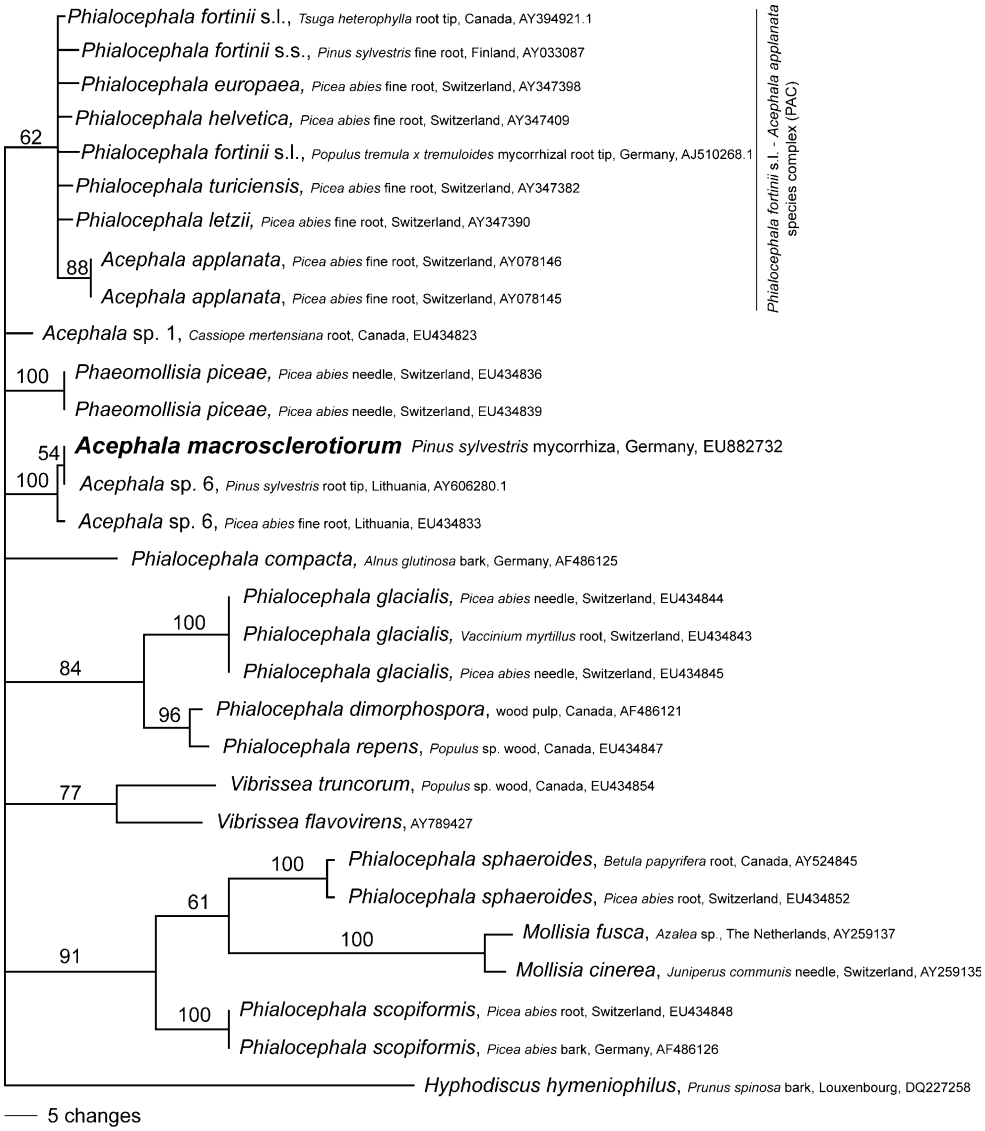

Fig. 7 Phylogenetic position of *A. macrosclerotiorum* sp. nov. among its closest relatives based on ITS sequence data. The bootstrap 50% majority-rule consensus tree (100 replicates) was constructed based on 100 trees generated using parsimony as the optimality criterion. *H. hymeniophilus* served as the outgroup. Bootstrap values ≥50% are indicated *above* branches. Species names are followed by host (substrate) and tissue, country of origin, and GenBank accession number

tum, lanosum, rubrofuscum; pars externa coloniae fusca mycelioque aereo deserta; reverso etiam atrofusco; sporulatio ignota. *Phaeomollisiae piceae* T.N. Sieber & C.R. Grünig similis sed ab hac specie ob transitionem in acido DNA vocato in 31 positione ut in *Phaeomollisiae piceae* sequentia in deposito GenBank vocato praesentem differt. Species pinirrhizae sclerotibus praeditam ectomycorrhizam in *Pino sylvestri* format, tenui tunica myceliari sclerotibusatrobrunneis crassitunicatis hemisphaericis 100–300 µm diametro in radicibus composita.

HOLOTYPUS: cultura sicca ex radicibus vivis *Pini sylvestris*, Germania, in loco dicto "Hubertusstock" 18.5.2004, B. Münzenberger, herbarium ZT (Myc 1239), cultura viva numero CBS 123555 deposita Centraalbureau voor Schimmelcultures, Utrecht, Batavia.

A. macrosclerotiorum sp. nov. B. Münzenberger & B. Bubner

MycoBank, no. MB 513098

Colonies on malt extract agar (20 g L^{-1} malt extract [DIFCO], 20 g L^{-1} agar) measure 40–44 mm in diameter after 20 days at 20°C; mycelium in the agar dark brown; aerial mycelium cottony, red brown; outermost 3–5 mm of the colony brown without aerial mycelium; margin fringed; reverse dark brown. Sporulation not observed. The internal transcribed spacers (ITS) of the rDNA (GenBank accession number EU882732) differ in 31 nucleotide positions from those of the closest known relative, *P. piceae* T.N. Sieber & C.R. Grünig (GenBank accession number EU434836). On *P. sylvestris*, the fungus forms *Pinirhiza sclerotia* ectomycorrhiza which is characterized by a thin fungal mantle covered with melanized, hemispherical sclerotia of 100–300 µm in diameter.

HOLOTYPUS: Dried culture of an isolate of *Pinirhiza sclerotia* ectomycorrhiza on *P. sylvestris*, Hubertusstock,

Germany, 18.5.2004, B. Münzenberger, herbarium ZT (Myc 1239), living culture (CBS 123555; alternative strain no. EW76) deposited at the Centraalbureau voor Schimmelcultures, Utrecht, Batavia.

Etymology. macrosclerotia: the ectomycorrhizal mantle formed by the fungus is covered by large sclerotia.

Specimens examined: EW 76.

Discussion

This study showed that *A. macrosclerotiorum* sp. nov. formed true ectomycorrhizas on *P. sylvestris* in the field and under axenic conditions. The significance of the DS fungal infection pattern can only be determined after isolation of the fungus and subsequent reinoculation of aseptically grown plants (Haselwandter and Read 1980) because DS fungi are often isolated from ectomycorrhizas without knowing whether the DS fungus is the ectomycorrhiza-forming fungus itself or only a resident in an ectomycorrhiza formed by another fungus (Holdenrieder and Sieber 1992). Similarly, DNA extracted from ectomycorrhizas collected in the field can originate either from an ectomycorrhizal fungus or another root endophyte. Thus, only resynthesis experiments under axenic conditions will establish the "mycorrhizal" status of a fungal isolate from mycorrhizas.

P. sylvestris–A. macrosclerotiorum mycorrhizas collected from the field and those formed in aseptic culture showed exactly the same morphology and anatomy, confirming that the culture system used was appropriate (Stoyke and Currah 1993; Jumpponen and Trappe 1998). The hybrid aspen clone did not form ectomycorrhiza either with *A. macrosclerotiorum* or *P. fortinii* using the same culture system. This is inconsistent with the findings of Kaldorf et al. (2004) who described a dark brown ectomycorrhiza on the same hybrid clone in the field, which they attributed to *P. fortinii* based on ITS sequence information from DNA extracted from ectomycorrhizas; however, they did no resynthesis experiments. Thus, *P. fortinii* may have been the ectomycorrhiza-forming fungus but could also have cooccurred inside the hyphal mantle and/or the Hartig net of a dark mycorrhiza formed by another fungus (Kaldorf, personal communication).

Fox (1986a) described sclerotia as parts of the hyphal mantle of a basidiomycetous ectomycorrhizal fungus, *H. sacchariolens*, for the first time. We have shown that sclerotia occur in the mantle of an ascomycetous fungus. These sclerotia are released into the substrate in a later ontogenetical stage.

As this sclerotia-forming morphotype is easy to recognize, the question arises as to why it was overlooked for so long, particularly as it has an abundance of 2.7% in the organic layer and 3.8% in the mineral soil layer in the forest area studied. One possible explanation could be that it is restricted to dry and sandy soils typical of the north eastern lowlands of Germany. However, further investigations are necessary to provide a clearer distribution of the fungus within Germany and abroad.

Fixation of sclerotia is generally difficult (Grenville et al. 1985a); in this case, due to the thickened cell walls especially in the outer rind of the sclerotia. These cell walls were heavily impregnated with melanins indicative of a protective function of the inner cortex filled with plenty of lipids. Lipid bodies in the cortex could serve as energy-rich carbon reserves to sustain fungal cells during extended droughts or for the development of melanized hyphae and sclerotia (Barrow 2003). Lipids were the only storage substance found inside the sclerotia in the present investigation. This is not surprising as it is well known that DS fungi store lipids (Peterson et al. 2008). However, we did not search for polyphosphates that are known to be inside of the vacuolar system of these fungi (Yu et al. 2001; Saito et al. 2006).

DS fungi such as *P. fortinii* are thought to vary from mutualistic to neutral and parasitic, but their ecological functions are little understood (Grünig et al. 2008b; Peterson et al. 2008). To date, only the formation of so-called microsclerotia was known. They are formed intracellularly and intercellularly and are released into the soil as propagules. The sclerotia formed by the newly described DS fungus were much larger and were part of the hyphal mantle. They break through the mantle at maturity and are released into the soil. Therefore, we called this type of sclerotia "macrosclerotia." The tissues of the sclerotia were formed by melanized or nonmelanized hyphae. The common occupation of the same tissue by melanized and hyaline structures is described in detail by Mandyam and Jumpponen (2005).

The fungus was placed in *Acephala* in accordance with Grünig and Sieber (2005) who erected this genus to accommodate nonsporulating, dematiaceous hyphomycetes with strong relationships to *Phialocephala* spp. The new species is closely related to *P. piceae*, *P. compacta*, and species of the PAC, but its ITS1–5.8S–ITS2 sequence deviated in more than 30 (≤6.4%) nucleotide positions from those of these closest relatives, and topology of the phylogenetic tree (Fig. 7) was uncertain. Figure 7 depicts a section of a larger tree that was shown to form a strongly supported clade within the Helotiales (Grünig et al. 2009; Wang et al. 2006). This clade contains morphologically highly dissimilar ascomycete species of the genera *Vibrissea*, *Loramyces*, and *Mollisia*, supporting evolutionary relationships between aquatic fungi, i.e., *Vibrissea* and *Loramyces* species, root endophytes, and ectomycorrhiza-forming ascomycetes. While most terminal nodes in the clade were strongly supported, internal nodes remained largely

unresolved, indicating either (1) niche specialization of an ancestral species into aquatic, soil, and root environments, (2) homoplasious rDNAs, or (3) inclusion of a limited number of species into phylogenetic analysis partly due to incomplete knowledge of the taxa deserving inclusion. The last is the most probable reason for the weak resolution within the phylogenetic tree because the number of species was limited by the availability of molecular data. In addition, many helotialean species including the "missing links" are still "out there" awaiting their discovery. It is important to note that morphologically highly dissimilar species need to be included in attempts to reconstruct phylogenies in the Helotiales based on molecular data.

DS fungi might function as mutualistic fungi, taking part in nutrient and water acquisition (Jumpponen and Trappe 1998; Grünig et al. 2008b), especially in unfavorable environments. It is possible that these fungi may provide plants with access to more recalcitrant pools of carbon, nitrogen, and phosphorus in litter (Peterson et al. 2004). In a growth-room experiment, *P. fortinii* did not affect growth of *Pinus contorta* but significantly increased the foliar phosphorus concentration (Jumpponen et al. 1998); and so, a mycorrhiza-like relationship was postulated. Bartholdy et al. (2001) demonstrated that isolates of *P. fortinii* secrete siderophores into the culture medium, suggesting that this fungus may be able to compete with other soil organisms for iron. There should be future investigations at the functional level to demonstrate any important benefits of this newly described fungal species.

Acknowledgements The authors are indebted to the German Research Foundation (DFG), project Mu 1035/9-3 for the financial support. We thank Orlando Petrini for the Latin diagnosis. We are grateful to M. Roth, R. Krüger, and G. Vogt for the excellent technical assistance.

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