The taxonomic position of *Asterodon*, *Asterostroma* and *Coltricia* inferred from the septal pore cap ultrastructure

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The ultrastructure of the septal pore cap (SPC) of *Asterodon*, *Asterostroma* and *Coltricia* were examined to establish the taxonomic position of these genera. *Asterostroma* has dolipores with perforate SPCs and is classified in the *Lachnocladiaceae*. In contrast, *Asterodon* and *Coltricia* have dolipores with imperforate SPCs and belong to the *Hymenochaetaceae*. Other selected species of genera belonging to the *Hymenochaetaceae* like *Hydnochaete*, *Coltriciella*, *Inonotus*, *Onnia*, and *Cyclomyces* also contained imperforate SPCs. *Coltriciella*, *Inonotus* and *Cyclomyces* moreover presented a lamella of endoplasmic reticulum above the imperforate SPC after chemical fixation. Such a lamella could rarely be observed in *Coltricia* only after high-pressure freezing and freeze substitution. Cryofixed fungal cells of *Cyclomyces* and *Coltricia* showed differences in the architecture of the matrix of the SPC. *Coltricia* showed a more layered matrix structure than the SPC of *Cyclomyces*. In addition, transmission- and scanning electron microscopy revealed an indent in the centre of the imperforate SPC of *Cyclomyces*, indicating a reduced thickness, and resulting into a tented profile in cross-sections.

INTRODUCTION

The *Hymenochaetaceae* were recognized as a natural group by Patouillard (1900) as 'Série des Igniaires' and formally described as a family by Donk (1948), characterized by the occurrence of brown setae, a brown trama which turns blackish brown to black in potassium hydroxide (xanthochroic reaction), and the absence of clamp connections. Patouillard included the genera *Cyclomyces*, *Hydnochaete*, *Hymenochaete*, *Phellinus* and *Xanthochrous*. The genera with brown, thick-walled, stellate structures were placed in the 'série des Astérostromes' (Patouillard 1900). In 1964 Donk distinguished three subfamilies: the *Vararioideae*, the *Asterostromatoideae* (including *Asterostroma*) and the *Hymenochaetoideae* (including *Asterodon*). In his concept, *Scytinostroma* was left in the then polyphyletic family *Corticiaceae*. Reid (1965) raised the *Vararioideae* to the family level by describing the *Lachnocladiaceae*, in which he also accommodated his new genus *Dichopleuropus*, as well as *Dichantharellus*, *Asterostroma*, *Vararia* and *Scytinostroma*. Pouzar (1983) proposed the family *Asterostromataceae* for *Asterostroma* only, and classified the family next to the *Lachnocladiaceae*. He explicitly excluded *Asterodon* and considered the stellate structures as analogous. Pegler (1995) considered the stellate structures in *Asterostroma* and *Asterodon* as homologous and accommodated both genera in the *Asterostromataceae* in the order *Hymenochaetales*.

Oberwinkler (1977) raised the *Hymenochaetaceae* to the ordinal level, but left the position of the *Lachnocladiaceae* open. He suggested a closer relationship of the *Lachnocladiaceae* to the 'aphyllophoralean *Russulales*', while Stalpers (1974, 1979) suggested a close relation with *Heterobasidion* and the *Bondarzewiaceae*. Both suggestions have been confirmed by r-DNA sequences analysis, as the *Bondarzewiaceae* is close to the *Russulales* (Hibbett & Donoghue 1995, 1997).

The *Lachnocladiaceae* is generally regarded as a small, well-founded family at the margin of the aphyllophoralean system. Members of this family are mainly characterized by the possession of dextrinoid, thick-walled dichohyphidia combined with the occurrence of a gloeoplerous system, which only in a very few cases could not be demonstrated, for example, *Vararia cinnamomea* Boidin et al. (Boidin & Lanquetin 1984). The variation of the basidiome is highly reminiscent of that of the *Thelephoraceae* (Stalpers 1993). The family thus contains: (a) corticioid to stereoid genera (*Asterostroma*, *Dichostereum*, *Scytinostroma*, *Stereofomes*, and *Vararia*); (b) stipitate stereoid to cantharelloid genera (*Dichopleuropus*, *Dichantharellus*), and (c) a ramarioid genus (*Lachnocladium*).

A useful taxonomic character in the basidiomycetes is the

ultrastructure of the septal pore cap (SPC). Moore (1978, 1980) described several morphological types, which were considered to be correlated with larger taxonomic units. Although the SPC of the homobasidiomycetes is usually perforated, imperforate SPCs occur also, for example, in the *Cantharellaceae* and isolated genera like *Typhula*, *Radulomyces*, and *Phanerochaete* (Keller 1997). Also the *Hymenochaetaceae* have dolipores with imperforate SPCs (Moore 1980), with the exception of *Phaeolus* and *Coltricia*. *Phaeolus* deviates in other respects as well, and is now considered to belong to the *Polyporaceae* sl., close to *Laetiporus* as suggested by Ryvarden (1991) and confirmed on the basis of r-DNA sequences by Hibbett & Donoghue (1995). *Coltricia*, however, fits morphologically very well in the *Hymenochaetaceae*: it has a xanthochroic reaction, lacks clamp connections, and at least some tropical representatives have extrahymenial setae, for example, *Coltricia duostratosa* and *Coltricia hamata*. It is somewhat deviating, because it is mycorrhizal (Danielson 1984), and thus soil-inhabiting. Nevertheless, Moore (1996) proposed the order Coltriciales to accommodate *Coltricia*, on the exclusive basis of the structure of the dolipore and the perforate septal pore cap. Moore (1980) based his findings on a culture of *C. perennis* (CBS 372.52), which was removed from that collection in 1992, because the identity was then considered doubtful. To solve the problem, all culture collections, which had cultures of *Coltricia*, as listed in the 'World Directory of Collections of Cultures of Microorganisms' were addressed for strains, and fresh material was collected.

To determine the classification of *Asterodon*, *Asterostroma* and *Coltricia*, the SPCs of these genera were examined next to the SPCs of *Hydnochaete*, *Coltriciella*, *Inonotus*, *Onnia*, and *Cyclomyces*. The possible analogy or homology of asterosetae and asterohyphidia is discussed.

MATERIALS AND METHODS

Organisms, media and culture conditions

Hydnochaete japonica (CBS 499.76), *Coltriciella dependens* (CBS 247.50), *Inonotus weirii* (CBS 663.85), *Onnia tomentosa* (CBS 278.55), *Asterostroma medium* (CBS 139.55), *Cyclomyces fuscus* (IFO 9789), and *Coltricia perennis* (CBS 101081) were maintained on yeast-malt extract agar (YMA, 0.3% yeast extract + 0.3% malt + 0.5% peptone + 1% glucose + 2% agar). Hyphae were scraped from a slant culture and grown on YMA in Nunclon sterile dishes 60 mm in diameter and 15 mm high at 24 °C.

Coltricia perennis and *Cyclomyces fuscus* were also grown at room temperature on YMA between two perforated polycarbonate filters (Poretics: Polycarbonate Track Etching (PCTE) filters, Ankersmit, Breda, The Netherlands, 0.6 µm pore size and 37 mm filter size).

Basidiomes of *Coltricia perennis* (CBS H-001461, air-dried) and *Asterodon ferruginosus* (O, freeze-dried) were examined with transmission electron microscopy.

Transmission electron microscopy

Colonies grown on YMA were fixed in 3% glutaraldehyde (EM grade, Polysciences Inc.) buffered with 50 mM sodium cacodylate buffer (SCB), pH 7.4, for 16 h at 4 °C. After washing in SCB and distilled water 4 × 6 mM samples were removed from the peripheral part of the colony and subsequently post-fixed in 1% aqueous potassium permanganate for 90 min at room temperature. The samples were washed with distilled water, dehydrated in a graded series of acetone, infiltrated and embedded in Spurr's resin (Spurr 1969).

Samples from dried material of *Coltricia perennis* were immersed in 5% RBS 50 (Hicol) for 2 d., rinsed with SCB, fixed in 1.5% aqueous potassium permanganate for 2 h, dehydrated in a graded series of acetone and infiltrated and embedded in Spurr's resin (Spurr 1969). *Asterodon ferruginosus* (Herbarium Norway) samples were immersed in a mixture of 0.5% Tween-20 and 3% glutaraldehyde in SCB at 4 °C. After rinsing with SCB the samples were post fixed in a mixture of 1% osmium tetroxide and 1.5% potassium hexacyanoferrate (II) trihydrate in SCB for 16 h at 4 °. After rinsing with SCB the samples were further prepared as described above.

After polymerisation for 24 h at 65 ° and subsequently ultramicrotomy, 80 nm sections of hyphae were mounted on 1.1% pioloform and carbon-coated single-hole copper grids, dried for 16 h, and stained with 4% aqueous uranyl acetate for 45 min and with lead citrate for 8 min according to Venable & Coggeshall (1965).

Samples from *Coltricia perennis* and *Cyclomyces fuscus* were also cryofixed and freeze-substituted. Peripheral mycelial parts of colonies cultured between PCTE-filters were placed in specimen holders with 1-hexadecene (Müller & Moor 1984, Studer *et al*. 1995) and subsequently high-pressure frozen with a high-pressure freezer (Leica EM HPF) according to the supplier's manual, and used for freeze substitution. Hyphal material was transferred in liquid N_2 to a CS auto substitution-chamber (Reichert-Jung, Vienna) at −90 ° containing a mixture of 1% osmium tetroxide, 3% glutaraldehyde and 0.3% uranyl acetate in methanol (modified from Müller, Marti & Kriz 1980). After 5 days the temperature was raised from −90 ° to 4 ° at a rate of 10 ° h^{-1}. The specimens substituted with the complex FS-medium were rinsed with methanol, followed by anhydrous acetone. After raising the temperature to room temperature, the specimens were infiltrated and embedded with Spurr's resin (Spurr 1969), and further processed for transmission electron microscopy as described above.

Scanning electron microscopy

The *Cyclomyces fuscus* colonies grown between PCTE-filters were fixed in a mixture of 2% (v/v) glutaraldehyde (EM grade 8%; Polysciences Inc., Warrington, PA 8976) and 50 mM sodium cacodylate buffer, pH 7.4, for 16 h at 4 °. Thereafter postfixed with 1% (w/v) osmium tetroxide, buffered with 66 mM phosphate buffer (PB), pH 7.4, for 16 h at 4 °. After a three times wash in PB, 4 mm × 6 mm rectangles were cut with a razor blade from the colony, and subsequently immersed in a series of 15, 30 and 50% (v/v) aqueous dimethyl sulfoxide (DMSO), for at least 15 min each, then frozen on a liquid nitrogen-precooled metal block and fractured with a razor blade (Müller *et al*. 1994). After thawing in 50%

DMSO and washing in PB, the fractured fungal pieces were macerated in 0.2% osmium tetroxide/ PB for 10 days at room temperature, immersed in 30% (v/v) aqueous *N*, *N*-dimethylformamide (Meissner & Schwarz 1990), and subsequently plunge-frozen in liquid propane. After freeze substitution in methanol for 1 d at −90 °C the methanol was changed with anhydrous acetone, and the fungal pieces were gently placed in baskets, subsequently critical-point-dried, mounted on stubs with conductive carbon cement (Neubauer), 2 nm platinum magnetron sputter-coated (Müller *et al*. 1994), and examined in a SEM equipped with a field emission gun (JSM 6300F, JEOL) at an acceleration voltage of 8 kV and a work distance of 10 mm.

RESULTS

Culturing of *Coltricia perennis* turned out to be difficult. Extensive trials (30 inocula on different media) for three different collections resulted in only one isolate of *Coltricia*. There was a large variety of contaminants, including other basidiomycetes. Also the material received from various collections was heterogeneous and only one strain actually belonged to *Coltricia* s. str.

After transmission electron microscopy of chemically fixed fungal material two types of SPCs were observed (Fig. 1 a–i). An imperforate SPC was present in *H. japonica* (Fig. 1a), *Coltriciella dependens* (Fig. 1b), *I. weirii* (Fig. 1c), *O. tomentosa* (Fig. 1d), *Coltricia perennis* (both fresh and dried material, respectively Fig. 1e and 1f), *Cyclomyces fuscus* (Fig. 1g), and *Asterodon ferruginosus* (Fig. 1h, dried material). A perforate SPC was only visualized in *Asterostroma medium* (Fig. 1i). In addition, imperforate SPCs with the occasional presence of a lamella of ER above the SPCs were observed in *I. weirii* (Fig. 2a), *Coltriciella dependens* (Fig. 2b), and *Cyclomyces fuscus* (Fig. 2c).

Two representative species with either a lamella of ER or without such lamella above the imperforate SPCs were high-pressure frozen and freeze-substituted to show the presumed

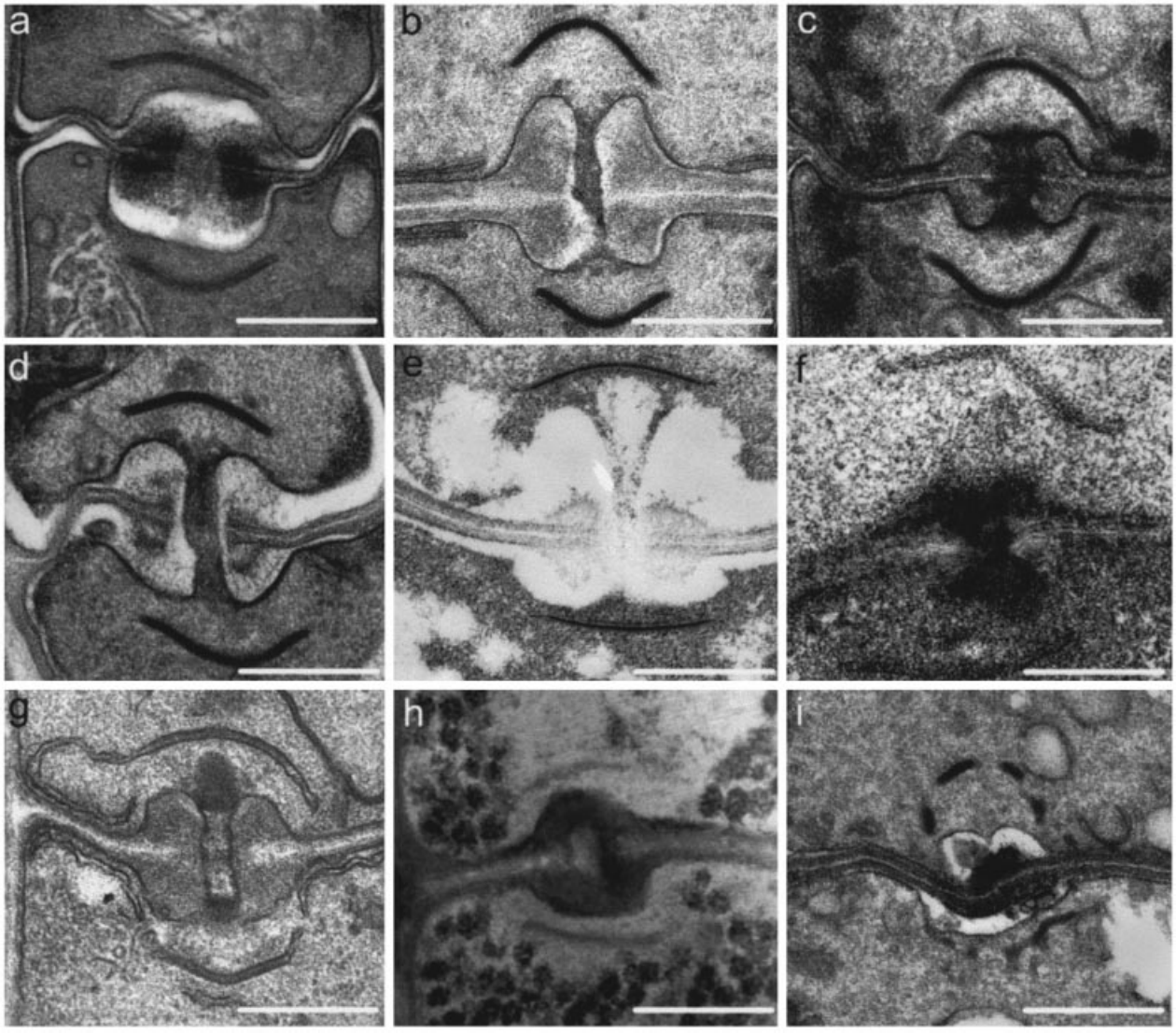

Fig. 1. Electron micrographs of septal pore caps after chemical fixation. (a) *Hydnochaete japonica*. (b) *Coltriciella dependens*. (c) *Inonotus weirii*. (d) *Onnia tomentosa*. (e) *Coltricia perennis*. (f) *Coltricia perennis* (dried material). (g) *Cyclomyces fuscus*. (h) *Asterodon ferruginosus* (dried material). (i) *Asterostroma medium*. *Note*: imperforate septal pore caps in Figs a–h, and a perforate septal pore cap in Fig. i. Bars = 500 nm.

Fig. 2. A lamella of endoplasmic reticulum (arrowheads) is present above the septal pore cap after chemical fixation. (a) *Inonotus weirii*. (b) *Coltriciella dependens*. (c) *Cyclomyces fuscus*. Bars = 250 nm.

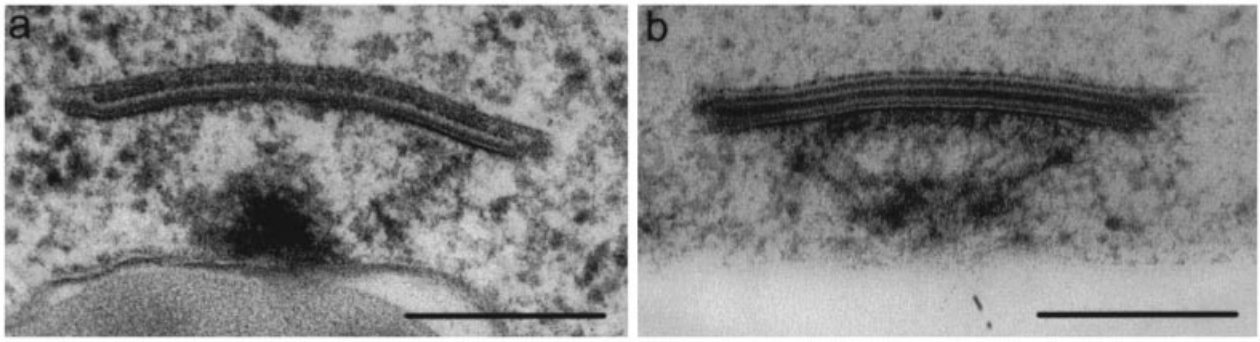

Fig. 3. Electron micrographs of septal pore caps after high-pressure freezing and freeze substitution. Note the difference in the matrix layers of the septal pore cap in *Cyclomyces fuscus* (Fig. a) compared with that in *Coltricia perennis* (Fig. b). Bars = 250 nm.

in vivo situation. This resulted in an ultrastructure of the dolipore-SPC complex different from the chemically fixed fungal samples.

High-pressure freezing and freeze-substitution revealed a wealth of details of the dolipore-SPC complex with associate subcellular structures. The imperforate SPC of *Cyclomyces fuscus* (Fig. 3a) showed a less complicated layered structure than the imperforate SPC of *Coltricia perennis* (Fig. 3b). The SPC of *Cyclomyces fuscus* showed an outer and an inner membrane enclosed an electron-dense layer positioned at the outer membrane and a less dense layer present at the inner membrane. In contrast, in *Coltricia perennis* a prominent electron dense layer was present in the middle of the SPC matrix, and a less dense layer near the outer membrane. Both SPCs show a thin electron dense layer against the outer side of the inner membrane.

Cyclomyces fuscus showed a lamella of ER above its imperforate SPC (Fig. 4a). However, this lamella could not always be demonstrated as can be seen in the lower SPC (Fig. 4a). The ER along the dolipore septa was connected with the base of the SPC (Fig. 4a). Occasionally, the SPC revealed a clear spot in its centre (Fig. 4b), or the outer and inner membrane made intimate contact, excluding the SPC matrix (Fig. 4c, d). The plugging material that blocked the entrance of the dolipore channel was connected with the inner side of the SPC by electron dense filamental like structures and perpendicular orientated, thicker electron dense structures (Fig. 4b, d). These connections were also observed in *Coltricia perennis* (Fig. 5a, b). Rarely, an ER lamella was observed above the imperforate SPC of *C. perennis* (Fig. 5c).

After scanning electron microscopy the imperforate SPC of *Cyclomyces fuscus* showed differences in thickness (Fig. 6a, b). The connection of the ER along the dolipore septum with the base of the SPC (Fig. 6c) and the lamella of ER above the SPC (Fig. 6d) could easily be visualized. The outer membrane (Fig. 6e) as well as the inner membrane (Fig. 6f) showed an inward growth in the centre of the cap, indicating a reduced thickness of the SPC. The same can be concluded from the SPC of *Hirschioporus abietinus* as presented in figure 5 of Moore (1996).

DISCUSSION

Members of the *Hymenochaetaceae* have been studied after chemical fixation and dehydration in a graded series of an organic solvent, followed by embedding in resin. This method is fast and resulted in the visualization of an imperforate SPC in the following species: *Hymenochaete rubiginosa* (Oberwinkler 1985); *Phellinus torulosus* (Moore 1980); *Onnia tomentosa* (Moore 1980; Fig. 1); *Onnia circinata* (Moore 1980); *Onnia leporina* (Moore 1980); *Inonotus hispidus* (Moore 1980); *Inonotus weirii* (Fig. 1); *Hydnochaete japonica* (Fig. 1); *Coltriciella dependens* (Fig. 1); *Coltricia perennis* (Fig. 1); *Cyclomyces fuscus* (Fig. 1); and *Asterodon ferruginosus* (Fig. 1).

In addition, a lamella of endoplasmic reticulum (ER) above the imperforate SPC was observed in the following species: *Inonotus weirii* (Fig. 2), *Coltriciella dependens* (Fig. 2), *Cyclomyces fuscus* (Fig. 2), *Onnia circinata* (Moore 1980) and *Onnia leporina* (Moore 1980). This lamella of ER above the imperforate SPC

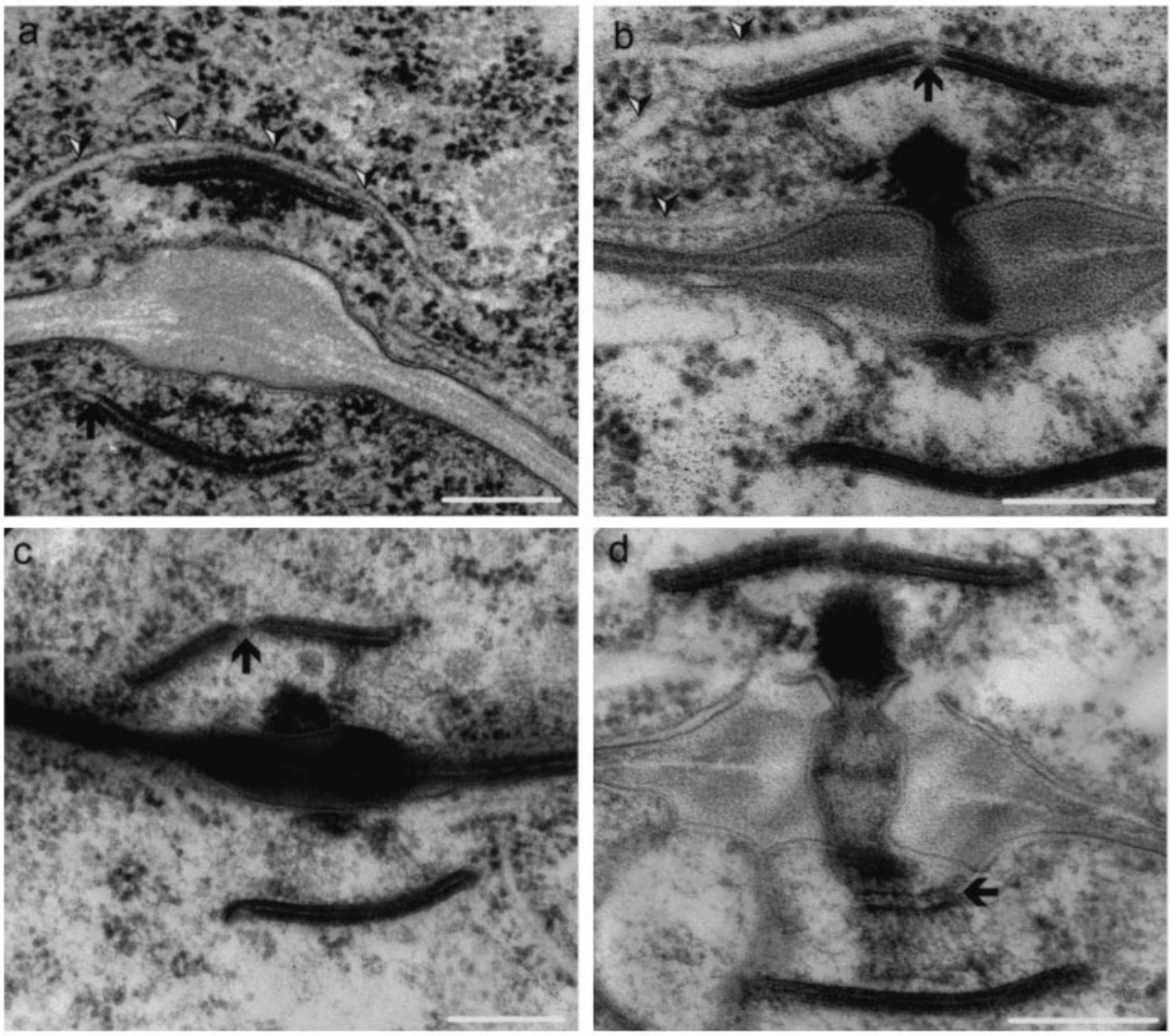

Fig. 4. High-pressure frozen and freeze-substituted septal pore caps in *Cyclomyces fuscus*. (a) A lamella (arrowheads) of endoplasmic reticulum (ER) above the imperforate septal pore cap (SPC), and ER connected with the SPC (arrow). (b) A clear spot (arrow) in the centre of the SPC. ER along the septum, towards the SPC and above the SPC (arrowheads); a plug blocks the entrance of the septal pore channel. (c) The centre of the SPC is thinner than the remainder of the cap (arrow). (d) Filamental like structures are present between the SPC and the plugging material. Note the electron denser perpendicular orientated structures (arrow). Bars = 250 nm.

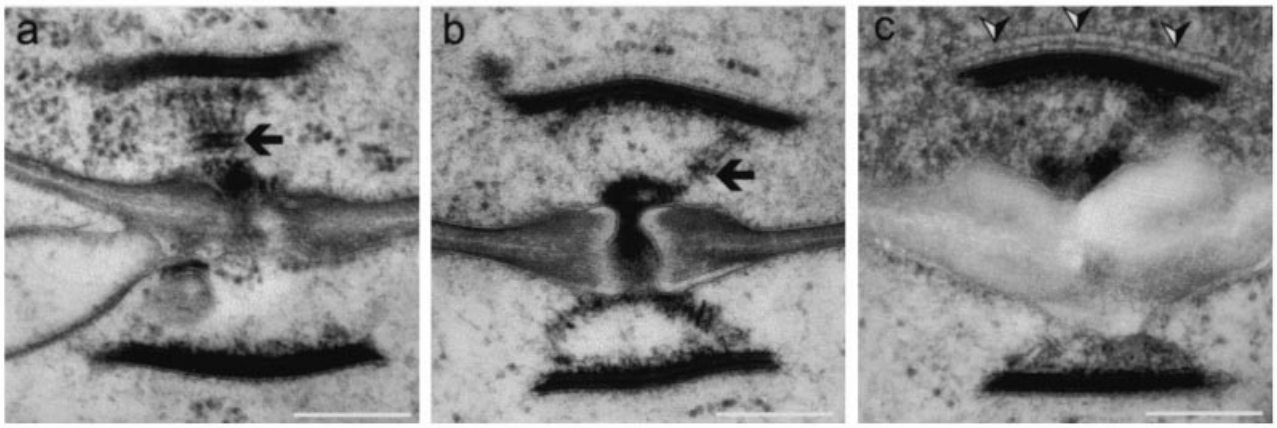

Fig. 5. High-pressure frozen and freeze-substituted septal pore caps in *Coltricia perennis*. (a–b) Filament-like structures and perpendicular orientated structures (arrow) are present between the septal pore cap (SPC) and the plugging material. (c) A lamella (arrowheads) of endoplasmic reticulum is present above the SPC. Bars = 250 nm.

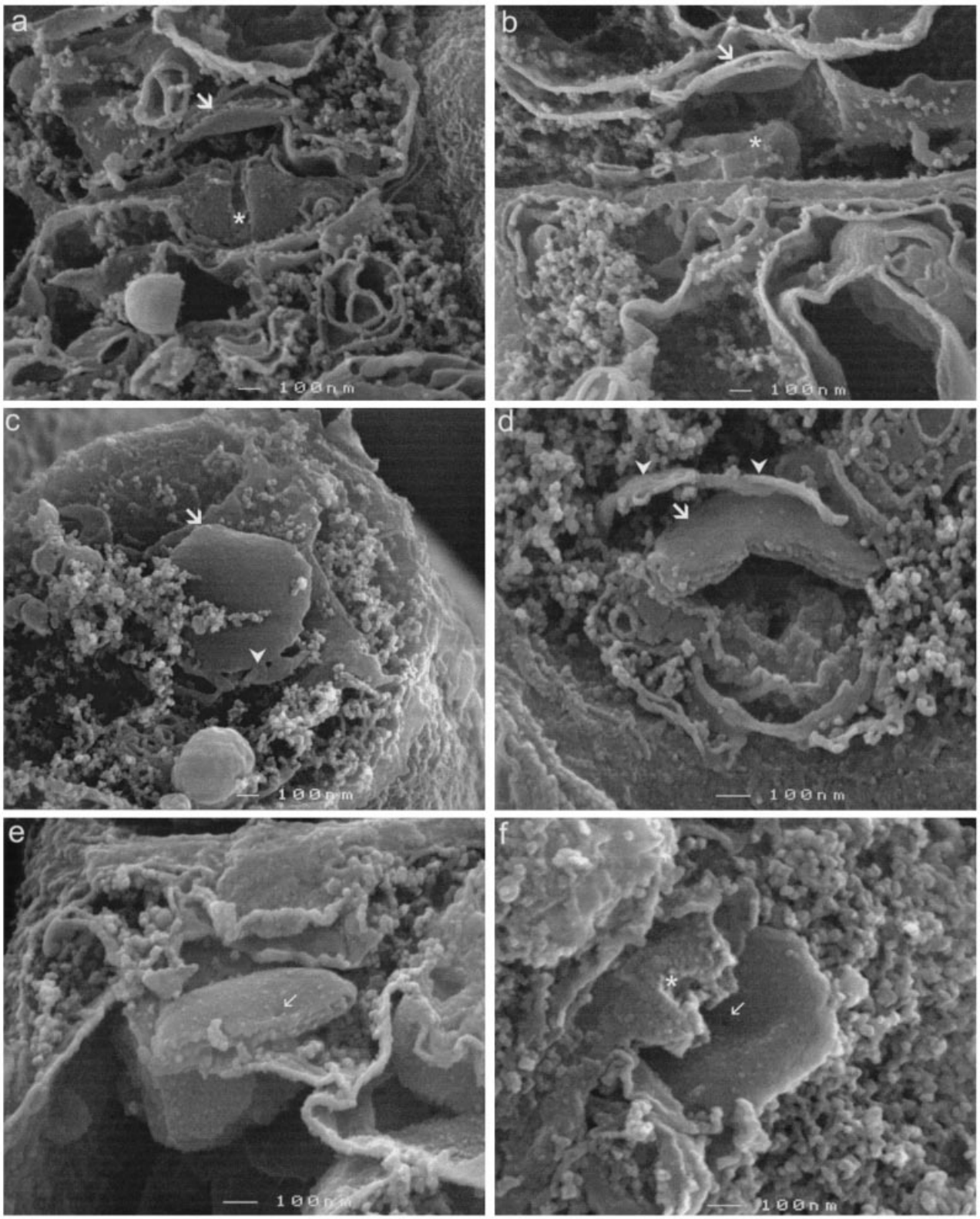

Fig. 6. Scanning electron micrographs of septal pore caps in *Cyclomyces fuscus*. (a) Median view of the septal pore cap (SPC) (arrow) and the septal pore channel (asterisk). (b) Near median view of the SPC (arrow) and the septal swelling (asterisk). (c) Connection of the endoplasmic reticulum (arrowhead) with the SPC (arrow). (d) A lamella (arrowheads) of endoplasmic reticulum is above the SPC (arrow). (e) An indentation in the outer surface of the SPC (small arrow). (f) An indentation in the inner surface of the SPC (small arrow). Bars = 100 nm.

does not only occur in the *Hymenochaetaceae*, but also in species of other families like the *Botryobasidiaceae* (Langer 1994) and the *Exidiaceae* (Currah & Sherburne 1992, Müller *et al*. 1998). It is not an introduced artefact of the specimen preparation by chemical fixation, as more reliable methods such as high-pressure freezing and freeze substitution also

resulted in the visualization of the lamella of ER above the imperforate SPC of *Cyclomyces fuscus* (Fig. 4) and *Coltricia perennis* (Fig. 5). The function of this lamella of ER above the imperforate SPC is not known. Younger fungal cells exhibit frequently such a lamella. This lamella of ER seems represented in some *Hymenochaetaceae* genera, and not in others. This may suggest that species without a lamella of ER above the imperforate SPC are more closely related than those with a lamella of ER above the imperforate SPC.

Asterodon

In the *Aphyllophorales*, setae and setal hyphae are restricted to the *Hymenochaetaceae*. Typical setae are straight, brown, thick-walled structures; they are rarely curved (e.g. *Inonotus triqueter*) or branched (e.g. *I. cuticularis*). The asteroseta, which is only known from *Asterodon*, represents a further stage of this development. An asteroseta develops from an unbranched seta, from whose base a number of branches sprout. The fully developed structure usually has a distinct main axis, the original seta. This is described and illustrated in detail by Corner (1948).

The asterosetae of *Asterodon ferruginosus*, the only species of the genus, resemble at first sight asterohyphidia, but the development is different (see also below). For this reason Donk (1964), followed by Stalpers (1996), considered *Asterodon* to belong to the *Hymenochaetaceae*. This is consistent with the fact that *Asterodon ferruginosus* has an imperforate SPC.

Asterostroma

The *Lachnocladiaceae* are characterized by the presence of dichohyphidia, which are terminal cells with a repeatedly dichotomously branched hyphoid base (stipe). These branches are at least partly thick-walled and dextrinoid. Occasionally the hyphoid base becomes thick-walled or somewhat inflated (trunk) or both. Boidin *et al*. (1980) classified the dichohyphidia according to the shape of the base, and the density, rigidity and geometry of the branches.

Asterohyphidia are only a variation of dichohyphidia. They do not branch from a central point, although the branching can be very compact, and the terminal branches are relatively long. More rarely branches of asterohyphidia show a terminal bifurcation and such asterohyphidia have been called 'stellate dichohyphidia', indicating a continuum between the two types. Compare, for example, Hallenberg's drawings of a dichohyphidium of *Vararia gallica* (Fig. 31c) and an asterohyphidium of *Asterostroma laxum* (Fig. 6b) (Hallenberg 1985).

The fact that the septal pore cap (SPC) of *Asterostroma medium* is perforated is consistent with the classification of *Asterostroma* in the *Lachnocladiaceae*. Therefore, Pegler's (1995) proposal to classify the genus in the *Hymenochaetaceae* has to be rejected.

Coltricia

Both basidiomes and the cultures of *Coltricia perennis* were found to have an imperforate SPC. An earlier report of a perforate SPC (Moore 1980) was very likely based on a misidentified culture. Consequently, both morphological and ultra structural characters of *Coltricia* support its classification in the *Hymenochaetaceae*. Small subunit ribosomal DNA sequences also sustain this conclusion (Hibbet & Donoghue 1995). This means that the presence of an imperforate SPC is an ultrastructural character shared by all *Hymenochaetaceae*, in addition to the occurrence of brown setae and setal hyphae, a xanthochroic reaction of the trama, and the absence of clamp connections.

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