Studies on the ultrastructure of *Lachnum* and related genera (Hyaloscyphaceae, Helotiales, Ascomycetes)

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The ultrastructural characters of hairs and asci of six genera of the Hyaloscyphaceae, *Albotricha, Incrucipulum, Brunnipila, Capitotricha, Dasyscyphella* and *Lachnum* were studied using transmission electron microscopy. Clear intergeneric differences exist in the hair wall structure between the studied genera. Both hair and ascal ultrastructural features support the narrow genus concept in the group of *Lachnum* and its segregates. The genus *Albotricha* is found to be closely related to *Capitotricha*.

Keywords: Hyaloscyphaceae, ultrastructure, taxonomy.

The genus *Lachnum* Retz. is a central genus of Hyaloscyphaceae, subfamily Lachnoideae (Raitviir, 1987). Dennis (1949) already noted its heterogeneity and proposed 15 sections within it. Later Raitviir (1970), Baral (Baral & Kriegelsteiner, 1985) and Haines (1989) raised most of these sections to the generic rank. Raitviir (1987), however, hold a rather conservative position enclosing all rough-haired species in the *Lachnum* and reducing Baral’s (Baral & Kriegelsteiner, 1985) genera *Brunnipila* Baral, *Capitotricha* (Raitv.) Baral and *Incrucipulum* Baral to the subgeneric and section rank. There exists no consensus about the generic concept in the Lachnoideae and *Lachnum* has been defined in its old, very wide sense (Hawksworth & al., 1995). Evidently the consensus cannot be found on the basis of morphological characters only and some additional proof is needed.

Ultrastructural and molecular taxonomy studies may be used to check the morphology-based taxonomy. Cantrell & Hanlin (1997) devoted their recent study on the molecular taxonomy of the Hyaloscyphaceae primarily to the delimitation of subfamilies and, unfortunately, did not touch the generic delimitation. They only con-
eluded that "Lachnum is either paraphyletic or polyphyletic as currently delimited and might be divided into several genera" (Cantrell & Hanlin, 1997: 755). In our opinion their results support well the segregation of Brunnipila and Capitotricha, as the species of Brunnipila (as L. clandestinum and L. fuscescens) and Capitotricha (as L. bicolor) discussed as third and fourth group (Cantrell & Hanlin, 1997: 753), form groups of the generic rank in the consensus tree based on molecular data (Cantrell & Hanlin, 1997, fig. 3).

Ultrastructural characters have been found to be useful in delimiting families and genera of Ascomycetes (Bellemère, 1994; Kimbrough, 1994; Verkley, 1995; Brummelen, 1998; Hyde & Wong, 1999). Particularly Verkley's (1995) results suggest that ultrastructural studies offer a promising approach to solve several taxonomic problems in the Helotiales. The Hyaloscyphaceae, however, have practically never been the subject of TEM studies. Only Verkley (1996) has studied the ascus apical apparatus of the two genera Lachnum and Trichopeziza and concluded that there are differences at the generic level within the same type of ascal apparatus structure.

In the present investigation we have studied ultrastructural characters which could be used at the generic level to test some splitting hypotheses based on characters observable at the light microscopy level and to describe and discuss the differences between Lachnum and some of its segregates at the ultrastructural level. We concentrated our attention to the genera Brunnipila, Capitotricha and Incrucipulum, which are segregates of Lachnum characterised by thick-walled hairs, and to Dasyscyphella Tranzsch. and Albotricha Raitv., which have been treated as synonyms by Weber (1992).

The hair wall was chosen for ultrastructure studies because the taxonomy of the Hyaloscyphaceae is mainly based on hair characters, which show a great diversity under the light microscope. The hairs of the genera studied differ in wall thickness, pigmentation and their being totally or only partly warty. The size, shape and persistence of the hair wall ornamentation, generally called 'granulation' cannot be observed adequately enough using the light microscope. The SEM studies contributed very little to the evaluation of hair ornamentation as a character of taxonomic value. Raitviir (1971) stressed only the difference between smooth-haired and rough-haired genera. Hein (1980) remarked that the ornamentation of Lachnum (Capitotricha) bicolor hairs differ from those of other species studied by him. From the SEM pictures presented by Järv (1995), Incrucipulum, Brunnipila and Capitotricha have each their characteristic hair ornamentation. All these considerations provide good reasons for studying the hairs by TEM. The ascus apex was also studied following Verkley's (1995) ideas on its taxonomic and phylogenetic value.
Materials and methods

Fresh material was collected in the field and kept living in small plastic boxes until fixation. In the winter season plant material, particularly *Rubus idaeus* canes, were incubated in vegetation chambers under artificial light and humid conditions to get living apothecia for fixation. After identification and removal of several apothecia for fixation, part of the material was dried and deposited in the herbarium of TAA.

The methods used were those described by Curry & Kimbrough (1983) and Samuelson & Kimbrough (1978). For transmission electron microscopy, the fruit bodies were fixed for 2 h using 2% paraformaldehyde, 2.5% glutaraldehyde and 2 mM calcium chloride in 0.1M sodium cacodylate buffer (pH=7.2). The material was rinsed in 0.1M cacodylate buffer (pH=7.2) and fixed afterwards for 45 min in 1% osmium tetroxide in the same buffer (pH=7.2). The material was buffer rinsed and dehydrated in an ethanol series followed by acetone (each 10 min: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 3 x 96%, acetone). Fixation and dehydration were performed at room temperature. The material was embedded in Spurr's resin (ERL 4206) using infiltration series resin and acetone in 1:3, 1:1 and 3:1 proportions for 3 hours each. The material was thin-sectioned on a Reichert ultramicrotome Om2U using glass knives and the sections were stained with uranyl acetate and lead citrate. The material was examined using a JEOL-100S electron microscope.

For each species, the hair wall structure and the apical apparatus of the ascus were studied using magnifications from 10,000 X to 20,000 X. Hair wall stratification is defined according to Gooday (1995). ‘Inner layer’ is used for the stratum forming the septa, ‘outer layer’ for the stratum not involved in the septum formation (the boundary is the electron-transparent middle lamella) and ‘fibrillar layer’ for the most external covering layer often composed of more or less loose material, which may sometimes become disrupted or eroded. The terminology of Verkley (1995) is followed for the ascus apical apparatus.

Results

The inner structure of the hair wall is visible in TEM as strata composed of material variable in its electron-density and arranged in different patterns. Two basic layers – inner and outer – and an external fibrillar layer can be distinguished. All studied taxa are characterised by hairs totally or partly bearing warts on their surfaces. The warts are different in size, shape and persistence. Traditionally the ornamented hairs of the Hyaloscyphaceae have been described as “granulated”, but taking into account the mode how the fine structures causing the roughness of the hair surface originate from the hair wall we prefer to call the structures present on the hair wall “warts”. The same term is generally used to describe the ascospore ornamentation of the Pezizales (Dennis, 1978). “Granules” or “granulated” is a more adequate term for the pieces of material of external origin loosely attached to the hair wall and not being an organic part of hair wall.

Species belonging to the same genus (Brunnipila clandestina and B. calyculaeformis; Lachnum virgineum and L. brevipilosum) are very similar in their hair wall ultrastructure: the differences appear at the intergeneric level. It was possible to distinguish four types of hair wall ultrastructure in the eight species studied and described in detail in the discussion.

1. The Lachnum type (Figs. 1-7)

*Lachnum brevipilosum* and *Lachnum virgineum*

Hairs under the light microscope hyaline, thin-walled, totally covered by warts. – Hair wall under the TEM 0.4 \( \mu \)m thick, warty, not very clearly stratified. The outer and inner layers are very similar and relatively homogeneous, composed of finely granular material. The outer layer is a little more electron dense. The up to 1.5 \( \mu \)m high and 0.8 \( \mu \)m wide warts are the most electron dense parts of the wall. Fibrillar layer electron dense and even, very well observable also on the surface of the warts (Figs. 2 and 3). – Ascus tip conical to subpapillate, rounded, but somewhat flattened by the pore (Fig. 1). Apical thickening relatively narrow, containing a pattern of irregularly oriented granules and fibrils. The apical ring consists of uni-
Figs. 1–7. – Lachnum brevipilosum. 1. ascus apical apparatus. – 2. hair wall. – Lachnum virgineum. 3. hair wall. – Dasyscyphella cassandrae. 4. ascus apical apparatus. – 5–7. hair wall. – A: annulus; ac: apical chamber; ap: annular protrusion; at: apical thickening; c: central cylinder; gc: glycogen, sp: ascospore. – Bar equals 1 μm.
form granular matter and is the most electron dense part of the apical apparatus. The annular protrusions are relatively long and tapering toward their lower end. Apical canal electron transparent, thinner than the lateral wall of the ascus. Apical chamber large and high.

Dasycyphella cassandrae

Hairs under the light microscope hyaline, thin-walled, with two completely smooth apical cells, with warts in their lower part. – Hair wall under the TEM 0.4 μm thick in their central and lower part, warty, poorly differentiated, moderately electron dense, becoming a little more electron transparent inside, containing small fibrils and granules which are parallel to the inner boundary of the wall. The outer and inner layer have no clear boundary between them (Figs. 5–7). Fibrillar layer well differentiated, separated from the outer layer by an electron dense line, variable in thickness, sometimes wavy, consisting of finely structured material. Warts up to 1 μm high and 1.5 μm wide, more or less regularly heap-shaped, sometimes slightly eroding (Figs. 5 and 6). The material inside outgrowths is heterogeneous and more electron dense coiled lines delimit ‘chambers’ surrounded by an electron transparent matrix. – Ascus tip conical-rounded, not flattened by the relatively high pore, electron transparent (Fig. 4). Apical thickening narrow and high. Apical ring quite electron transparent, becoming more electron dense in the relatively long and a little tapering downwards the annular protrusions. Apical canal thinner than the lateral wall of the ascus, electron transparent. Apical chamber high, narrowly ovate.

2. The Brunnipila type (Figs. 8–12)

Brunnipila calyculaeformis and Brunnipila clandestina

Hairs under the light microscope with dark brown thick walls covered by papilliform warts. – Hair wall under the TEM 0.6 μm thick, with elongated warts, very electron dense. The walls contain melanin and the high electron density is the result of this pigment (Verkley, pers. comm.). Inner and outer layer similarly composed of homogeneous finely granular matter, the layers and their strata distinguishable on the basis of their electron density. The inner stratum of the inner layer electron transparent, clearly observable (Figs. 11 and 12). The outer stratum of the inner layer is highly electron dense. The inner stratum of the outer layer observable as slightly more electron transparent band, with the electron density increasing gradually below the warts, whilst the outer stratum is more electron
Figs. 8–12. — *Brunnipila clandestina*. 8. ascus apical apparatus. — 9. ascus apical apparatus (semi–median section, the central cylinder has not been grazed fully). — 10. hair wall. — *Brunnipila calyculaeformis*. 11–12. hair wall. — a: annulus; ac: apical chamber; at: apical thickening; c: central cylinder; gc: glycogen; ml: middle lamella; n: nucleus; s: septum; sp: ascospore. — Bar equals 1 μm.
dense, especially below the warts. Warts clavate, capitate or pustulat e, highly electron dense, up to 0.3–1.3 μm high and 0.3–0.5 μm wide. Fibrillar layer indistinct (Figs. 10–12). – Ascus tip truncate, and characteristically becoming rapidly broader in the sub-apical region (Figs. 8 and 9). Apical thickening well differentiated, composed of sparse irregular fibrillar material. Apical ring well differentiated, electron-dense, consisting of fibrillar material orientated at a more or less right angle to its longitudinal axis. Apical canal cylindrical, as thick as the lateral wall. Annular protrusions point straight downwards. Apical chamber small.

3. The Incrucipulum type (Figs. 13–15)

Incrucipulum ciliare

Hairs under the light microscope hyaline with thick, finely w arted, walls. – Hair wall under the TEM 0.7 μm thick, with papilliform warts, well differentiated (the boundary between the outer and inner layer is distinct: Fig. 14), moderately electron dense. The inner layer is divided into a thin, electron transparent inner stratum showing coarse granulation, and a slightly more electron dense outer stratum showing fine granulation. The outer layer becomes gradually more electron dense outwards. The inner stratum of the outer layer is similar in its structure to the inner layer but slightly more longitudinally fibrillar; the outer stratum almost homogeneous, more electron-dense (Figs. 14 and 15). Warts papilliform, 0.5–0.8 μm high and 0.2–0.5 μm wide, electron dense, consisting of the same material as the outer part of the outer layer. Fibrillar layer regular, thin, electron dense. Septal pore has a pulley-wheel shaped plug, no Wor onin bodies present (Fig. 15). – Ascus tip conical-truncate. Apical thickening medium sized, composed of sparse fibrillar material. Apical ring consisting of electron dense material, wing-shaped electron dense striations present throughout the apical thickening. Apical canal cylindrical, thicker than the lateral wall, filled with sparse fibrillar material. Apical chamber convex and wide. Annular protrusions short, slightly bent outwards (Fig. 13).

4. The Capitotricha type (Figs. 16–20)

Albotricha acutipila

Hairs under the light microscope hyaline, thin-walled, irregularly w arted in their lower part and smooth apically. The warts become easily detached in KOH followed by treatment with Melzer’s reagent or in ammoniacal Congo Red giving the hairs an almost completely smooth outlook. – Hair wall under the TEM about 0.4 μm thick, bearing irregular eroding warts. The overall electron
Figs. 13–15. *Incrucipulum ciliare.* 13. ascus apical apparatus. – 14. hair wall. – 15. hair wall with a septum and pore plug. – a: annulus; ac: apical chamber; ap: annular protrusion; at: apical thickening; c: central cylinder; gc: glycogen; ml: middle lamella; p: septal pore; rer: rough endoplasmatic reticulum; s: septum; sp: ascospore; v: vacuole; w: Woronin body. – Bar equals 1 μm.
Figs. 16-20. **Albotricha acutipila.** 16. ascus apical apparatus. - 18. hair wall, the arrow points to an eroding outgrowth. - 19. hair wall and septum with pore plug and Woronin bodies. - **Capitotricha bicolor.** 17. ascus apical apparatus. - 20. hair wall and septum, the arrow points to an eroding outgrowth. - a: annulus; ac: apical chamber; ap: annular protrusion; at: apical thickening; c: central cylinder; gc: glycogen; ml: middle lamella; p: septal pore; rer: rough endoplasmatic reticulum; s: septum; sp: ascospore; w: Woronin body. - Bar equals 1 μm.
density in the wall is fairly low and quite evenly arranged. Inner layer homogeneously finely fibrous, changing to slightly more electron dense outwards. Inner stratum of inner layer as observed in the septa is more electron transparent and composed of more coarsely granular material than the outer stratum (Figs. 18 and 19). In the lateral walls the inner layer becomes very thin and the strata are not distinct. Inner stratum of the outer layer homogeneous, finely granular; the outer stratum consists of fibrillar, erodible, irregular material, becoming gradually slightly more electron dense outwards. Warts of irregular shape, sometimes basally constricted, up to 0.4 \( \mu \text{m} \) high and up to 0.8 \( \mu \text{m} \) wide, composed of material similar to that in the outer layer, becoming hollow inside and finally disintegrating. Fibrillar layer thin, electron dense, disrupted in some places. Pore in the hair septum with a pulley-wheel shaped plug with surrounding Woronin bodies (Fig. 19) similar to those observed in Pseudopeziza trifolii (Meyer & Luttrell, 1986). – Ascus tip umbonate, flattened by the pore. Apical thickening comparatively narrow and, in comparison to the other parts of the ascus wall, composed of sparse material. Apical ring electron dense and well differentiated with regard to the apical canal and the ascus wall. Apical canal cylindrical, as thick as the ascus lateral wall, composed of fibrous, sparse and relatively electron transparent material. Annular protrusions point straight downwards. Apical chamber convex (Fig. 16).

**Capitotricha bicolor** (Figs. 17 and 20)

Hairs under the light microscope hyaline, moderately thick-walled, covered by coarse warts. – Hair wall under the TEM about 0.5 \( \mu \text{m} \) thick, covered with irregular warts, moderately electron dense, clearly stratified, containing a fine pattern of fibrils oriented parallel to the inner boundary of the wall. Inner layer composed of fibrillar material, which is more electron dense in the outer stratum of the layer. Inner stratum of the outer layer finely fibrillose-granular, electron dense; outer stratum more homogeneous and electron transparent. Warts up to 1 \( \mu \text{m} \) high and wide, sometimes basally constricted, often ragged or wavy externally, basally composed of material very similar to that of the outer stratum of the outer layer, in central and upper part containing sparse material eroding into large hollow cavities. Fibrillar layer thick, electron dense. The septal middle lamella is often W-shaped (Fig. 20). – Ascus tip conical-truncate. Apical thickening consisting of sparse material having a pattern of irregularly oriented fibrils and fine granules. Apical ring consisting of fine fibrils oriented perpendicularly to its longitudinal axis. Apical canal cylindrical, as wide as the ascus wall. Annular protrusions point straight downwards. Apical chamber medium-sized, convex. Fibrillar layer of ascus wall well developed (Fig. 17).
Discussion

The four types of hair wall ultrastructure can be shortly described as follows.

1. **The Lachnum type** (Fig. 21A). – Relatively thin wall, with poorly differentiated outer and inner layer and distinct electron dense fibrillar layer. Warts more or less conical, obtusely rounded, homogeneous, not erodible.

2. **The Brunnipila type** (Fig. 21B). – Thick, well stratified, very electron dense wall. Fibrillar layer indistinct. Warts elongated, clavate or capitate, often basally constricted, homogeneous, not erodible.

3. **The Incrucipulum type** (Fig. 21C). – Very thick, well stratified, moderately electron dense wall with elongated papilliform outgrowths. Fibrillar layer thin, regular, electron dense. Warts elongated, more or less cylindrical, homogeneous, electron dense, not erodible.

4. **The Capitotricha type** (Fig. 21D). – Thick, well stratified wall of low to moderate electron density. Fibrillar layer electron dense, sometimes discontinuous. Warts of irregular shape and heterogeneous texture, erodible.

The four types differ from each other in hair wall thickness, stratification and electron density, as well as in shape and composition of warts growing out from the outer layer. In all types two layers and an external fibrillar layer are present.

In the **Lachnum type**, which is the most simple, the inner and outer layer are poorly differentiated and no stratification can be seen within them. The remaining three types are characterised by thick, well stratified walls.

The **Brunnipila type** is distinct because of its extremely high electron density, which masks some fine details of complex stratification within the hair wall layers, particularly in the outer layer. This effect is due to the high uptake of uranyl-lead contrasting by the melanin containing parts of the hair wall (Verkley, pers. comm).

The **Incrucipulum type** shows the clearest stratification combined with comparatively low overall electron density. In both inner and outer layer two strata and lines separating them are clearly visible.

In the first three types the warts have a homogeneous and persisting structure. They protuberate from the outer stratum of the outer layer and have some characteristic features in each type. In the **Lachnum type** their electron density may be slightly lower or slightly higher than that of the hair wall, but they are always separated from the wall itself by a very thin electron dense line.

In the **Brunnipila** and **Incrucipulum** types the warts are distinctly more electron dense than the hair wall itself; in the In-
Fig. 21. – Diagrammatic schemes of the types of the hair walls. – A: *Lachnum*, B: *Brunnipila*, C: *Incrucipulum*, D: *Capitotricha*. – **IL**: inner layer; **OL**: outer layer; dotted line: boundary between inner and outer strata of the hair wall layers.
crucipulum type they clearly originate as protrusions from the outermost stratum of the hair wall, whereas in the Brunnipila type the transverse electron dense bands extend from the warts to the plasmalemma, thus creating a peculiar rooting effect.

The Capitotricha type has thick walls of complex stratification. The strata of the outer layer are, however, not as clearly distinct as in the Brunnipila type. The most original feature of this type, however, lies in the warts, which are composed of heterogeneous, loose, erodible material and eventually disintegrate. The warts are separated from the hair wall by an electron dense line as in Lachnum type.

The hair morphology is not uniform in the taxa having hairs of Lachnum or Capitotricha type and the morphogical differences are easily observed under the light microscope. In all species of Lachnum the hairs are evenly warted, but in the species of Dasyscyphella one or two often slightly swollen apical cells are completely smooth and devoid of any warts (Raitviir, 1977). The hair morphology of Capitotricha and Albotricha is even more divergent. The totally coarsely warted cylindrical hairs of Capitotricha look thick-walled under the light microscope, whereas the conical pointed hairs of Albotricha are thin-walled, always smooth in their upper third and covered with non-persisting warts, dissolving in Melzer's after KOH pre-treatment or in ammoniacal Congo Red, in their lower two thirds.

The ultrastructure of the ascus apical apparatus in the taxa investigated shows some peculiar features that correlate with the hair wall ultrastructure typology. In its general morphology the apical apparatus of genera related to Lachnum belongs to the Verkley’s preliminary type VIII ‘Chlorociboria-Pezizella-Calyccina’ (Verkley, 1995) and agrees to that found in two earlier studied hyaloscyphaceous species, Lachnum virgineum and Trichopeziza mollissima (Verkley, 1996). As noted already by Verkley (1996), however, within this type some fine details are specific to each genus, thus supporting segregation at the generic level and at the same time pointing to the relationships between the genera. In both Lachnum and Dasyscyphella, having the same hair wall structure, the apical apparatus (Figs. 1 & 4) is characterised by long pointed annular protrusions which are short and blunt in the other four genera. At the same time the intergeneric difference lies in more strongly developed apical thickening of Dasyscyphella. In Incrucipulum the apical apparatus is thinner and wider than in other genera and remarkably truncate (Fig. 13). The Brunnipila ascus (Figs. 8 & 9) is also truncate and comparatively wide, but thicker and with even shorter annular protrusions than the Incrucipulum ascus. Capitotricha and Albotricha possess the same ascus wall type with eroding warts and show again remarkable similarity in their apical apparatus. In both the annulus
is long and wide with a wide canal, but in *Albotricha* (Fig. 16) the apical thickening is not so strongly developed as in *Capitotricha* (Fig. 17).

The combined pattern of hair morphology and hair wall ultrastructure complemented with features of the ascus apical apparatus provide a good support to identify the genera *Albotricha*, *Capitotricha*, *Brunnipila*, *Dasyscyphella* and *Incrucipulum* as good taxa, clearly distinct from *Lachnum* s. str. *Dasyscyphella* and *Lachnum* show similarities that suggest a closer relationships between them than to the other genera. *Albotricha* appears to be closely related to *Capitotricha* and its placement into the subfamily Trichopezizelloideae (Raitviri, 1987) as well as the proposed identity of *Albotricha* and *Dasyscyphella* (Weber, 1992) should be rejected.

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**References**


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