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Phylogenetic and structural studies in the Thelebolaceae (Ascomycota)

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Several minute dung-inhabiting discomycetes have been classified in the family Thelebolaceae, which has traditionally been included in the order Pezizales. The non-operculate type-genus *Thelebolus* has recently been excluded from the Pezizales. The phylogenetic distribution of other genera associated with Thelebolaceae is still obscure. We have analysed ca. 580 bp from a variable part of the nuclear SSU rRNA gene from *Ascozonus*, *Caccobius*, *Lasiobolus* and *Thecotheus*, and compared these with ca. 1700 bp sequences from *Thelebolus*, *Pleospora*, Pezizales, Leotiales and Leotiales-related taxa. In the resulting trees, *Ascozonus* and *Caccobius* group with *Thelebolus* and the inoperculate discomycetes; *Lasiobolus* groups with *Ascodesmis*, and *Thecotheus* with *Ascobolus* within Pezizales. SEM pictures of fruit-bodies and ascus apices of *Ascozonus*, and ascospores from *Thecotheus* are presented to illustrate characteristic features of these taxa.

Key Words——coprophilous ascomycetes; fungal systematics; SSU rDNA phylogeny.

Several discomycete genera possess a coprophilous lifestyle. Many of them have minute ascomata with a hyaline, poorly developed excipulum and often with multispored, thick unitunicate or bitunicate asci. An assemblage of these taxa has traditionally been referred to the operculate order Pezizales: to the "Ascobolei spurii" group (Boudier, 1869), the tribe Theleboleae (Kimbrough and Korf, 1967), the subfamily Theleboloideae (van Brummelen, 1967), or the family Thelebolaceae (Eckblad, 1968). The group consists of members with operculate asci, e.g., *Lasiobolus* Sacc., *Coprotus* Korf & Kimbr. and *Thecotheus* Boud., as well as representatives having uni-or bitunicate asci which open with an irregular, vertical split, e.g., *Ascozonus* (Renny) E. C. Hansen, *Caccobius* Kimbr., *Coprobolus* Cain & Kimbr., *Trichobolus* (Sacc.) Kimbr. & Cain and *Thelebolus* Tode. The latest Dictionary of the fungi (Hawksworth et al., 1995) accepts 14 genera in the emended family Thelebolaceae, also including *Chalazion* Dissing & Sivertsen (Dissing and Sivertsen, 1975), *Leptokalpion* Brumm. (van Brummelen, 1977), *Pseudascozonus* Brumm. (van Brummelen, 1987), *Ramgea* Brumm. (van Brummelen, 1992), *Coprotiella* Jeng & J. C. Krug, *Dennisiopsis* Subram. & Chandrash., *Mycoarctium* K. P. Jain & Cain, and *Ochotrichobolus* Kimbr. & Korf (van Brummelen in Dissing and Schumacher, 1994: 400).

The type genus of the family, *Thelebolus*, has been seen as a non-pezizalean taxon based on fruit-body ontogeny and ascus characters. A relationship with Erysiphales (Zukal, 1886; Cooke and Barr, 1964), or, Pleosporales (Samuelson and Kimbrough, 1978) has been suggested. Recent molecular analyses indicate a relationship with Leotiales and Erysiphales (Momol et al., 1996; Landvik et al., 1997).

We have sequenced parts of the nuclear SSU rDNA from another four coprophilous species which have been associated with the family Thelebolaceae. Two of these release their spores through an ascus apical split, *Ascozonus woolhopensis* (Berk. & Br.) Boud. and *Caccobius minusculus* Kimbr.; and two are operculate, *Lasiobolus papillatus* (Pers.) Sacc. and *Thecotheus holmskjoldii* (E. C. Hansen) Chenant. The sequences were compared to homologous sequences from *Thelebolus* and from a selection of six pezizalean, one "loculoascomycete" and nine leotialean and Leotiales-related taxa, addressing the following question: Are the studied species related to *Thelebolus* or to the true pezizalean members as defined in recent classifications?

Additionally, we present scanning electron (SEM) micrographs of fruit-bodies and ascus tops from *A. woolhopensis* and from *T. holmskjoldii* ascospores, to illustrate the characteristic ring-like zone (*Ascozonus*) and the outer spore-coating layer (*Thecotheus*) in these genera.

Materials and Methods

The SSU rDNA sequences included in this study are listed in Table 1. Sequences from *Ascozonus* were obtained both from fresh fruit-bodies and from a culture isolate

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derived from ascospores. The two sequences were identical and, therefore, only one sequence was included in the DNA analysis matrix. One specimen of *T. holmskjoldii* was sequenced, and one was prepared for viewing of ascospores by SEM. Sequencing of the material was performed at two different laboratories: at Umeå University, Sweden (*Ascozonus* and *Thecotheus*) and at the University of Oslo, Norway (*Caccobius* and *Lasiobolus*). In an earlier study we analysed short SSU rDNA sequences from a number of "Pezizalean species" in order to determine their higher systematic position (Landvik et al., 1997, data not shown). In this manuscript we include a phylogenetic analysis based on partial SSU rDNA sequences, to show the usefulness of this genomic region.

DNA extraction and sequencing DNA was extracted from 2–10 fruit-bodies of *Ascozonus* and *Caccobius* collected from fresh dung, from spores of *Thecotheus* that were shot on a cover glass, and from axenic culture isolates of *Ascozonus* and *Lasiobolus*. The extractions followed the instructions of Dynabeads DNAdirect (Dynal) or the silica method described in Landvik et al. (1996).

SSU rDNA from *Caccobius* and *Lasiobolus* was amplified and sequenced manually with the primers 18Sint3 and c20scl (Holst-Jensen et al., 1997). The sequenced fragment corresponds to position numbers 553–1,150 in *Saccharomyces cereviseae* Meyen ex E. C. Hansen, acc. nr. V01335. DNA from *Ascozonus* and *Thecotheus* was amplified with the primer pairs SL1 (Landvik et al., 1996) - KW65 (K. Winka, pers. comm.), covering a fragment corresponding to positions 5–1,306 in *S. cereviseae*, and the primer pairs KW3 (K. Winka, pers. comm.) – NS8

Table 1. Materials used in the study.

Acc.nr	Species
AF010590	*Ascozonus woolhopensis* (Berk. & Br.) Boud.
	Norway, Östfold, Hvaler comm., N. Kirköy, Utengen. 961205.
	On roe-deer dung. Leg. Roy Kristiansen. ARON[a] 2535.
AF010587	*Caccobius minusculus* Kimbr.
	Norway, Östfold, Hvaler comm., N.Kirköy, Utengen. 961205.
	On rabbit dung. Leg. Roy Kristiansen. ARON 2536.
AF010588	*Lasiobolus papillatus* (Pers.) Sacc.
	Norway, Östfold, Hvaler comm., Vesteröy, Kuvauen. 950401.
	On badger dung. Leg. Roy Kristiansen. ARON 2128.
AF010589	*Thecotheus holmskjoldii* (E. C. Hansen) Chenant.
	Norway, Östfold, Hvaler comm., Asmalöy, Skipstadkilen. 9212x.
	On sheep dung. Leg. Roy Kristiansen. RK[b] RK 92.93.
For SEM	*Thecotheus holmskjoldii* (E.C. Hansen) Chenant.
	Norway, Östfold, Hvaler comm., Asmalöy, Viker. 921108.
	On sheep dung. Leg. Roy Kristiansen. RK. 92.84.
Retrieved from GenBank/EMBL	
U45438	*Amylocarpus encephaloides* Currey
L37533	*Ascobolus lineolatus* Brumm.
U53372	*Ascodesmis sphaerospora* Obrist
L26253	*Blumeria graminis* (DC.) Speer f.sp. *hordei*
U53369	*Cyttaria darwinii* Berk.
Z30240	*Cudonia confusa* Bres.
U53379	*Hydnotrya tulasnei* (Berk. & Br.) Berk. & Br.
L37536	*Leotia lubrica* Pers.
U46031	*Microglossum viride* (Pers.) Gillet
Z27393	*Neolecta vitellina* (Bres.) Korf & J.K.Rogers
U53381	*Otidea leporina* (Batch) Fuckel
U53384	*Peziza succosa* Berk.
V05201	*Pleospora herbarum* (Pers.) Rabenh.
U53370	*Rhytisma salicinum* Fr.
L37541	*Sclerotinia sclerotiorum* (Lib.) de Bary
U49936	*Thelebolus stercoreus* Tode
Z49755	*Tuber* cf. *rapaeodorum* Tul.

a) ARON=Herbarium and culture collections of the Ascomycete Research Group, University of Oslo, Norway.
b) Herbarium of Roy Kristiansen.

(White et al., 1990), covering positions 582–1,788. The PCR products were purified by filtration in Centricon 100 concentrators (Amicon). Aliquots of 75 ng of the PCR products were cycle-sequenced using the primers NS1 (pos. 20–38, White et al., 1990), KW3 (pos. 582–601), SL 344 (pos. 1,115–1,134, Landvik et al., 1996), and KW7 (pos. 1,417–1,436, K. Winka, pers. comm.), and purified by Centri-sep columns (Princeton Separations, Inc.) before being run on an ABI 377 Automatic sequencer (Perkin Elmer).

Phylogenetic analyses Partial sequences (ca. 580 bp) from *A. woolhopensis*, *C. minusculus*, *L. papillatus* and *T. holmskjoldii* were manually aligned with the SSU rDNA sequences (ca. 1,700 bp long) from 17 ascomycetes in GenBank, including 15 discomycetes and the bitunicate species *Pleospora herbarum*. *Neolecta vitellina* was used as an outgroup. The matrix was subjected to heuristic parsimony analyses and bootstrap tests of 1,000 replicates using PAUP 3.1.1. (Swofford, 1993). Ten stepwise random taxon addition replicates were performed for each heuristic search. Two parsimony constraints were tested: one grouping the four studied species with *Thelebolus*, and one grouping the four species with Pezizales. Gaps were treated as missing characters. We also studied the effect on the tree topology of including either the full-length sequences from *Ascozonus* and *Thecotheus* or a data matrix limited to ca. 580 bp from all 21 species. In these tests, we used a heuristic search and 100 bootstrap replicates. Finally, using the PAUP* test version 4d59 (Swofford, pers. comm.), we performed a neighbor-joining analysis on the data matrix including the shorter sequences from the four studied species and compared the unconstrained and constrained trees yielded by maximum likelihood (ML). The ML analyses were based on two slightly different matrices 1) the matrix including the short sequences from the four studied species, and 2) a similar matrix, but this time including the full sequence from *Ascozonus*. The constraints forced *Ascozonus* to merge with the included pezizalean taxa. All ML trees were obtained using the maximum likeliihood heuristic search option and the transition/transversion ratio of 2.

The alignment, including the full-length sequences of *Ascozonus* and *Thecotheus*, is available on the Internet at the adress: "http://biologi.uio.no/Saras.html."

Cultivation of *Ascozonus* Axenic culture isolates of *A. woolhopensis* were obtained by pressing an insect needle into a dung-inhabiting fruit-body and transferring the tissue via the needle onto a PDA plate (potato dextrose agar, Difco), with tetracyclin (12.5 mg/L) and streptomycin (25.0 mg/L) added. Pure somatic cultures were transferred to new plates and cultivated at room temperature in daylight. Similar culture procedures with *Caccobius* were not successful.

Scanning electron microscopy *Ascozonus woolhopensis* material from fresh and cultivated fruit-bodies was prepared for SEM. Whole fruit-bodies were detached from the dung with a pair of forceps and transferred to a piece of filter paper folded into a small bag. The bag was closed with a string of sewing thread. Also, a piece of agar containing fruit-bodies was cut out. One fruit-body was squashed to better expose the asci. The bag containing detached fruit-bodies, and fruit-bodies on agar pieces were immersed in 2% glutaraldehyde for 3 d at 4°C, then in 1% osmium tetroxide for 2 h at 4°C prior to stepwise alcohol dehydration (10 min in 70%, 90%, 96% and four × 15 min in 100% ethanol), followed by dehydration in a Balzers Critical Point Dryer. The material was carefully mounted on aluminium stubs with silver conductive paint, sputter-coated with gold-palladium in a Polaron SEM coating unit E 5000 and studied in a JSM 6400 scanning electron microscope. The scanty material of *Caccobius* did not allow a SEM study of the ascocarp, and fresh fruit-bodies of *Lasiobolus* or *Thecotheus* were no longer available. Ascospores from *Thecotheus* were shot onto a cover glass at the time of collection. The cover glass with spores was mounted on a stub and sputter coated with gold-palladium before examination.

Results

The phylogram in Fig. 1 shows one out of four most parsimonius trees (MPTs). *Ascozonus* and *Caccobius* group with *Thelebolus* (supported by 19 synapomorphies and a bootstrap value of 83%) in the Leotiales group, while *Lasiobolus* groups with *Ascodesmis* (11 synapomorphies, 99% bootstrap), and *Thecotheus* with *Ascobolus* (41 synapomorphies, 92% bootstrap) in Pezizales (tree length=484; CI=0.53; RI=0.50). One clade leads to a paraphyletic group of Leotiales and related taxa (9 synapomorphies, 57% bootstrap), and one clade leads to Pezizales (12 synapomorphies, 54% bootstrap). The four trees differed by unresolved branching patterns within the group of *Amylocarpus*, *Sclerotinia* and *Blumeria*, and in the group of *Thelebolus*, *Ascozonus* and *Caccobius*. No differences in tree topology were seen with the inclusion of the full sequences of *Ascozonus* and *Thecotheus* (data not shown). Eight trees, each 29 steps longer than the unconstrained MPT, were obtained by forcing *Lasiobolus* and *Thecotheus* into the *Thelebolus* clade. Forcing *Ascozonus* and *Caccobius* into the Pezizales clade gave two trees, 9 steps longer than the MPT.

A neighbor-joining analysis of the unconstrained matrix yielded a tree that, like the MPT in Fig. 1, also grouped *Ascozonus* and *Caccobius* with *Thelebolus*, *Thecotheus* with *Ascobolus*, and *Lasiobolus* with *Ascodesmis*. The differences between these trees were concentrated on the branching pattern of *Pleospora* and *Peziza* (data not shown).

A parsimony analysis using the limited character set of 580 bp from all species resulted in a changed topology regarding the position of Leotiales and Leotiales-related taxa, whereas the pezizalean lineages maintained identical topology (data not shown). The branch leading to *Lasiobolus* and *Ascodesmis* was supported by a bootstrap value of 98%, the *Thecotheus* and *Ascobolus* branch by 95% bootstrap. The group consisting of *Thelebolus*, *Caccobius* and *Ascozonus* (69% bootstrap

value) appeared as a sister group to the rest of the ingroup and did not cluster within Leotiales.

In a ML test, trees forcing either the partial or the full sequence of *Ascozonus* to merge with pezizalean genera were, in both cases, considered significantly worse than the unconstrained trees (Table 2).

The cultures of *Ascozonus* formed plenty of primordia and fruit-bodies after ca. 3 wk at room temperature (18–21 °C) in daylight. Each ascoma contained many times more asci than did those on dung. We have seen that the ascus top ruptures down to the ring zone in a bilabiate manner (Figs. 6, 7). One ascus, where the opened, split walls were kept together by the apical disc, was also observed (Fig. 7). A single ascoma primordium is shown in Fig. 5.

Discussion

A major goal in this study was to use SSU rDNA sequences to establish the ordinal/familial position of the studied genera. A second objective was to investigate whether a shorter, variable part of the gene sequence would yield sufficient characters for classification of "pezizalean" species at family level or higher. Once the

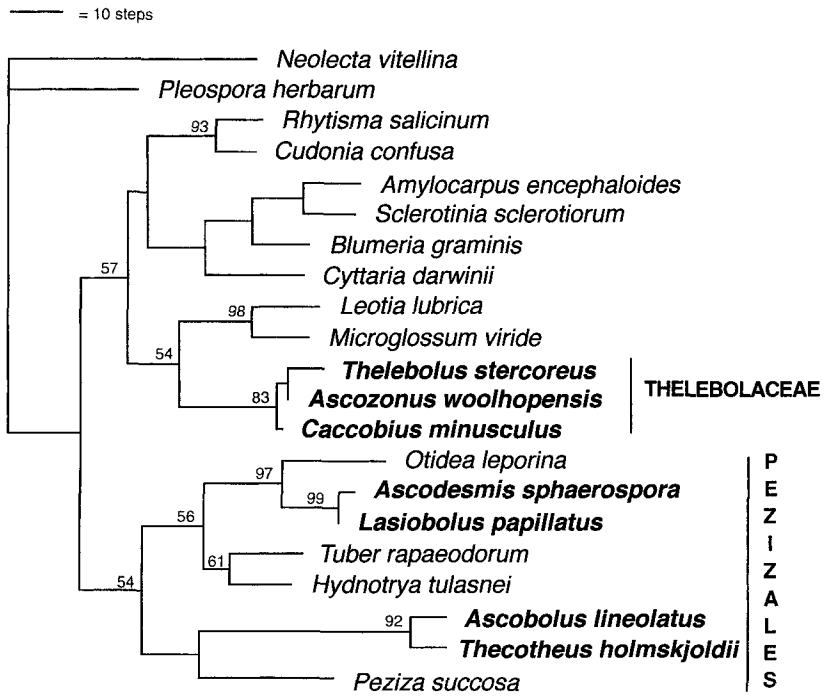


Fig. 1. One of four trees obtained after a heuristic search of ca. 580 bp of SSU rDNA from *Ascozonus woolhopensis*, *Caccobius minusculus*, *Lasiobolus papillatus* and *Thecotheus holmskjoldii* aligned to the whole SSU rDNA gene from the rest of the study group.

Bootstrap values above 50%, from 1,000 bootstrap replicates, are shown above the branches. The non-operculate *Ascozonus* and *Caccobius* group with *Thelebolus* and inoperculate taxa. The operculate genera *Lasiobolus* and *Thecotheus* group with *Ascodesmis* and *Ascobolus*, respectively, of the order Pezizales. The other three MPTs differed in topology within Thelebolaceae and in the internal topology of *Amylocarpus*, *Sclerotinia* and *Blumeria*.

Table. 2. Two data sets, tested by Maximum Likelihood, with constraints forcing *Ascozonus* to merge with the included pezizalean taxa: 1) SSU rDNA sequences (ca. 1,700 bp) from 17 ascomycetes in GenBank, and the NS3-4 region from the studied species. 2) The same matrix as in the previous set, but this time including the full sequence from *Ascozonus* (1,729 instead of 574 bp). Forcing *Ascozonus* to merge with the pezizalean taxa resulted in a significantly worse tree in both cases.

Tree	−ln L	Diff. (−ln L)	S.D. (diff.)	T	*P**	Sign. worse?
Ascozonus NS3-4						
No constraint	6941.60921	(best)				
Constraint	7122.93275	181.32353	34.16953	5.3066	<0.0001	Yes
Ascozonus full SSU						
No constraint	7044.39724	(best)				
Constraint	7248.99406	204.59682	35.73572	5.7253	<0.0001	Yes

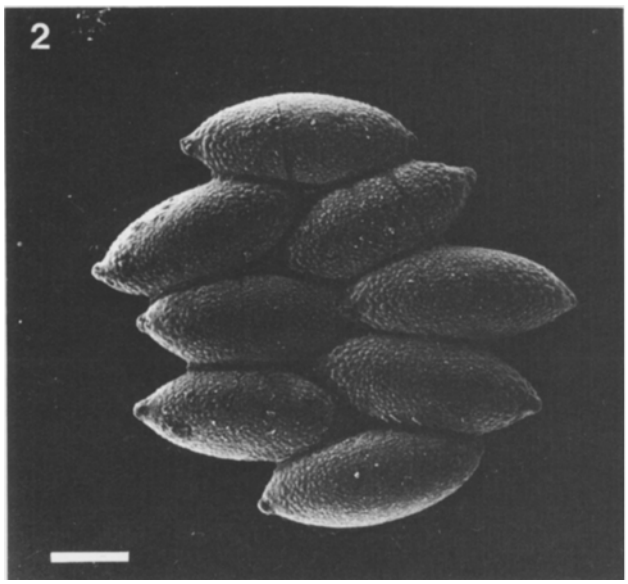
higher position is established, a more variable gene than the SSU rDNA may be chosen for the finer distinctions within group phylogeny. In a previous study of the subordinal grouping of Pezizales taxa, we found that by including the partial SSU rDNA sequence of ca. 580 bp, including helices 22–31 in a SSU rRNA secondary structure model (Van de Peer et al., 1997) (primer area NS3-NS4, White et al., 1990), we obtained sufficient characters to solve most familial associations (Landvik et al., 1997, data not shown). This region, which has been selected and utilized in other fungal evolutionary studies (e.g., Bruns and Szaro, 1992), also provided enough characters to establish the subordinal position of the four "thelebolacean" members included in the present study. By including the restricted sequence region from *Ascozonus* (into a ca. 1,700 bp data set), the same topology was obtained as when the full *Ascozonus* sequence was included, and trees where these sequences were forced to merge with the pezizalean sequences were in both cases considered significantly worse than the unconstrained trees.

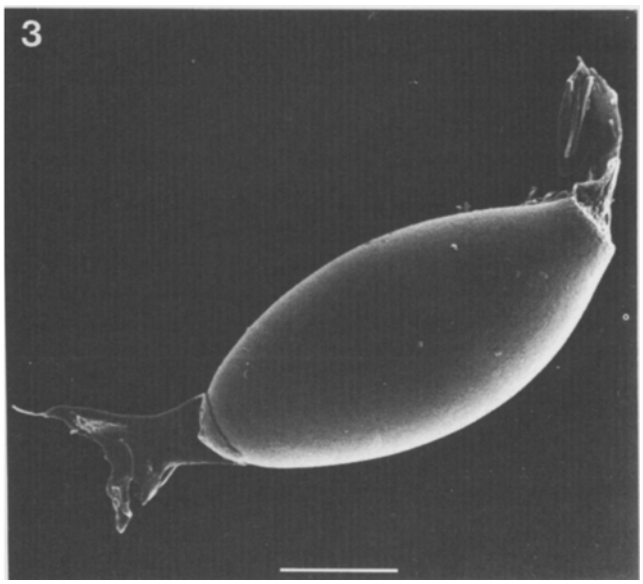
We were not able to resolve the phylogeny within the family Thelebolaceae, neither did we find a stable position for the genus *Thelebolus* (Thelebolaceae sensu stricto). As reported earlier (Momol et al., 1996; Landvik et al., 1997), the genus *Thelebolus* groups with leotialean species, but with low confidence. *Thelebolus*, *Ascozonus*, and *Caccobius* differ from leotialean species by the release of ascospores through a split in the apical ascus instead of the "leotialean pore." Unfortunately, few SSU rDNA sequences are presently available from the Leotiales. Based on available sequences, this order, as classified today, appears paraphyletic. It mixes with members of Erysiphales (Saenz et al., 1994), and probably also with Rhytismatales and Cyttariales (Landvik, 1996).

In our trees, the operculate genera *Lasiobolus* and *Thecotheus* group within the Pezizales, while the genera *Ascozonus* and *Caccobius*, which lack operculum, group with *Thelebolus* and other inoperculate taxa. The possession of an ascus apex that opens with a split, rather than an operculum, seems to be an important character to distinguish the true thelebolacean representatives from the Pezizales. This contrasts the earlier classifications where operculate and non-operculate genera are included in the single family Thelebolaceae.

Lasiobolus *Lasiobolus* was erected for the setose species of *Ascophanus* included in Boudiers "Ascobolei spurii" (Saccardo, 1884). The genus was accepted in the thelebolacean group by Kimbrough and Korf (1967), van Brummelen (1967; in Dissing and Schumacher, 1994: 400) and Eckblad (1968). Conway (1975b) recommended a transfer of *Lasiobolus* to the family Pyronemataceae (Pezizales) tribe Scutellinieae, based on ontogenetic studies of *Lasiobolus ciliatus* (Schmidt ex Pers) Boud. Landvik et al. (1997) suggested that "the genus *Ascodesmis* should be compared with other taxa of the Otideaceae possessing small ascomata and protruding asci, and having a fimicolous habit (e.g., *Lasiobolus* Sacc.)." The finding that *Lasiobolus* and *Ascodesmis* group together on a well-supported branch (99% bootstrap) within the Pezizales, strongly supports such a relationship.

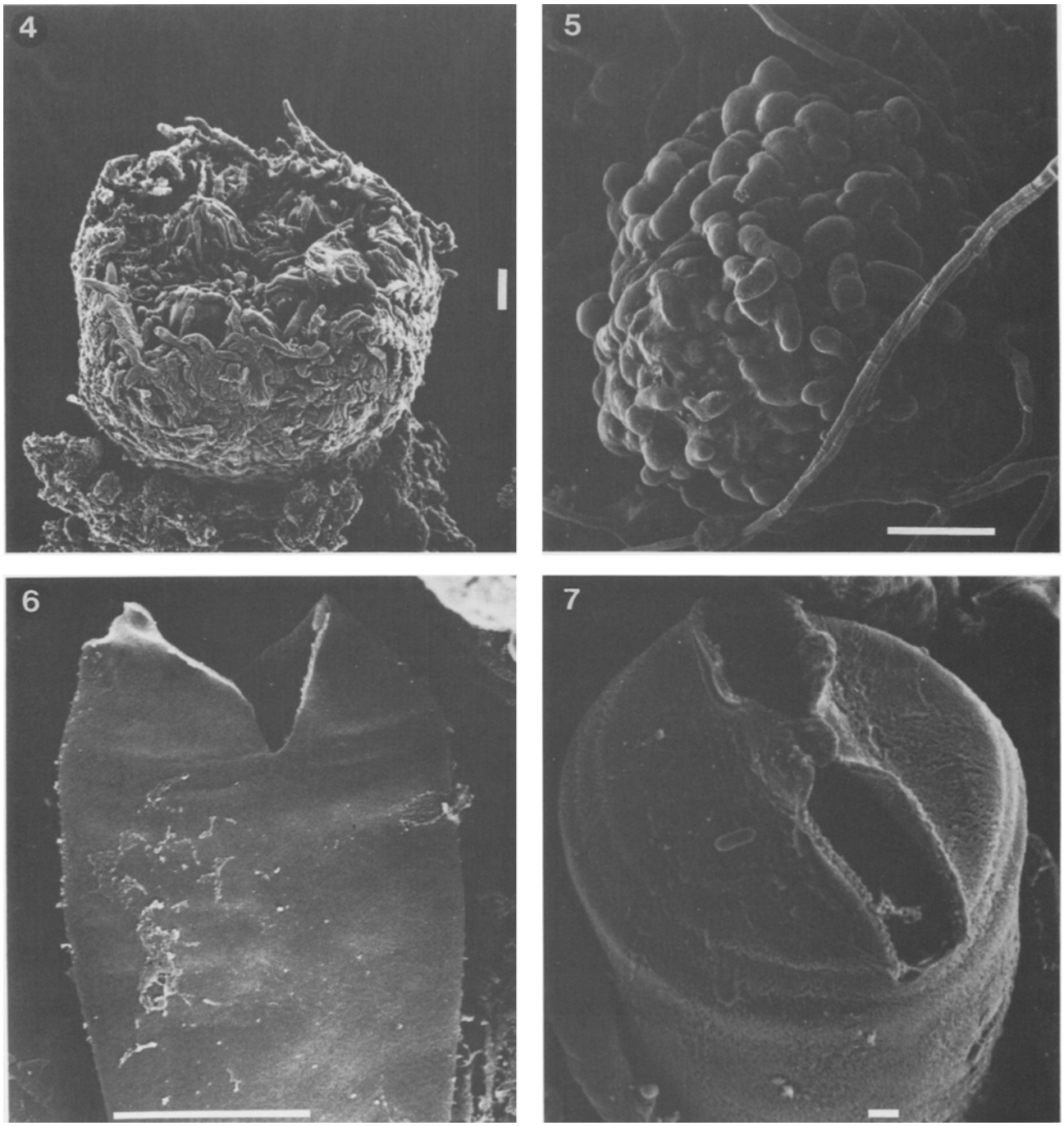
Thecotheus *Thecotheus* is a conspicous member of the Pezizales, having its own anamorphic state and a thick, gelatinous ascospore coating which expands after spore liberation (Conway, 1975a) (Figs. 2, 3). The genus was placed in "Ascobolei spurii" by Boudier (1869) but referred to the tribe Pezizeae by Kimbrough and Korf (1967) because of asci that turn blue in iodine and ascospores with callose-pectic ornamentation. Eckblad (1968) re-introduced *Thecotheus* among the thelebolace-





Figs. 2, 3. Scanning electron micrographs of ascospores of *Thecotheus holmskjoldii*.
2. Ascospores before expansion of the gelatinous coating layer. 3. Ascospore with a detached coating layer. Scale bars: 10 μm.

an taxa on the basis of the diffuse blueing of the asci, the often multispored and protruding asci, similar to the rest of the Thelebolaceae, and the simple excipular structure being unlike that found in the *Peziza* group. Subsequent authors have re-emphasized the relationship of *Thecotheus* to either Pezizaceae (Kimbrough, 1972) or Ascobolaceae (Korf, 1972, 1973; Conway, 1975a; Kimbrough and Curry, 1985; Aas, 1992; van Brummelen in Dissing and Schumacher, 1994: 398). Conway (1975a), while accepting the assignment to Ascobolaceae, also suggested a possible relationship with the Pyronemataceae *sensu* Korf (1972). Our analyses strongly support the inclusion of *Thecotheus* in Ascobolaceae. The clade containing *Ascobolus* and *Thecotheus*



Figs. 4–7. Scanning electron micrographs of *Ascozonus woolhopensis*.
4. Fruit-body removed from roe-deer dung. The marginal hairs are deformed during the fixation procedure. 5. A primordium of a fruit-body formed in culture. 6. An open, split ascus top after spore release. A small apical disc is visible. 7. An incompletely opened ascus top, from above. Scale bars: 10 μm in Figs. 4–6; 1 μm in Fig. 7.

shares as many as 41 synapomorphies and has bootstrap support of 92%.

Ascozonus *Ascozonus* has been included in Ascobolaceae (Kimbrough, 1966; Hawksworth et al., 1995), but has been re-classified in Thelebolaceae in most recent works (e.g., Kimbrough and Korf, 1967; van Brummelen, 1967; van Brummelen in Dissing and Schumacher, 1994: 401; Eckblad, 1968). Our molecular data support the placement of *Ascozonus* within the family Thelebolaceae.

The upper part of the clavate ascus in *Ascozonus* has a characteristic ring-like zone and a papilla with a small apical disc (van Brummelen, 1974). Van Brummelen (1974) showed that the ascus top is first disrupted at the margin of the apical disc, followed by a splitting of the wall in the upper part of the ascus. In this paper, we show a SEM picture of an ascus from *Ascozonus* (Fig. 7), where the split walls are kept together by the apical disc. This may well represent a fixation artefact.

Caccobius *Caccobius* has been seen as an intermediate taxon between *Ascozonus* and *Thelebolus* (Kimbrough and Korf, 1967). It differs from these genera mainly in fruit-body morphology and ascus apical apparatus. In *Caccobius* the ascus opens by an "irregular tear at the (apical) plug" (Kimbrough and Korf, 1967). While *Ascozonus* and *Thelebolus* are common and widespread, *Caccobius* has only been reported once before (from North America). Our *Caccobius* collection was found on rabbit dung in late autumn. At the time of collecting, the weather had been mild for a few days preceded by a period of low temperature (ca. −5°C) for several wk. The fruit-bodies were maintained in a moist chamber at room temperature. Only a few ascomata per fecal pellet were observed. Our specimens match well with the description of *C. minusculus* in Kimbrough and Korf (1967), except that the reddish colour of the fruit-bodies seems to be permanent.

We find strong support for a close relationship of the genera *Ascozonus* and *Caccobius* with *Thelebolus*. These non-operculate genera are all to be excluded from the order Pezizales.

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