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# *Leohumicola*, a new genus of heat-resistant hyphomycetes

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**Abstract:** The new anamorph genus *Leohumicola* (hyphomycetes) is described for four species, including three new species isolated after heat treatment of soils collected in Canada. The species produce slow-growing agar colonies that eventually produce lateral or terminal aleurioconidia, with a dark brown terminal cell, and the remains of a paler basal cell that fractures during secession. The genus is compared with *Humicola*, *Trichocladium*, *Thermomyces*, *Complexipes* and some other morphologically similar genera. Nuclear ribosomal small subunit (SSU) ribosomal DNA sequences demonstrate that *Leohumicola* is a monophyletic group in the *Leotiomycetes*, distinct from *Humicola* and *Trichocladium* (*Sordariales*), and *Thermomyces* (*Eurotiales*). Internal transcribed spacer sequences (ITS) support our recognition of four species of *Leohumicola*, each with distinct colony and micromorphological characters. The existence of additional species is probable based on our own ITS sequences and some retrieved from GenBank. The type species *L. verrucosa*, was recovered from a variety of soil types across Canada, and has sympodially proliferating conidiogenous cells that produce conidia with verrucose terminal cells that measure 4–5.5 x 4–5.5 µm. The SSU of some strains of this species have five long Group I introns that extend the length to more than 3700 nt. *Leohumicola lenta* produces very slowly growing colonies on agar media and larger conidia than *L. verrucosa*, and *L. terminalis* produces only terminal conidia. The latter two species are represented by single strains. The fourth species, *L. minima* is based on *Trichocladium minimum*, originally isolated from volcanic ash soil from Chile. Internal transcribed specer (ITS) sequences suggest that *Humicola* is a synonym of *Trichocladium*, a finding that may require conservation of *Humicola*. Dichotomous keys are provided to the accepted species of *Leohumicola*, and to morphologically similar aleurioconidial genera.

**Taxonomic novelties:** *Leohumicola* N.L. Nickerson, Hambleton & Seifert gen. nov., *Leohumicola verrucosa* N.L. Nickerson, Hambleton & Seifert sp. nov., *Leohumicola lenta* Hambleton, Seifert & N.L. Nickerson sp. nov., *Leohumicola minima* (de Hoog & Grinbergs) Seifert & Hambleton comb. nov., *Leohumicola terminalis* Hambleton, Seifert & N.L. Nickerson sp. nov.
**Key words:** aleurioconidium, chlamydospore, *Culcitalna*, Group I introns, *Humicola*, *Leotiomycetes*, *Thermomyces*, *Trichocladium*.

## INTRODUCTION

For several years, one of us (NLN) has been isolating fungi from soils of agricultural sites exposed to high heat during periodic burning of crop debris. This is done, for example, with commercial lowbush blueberry fields in eastern Canada. After this treatment, soils are enriched for heat-resistant fungi that survive the heat levels produced by fires. Concentrations of propagules often exceed 1000 colony-forming units (cfu)/g soil. Similar or identical species occur in forest soils and other non-agricultural settings (Nickerson, unpubl. data). For many of these microbes, the heat-resistant structures seem to be thick-walled, melanised “chlamydospores” or “aleurioconidia.” Some species produce synanamorphs that facilitate identification, such as the recently described genus *Devriesia* Seifert & N.L. Nickerson, which includes species with *Cladosporium*-like mononematous anamorphs in addition to multi-celled “chlamydospores” (Seifert *et al*. 2004).

Many cultures, however, lack distinctive synanamorphs and must be characterised on the minimal morphology of their “chlamydospores” or “aleurioconidia”.

Some clarification of terms is necessary for studies of such morphologically reduced anamorphs. Both “chlamydospores” and “aleurioconidia” have been used for thick-walled, lateral or terminal conidia that secede by dissolution of part of the wall of a basal cell, a process now called “rhexolytic secession”. Participants of the influential “first Kananaskis conference” (Kendrick 1971) were proponents of terminology reflective of ontogenetic events, as opposed to terms indicating general morphological similarities. They provided the following definition of chlamydospore: “a thick-walled, thallic, terminal or intercalary spore,” with the implication that the resulting spores are not dispersed and do not secede until the adjacent hyphal cells dissolve away, and are hence “resting spores”. Hughes (1985) provided a precise ontogenetic definition for chlamydospore,

based on the structures originally denoted by the term, produced by agarics now classified in *Asterophora* Ditmar (anamorph: *Ugola* Adanson). By these criteria, true chlamydospores do not occur in the *Ascomycetes* but no alternative term has been proposed. Thus, we will use the term in the sense of Kendrick (1971). "Aleurioconidium"was rejected during the Kananaskis discussions (Kendrick 1971), but we will use the term for solitary, holothallic or monoblastic conidia that are released by rhexolytic secession. These are propagules, i.e., they are dispersed. They tend to be produced on somewhat differentiated conidiophores or from conidiogenous cells, rather than in or on otherwise vegetative mycelium. Perhaps careful ontogenetic studies of these structures, as undertaken by Hughes (1985) for *Asterophora*, combined with phylogenetic analysis, will provide more taxonomic resolution than is possible through consideration of mature spores alone.

Should such reduced phenotypes be used as the morphological basis for generic names of fungi that are known to be phylogenetically distinct? Most anamorph generic names refer to conidial morphs, but there is a long history of applying anamorph names to vegetative mycelium, and structures such as cystidia or sclerotia. Some of these well-known anamorph generic names set precedents for our consideration of chlamydosporic or aleurioconidial genera. The important anamorph genus *Rhizoctonia* DC is based on characteristically swollen, sterile mycelium. *Rhizoctonia sensu lato* now is subdivided into segregate genera that correlate with teleomorph groups (Roberts 1999) in the basidiomycetes, and ascomycetes, with *Rhizoctonia sensu stricto* restricted to some anamorphs of the *Ceratobasidiaceae*. All of these genera have *Rhizoctonia*-like hyphae, and are distinguished by ultrastructural characters, teleomorph connections, and molecular phylogenies, and to a certain extent by the presence or absence of clamp connections and by ecological characters. The segregates were proposed and accepted because there was a need to distinguish ascomycetes and basidiomycetes, and pathogenic from mycorrhizal and saprobic species. The parallels between this situation and that pertaining to chlamydosporic or aleurioconidial morphs should become obvious in this paper. In particular, our study focuses on fungi placed in, or potentially compatible with, two of the largest such traditional genera, *Trichocladium* and *Humicola*.

Many fungi isolated from soils can be described as *Humicola*- or *Trichocladium*-like, on the basis of the lateral or terminal production of brown aleurioconidia on minimally differentiated conidiophores. *Humicola* Traaen includes about 20 described species (Nicoli & Russo 1974, Bertoldi 1976), many poorly understood in modern terms. The type species, *H. fuscoatra* Traaen[1], and a second species, *Humicola grisea* Traaen[1], are common, cosmopolitan soil fungi (Domsch *et al*. 1980). Both species produce spreading colonies on agar media and usually form single-celled, brown or blackish aleurioconidia in the aerial mycelium and also within the medium (Fig. 1G–K). *Humicola grisea* also produces chlamydospores that are solitary or in chains. They are similar in size and pigmentation to the aleurioconidia. The frequency of occurrence of *H. fuscoatra* and *H. grisea*, combined with the ability of these species to produce cellulases and some antibiotics, has meant that the generic name *Humicola* has become relatively well-known. *Trichocladium* Harz includes 18 species with septate, dark brown conidia (Goh & Hyde 1999). The type species, *Tr. asperum* Harz, produces spreading colonies on agar media, and the rough-walled, 2- to 3-celled aleurioconidia have germ pores (Fig. 1L–P). *Humicola fuscoatra*, *H. grisea* and *Tr. asperum* all have acremonium-like synanamorphs, or more accurately, synanamorphs resembling the heterogeneous, non-hypocrealean *Acremonium* section *Chaetomioidea* G. Morgan-Jones & W. Gams, characterised by broad-based phialides and catenulate conidia. A third similar genus is *Thermomyces* Tsiklinsky, with four known species (Domsch *et al*. 1980). In the type species, *T. lanuginosus* Tsiklinsky, rough-walled, dark, aseptate aleurioconidia are produced at the end of a hyaline conidiogenous cell (Fig. 1Q–U). This thermophilic species lacks a synanamorph, and the conidia lack germ pores. However, some other species of *Thermomyces* have acremonium-like synanamorphs (Hennebert 1971).

The present paper deals with a set of aleurioconidial, humicola-like and trichocladium-like isolates that form ericoid mycorrhizas (Nickerson, unpubl. data). They are shown to belong to the *Leotiomycetes*, clustering around a set of strains isolated from soil or plant roots in other studies. To provide the context for the recognition of these strains and some related species as a new genus, morphological and phylogenetic reconsiderations of *Humicola*, *Trichocladium* (plus one of its synonyms, *Culcitalna* S.P. Meyers & R.T. Moore) and *Thermomyces* were undertaken. The morphological studies compared type species of the relevant genera, and included new studies of the ontogeny of their aleurioconidia. The phylogenetic studies employed parsimony analysis of aligned small subunit (SSU) and internal transcribed spacer (ITS) ribosomal DNA sequences.

[1]Both *H. fuscoatra* and *H. grisea* have several described varieties or *formae*. In this paper, our use of these names always refers to the type variety and/or *forma* of these species.

## MATERIALS AND METHODS

Cultures from heated soil (Canadian Collection of Fungal Cultures, Ottawa, Canada [CCFC] cultures with "DAOM" accession numbers DAOM 226889, 231142–231145, and 231147–231149) were isolated from soil samples that were exposed to 75 ºC for 30 min, followed by dilution plating using the methods described by Seifert *et al*. (2004). Cultures from unheated soil, DAOM 230084 and 230085, were isolated by using soil washing (10 cycles of washing a small amount of soil in 1 mL sterile distilled water in 2 mL microfuge tubes, centrifugation, and resuspension of the pellet) followed by plating of individual soil particles. A single culture from surface sterilised ericoid mycorrhizal roots, DAOM 231141, was isolated following the methods outlined in Hambleton & Currah (1997) by G. Hill-Rackette at the University of Alberta.

Ten isolates from soil and one from roots were examined and compared to reference cultures of the type species of the genera *Culcitalna*, *Humicola*, *Trichocladium*, and *Thermomyces*, and to *H. grisea* and *Trichocladium minimum*, obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands (Table 1). Cultures were deposited in CCFC; representative strains of the new species were also deposited in CBS. Colony and microscope descriptions are based on malt agar (MA, 2 % malt extract and 0.75 % agar, Difco Laboratories, Detroit, MI), potato-dextrose agar (PDA, Difco Laboratories, Detroit, MI), and oatmeal agar (OA, Samson *et al*. 2000) at room temperature (about 25 ºC) under ambient light conditions in the laboratory. Cultures were inoculated using three agar plugs per plate. For *Leohumicola* spp. only, additional PDA plates were inoculated with a suspension of mycelium macerated in sterile water in a 1.5 mL microfuge tube using a micropestle, and then spread over the agar surface. Microscopic measurements were made in lactic acid. Means and standard errors presented are based on 25 measurements. Colour codes and capitalised colour names refer to Kornerup and Wanscher (1978).

Small subunit and / or ITS sequences were determined for the strains listed in Table 1. Genomic DNA was extracted from mycelium grown on PDA or MA using an UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA, U.S.A.). DNA amplification and cycle sequencing reactions were performed on a Techne Genius™ thermocycler (Techne Inc., Burlington, NJ, U.S.A.). PCR reactions were performed in 25 ìL volumes using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway NJ, U.S.A.) and 2 µL of template DNA. PCR cycling parameters included 30 cycles of denaturation at 95 ºC for 1.5 min, annealing at 56 ºC for 1 min, and extension at 72 ºC for 2 min, with an initial denaturation of 4 min and a final extension step of 10 min. Primers NS1 and ITS4 (White *et al* 1990) were used to amplify the SSU and the complete ITS region, including the ITS1, 5.8S and ITS2. Amplified products were purified using the UltraClean™ Microbial PCR Purification Kit (Mo Bio Laboratories) and DNA concentrations were estimated from fragments stained by ethidium bromide and separated by agarose gel electrophoresis.

Sequencing reactions were performed using the BigDye™ Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA, U.S.A.) with the recommended cycling parameters. Reactions were purified by ethanol/ sodium acetate precipitation and resuspended as recommended for processing on an ABI PRISM® 3100 DNA Analyzer (Applied Biosystems). Sequencing primers were selected from the SSU and ITS primers given in White *et al*. (1990) and Landvik *et al*. (1997) plus two additional primers, NS18mun (5' CTTGTTACGACTTTTACTTCC) and NS151mun (5'GAAACTCACCAGGTCCAGACA) primer sequences courtesy of Keith Egger, University of Northern British Columbia). Consensus sequences were determined from overlapping sequence data for both DNA strands, except where noted, using the software Sequencher™ (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

To examine phylogenetic relationships, DNA sequences were manually aligned in two separate data matrices using Se-Al (Sequence Alignment Program v1.d1; Rambaut 1996). Seven new SSU sequences were aligned with 77 sequences retrieved from GenBank, chosen from seven classes of *Pezizomycotina* to represent the phylogenetic diversity of ascomycetes. Representatives of the *Saccharomycotina* and *Taphrinomycotina* served as outgroup taxa. Twelve new ITS sequences for *Leohumicola* spp. were aligned in a second data matrix with 23 GenBank sequences, identified as being closely related based on searches using Gapped-BLAST (Altschul *et al*. 1997). The only sequences with high similarity to *Leohumicola* were for unidentified fungi isolated from mycorrhizal roots into pure culture or from cloned PCR products amplified from DNA extracted directly from roots or soil. *Bisporella citrina* (Batsch) Korf & S.E. Carp. AF335454 and *Neofabraea malicorticis* (H. Jacks.) Nannf. AF281386 were selected as outgroup taxa because they were among the few sequences of named teleomorphs in the BLAST search list. Accession numbers for the sequences retrieved from GenBank are given in Figs 3 and 4, and the alignments are deposited in TreeBASE (http://www.treebase.org/treebase/), Study Accession No. S1395. Both data matrices were subjected to parsimony analysis using the heuristic search option of PAUP* v. 4.0b10 (Swofford, 1999)

with simple stepwise addition of taxa, tree bisection-reconnection (TBR) branch swapping, and gaps treated as missing data. Bootstrap percentages used to assess support for the branching topologies were determined from 1000 resamplings of each dataset using the full heuristic search option (ITS) or the "fast" stepwise-addition option (SSU), with simple stepwise addition.

## RESULTS

### Development of aleurioconidia

We compared the morphology and ontogeny of conidia of our strains with that of the type species of *Humicola*, *Trichocladium*, and *Thermomyces*, and the common soil fungus *Humicola grisea*. In the species for which we have the most cultures (described as *Leohumicola verrucosa* below), initials were more or less cylindrical and hyaline, then swelled into clavate to ellipsoidal cells (Fig. 1A). The initials sometimes emerged directly from the conidiogenous hyphae and were delimited by a basal septum, but a cylindrical extension of the conidiogenous hypha was often produced before the delimiting septum of the conidium was laid down (Fig. 1B). Pigmentation was initially uniform. As the central septum of the conidium developed, the terminal cell began to swell (Fig. 1C). The terminal cell of the conidium continued swelling, became darkly pigmented (Fig. 1D) and usually conspicuously roughened (Fig. 1E–F). Conidial secession was rhexolytic, with the rupture occurring at any point in the basal cell, but usually very close to the bottom. The empty remains of the body of the basal cell usually remained attached to the detached terminal cell (Fig. 1F). Therefore, the conidia were two-celled during development, but one-celled after secession. After secession, the cylindrical extension of the conidiogenous hyphae remained like a broad, flat-topped denticle (Fig. 6F, arrows). In some cultures, 1–5 sympodial proliferations of this cell occurred, resulting in a cluster of 2–6 conidia arising from a compact cluster of broad denticles (Fig. 6C–D).

In both *Humicola* species, the distinction between aleurioconidia and chlamydospores was difficult to make, because the pigmentation, dimensions and shapes of the structures were comparable. In *H. fuscoatra*, the aleurioconidia were light olivaceous brown, apparently blastic, and terminal or lateral on hyphae, whereas the chlamydospores were intercalary and apparently thallic. The structures of *H. grisea* were larger than those of *H. fuscoatra*. They were therefore chosen for illustration. In *H. grisea*, the aleurioconidial initials were cylindrical, hyaline, and initially 1–2–septate. The terminal cell swelled, was clavate at first (Fig. 1G), and then became globose (Fig. 1H); sometimes, lateral conidial initials arose directly from conidiogenous hyphae, enlarging to become globose. The thickening of the wall was synchronous with the development of pigmentation (Fig. 1I–J). Germ pores or wall thinnings became visible only when pigmentation and wall thickening were complete, but before secession occurred (Fig. 1K, arrow). The behaviour of the basal cells of *H. grisea* was variable, which resulted in a great variety of conidial forms. Usually, the basal cells were stalks that sometimes developed secondary septa. They remained hyaline or became pigmented and thick-walled. In the latter case, one or more of these cells sometimes became incorporated into the material differentiating as conidia, which then developed as a monilioid chain of brown cells. Sometimes two closely spaced septa developed (as in *Tr. asperum*, see below), and this was the 'separating cell' where secession occurred. Rhexolytic secession usually occurred below the most basally situated pigmented cell. In most strains, the majority of the conidia were single-celled during development and after secession.

In *Tr. asperum*, development of aleurioconidia (Fig. 1L–P) was initially very similar to the process observed in *H. grisea*. The two closely spaced septa that delimited the 'separating cell' (Fig. 1M, O) were more regularly produced in the former fungus than in the latter, and the number of cells in the conidia (usually 2, but from 1–3) was the same as the number of septa above the separating cell. The development of roughening on the conidial cells (Fig. 1O) began later than the pigmentation and thickening processes, but was complete before secession. After rhexolytic secession, the conidium carried a short, tubular remnant of the separating cell (Fig. 1P). In contrast to *L. verrucosa* and in common with *H. grisea*, *Tr. asperum* formed aleurioconidia that had the same number of cells in the conidial initial as was seen in the detached conidium.

The ex-type strain of *Th. lanuginosus* (CBS 632.91) had discrete, lateral conidiogenous cells giving rise to solitary, single-celled, blastic conidia. This conidium was initially hyaline (Fig. 1R), but became rough-walled (Fig. 1S), and then pigmented (Fig. 1T) while still attached to the conidiogenous cell. We observed both apparently schizolytic and rhexolytic secession of mature conidia in this strain. The part of the conidiogenous cell that remained after rhexolytic conidial secession was quite short (Fig. 1 U), generally a frill less than 1 µm long. The conidia of *Th. lanuginosus* remained single-celled from their initiation until their secession.

### Phylogenetic analysis

SSU data were determined for four strains of *Leohumicola*, and five strains representing morphologically similar genera (Table 1). The total length of the SSU sequence determined for DAOM

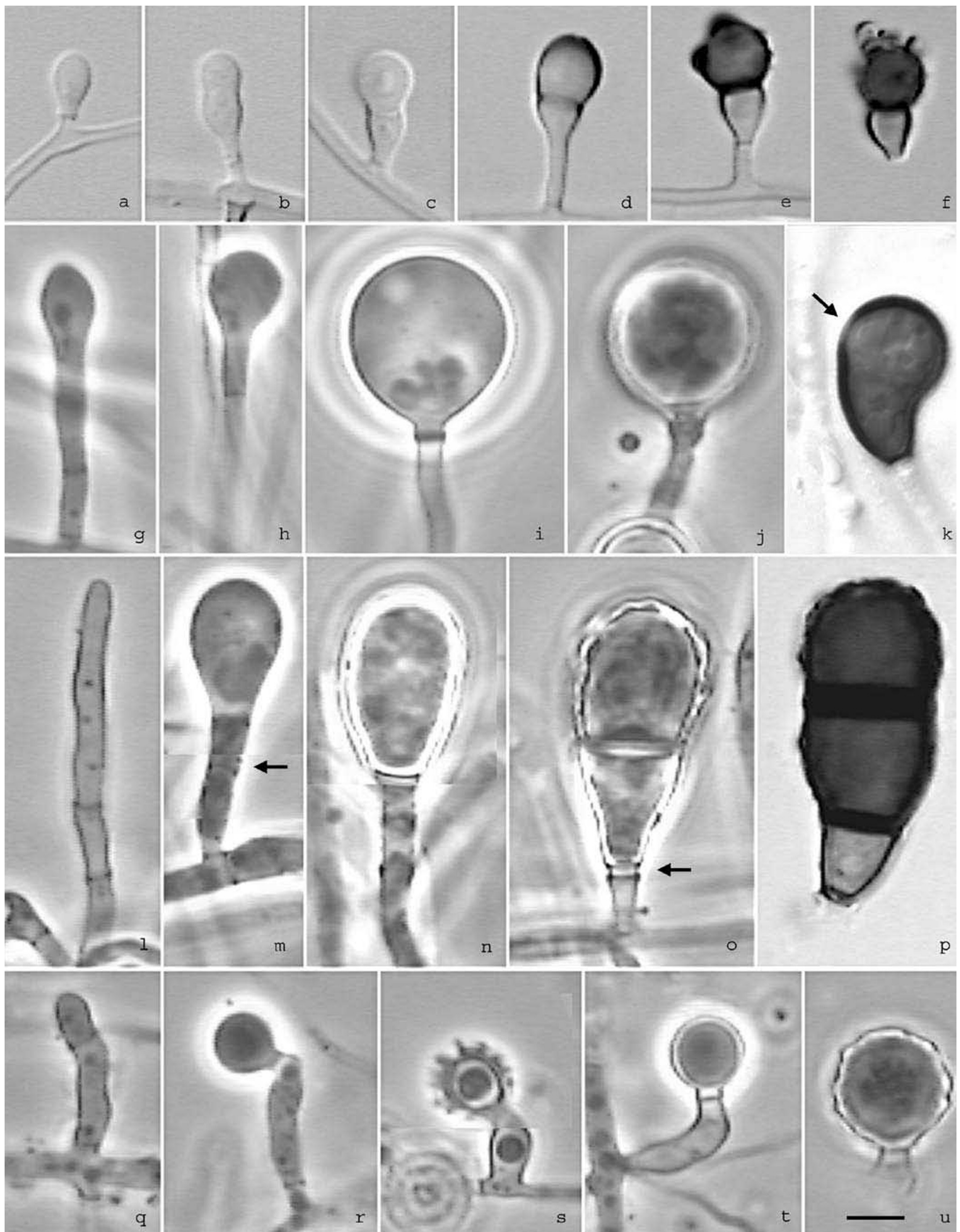


**Fig. 1.** Conidial ontogeny in the four genera of aleurioconidial fungi considered in this study, showing progression of initials, delimitation of conidia, development of septa, pigmentation and roughening, and freed mature conidia. A–F. *Leohumicola verrucosa* DAOM 231143 on PDA. G–K. *Humicola grisea* DAOM 232586, arrow in K indicating germ pore or area of wall thinning. L–P. *Trichocladium asperum* DAOM 67952, arrows in m and o indicating the location of the double septa of the eventual site of rhexolytic secession. Q–U. *Thermomyces lanuginosus* DAOM 232588. Scale bar = 5 µm (shown in U).

226889, the ex-type culture of the fungus described below as *L. verrucosa*, was 3571 nt, and included five Group I introns that were removed prior to phylogenetic analysis. The edited sequence was 1726 nt long and was complete to the 3´ end. The SSU data for DAOM 230085 were identical to those of 226889, and included the same five Group I intron sequences. The edited SSU data for DAOM 230084 differed at three positions and the complete sequence contained 4 insertions of similar size in the same locations as introns 2–5 of DAOM 226889 and 230085, but the intron sequences themselves were substantially different.

Introns were located by comparing the sequence with a range of SSU sequences using the large gap alignment function of Sequencher™ and then delimited based on the typical Group I splice junctions (Holst-Jensen *et al*. 1999) observed at the insertion sites. The positions of the intron sites, defined by the number of the nucleotide located on the 5´ side of their insertion positions in the complete SSU rDNA sequence *Escherichia coli* Migula (J01695) were: 516, 789, 943, 1199, and 1506.

Fig. 2 shows the lengths of the amplified NS1-ITS4 PCR products for strains of *Leohumicola* spp. The total NS1-ITS4 sequence determined for DAOM 226889 was 4064 nt (Fig. 2, lane 5). By inference, the ex-type of our new species *L. terminalis* (lane 4) and all strains of *L. verrucosa* have five SSU introns (lanes 5–10), except DAOM 231141 (lane11), which is shorter and appears to have three. The PCR amplicon for the ex-type of the new species *L. lenta* (lane 3) is longer, which suggests the presence of an additional intron, but may also reflect increased length(s) of individual introns. Intron 1 was lacking in DAOM 230084, accounting for the slightly shorter PCR fragment visible in Fig. 2 (lane 12), while that of 231148 is of similar length (lane 13).

No introns were detected in the SSU sequences determined for the other species, all complete to the 3´ end. The comparative size of the PCR amplicon for these fungi is illustrated by *L. minima*, total NS1-ITS4 sequence determined of 2,218 nt (Fig. 2, lane 2). The SSU sequence alone for *L. minima*, was 1726 nt long, differing from *L. verrucosa* DAOM 226889 at three positions. *Culcitalna achraspora*, and *H. fuscoatra* were 1723 nt; *Th. lanuginosus* was 1724 nt; *Tr. asperum* and *H. grisea* were 1723 nt and identical.

The complete ITS1/5.8S/ITS2 sequences for *Leohumicola* spp. were 463 nt, except for DAOM 230084 which was 462 nt. Strains of *L. verrucosa* differed among themselves at nine positions, of which seven were in the ITS1; DAOM 226889, 230085 and 231143 were identical. The complete ITS sequence for *Th. lanuginosus* was 537 nt, while those of *H. grisea* and *Tr. asperum*, were 472 nt and identical. Additional strains of *H. grisea* (DAOM 187695, 226840) and *Tr. asperum* (DAOM 67952, 226842) were sequenced to confirm this result and all had identical ITS sequences. ITS sequences were deposited in GenBank with the corresponding SSU data for each strain, where determined, as one accession per strain. For *Leohumicola* spp., only the edited SSU sequences for DAOM 226889 and 230084 were deposited, because of the poor double stranded coverage of the intron regions.

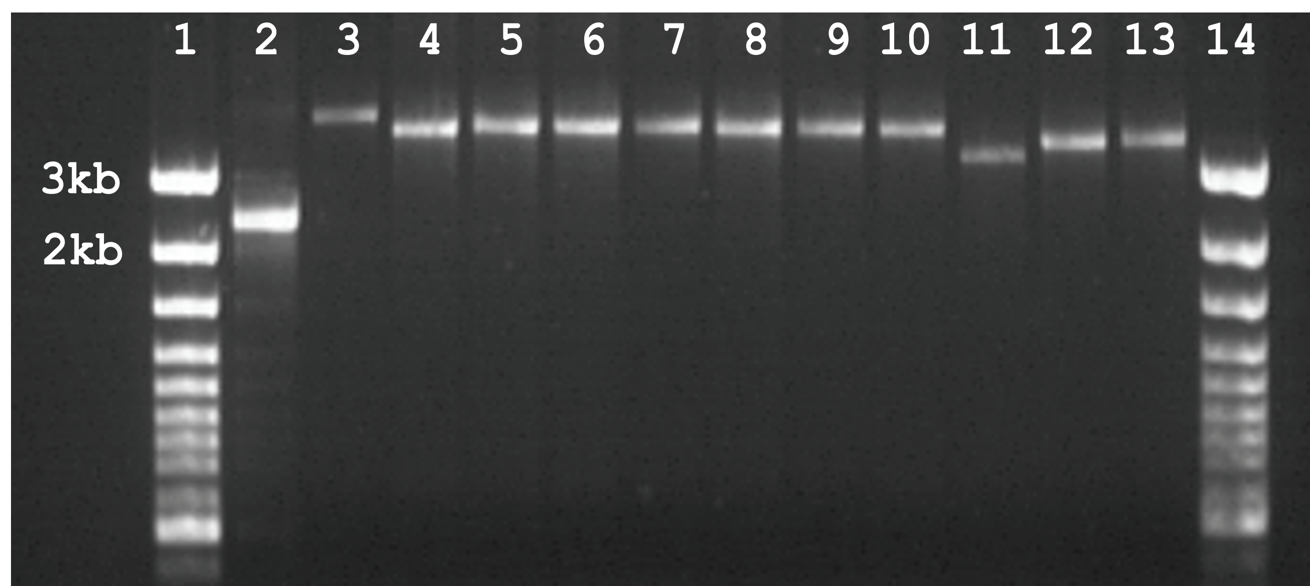


**Fig. 2.** PCR products from DNA extracted from *Leohumicola* spp., amplified using primers NS1 and ITS4, stained by ethidium bromide and separated by agarose gel electrophoresis. Lanes 1 and 14 give a size standard in kilobases. Lanes 2 to 13 were loaded as follows: 2. *L. minima* (NS1-ITS4 sequence determined = 2,218 nt); 3. *L. lenta*; 4. *L. terminalis*; 5. to 11. *L. verrucosa* 226889 (4,064 nt), 230085, 231147, 231144, 231143, 231142, 231141; 12. *Leohumicola* sp. 230084; 13. *Leohumicola* sp. 231148.

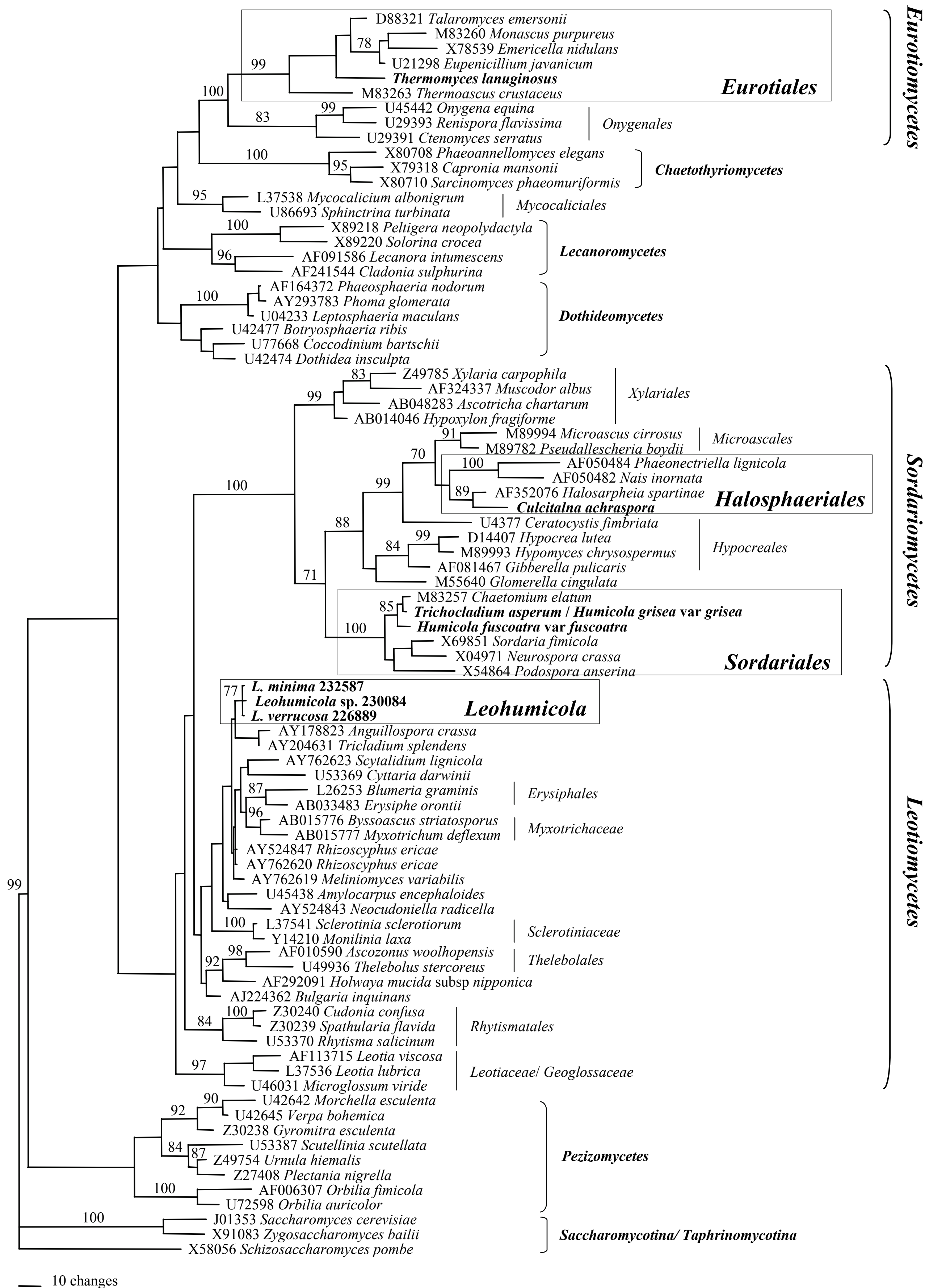


**Fig. 3.** One of 128 equally parsimonious trees based on a heuristic analysis of small subunit rDNA sequences of *Leohumicola* spp. with 81 representatives from seven classes of *Pezizomycotina* (2115 steps, CI 0.451, RI 0.727). The phylogenetic hypothesis shows the placement of *Leohumicola* and the type species of *Humicola*, *Culcitalna*, *Thermomyces*, and *Trichocladium* (names in bold type) among three classes of ascomycetes. Bootstrap support values over 70 % from 1000 replicates of a "fast" stepwise-addition search are shown.

The SSU data matrix comprised 84 taxa and 1793 aligned characters, of which 461 were parsimony-informative, 1101 were constant, and 197 were parsimony-uninformative; 34 ambiguously aligned characters were excluded. Parsimony analysis resulted in 128 equally parsimonious trees (MPTs) of 2115 steps. Results of a bootstrap analysis are shown on one MPT (Fig. 3). *Leohumicola* spp. formed a monophyletic group with moderate bootstrap support of 77 %, within a larger clade corresponding to the *Leotiomycetes*, comprising inoperculate discomycetes, related anamorphs and species of *Myxotrichaceae* and *Erysiphales*. Several subclades were supported by bootstrap, those corresponding to the *Myxotrichaceae*, *Erysiphales*, *Leotiaceae*/*Geoglossaceae*, *Sclerotiniaceae*, *Rhytismatales*, and *Thelebolales*. The *Leotiomycetes* clade and all branches within it were retained in the strict consensus except the ones defining the *Bulgaria*/*Holwaya*/*Thelebolales* group and the *Scytalidium*/*Cyttaria* group (data not shown).

*Trichocladium asperum* and the two species of *Humicola* examined grouped with *Chaetomium elatum* Kunze & J.C. Schmidt M83257 with 85 % support in a well-supported clade (100 %) corresponding to the *Sordariales*. *Culcitalna achraspora* grouped with *Halosarpheia spartinae* (E.B.G. Jones) Shearer & J.L. Crane AF352076 with moderate support (89 %) and, together with a clade comprising two other species in the *Halosphaeriales* (100 %), was sister to the *Microascales* (91 %). These taxa were nested within

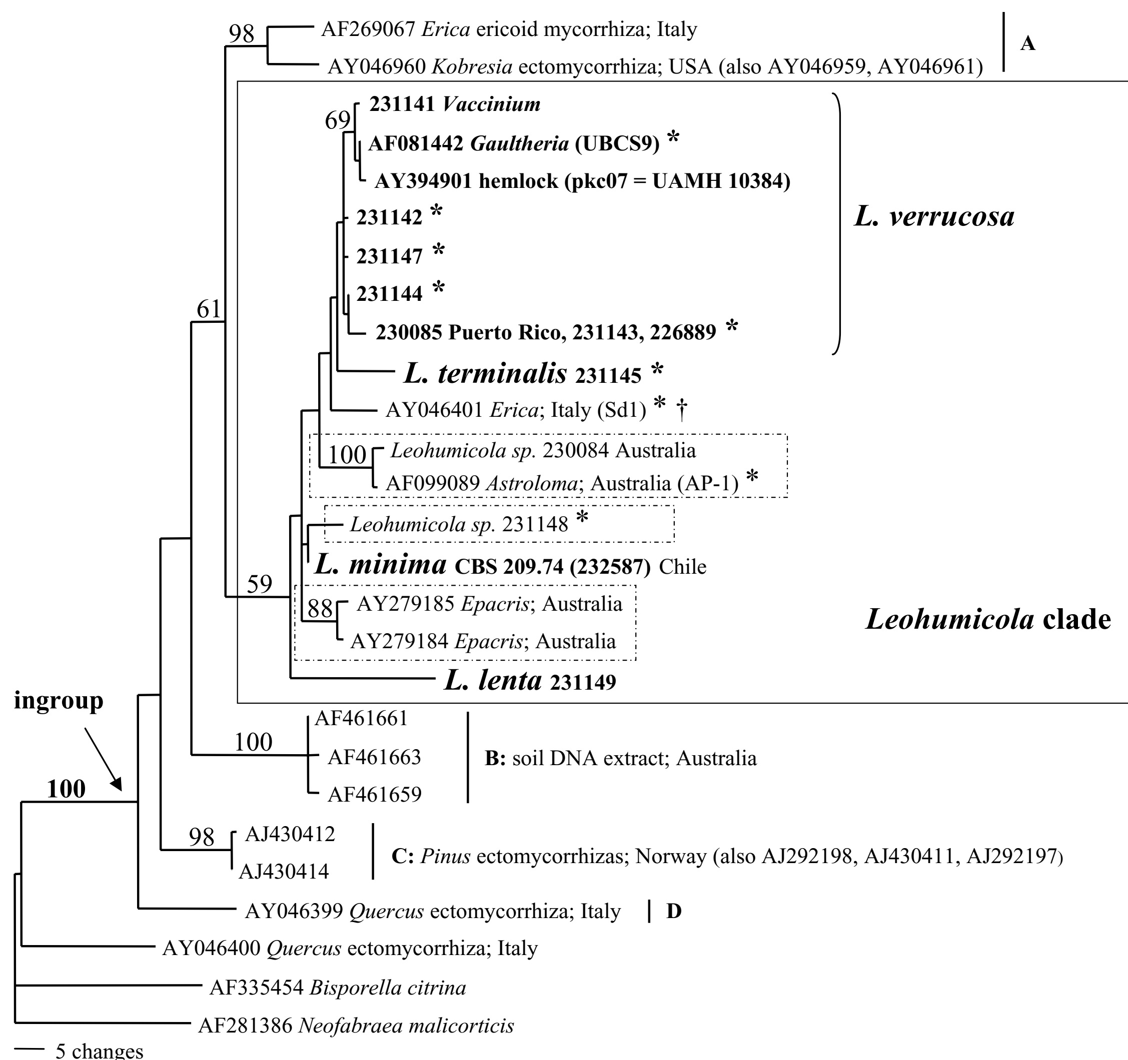


**Fig. 4.** One of 106 equally parsimonious trees based on a heuristic analysis of ITS sequences for four species of *Leohumicola* with taxa hypothesized to be related (250 steps, CI 0.732, RI 0.756). Not shown is sequence AY394888 from hemlock (*Tsuga*) roots, which is identical to included sequence AF081442 from salal (*Gaultheria*) roots. Six-digit numbers are DAOM culture accession numbers (Table 1). Bold type indicates isolates in named *Leohumicola* species. Boxes with dashed lines indicate taxa that may be additional undescribed species of *Leohumicola*. A dagger (†) indicates a sequence that varied in position among different trees. An asterisk indicates the sequence is based on a culture that has formed ericoid mycorrhizas in resynthesis experiments. Note that mycorrhiza formation by two isolates not belonging to *L. verrucosa* is confirmed here but not discussed in the text. Within the *Leohumicola* clade, plant hosts are indicated where applicable for isolates from roots; isolates with no host name are from soil. Isolates for which no country of isolation is indicated are from Canada. Bootstrap support values over 50 % from 1000 replicates of a full heuristic search are shown.

the well-supported *Sordariomycetes* (100 %), which also included representatives of the *Xylariales* and *Hypocreales*. *Thermomyces lanuginosus* grouped within the *Eurotiales* with 99 % support which, together with the *Onygenales* (83 %), formed the highly-supported *Eurotiomycetes* (100 %). Clades corresponding to the *Mycocaliciales*, *Chaetothyriomycetes*, *Lecanorales*, *Pleosporales* (*Dothideomycetes*), and groupings within the *Pezizales* (*Pezizomycetes*), all received moderate to high bootstrap support within the well-supported ingroup, *Pezizomycotina*.

The aligned ITS data matrix comprised 35 taxa and 485 characters. Eight sequences, including two strains of *L. verrucosa*, were excluded from the final analyses because they were identical to other sequences in the alignment. This reduced the number of MPTs that differed only by arrangements of zero length branches. Of the aligned characters, 75 were

**Table 1.** Isolates and GenBank accession numbers.

| Name | Accession No.[a] | Source | Region Sequenced | GenBank Accession No. |
|---|---|---|---|---|
| *Leohumicola verrucosa* | 226889[T] (CBS 115880) | heated soil, commercial lowbush blueberry field, Nova Scotia, Canada | SSU-ITS | AY706320 |
| | 231141 | roots, *Vaccinium myrtilloides*, fire-disturbed stand of jack pine–aspen/blueberry–bearberry, Alberta, Canada | ITS | AY706321 |
| | 231142 | heated soil, commercial lowbush blueberry field, Nova Scotia, Canada | ITS | AY706322 |
| | 231143 (CBS 115881) | heated soil, stand of red and white pine, Nova Scotia, Canada | ITS | AY706323 |
| | 231144 | heated soil, stand of white pine, Nova Scotia, Canada | ITS | AY706324 |
| | 231147 (CBS 115947) | heated soil, lodgepole pine forest, Alberta, Canada | ITS | AY706325 |
| | 230085 | soil, Puerto Rico | ITS (SSU = 226889) | AY706326 |
| *Leohumicola terminalis* | 231145[T] (CBS 115946) | heated soil, stand of sugar maple, Nova Scotia, Canada | ITS | AY706327 |
| *Leohumicola lenta* | 231149[T] (CBS 115945) | heated soil, native tallgrass prairie, Manitoba, Canada | ITS | AY706328 |
| *Leohumicola minima* | CBS 209.74[T] (232587) | volcanic ash soil, Valdivia, Chile | SSU-ITS | AY706329 |
| *Leohumicola* sp. | 231148 | heated soil, native tallgrass prairie, Manitoba, Canada | ITS | AY706330 |
| | 230084 | soil, under *Eucalyptus*, New South Wales, Australia | SSU-ITS | AY706331 |
| *Culcitalna achraspora* | CBS 163.60 (231161) | driftwood in seawater, Florida, U.S.A. | SSU | AY706332 |
| *Humicola fuscoatra* var. *fuscoatra* | CBS 118.14[T] (35882) | soil, Norway | SSU | AY706333 |
| *Humicola grisea* var. *grisea* | CBS 119.14[A] (232586) | soil, Norway | SSU-ITS | AY706334 |
| *Thermomyces lanuginosus* | CBS 632.91[T] (232588) | rotting guayule shrub, California, USA | SSU-ITS | AY706335 |
| *Trichocladium asperum* | 232342 | tubers, *Solanum tuberosum*, PEI, Canada | SSU-ITS | AY706336 |

[a]Numbers unless otherwise noted DAOM, Canadian Collection of Fungal Cultures, Ottawa, Canada; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[T]Ex-type strain.

[A]Authentic strain.

parsimony-informative, 333 were constant, and 77 were parsimony-uninformative. Parsimony analysis resulted in 106 equally parsimonious trees of 250 steps. One MPT is shown in Fig. 4 with the results of a bootstrap analysis. Most of the sequences formed a well-supported ingroup (100 %) comprising the *Leohumicola* clade and four other groups of sequences of unidentified fungi, Groups A–D, (discussed below), of which three were well-supported by bootstrap values, and one was a single sequence on its own branch. All strains and species of *Leohumicola* formed a monophyletic group, with low bootstrap support, that also included some GenBank sequences for unidentified fungi sampled from mycorrhizal roots.

The four species described below were phylogenetically distinct within the *Leohumicola* clade. Strains of *L. verrucosa*, the only one of our species represented by more than one strain, formed a subclade in all trees that included two GenBank sequences AF081442 (Monreal *et al*. 1999, *ex Gaultheria* roots; identical to AY394888, Lim *et al*. unpublished, *ex* hemlock [*Tsuga*] roots, not shown) and AY394901 (Lim *et al*. unpublished, *ex* hemlock roots), and sometimes a third, AY046401 (Bergero *et al*. 2000, *ex Erica* roots). Three other potential species of *Leohumicola* were revealed (shown on Fig. 4 in boxes with dashed lines). DAOM 231148 was on its own branch and two pairs of sequences each formed bootstrap-supported subclades, one consisting of DAOM 230084 and AF099089 (McLean *et al*. 1999, *ex Astroloma* roots), 100 % supported, and AY279184 with AY279185 (Williams *et al*. unpublished, *ex Epacris* roots), 88 % supported. AY046401, the Bergero *et al*. (2000) sequence from *Erica* roots, was mobile within the *Leohumicola* clade, contributing to the large number of MPTs. When it was excluded from the analysis, only ten MPTs were found and they were shorter than the original MPTs by nine steps. Bootstrap support for the monophyletic *Leohumicola* clade, present in all ten trees, increased to 82 % (results not shown).

The presence of four additional species in the ingroup was suggested by the ITS analysis. Group A comprised sequences for one culture isolated from *Erica* ericoid mycorrhizas from Italy (Bergero *et al*. 2003) and cultures isolated from *Kobresia* ectomycorrhizas (Schadt & Schmidt unpublished). Group B comprised sequences derived from DNA extracted from soil collected in Australia (Chen & Cairney 2002). Group C comprised sequences from cultures, as well as from direct root tip DNA extractions, obtained from *Pinus* ectomycorrhizas from Norway (Vrålstad *et al*. 2002). Group D was a single sequence, on its own branch, from a culture isolated from *Quercus* ectomycorrhizas in Italy (Bergero *et al*. 2003).

## TAXONOMIC PART

### Generic concepts

Our phylogenetic studies of SSU sequences from our new isolates, authentic strains of the type species of *Humicola*, *Thermomyces* and *Culcitalna*, and reliably identified isolates of the type species of *Trichocladium* demonstrate the phylogenetic divergence of these fungi. Our heat-resistant strains assigned to the *Leotiomycetes* are here circumscribed in a new genus, *Leohumicola*. Additional pertinent genera are morphologically compared below. A key to similar genera is also provided.

Despite morphological similarities with the broad concepts now prevalent for *Humicola* and *Trichocladium*, the four species of *Leohumicola* are phylogenetically distant from the type species of these genera. The *Leohumicola* species are morphologically and ontogenetically homogenous. All have holothallic or monoblastic conidial initials that are two-celled, with the terminal cell becoming increasingly pigmented and swollen, and also often roughened, during development. After rhexolytic secession, the conidia are one-celled. The class *Leotiomycetes* that these species belong to currently includes no genera described as having similar chlamydosporic or aleurioconidial anamorphs. Also, no teleomorphic or other named conidial fungi fall into the ITS clade that encompasses these species. In classical anamorph taxonomy, these species could be classified in the form genera that they fit best based on morphological characters. In modern systematics, however, where monophyletic, phylogenetically meaningful genera are preferred, this is an unsatisfactory solution. The phylogenetic rearrangement of the sterile fungi formerly included in *Rhizoctonia* makes an interesting parallel case, as is outlined in the Introduction. However, given the often sporadic scattering of reduced anamorphs among morphologically more differentiated fungi, how rigidly should we apply monophyly in these cases? It is probably not helpful to populate fungal nomenclature with a large number of monotypic anamorph genera with minimal morphology. Perhaps it would be appropriate to propose single generic names for the chlamydosporic or aleuriosporic anamorphs of a particular order or family, consistent with the diversity of morphologically reduced species in the taxon under consideration. Although *Leohumicola* as described here appears to be monophyletic, it might be pragmatic to include similar aleurioconidial anamorphs (yet to be discovered) allied with different groups of *Leotiomycetes*.

The two species of *Humicola* included in our phylogenetic analysis, the type *H. fuscoatra*, and *H. grisea*, and the type species of *Trichocladium*, *Tr. asperum*, are phylogenetically related to the

*Chaetomiaceae* (*Sordariales*) rather than the *Leotiomycetes*. This was expected, given the tentative teleomorph connections reported for both genera (Gams 1971). We were surprised that the ITS sequences of *H. grisea* and *Tr. asperum* were identical. We confirmed this by sequencing several strains of each species. These two species are morphologically distinct based on conidial characters (dimensions, number of septa, wall roughening, see Fig. 1G–K and L–P), but there are several similarities that support the close phylogenetic relationship. The aleurioconidia have very similar ontogeny, and both have multiple areas of wall-thinning that often have been overlooked, but were reported as germ pores in *Tr. asperum* by Hughes (1972) and in *H. grisea* by Griffiths (1974). Griffiths (1974) studied the ultrastructure of the walls of the aleurioconidia of *H. grisea*, illustrating an electron-dense, melanised outer zone, and an electron-transparent, apparently nonmelanised, inner zone. Some areas of the outer zone had thin regions that seemed to represent germ pores. In light microscopy, these regions are seen in face view as a pale area within the pigmented part of the wall. In optical cross-section, they are seen as a discontinuity in the outer, pigmented part of the wall (Fig. 1K). They are apparently identical to the so-called germ pores of *Tr. asperum*. We have not observed germination through these putative pores, which do not seem to occur in the pigmented, intercalary chlamydospores of *H. grisea*. Both species have acremonium-like synanamorphs, and the growth rates in agar culture are similar.

It is possible that the aleurioconidial genera *Humicola* and *Trichocladium* should be considered synonyms. The identical ITS sequences for *H. grisea* and *Tr. asperum* certainly suggest they should be classified in the same genus. Normally, the older name *Trichocladium* would take precedence, but *Humicola* is a much more widely-used generic name. Therefore, consideration will be given to conserving the latter name. In the meantime, there is little point in transferring species of *Trichocladium* to *Humicola*, or vice versa, because there is likely to be considerable phylogenetic heterogeneity among the current species. This is illustrated by our results with *C. achraspora*, the type species of *Culcitalna*, still generally known as *Trichocladium achrasporum* (Goh & Hyde 1999). Small subunit analysis of this species places it in the *Halosphaeriaceae*, *Halosphaeriales*, consistent with the reported teleomorph *Halosphaeria mediosetigera* Cribb & J.W. Cribb (Shearer & Crane 1977). Clearly, *Culcitalna* cannot be considered a synonym of *Trichocladium*.

Based on SSU sequences, *Th. lanuginosus* is phylogenetically related to the *Trichocomaceae* (*Eurotiales*). A BLAST search using the ITS sequence of the type strain supports the inclusion of this species in the *Trichocomaceae*, with *Talaromyces thermophilus* Stolk the nearest neighbour for which sequences exist. Conidial secession of *Th. lanuginosus* is usually rhexolytic (although we saw that it can also be schizolytic), differing from the schizolytic secession of the other anamorphs in this family. Some species, however, of the anamorph genus *Paecilomyces* Bain. and the related teleomorph *Byssochlamys* Westling produce stalked "chlamydospores" (Samson 1974) that are generally similar to the structures produced by *Th. lanuginosus*. We do not know whether these structures secede.

There are other morphologically similar genera not included in our phylogenetic or ontogenetic analyses. The characters of these genera are summarised in Table 2, and the genera are included in a morphological key given below. Of particular relevance is *Complexipes* C. Walker, originally described as a member of the zygomycetous family *Endogonaceae* (Walker 1979) but later recognised as an ascomycete and emended to include anamorphs of *Tricharina* Eckblad (*Pezizales*, Yang & Korf 1985). These fungi form ectendomycorrhizae of conifers, and are known as E-strain fungi (Wilcox *et al*. 1974, Yu *et al*. 2001). They produce thick-walled, brown aleurioconidia that are up to 100 µm in diam, but have no reported synanamorphs. Apart from the differences in size and phylogenetic affinities, the overall morphological similarity with some species of *Leohumicola* is striking, considering the apparent similarities in ecology. The sympodially proliferating conidiogenous denticles that occur in *L. verrucosa* also suggest a comparison with *Scolecobasidium* Abbott (de Hoog & von Arx 1973) and its segregate *Ochroconis* de Hoog & von Arx (de Hoog 1985). Species of these two genera have lightly pigmented, sympodially proliferating conidiogenous cells, and dry, septate conidia. In contrast to *L. verrucosa*, all species produce distinct, elongated conidiogenous cells, and the connection between the conidia and the conidiogenous cell is very narrow. Also, the conidia lack distinctly different terminal and basal cells. The conidia of the species of *Scolecobasidium* and *Ochroconis* would not be considered aleurioconidia or chlamydospores. A molecular revision of this complex is underway by de Hoog *et al*. (pers. comm.), and we have determined that there is little phylogenetic relationship between *Leohumicola* and either *Scolecobasidium* or *Ochroconis*.

**Species concepts**

Our analysis of ITS sequences reveals the existence of a monophyletic clade that includes all of our isolates, as well as many unnamed sequences obtained from cultures or DNA clones originating from mycorrhizas of different plants in several continents and countries. Based on micromorphology, most of our strains seem to belong to one species, which we describe below as *Leohumicola verrucosa*.

**Table 2.** Hyphomycete genera resembling *Leohumicola* in morphology and (except *Culcitalna*) rhexolytic secession.

| Genus | Teleomorph affinity | Growth *in vitro* | Conidiogenous cell | Conidial initial | Mature aleurioconidia | Synanamorphs | Reference |
|---|---|---|---|---|---|---|---|
| *Carmichaelia* Sharma | unknown | unknown | monoblastic | 1-celled | 1-celled<br>dark brown<br>star-like vacuole | unknown | Sharma 1980 |
| *Chlamydomyces* Bain. | Unknown. Perhaps *Ceratostomataceae*, *Hypocreales* based on synanamorph | rapid | monoblastic | 2-celled | 2-celled<br>brown | *Aspergillus*-like (*Proteophiala* Cif.) | Ellis 1976 |
| *Complexipes* C. Walker | *Tricharina* (*Pezizales*) | unknown | monoblastic | 1-celled | 1-celled<br>dark brown<br>very large | unknown | Yang & Korf 1985 |
| *Culcitalna* S.P. Meyers & R.T. Moore | *Halosphaeria* (*Halosphaeriaceae*, *Halosphaeriales*) | slow | ?thallic | many-celled | many-celled<br>dark brown | none | Ellis 1976 |
| *Desertella* Mouchacca | Unknown | unknown | ?thallic; multilobed then becoming septate | 1-celled | 1-celled<br>hyaline | unknown | Mouchacca 1979 |
| *Humicola* Traaen | *Chaetomiaceae*, *Sordariales* | rapid | monoblastic | 1-celled | 1-celled<br>dark brown<br>germ pores | *Acremonium*-like | Domsch *et al*. 1980 |
| *Leohumicola* | *Leotiomycetes* | slow | monoblastic or sympodial | 2-celled | 1-celled | none | This paper |
| *Mycogone* Link | *Hypomyces* (Fr.) Tul. (*Hypocreaceae*, *Hypocreales*) | rapid | monoblastic | 2-celled | 2-celled or more<br>yellow or pink | *Verticillium*-like | Põldmaa *et al*. n.d. |
| *Sepedonium* Link | *Hypomyces* (Fr.) Tul. (*Hypocreaceae*, *Hypocreales*) | rapid | monoblastic | 1-celled | 1-celled<br>brown | *Verticillium*-like | Sahr *et al*. 1999 |
| *Thermomyces* Tsilinksy | *Trichocomaceae*, *Eurotiales* | moderate | monoblastic | 1-celled | 1-celled | none or *Acremonium*-like | Domsch *et al*. 1980 |
| *Trichocladium* Harz | *Chaetomiaceae*, *Sordariales* | rapid | monoblastic | 2-celled or more | 2-celled or more<br>dark brown<br>germ pores | *Acremonium*-like | Goh & Hyde 1999 |

The four species of *Leohumicola* that we currently recognise are distinct in their growth rates and in their microscopic characters (Table 3). Growth rates in culture are helpful for identification and are relatively constant on the three media we have used, PDA, MA and OA. The species differ in the dimensions and shapes of conidia and their component cells, as well as in the degree of terminal cell roughening, the proliferation or lack thereof of the conidiogenous cells, and the position of the conidia on the conidiogenous hyphae. *Leohumicola terminalis* is described here based on a single strain that may not have been sporulating optimally. Additional diagnostic characters may be discovered when new cultures become available.

Most *Leohumicola* strains produce soluble pigments. In young colonies, these pigments are usually pale yellow or reddish, increasing in intensity as growth continues. In old colonies, the pigments are dark olive, brown or purplish. The intensity of the pigments varied among strains, and among media, and tended to be more pronounced when macerated inocula were used. The colour and intensity of these soluble pigments does not seem to be species-specific.

Strains of *Leohumicola* tend to produce sterile colonies, or only produce conidia after up to 6 mo cultivation. Colonies derived from macerated inocula were more apt to sporulate than colonies from point inocula. The latter tended to develop conidia mainly on aerial mycelium or fascicles that emerged from the surface or in the near vicinity of the inoculum block. Of the strains of *L. verrucosa* examined, DAOM 231143 was the most prolific sporulator, producing conidia after 2 wk on PDA or OA. DAOM 231147 sporulated after 3–4 wk on PDA and OA, also relatively abundantly. DAOM 226889 sporulated abundantly, but took much longer. The other *L. verrucosa* strains eventually sporulated but only after 6 mo or more of incubation. Conidia were then only sparsely formed. We suggest that when cultures that seem to belong to this clade are analysed, extended cultivation be done on several media, using a macerated inoculum. This procedure will increase the probability of detecting conidia.

In ITS sequences, one species, *L. verrucosa*, showed a fair degree of divergence (nine single-nucleotide substitutions, mostly in the ITS1). Near the completion of this study, we obtained the strain represented by the sequence AY394901 (Lim *et al*. unpublished, *ex* hemlock roots), UAMH 10384 (University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada). After 6 mo growth, conidia had formed sparsely on mycelium submerged in the agar. Colony growth rate and pigmentation, and conidial measurements, were typical of *L. verrucosa*, though conidia lacked ornamentation. We have not seen the strain represented by AF081442 (Monreal *et al*. 1999, *ex Gaultheria* roots).

It is also obvious from Fig. 4 that additional species of *Leohumicola* have been isolated or that their DNA has been detected in other studies. We isolated one strain (DAOM 230084) that may be conspecific with a fungus recorded on *Astroloma pinifolium* (R. Br.) Benth. (*Epacridaceae*) roots in Australia (AF099089), but our strain did not sporulate and we have not described the species here. There appear to be at least two other species within the monophyletic *Leohumicola* clade, DAOM 231148 and one strain from *Epacris* sp. in Australia, with a possible third species from *Erica arborea* roots in Italy. It is possible that the other four clades in the ingroup in Fig. 4 are also *Leohumicola* spp., and it will be intriguing to see whether similar conidia can be demonstrated for those fungi.

## Taxonomic Descriptions

***Leohumicola*** N.L. Nickerson, Hambleton & Seifert, **gen. nov.** MycoBank MB500248

*Etymology*: *Leo*- an abbreviation of *Leotiomycetes*, the class in which these fungi are classified; -*humicola*, for the morphological similarity of these fungi to the hyphomycete genus *Humicola*.

Conidiophora absentia vel inconspicua; hyphae conidiogenae subhyalinae vel dilute brunneae, in hyphis aeriis simplicibus vel fasciculatis. Conidia sicca, singula vel pauca aggregata, plerumque bicellularia, e cellula terminali et basilari composita, nonnumquam ambo cellulae septatae. Cellula terminalis fusca, modice crassitunicata, verrucosa vel levis, poro germinationis carens. Cellula basilaris obconica, crateriformis vel cylindrica, subhyalina vel dilute brunnea. Conidia monoblastica oriuntur, primum plus minusve cylindrica, hyalina, intumescentia, fuscescentia et in medio septata; rhexolytice liberata cellula basilari iuxta septum corrupta; denticulum truncatum in hypha conidiogena relinquentia. Denticuli in nonnullis speciebus semel vel bis sympodialiter proliferentia. Coloniae in agaro PDA dicto plus minusve restrictae (minus quam 25 mm diam post 14 dies), saepe pigmento luteo, viridi, brunneo vel rubro diffundente. Synanamorphe absens.

*Typus*: *L. verrucosa* N.L. Nickerson, Hambleton & Seifert

Conidiophores absent or scarcely developed; conidiogenous hyphae subhyaline to pale brown, in aerial mycelium or in hyphal fascicles. Conidia dry, single or in small groups, initially two-celled, with a terminal cell and a basal cell, sometimes with an extra division in either cell. Terminal cell dark brown, with walls slightly thickened and smooth or with conspicuous warts or projections; germ pores lacking. Basal cell obconical, cupulate or cylindrical, subhyaline to pale brown. Conidium ontogeny monoblastic; the initial more or less cylindrical, hyaline, swelling and becoming pigmented simultaneously, then developing a central septum. Secession rhexolytic; rupturing occurring at any point in the basal cell, the body of

which remains attached to the detached terminal cell, making the seceded conidium functionally single-celled. Conidiogenous hyphae after secession retaining a flat-topped denticle, or in some species up to five sympodial proliferations of this remnant; where proliferation occurs, the 2–6 conidia produced accumulate in a cluster.

Colonies on PDA restricted to moderately slow (less than 25 mm in 14 d), often with yellow, green, brown or red soluble pigments.

Synanamorphs none.

**Key to *Leohumicola* and similar genera**

1. Ameroconidia or basal cells of pluricellular conidia usually forming attached to lateral or terminal stalks, enticles or conidiogenous cells ............ 2
1. Basal cell of conidium or conidial chain integrated in vegetative hyphae ............ *Culcitalna*

2. Conidia large, usually > 50 µm diam ............ *Complexipes*
2. Conidia < 50 µm diam ............ 3

3. Aleurioconidia with germ pores or conspicuous thin spots in the wall; acremonium-like synanamorph produced ............ 4
3. Aleurioconidia lacking germ pores ............ 5

4. Conidia rough-walled, 1 or more septate ............ *Humicola* (*Trichocladium*)
4. Conidia smooth-walled, usually aseptate, or sometimes consisting of a constricted chain of cells ............ *Humicola*

5. Conidium initial 1-celled ............ 6
5. Conidium initial 2-celled ............ 9

6. Conidia rough-walled............ 7
6. Conidia smooth ............ 8

7. Synanamorph verticillium-like; mesophilic; occurrence typically on other fungi ............ *Sepedonium*
7. Synanamorph absent or, if present, *verticillium*-like; thermophilic; occurrence typically in self-heating material ............ *Thermomyces*

8. Conidia hyaline to pale yellow-brown; occurrence typically in soil ............ *Desertella*
8. Conidia dark brown with a star-like vacuole; occurrence typically on wood ............ *Carmichaelia*

9. Colony growth on agar restricted; conidia mostly 1-celled after secession but with conspicuous remnants of the separating cell; no synanamorph produced ............ *Leohumicola*
9. Colonies fast-growing; conidia remaining 2-celled after secession; synanamorph present ............ 10

10. Synanamorph *aspergillus*-like (*Proteophiala*); terminal cell of conidia dark brown; occurrence typically not on fungi ............ *Chlamydomyces*
10. Synanamorph *verticillium*-like; terminal cell of conidia not dark brown; occurrence typically as parasites of macrofungi ............ *Mycogone*

**Key to the species of *Leohumicola*.**

1. Colony diam > 20 mm in 2 wk on PDA; terminal cell of conidium mostly ellipsoidal ............ *L. minima*
1. Diam < 20 mm in 2 wk on PDA; terminal cell mostly globose ............ 2

2. Colony diam < 5 mm in 2 wk on PDA; terminal cell of conidia 7–10 µm long ............ *L. lenta*
2. Diam 10–20 mm in 2 wk on PDA; terminal cell < 7.5 µm long ............ 3

3. Conidia lateral and terminal, often rough-walled, terminal cell 4–5.5 µm long; sympodial proliferation sometimes occurring ............ *L. verrucosa*
3. Conidia only terminal, usually smooth-walled, terminal cell 5–7.5 µm long; sympodial proliferation not occurring ............ *L. terminalis*

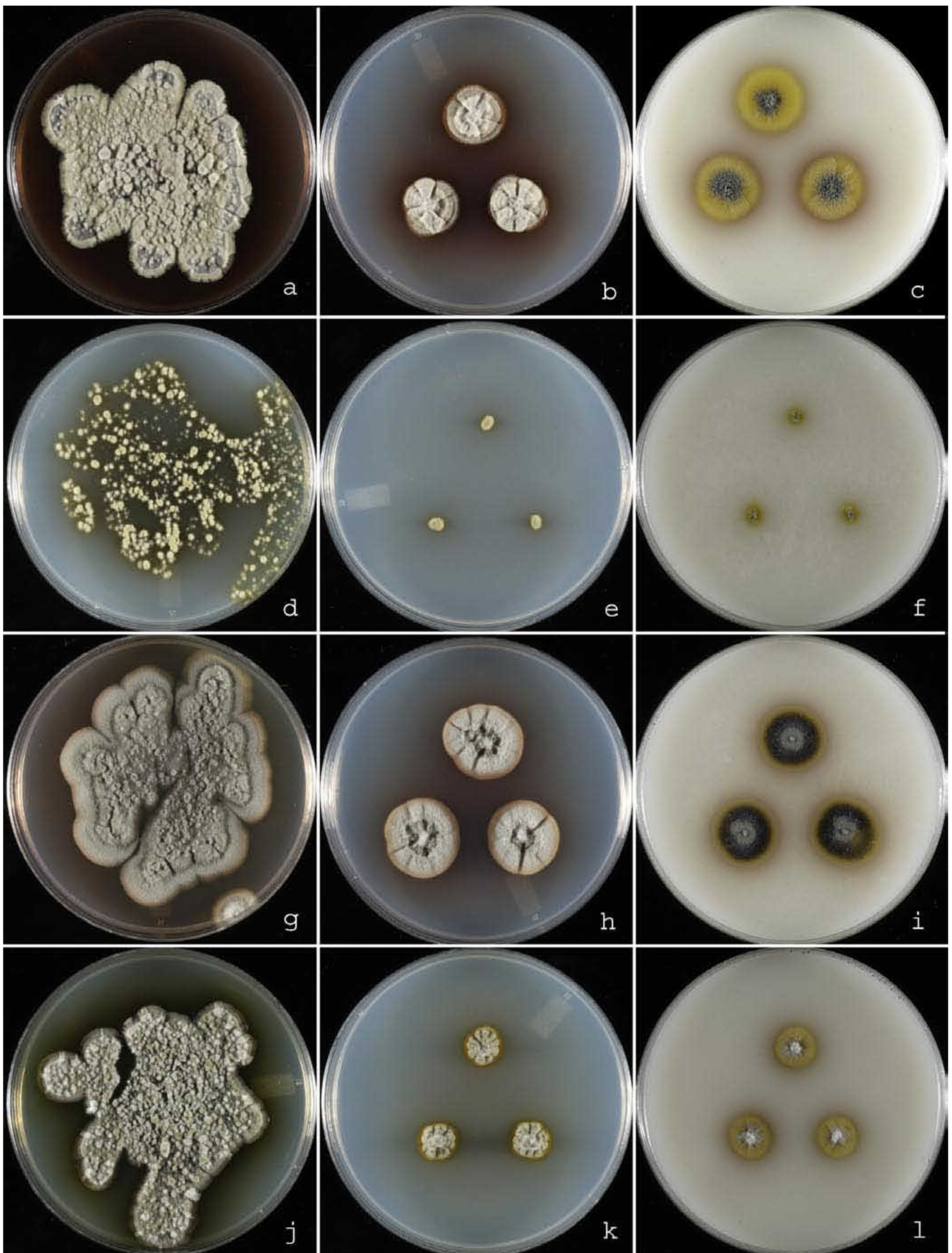


**Fig. 5.** Ex-type strains of four *Leohumicola* species growing from macerated inocula on PDA, and on PDA and OA with point inocula, after 14 d at room temperature. A–C. *L. verrucosa*. D–F. *L. lenta*. G–I. *L. minima*. J–L. *L. terminalis*.

**Table 3.** Summary of colony and microscopic characters of four species of *Leohumicola*.

| Species | Colony characters after 14 d | | | | | Conidia | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PDA | | | MA | OA | | | | |
| | diam (mm) | mycelial colour | soluble pigments | diam (mm) | diam (mm) | position | terminal cell (µm) | wall | basal cell (µm) |
| *verrucosa* | 12–18 | greyish yellow olive yellow gray | red reddish brown olive brown | 12–18 | 15–19 | lateral + terminal | 4–5.5 × 4–5.5 | verrucose (usually) | 2–4.5 × 2.5–3 |
| *lenta* | 1 | olive | minimal | 2 | 3–4 | lateral + terminal | 7–10 × 6.5–8.5 | smooth | 4–11 × 2–5 |
| *minima* | 20–22 | grey olive gray brownish gray | olive grey brownish grey | 16–18 | 18 | lateral + terminal | 4.5–8 × 3.5–5 | smooth or partly verrucose | 2–5.5 × 2–3 |
| *terminalis* | 10 | grayish yellow | olive | 8–9 | 11–12 | terminal only | 5–7.5 x 5–8.5 | slightly rough | 3–8.5 × 2.5–4.5 |

***Leohumicola verrucosa*** N.L. Nickerson, Hambleton & Seifert, **sp. nov.**, MycoBank MB500249, Figs 1A–F, 5A–C, 6

*Etymology*: Derived from *verrucosus* (L), referring to the ornamentation of conidial terminal cells.

Conidia lateralia vel modice terminalia, cellula terminali 4–5.5 × 4–5.5 µm, (sub)globosa, ellipsoidea vel ovata, brunnea, verrucosa vel laevi; cellula basilari 2–4.5 × 2.5–3 µm, crateriformis, obconica vel cylindrica. Coloniae in agaro PDA dicto 14–18 mm diam post 14 dies.

Holotypus: cultura ex solo isolata, exsiccata in herbario DAOM 226889, viva ex-typo in CCFC.

Conidiogenous hyphae subhyaline to pale brown, 1–2.5 µm wide, often in fascicles in aerial mycelium. Conidiogenous cells reduced to a single denticle 1–5 µm long and 1–2 µm wide (Figs 1A–C, 6 F), or with a discrete conidiogenous cell up to 7.5 µm long, the denticles single or the cell proliferating sympodially up to five times to produce a node or elongated cluster of divergent denticles (Fig. 6C, D, F), or sometimes with once- or twice-branched structures resembling conidiophores, with 2–3 branches per branch point. Conidia initially two-celled, single or side by side in small clusters, or with up to six successively produced conidia emerging from sympodially proliferating denticles; terminal cell 4–5.5 × 4–5.5 µm excluding the roughening (mean +/- SE = 4.8–5.1 +/- 0.1 × 4.6–5.0 +/- 0.1), globose, subglobose to ellipsoidal or ovate, at first the same colour as the basal cell (Fig. 1A–C), then becoming dark brown while still attached (Fig. 1D–E), with walls slightly thickened, usually verrucose or echinulate (Figs 1E, 6A–B, E); older conidia sometimes remaining smooth in some strains but more often with finger- or bubble-like projections or spines about 1–2.5 µm long, 0.5–2.5 µm wide at the base, either concentrated at the apex of the terminal cell or in some strains covering the whole exposed portion of this cell (Figs 1F, 6G); connection to basal cell 2–3 µm wide, often constricted, with connection between the two cells reduced to a minute central pore; basal cell 2–4.5 µm long (mean +/- SE = 3.1–3.4 +/- 0.1), 2.5–3 µm wide, obconical, cupulate or cylindrical, symmetrical or asymmetrical, subhyaline to pale brown, eventually becoming almost as dark as the terminal cell (Fig. 6 E); ratio of lengths of terminal:basal cell 1.1–2.3 µm (mean +/- SE = 1.5–1.6 +/- 0.1). Three-celled conidial initials, with either the terminal or basal cell developing an internal septum, seen rarely. Basal cell of conidium rupturing during secession, resulting in a functionally single-celled conidium bearing the remnant of the basal cell (Fig. 6G). Flat-topped denticles 1–3.5 µm long remaining on conidiogenous hyphae (Fig. 6F, arrow). Chlamydospores sparsely produced, intercalary and single in hyphae of older cultures, cylindrical to ellipsoidal or globose, concolorous with or paler than conidial terminal cells, 5–6.5 × 3–5 µm, walls thin or slightly thickened. Vegetative mycelium often with swollen, monilioid, hyaline or subhyaline hyphae 3–8 µm wide, constricted at septa, with slightly thickened walls and oily cellular contents.

Colonies on PDA after 14 d under ambient light at room temperature (Fig. 5A–B) 12–18 mm diam (mean +/- SE = 14.8 ± 0.04, n=21), greyish yellow (2C-D2-4) to olive yellow (2D6), or in the absence of yellow pigments, white to grey (2C–D1), relatively uniformly coloured or with concentric rings, sometimes sectoring, sometimes darkest near the inoculum, usually with

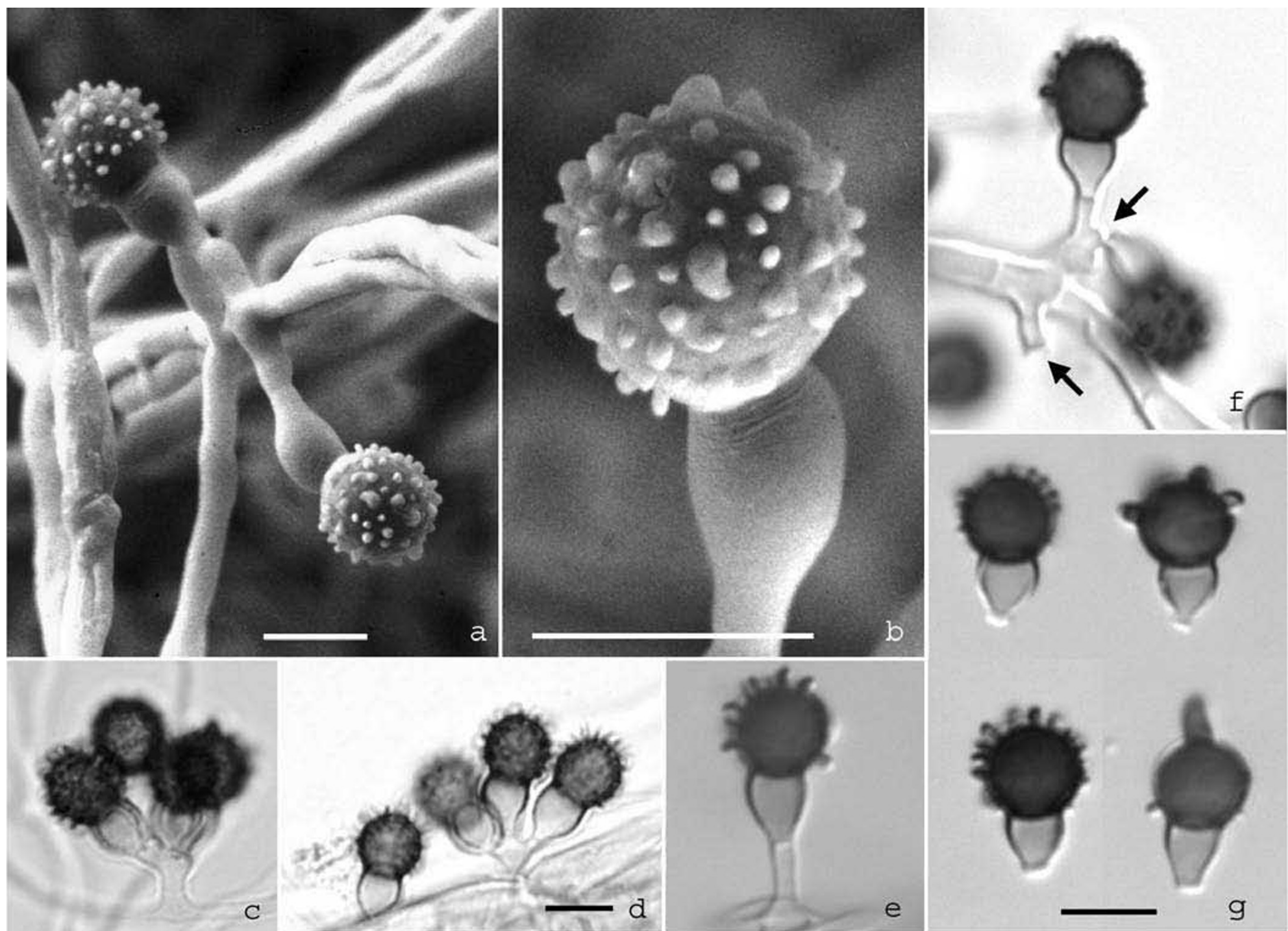

**Fig. 6.** *Leohumicola verrucosa*. A–B. SEM micrographs of DAOM 226889, showing conidiogenous hyphae, conidia, and roughening of the terminal cell. C–D. Proliferating conidiogenous cells in DAOM 231147 in artificial culture with *Vaccinium* roots. E–G. Attached and seceded conidia from cultures on PDA. E and G. DAOM 226889. F. DAOM 231143, arrows indicting the flat-topped denticle that remains after the conidium secedes. Scale bars = 5 µm. Scale bar in D applies to C, D; in G applies to E–G. SEM photographs courtesy of S. Carbyn and P. Allan-Wojtas.

more or less white margin, planar or convex, wrinkled or sulcate, sometimes splitting the agar near the colony centre, with low, felty, slightly lanose, or radiating funiculose aerial mycelium. Exudates not produced; soluble pigment production variable, starting yellow and becoming either dull red (8B3) to reddish brown (8–10E8), or, in the absence of red pigments, becoming olive brown (4E5). Margin smooth and entire. Colony reverse olive brown (4E6–8) in the absence of soluble pigments, or dark brown (7F6–8) when soluble pigments are produced.

*Substrate and distribution*: soil, roots; Canada (Alberta, Nova Scotia). Puerto Rico.

*Living cultures examined*: **Canada**, Nova Scotia, from heated soil, commercial lowbush blueberry field, 19 Nov. 1997, N.L. Nickerson S79.2 (ex-type culture DAOM 226889 = CBS 115880, **holotype** is a dried culture under the same name in herb. DAOM); from heated soil, commercial lowbush blueberry field, 17 Mar. 2002, N.L. Nickerson S117T-9 (DAOM 231142); from heated soil, stand of red and white pine, 18 Sept. 2002, N.L. Nickerson S161R5-3 (DAOM 231143 = CBS 115881); from heated soil, stand of white pine, 29 Apr. 2002, N.L. Nickerson S211R6-2 (DAOM 231144); Alberta, from heated soil, lodgepole pine forest, 2 Aug. 2000, N.L. Nickerson S281R1-10 (DAOM 231147 = CBS 115947); from roots of *Vaccinium myrtilloides*, fire disturbed stand of jack pine–aspen/blueberry–bearberry, July 1998, G. Hill-Rackette S1-P2-P-2 (DAOM 231141). **Puerto Rico**, from soil, June 1998, W. Burpee W-Pr-12a (DAOM 230085).

*Comments*: *Leohumicola verrucosa* was frequently isolated from soils from lowbush blueberry fields in Canada, with densities exceeding 10 000 cfu/g recorded in some soils (Nickerson, unpubl. data). It is distinguished from the other *Leohumicola* species by its relatively small conidia, with terminal cells less than 5.5 µm diam and usually bearing conspicuous roughening. It is also the only species in which we have relatively frequently seen sympodial proliferation

of the conidiogenous cell. As detailed above, three (DAOM 226889, 231143, 231147) of the seven cultures of this fungus we have examined sporulated relatively abundantly, while the others sporulated only sparsely after prolonged incubation. As noted on Fig. 4, four of the strains examined were tested and all formed morphologically typical ericoid mycorrhizas *in vitro* in resynthesis experiments (Nickerson, unpubl. data). Based on the recovery of *L. verrucosa* from ericoid roots from Alberta and unheated soil from Puerto Rico, we expect that this fungus may have a much broader distribution than we have so far determined. One sequence obtained from hemlock roots, AY394901, is based on a culture, UAMH 10384, that formed conidia under our growth conditions. Two other sequences, one from salal (*Gaultheria shallon*, AF081442) and a second from hemlock roots (AY394888; not shown in Fig. 4 but identical to AF081442) may also represent this species.

***Leohumicola lenta*** Hambleton, Seifert & N.L. Nickerson, **sp. nov.**, MycoBank MB500250, Figs 5D–F, 8A–L

*Etymology*: *lenta* (L), slow, for the slow growth of this fungus in agar culture.

Conidia lateralia vel raro terminalis, cellula terminali 7–10 µm × 6.5–8.5, (sub)globosa, ellipsoidea vel ovata, raro cylindrica, brunnea, laevia vel modice *verrucosa*; cellula basilari 4–11 × 2–5 µm, obconica, doliiformis vel cylindrica. Coloniae in agaro PDA dicto 2 mm diam. post 14 dies.

*Holotypus*: cultura ex solo isolata, exsiccata in herbario DAOM 231149, viva ex-typo CCFC.

Conidiogenous hyphae subhyaline to pale brown, 1.5–2 µm wide. Conidiophores reduced to a single denticles 1–2.5 µm long and 1–1.5 µm wide (Fig. 8A, G, arrows). Conidial initials usually two-celled, single, either lateral or more often terminal on conidiogenous hyphae; terminal cell 7–10 × 6.5–8.5 µm (mean +/- SE

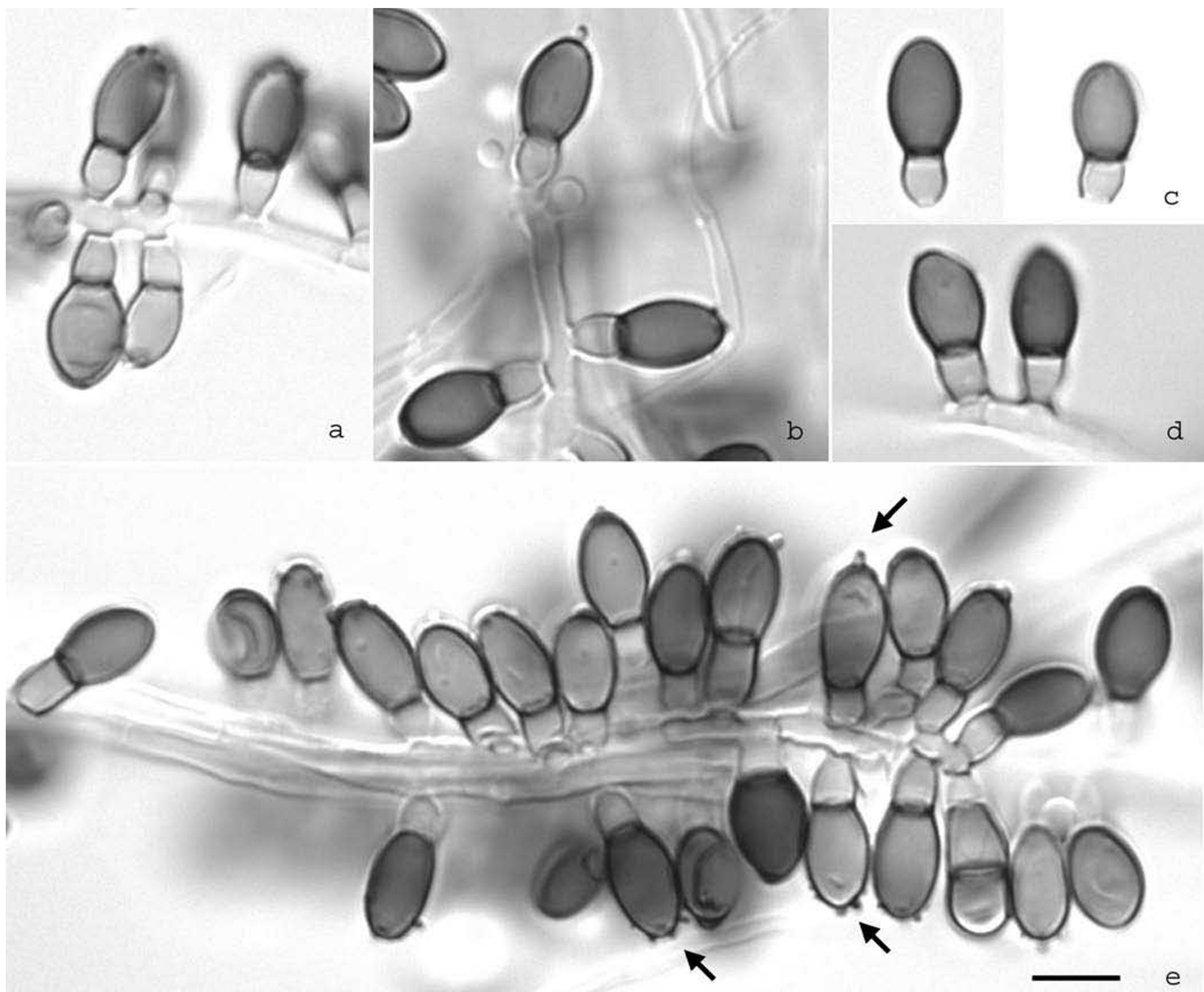


**Fig. 7.** *Leohumicola minima*, ex-type strain on PDA. Conidia emerging from individual conidiogenous hyphae (A, B, D) or hyphal fascicles (E). Arrows in E indicating roughening at apex of the terminal conidial cell. C. Seceded conidia. Scale bar = 5 µm (shown in E).

= 8.4 +/- 0.1 × 7.5 +/- 0.1), globose, subglobose to ellipsoidal or ovate, rarely cylindrical, at first the same colour as basal cell (Fig. 8A, H), becoming dark brown while still attached (Fig. 8I); conidial walls thickened slightly more than 0.5 µm, remaining smooth or in a minority of conidia becoming verrucose at the apex after > 6 mo; warts about 0.5 µm high. Conidial connection to basal cell 1.5–3 µm wide, usually not constricted, somewhat flaring; when constricted, forming with a 'shoulder' in the basal cell where the narrowing occurs; a minute central pore connects the two cells. Basal cell 4–11 × 2–5 µm, obconical, cylindrical, or deiform, symmetrical or often asymmetrical or irregular, subhyaline to pale brown, paler than the terminal cell. Ratio of lengths of terminal:basal cell 0.8–1.8 (mean +/- SE = 1.2 +/- 0.1). Conidia after secession functionally single-celled, with the cylindrical remnant of the former basal cell remaining attached (Fig. 8C–F, I–L). Three-celled initials with septate basal cell occurring rarely. Chlamydospores sparsely produced in submerged mycelium, intercalary, single or in short chains, concolorous with conidial terminal cell,

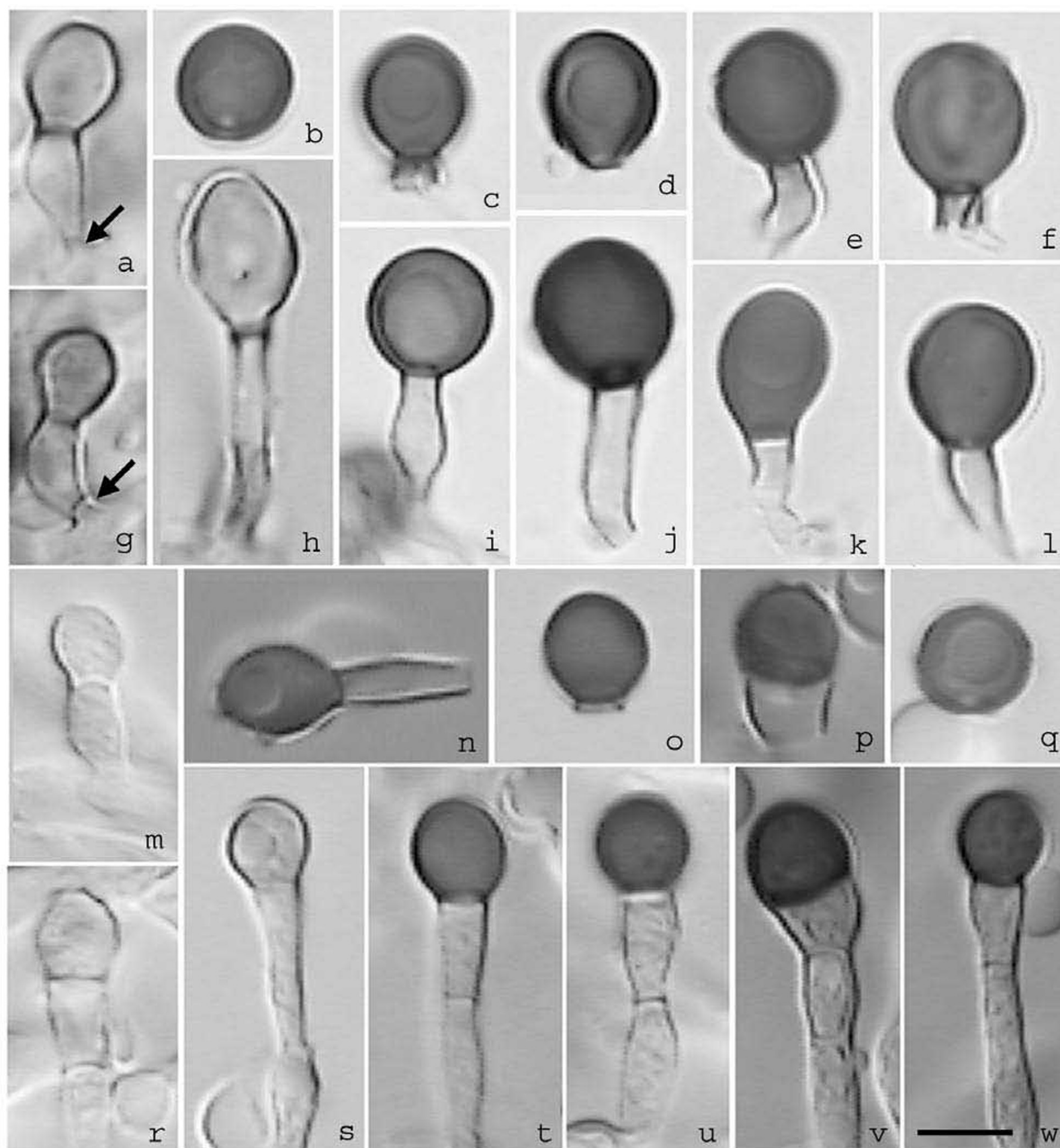


**Fig. 8.** *Leohumicola lenta* and *L. terminalis*, conidiogenous hyphae and seceded conidia, ex-type strains on PDA. A–L. *L. lenta*. A, G–I. Conidial development, arrows indicating the conidiogenous denticle that remains after the conidium secedes in A and G. B–F, J–L. Seceded conidia, showing variable length of basal cell remaining with the terminal cell after secession. M–W. *L. terminalis*. M, R–W. Conidial development. N–Q. Seceded conidia, showing variable length of basal cell remaining with the terminal cell after secession. Scale bar = 5 µm (shown in W).

cylindrical to ellipsoidal, sometimes with irregular constrictions, 7.5–14 × 4–5 µm, with walls up to 0.5 µm thick.

Colonies on PDA after 14 d under ambient light at room temperature (Fig. 5D–E) extending around 1 mm from inoculum block; colour and aerial mycelium mostly confined to inoculum. Surface of new growth smooth with sparse aerial mycelium; exudates and soluble pigment not produced. Margin smooth and entire; colony reverse pale coloured to dark Olive (3F8).

*Living culture examined*: **Canada**, Manitoba, from heated soil, native tallgrass prairie, 5 July 2002, N.L. Nickerson S285R4-6 (ex-type culture DAOM 231149 = CBS 215945, **holotype** a dried culture under the same number in herb. DAOM).

*Comments*: Morphologically, *L. lenta* is similar to *L. verrucosa*, but the conidia are larger and the terminal cell is only rarely roughened. The basal cell of the conidium initial has a more cylindrical shape than the typically cupulate structure seen in *L. verrucosa*. No sympodial proliferation of the conidiogenous cell has been observed.

***Leohumicola minima*** (de Hoog & Grinbergs) Seifert & Hambleton, **comb. nov.**, MycoBank MB500251, Figs 5G–I, 7.
≡ *Trichocladium minimum* de Hoog & Grinbergs, Trans. Br. Mycol. Soc. 64: 341. 1975 (basionym).

Conidiogenous hyphae hyaline to pale brown, 1.5–2 µm wide, solitary (Fig. 7A–B, D) or in fascicles in the aerial mycelium (Fig. 7E). Conidiogenous cells reduced to a single denticle, usually less than 0.5 µm but sometimes up to 3 µm long. Conidial initials usually two-celled, single, in pairs or in clusters along the hyphae; terminal cell (4.5–)6–8 × 3.5–5 µm (mean +/– SE = 6.8 +/– 0.1 × 4.1 +/– 0.1), ellipsoidal, sometimes with an acute apex, at first the same colour as basal cell, becoming brown while still attached; walls slightly thickened, with a few finger-like projections or spines that are about 1 µm long, thin at the base, ≤ 0.5 µm wide, and mostly found at the apex of the terminal cell; connection of basal cell to terminal cell 2–2.5 µm wide, often constricted, with a minute central pore between the two cells; basal cell 2–5.5 µm long (mean +/– SE = 2.8 +/– 0.1), 2–3 µm wide, cylindrical or doliform, usually radially symmetrical, subhyaline to pale brown, remaining paler than the terminal cell, rupturing during secession with much of the cell wall remaining attached to the terminal cell as a remnant (Fig. 7C); ratio of lengths of terminal:basal cell 1.3–3.2 (mean +/– SE = 2.5 +/– 0.1). Terminal cells sometimes developing directly on the conidiogenous hypha, with no intervening basal cell; conidia so formed appear not to secede. Chlamydospores intercalary in hyphae or fascicles, sometimes terminal, cylindrical and same width as hyphae, or swelling to become ellipsoidal, pyriform, or a lopsided ellipsoidal shape, single or in short chains, same brown colour as conidial terminal cell, (4–)6–10 × 2.5–6.5 µm.

Colonies on PDA after 14 d under ambient light at room temperature (Fig. 5G–H): 20–22 mm diam, grey (3-4BD1), darkest around the inoculum block, paler towards the margin, slightly convex or wrinkled, with a few radial sulcae, sometimes splitting the agar near colony centre, with low, lanose, white aerial mycelium. Exudates not produced; soluble pigment at first orange grey (6B3), becoming brownish grey (6CD3). Margin smooth and entire; colony reverse light to dark brown (6DF5–6).

*Living culture examined*: Isolated *ex* volcanic ash soil, Valdivia, **Chile**, 1972 (ex-type strain CBS 209.71 = DAOM 232587).

*Comments*: The original description of this fungus as a *Trichocladium* species included sufficient detail to make us suspect that the species was related to *Leohumicola*. The conidia were noted as being smaller than those of other described *Trichocladium* species (hence the epithet *minimum*), and the ontogeny, although not explicitly described by de Hoog & Grinbergs (1975) seemed similar to what we had observed in *Leohumicola* species. The phylogenetic affinities of this species with other *Leohumicola* species were confirmed via ITS sequencing. In contrast to many strains of the other species, *L. minima* forms conidia readily on both PDA and OA. Morphologically, it differs from other *Leohumicola* species in the more rapid growth of its colonies and in the more elongated shape of the conidial terminal cell. Although de Hoog & Grinbergs (1975) did not describe roughening of the conidia, we observed distinct projections at the apex of terminal cells of numerous conidia on colonies grown on PDA (Fig. 7E, arrows). Given that this fungus was originally isolated from volcanic ash soil, its conidia might be heat-resistant.

***Leohumicola terminalis*** Hambleton, Seifert & N.L. Nickerson, **sp. nov.**, MycoBank MB500252, Figs 5J–L, 8M–W.

*Etymology*: *terminalis* (L), terminal, for the terminal position of the conidia on the conidiogenous hyphae.

Conidia praecipue terminalia, cellula terminali 5–7.5 × 5–8.5, (sub)globosa, ellipsoidea vel ovata, brunnea, laevia vel modice echinulata; cellula basilari 3–8.5 × 2.5–4.5 µm, obconica, crateriformis vel cylindrica. Coloniae in agaro PDA dicto 10 mm diam. in 14 dies.

*Holotypus*: cultura ex solo isolata, exsiccata in herbario DAOM 231145, viva ex-typo CCFC.

Conidiogenous hyphae often monilioid, with individual cells hyaline and 5–10 µm long and 7–10 µm wide. Conidiophores and discrete conidiogenous cells generally not produced. Conidia arising singly at the ends of individual hyphae, usually with two-celled initials; terminal cell 5–7.5 × 5–6(–8.5) (mean +/– SE = 6.1 +/– 0.1 × 5.6 +/– 0.1), globose, subglobose to ellipsoidal or ovate, at first the same colour as basal cell (Fig. 8M, R), becoming dark brown while still attached (Fig. 8S–T); walls slightly thickened, rarely with narrow spines about 1 µm long, especially at the apex of the cell; connection to the basal cell 2–4.5 µm wide, usually not constricted, with a minute central pore connecting the two cells; basal cell (1–)3–8.5 µm long, 2.5–4.5 µm wide at the broadest part, obconical, cupulate or cylindrical, usually radially symmetrical, subhyaline to pale brown, remaining lighter than the terminal cell (Fig. 8U–W), rupturing during secession with a varying proportion of the cell wall remaining attached to the terminal cell as a remnant (Fig. 8N–Q). Ratio of lengths of terminal:basal cell 0.8–1.9 (mean +/– SE = 1.3 +/– 0.1). Some conidia producing extra cells, generally through the division of the terminal cell, resulting in a globose structure with a central septum, or in a large cell homologous with the normal terminal cell surmounted by a small, dark dome-like additional cell (Fig. 8V). Chlamydospores sparse, intercalary and solitary in hyphae of older cultures, cylindrical or slightly swollen, pale brown or darker and concolorous with the conidial terminal cell, 6.5–9 × 4.5–5 µm, with walls up to 0.5 µm thick. Vegetative mycelium normally thin, about 1–1.5 µm diam, but often consisting at least in part of hyaline, swollen, monilioid hyphae 3–9 µm wide, constricted at the septa or with segments of narrow hypha between the enlarged cells.

Colonies on PDA after 14 d under ambient light at room temperature (Fig. 5J–K): 10 mm diam, greyish yellow to yellowish green (3C–D1–2), most deeply grey around the inoculum block, most greenish in tone at the margin, convex, wrinkled, restricted, with low, lanose to felty aerial mycelium. Exudates not produced; soluble pigment at first light yellow and eventually becoming dark olive (3F5–8). Margin smooth and entire, tending to be sulcate as a result of depression of the agar by the colony. Colony reverse dark olive (3F8).

*Living culture examined*: **Canada**, Nova Scotia, from heated soil, stand of sugar maple, 29 Apr. 2002, N. L. Nickerson S267R6-1 (ex-type culture DAOM 231145 = CBS 115946, the **holotype** a dried culture in herb. DAOM).

*Comments*: *Leohumicola terminalis* is known from only a single strain. Conidia are typical of the genus, though less regularly shaped than those of *L. verrucosa* and *L. minima*. The species is distinguished morphologically by the exclusively terminal position of its conidia, a feature contrasting with the often laterally developing conidia of the other species.

## DISCUSSION

Based on the substantial number of compatible but unnamed sequences and cultures recovered from studies of soil and plant roots (detailed above), *Leohumicola* appears to be a genus that was waiting for a name. Both SSU and ITS analyses demonstrate that it is phylogenetically distinct and related to the *Leotiomycetes*. The closest relatives based on ITS BLAST searches were all unidentified fungi, sequences of which were derived from pure cultures isolated from mycorrhizal roots, or from DNA extracted directly from roots or soil. These sequences came from studies on the genetic diversity of endophytes in ectomycorrhizal and ericoid mycorrhizal roots in Italy (Bergero *et al*. 2003), Norway (Vrålstad *et al*. 2002), the west coast of Canada (Monreal *et al*. 1999) and Australia (McLean *et al*. 1999, Chen & Cairney 2002).

Additional studies of soils and ericoid mycorrhizas from other parts of the world are likely to reveal new species. As mentioned above, in addition to the four species described here, several other species have apparently been detected in the molecular studies cited above. Of particular interest are the ITS sequences that grouped in the *Leohumicola* clade (Fig. 4). Although some are not associated yet with a published study, three are based on cultures isolated from roots of ericaceous hosts, and are discussed but not identified. They are, however, shown to form ericoid mycorrhizas *in vitro*.

Looking at these sequences in more detail than was done above in the Results section, we see that putative *L. verrucosa* isolate UBCS9 (called Unknown 1 in Xiao and Berch 1996 and listed under GenBank number AF081442 by Monreal *et al*. 1999 and Berch *et al*. 2002), isolated from ericaceous (*G. shallon*) roots in British Columbia, Canada, grouped with low bootstrap support with *L. verrucosa* isolate DAOM 231141 from Alberta, Canada *Vaccinium* roots. Isolate AP-1 (McLean *et al*. 1998; accessed as GenBank number AF099089 in McLean *et al*. 1999) was isolated from epacridaceous *A. pinifolium* roots in Australia and grouped with strong support with our Australian soil isolate DAOM 230084, identified only as *Leohumicola* sp. Isolate Sd1 (Bergero *et al*. 2000; accessed as AY046401 in Bergero *et al*. 2003), isolated from *E. arborea* roots in Italy, grouped in the *Leohumicola* clade in all MPTs but its position varied. The culture was described as releasing a dark green diffusible pigment and producing stalked brown

"chlamydospores", 6–8 um in diameter, observations consistent with the generic diagnosis of *Leohumicola*. Most of our *Leohumicola* strains were isolated from soil, with only one deriving from roots. *In vitro* experiments have demonstrated that these isolates can form a typical ericoid mycorrhizal association (Nickerson, unpublished), although the precise ecological and physiological status of this association is undetermined.

The relatively slow growth of the species we have seen to date, combined with their apparent reluctance to sporulate in agar culture, suggests that demonstrating the morphological characters of additional species might be a challenge, especially if only a small number of isolates are available. As mentioned above, we recommend use of the macerated inoculum technique as a way of stimulating more rapid sporulation in these strains.

Research aimed at documenting the range of fungi endophytic in roots of the *Ericales* has already uncovered a large assemblage of nonsporulating cultural morphotypes with divergent phylogenetic affinities (Chambers *et al*. 2000, Vrålstad *et al*. 2002, Allen *et al*. 2003). Notable among the groups of closely related sequences is the one comprising the well-known ericoid mycorrhizal fungus *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang & Korf (≡ Hymenoscyphus *ericae* (Read) Korf & Kernan), known as the "*Hymenoscyphus ericae* aggregate" (Vrålstad *et al*. 2000). Much experimental work has been done examining the ecological importance of *R. ericae* and the physiological basis of the plant-fungus interaction (see Read 1983, Read & Bajwa 1985). *Rhizoscyphus ericae* is one of the few ericoid endophytes to have been studied for ecological function but, even though well-studied, the species is not readily identified. The inoperculate discomycetous teleomorph is known only from cultures and only two strains are documented as having formed apothecia (Hambleton *et al*. 1999). Appropriate cultural conditions and media, such as cereal agar, will generally induce production of the narrow, hyaline to subhyaline arthroconidia of the anamorph, *Scytalidium vaccinii* Dalpé, Sigler & Litten but otherwise cultures are often sterile, especially on the more commonly used potato dextrose and malt agars (Egger & Sigler 1993). Colonies are slow-growing on all media, with growth rates close to those of *L. verrucosa*, but they lack soluble pigments, and are characterised by the presence of fasciculate strands, deep radial furrows and narrow hyphae often forming loops and aggregating into melanised strands. Colony colour varies from pale grey to greyish or reddish brown to steel grey, typically with a narrow light-coloured colony margin on PDA (Egger & Sigler 1993, Hambleton & Currah 1997, Hambleton & Sigler 2005–this volume). *Rhizoscyphus ericae* and one nonsporulating species of "*H. ericae* aggregate", *Meliniomyces variabilis* Hambleton & Sigler, grouped together and were distinct from the *Leohumicola* clade within the *Leotiomycetes* in our SSU analyses (Fig. 3; see also Hambleton & Sigler 2005–this volume).

The multiple large Group I intron insertions in *Leohumicola* SSU sequences occurred at insertion sites previously reported in ascomycetes (Gargas *et al*.1995, Gargas & DePriest 1996, Perotto *et al*. 2000). Within the fungi relevant to ericoid mycorrhizal research, Perotto *et al*. (2000) found that Group I introns were commonly found in the root endophytes sampled but appeared only in the 3′ half of the SSU; none were found in the 5′ half. Some introns were sporadically detected among the different isolates of particular species, but studies ultimately also showed that insertions were not always amplified consistently even for individual isolates, indicating that tandem rDNA repeats are heterogeneous for their presence. The authors suggested that the number of intron-free repeats could be underestimated unless specific primers were used for screening. In our study, the size of the PCR amplicons varied within *L. verrucosa* but the results were reproducible with the primers and conditions used. Introns were located at four of the five sites noted in previous studies and at one additional site in the 5′ end, site 516. The occurrence of five SSU introns in a single strain of *L. verrucosa* contrasts with most studies that report one to three introns in a strain. Gargas *et al*. (1995) reported eight large insertions, of which 4 were analysed and identified as Group I introns, in the SSU of a single strain of *Lecanora dispersa* (Pers.) Sommerf. (*Lecanoromycetes*). Such results indicate that obtaining complete SSU sequences for future phylogenetic studies of unidentified fungal associates of ericoid mycorrhizas may be challenging.

It remains to be seen whether the type of multifaceted study used here with *Leohumicola* will allow the development of phylogenetically accurate, morphology-based generic concepts for the broad range of species presently classified in *Humicola* and *Trichocladium*, as well as in other poorly understood genera such as *Carmichaelia* and *Desertella*. It is possible that routine DNA sequence analysis may be necessary for the accurate identification of at least some such fungi.

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