Phylogenetic relationships among species of *Leotia* (Leotiales) based on ITS and RPB2 sequences

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Thirty-three collections of *Leotia* were used to investigate inter- and infra-specific relationships in the genus. Collections were obtained from various parts of the world and represent the ascomatal color forms typical in species of the genus. The ITS rDNA and a variable region of the RNA polymerase II (RPB2) gene were sequenced and analyzed using parsimony and maximum likelihood methods. Although ITS and RPB2 tree topologies differed in regard to the position of two clades of *L. lubrica* and *L. atrovirens*, no significant conflict between ITS and RPB2 data or trees was found as determined by the partition homogeneity test. RPB2 sequences in general gave results comparable to ITS; the RPB2 sequences were more easily aligned. Phylogenetic analysis of the sequence data indicates that *L. viscosa*, *L. lubrica* and *L. atrovirens* are polyphyletic species. This suggests that ascomatal color in fresh specimens is not a reliable character alone for determining species in this group. Four major well-supported groups were found; these do not fully correspond to the commonly recognized species. Stipe color, in both fresh and dry condition, seems to correlate with the major recognized groups but features of the ascospores, asci and paraphyses prove too variable to be informative. The most basal group of *Leotia* species, identified as *L. atrovirens,* differ from all others by having stipes without gel tissue in their outer layers.

The genus *Leotia* Pers. contains species of inoperculate discomycetes with stipitate-capitate ascomata that are constructed, at least in part, of tissues built-up of hyphae imbedded in a gelatinous matrix. The genus has been placed in the Geoglossaceae (DURAND 1908) but later was moved to a broadly conceived family Helotiaceae (KORF 1958). The family Helotiaceae was later reformulated as the Leotiaceae (KORF 1973). The family Leotiaceae and the order Leotiales have undergone several reinterpretations (KORF, ITURRIAGA & KIZON 1996, LIZON, KORF & ITURRIAGA 1998, KORF & LIZON 2000, 2001). These revisions were aimed at restricting the family Leotiaceae to a small number of genera that had gelatinous tissues associated with the outer excipulum. Ultimately, KORF & LIZON (2001) recognize the order, Leotiales, with a single family Leotiaceae for *Leotia* and several genera with this gelatinous construction. The position of the genus *Leotia* among the Ascomycota is not fully resolved. BARAL (1999) found the grouping of taxa suggested by Korf and co-authors unsupportable on morphological grounds. Ultrastructural studies by VERKLEY (1994) indicate that asci of *Leotia lubrica* are distinctive among the inoperculate discomycetes. Molecular phylogenetic studies have shown that *Leotia* species form one of several lineages among the inoperculate discomycetes (LANDVIK, SHAILER & ERIKSSON 1996, LANDVIK, KRISTIANSEN & SCHUMACHER 1998, GERNANDT et al. 2001). LSU and SSU rDNA sequence analyses revealed a close relationship between *Leotia* and *Microglossum* Gillet (GERNANDT et al. 2001), a taxon traditionally placed in the Geoglossaceae.

Microscopic characters show little consistent variation among species of *Leotia*; species delimitation has been based largely on ascomatal color patterns. Most authors have followed the nearly century old concepts developed by DURAND (1908). He named the species with entirely yellow ascomata *L. lubrica* (Scop.) Pers. Those species with green hymenia and yellow stalks were called *L. stipitata* (Bosc) J. Schröt. (now named *Leotia viscosa* Fr.) and those species with entirely green ascomata were referred to as *L. chlorocephala* Schw.: Fr. (now called *L. atrovirens* Pers.: Fr.). These widely used concepts have been modified only slightly by subsequent investigators (NANNFELDT 1942, MAINS 1956, GRUND & HARRISON 1967). These authors noted ambiguities and difficulties regarding species delimitations, as did DURAND (1908), who stated that identification of these fungi should only be done from fresh, living material.

A different approach to species delimitation was taken by IMAI (1936). He stated that the variability in ascomatal color, size and form was related to habitat and developmental stage. He recognized a single species, *L. lubrica*, and treated *L. viscosa* and *L. atrovirens,* as forms of *L. lubrica*. Later, he (IMAI 1941) recognized eleven forms of *L. lubrica* and one species, *L. rutilans* (S. Imai & Minakata) S. Imai. TAI (1944) raised

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six of Imai's forms to species level and added four new species. In general, new species and forms of *Leotia* have been described based on limited anatomical or morphological features (VELENOVSKY 1934, IMAI 1936, 1941, TAI 1944, BENEDIX 1955, MAINS 1956, OTANI 1982).

The purpose of our study was to investigate the inter- and infraspecific variation in the more common forms of *Leotia* using molecular characters. To assess this variation we used a series of geographically disjunct collections. Specifically, molecular sequence data were used to assess the utility of ascomatal color as a species character. We sequenced ITS rDNA and a variable region of the RPB2 gene. The ITS region has been widely used in phylogenetic studies at the species level; the protein-coding gene RPB2 has been used at the level of genus and above (LIU, WHELEN & HALL 1999, ZHANG & BLACKWELL 2001). As part of this study we were able to test the utility of this variable region of the RPB2 gene in a species level study.

Material and methods

Material studied

33 collections of *Leotia* species from localities worldwide were studied (Tab. 1). Collections were identified following DURAND'S (1908) classification that primarily uses ascomatal color to delimit species. *Microglossum viride* (Pers.: Fr.) Gillet and *M. rufum* (Schw.) Underw. were used as an outgroup. The selection of outgroup taxa was based on results indicating that *Leotia* forms a sister group to *Microglossum* (LANDVIK, SHAILER & ERIKSSON 1996, GERNANDT et al. 2001).

Morphological methods

All material was studied by light microscopy. Sections were made using a freezing microtome (Physitemp Inst. Inc., Saddle Brook, New Jersey). Congo Red in ammonia, Melzer's reagent and Cotton Blue in lactic acid (HANSEN, PFISTER & HIBBETT 1999) were used as mounting media. Morphological studies focused on characters such as spore size and shape; form and width of paraphyses; and presence and distribution of gel tissue.

Molecular methods

Total genomic DNA was extracted from fresh or dried specimens as follows: Small pieces (ca 3–5 mm) of ascomata were surface sterilized in 30 % bleach for one minute, followed by 70 % ethanol for one minute. Cleaned material was ground in liquid nitrogen and suspended in 800 µl SDS DNA extraction buffer [1 % polyvinyl polypyrolidone (PVPP), 1 % SDS, 200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, buffer adjusted to pH 6.8]. The suspension was incubated at 65 °C for about 60 minutes. Extraction was further carried-out using the DNease tissue kit (QIAGEN Inc., Balencia, California) following the manufacturer's instructions. PCR in 50 µl reactions, using Taq DNA polymerase (Invitrogen), was conducted in a Perkin Elmer Cetus' 9600 GeneAmp PCR Instrument system (Applied Biosystems, Foster City, California) under the following parameters: 94 °C for 35 seconds, 52 °C for 55 seconds, 72 °C for 1 minute and 52 seconds, for a total of 36 cycles, and then 72 °C for 10 minutes for final elongation. The ITS region, including the 5.8S rDNA, was amplified using the primers ITS4 and ITS5 (WHITE et al. 1990). The following RPB2 primers were specifically designed for *Leotia* species based on the *Leotia* sequence published by LIU, WHELEN & HALL (1999): RPB2-6FL (5' TGGGGTCTCGTCTGTCCTGC 3') and RPB2-7RL (5' CCCATAGCTTGCTTACCCAT 3'). These two primers amplified a region in the RPB2 gene between motif 6 and 7, the most variable region among different groups of ascomycetes (LIU, WHELEN & HALL 1999). PCR products were purified using the Gel Extraction Kit (QIAGEN Inc., Balencia, California). Cycle sequencing was done using BigDye Terminator (Perkin-Elmer Applied Biosystems, Foster City, California) as follows: 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes, for a total of 25 cycles. In addition to the primers used for PCR, internal primers for ITS, ITS 2 and ITS 3, were used for cycle sequencing (WHITE et al. 1990). Based on the one *Leotia* sequence in LIU, WHELEN & HALL (1999), two internal RPB2 primers were designed specifically for *Leotia* species: RPB2-6.1L (5' TCTTGTGCT AACAAGGG 3') and RPB2-6.3LR (5' AGTATCCACGTT CTTGTTTC 3'). Sequencing reactions were purified using ethanol/ammonium acetate precipitation. The purified sequence reactions were electrophoresed on an ABI PRISM 377 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, California).

Data analyses

DNA sequences were edited and contigs assembled using Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan). Sequences have been deposited in GenBank TABLE I. The sequences were aligned with the program Clustal X (THOMPSON et al. 1997) and optimized manually in the data editor of PAUP* (SWOFFORD 2001). Sequences of the ITS rDNA and RPB2 gene as individual and combined data sets were analyzed for all 33 taxa using maximum parsimony and maximum likelihood methods performed in PAUP* (SWOFFORD 2001).

Maximum parsimony analyses were conducted using heuristic searches with 1000 random taxon addition, tree bisection reconnection (TBR) branch swapping, MULPARS effective, MaxTrees set to auto-increase and branches collapsed if maximum branch length is zero. The support of branches was determined using 1000 bootstrap replicates with simple stepwise addition (FELSENSTEIN 1985). Phylogenetic trees were generated with all characters included, equally weighted, and unordered. Some regions of the ITS could not be unequivocally aligned and therefore, the sensitivity of the data were explored in parsimony analysis with gaps treated in different ways and with inclusion or exclusion of ambiguous regions. Phylogenetic analyses of the data set were performed under the fol-

Tab. 1: Specimens of *Leotia* and *Microglossum* species used in this study and their GenBank accession numbers.

Species names and numbers	Geographic origins	GenBank number ITS/RPB2
L. lubrica 1MA	Greenhill conservation land, Acton, MA, USA. 7-VII-98 D. Hibbett (FH)	AY144551, AY144516
L. lubrica 2MA	Fort Devens, MA, USA. 10-IX-98 G. A. Riner ZZ D103 (FH)	AY144554, AY144519
L. lubrica 3MA	Great Brook State Park, Carlisle, MA, USA. 14-VIII-96. D. H. Pfister (FH)	AY144544, AY144509
L. lubrica 4Chi	Dongling Mountain, Beijing, China, 4-IX-99, Z. Wang WZ 76063 (FH)	AY144556, AY144521
L. lubrica 5Chi	Dongling Mountain, Beijing, China, 19-VIII-98, Z. Wang WZ 75863 (FH)	AY144550, AY144515
L. viscosa 1Chi	Hailuogou, Gongga Mountain, NW Sichuan, China, 16-VIII-97, D. Hibbett, WZ 2030 (FH)	AY144537, AY144502
L. viscosa 2MA	Fort Devens, MA, USA, 30-VIII-98, G. A. Riner (FH)	AY144538, AY144503
L. viscosa 3Chi	Gongga Mountain, NW Sichuan, China, 23-VIII-97, Z. Wang WZ 2144 D. Hibbett (FH)	AY144535, AY144500
L. viscosa 4Chi	Gongga Mountain, NW Sichuan, China, 20-VIII-97 Z. Wang WZ 2107 (FH)	AY144536, AY144501
L. viscosa 5VT	Newfane Hill, Newfane, VT, USA, 22-VIII-82, D. H. Pfister (FH)	AY144539, AY144504
L. atrovirens 1Chi	Gongga Mountain, NW Sichuan, China, 23-VIII-97, Z. Wang WZ 2145 (FH)	AY144567, AY144532
L. atrovirens 2NH	Lincoln, NH, USA, 25-VII-99, W. Neill ZZ 99-20 (FH)	AY144565, AY144530
L. atrovirens 3CAN	Cory Lake, Chalk River, Ontario, Canada, IX-41, J. W. Groves, 7653 (FH)	AY144566, AY144531
L. lubrica 4PA	Alan Seeger State Park, PA, USA, 7-VIII-82, D. H. Pfister (FH)	AY144557, AY144522
L. atrovirens 5NJ	Newfield, NJ, USA, 18-VIII-84, D. H. Pfister (FH)	AY144563, AY144528
L. lubrica 6VT	Powerline, Galistonbury, VT, USA, 29-VIII-81, D. H. Pfister (FH)	AY144558, AY144523
L. atrovirens 02VT	Middleberry Gap, Green Mountain National Park, VT, USA, 3-VIII-00, D. H. Pfister ZZ 00-02 (FH)	AY144564, AY144529
L. lubrica 04VT	Middleberry Gap, Green Mountain National Park, VT, USA, 3-VIII-00, D. H. Pfister ZZ 00-04 (FH)	AY144559, AY144524
L. lubrica 14VT	Norwich, VT, USA, 4-VIII-00, K. Peterson ZZ 00-14 (FH)	AY144540, AY144505
L. lubrica 15VT	Norwich, VT, USA, 4-VIII-00, K. Peterson ZZ 00-15 (FH)	AY144560, AY144525
L. lubrica 21ME	Eagle Hill, Steuben, ME, USA, 5-IX-00, K. Peterson ZZ 00-21 (FH)	AY144561, AY144526
L. lubrica 25VT	Indian Brook Conservation, VT, USA, 30-VII-00, Z. Zhong ZZ 00-25 (FH)	AY144555, AY144520
L. lubrica 32VA	Jefferson National Forest, Giles County, VA, USA, 13-VIII-00, H. Miller ZZ 00-32 (FH)	AY144545, AY144510
L. lubrica 41CA	Santa Cruz Co., Soquel State Forest, CA, USA, 5-III-94, K. Shanks DD KMS 441 (SFSU)	AY144552, AY144517
L. lubrica 80CA	Wunderlich Park, San Mateo Country, CA, USA, 26-I-86, M. Seidl, DD MTS 880 (SFSU)	AY144553, AY144518
L. lubrica 1Eng	Pruteland, Northumberland, Northeastern England, 28-IX-00, G. Beakes (FH)	AY144546, AY144511
L. lubrica 2Eng	Allen Valley, Northumberland, Northeastern England, England, 14-X-00, G. Beakes (FH)	AY144541, AY144506
L. lubrica 3Eng	Kielder castle, Northumberland, Northeasten England, England, 8-X-00, G. Beakes (FH)	AY144548, AY144513
L. lubrica 4Eng	Kingston Surrey, near Kew, Southeastern England, England, 21-X-00, G. Beakes (FH)	AY144547, AY144512
L. lubrica 1Aus	Mt. Field National Park, Russel Falls, Tasmania, Australia, 27-V-87, P. K. Buchanan PDD55184 (PDD)	AY144542, AY144507
L. lubrica 2New	Otago Lakes, vic. Makarora, Cameron Flat, New Zealand, 3-IV-00, E. Horak PDD72250 (PDD)	AY144543, AY144508
L. lubrica 1Den	Jutland, NW of Aars, Jenle plantage, Denmark, 12-VIII-00, T. Laessoe TL-5950 (C)	AY144562, AY144527
L. lubrica 1Nor	Sogndal, Hella, nr Ferry port, Norway, 9-IX-00, T. Laessoe, TL-5985 (C)	AY144549, AY144514
M. viridi	Gongga Mountain, China, 23-VIII-97, Z. Wang WZ 2149 (FH)	AY144534, AY144499
M. rufum	"Ballycroy" near Toronto, Ontario, Canada, 5-VIII-89, D. H. Pfister (FH)	AY144533, AY144498

lowing options: all characters included with gaps treated as missing data; regions with missing and ambiguous sequences (as indicated through PAUP) deleted; all sequence characters included with gapped positions coded (BAUM, SYTSMA & HOCH 1994); a transition transversion ratio Ti/Tv = 1:3 (estimated from maximum likelihood methods).

For maximum likelihood analyses the model with the highest maximum likelihood score was selected by Modeltest version 3.06 (POSADA & CRANDALL 1998). The best-fit models selected were TrNef + G and TrN + G, which are submodels of GTR + G, for ITS and RPB2 data, respectively. The parameters estimated from the TrNef + G model were as follows: -lnL = 1740.3920; equal base; substitution model: rate matrix: R(a) [A-C] = 1.0000, R(b) [A-G] = 2.9619, R(c) [A-T] = 1.0000, R(d) [C-G] = 1.0000, R(e) [C-T] = 7.4791, R(f) [G-T] = 1.0000; among-site rate variation: proportion of invariable sites = 0; variable sites (G): Gamma distribution shape parameter = 0.3720. The parameters for TrN + G model were as follows: -lnL = 2349.8379; Base frequencies: freqA = 0.2901, freqC = 0.1964, freqG = 0.2511, freqT = 0.2623; Substitution model: Rate matrix: R(a) [A-C] = 1.0000, R(b) [A-G] = 9.6700, R(c) [A-T] = 1.0000, R(d) [C-G] = 1.0000, R(e) [C-T] = 17.5263, R(f) [G-T] = 1.0000; Among-site rate variation: Proportion of invariable sites = 0; Variable sites (G): Gamma distribution shape parameter = 0.1970. The general time-reversible (GTR) model (LANAVE et al. 1984, TAVARE 1986, RODRIGUEZ et al. 1990) allows unequal base composition and various probabilities for each of the six substitution types. G accommodates heterogeneous rates of change at all sites assumed to follow a discrete approximation of gamma distribution (YANG 1994). A molecular clock (SANDERSON 1998) was enforced under the best models, and a likelihood ratio test (GOLDMAN 1993a, 1993b) was conducted to evaluate whether the clock model could be accepted given the data. To lower the search time some simple models were also carried out to generate trees. The models were Jukes-Cantor (JC) (JUKES & CANTOR 1969), Felsenstein 81 (F81) (FELSENSTEIN 1981), Kimura 2-parameter (K2P) (KIMURA 1980), Hasegawa-Kishino-Yano 85 (HKY 85) (HASEGAWA, KISHINO & YANO 1985) and K2P + γ (KIMURA 1980, YANG 1994). In these analyses missing and ambiguous regions, as indicated by PAUP, and taxa with identical or almost identical sequences were excluded. Parameters were set to "estimate" in the analyses. One of the most parsimonious trees was used as the starting tree to enhance the speed of heuristic searches using TBR branch swapping.

To test the congruency of the ITS and RPB2 data sets, the partition homogeneity test, also called incongruence length difference (ILD) test (FARRIS et al. 1994), was performed using parsimony. To test the congruency of ITS and RPB2 trees several tree-based comparisons were conducted. These tests were the non-parametric Winning-sites tests and Templeton test (TEMPLETON 1983) in parsimony analysis and parametric Kishino-Hasegawa tests (KISHINO & HASEGAWA 1989) under the best models in likelihood analyses.

Results

RPB2 and ITS trees

In the 865 bp RPB2 region sequenced only three gaps were needed to allow alignment and these gaps were uninformative. A single most parsimonious tree (Fig.1, A) was generated with all characters included, equally weighted, and gaps treated as missing data (number of informative characters = 112, tree length = 262 steps, CI = 0.844, RI = 0.876). On the contrary, there were many insertions and deletions in the ITS sequences. Four equally most parsimonious trees were generated from the ITS data with the same setting (number of characters = 634, number of informative characters = 122, tree length = 259 steps, CI = 0.826, RI = 0.898). The four ITS trees showed the same groupings and the topologies were nearly identical except for the position of one subgroup of two collections of *L. lubrica* from California. The topology of the consensus tree was identical to one of the most parsimonious trees (Fig. 1B). Maximum likelihood and maximum parsimony analyses with all characters equally weighted gave identical trees for ITS and RPB2 data respectively. The molecular clock was rejected for the ITS data ($P = 0.000$) under the best model, while it was not rejected for the RPB2 data ($P = 0.995$).

The molecular phylogenetic analysis showed para- and polyphyly among the species as recognized morphologically. Four well-supported groups (I–IV) were recognized in all trees with bootstrap values ranging from 74 % to 100 % (Figs. 1 A, B). Within the largest group, group I, six subgroups were recognized, indicated as a-f in Fig. 1; the groupings were identified in both the ITS and RPB2 trees (Figs. 1 A,B). Topologies of the single most parsimonious RPB2 tree (Fig. 1B) and the ITS consensus are similar in most respects. They differ in the positions of groups II and III. In the RPB2 tree group III forms a sister group to groups I and II; in the ITS tree group II is sister to groups I and III. Arrows indicate the nodes in question.

ITS - RPB2 congruency

The partition homogeneity test showed that there was no significant conflicts between ITS and RPB2 data (100 replicates, $P = 0.06$) even though the tree topologies differed. Non-parametric tests and the Kishino-Hasegawa test did not indicate significant disagreement between the ITS and RPB2 trees (RPB2 data set: Templeton $P = 0.046$, Winning-sites $P = 0.125$; ITS data set: Templeton $P = 0.564$, Winning-sites $P = 1.000$; Kishino-Hasegawa tests: RPB2 data set using model TrN + \ with molecular clock enforced: $P = 0.053$, ITS data set using model TrNef + γ with no clock enforced: $P = 0.075$). These tests indicated that RPB2 and ITS data could be combined. A single most parsimonious tree from the combined data was found (Fig. 2, number of characters = 1499, number of informative characters = 234, tree length = 522 steps, CI = 0.833, RI = 0.887) with a topology identical to the RPB2 tree (Fig. 1A). Bootstrap support increased for most branches of

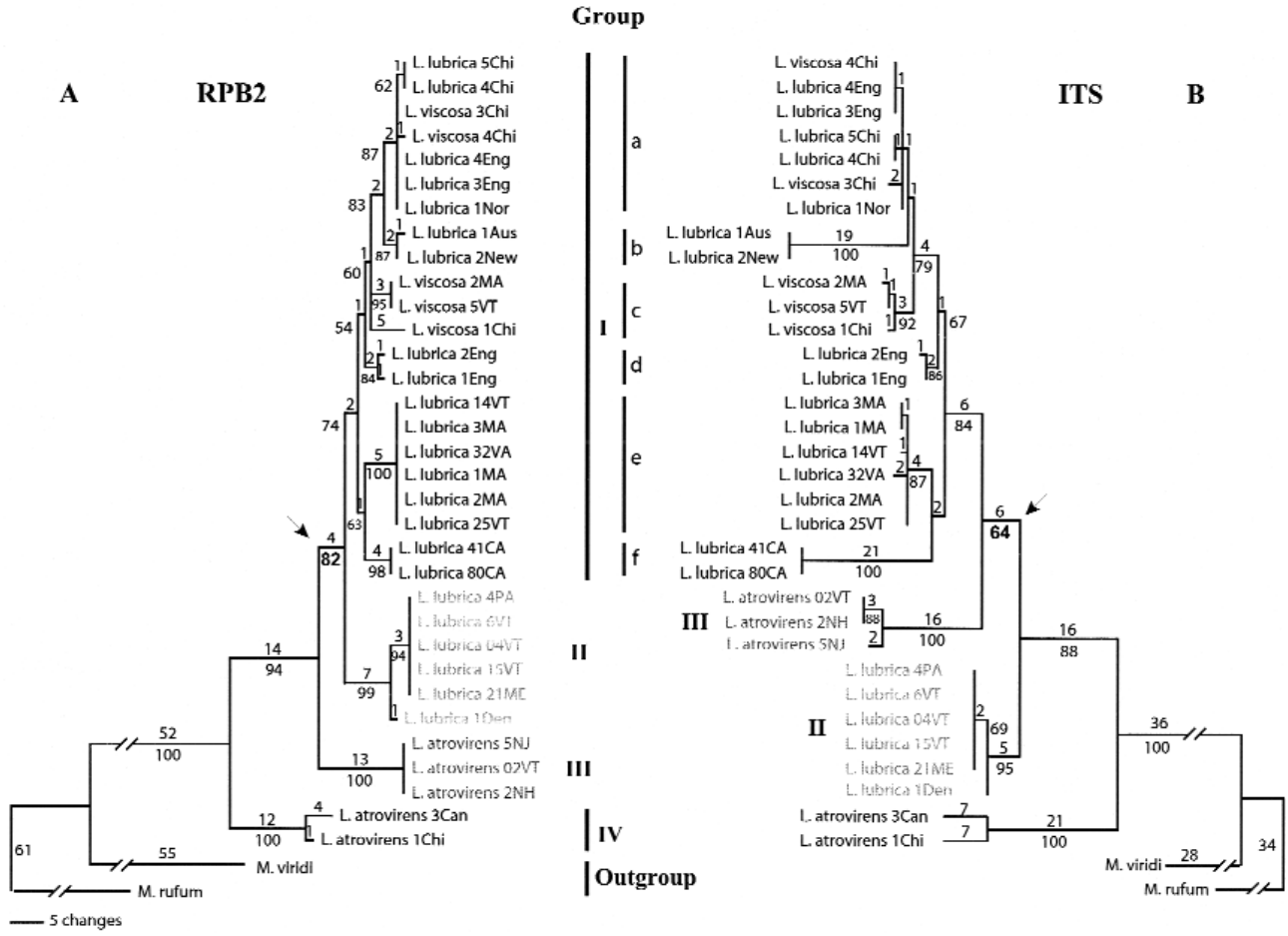

Fig.1: RPB2 and ITS trees with species of *Leotia* (*L.*) and *Microglossum* (*M.*). Numbers above the branches indicate the branch length, and those below are bootstrap values. Arrows indicate the branches involved in the position of groups II and III. Collections are numbered and geographical details are as follows: MA, VT, VA, CA, PA, ME, NJ, NH for states in the USA; Aus = Australia; Can = Canada; Chi = China; Den = Denmark; Eng = England; New = New Zealand. – **A**. RPB2 phylogram of the single most parsimonious tree generated using heuristic search with 1000 replicates. Of 865 characters, all are unordered and equally weighted; 112 are parsimony-informative. Gaps are treated as 'missing'. The length = 262; CI = 0.844; RI = 0.876. – **B**. One of the four most parsimonious trees from ITS data using heuristic search with 1000 replicates. Of 634 characters, all are unordered and equally weighted; 122 are parsimony-informative. Gaps are treated as 'missing'. The length = 259; CI = 0.826; RI = 0.898.

the combined tree compared to the non-combined trees. Support for the monophylly of group I plus II (the *L. lubrica – viscosa* group) is low (see arrow, Fig. 2).

Maximum parsimony analyses of the ITS data under different character treatments produced different topologies. When gap positions and ambiguous sequences (defined by PAUP as missing ambiguous data) were omitted, the topology produced was identical to the ITS tree generated from all sequence characters included and gaps treated as missing data (Fig. 1 B). However, when the gap coding was added or Ti/Tv was defined as 1:3 groups II and III switched positions in the tree and produced an ITS tree topology identical to the RPB2 tree (Fig. 2).

Morphology and groups

Morphological studies allowed placement of taxa in the morpho-species generally accepted. The size and shape of spores, asci, and paraphyses were recorded and studied but these characters were not found to distinguish the lineages identified by molecular data. Although the size and shape of the spores varied (ranging from 16–29 µm long and 3–8 µm wide), the variation was continuous and no disjunct patterns were found.

Based on our analyses none of the three morphologically defined species, *L. lubrica*, *L. viscosa* and *L. atrovirens,* were monophyletic (Figs. 1,2). Group I consisted of the "typical collections" of *L. lubrica* (uniformly yellow throughout and stipes usually remaining yellow when dried) and collections of *L. viscosa* (green hymenium and yellow stipe). Ascomata of typical *L. lubrica* and *L. viscosa* shared stipe color and stipe tissue morphology. Group II included *L. lubrica* collections with entirely yellow ascomata that became entirely dark green when dried. It was difficult to distinguish these collections from the typical *L. lubrica* collections in fresh conditions. In group III all collections contained at least some green asco-

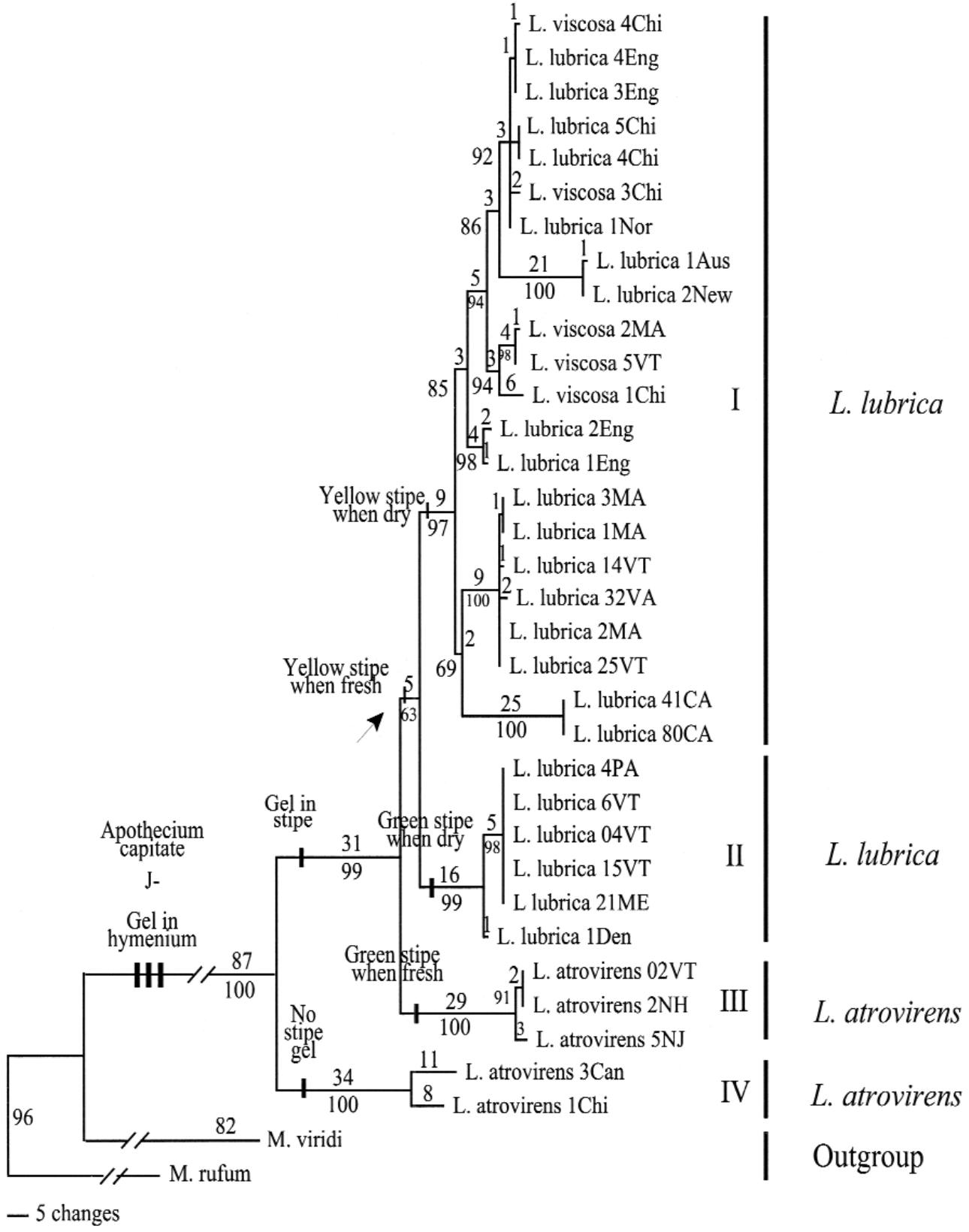

Fig. 2: Phylogram of the single most parsimonious tree generated from combined ITS and RPB2 data using heuristic search with 1000 replicates. Of 1499 characters, all are unordered and equally weighted; 234 are parsimony-informative. Gaps are treated as 'missing'. The length = 522; CI = 0.833; RI = 0.887. Abbreviations and notations are as in Fig. 1.

mata when fresh and all ascomata in this group became green when dried. These collections were identified as *L. atrovirens.* The presence of green ascomata distinguishes group III from groups I and II. Collections in group IV, also identified as *L. atrovirens*, were entirely green or dark green when fresh and remained green when dried. Members of group IV have no gelatinous tissue in the stipe. In these collections an outer layer composed of parallel or someway interwoven hyphae is found but there is no gelatinous matrix (Fig. 3 B, D). In collections from groups I, II and III one or more layers of gelatinous tissue were noted in the stipes (Figs. 3 A, C). Four collections (*L. lubrica* 1Aus, *L. lubrica* 32VA, *L. lubrica* 80CA and *L. atrovirens* 2NH) showed only a single outer gel layer. All the collections of *Leotia* we studied had gelatinous material in the swollen head. Several morphological characters were mapped on the tree generated from the combined RPB2 and ITS data sets (Fig. 2).

Disscusion

This study casts doubt on the use of ascomatal gel as the single character to distinguish a restricted family Leotiaceae in the order Leotiales (KORF & LIZON 2001). We would refer group IV collections, those lacking gel in the stipe, to *Leotia* based on the presence of gel in the hymenium and the inamyloid asci, as well as because of their morphology. They have more or less globose hymenial heads as is characteristic of *Leotia* species as opposed to *Microglossum* species that characteristically have elongate heads. *Microglossum* species have an amyloid ascus pore that further distinguishes them from *Leotia* species.

The results of this study bring into question the morphological system of species identification that is currently in use. Color characters may be useful but need to be recorded in both fresh and dried condition. The general view (DURAND 1908, IMAI 1936, 1941) that microscopic features are of minor importance for the taxonomy of these fungi has been confirmed but some characters, particularly in the pigmentation of fresh and dried ascomata and in distribution of gelatinous tissues are shown to be of importance. In particular, the pigmentation of the stipe provides some support for the four groups. It should be noted that the composition and function of the green pigment is not known. ARPIN (1969) studied the carotenoids in discomycetes and used presence, absence and type of carotenoids in his classification. He reported the presence of carotenes in both *L. lubrica* and *Microglossum olivaceum*. Characterization of the green pigment and its further study would be helpful. *Leotia lubrica* forma *stevensoni* DURAND (1908) is distinguished from typical *L. lubrica* by the production of uniformly greenish or olivaceous ascomata in age or in drying. In our study, collections with this type of coloration form a monophyletic group (group II).

This study does not provide a complete morphological analysis of the species treated. Such a study, involving ascus and crozier formation, histochemical study of asci in living condition and other features called for by BARAL'S (1992) vital taxonomy would greatly contribute to the understanding of species in this genus. Such a study is beyond the scope of the present work.

One of the significant findings in our study was the absence of gelatinized tissue in stipes of two collections (group III) identified as *L. atrovirens* (Fig. 3 B, D). These formed a sister group to the rest of the ingroup. DURAND (1908), BENEDIX (1955) and MAINS (1956) described two gel layers in the stipe of *Leotia* species. The presence of gelatinous layers, along with inamyloid asci and more or less globose apothecial heads, distinguish *Leotia* species from species of *Microglossum*. MOORE (1965) summarized the previous studies of gel tissue ontogeny in *L. lubrica* and detailed its development in *L. viscosa*. Our studies confirm the presence of stipe gel in all collections except those of group IV. Further study will be necessary to resolve the proper names for the two species with entirely green ascomata.

Recognition of multiple taxa within *Leotia* by IMAI (1936, 1941) and BENEDIX (1955) has been followed by few workers, but our studies indicate that there may be more genetic variation among *Leotia* collections than is commonly recognized. These alternative systems will need to be re-evaluated. Although sequence differences have been shown in this work, providing a formal classification will require reconciliation of the limited morphological data with the sequence data and will require study of the ecology and biology of the species involved.

The ITS rDNA and RPB2 gene sequences both provided useful information for inferring phylogenetic relationships in this study. The RPB2 and ITS trees (Fig. 1) generated with characters equally weighted and gaps treated as missing data differed from each other in the positions of group II and III and in the monophylly of subgroup c of group I. The bootstrap values supporting the positions of groups II and III were much higher in the RPB2 tree than in the ITS tree (82 % versus 64 %, indicated by arrows in Figs. 1 A, B). In the combined tree (Fig. 2), which agrees with the RPB2 tree in the position of group III the support is also relatively low (indicated by arrows).

In addition to the use of RPB2 sequences in broad scale phylogeny of fungi at ordinal level (LIU, WHELEN & HALL 1999), RPB2 sequence analyses have been successfully applied at family and genus levels (ZHANG & BLACKWELL 2001). In their studies the region of the RPB2 gene between motif 6 and 7 (LIU, WHELEN & HALL 1999), the same region we used, was sequenced in conjunction with sampling of the SSU and LSU rDNA. They converted sequences to amino acids, which were in turn used to infer higher-level relationships. Our study has shown that nucleotides in this region provide good resolution at the inter- and infra-specific level in *Leotia.*

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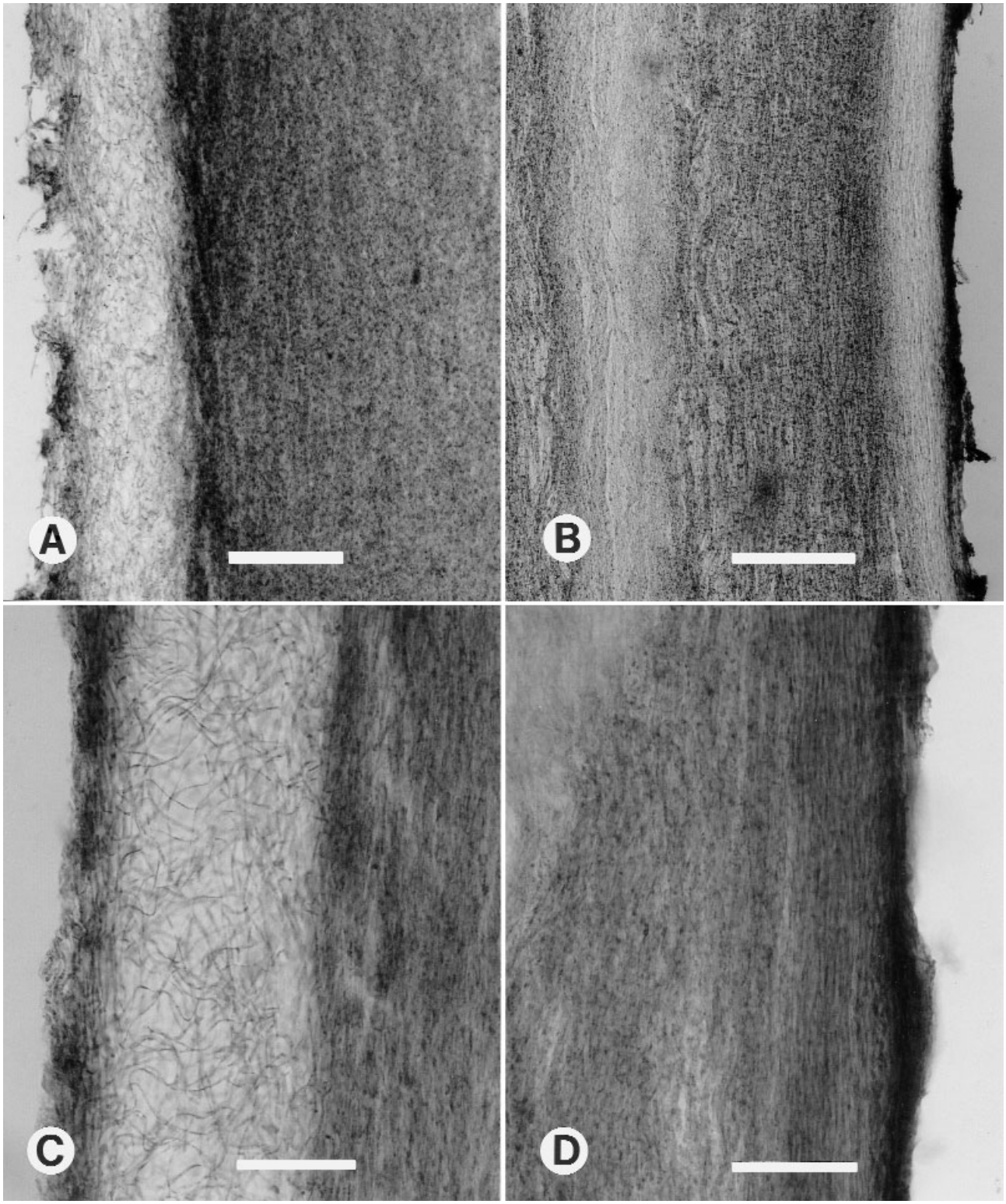

Fig. 3 A–D: Longitudinal sections through stalks of *Leotia* species showing outer most layers of the stalk. All material mounted in Cotton Blue in lactic acid. **A**. *L. atrovirens* 2NH, one of the group III collections, showing gelatinous layer on the outside of the stalk. Scale = 200 µm. **B**. *L. atrovirens* 3Can, one of the group IV collections, no gelatinous layer is present. Scale = 200 µm. **C**. *L. lubrica* 6VT, a member of group II, illustrating gelatinous layer. Scale = 100 µm. **D**. *L. atrovirens* 1Chi, another of the group IV collections, no gelatinous layer is present. Scale = 100 µm.

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