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FULL PAPER

Molecular phylogenetic studies of *Lachnum* and its allies based on the Japanese material

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Abstract Molecular phylogenetic studies were carried out based on ITS-5.8S rDNA, the D1–D2 region of the large subunit rRNA gene, RPB2, and combined data of D1–D2 and RPB2 as well as these three genes on 36 species among 7 genera for *Lachnum* and allied genera in the family Hyaloscyphaceae. In the combined data of all three regions, seven strongly supported clades were obtained. The same clades were also recognized in most of the trees based on each gene, and the combined data of D1–D2 and RPB2, although some of them were not strongly supported. Four clades represented *Albotricha*, *Brunnipila*, *Incrucipulum*, and *Lachnellula*, respectively, whereas *Lachnum* was distributed to the remaining three clades. The molecular phylogenies strongly supported a group of species with granulate hairs, and we suggest the concept of Lachnaceae should be restricted to these species. Based on the molecular phylogenetic analysis, three new combinations—*Incrucipulum longispineum*, *I. radiatum*, and *Lachnellula pulverulentum* from *Lachnum*—are proposed.

Keywords Generic concept · Hyaloscyphaceae · Lachnaceae · Lachnoid fungi · Taxonomy

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Introduction

The genus *Lachnum* Retz. is currently included in the family Hyaloscyphaceae Nannf., Helotiales [(syn. Lachnaceae) Raitviir 2004; Lumbsch and Huhndorf 2007; Kirk et al. 2008], and embraces about 250 species (Kirk et al. 2008). *Lachnum* occur on various substrates including ferns, herbs, and wood or leaves of coniferous or broad-leaved trees. Morphologically, *Lachnum* is characterized by usually stipitate, less frequently sessile apothecia, having totally granulate hairs that are hyaline or brown colored, equipped with crystals or resinous matters at their apices. The genus also has paraphyses typically lanceolate to narrowly lanceolate or nearly cylindrical, usually extending over the asci at various degrees. The ectal excipulum is typically textura prismatica, but textura angularis is also included. The color of the hymenium varies greatly, from pure white to yellow or reddish. All these characters are used in combination in the taxonomy of *Lachnum.*

In the Hyaloscyphaceae, several genera similar in their morphology of hairs, asci, paraphyses, and ectal structures are included, e.g., *Albotricha* Raitv., *Dasyscyphella* Tranzschel, *Lachnellula* P. Karst., *Trichopeziza* Fuckel, and *Trichopezizella* Dennis ex Raitv. They are characterized by having either granulate hairs or lanceolate paraphyses and are here termed "lachnoid fungi." Lachnoid fungi were included in the tribe Lachneae Nannf. in the family Hyaloscyphaceae Nannf. until Raitviir (2004) raised Lachneae to the familial rank. Nannfeldt (1932) interpreted *Lachnum* in the broad sense, and the genus embraced many diverse species. Since Nannfeldt (1932), the generic taxonomy of lachnoid fungi has been revised by various authors based on different interpretations of the diagnostic characters (Dennis 1949, 1962; Korf 1973; Raitviir 1987),

and several new genera were proposed (Raitviir 1970; Baral and Krieglsteiner 1985). To stabilize the taxonomy of lachnoid fungi, examination of the morphological characters in the context of a molecular phylogeny is crucial.

Cantrell and Hanlin (1997) analyzed the Hyaloscyphaceae including 13 species of *Lachnum* and allied genera (1 species each from *Lachnellula*, *Trichopeziza*, and *Trichopezizella*) based on internal transcribed spacer region and 5.8S ribosomal DNA (ITS-5.8S rDNA) sequences. In their analysis, *Lachnum* was suggested to be paraphyletic, and the foregoing genera formed a monophyletic group with *Perrotia* Boud., *Solenopezia* Sacc., and *Proliferodiscus* Spooner, but not with strong support. They interpreted this clade to represent the subfamily Lachnoideae.

Inspired by Cantrell and Hanlin (1997); Raitviir (2004) raised the concept of the tribe Lachneae (Nannfeldt 1932) to familial rank and established the family Lachnaceae, defined as "apothecia medium-sized to large, superficial, stipitate, sometimes sessile, externally covered with long hairs. Ectal excipulum of textura prismatica, in some genera of textura angularis or textura globulosa, cells hyaline or brownish, thin-walled, sometimes thick-walled. Hairs generally cylindrical, but sometimes tapering to conical, usually multiseptate, with thin to thick hyaline or brownish walls, warted or smooth. Hair wall layers are clearly stratified in TEM. Asci cylindrical or cylindric-clavate, ascus apparatus well developed, pore mostly blue in Melzer's reagent (MLZ), or not blue in MLZ. Spores hyaline, of very variable size and shape, mostly aseptate, but in a few species up to 15-septate." His idea to include all the lachnoid genera in Lachnaceae was based on the data suggested by Cantrell and Hanlin (1997), but monophyly of the lachnoid genera was not strongly supported by molecular phylogeny (Cantrell and Hanlin 1997).

The analysis of Cantrell and Hanlin (1997) was solely based on ITS-5.8S rDNA, and taxon sampling and bootstrap values were not sufficient to discuss the generic taxonomy. Deeper structures, including the relationship between genera, were not resolved.

Recently, molecular phylogeny based on multiple genes, including protein-coding genes, has been used to increase phylogenetic resolution (Liu and Hall 2004; Lutzoni et al. 2004; Hansen et al. 2005; James et al. 2006; Spatafora et al. 2006). Molecular phylogenetic analysis based on multiple genes incorporating multiple lachnoid genera would clarify the generic and familial delimitation and reveal morphological characters important for taxonomy. In the present article, we carried out molecular phylogenetic analysis of the lachnoid fungi using multiple genes. Because the generic taxonomy of lachnoid fungi is still confused, we tentatively followed the generic concept of Raitviir (1987) for identification, and then considered their taxonomic position based on molecular phylogeny.

Materials and methods

Collection and isolation

Fifty-eight specimens, 36 species distributed in 7 genera excluding the outgroup, were collected (Table 1). Collection, isolation, and observation techniques followed Hosoya and Otani (1997). The specimens obtained included 12 unidentified species new to science. Descriptions of these fungi will be presented elsewhere (in preparation). The specimens used in the present study were deposited in the mycological herbarium of the National Museum of Nature and Science (TNS). Isolates were deposited to Biological Resource Center, National Institute of Technology and Evaluation (NITE-BRC).

DNA extraction, polymerase chain reaction (PCR), and sequencing

Isolates were cultivated in 2 ml 2% malt extract for 2 weeks, and the mycelia were harvested and frozen at –80°C. About 50 mg mycelium was mechanically lysed by a Qiagen TissueLyser, using ceramic beads. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. To amplify internal transcribed spacer (ITS1 and ITS2) and 5.8S ribosomal regions, the primer pair ITS1F and ITS4 (White et al. 1990) was used. To amplify the D1–D2 region of large subunit rDNA (28S rDNA), the primer pair NL1 and NL4 (O'Donnell 1993) was used. To amplify the partial sequence of the RNA polymerase II second largest subunit (RPB2), primer pairs fRPB2-5F and RPB2-P7R, RPB2-P6F and RPB2-P7R, or RPB2-P6Fa and fRPB2-7cR (Liu et al. 1999; Hansen et al. 2005) were used.

DNA was amplified using 40 μl PCR reactions, containing 0.2 μM each primer, 1 U TaKaRa Ex Taq DNA polymerase (TaKaRa, Tokyo, Japan), and a deoxynucleoside triphosphate (dNTP) mixture containing 2.5 mM each dNTP and ExTaq buffer containing 2 mM Mg^{2+}. PCR was carried out using a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). For ITS-5.8S rDNA and the D1–D2 region of 28S rDNA, DNA was denatured for 3 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min. For RPB2, DNA was denatured for 3 min at 95°C, followed by 25 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 40 s, and extension at 74°C for 2 min. Then, another 25 cycles were carried out by increasing the extension step for 5 s in every cycle at the same temperature. The final extension of 10 min at 72°C was added at the end of the reaction.

Table 1 Taxa analyzed in the present study

Isolate no.	TNS-F-no.	Species	Locality	Substrate	Collecting date	GenBank accession number		
						ITS-5.8S	D1–D2	RPB2
FC-2243	16682	*Albotricha acutipila* (P. Karst.) Raitv.	Oirase, Aomori	Stem of bamboo	5/26/2006	AB481233		
FC-2262	16740	*Albotricha acutipila* (P. Karst.) Raitv.	Sugadaira, Nagano	Stem of bamboo	6/17/2006	AB481234	AB481317	AB481354
FC-2094	16497	*Albotricha albotestacea* (Desm.) Raitv.	Sugadaira, Nagano	Stem of *Miscanthus*	5/18/2005	AB481235	AB481303	AB481340
FC-2261	16730	*Albotricha albotestacea* (Desm.) Raitv.	Sugadaira, Nagano	Stem of *Miscanthus*	6/17/2006	AB481236		
FC-2190	16624	*Albotricha* sp.1	Sugadaira, Nagano	Stem of bamboo	9/29/2005	AB481237	AB481310	AB481347
FC-2282	16771	*Albotricha* sp.1	Sugadaira, Nagano	Stem of bamboo	9/24/2006	AB481238		
FC-2013	16439	*Dasyscyphella longistipitata* Hosoya	Yamakita, Kanagawa	Cupule of *Fagus crenata*	4/17/2005	AB481239	AB481294	AB481331
FC-2018	16433	*Dasyscyphella longistipitata* Hosoya	Mt. Tanzawa, Kanagawa	Cupule of *Fagus crenata*	4/16/2005	AB481240		
FC-2031	16466	*Dasyscyphella montana* Raitv.	Sugadaira, Nagano	Wood	4/29/2005	AB481241		
FC-2070	16527	*Dasyscyphella montana* Raitv.	Sugadaira, Nagano	Wood	5/21/2005	AB481242	AB481299	AB481336
FC-2209	16701	*Dasyscyphella* sp.1	Mayagatai, Aomori	Wood	5/27/2006	AB481243	AB481313	AB481350
FC-2034	16462	*Lachnellula occidentalis* (G.G. Hahn & Ayers) Dharne	Kakuma, Nagano	Twig	4/29/2005	AB481244	AB481296	AB481333
FC-2067	16513	*Lachnellula occidentalis* (G.G. Hahn & Ayers) Dharne	Sugadaira, Nagano	Twig	5/19/2005	AB481245		
FC-2304	16450	*Lachnellula resinaria* (Willd.) Dharne	Itako City, Ibaraki	Wood	2006	AB481246		
FC-2354	16812	*Lachnellula subtilissima* (Cooke) Dennis	Mt. Shirane, Gunnma	Twig	6/8/2007	AB481247	AB481326	AB481363
FC-2110	16529	*Lachnellula suecica* (de Bary ex Fuckel) Nannf.	Sugadaira, Nagano	Twig of *Larix kaempfer*	5/21/2005	AB481248	AB481304	AB481341
FC-2147	16582	*Lachnum abnorme* (Mont.) J.H. Haines & Dumont	Yamakita, Kanagawa	Wood	7/2/2005	AB481249		
FC-2172	16617	*Lachnum abnorme* (Mont.) J.H. Haines & Dumont	Yamakita, Kanagawa	Twig	7/3/2005	AB481250	AB481309	AB481346
FC-2056	16494	*Lachnum asiaticum* (Y. Otani) Raitv.	Sugadaira, Nagano	Stem of bamboo	5/18/2005	AB481251	AB481297	AB481334
FC-2294	16758	*Lachnum ciliare* (Schrad.) Rehm	Sugadaira, Nagano	Leaf of *Quercus crispula*	9/22/2006	AB481252	AB481324	AB481361
FC-2295	16759	*Lachnum ciliare* (Schrad.) Rehm	Sugadaira, Nagano	Leaf of *Quercus crispula*	9/22/2006	AB481253		
FC-2197	16637	*Lachnum fuscescens* (Pers.) P. Karst.	Agatsuma, Gunnma	Leaf of *Lindera obtusiloba*	4/27/2006	AB481254		
FC-2200	16635	*Lachnum fuscescens* (Pers.) P. Karst.	Agatsuma, Gunnma	Leaf	4/27/2006	AB481255	AB481311	AB481348
FC-2323	17632	*Lachnum longispineum* Hosoya & Issh. Tanaka	Sendai, Miyagi	Leaf of *Lyonia ovalifolia*	7/29/2006	AB481256	AB481325	AB481362
FC-2212	16651	*Lachnum nudipes* (Fuckel) Nannf.	Mt. Iwaki, Aomori	Stem of *Fallopia*	5/24/2006	AB481257	AB481314	AB481351
FC-2216	16709	*Lachnum nudipes* (Fuckel) Nannf.	Yuzawa, Niigata	Stem of bamboo	5/12/2006	AB481258		
FC-2058	16501	*Lachnum pudibundum* (Quél.) J. Schröt.	Sugadaira, Nagano	Wood	5/18/2005	AB481259	AB481298	AB481335
FC-2025	16451	*Lachnum pulverulentum* P. Karst.	Itako, Ibaraki	Leaf of *Pinus densiflora*	4/19/2005	AB481260	AB481295	AB481332
FC-2283	16769	*Lachnum radiatum* Issh. Tanaka & Hosoya	Sugadaira, Nagano	Leaf of *Fagus crenata*	9/24/2006	AB481261	AB481322	AB481359
FC-2286	16764	*Lachnum radiatum* Issh. Tanaka & Hosoya	Sugadaira, Nagano	Leaf of *Fagus crenata*	9/23/2006	AB481262		
FC-2093	16545	*Lachnum rhytismatis* (W. Phillips) Nannf.	Sugadaira, Nagano	Leaf of *Symplocos coreana*	5/23/2005	AB481263	AB481302	AB481339
FC-2165	16544	*Lachnum rhytismatis* (W. Phillips) Nannf.	Sugadaira, Nagano	Leaf of *Symplocos coreana*	5/23/2005	AB481264		
FC-2054	16487	*Lachnum soppittii* (Massee) Raitv.	Kakuma, Nagano	Leaf of *Quercus serrata*	5/17/2005	AB481265		

Table 1 continued

Isolate no.	TNS-F-no.	Species	Locality	Substrate	Collecting date	GenBank accession number		
						ITS-5.8S	D1–D2	RPB2
FC-2160	16551	*Lachnum soppittii* (Massee) Raitv.	Mt. Tsukuba, Ibaraki	Leaf	5/28/2005	AB481266	AB481308	AB481344
FC-1290	17631	*Lachnum varians* (Rehm) Spooner	Yakushima, Kagoshima	Fern	10/23/2005	AB481267	AB481293	AB481330
FC-2137	16583	*Lachnum virgineum* (Batsch) P. Karst.	Yamakita, Kanagawa	Wood	7/2/2005	AB481268	AB481306	AB481343
FC-2143	16588	*Lachnum virgineum* (Batsch) P. Karst.	Yamakita, Kanagawa	Wood	7/2/2005	AB481269		
FC-2117	16442	*Lachnum* sp.1	Sugadaira, Nagano	Wood	5/18/2005	AB481270	AB481305	AB481342
FC-2211	16642	*Lachnum* sp.1	Mt. Tsukuba, Ibaraki	Stem of bamboo	5/2/2006	AB481271		
FC-2248	16690	*Lachnum* sp.4	Towada-ko, Akita	Stem of *Fallopia*	5/26/2006	AB481272		
FC-2273	16691	*Lachnum* sp.4	Towada-ko, Akita	Stem of *Fallopia*	5/26/2006	AB481273	AB481320	AB481357
FC-2202	16634	*Lachnum* sp.5	Agatsuma, Gunnma	Leaf of *Fagus crenata*	4/27/2006	AB481274	AB481312	AB481349
FC-2076	16520	*Lachnum* sp.9	Sugadaira, Nagano	Stem of bamboo	5/21/2005	AB481275	AB481300	AB481337
FC-2260	16715	*Lachnum* sp.9	Mt. Tsukuba, Ibaraki	Leaf	6/14/2006	AB481276		
FC-2264	16722	*Lachnum* sp.11	Sugadaira, Nagano	Petiole of *Pterocarya*	6/17/2006	AB481277	AB481318	AB481355
FC-2252	16681	*Lachnum* sp.12	Oirase, Aomori	Leaf	5/26/2006	AB481278		
FC-2253	16675	*Lachnum* sp.12	Oirase, Aomori	Leaf	5/25/2006	AB481279	AB481316	AB481353
FC-2355	16838	*Lachnum* sp.13	Tsukuba, Aomori	Leaf of evergreen wood	6/15/2006	AB481280	AB481327	AB481364
FC-2358	16841	*Lachnum* sp.13	Mt. Tsukuba, Ibaraki	Leaf of evergreen wood	6/23/2007	AB481281		
FC-2244	16680	*Lachnum* sp.14	Oirase, Aomori	*Equisetum hyemale*	5/26/2006	AB481282	AB481315	AB481352
FC-2079	16535	*Lachnum* sp.15	Sugadaira, Nagano	Leaf of *Quercus crispula*	5/21/2005	AB481283	AB481301	AB481338
FC-2270	16667	*Lasiobelonium lonicerae* (Alb. & Schwein.) Raitv.	Seisyuurindou, Aomori	Wood	5/24/2006	AB481284	AB481319	AB481356
FC-2285	16760	*Trichopeziza mollisima* (Lasch) Fuckel	Sugadaira, Nagano	Stem of herb	9/22/2006	AB481285	AB481323	AB481360
FC-2288	16763	*Trichopeziza mollisima* (Lasch) Fuckel	Sugadaira, Nagano	Stem of herb	9/23/2006	AB481286		
FC-2156	16579	*Trichopezizella otanii* J.H. Haines	Tyoujyabaru, Oita	Stem of *Fallopia*	6/22/2005	AB481287	AB481307	AB481345
FC-2345	16833	*Trichopezizella otanii* J.H. Haines	Sugadaira, Nagano	Stem of herb	6/10/2007	AB481288		
FC-2255	16686	*Trichopezizella* sp.1	Towada-ko, Akita	Stem of herb	5/26/2006	AB481289		
FC-2274	16684	*Trichopezizella* sp.1	Towada-ko, Akita	Stem of herb	5/26/2006	AB481290	AB481321	AB481358
FC-2038	16472	*Hymenoscyohus varicosporoides* Tubaki	Kasumigaura, Ibaraki	Wood	5/5/2005	AB481291	AB481292	AB481329

PCR products were purified using an ExoSAP-IT purification kit (USB, Cleveland, OH, USA). Total DNA samples were deposited in the Molecular Biodiversity Research Center in the National Museum of Nature and Science and are available for research upon request. Sequencing was carried out using a BigDye Terminator v 3.1 Cycle Sequencing Kit on a DNA auto sequencer 3130x (Applied Biosystems) following the manufacturer's instructions. The obtained sequence was assembled and edited by SeqMan (Lasergen v6 DNAstar), and congruent sequences obtained from both strands were saved. Ambiguously aligned sites were excluded from the analysis.

Phylogenetic analysis

The obtained DNA sequences were aligned by Clustal W (Thompson et al. 1994), and edited manually when necessary using BioEdit v. 7.0.5.2 (Hall 1999). The obtained alignments were analyzed by neighbor-joining (NJ), maximum-parsimony (MP), and Bayesian analyses. NJ and MP analyses were carried out using PAUP* 4.0b10 (Swofford 2002). For MP analysis, the heuristic search option with tree bisection-reconnection (TBR) and Multrees option on and 1000 replicates of random addition sequence were conducted. Support for the individual nodes was tested with bootstrap analysis under the equally weighted parsimony criterion. Bootstrap analysis was based on 1000 bootstrap replicates using the heuristic search option (TBR and Multrees options on), with ten random addition sequences. NJ analysis was conducted with the Kimura two-parameter model, with a transition/transversion ratio set to 2 and a gamma shape set to 0.5. Support for individual nodes was tested by bootstrap analysis based on 1000 bootstrap replicates under the same settings. NJ and MP analyses were conducted with individual dataset of ITS, D1–D2, and RPB2 sequences to determine if there are any hard conflicts among datasets. The analyses were also conducted with a combined dataset of D1–D2 + PRB2 as well as all three-gene datasets.

Bayesian analysis was carried out using MRBAYES 3.1.2b (Huelsenbeck and Ronquist 2001), with a combined dataset of ITS, D1–D2, and PRB2. Five data partitions, including ITS, D1–D2, and one for each codon position for RPB2, were delimited. To determine which model of nucleotide substitution best fit the analyzed data, hierarchical likelihood ratio tests were carried out in MODELTEST v3.7 (Posada and Crandall 1998), and the GTR + G + I model was selected for each data partition. The GTR + G + I model was employed separately for each of the five data partitions. Four simultaneous chains of Markov Chain Monte Carlo were run starting from random trees for 5,000,000 generations, sampling every 500 generations. The average standard deviation of split frequencies (ASDSF) was observed to verify that the values dropped below 0.01. Support for individual nodes was tested by posterior probabilities (PP), obtained from a 50% majority rule consensus after deleting the trees in the burn-in period.

Trees were drawn using MEGA4 (Tamura et al. 2007) and Treeview (Page 1996).

Because phylogenetic analyses of Helotiales or Leotiomycetes (Yu and Zhuang 2003; Lutzoni et al. 2004; Spatafora et al. 2006; Wang et al. 2006) suggest a close relationship between Hyaloscyphaceae and Helotiacaceae, *Hymenoschyphus varicosporoides* Tubaki, was used as the outgroup taxon in the present study, and genes of the same sites were sequenced.

Results

Trees based on single gene sequences

The aligned ITS-5.8S rDNA sequence was composed of 481 pb, and 93 ambiguously aligned sites (sites no. 9–11, 29–31, 56–104, 118–121, 134–136, 341–342, 366–369, 418–423, and 463–481) were excluded from the analysis. In the tree inferred from the ITS-5.8S rDNA sequence, 68 most parsimonious trees were obtained (Table 2). Taxa morphologically identified as the same species always grouped together and were supported with high bootstrap probability (BP) (BP > 80%, mostly 100%) because of the high similarity of the sequences. In the NJ tree (Fig. 1), four clades (clades 1, 2, 4, and "*Brunnipila*") including more than two species were supported by >80% BP (Table 3). Clades 1 and 4 were also supported by high BP (>80%) in MP trees, but the other two clades received only moderate support (66% and 65% BP, respectively) (Table 3, Fig. 1). To reduce complexity in the analysis, one sequence was randomly chosen from each species for further analysis in other genes.

Although the deeper branching orders differed from each other depending on the analyzed gene, each gene produced seven clades with the same taxa (Table 3). The only exceptions include the *Lachnellula* clade with the D1–D2 dataset and clade 2 with the RPB2 dataset, which were not generated in our analyses of individual genes (Table 3).

The maximum number of parsimony-informative sites was obtained in RPB2 whereas the least were found in the D1–D2 region (see Table 2). Sequences in the D1–D2 region were identical among different species or possessed only several variable sites, which implies that it is too

Table 2 Properties of the datasets and phylogenetic trees inferred from each dataset

Gene	Genus	Taxa[a]	Isolates[b]	Sequence Length (bp)[c]	Informative sites in MP (bp)[d]	Informative sites in MP (%)	Number of MP trees	Tree length in MP	Consistency index (CI)	Retention index (RI)
ITS-5.8S rDNA	7	36	58	388	97	20.2	68	480	0.3542	0.7189
28S D1–D2	7	36	36	510	63	12.4	1332	234	0.4274	0.6927
RPB2	7	36	36	624	245	38.1	2	1580	0.2741	0.4695
D1–D2 + RPB2	7	36	36	1134	308	26.7	1	1839	0.2898	0.4973
ITS + D1–D2 + RPB2	7	36	36	1530	402	24.6	2	2367	0.2949	0.4846

[a] Number of species analyzed excluding the outgroup

[b] Number of isolates analyzed excluding the outgroup

[c] Analyzed after ambiguously aligned sites were excluded

[d] After ambiguously aligned sites excluded

conservative to resolve phylogenetic relationships of this group.

Phylogenetic trees based on combined sequences

Because the MP and NJ trees based on ITS-5.8S rDNA, D1–D2, and RPB2 did not conflict with each other in clades with BP > 80%, the sequence data were combined and analyzed by MP and Bayesian methods. From the resulting combined sequence composed of 1637 bp (484 bp for ITS-5.8S rDNA, 510 bp for D1–D2, 643 bp for RPB2), 88 ambiguously aligned sites for ITS (sites no. 31–41, 60–109, 370–373, 422–428, and 469–484) and 19 ambiguously aligned sites for RPB2 (sites no. 1352–1369) were excluded from the analysis. Based on the combined data of all three genes, two MP trees were generated in which all the seven clades recognized in other trees inferred from other genes were strongly supported (see Table 3, Fig. 2). The two trees only differed in the topology of nonstrongly supported branches. Bayesian analysis generated 50,000 trees, and the first 10,000 trees with ASDSF greater than 0.01 were discarded as the burn-in phase. A 50% majority rule consensus of the remaining 40,000 trees showed the average log-likelihood of –12885.11 (harmonic mean). The potential scale reduction factor was 1.000–1.001 for all parameters, indicating that the analyses were run for a sufficient number of generations. Bayesian trees provided high PP to the deeper nodes, but they were collapsed in strict consensus in MP. Nonetheless, all seven major clades were supported both by BP values (98–100%) and PP (1.00) (Table 3, Fig. 2). All the clades except for clade 3 were also recognized in the phylogenetic analysis based on ITS-5.8S rDNA (see Fig. 1), although not all of them were strongly supported. Because the combined data gave the largest numbers of highly supported clades, the following discussion was based on this tree (Fig. 2).

Discussion

Phylogenetic assessment of *Incrucipulum*

The clade composed of *Lachnum ciliare*, *L. longispineum*, and *L. radiatum* was recognized in all the trees obtained and was strongly supported (see Table 3, Figs. 1, 2). These three species grow on leaves and show strong host selectivity. They produce stipitate, white apothecia. The morphology of the hairs in these species differs considerably from one species to another (Tanaka and Hosoya 1999), but they commonly bear crystals at the apex of the hairs. They also have thick-walled ectal excipulum composed of cubic cells with granulate surface.

Baral and Krieglsteiner (1985) proposed the genus *Incrucipulum* Baral for lachnoid fungi with thick-walled, cubic ectal excipular cells with granulated surface and hairs with crystals at their apices. The genus currently includes four species [*I. ciliare* (Schrad.) Baral (type species), *I. virtembergense* (Matheis) Baral, *I. capitatum* (Peck) Baral, and *I. sulphurellum* (Peck) Baral]. High morphological similarity was pointed out for *I. virtembergense* and *I. longispineum* (Tanaka and Hosoya 1999). Because the molecular phylogeny is congruent with morphology, the genus *Incrucipulum* is justified, and the following two new combinations are proposed here.

Incrucipulum radiatum (Issh. Tanaka & Hosoya) Sasagawa & Hosoya, comb. nov.

MycoBank no.: MB 515282

≡*Lachnum radiatum* Issh. Tanaka & Hosoya, Mycoscience 42: 606, 1999 (basionym).

Incrucipulum longispineum (Hosoya & Issh. Tanaka) Sasagawa & Hosoya, comb. nov.

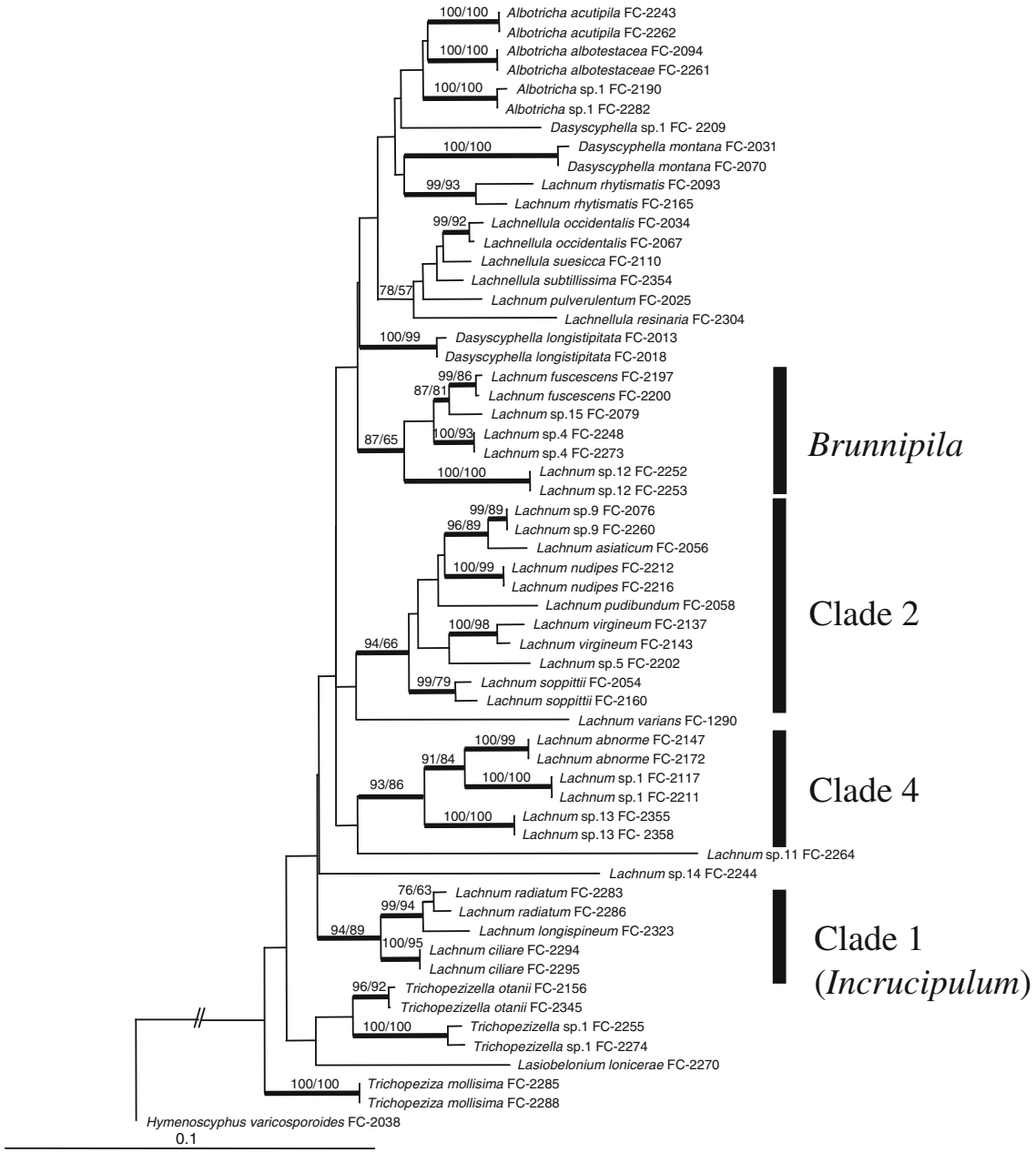

Fig. 1 A neighbor-joining (NJ) tree of *Lachnum* and its allied fungi inferred from sequences of ITS-5.8S rDNA. Bootstrap values (BP) of 1000 replications in NJ analysis are indicated on the nodes followed by BP of 1000 replications in maximum parsimony analysis. Clades supported by BP above 80% in NJ analysis are shown with *thickened branches*. The names of the clades are given so that they are congruent with those in Fig. 2 for convenience

MycoBank no.: MB 515283

≡*Lachnum longispineum* Hosoya & Issh. Tanaka, Mycoscience 42: 598, 1999 (basionym).

Phylogenetic assessment of *Lachnellula*

Lachnellula species formed a monophyletic group with *Lachnum pulverulentum* (see Table 3, Fig. 2). All the members included in this clade share the following remarkable features: short stipes, yellow hymenium, and coniferous habitat.

Lachnum pulverulentum, supported as a sister group to the *Lachnellula* species, has several atypical characters for *Lachnum*, such as a short stipe or almost no stipe (Dennis 1949; Tanaka and Hosoya 1999) and narrow, almost cylindrical paraphyses not exceeding the asci. The morphology of the paraphyses and the yellow hymenium are

Table 3 Summary of the lachnoid fungi analyzed in the present study and reliability of clades commonly generated in the phylogenetic analysis based on three genes and two combined data sets

Clade	Members	ITS-5.8S		D1–D2		RPB2		D1–D2 + RPB2			ITS + D1–D2 + RPB2		
		NJ	MP	NJ	MP	NJ	MP	NJ	MP	Bayesian	NJ	MP	Bayesian
Incrucipulum (Clade1)	*Lachnum ciliare, L. longispineum, L. radiatum*	94	89	99	99	100	100	100	100	1.00	100	100	1.00
Brunnipila	*Lachnum fuscescens*, *Lachnum* sp.4, *Lachnum* sp.12, *Lachnum* sp.15	87	65	97	92	99	99	100	100	1.00	100	100	1.00
Lachnellula	*Lachnellula occidentalis, L. resinaria, L. subtilissima, L. suecica Lachnum pulverulentum,*	78	57	<50	<50	98	70	99	93	1.00	100	98	1.00
Albotricha	*Albotricha acuipila*, *A. albotestacea*, *Albotricha* sp.1	<50	<50	63	<50	100	99	100	100	1.00	100	100	1.00
Clade 2	*Lachnum asiaticum,L. nudipes, L. pudibundum,L. soppittii, L. virgineum, Lachnum* sp.5, *Lachnum* sp.9, *Lachnum* sp. 11[a]	94	66	99	97	ND	ND	95	94	1.00	99	100	1.00
Clade 3	*L. rhytismatis, Lachnum* sp.14	ND	ND	96	90	100	100	100	100	1.00	100	100	1.00
Clade 4	*Lachnum abnorme*, *Lachnum* sp.1, *Lachnum* sp.13	93	86	96	91	69	58	97	96	1.00	100	100	1.00

For neighbor-joining (NJ) and maximum-parsimony (MP) trees, bootstrap values are presented; for Bayesian analysis, posterior probability is presented

ND, not generated in the given analysis

[a] *Lachnum* sp. 11 did not group with other members in ITS-5.8S analysis

congruent with *Lachnellula*. Because external morphology and paraphyses morphology, hymenium color, and the habitat of *L. pulverulentum* agreed with those of *Lachnellula*, and were strongly supported by molecular phylogenetic data, we propose to transfer *Lachnum pulverulentum* to *Lachnellula*.

Lachnellula pulverulenta (P. Karst.) Sasagawa & Hosoya, comb. nov.

MycoBank no.: MB 515284

≡*Peziza pulverulenta* Lib., Pl. Cryp. Ardienn. no. 125, 1832 (basionym).

≡*Lachnum pulverulentum* (Lib.) P. Karst., Myc. Fenn. i. p. 175. 1871.

See Tanaka and Hosoya (1999) for other synonyms.

Assessment of *Albotricha*

The clade was found to group all the *Albotricha* species analyzed in the present study, including *A. acutipila* (type species), *A. albotestacea*, and the unidentified *Albotricha* sp. 1, which should be described as a new species based on morphology (see Table 3, Fig. 2). The genus *Albotricha* Raitv. was established for lachnoid fungi with smooth, acute hairs granulate at the base and long extending paraphyses. *Albotricha* resembles *Dasyscyphella* in having hairs with smooth apices, but *Albotricha* differs in tapered apices in contrast to *Dasyscyphella*, which typically has enlarged apices. *Albotricha* also differs from *Dasyscyphella* in wider, lanceolate paraphyses, extending longer than those in *Dasyscyphella*.

Assessment of *Dasyscyphella*

In contrast to *Albotricha*, strongly supported by molecular phylogeny, *Dasyscyphella* was suggested to be polyphyletic. The three species analyzed in the present study were phylogenetically apart from each other and from *Albotricha*, suggesting granulate hairs with smooth apices is a convergent character. To know the proper position of *Dasyscyphella*, analysis with more members including the type (*Dasyscyphella nivea* (R. Hedw.) Raitv.) is required.

Delimitation of *Brunnipila*

A group of lachnoid fungi with brown hairs equipped with a resinous matter at their apices (*L. fuscescens*, *Lachum* sp. 4, sp. 12, and sp. 15) was strongly supported (see Table 3, Fig. 2). These species should be classified in section *Bruneolae* (Dennis 1949) of *Dasyscyphus* Gray (the former name of *Lachnum*) because of the hair character. Baral and Krieglsteiner (1985) raised this group to the genus *Brunnipila* Baral, which was not accepted by Raitviir (1987).

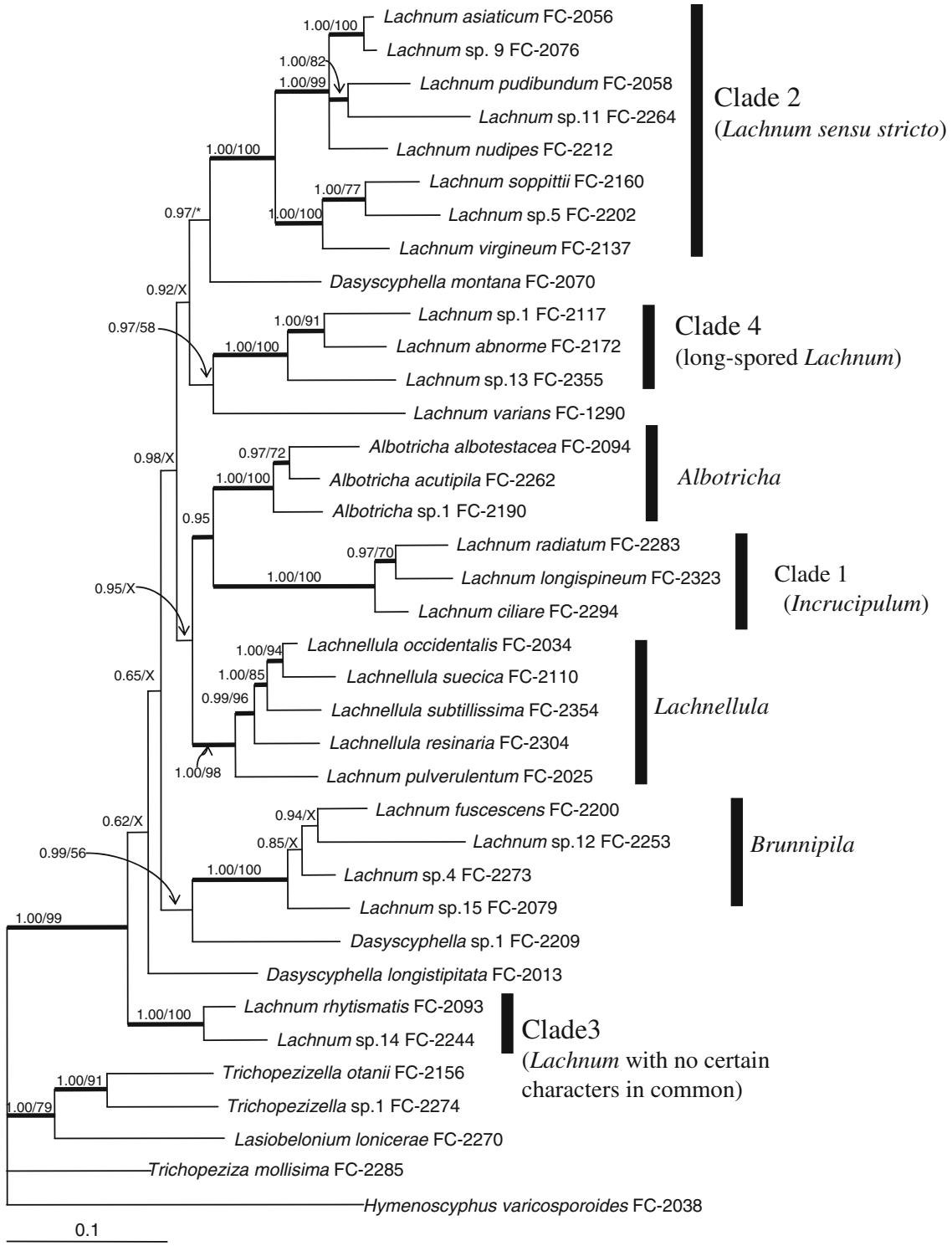

Fig. 2 A 50% majority consensus tree based on Bayesian analysis of *Lachnum* and its allied fungi inferred from sequences of combined data of ITS-5.8S rDNA, D1–D2 region of 28S rDNA, and RPB2 regions. Posterior probability (PP) in Bayesian analysis is indicated on the nodes, followed by the bootstrap value (BP) of 1000 replications in maximum-parsimony analysis when BP exceeds 50%. Clades with PP $\geq$ 0.95 with BP $\geq$ 70% are shown with *thickened branches*. *X* shows that the nodes are not present in MP trees

Browning of the hairs sometimes occurs in *Lachnum abnorme*. However, in *L. abnorme* hair coloration occurs only occasionally and gradually in older hairs. Hair coloration is more distinct from hyaline ectal cells in members in this clade, and coloration occurs in young hairs. Because distinct hair coloration is supported by molecular phylogeny, it is appropriate to accept the generic concept of *Brunnipila*. Other unidentified *Lachnum* are to be treated as *Brunnipila*. These fungi were new to science (Hosoya et al. in preparation).

Delimitation of *Lachnum*

The rest of *Lachnum* analyzed in the present study were distributed among clades 2, 3, and 4. Members in clade 4 (*Lachnum abnorme*, *Lachnum* sp. 1 and sp. 13; see Fig. 2) were characterized by long ascospores (>30 μm), either aseptate or septate, and narrow, delicate hairs. They also shared narrowly lanceolate paraphyses. No genus has ever been proposed for long-spored *Lachnum*, although many long-spored lachnoid fungi have been described, especially in tropical areas of the world (e.g., Dennis 1954; Haines and Dumont 1984; Spooner 1987). The present study suggests that these fungi form a phylogenetically distinct group. Because only three species were included in the present study in comparison to the numbers of described species with long ascospores in *Lachnum*, it is premature to establish a new genus solely based on long ascospores. Hence, they should be tentatively retained in the genus *Lachnum*.

Lachnum virgineum, the type species of *Lachnum*, was included in clade 2, and members of this clade were characterized by stipitate apothecia, hairs without conspicuous crystals at their apices, clearly lanceolate paraphyses, and elliptical to fusiform, aseptate ascospores. *Lachnum virgineum*, *L. nudipies*, *L. pudibundum*, and *L. soppittii* were included in the section Typicae in the taxonomy of Dennis (1949), the character of which partially agreed with those in clade 2, but more strictly delimited in hairs without crystals. The unidentified members in clade 2 also show similar characters. Hence, this morphologically based group was well supported by the molecular phylogeny. Dennis (1949) did not mention the presence of crystals or resinous matters on the hair apices to define the section Typicae, and included *Lachnum rhytismatis* with conspicuous crystals at the hair apices. However, *L. rhytismatis* formed a separate group in clade 3 with *Lachnum* sp. 14 in the present study. The molecular phylogeny suggests that the presence of hair crystals is taxonomically important. However, no common morphological characters were found in clade 3 in the present study because *Lachnum* sp. 14 was characterized by short, less granulate hairs with unique curvature, and narrowly lanceolate paraphyses. Our results suggest that the presence of hair crystals alone does not indicate a phylogenetic relationship.

Even after segregation of *Incrucipulum* and *Brunnipila*, the diverse morphological variation in hairs, ascospores, paraphyses, and ectal excipulum, as well as a high level of molecular variation within *Lachnum*, suggest the requirement for emendation of generic circumscription. Although the present study showed at least three strongly supported phylogenetic groups in the genus *Lachnum*, their morphological support was not sufficient for clear delimitation. Hence, the genus should be retained for taxonomic practicality at present. For better resolution of the genus *Lachnum*, more genes should be incorporated in the analysis.

Delimitation of Lachnaceae

Raitviir (2004) raised the tribe Lachneae to the family Lachnaceae. He included all the lachnoid fungi that have either granulate hairs or lanceolate paraphyses. He believed that these fungi formed a monophyletic group, based on Cantrell and Hanlin (1997). In the present analysis, the majority of the genera included in Lachnaceae sensu Raitviir were incorporated. We believe the taxon sampling is sufficient to discuss the taxonomy of lachnoid fungi. A group of fungi excluding *Lasiobelonium*, *Trichopeziza*, and *Trichopezizella* formed a strongly supported phylogenetic group (see Fig. 2). The potential synapomorphic character in this phylogenetically strongly supported group is hairs having granulation at their base, whereas *Lasiobelonium*, *Trichopeziza*, and *Trichopezizella* have totally smooth-walled hairs. No other characters currently used for the taxonomy of lachnoid fungi, such as ascospore or paraphyses morphology and ectal structure, were sufficient for segregation because the same character state occurred in both granulate-haired members and smooth-walled members. No studies ever showed strong molecular phylogenetic support for monophyly of the lachnoid fungi. Hence, we propose here to emend the familial concept of Lachnaceae to include only granulate-haired members.

Lachnaceae Raitv., Scripta Mycologica, Tartu 20: 7, 2004. emend. Hosoya & Sasagawa

Apothecia usually 0.3–3 mm in diameter, sessile, subsessile to stipitate. Hairs granulate at least at the base, hyaline or brown colored, crystals present or absent at their apices. Ectal excipulum of *textura globulosa*, *prismatica*, or *intricata*, having granulation on the surface or not. Asci usually cylindrical clavate with conical or hemispherical apex; pore usually stained clearly by MLZ. Ascospores globose, elliptical to fusiform, cylindrical to filiform, aseptate to multiseptate.

Table 4 Summary of characters of the clades in Lachnaceae analyzed in the present study generated by the combined data of ITS-5.8S rDNA, D1–D2 region of 28S rDNA, and RPB2 regions

Clade	Ectal excipulum			Hairs		Ascospores		Paraphyses	Hymenium color	Mode of life	Substrate
	Structure	Surface	Cell wall	Apex	Crystal	Color					
Albotricha	t. prismatica to t. angularis	Smooth	Thin	Smooth	–	Hyaline	Fusiform	Lanceolate, long exceeding over the asci	White to yellowish brown	Saprophytic	Herbs
Brunnipila	t. prismatica to t. angularis	Smooth	Thin	Granulate	+	Brown	Ellipsoid to fusiform	Lanceolate, long exceeding over the asci	White to pale brown	Saprophytic	Deciduous leaves, herb
Incrucipulum (Clade 1)	t. prismatica	Granulate	Thick	Granulate	+	Hyaline	Fusiform	Narrowly lanceolate	White	Saprophytic	Deciduous leaves
Lachnellula	t. prismatica, t. intricata, t. globulosa	Smooth	Thin	Granulate	–	Hyaline	Globose, ellipsoid to tear-shaped	Cylindrical, as long as asci or slightly exceeding	Yellow to orange	Saprophytic to parasitic	Coniferous wood and leaves
Clade 2	t. prismatica to t. angularis	Smooth	Thin	Granulate	–	Hyaline	Ellipsoid to fusiform	Lanceolate	White to pale brown	Saprophytic	Herb, deciduous tree wood and leaves
Clade 3	t. prismatica to t. angularis	Smooth	Thin	Granulate	–, +	Hyaline	Ellipsoid to fusiform	Lanceolate	White to pale yellow	Saprophytic	Deciduous leaves, ferns
Clade 4	t. prismatica	Smooth	Thin	Granulate	–	Hyaline	Filiform	Narrowly lanceolate	Pale yellow to orange	Saprophytic to parasitic	Herb, deciduous tree wood, leaves

t, textura

Type genus: *Lachnum* Retz., Flor. Scand. Prodromus p. 256, 1779.

The family placement of the genus *Lachnellula* has been a matter of debate for a long time. When Nannfeldt (1932) established the family Hyaloscyphaceae (in which members of Lachnaceae were included), *Trichoscyphella* Nannf. (former name for *Lachnellula*) was placed in the family Helotiaceae because of the ectal excipulum structure of *textura intricata*. This taxonomy was followed by Dennis (1949). However, other researchers of the Hyaloscyphaceae (Raitviir 1970, 1987; Korf 1973; Baral and Krieglsteiner 1985) placed the genus in the family Hyaloscyphaceae, noting the morphological similarity of the hairs and paraphyses. In the present study, the placement of *Lachnellula* in the Lachnaceae was justified by molecular phylogeny.

Hairs, asci, paraphyses, ectal excipular structure, and ecology have been used for the taxonomy of Lachnaceae. However, no single character readily distinguishes the genera, except for hair coloration (*Brunnipila*) and excipular surface (*Incrucipulum*) (Table 4). This observation suggests that there has been extensive homoplasy in most of the morphological characters that previously have been used to delimit taxa and that these should therefore be used in combination to distinguish genera. Hair characters, in combination with paraphyses characters, are most important because they distinguish most of the genera. Based on the criteria in order of priority, the key to the genera examined in the present study follows:

Key to the lachnoid genera based on phylogeny using diagnostic characters

1	Hairs brown	*Brunnipila*
1	Hairs hyaline	2
	2 Hairs apex smooth	3
	2 Hairs totally granulate	4
3	Paraphyses long exceeding the asci	*Albotricha*
3	Paraphyses not inconspicuously exceeding the asci	*Dasyscyphella*
	4 Hair crystals present, ectal excipular surface granulate	*Incrucipulum*
	4 Hair crystals present or absent, ectal excipular surface smooth	5
5	Hymenium yellow, on conifers	*Lachnellula*
5	Hymenium of various color, on various substrates	*Lachnum* (still paraphyletic)

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