

**Phylogenetic relationships among species  
and genera of Geoglossaceae (Helotiales)  
based on ITS and LSU nrDNA sequences**

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## ABSTRACT

The phylogenetic relationship in Geoglossaceae ss. lato (*Geoglossum* Pers., *Trichoglossum* Boud., *Microglossum* Gillet., *Cudonia* Fr., *Spathularia* Pers., *Mitrula* Fr. and *Bryoglossum* Redhead), as proposed by Nannfeldt (1942) and Ohenoja (2000), was investigated by applying sequences of rDNA, including ITS and partial large subunit (LSU) rDNA into phylogenetic methods. The phylogenetic methods used in this study were Maximum Parsimony and Bayesian analysis with Markov chain Monte Carlo algorithms. In the light of the obtained molecular phylogenies, the evolution of morphological characters was analyzed and evaluated.

Our data show that the molecular phylogeny of Geoglossaceae ss. lato. is inconsistent with the classification system of Nannfeldt and Ohenoja. The results demonstrate that *Geoglossum* together with the genera *Trichoglossum* and *Sarcoleotia* constitute the family Geoglossaceae ss. stricto. Phylogenetic analyses in this study show that the close relationship between *Geoglossum* and *Microglossum* based on morphology is artificial. *Microglossum* should no longer be included in the family Geoglossaceae, but better be re-moved to Leotiaceae. As suggested by earlier studies, there is a close relationship between *Cudonia* and *Spathularia*, which should be excluded from the Geoglossaceae.

Our study shows that the set of morphological characters used to circumscribe Geoglossaceae ss. lato. have to be abandoned and a new set of morphological characters have to be considered in the delimitation of the family.

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## INTRODUCTION

The family Geoglossaceae Corda is a group of inoperculate discomycetes of the order Helotiales, the largest order of inoperculate discomycetes. The Geoglossaceae ss. lato (lat.) (Ohenoja 2000, Nannfeldt 1942) includes the genera, *Geoglossum* Pers., *Trichoglossum* Boud. and *Microglossum* Gillet, commonly referred to as earth tongues, and *Mitrula* Fr., *Cudonia* Fr., *Spathularia* Pers., and *Bryoglossum* Redhead. Taxonomically important macro-morphological characters include ascocarp shape, colour and surface consistency of head (dry, viscid) and stipe (smooth, hairy, or covered with scales). The ascocarp is tongue-, club-, or fan shaped, and the colour varies from black, brownish black, brown, reddish brown, green, orange to yellow.



Figure 1.a. *Geoglossum difforme*. Ascocarp.



b. *Geoglossum glutinosum*. Ascocarp

In Geoglossaceae, asci are usually 8-spored, more rarely 4-6 spored, with  $\pm$  amyloid pore. The ascospores vary in shape; i. e. curved, fusoid, cylindric to filiform, clavate, oblong, acicular and straight; and colour, i. e. hyaline, brown and greyish brown, with 0 (non-septate) up to 15 septae (multi-septate). The paraphyses vary in shape, i.e. straight, curved, coiled, filiform, few- to many-septate,  $\pm$  enlarged at the apex, and colour, hyaline to coloured (Ohenoja 2000). Examples of *Geoglossum* ascocarps, paraphyses and ascospore are shown in Fig. 1 and 2.

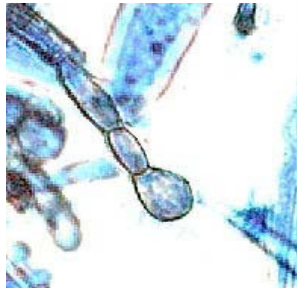


Figure 2. a. *Geoglossum cookeianum*.  
Paraphysis.



b. *Geoglossum umbratile*.  
Ascospore.



c. *Geoglossum uliginosum*.  
Paraphyses.

The members of the family Geoglossaceae can be found in forests, growing in soil, decaying leaves, wood, or in moist pastures rich in organic matter (Alexopoulos *et al* 1996). The ascocarps usually appear late in summer and autumn, often after heavy rains (Nannfeldt 1942).

The members of the family Geoglossaceae have received extensive attention, and several morphotaxonomical studies have been undertaken (Durand 1908, 1921, Nannfeldt 1932, 1942, Imai 1934, 1940, Bille-Hansen 1954, Mains 1954, 1955, 1956, Eckblad 1963, Maas Geesteranus 1964, 1965, 1966, Nitare 1982, 1983, 1984, Nitare & Ryman 1984). Boudier (1885) divided the discomycetes into two distinct groups based on the structure of the ascus, those with operculate asci and those with inoperculate asci. In 1907, Boudier proposed a system where the family Geoglossaceae was transferred to the inoperculate discomycetes, in which the ascus opens by a pore. Durand (1908) published a monograph of the Geoglossaceae of North America and at the same time divided the family into two subfamilies, Geoglosseae and Cudonieae. *Mitrula*, *Microglossum*, *Corynetes* Hazsl., *Gloeoglossum* Durand, *Geoglossum*, *Trichoglossum*, *Spathularia* were included in the subfamily Geoglosseae, and *Leotia* Pers., *Vibrissea* Fr., *Apostemidium* Karst. and *Cudonia* were included in the subfamily Cudonieae. In “*Geoglossaceae of Sweden*”, Nannfeldt (1942), when treating the Nordic and Swedish genera and species, followed Durand’s classification, with only a few changes of the generic delimitations. While Durand (1908) distinguished *Gloeoglossum* from *Geoglossum* by a viscid consistency of ascomata and the presence of paraphyses down the whole stem in the former genus, Nannfeldt (1942) included *Gloeoglossum* in *Geoglossum*. Furthermore, Nannfeldt (1942) excluded the genera *Vibrissea* and *Apostemidium* from the family and transferred them to the order Ostropales.

In 1964, Maas Geesteranus reduced *Ochroglossum* Imai (1955), originally introduced as a section of *Microglossum* with yellow-brown colours, to a synonym of *Microglossum*. Maas Geesteranus (1964) regarded *Mitrula* as a monotypic genus. Durand (1908) placed the genus *Leotia* in the family Geoglossaceae, but it was later re-moved to the family Helotiaceae by Korf (1958). Korf maintained that *Leotia* was unrelated to other members of the Geoglossaceae and, in agreement with Imai (1956), transferred the subfamily Leotioideae Imai (with *Leotia*) to the Helotiaceae.

Nannfeldt (1932) placed the Geoglossaceae in the order Helotiales and regarded the family as closely related to Helotiaceae. Geoglossaceae was kept separate by the clavate, capitate or pileate ascocarps with a hymenium covering the convex upper portion; in contrast to the Helotiaceae with discoid, saucer-shaped or cupulate ascocarps.

The macromorphology of the ascocarp has always been important in the taxonomy of Geoglossaceae, but microanatomical characters have also been used. Geoglossaceae has been separated into two groups based on the colour of the ascospores and the reaction of the ascus pore in Melzer's reagent, the first group having hyaline ascospores and a negative reaction (J-) in Melzer's reagent; i. e. *Cudonia*, *Spathularia* and *Spathulariopsis*, and the other group, including *Geoglossum* and *Trichoglossum*, having dark ascospores and a positive reaction of the ascus pore (J+) in Melzer's reagent. *Mitrula*, *Nothomitra* and *Microglossum* do not fit into either of these groups (Korf 1973, and Verkley 1994, Wang *et al* 2002).

The introduction of molecular methods in the biosystematics during the last two decades has opened a variety of new possibilities in "mapping the systematic position" and studying the phylogeny of taxa at different taxonomic levels.

Phylogenetic analyses of the LSU and SSU rDNA regions showed that there is a close relationship between *Leotia* and *Microglossum* (Gernandt *et al* 2001 Hambleton *et al* 2003, Wang *et al* 2005). Recent molecular studies (Platt & Spatafora 1999, Bhattacharya *et al* 2000, Gernandt *et al* 2001, Lutzoni *et al* 2001, Wang *et al* 2002, Tehler *et al* 2003) have also concluded that *Cudonia* and *Spathularia* are closely related and are to be excluded from Geoglossaceae ss. stricto (str.) Phylogenetic analyses have further indicated that Helotiales is not a monophyletic group (Vrålstad *et al* 2001, Gernandt *et al* 2001) and that Helotiales and

Rhytismatales together form a monophyletic group, with Geoglossaceae excluded from the former (Tehler *et al* 2003, Lutzoni *et al* 2004, Wang *et al* 2005).

The diversity of phylogenetic inferences has increased over the past few years. Maximum Parsimony (MP) analysis that traditionally has been the predominant phylogenetic (cladistic) method has been challenged by other phylogenetic methods, e. g. maximum likelihood methods. One of these methods is the likelihood based method called MrBayes (Huelsenbeck & Ronquist 2001). The Bayesian method is, like Maximum Parsimony and Maximum Likelihood (ML), character based (Hall 2004). Bayesian analysis is based upon a quantity called posterior probability (PP) of a tree. The posterior probability involves a summation of all trees obtained, and for each tree, integration over all possible combinations of branch lengths and substitution model parameter values. Fortunately, a number of numerical methods are available that allow the posterior probability of a tree to be approximated, of which the most useful one is Markov Chain Monte Carlo (MCMC) (Huelsenbeck *et al* 2001). Being a maximum likelihood related approach; Bayesian analysis requires probabilistic models of the nucleotide substitution. The best fit models for the Bayesian analysis can be estimated using the software MODELTEST (Posada & Crandall 1998). An advantage of the Bayesian approach is its robustness over traditional tree building methods. It considers all potential trees, weighted according to the probability that each is correct, while other phylogenetic methods, such as ML and MP, select only a single or several equally optimal trees (Schmitt *et al* 2003). MrBayes is easy to use, it is quite fast, and it is capable of dealing with a very large number of operational taxonomical units (OUTs).

As already mentioned above, the circumscription of Geoglossaceae has been disputed, and a number of revisions have been done. The aim of our study has been to uncover the main evolutionary lineages in Geoglossaceae ss. lat. and its close relatives, based on the classification introduced by Nannfeldt (1942) and later reinforced by Ohenoja (2000). For this purpose, we have used molecular markers (ITS and LSU nrDNA sequences) and various computer-based phylogenetic analyses. The sample to be included has been chosen based on the intrafamilial classification proposed by Nannfeldt and Ohenoja. Furthermore, another aim has been to analyze and evaluate the evolution of morphological characters in light of the obtained molecular phylogenies.



## **MATERIALS AND METHODS**

### **Specimens examined**

Fresh specimens were collected in western Norway, Møre og Romsdal county in October 2003. Dried specimens were obtained from various University herbarias. The specimens included in the study are listed in Table 1.

### **Molecular methods**

DNA extraction was performed using a 2% CTAB miniprep method described by Murray and Thompson (1980) with a few modifications: DNA was re-suspended in 100 µl dsH<sub>2</sub>O at the final step of extraction, and DNA templates were diluted 20x or 50x prior to PCR amplification. PCR amplification was accomplished using the primers ITS1 and ITS4 (White *et al* 1990) for the nuclear ITS1-5.8S-ITS2 rDNA region and the primers LROR and LR5 (White *et al* 1990) for the partial large subunit (LSU) 28S region. PCR was performed in 30 µL reactions containing 17.5 µL 50 x diluted template DNA and 12.3 µL reaction mix (final concentrations: 4 X 250 mM dNTPs, 0.625 mM of each primer, 2 mM MgCl<sub>2</sub> and 1 unit DyNazyme™ II DNA polymerase [Finnzymes Oy, Espoo, Finland] on a Biometra PCR machine. The ITS and LSU amplification programs were initiated by a 4 min denaturation step, followed by 38 cycles of 25 s at 94°C, 25 s at 54°C (ITS) or 50°C (LSU) and 35 s at 72°C, and terminated by a 10 min elongation step at 72°C before storage at 4°C. Negative controls were included in each run. Amplification products were visualized with 1% agarose gels stained with ethidium bromide in a 0.5 x TBE buffer system. Gels were run at 80 V for 25 minutes and photographed over an UV transilluminator.

Automated sequencing was performed on a Mega Bace™500 DNA Analysis System (Amersam Biosciences, Ohio, USA) using the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Buckinghamshire, England) according to the manufacturers recommendations. PCR products and cycle sequencing products were purified with the ExoSAP-IT and AutoSeq96™ Dye Terminator Clean-up Kits, respectively, according to the manufacturers' recommendations (Amersham Biosciences, Ohio, USA).

Table 1. Specimens included in the study.

Taxon <sup>o</sup>	Author	Reference no.
<i>Geoglossum glutinosum</i>	Pers.	O-F 73261
<i>Geoglossum glutinosum</i>	Pers.	O-F 105458
<i>Geoglossum spec. 1</i>		A63 <sup>1</sup>
<i>Geoglossum spec. 2</i>		O-F 170758
<i>Geoglossum vleugelianum</i>	Nannf.	O-F 72126
<i>Geoglossum simile</i>	Peck	O-F 70873
<i>Geoglossum umbratile</i>	Sacc.	O-F 173025
<i>Geoglossum montanum</i>	Nannf.	Ups-A43
<i>Geoglossum spec. 3</i>		O-F 63985
<i>Geoglossum difforme</i>	Fr.	A48 <sup>1</sup>
<i>Geoglossum sphagnophilum</i>	Ehrenb.	B10 <sup>§</sup> (97/048)
<i>Geoglossum spec. 4</i>		O-F 175026
<i>Geoglossum littorale</i>	(Rostr.) Nannf.	C-A24
<i>Geoglossum fallax</i>	E.J. Durand	A60 <sup>1</sup>
<i>Geoglossum starbaeckii</i>	Nannf.	O-F 64317
<i>Geoglossum starbaeckii</i>	Nannf.	O-F 73035
<i>Geoglossum uliginosum</i>	Pers.	O-F 64552
<i>Geoglossum cookeianum</i>	Nannf.	3868F <sup>§</sup>
<i>Geoglossum lineare</i>	Hakelier	Ups-B11
<i>Geoglossum arenarium</i>	(Rostr.) Lloyd	A26 <sup>1</sup>
<i>Geoglossum nigrinum</i>	(Fr.) Cooke	∞
<i>Geoglossum glabrum</i>	Pers.	∞
<i>Geoglossum alpinum</i>	Eckblad	A101 <sup>§</sup> (2790)
<i>Trichoglossum velutipes</i>	(Peck) E.J. Durand	O-F 173022
<i>Trichoglossum walteri</i>	(Berk.) E.J. Durand	O-F 63986
<i>Trichoglossum hirsutum</i>	(Pers.) Boud.	A62 <sup>1</sup>
<i>Microglossum atropurpureum</i>	(Batsch) P. Karst.	A58 <sup>1</sup>
<i>Microglossum fuscobubens</i>	Boud.	O-F 161832
<i>Microglossum olivaceum</i>	(Pers.) Gillet	O-F 161855
<i>Microglossum viride</i>	(Schrad.) Gillet	O-F 171030
<i>Microglossum novo species</i>		A52 <sup>1</sup>
<i>Sarcoleotia globosa</i>	(Sommerf. ex Fr.) Korf	S31 <sup>1</sup>
<i>Cudonia confusa</i>	Bres.	O-F 63112
<i>Cudonia circinans</i>	(Pers.) Fr.	O-F 60763
<i>Cudonia lutea</i>	(Peck) Sacc.	∞
<i>Cudonia sichuanensis</i>	Zheng Wang	∞
<i>Spathularia rufa</i>	Schmidel	O-F85528
<i>Spathularia flavida</i>	Pers.	O-F100097
<i>Leotia viscosa</i>	Fr.	A35 <sup>(g - ITS/∞ - LSU)</sup>
<i>Leotia lubrica</i>	(Scop.) Pers.	∞
<i>Heyderia abietis</i>	(Fr.) Link	O-F 183258
<i>Mitruia paludosa</i>	Fr.	O-F64862
<i>Cudoniella sp.</i>		H-A32 <sup>3</sup>
<i>Cudoniella sp.</i>		H-A8 <sup>3</sup>
<i>Piceomphale bulgarioides</i>	(Rabenh.) Svrček	H-A37 <sup>3</sup>
<i>Rhytisma acerinum</i>	(Pers.) Fr.	∞
<i>Hymenoschyphus fructigenus</i>	(Bull.) Fr.	A30 <sup>3</sup>
<i>Hymenoschyphus conscriptus</i>	(P. Karst.) Korf ex Kobayasi, Hirats. f., Aoshima, Korf, Soneda, Tubaki & Sugiy.	B16 <sup>3</sup>
<i>Bryoglossum gracile</i>	(P. Karst.) Redhead	A40 <sup>3</sup>

O = herbarium specimen from the Botanical Museum of Oslo,  
 Ups = herbarium specimen from the Botanical Museum of Uppsala  
 C = herbarium specimen from the Botanical Museum of Copenhagen

<sup>1</sup> Specimen collected by the author

<sup>§</sup> DNA available at ARON (Ascomycete research group, Oslo)

<sup>3</sup> Specimen from Herb. Trond Schumacher

∞ Sequences retrieved from EMBL/GenBank/DDBJ (<http://www.ncbi.nlm.nih.gov>)

## Alignment and phylogenetic analyses

In addition to the sequences generated in this study, three ITS sequences of *Cudonia lutea*, *C. sichuanensis* and *Leotia lubrica* (AF433150, AF 433148, AY144594) and seven LSU sequences of *Geoglossum glabrum*, *G. nigrinum*, *Cudonia lutea*, *C. sichuanensis*, *Leotia lubrica*, *L. viscosa* and *Rhytisma acerinum* (AY533015, AY544650, AF433138, AF433136, AY544644, AF113737 and AF356696) were retrieved from EMBL/GenBank (<http://www.ncbi.nlm.nih.gov>) and included in the analyses. Sequences were aligned using the program BioEdit Sequence Alignment Editor Version 5.0.9 (Hall 1999). Three different alignments were established for ITS, LSU and a combined ITS and LSU dataset. *Hymenoscyphus fructigenus* and *H. conscriptus* were used as outgroup in all analyses. The ITS datamatrix included 46 taxa. The taxa *Geoglossum lineare*, *G. starbaeckii* (O-F 73035), and *Bryoglossum gracile* were unique for the ITS data set. Forty-six taxa were included in the LSU data set. *Geoglossum nigrinum*, *G. glabrum* and *Rhytisma acerinum* were unique for the LSU data set. The combined ITS and LSU alignment included 43 taxa.

Phylogenetic analyses of sequence data were performed using (i) the maximum-parsimony (MP) criterion as implemented in PAUP\* version 4.0b10 (Swofford 2003) and (ii) Bayesian analyses as implemented in MrBayes version 3.0 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). In the parsimony analyses, gaps were treated as both coded characters and as missing characters. Gaps were coded using the “simple indel coding method” as described by Simmons and Ochoterena (2000). Simple indel coding is implemented by coding all gaps that have different 5' and/or 3' termini separate as presence/absence characters. Whenever gaps from different sequences were a subset of other gaps, sequences possessing the longer, completely overlapping gaps were coded as inapplicable for the gap being coded (Simmons & Ochoterena, 2000). The MP analyses were performed using heuristic searches with 10 RAS (random addition of sequences) replicates, TBR (tree-bisection-reconnection) swapping and maxtrees = unlimited. All other settings were default. Bootstrap support was estimated with 1000 replicates and 10 RAS per bootstrap replicate. The best fit models for the Bayesian analysis were estimated using the software MODELTEST (Posada & Crandall 1998) version 3.06, and the GTR+I+G model was selected for all datasets. Settings for the Bayesian analyses were as follow: the generations were set at 1000,000, rates=gamma, nset=6 and savebrlens=yes. All other settings were default. Four

chains were ran simultaneously for 1000,000 generations. Trees were sampled every 100 generation which gave a total of 10,000 trees. The chains reached stationarity around the 10,000 generation. Thus, the first 1000 trees were deleted as the “burn in period” of the chain.

### **Morphological studies**

For morphological analyses, the same taxa were used as for the molecular analyses (see Table 1). Microanatomical characters are based on observations of dried or fresh material squashed in cotton blue and water and photographs were made using a Leica DMR microscope. Macroscopic morphological characters were based on direct observations. Literature was consulted to complement these observations (Nannfeldt 1942, Mains 1956, Verkley 1994, Dissing 2000a, 2000b, 2000c, 2000d, Ohenoja 2000, Schumacher 2000, Vesterholt 2000). A total of 19 characters were scored and the characters were coded using binary states (Table 2). The morphological characters were scored in a matrix and the evolution of each character was analysed by tracing the characters on a combined ITS and LSU strict consensus tree in McClade (Maddison & Maddison, 2001).

Table 2. Morphological characters investigated.

No.	Character type	Character state	Character description
1	Ascospore (length)	0	Not
		1	< 60
2		0	Not
		1	> 60
3	Ascospore-septation	0	Septate
		1	One-celled
4		0	Not
		1	0-7 septa
5		0	Not
		1	8-15 septa
6	Ascospore-shape	0	Non-clavate
		1	Clavate
7	Ascospore-colour	0	Hyaline
		1	Brown, light brown
8	Ascus-length	0	Not
		1	<150
9		0	Not
		1	>150
10	Number of spores	0	<8
		1	8
11	Paraphyse-shape	0	Filiform, sparingly septate
		1	Curved, septate, enlarged at the apex and constricted at septa
12	Paraphyse-colour	0	Hyaline
		1	Coloured
13	Agglutination	0	Not agglutinated
		1	Agglutinated
14	Apical pore in ascus	0	Non-amyloid
		1	Amyloid
15	Colour of ascocarp	0	Other colours
		1	Black/ dark brown
16	Ascocarp-size	0	Not
		1	<3
17		0	Not
		1	>3
18	Ascocarp-shape	0	Not clavate
		1	Clavate
19	Hymenium	0	Discontinuous with stipe
		1	Continuous with stipe

## RESULTS

### ITS phylogeny

A parsimony analysis of the ITS dataset with gaps treated as 'new state', yielded five equally most parsimonious trees (MPTs), 2157 steps in length. As observed in Fig. 3, three main clades were identified (designated as clade 1-3 in Fig. 3). **Clade 1** (100% bootstrap support (bts)) included all species that have been referred to as black earth tongues. Clade 1 was divided into two subclades; **subclade 1a** (100% bts) including the three species of *Trichoglossum* (*T. walteri*, *T. hirsutum*, and *T. velutipes*) and **subclade 1b** including all the species from the genus *Geoglossum*. *Geoglossum littorale* clustered basically in the 1a subclade, and *Sarcoleotia globosa* clustered basically in clade 1 on a separate lineage. **Clade 3** (100% bts), the sistergroup of clade 1, embraced species from the genera *Spathularia* and *Cudonia*, where *Spathularia* species (*S. rufa* and *S. flavida*) constituted one subclade (94 % bts), and the *Cudonia* species (*C. circinans*, *C. confusa*, *C. lutea*, and *C. sichuanensis*) another subclade (81 % bts). **Clade 2** (88% bts) consisted of species from the genera *Microglossum* (*M. atropurpureum*, *M. olivaceum*, *M. fuscorubens*, *M. novo species* and *M. viride*) and *Leotia* (*L. viscosa* and *L. lubrica*).

Largely, the same tree topology was obtained in a parsimony analysis, when gaps were scored as 'missing data' (data not shown). This analysis yielded 1 MPT, 1677 steps in length. **Clades 1** (100% bts), **3** and **2** included the same species as above with only slightly lower bootstrap support (Table 3). Some rearrangements of taxa of the subclades occurred, e. g. *Geoglossum littorale*, *G. glutinosum* and *G. arenarium* grouped together with *Trichoglossum* in subclade 1a.

A Bayesian analysis gave the same main tree topology as in the parsimony analyses. **Clades 1** and **3** had a posterior probability (PP) of 100% and **clade 2** 99% PP (Table 3). Compared to the parsimony analyses, clade 2 constituted the sistergroup to clade 1, in stead of clade 3.

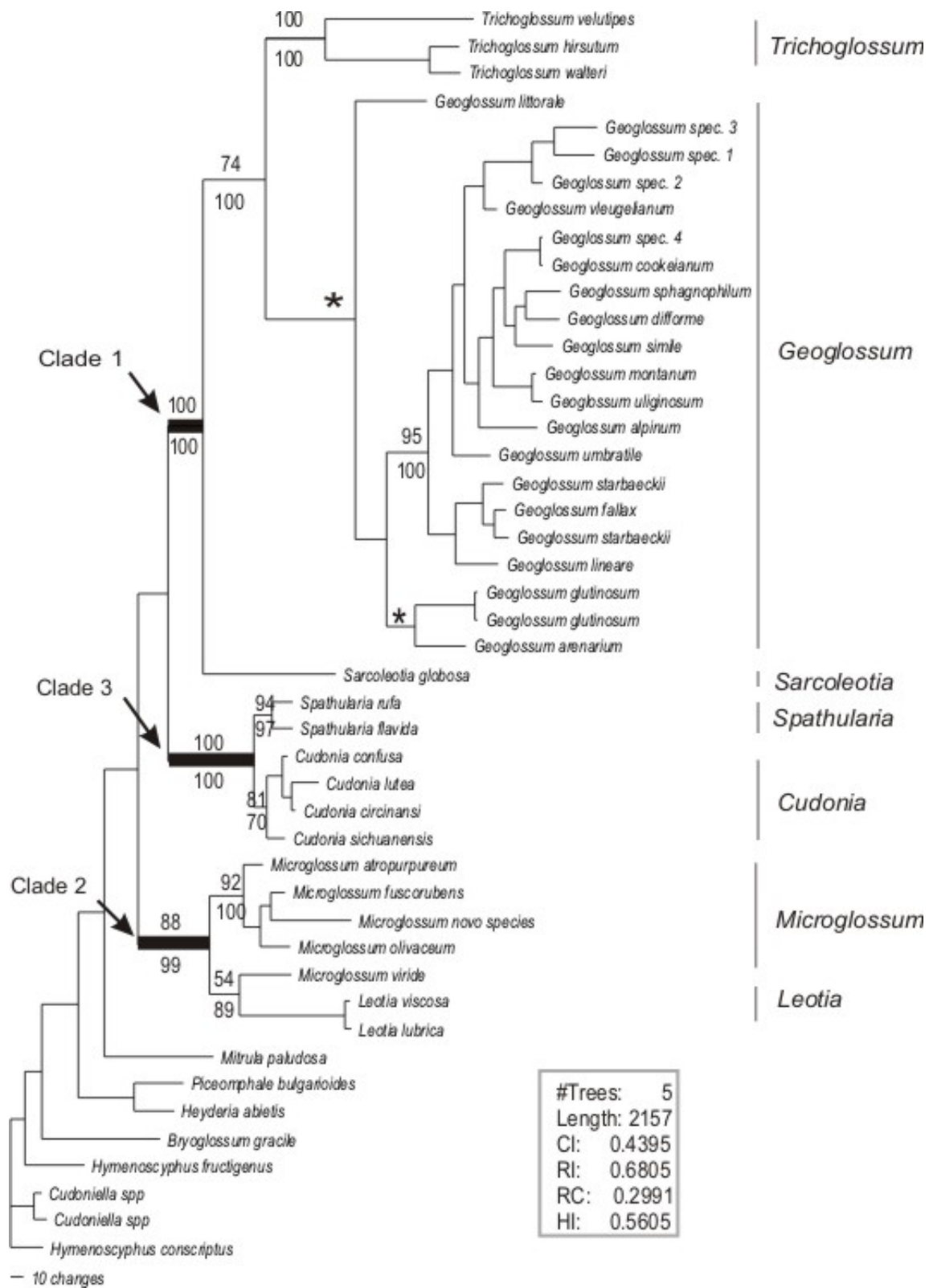


Figure 3. Geoglossaceae MP (most parsimonious) phylogeny of the ITS dataset (using gaps as 'new state'). The analysis gave five MPTs of which one is shown. The asterisk symbol indicates branches that collapsed in the strict consensus tree. Bootstrap support values are marked above the branches and posterior probability values below branches.

Table 3. Support values for the three main clades of the phylogenetic analyses (cf. Figs. 3-5). In the maximum parsimony analyses (MP), bootstrap support values are given; in the Bayesian analyses, posterior probability values are given.

Clades	ITS			LSU			ITS + LSU		
	MP gap	MP - gap	Bayes	MP gap	MP - gap	Bayes	MP gap	MP - gap	Bayes
1	100	100	100	100	100	86	100	100	100
2	88	59	99	99	99	100	100	98	100
3	100	100	100	100	100	100	100	100	100

### LSU phylogeny

A parsimony analysis of the LSU rDNA dataset with gaps treated as 'new state', yielded 340 equally MPTs, 746 steps in lengths, of which the strict consensus tree is shown in Fig. 4. The same three main clades as in the ITS phylogeny were identified: **Clade 1** (100% bts) included species that belong to the genera *Geoglossum*, *Trichoglossum* and *Sarcoleotia*. As in the ITS phylogeny, this clade was divided into two subclades, with a few rearrangements: **subclades 1a** with *Trichoglossum velutipes*, *T. walteri* and *T. hirsutum*, and two *Geoglossum* species; *G. glutinosum*, and *G. arenarium*; and **subclade 1b** (76% bts) with the rest of the *Geoglossum* species (17), except *G. littorale*. *Geoglossum littorale* and *S. globosa* clustered basically in clade 1 on separate lineages. **Clade 2** (99% bts) included species from the genera *Microglossum* (*M. atropurpureum*, *M. fuscorubens*, *M. novo species*, *M. olivaceum* and *M. viride*) and *Leotia* (*L. lubrica* and *L. viscosa*). **Clade 3** (100% bts) included the genera *Cudonia* (*C. circinans*, *C. confusa*, *C. lutea*, and *C. sichuanensis*) and *Spathularia* (*S. rufa* and *S. flavida*).

In a parsimony analysis where gaps were scored as 'missing data' (data not shown), the analysis yielded 330 equally MPTs, 690 steps in length an identical tree topology was obtained. The main clades had the same bts (Table 3) as in the analysis above, and there were only minor positional rearrangements within the clades.

A Bayesian analysis gave largely the same results as for the parsimony analyses with one exception: **subclade 1a** was divided into two groups with *G. glutinosum* and *G. arenarium* in one and the *Trichoglossum* species in the other. A high support was obtained for the main clades in the Bayesian analysis (Table 3).



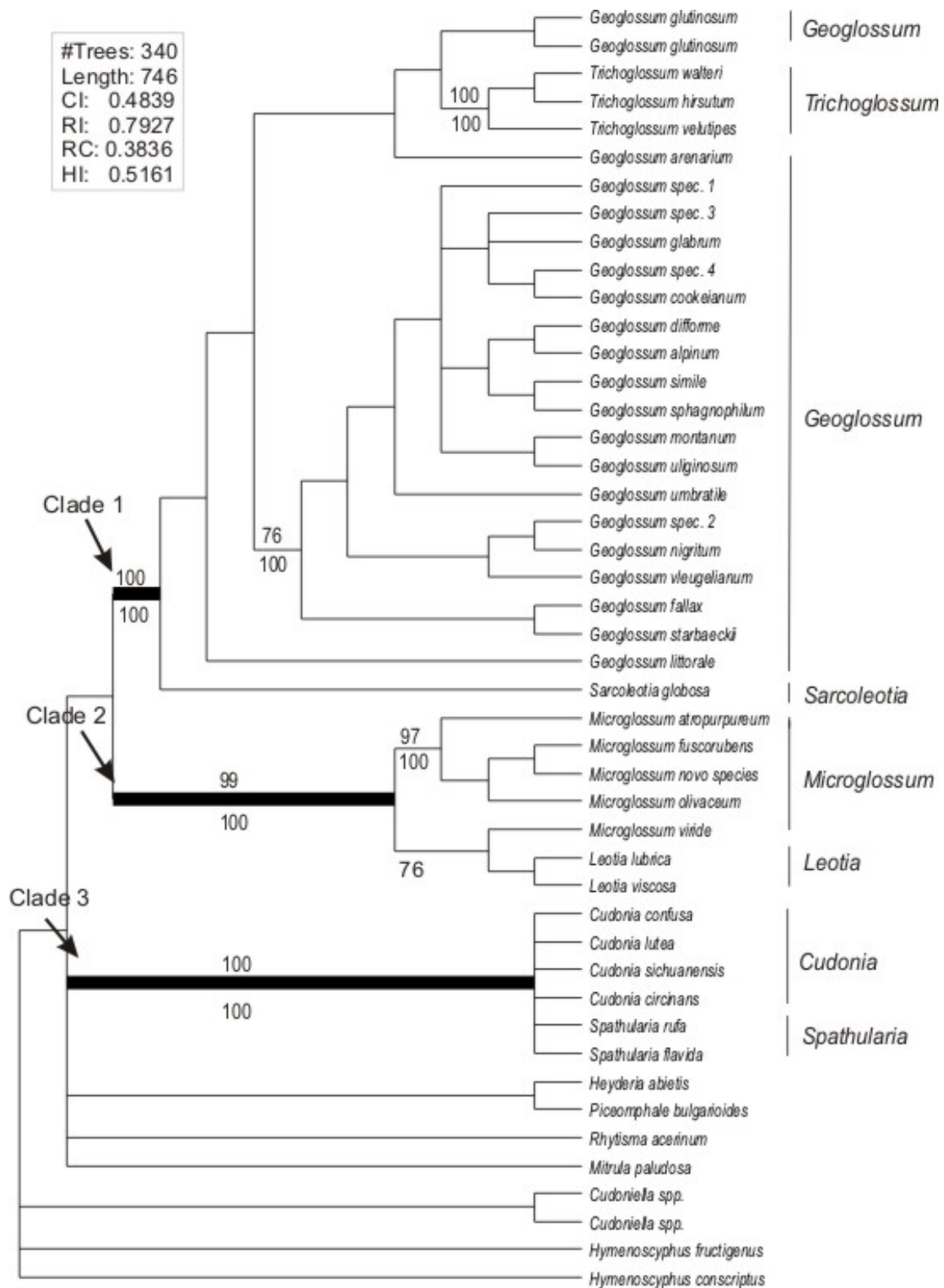


Figure 4. Geoglossaceae MP phylogeny based upon parsimony analysis of the LSU dataset (using gaps as 'new state'). The analysis gave 340 MPTs, of which the strict consensus tree is shown. Bootstrap support values are marked above the branches and posterior probability values below branches.

### Combined ITS and LSU phylogeny

A high congruence was observed between the ITS and LSU phylogenies, and a combined analysis of the two datasets was undertaken. A parsimony analysis of the combined ITS and LSU dataset with gaps treated as 'new state', yielded four equally MPTs, 2763 steps in length, of which one is shown in Fig. 5. As for the combined ITS and LSU phylogenies, the same three main clades were identified. **Clade 1** (100% bts) included the genera *Geoglossum*, *Trichoglossum* and *Sarcoleotia* and was divided into two subclades, equal the ITS phylogeny; **subclade 1a** (100% bts), which included the *Trichoglossum* species (*T. velutipes*, *T. hirsutum* and *T. walteri*) and **subclade 1b**, which included all the *Geoglossum* species. *Geoglossum littorale* constituted a basal separate lineage in the 1b subclade and *Sarcoleotia globosa* clustered basically in clade1 separate from the rest of the species. **Clade 2** (100% bts) included the *Microglossum* species (*M. atropurpureum*, *M. fuscorubens*, *M. novo species*, *M. olivaceum* and *M. viride*) and the *Leotia* species (*L. viscosa* and *L. lubrica*). **Clade 3** (100% bts) included the *Spathularia* species (*S. rufa* and *S. flavida*) in one subclade (94% bts) and the *Cudonia* species (*C. confusa*, *C. lutea*, *C. sichuanensis* and *C. circinans*) in another subclade (81% bts).

The combined ITS and LSU dataset was also analysed treating gaps as 'missing characters', which gave three equally MPTs, 2232 steps in length. In this analysis **Clades 2** (98% bts) and **3** (100% bts) had the same tree topology as in the previous analysis. In **Clade 1** (100% bts), **subclade 1a** included *Trichoglossum*, *Geoglossum glutinosum* and *G. arenarium* and **subclade 1b** (97% bts) the rest of the *Geoglossum* spp. *Geoglossum littorale* constituted a lineage of its own, being a sistergroup to subclades 1a and 1b.

A Bayesian analysis gave the same main tree topology as for the parsimony analysis and all the three main clades obtained 100% PP (Fig. 5 and Table 3).

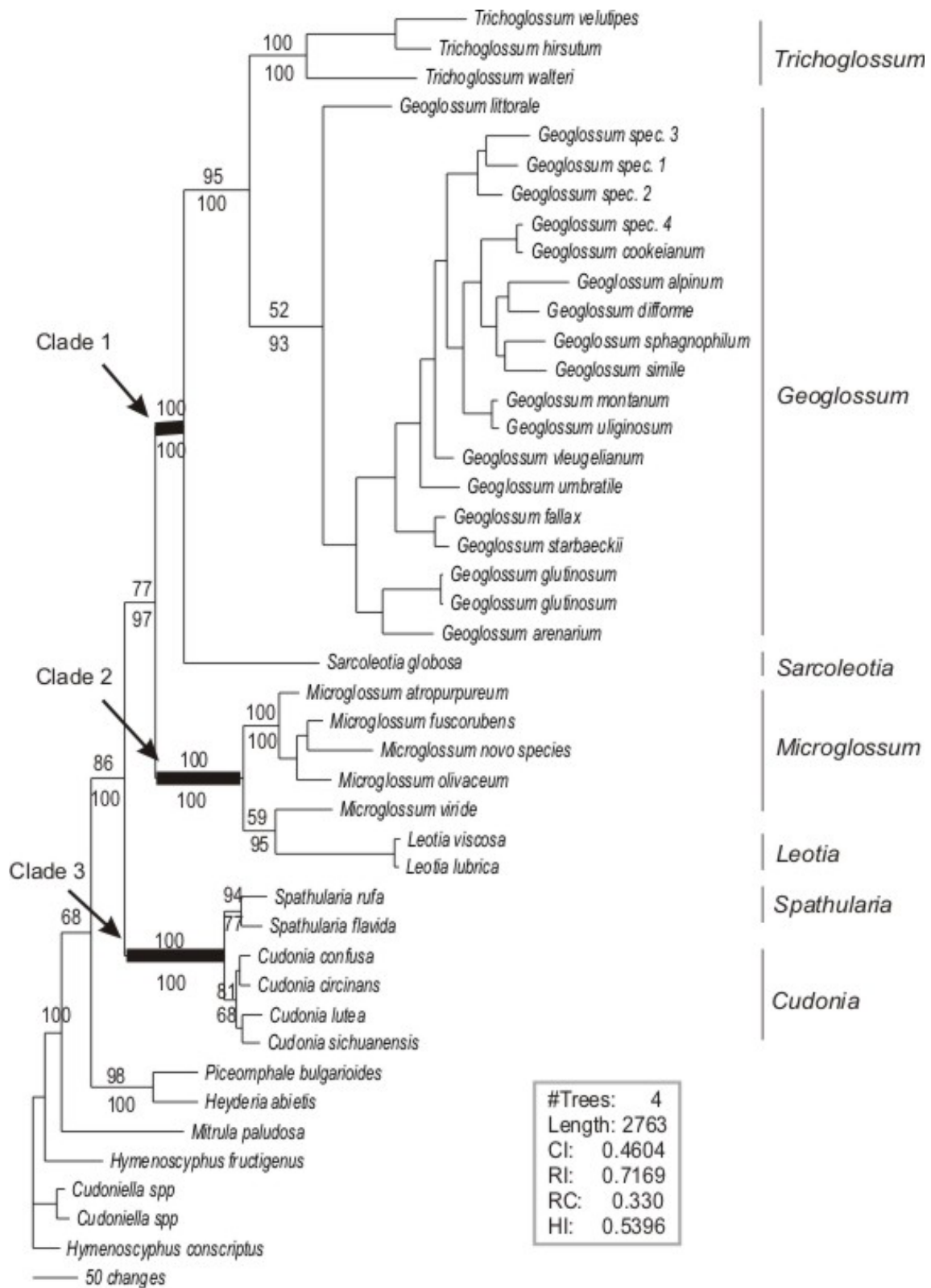


Figure 5. Geoglossaceae MP phylogeny of the combined ITS and LSU dataset (using gaps as 'new state'). The analysis gave four MPTs, of which one is shown. Bootstrap support values are marked above the branches and posterior probability values below branches.

## Evolution of morphological characters

In order to analyse the evolution of morphological characters, each character was traced on the phylogenetic trees obtained from the parsimony analysis of the combined ITS and LSU dataset. Most characters were highly homoplasious when superimposed on the phylogeny (data not shown), however, some characters gave significant phylogenetic information. A phylogenetic tree from the combined ITS and LSU dataset, where these latter characters are superimposed, is presented in Fig. 6. The character 'ascospore length', did not partition the taxa into significant groups, although a majority of *Geoglossum* and *Trichoglossum* species have ascospores greater than 60  $\mu\text{m}$  (data not shown). 'Ascospore shape' and 'ascospore colour' separated most *Geoglossum* and *Trichoglossum* species from the rest of the taxa, except for *G. littorale* and *G. arenarium*, which have subhyaline ascospores (Fig. 6). The characters 'ascus length' and 'ascospore septation' (data not shown) were homoplasious. The character 'paraphysis shape' largely divided the species into two groups, i.e. *Geoglossum*, *Trichoglossum* and *Sarcoleotia globosa* with curved, septated paraphyses,  $\pm$  constricted at septa, and the rest of the species having filiform and sparingly septate paraphyses (Fig. 6). The character 'coloured paraphyses' is a synapomorphy shared by the taxa in the monophyletic group of *Geoglossum*, *Trichoglossum* and *Sarcoleotia globosa* (Fig. 6). The character 'ascus pore amyloid or non-amyloid' is homoplasious, being a result of convergent evolution of clade 1 and *Microglossum* of clade 2, and the more basally clustered taxa like *Piceomphale bulgarioides*, *Heyderia abietis*, *Mitrulea paludosa* and *Hymenoscyphus* (data not shown). The character 'ascocarp colour' unites *Geoglossum*, *Trichoglossum* and *Sarcoleotia globosa* of clade 1 (Fig. 6). The character 'ascocarp shape clavate' is homoplasious, it is shared by *Geoglossum* and *Trichoglossum* of clade 1, and *Microglossum* of clade 2 (Fig 6). The character of 'hymenium continuous or discontinuous with the stipe' is homoplasious as well and shared by *Geoglossum* and *Trichoglossum*, of clade 1, *Microglossum*, of clade 2, *Spathularia*, of clade 3, and *Mitrulea*, which clustered more basically in the tree (data not shown).

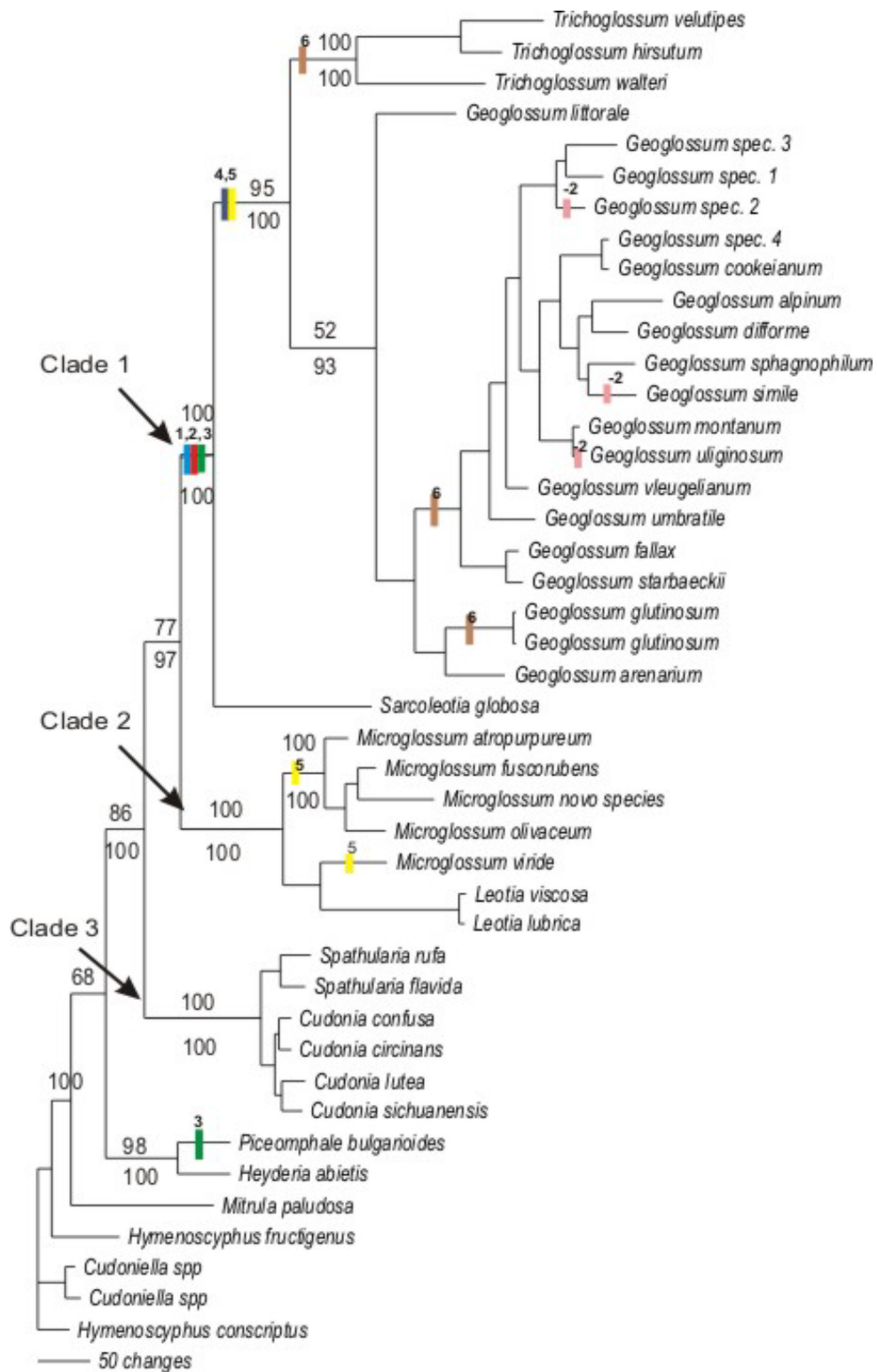


Figure 6. The evolution of selected morphological characters marked on a combined ITS and LSU phylogenetic tree. Characters indicated, 1 (blue): 'paraphyse shape - curved, multiseptate, enlarged at the apex, constricted at septa; 2 (red): paraphyses coloured (-2 (pink) paraphyses hyaline); 3 (green): the colour of ascocarp black/dark brown; 4 (dark blue): ascospores with a clavate shape; 5 (yellow): ascocarp with a clavate shape; 6 (brown): coloured ascospores.

## DISCUSSION

A main result of this study is that the molecular phylogeny of the Geoglossaceae and its allies is incongruent with the classification system, as proposed by Nannfeldt (1942), Maas Geesteranus (1966) and Ohenoja (2000).

The ITS and LSU phylogenies gave largely the same tree topology and worked well to study relationships within Geoglossaceae ss. lat. and related taxa of the Helotiales. Furthermore, the phylogenetic methods employed, i. e. Maximum Parsimony (MP) and MrBayes, gave largely compatible topologies for the three datasets (ITS, LSU and combined ITS and LSU). The Bayesian analyses gave slightly higher support for the inferred phylogenies than the parsimony analyses (Table 3). The combined ITS and LSU dataset performed best overall, with more resolved clades, higher bootstrap support and higher Bayesian posterior probability (Figs. 3-5, and Table 3). Since the phylogenies were largely congruent, most of the discussion rests on the more robust combined ITS and LSU dataset.

Our results to some extent contradict the various classification “systems” of Geoglossaceae proposed, based on morphological characters alone. Maas Geesteranus (1966) claimed that there was only one ‘good’ microanatomical character that separated the Helotiaceae from the Geoglossaceae the latter family containing *Geoglossum*, *Trichoglossum*, *Microglossum*, *Thuemenidium* Kuntze, *Nothomitra* Maas G., *Mitrula* and *Spathularia*, and that was the profile line that can be drawn by following the contour of the stipe upward to the hymenium; in the Geoglossaceae this line is continuous, but in the Helotiaceae discontinuous. Based on this character, Maas Geesteranus (1966) transferred the genera *Cudonia* and *Sarcoleotia* to Helotiaceae, and restricted the Geoglossaceae to those genera of the Helotiales in which, at least in the younger stages, the fertile head and stipe form one continuous line.

However, such a delimitation of genera and families gets no support from our study. In all molecular phylogenies Geoglossaceae ss. lat. is polyphyletic. In a phylogenetic context, the Geoglossaceae should be restricted to encompass the genera *Geoglossum*, *Trichoglossum* and *Sarcoleotia*, i.e. the Geoglossaceae ss. str.

(clade 1, Fig. 5). While *Geoglossum* and *Trichoglossum* share this continuous profile line, *Sarcoleotia globosa* is in lack of it. The monophyletic group of *Microglossum* and *Leotia*, which gets high support in all molecular analyses (clade 2, Fig. 5), is also split based on this morphological character, *Microglossum* having a continuous line from the hymenium to the stipe and *Leotia* a discontinuous line. The character separates the monophyletic group of *Spathularia* and *Cudonia* as well (clade 3, Fig. 5).

Another morphological character which has been extensively used by some authors is the reaction of the ascus pore in Melzer's reagent (Maas Geesteranus 1964, 1966, Verkley 1994, Wang *et al* 2002). Maas Geesteranus (1966) stated that the Melzer reaction was a 'good' character in order to separate genera, but unsuited to separate the Geoglossaceae from the Helotiaceae. This is in accordance with the results obtained in this study (data not shown), where Geoglossaceae ss. str. (clade 1) shares the positive (J+) reaction in Melzer's reagent with *Microglossum* of clade 2 and also with some of the taxa clustered basically, such as *Piceomphale bulgarioides*, *Heyderia abietis*, *Mitrla paludosa*, and *Hymenoscyphus*.

Another important result from our study is that some morphological characters that have been abandoned by some recent authors should be reconsidered in classification work. Maas Geesteranus (1966) regarded the characters of paraphysis shape and colour as inadequate to distinguish between the Geoglossaceae and the Helotiaceae. Nevertheless, the curved, multiseptate and coloured paraphyses, usually constricted at septa, represent a synapomorphy for the Geoglossaceae ss. str. (clade 1), when superimposed on the molecular phylogeny (Fig. 6). Tracing the morphological characters superimposed on the molecular phylogenies also reveal 'ascospore colour' and the 'clavate shape of the ascospore' as unique characters for *Trichoglossum* and most species of *Geoglossum* (Fig. 6), except for the *Geoglossum littorale* and *G. arenarium* of subclade 1b of Geoglossaceae, which have subhyaline ascospores .

Ascocarp features have been considered as important characters in the circumscription of genera of the families Geoglossaceae and Helotiaceae (Nannfeldt 1942, Maas Geesteranus 1966, Eckblad 1963). The clavate shape of the ascomata unites the group commonly referred to as earth tongues (*Geoglossum*, *Trichoglossum* and *Microglossum*) (Eckblad 1963). However, the molecular

phylogeny shows that this grouping is artificial, with *Geoglossum* and *Trichoglossum* in clade 1 and *Microglossum* in clade 2 (Fig. 6).

There is another ascocarp feature uniting Geoglossaceae ss. str. The black and dark brown colours of the ascocarp are another synapomorphy of the monophyletic group of Geoglossaceae ss. str. (Fig. 6).

The genus *Corynetes* was erected by Hazsl. (1881) on the basis of ascocarp morphology and hyaline ascospores to include *C. arenarius*, *C. atropurpureus* and *C. globosus*. *Corynetes* was included in the Geoglossaceae by Durand (1908), who distinguished *Corynetes* from *Microglossum* based on the black/purplish-black/brown-black colours of the ascocarp in the former. Nannfeldt (1942) concluded that *Geoglossum*, *Trichoglossum* and *Corynetes* constitute a closely related group of taxa. Hyaline ascospores separate *Corynetes* from *Geoglossum*, although tardily and slightly coloured ascospores also occur in some species of *Geoglossum*. Mains (1955) united *Microglossum* and *Corynetes* based on the fact that both genera contain representatives with subhyaline ascospores. The colour of the ascocarp was also maintained as insufficient for a generic separation (Mains 1955). Eckblad (1963) also emphasized the close relationship between *Geoglossum*, *Trichoglossum* and *Corynetes* and concluded that if the latter was to be united with *Microglossum*, *Geoglossum* and *Trichoglossum* should be included as well.

The recognition of *Corynetes* in its original sense, to include *Geoglossum arenarium*, *G. atropurpureum* and *Sarcoleotia globosa*, got no support from the molecular phylogeny presented here. *Corynetes* in its original sense, is polyphyletic, e.g. *Geoglossum arenarium* and *S. globosa* are included in clade 1 (Fig. 5) and *G. atropurpureum* in clade 2 (with *Microglossum*) (Fig. 5).

Maas Geesteranus (1964) rejected *Corynetes* Hazsl. because it was an orthographic variant and homonym of *Corynites* Berk. & M.A. Curtis, and introduced in stead *Thuemenidium* Maas Geesteranus (1964) to accommodate the single species *T. atropurpureus*. According to Eckblad (1963), *Thuemenidium* mainly differs from *Geoglossum* by its colourless ascospores, but is obviously closely related to *Geoglossum* (Eckblad 1963). Læssøe and Elborne (1984) found *T. atropurpureus* to be intermediate between *Geoglossum* and *Microglossum*, because of hyaline ascospores which separate it from the former genus and the size of ascospores which separates it from the latter genus.



*Geoglossum atropurpureum* should, according to the molecular phylogeny presented here, be included in the genus *Microglossum* and transferred to the family Leotiaceae. The hyaline ascospores and purplish brown colour of the ascocarp are features shared with *Microglossum*. The combination in *Microglossum* has already been proposed by Karsten (1885), as *M. atropurpureum*, and was accepted by Mains (1955). In the molecular phylogeny the genera *Microglossum* and *Leotia* constitute a 100% bts and 100% PP monophyletic group (clade 2), which is the sistergroup to Geoglossaceae ss. str. (clade 1) (Fig. 5).

*Sarcoleotia globosa* was first described by Sommerfelt in 1826 as *Mitrula globosa* (cf. Schumacher & Sivertsen 1987). Fries received specimens from Sommerfelt and referred the species to *Geoglossum*; Durand (1908) transferred it to *Corynetes* and Maas Geesteranus (1966) and Korf (1971) to *Sarcoleotia*. Imai and Korf (1958) referred *Leotia* and *Neocudoniella* Imai to the Helotiaceae, and later accommodated *Sarcoleotia* in this latter family.

Our analyses confirm that *Sarcoleotia globosa* is to be included in the Geoglossaceae ss. str. *Sarcoleotia globosa* forms a separate lineage within the Geoglossaceae ss. str. (clade 1, Fig. 5), a position strongly supported (100% bts and 100% PP) in all analyses (Figs. 3-5 and Table 3). Nannfeldt (1942) and Nitare (1982) both suggested to include *S. globosa* in the Geoglossaceae. *Sarcoleotia globosa* is rather distinct from the rest of the clade; in addition to having a discontinuous profile line between hymenium and stipe, *S. globosa* has hyaline ascospores and stipitate ascocarp, which clearly distinguish *Sarcoleotia* from *Geoglossum* and *Trichoglossum* (Fig. 6). However, other morphological features such as, shape and colour of paraphyses and the dark colour of ascocarp, corroborate the grouping of *Sarcoleotia* with *Geoglossum* and *Trichoglossum* (Fig. 6). *Geoglossum arenarium*, a rather aberrant species of the genus *Geoglossum*, which is also suggested by the molecular phylogeny, is encompassed as a separate lineage of *Geoglossum* (clade 1, Fig. 5).

The genera *Cudonia* and *Spathularia* were originally placed in the family Geoglossaceae ss. Durand (1908), where they were included in different subfamilies, i. e. *Cudonia* in Cudonieae and *Spathularia* in Geoglosseae. *Cudonia* was later transferred to the Helotiaceae by Maas Geesteranus (1966). Korf (1973) included *Cudonia* in his concept of Leotiaceae (= Helotiaceae) based on macromorphology. Later, the concept of the Leotiaceae ss. Korf was restricted and *Cudonia* excluded

from the family (Lizon *et al* 1998) and again included in the Geoglossaceae (Ohenoja 2000).

Through the years, many authors have recognized the close relationship between *Cudonia* and *Spathularia* (Nannfeldt 1942, Mains 1956, Eckblad 1963). Nannfeldt (1942) found no microscopical differences between the two groups, an observation also supported by Mains (1956). *Mitrula*, *Cudonia*, *Spathularia* and *Leotia* were by Eckblad (1963) separated from *Geoglossum*, *Trichoglossum* and *Microglossum* based on macromorphology, form and colour.

The close relationship between *Cudonia* and *Spathularia* has got support from recent molecular studies (Platt & Spatafora 1999, Bhattacharya *et al* 2000, Gernandt *et al* 2001, Lutzoni *et al* 2001, Wang *et al* 2002, Tehler *et al* 2003). Based on these observations, Cannon (2001) established the new family Cudoniaceae of the Helotiales to include the genera *Cudonia* and *Spathularia* (Kirk *et al* 2001). Wang *et al* (2002) showed that *Cudonia* and *Spathularia* together form a strongly supported monophyletic group, an observation also supported in this study (Figs. 3-5, Table 3). The ITS and the combined ITS and LSU phylogeny strongly support a separate clade of *Cudonia* and *Spathularia*, where *Cudonia* spp. clustered in one group and *Spathularia* in another group. In the LSU analyses (in both Parsimony and Bayesian analyses) (Fig. 4), most branches within this clade collapsed. It is impossible to conclude whether *Spathularia* and *Cudonia* should be separated into two different genera or not, based on the present study, however, the two genera are apparently distantly related to the Geoglossaceae ss. str. This is also in accordance with the study of Wang *et al* (2002).

*Cudonia* and *Spathularia* are similar in morphological as well as molecular characters, however, the continuous profile line from hymenium to the stipe, separates *Spathularia* from *Cudonia*. Both genera possess ascocarps in the range of yellow, brownish and ochraceous colours, hyaline ascospores which in some species produce conidia on sterigma, hyaline and filiform paraphyses, and a non-amyloid reaction of ascospore in Melzer's reagent (J-). The paraphyses of *Cudonia* and *Spathularia* are filiform and sparingly septate, while paraphyses characteristic for Geoglossaceae ss. str. are curved, multiseptate and constricted at septa, characters that neatly separate Geoglossaceae ss. str. from the rest of the taxa in the study. Eriksson *et al* (2001), based on the results obtained by Gernandt *et al* (2001), where *Cudonia* and *Spathularia* formed a strongly supported monophyletic group within

Rhytismataceae, transferred Cudoniaceae from the Helotiales to the Rhytismatales. Such an association between *Cudonia*, *Spathularia* and the Rhytismataceae was already suggested by Nannfeldt (1942). He concluded that *Cudonia* and *Spathularia* share important morphological characters with Phacidiaceae (including *Rhytisma*), such as filiform, branched and circinate paraphyses, and a stromatic layer that covers the hymenium in the early stage of ascoma development (Nannfeldt 1942). Recent molecular studies support a close relationship between Cudoniaceae and Rhytismataceae (Gernandt *et al* 2001, Lutzoni *et al* 2001, 2004, Wang *et al* 2002, 2005, Tehler *et al* 2003, Miadlikowska & Lutzoni 2004). Wang *et al* (2005) also demonstrated that the Helotiaceae and Rhytismataceae together form a monophyletic group.

The genus *Leotia* was originally placed in the family Geoglossaceae ss. Durand (1908), but was later transferred to the family Helotiaceae by Korf (1958). The Leotiaceae and Leotiales have undergone several reinterpretations (Korf *et al* 1996, Lizon *et al* 1998, Korf & Lizon 2000, 2001). Korf (1999) recognized *Leotia* and the restricted family Leotiaceae ss. Korf as separate from the Helotiaceae, and established the new order Leotiales. Eriksson (1999) supported Korf and recognized Leotiaceae as a separate family, but retained both families in the Helotiales. Our study supports the segregation of *Leotia* from a restricted, monophyletic Helotiaceae. However, further analyses, including additional taxa of *Leotia* and representatives of the Helotiaceae, are needed in order to define the two groups, Helotiaceae ss str. and *Leotia* and their allies, respectively.

Recent molecular studies have shown that *Microglossum* and *Leotia* are closely related (Gernandt *et al* 2001, Tehler *et al* 2003, Hambleton *et al* 2003, Wang *et al* 2005), an observation which also get support from the present study. *Microglossum* was originally placed in the family Geoglossaceae ss. Durand (1908). Later Kirk *et al* (2001) omitted *Microglossum* from the Geoglossaceae, but was uncertain about where to place it. *Microglossum* and *Leotia* constitute a relatively strongly supported monophyletic group (Figs. 3-5, Table 3). In all analyses *Microglossum viride* forms a monophyletic group with *Leotia* (the combined ITS and LSU dataset: 59% bts and 95% PP, LSU dataset: bts < 50% and 76% PP, and ITS dataset: 54% bts and 89% PP, Figs. 3-5), as a sister group to a moderately supported clade of the remaining *Microglossum* species. Despite differences in

morphological features, such as presence of gelatinous layers, along with clavate shape of the ascocarp in *Microglossum*, opposite to a stipitate shape in *Leotia*, the J+ reaction in *Microglossum* and the J- reaction in *Leotia* (Zhong & Pfister 2004), they both contain hyaline multiguttulate ascospores, hyaline and filiform paraphyses, and brightly coloured ascocarps, which together with the molecular characters, corroborate the grouping of *Microglossum* and *Leotia*.

Recent molecular studies have also demonstrated that Geoglossaceae ss. str. probably is to be excluded from the Helotiales in future classifications (Tehler *et al* 2003, Lutzoni *et al* 2004, Wang *et al* 2005). Tehler *et al* (2003) showed that *Geoglossum* represents the sistergroup to the inoperculate euascomycetes (Orbiliomycetes excluded). The basal position of *Geoglossum* was also confirmed by Lutzoni *et al* (2004). By the use of nucSSU, nucLSU, mitSSU rDNA and RPB2, phylogenetic analyses revealed that the Helotiales was divided in two lineages, e.g. one lineage leading to the Erysiphales, Dermataceae, Helotiaceae, Hyaloscyphaceae, Leotiaceae, Sclerotiniaceae, Cudoniaceae and Rhytismataceae (Rhytismatales), and a second lineage leading to *Geoglossum* and *Trichoglossum*. The separation of *Geoglossum* and *Trichoglossum* from other genera of the Helotiales has been observed in other rDNA based phylogenies as well (Wang *et al* 2005), and is consistent with the results from this study. However, additional sampling is needed to resolve this issue with greater confidence.

## CONCLUSIONS

This study has shown that Geoglossaceae should include the group of taxa referred to as *Geoglossum*, *Trichoglossum* and *Sarcoleotia*. In the light of the molecular phylogenies presented, *Microglossum* must be excluded from the Geoglossaceae ss. str. and should better be referred to the Leotiaceae, such as circumscribed by Korf (1999). *Cudonia* and *Spathularia*, *Mitrula* and *Bryoglossum* constitute clades that should also be excluded from the Geoglossaceae.

Morphological characters traditionally used to delineate genera and families of the clavate and stipitate ascocarps in this group of fungi, such as a continuous or discontinuous between the fertile head and the stipe, and the ascus pore reaction in Melzer's reagent should be abandoned, and instead morphological characters to be re-considered in classification work are paraphyses (shape, colour, septation,  $\pm$  constricted at septa), ascospores (coloured/hyaline, clavate/ellipsoid) and colours of ascocarp should be reconsidered in classification work.

## REFERENCES

**Alexopoulos, C. J., Mims, C. W., Blackwell, M. (1996).** *Introductory Mycology*.  
Forth Edition.

**Bhattacharya, D., Lutzoni, F., Reeb, V., Simon, D., Nason, J. & Fernandez, F. (2000).** *Widespread Occurrence of Spliceosomal Introns in the rDNA Genes of Ascomycetes*. *Mol. Biol. Evol.* 17(12), 1971-1984.

**Bille-Hansen, E. (1954).** *The Danish Species of Geoglossum and Related Genera*.  
*Bot. Tidsskr.* 51, 7-18.

**Boudier, E. (1885).** *Nouvelle classification naturelle des Discomycetes charnus*.  
*Bull. Soc. mycol. Fr.:* 1:91-120.

**Cannon, P. F. (2001).** *Cudoniaceae*. In *Ainsworth & Bisby's Dictionary of the Fungi*,  
ix. Edited by Kirk P.M., Cannon, P.F., David J.C. & Stalpers J.A. 9th Ed. - CAB  
International.

**Dissing, H. (2000)a.** *Sarcoleotia*. In *Nordic macromycetes*, vol 1, 159-160. Edited by  
L. Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Dissing, H. (2000)b.** *Leotia*. In *Nordic macromycetes*, vol 1, 157. Edited by L.  
Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Dissing, H. (2000)c.** *Heyderia*. In *Nordic macromycetes*, vol 1, 151-152. Edited by L.  
Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Dissing, H. (2000)d.** *Cudoniella*. In *Nordic macromycetes*, vol 1, 145. Edited by L.  
Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Durand, E. J. (1908).** *The Geoglossaceae of North America*. *Ann. Mycol.* VI (5),  
387-477.

**Durand, E. J. (1921).** *New or noteworthy Geoglossaceae.* Mycologia 13, 184-187.

**Eckblad, F-E. (1963).** *Contributions to the Geoglossaceae of Norway.* Nytt Mag. Bot. 10, 137-158.

**Ericsson, O. E., (ed.). (1999).** *Outline of Ascomycota – 1999.* Myconet 3, 1-88.

**Eriksson, O.E., Baral, H.-O., Currah, R.S., Hansen, K., Kurtzman, C.P., Rambold, G. & Laessøe, T. (eds). (2001).** *Outline of Ascomycota – 2001.* Myconet 7, 1-88.

**Gernandt, D. S., Platt, J. L., Stone, J. K., Spatafora, J. W., Holst-Jensen, A., Hamelin, R. C. & Kohn, L. M. (2001).** *Phylogenetics of Helotiales and Rhytismatales based on partial small subunit nuclear ribosomal DNA sequences.* Mycologia 93, 915-933.

**Hall, B. G. (2004).** *Phylogenetic Trees Made Easy. A How-To Manual.* Second Edition.

**Hall, T. A. (1999).** *BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* BioEdit. Nucleic Acids Symposium Series 41, 95-98.

**Hambleton, S., Tsuneda, A. & Currah, R. S. (2003).** *Comparative morphology and phylogenetic placement of two microsclerotial black fungi from Sphagnum.* Mycologia 95(5), 959-975.

**Hazsl., M. T. (1881).** *Corynetes.* Természettud. Köréből Magyar Tud. Akad. Érték. 11(19), 7, [index].

**Huelsenbeck, J. P. & Ronquist, F. (2001).** *MRBAYES: Bayesian inference of phylogenetic trees.* Bioinformatics 17(8), 754-755.

- Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001).** *Bayesian Inference of Phylogeny and Its Impact on Evolutionary Biology*. Science 294, 2310-2314.
- Imai, S. (1934).** *Studies on the Geoglossaceae of Japan*. Trans. Sapporo Nat. Hist. Soc. 13, 179-184.
- Imai, S. (1940).** *The Geoglossaceae of Norway*. Ann. Myc. 38, 268-278.
- Imai, S. (1955).** *Ochroglossum*. Science Rep. Yokohama Nat. Univ., Section 2 (4), 6
- Imai, S. (1956).** *Contributiones ad studia monographica Geoglossacearum. III Studia classificationis phylogenicarum*. Sci. Rep. Yokohama Nat. Univ. 2(5), 1-8.
- Karsten, P. (1885).** *Microglossum atropurpureum*. Acta soc. Fauna Flora Fenn. 2(6), 110.
- Kirk, P.M., Cannon, P.F., David, J.C. & Stalpers J.A. (Eds). (2001).** *Ainsworth & Bisby's Dictionary of the Fungi*. 9th Ed. - CAB International.
- Korf, R. P. (1958).** *Japanese discomycete notes I-VIII*. – Science Reports of the Yohohama National University, Section II Biological and Geological Sciences 7, 7-35.
- Korf, R. P. (1971).** *Some new discomycete names*. Phytologia 21, 201-207.
- Korf, R. P. (1973).** *Discomycetes and Tuberales*. In Ainsworth GC, Sparrow FK, Sussmann AS (eds) *The Fungi – An advanced treatise*, 4A, pp 249-319. Academic press New York and London.
- Korf, R. P., Iturriaga, T. & Lizon, P. (1996).** *Proposal to conserve the family name Helotiaceae (Fungi)*. Taxon 45, 683-684.
- Korf, R. P. (1999).** *Pezoloma kathiae sp. nov. (Ascomycetes: Leotiales, Leotiaceae) and its placement in a new subgenus Phaeopezoloma*. Mycotaxon 73, 493-497.



**Korf, R. P. & Lizon, P. (2000).** *Validation of Nannfeldt's ordinal name Helotiales.* Mycotaxon LXXV, 501-502.

**Korf, R. P. & Lizon, P. (2001).** *The status of the ordinal name Leotiales.* Czech Mycology 52(4), 255-257.

**Lizon, P., Iturriaga, T., Korf, P. R. (1998).** *A preliminary discomycetes flora of Macaronesia: part 18, Leotiales.* Mycotaxon 67, 73-83.

**Lutzoni, F., Pagel, M. & Reeb, V. (2001).** *Major fungal lineages are derived from lichen symbiotic ancestors.* Nature 411, 937-940.

**Lutzoni, F., Kauff, F., Cox, C. J., McLaughlin, D, Celio, G., Dentinger, B., Padamsee, M., Hibbett, D., James, T. Y., Baloch, E., Grube, M., Reeb, V., Hofstetter, V., Schoch, C., Arnold, A. E., Miadlikowska, J., Spatafora, J., Johnson, D., Hambleton, S., Crockett, M., Shoemaker, R., Sung, G-H., Lücking, R., Lumbsch, T., O'Donnell, K., Binder, M., Diederich, P., Ertz, D., Guedian, C., Hansen, K., Harris, R. C., Hosaka, K., Lim, Y-W., Matheny, B., Nishida, H., Pfister, D., Rogers, J., Rossmann, A., Schmitt, I., Sipman, H., Stone, J., Sugiyama, J., Yahr, R. & Vilgalys, R. (2004).** *Assembling the Fungal Tree of Life: Progress, Classification, and Evolution of Subcellular Traits.* American Journal of Botany 91(10), 1446-1480.

**Læssøe, T. & Elborne, S. A. (1984).** *De danske jordtunger.* Svampe 9, 9-22.

**Maas Geesteranus, R. A. (1964).** *On some white-spored Geoglossaceae.* Persoonia 2(3), 81-96.

**Maas Geesteranus, R. A. (1965).** *Geoglossaceae of India and adjacent countries.* Persoonia 4(1), 19-46.

**Maas Geesteranus, R. A. (1966).** *On Helvella Platypus DC.* Koninkl. Nederl. Akademie van Wetenschappen, Amsterdam, Proc., Ser. C. 69, 191-203.

**Maddison, D. R., Maddison, W. P. (2001).** *MacClade 4: Analysis of phylogeny and character evolution.* Sinauer Associates, Sunderland, Massachusetts.

**Mains, E. B. (1954).** *North American species of Geoglossum and Trichoglossum.* Mycologia 46, 586-631.

**Mains, E. B. (1955).** *North American hyaline-spored species of the Geoglossaceae.* Mycologia 47, 846-877.

**Mains, E. B. (1956).** *North American species of the Geoglossaceae. Tribe Cudonieae.* Mycologia 48, 694-710.

**Miadlikowska, J. & Lutzoni, F. (2004).** *Phylogenetic classification of peltigeralean Fungi (Peltigerales, Ascomycota) based on Ribosomal RNA small and large subunits.* American Journal of Botany 91(3), 449-464.

**Murray, M. G. & Thompson, W. F. (1980).** *Rapid isolation of high molecular weight plant DNA.* Nucleic Acids Research 8, 4321-4325.

**Nannfeldt, J. A. (1932).** *Studien über die Morphologie und Systematik der nicht-lichenisierten inoperculaten Discomyceten.* N. Acta Reg. Soc. Sci. Upsal., Ser. IV, vol. 8, N:o 2.

**Nannfeldt, J. A. (1942).** *The Geoglossaceae of Sweden. (With regard also to adjacent countries).* Ark. Bot. 30A 4, 1-67.

**Nitare, J. (1982).** *Geoglossum arenarium, en problematisk jordtunga.* Göteborgs Svampklubbs Årsskrift 1981, 61-68.

**Nitare, J. (1983).** *Geoglossum hakelieri, ett nytt namn för G. fumosum Hakelier.* Windahlia 1982-83, 81-88.

**Nitare, J. (1984).** *Geoglossum asaeptatum n. sp., a new earthtongue with brown continuous spores.* Windahlia 14, 37-42.

**Nitare, J. & Ryman, S. (1984).** *Jordtungor av släktet Microglossum i Sverige*. Svensk Bot. Tidskr. 78, 63-69.

**Ohenoja, E. (2000).** *Geoglossaceae*. In *Nordic macromycetes*, vol 1, 177-183. Edited by L. Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Platt, J. L. & Spatafora, J. W. (1999).** *A re-examination of generic concepts of baeomycetoid lichens based on phylogenetic analyses of nuclear SSU and LSU ribosomal DNA*. Lichenologist 31(5), 409-418.

**Posada, D. & Crandall, K. A. (1998).** *Modeltest: testing the model of DNA substitution*. Bioinformatics 14, 817-818.

**Ronquist, F. & Huelsenbeck, J. P. (2003).** *MrBayes 3: Bayesian phylogenetic inference under mixed models*. Bioinformatics 19(12), 1572-1574.

**Schmitt, I., Lumbsch, H. T. & Söchting, U. (2003).** *Phylogeny of the lichen genus Placopsis and its allies based on Bayesian analyses of nuclear and mitochondrial sequences*. Mycologia 95(5), 827-835.

**Schumacher, T & Sivertsen S. (1987).** *Sarcoleotia globosa (Sommerf.: FR.) Korf, Taxonomy, Ecology and Distribution*. Arctic and Alpine Mycology II, 163-176.

**Schumacher, T. (2000).** *Piceomphale*. In *Nordic macromycetes*, vol 1, 159. Edited by L. Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Simmons, M. P. & Ochoterena, H. (2000).** *Gaps as Characters in Sequence-Based Phylogenetic Analyses*. Syst. Biol. 49(2), 369-381.

**Swofford, D.L. (2003).** *PAUP\*. Phylogenetic Analysis Using Parsimony (\*and other methods)*. Version 4. Sinauer Associates, Sunderland, Massachusetts.

**Tehler, A., Little, D. P. & Farris, J. S. (2003).** *The full-length phylogenetic tree from 1551 ribosomal sequences of chitinous fungi, Fungi.* Mycological Research 107(8), 901-916.

**Verkley G. J. M. (1994).** *Ultrastructure of the ascus apical apparatus in Leotia lubrica and some Geoglossaceae (Leotiales, Ascomycotina).* Persoonia. (Published by Rijksherbarium/Hortus Botanicus, Leiden) Volume 15(4), 405-430.

**Vesterholt, J. (2000).** *Hymenoscyphus.* In Nordic macromycetes, vol 1, 152-156. Edited by L. Hansen & H. Knudsen. Copenhagen, Nordsvamp.

**Vrålstad, T., Myhre, E. & Schumacher, T. (2001).** *ITS-sequence diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in disturbed boreal forests.* In the Ph.D. Thesis of Trude Vrålstad. University of Oslo.

**Wang Z., Binder M. & Hibbett D. S. (2002).** *A new species of Cudonia based on morphological and molecular data.* Mycologia 94 (4), 641-650.

**Wang Z., Binder M. & Hibbett D. S. (2005).** *Life history and systematics of the aquatic discomycete Mitrula (Helotiales, Ascomycota) based on cultural, morphological, and molecular studies.* American Journal of Botany 92(9), 1565-1574.

**White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990).** *Amplification and direct sequencing of ribosomal RNA genes for phylogenetics.* In: Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. eds. PCR Protocols, a Guide to Methods and Applications. San Diego, California, Academic Press, 315-322.

**Zhong Z. & Pfister D. H. (2004).** *Phylogenetic relationship among species of Leotia (Leotiales) based on ITS and RPB2 sequences.* Mycological Progress 3 (3), 237-246.