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Cover image — *Thuemenidium arenarium* and *Empetrum nigrum* on sand dune near the seashore by Jersey Trail, Newfoundland and Labrador, Canada, taken by Andrus Voitk. See article by Ohenoja et al. in this issue.

Northern species of earth tongue genus *Thuemenidium* revisited, considering morphology, ecology and molecular phylogeny

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Abstract: *Thuemenidium* is a small earth tongue genus with three recognized white-spored species. Within *Thuemenidium*, *T. atropurpureum* and *T. arenarium* have been reported only from the northern hemisphere while *T. berteroi* is known solely in the southern hemisphere. We reviewed the ecology, examined the morphology and inferred the systematic positions of northern species of *Thuemenidium* from LSU-rDNA gene phylogeny of 48 taxa in Pezizomycotina including recent collections. Our results suggest that *Thuemenidium* in its current sense is polyphyletic and that *T. atropurpureum*, closely related to *Microglossum* and *Leotia* species, is a member of Leotiaceae (Helotiales, Leotiomyces). Our phylogeny placed the other northern species, *T. arenarium*, in Geoglossaceae (Geoglossales, Geoglossomycetes), retaining genus *Thuemenidium*, pending further investigation.

Key words: ecology, Geoglossomycetes, Leotiomyces, phylogeny, *Thuemenidium*

INTRODUCTION

Early morphological classification of ascomycetes assigned terrestrial species with club-like fruiting bodies, commonly known as earth tongues, to *Geoglossum* (Persoon 1794). Since then the taxonomy of the earth tongues has been tempestuous. Many additional genera have come and gone. Myriad interpretations have been advanced for the genera

(Imai 1941, Maas Geesteranus 1964, Korf 1973, Pfister and Kimbrough 2001, Spooner 1987, Wang et al. 2006, Schoch et al. 2009b). Part of the difficulty stems from an attempt to erect a taxonomic ranking based on morphology for organisms whose key characters for defining genera, such as color, size and septation of ascospores, change with age (Spooner 1987). Diversity in ecology usually has been documented with little detail for many earth tongue fungi, leading to a lack of consideration as to its impact on classification, until very recently (Wang et al. 2006, Schoch et al. 2009a).

Saccardo (1884) split earth tongues on the basis of spore color, transferring white-spored species to *Microglossum*, and Kuntze (1891) further proposed a new genus, *Thuemenidium*, in honor of the Austrian mycologist Baron Felix von Thümen, for species of *Microglossum* with dark fruiting bodies. Five names for four species, *T. arenarium* (Rostr.) Korf, *T. atropurpureum* (Batsch) Kuntze, *T. berteroi* (Mont.) Gamundi, and *T. hookeri* (Cooke) Kuntze and *T. multifforme* (Henn.) Kuntze (= *T. hookeri*), had been used (<http://www.indexfungorum.org>), but the taxonomy of the four species is not straightforward. The type species, *T. hookeri*, was described on the basis of a single dried specimen from an unrecorded locality sent to Hookerian Herbarium of Kew Gardens. Neither fresh nor other dried specimens of the species were available at the time, and the species has never been identified. Masee (1897) attributed the matter to a misinterpretation of the original microscopic findings and thought that the specimen described as *Thuemenidium hookeri* is what we now know as *Thuemenidium atropurpureum*, an opinion shared by Durand (1908) and Nitare (pers comm 2009). This explanation however has not been universally accepted (<http://www.indexfungorum.org>). The other difficulty with the species is a controversy concerning the synonymization of *T. arenarium* and *T. atropurpureum* by Seaver (1951) and Cannon et al. (1985). Those authorities who do recognize the two as separate species do not always accept genus *Thuemenidium*; for example Lumbsch and Huhndorf (<http://www8.umu.se/myconet>) kept *T. arenarium* in *Geoglossum* (Note 271), based on the work of Nitare (1982), and assigned *T. atropurpureum* to *Microglossum* (Note 270) on the basis of the work of Wang et al. (2006). Index fungorum (<http://www.indexfungorum.org>), Nordic Macromycetes (Ohe-

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noja 2000), Dictionary of the Fungi (Kirk et al. 2008) and many other authorities support the assignment, retention or reassignment of all *Thuemenidium* species to *Geoglossum*. Despite a potentially straightforward construction of *Thuemenidium* based solely on morphology, the validity of its type species is doubtful, the separation of *T. arenarium* and *T. atropurpureum* in the genus has been invalidated by some workers, and the genus itself has not gained universal acceptance (Kirk et al. 2008).

The ability to determine phylogenetic ranking with DNA sequence data has introduced additional taxonomic unrest to mycology, but over the years a new sense of the validity of diverse morphological characters has begun to develop based on new rankings of fungi (Hibbett et al. 2007). An understanding is growing that some traditional taxonomic relationships defined by morphology appear to lack a singularly evolved genetic basis, while some potential morphological groupings thought to be divergently evolved seem to share unexpected genetic propinquity. Sorting out the rates of evolutionary change of fungal morphological traits has been key to recent advances. Those morphological traits evolving at the right rate (not too fast or too slow; Townsend 2007) also will be the traits that best define taxonomic groupings at various levels. Thus, instead of further complicating things, genetic sequencing sometimes has introduced a semblance of clarity into areas where confusion had remained because of unrecognized variability of the morphological characteristics, such as in Helotiales (Schoch et al. 2009a). In many instances it appears that confusion had arisen because genetically labile morphologic features were stressed and more conserved ones downplayed. The advent of genetic analysis has let us select and stress more accurately morphological characteristics that are evolving at appropriate rates to define coherent taxonomic groupings. For instance it has been demonstrated that two morphologic characteristics (the presence of hyaline spores and the presence of a distinct hymenial boundary) have a phylogenetic basis in earth tongue fungi, which now are recognized as at least two lineages, including a sole family in Geoglossomycetes (Schoch et al. 2009b) as well as Rhytismales and Leotiales in Leotiomycetes.

Of the four *Thuemenidium* species only *T. arenarium* and *T. atropurpureum* are known to occur in the northern hemisphere. *T. atropurpureum* is a grassland species. It is fairly common across the continent and is probably a saprotroph, although its ecology has not been determined with certainty. Durand (1908) mentioned distribution of *T. arenarium* in North America from Labrador and Newfoundland, and later it was recorded from Rankin Inlet and Baker Lake,

North West Territories, Canada, in 1971 and 1974 (Ohenoja 2010), and from Great Whale River area, Québec (Huhtinen 1985). In northern Scandinavia, where *T. arenarium* is more common, it grows in sand dunes near the seacoast, often with *Clavaria argillacea* Fr. (Ohenoja 1976, 2000), and has mycorrhizal relationships with *Empetrum nigrum* L. (Nitare, 1982). However association between *T. arenarium* and *E. nigrum* is neither ubiquitous nor strict; the plant has a much wider distribution in geography and ecology than the fungus. *E. nigrum*, also known as black crowberry, is a perennial shrub in order Ericales, of which many species live in mycorrhizal association with various fungi, and has been regarded as threatened or endangered in northern USA (<http://plants.usda.gov>). The plant has no significant commercial value, although its roots have been used as an eye medicine (Moerman 1998).

In this study we examined collections of *T. arenarium* from Labrador, Canada and Finland; and *T. atropurpureum* from Finland, Québec, Canada, and Northern Ireland for morphology. We also performed genetic sequencing and reviewed species ecology to (a) evaluate the question of possible synonymy of *T. arenarium* and *T. atropurpureum*, (b) assign both to a respective group, and (c) speculate on the validity of *Thuemenidium* as a genus.

MATERIALS AND METHODS

Taxon sampling.—*Thuemenidium* was studied from the Herbarium at the University of Oulu, Finland (OULU), the Fungarium of le Cercle des mycologues de Montréal (CMM), and from 2005 and 2008 forays conducted by Foray Newfoundland & Labrador and kept in the Herbarium of Gros Morne National Park, where morphology was assessed and molecular data were collected. A LSU-rDNA sequence data matrix was constructed containing 48 taxa of Pezizomycotina including 35 from Leotiomycetes and five from Geoglossomycetes. Species of Pezizales have been suggested to manifest a basal position in Pezizomycotina (Schoch et al. 2009a) and therefore were included to provide a root for trees.

Molecular techniques.—DNA was isolated from dried herbarium material following protocols of Wang et al. (2005). The LSU-rDNA region, bounded by primers LR0R and LR5, and the ITS1-5.8S rDNA-ITS2 region, bounded by primers ITS1F and ITS4, were successfully amplified from two isolates of *T. arenarium* at Yale University. The sequences are available from GenBank (GU324764, 324765, 324766, 324767). We used sequence data generated in Wang et al. (2006).

Phylogenetic analyses.—Sequences were aligned with Clustal X using the default setting (Thompson et al. 1997) and further adjusted by eye in the data editor of PAUP* 4.0b (Swofford 1999). Introns were deleted and ambiguously

aligned positions were excluded from the datasets before performing analyses. All datasets were analyzed in PAUP* 4.0b and MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001) with gaps treated as missing data. Parsimony analyses were performed with equal weighting of characters and transformations. Heuristic searches were performed with 1000 replicate searches, each with one random taxon addition sequence, MAXTREES set to auto-increase, and TBR branch swapping. Robustness of individual branches was estimated by maximum parsimony bootstrap proportions (BP) based on 500 bootstrap replicates, each consisting of a single heuristic search with 50 random taxon addition sequences, MAXTREES set to auto-increase, and TBR branch swapping. Bayesian phylogenetic analyses were performed with the Metropolis-coupled Markov chain Monte Carlo method (MCMCMC) under the GTR + Γ + I model in MrBayes 3.1.1 by running four chains for 2 000 000 generations. Trees were sampled every 100th generation. Likelihoods converged to a stable value after ca. 500 000 generations in the analysis, and all trees obtained prior to convergence were discarded before computing a consensus tree in PAUP. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. Clades with PP higher than 0.95 and BP higher than 80% were considered to be significantly supported (FIG. 1).

RESULTS

BRIEF MORPHOLOGY

Thuemenidium arenarium (Rostr.) Korf in Petersen and Korf, *Nordic JI Bot.*, 2:152, 1982.

Microglossum arenarium Rostr., *Med. Grönl.*, 3:606, 1892.

Corynetes arenarius (Rostr.) E. J. Durand, *Ann. Myc.*, 6:417, 1908.

Geoglossum arenarium (Rostr.) Lloyd, *Mycol. Notes*, 5, 1916.

Dry ascocarps brownish black to black, fertile head darker but not distinct from the stalk, club-shaped to spatulate, up to 35–40 mm high. Hymenium surface smooth while stalk surface furfuraceous. Asci J+, eight-spored, thin-walled, club-shaped, 165–180 \times 15–17 μ m. Paraphyses much longer than asci, well separated from each other, brown, curved and somewhat enlarged at apex, septate, branched, 3–6 μ m diam. Ascospores hyaline, thin-walled, smooth, cylindrical with one end slightly larger, (27–)31–34(–36) \times (4–)4.5–5.5(–6.0) μ m, rarely septate when mature.

Habitat. Dry, open, sandy heaths on dune areas or on shores. Associated with *Empetrum nigrum* and at times with *Clavaria argillacea*.

Phenology. Aug–Oct.

Distribution. Boreal-montane and arctic-alpine, occurs in Europe and N. America.

Specimens examined. Finland, Kalajoki, dry dune field along seashore, around *Empetrum nigrum* mats, 14-X-2007, OULU F77201; Labrador, Canada, Labrador Straits, near Forteau, sand dune near First Pond Trail, 8-IX-2005; sand dune by Jersey Trail, 9-IX-2005 (9335); sand dune near seashore close to Anse Amour, 16-VIII-2008.

Thuemenidium atropurpureum (Pers.) Kuntze, *Rev. Gen. Pl.*, 2:873, 1891.

Geoglossum purpurascens Pers. In Holmsk., *Coryph.*, 171, 1797.

Leotia atropurpurea (Pers.) Corda, *Ic. Fung.*, 5:79, 1842.

Microglossum atropurpureum (Pers.) Sacc., *Syll. Fung.*, 8:40, 1889.

= *Geoglossum atropurpureum* Cooke, *Mycographia*, 10, 1875.

Dry ascocarps brownish black to black, fertile head distinct from the stalk, club-shaped, up to 50 mm high. Hymenium surface smooth while stalk surface furfuraceous. Asci J+, mostly eight-spored, thin-walled, club-shaped, 90–130 \times 13–15 μ m. Paraphyses regular, slightly longer than asci in the hymenium, thin-walled, hyaline, no septa observed at apex; cell wall surface covered with amorphous purplish brown to brownish matter that unites paraphyses into an epithecium, 3 μ m diam. Ascospores hyaline, thin-walled, smooth, almost cylindrical or slightly curved, with rounded ends, (16–)23–33(–41) \times (3.8–)4.0–5.0 μ m, mostly three- to six-septate when matured.

Habitat. Grassy meadows and pastured forests, both on calcareous and acid soil.

Phenology. Sep–Oct to late Dec.

Distribution. The fungus is known to occur in North America, Europe and Macaronesia. In many countries of Europe it is considered a threatened fungus. It is used as an indicator species of meadows and pastured forests (Ohenoja 1995).

Specimens examined. Finland, Leivonmäki, on rock shelf covered with *Dicranum polysetum* Swartz, 26-VIII-1990, OULU F71890; Northern Ireland, County Down, on dune grassland, 5-XI-1998, 1100803 (J64235728), K(M)59206; Northern Ireland, Antrim, acidic upland, on grassland, 9-X-2001, 1136126 (D095515); Canada, Knowlton, 19-IX-1990, CMMF1235.

PHYLOGENETIC ANALYSES

Molecular inference from the LSU-rDNA dataset.— Relationships of the two northern species of *Thuemenidium* were investigated with LSU-rDNA (FIG. 1). The data matrix had an aligned length of 566 base pairs, including 257 parsimony informative positions and 87 uninformative variable positions. Bayesian and parsimony analyses on this dataset provided nearly identical topologies regardless of different measurements of the branch length, and the higher-level LSU-rDNA topology was congruent with the topology published recently with multilocus data (Schoch et al. 2009a). Two isolates of *T. atropurpureum* formed a clade (BP = 100%, PP = 1.0) with *Microglossum rufum* (Schwein.) Underw. in the well supported Leotia-Microglossum clade (BP = 98%, PP = 1.0). Two isolates of *T. arenarium* formed a clade (BP = 100%, PP = 1.0) in the Geoglossomycete

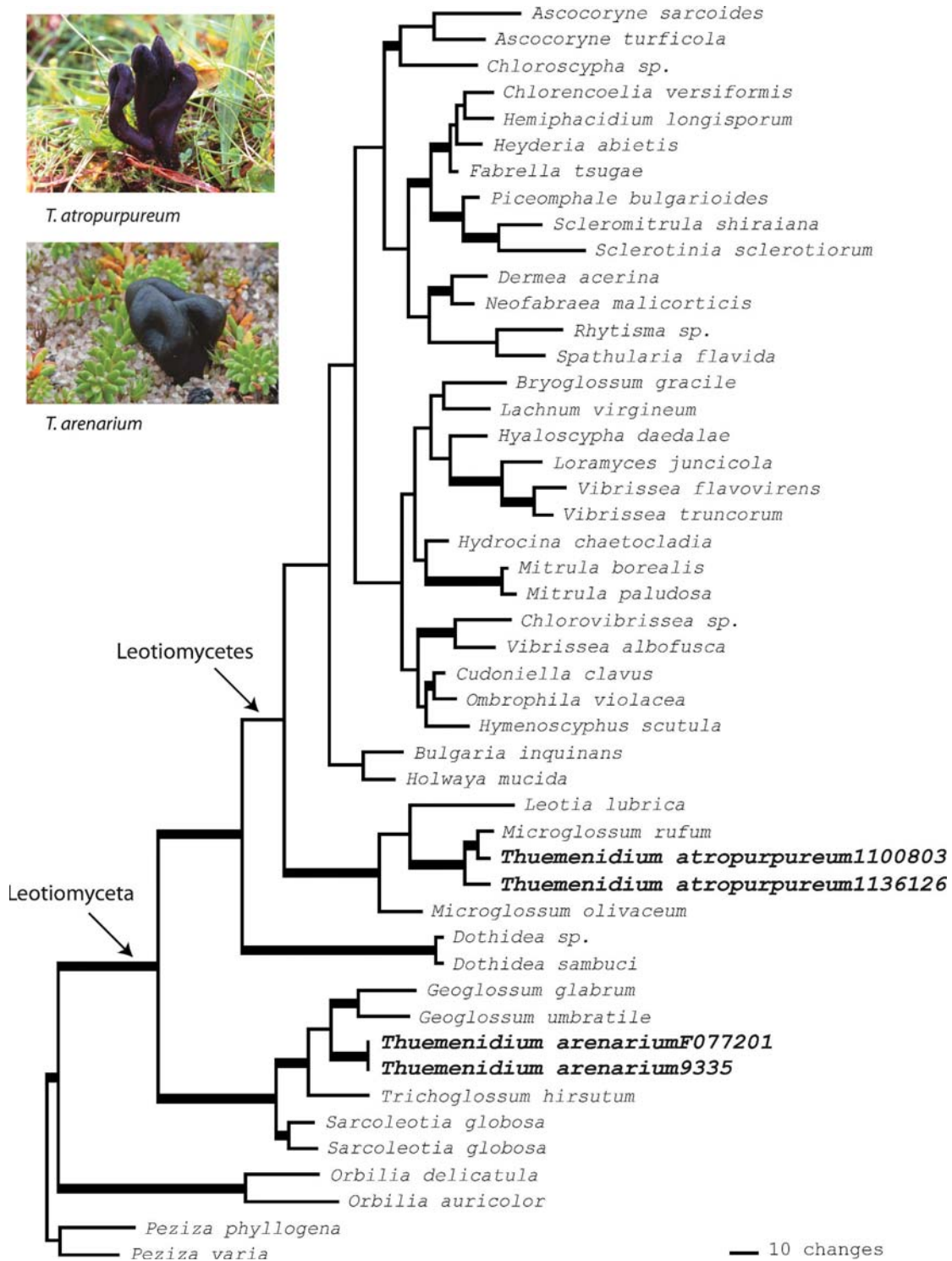


FIG. 1. Phylogenetic relationships of *Thuemenidium*. Parsimony and Bayesian analysis based on the LSU-rDNA sequences. One most parsimonious tree (length = 1247, CI = 0.425, RI = 0.614). Bootstrap values greater than 50% and posterior probability higher than 0.95 are indicated as bold branches. Inset: habitat pictures of *T. atropurpureum* (kindly provided by Roy Anderson) and *T. arenarium*.

clade (BP = 95%, PP = 1.0). A clade of *Geoglossum glabrum* Pers. and *G. umbratile* Sacc. (BP = 99%, PP = 1.0) was the sister group of *T. arenarium* without support in the Trichoglossum-Geoglossum clade (BP = 80%, PP = 1.0).

DISCUSSION

We have met our original goals with variable degrees of certainty: (i) Our studies demonstrate unambiguously that *T. atropurpureum* and *T. arenarium* are distinct and separate species. (ii) With respect to assigning them to appropriate groups, our work, combined with studies cited, support reassigning *T. atropurpureum* to the Leotia-Microglossum clade in Helotiales. We are less certain about the correct placement of *T. arenarium*; more robust phylogeny of the Geoglossum-Trichoglossum clade with proper sampling of type species is required to either confirm it as *G. arenarium* or suggest a new ranking. Pending further investigation, we leave it in *Thuemenidium* as a practical solution. (iii) Our results lacked the robustness and latitudinal scope to definitively confirm or reject *Thuemenidium* as a genus. Under these circumstances it seems most prudent to accept it pro tempore. If an examination of the type specimen of *T. hookeri* does confirm its synonymy with *T. atropurpureum* and if the latter's reassignment to *Microglossum* is established, *Thuemenidium* will be in the awkward position of having an extrageneric type species with *T. arenarium* as the sole species left in this now depauperate genus. Our preliminary analysis placed *T. arenarium* in the Geoglossomycete clade, raising the possibility that further work could lead to its reranking as a species of *Geoglossum* or a new genus, thus eliminating the need for *Thuemenidium* altogether.

Our examination of morphology in *Thuemenidium arenarium* and *T. atropurpureum* suggested that these fungi are distinct. However no key characters have been identified to link either confidently to *Microglossum* or *Geoglossum*. Morphological differences, especially in paraphyses, between the two northern *Thuemenidium* species have been identified by Dennis (1968) and Nannfeldt (1942). Also we observed that *T. arenarium* has larger asci and ascospores that are rarely septate even after release from the ascus. However classification of these two fungi based on morphology has been controversial because it is partially evident by the synonyms of these two species in *Microglossum* and *Geoglossum*. With doubt about Batsch's identification of *Corynetes arenarius* (Batsch 1783) we followed Seaver (1951) citing the synonyms here. Many mycologists argue that colorless ascospores alone should not separate *Thuemenidium* from *Geoglossum*, especially because some *Geoglossum* spe-

cies produce spores that exhibit an extended duration of hyaline coloration during development. Maas Geesteranus (1964) noted that the inflated longitudinal hyphae with narrower branches that compose a dense network in the stalk of *T. atropurpureum* were not found among other white-spored Geoglossaceae species, and he proposed to recognize *Thuemenidium* at the genus level as a nomenclatural solution, instead of as a taxonomic suggestion. For that reason Maas Geesteranus did not transfer *Corynetes arenarius* to *Thuemenidium*. He also pointed out that the hyphal structure in the stipe of *T. arenarium* is somewhat different from that of *T. atropurpureum*. The characteristic inflated hyphae in the stalk of *T. atropurpureum* probably are seen only in fresh specimens; in this study we did not see such structures in dried specimens of *T. atropurpureum* or *T. arenarium*.

Thuemenidium is distinguished from white-spored *Microglossum* by having dark apothecia, while those of species of *Microglossum* can be yellow, brown, green or dark green (Mains 1955). However favoring apothecial color over spore color in the classification of *Thuemenidium* is probably equally controversial. Because ascoma development is hemiangiocarpous in *Microglossum viride* but gymnocarpous in *Geoglossum* and *Trichoglossum* species some workers have suggested it as a criterion for placement of *Thuemenidium* (Nannfeldt 1942). Recent molecular phylogenies suggest that *Sarcoleotia*, *Geoglossum* and *Trichoglossum* species share a similar development type and are distantly related to other earth tongues with a distinct hymenial border (Wang et al. 2006). Unfortunately *Thuemenidium* specimens in early stages of development were not available for this study.

The ecology of earth tongue fungi in *Geoglossum*, *Trichoglossum*, *Microglossum* and *Thuemenidium* was once considered homogenous, not only because all were found commonly in more or less damp lawns or pastureland (Nannfeldt 1942) but also because the ecology of these fungi, indeed of most Leotiomycetes, has been both understudied and overlooked for a long time. Although there is no available hard evidence many species of *Geoglossum* and *Trichoglossum* are believed to be associated in some way with bryophytes. The ecology of *T. arenarium* is unique because it grows in sand dunes near the seacoast. Of note it often grows with *Clavaria argillacea* (Ohenoja 1995, 2000) and has been confirmed to form mycorrhizae with the black crowberry *Empetrum nigrum* (Nitare 1982). In contrast *T. atropurpureum* usually is collected from acidic grasslands where diverse mosses are common. So far no relationships between *T. atropurpureum* and specific mosses have been proposed. Lumbsch and Huhndorf kept *T. arenarium* in *Geoglossum* following Nitare (1982) and assigned only

T. atropurpureum to *Microglossum* on the basis of molecular evidence (<http://www8.umu.se/myconet>).

Molecular phylogenies with intensive samplings from the Leotiomycetes were rare until very recently with the development of the Assembling Fungal Tree of Life project. Only two sequences of LSU-rDNA of *T. atropurpureum* were deposited in GenBank before this study. This is the first study of the molecular phylogenetic relationships of the two northern *Thuemenidium* species in relation to all genera of other earth tongue fungi and present or previous Geoglossaceae in Pezizomycetes. The LSU-rDNA tree that we constructed had strong statistical support suggesting that (i) *T. atropurpureum* is closely related to *Microglossum rufum* and (ii) *T. arenarium* shares a clade with *Trichoglossum* and *Geoglossum*; these relationships are consistent with the ecological differences observed in previous studies. However the rDNA phylogenies in both the Leotia-Microglossum clade and the Geoglossum-Trichoglossum clade were not fully resolved, making it premature to propose a formal reclassification for these *Thuemenidium* species.

Although we did not answer all our questions definitively, this investigation did raise several interesting questions for further study. We did not have sufficiently fresh specimens of *T. berteroi* to allow reliable molecular studies, and morphologic re-examination of recently collected *Thuemenidium* species from New Zealand unfortunately showed them to be species of *Microglossum* (Peter Johnston pers comm). Based on Gamundi (1977) and Spooner (1987), *T. berteroi* is lignicolous, a saprotroph like *T. atropurpureum*, and resembles *T. atropurpureum* morphologically. We suspect that further investigations will reassign *T. berteroi* to the Leotia-Microglossum clade in Helotiales, alongside *T. atropurpureum*.

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