Inclusion of *Nothomitra* in Geoglossomycetes

Hustad VP\(^1\)\(^2\)\(^*,\) Miller AN\(^2\), Moingeon J-M\(^3\) and Priou J-P\(^4\)

\(^1\)Department of Plant Biology, University of Illinois at Urbana-Champaign, 505 S. Goodwin Ave., Urbana, IL 61801
\(^2\)Illinois Natural History Survey, University of Illinois, 1816 S. Oak St., Champaign, IL 61820
\(^3\)28 Grande Rue, 25520 Goux-les-Usiers, France
\(^4\)7 Rue De Picardie, F-56200 La Gacilly, France


*Nothomitra* is a small genus of earth tongues consisting of three species. Historically placed within the Geoglossaceae *sensu lato*, the genus is currently considered *incertae sedis* within the Helotiales. We reviewed the morphology and analyzed the phylogenetic relationships of *Nothomitra* using a combined dataset of ITS, LSU and Mcm7 DNA sequences representing 22 species. The placement of *Nothomitra* was strongly supported within the Geoglossomycetes clade, forming part of the ancestral base of the class with *Sarcoleotia globosa* and *Thuemenidium arenarium*. The inclusion of *Nothomitra* within the Geoglossomycetes is confirmed.

**Key words** – Ascomycota – earth tongues – Geoglossaceae – Leotiomycetes – phylogeny

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*Corresponding author: Vincent Hustad – e-mail – vhustad@illinois.edu

**Introduction**

Earth tongues are among the most widely distributed groups of fungi on earth and have been a subject of mycological inquiry since Persoon first described *Geoglossum* in the late 18\(^{th}\) century. Genera typically referred to as earth tongues include *Geoglossum*, *Trichoglossum*, *Microglossum*, *Leotia*, and *Spathularia*. During the last 200 years, numerous genera and species have been included and removed from this group based primarily on morphological data. Recent molecular studies (Pfister and Kimbrough 2001, Wang et al. 2006a and b, Schoch et al. 2009, Ohenoja et al. 2010) have suggested earth tongues are not a monophyletic group and this resulted in the introduction of the class Geoglossomycetes (Schoch et al. 2009), which contains four genera and approximately 50 species. Currently included within the Geoglossomycetes are *Geoglossum* (22 species), *Sarcoleotia* (4 species), *Thuemenidium* (5 species), and *Trichoglossum* (19 species) (Kirk et al. 2008). However, several genera formerly included within the Geoglossaceae *sensu lato* are currently considered *incertae sedis* and the placement of these taxa within the Pezizomycotina is unknown.

The monotypic genus *Nothomitra* was introduced by Maas Geesteranus (1964) to accommodate *N. cinnamomea* Maas Geest., which was described from specimens collected in Upper Austria during the autumn of 1962. Three species are accepted in the current concept of the genus following the additions of *Nothomitra kovalii* Raitviir (1971) from Kunashir in the Kuril Islands and *Nothomitra sinensis* Zhuang and Wang (1997) from China. At present, *Nothomitra* is only known to occur in Europe and Asia, though extensive distribution data is lacking. All species in *Nothomitra* are terrestrial with *N. cinnamomea* reported growing amongst *Sphagnum*, *N. kovalii* reported from rocky soil, and *N.
sinensis reported from mossy soil in coniferous forests. Nothomitra is found across a wide range of altitudes. N. cinnamomea is recorded from the European Alps from 670 to 1100 m elevation, Nothomitra kovalii is found between 400-800 m elevation on Mt. Mendeleyeva in the Kuril Islands, whereas N. sinensis is described from the Qilian Mountains in Northern China at 2850 m elevation.

Nothomitra is characterized by the distinct free edge of the hymenium at the junction of the stipe, unlike Microglossum in which the hymenium intergrades with the stipe on the flattened sides (see Fig 1C). Nothomitra is also differentiated from Microglossum in that the fertile head of the ascocarp is not flattened as in Microglossum, and the internal stipe hyphae of Nothomitra are parallel and easily separable versus the interwoven and agglutinated hyphae found in Microglossum. These morphological differences were cited by Maas Geesteranus (1964) as evidence that Nothomitra is not congeneric with Microglossum. However, Moingeon and Moingeon (2004) argued that these characters were not sufficient to support Nothomitra as a separate genus and advocated the placement of N. cinnamomea into Microglossum, thereby rendering the genus Nothomitra a synonym.

Since the importance of the morphological differences between Nothomitra and Microglossum are disputed as is the taxonomic placement of Nothomitra, it is necessary to evaluate molecular characters in order to determine the phylogenetic relationships of this genus. As such, the purpose of this study is to include Nothomitra in a modern phylogenetic analysis for the first time to determine its placement within the Pezizomycotina and to provide detailed insight into the systematics of the Geoglossomycetes using a multi-gene phylogeny.

Methods
Generation of Molecular Data

Total genomic DNA was extracted from dried ascomata using a QIAGEN DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, California) and gene fragments were PCR amplified and sequenced following the methods outlined in Promputtha and Miller (2010) and Raja et al. (2011). Gene fragments were amplified using the following sets of primers: ITS1 and ITS4 (White et al. 1990) for the internal transcribed spacer (ITS) region of nrDNA; JS1 (Landvik 1996) and LR6 (Vilgalys and Hester 1990) for the partial 28S nuclear ribosomal large subunit (LSU) of nrDNA; 709F and 1348R (Schmitt et al. 2009) for the DNA replication licensing factor MS456 (Mcm7).

These genes were chosen because: a) they provide appropriate resolution at various taxonomic levels (i.e. species to class), b) fungal and ascomycete–specific primers have been developed for these genes, c) a large number of available sequences are available from GenBank because previous researchers (e.g. Wang et al. 2006a and b, Schoch et al. 2009, Ohenoja et al. 2010, Hustad and Miller 2011) have used the nuclear ribosomal genes to effectively reconstruct phylogenies within Geoglossomycetes and neighboring groups, d) based on our preliminary data (Raja et al. 2011), Mcm7 shows promise for reconstruction of accurate species-level to class-level phylogenies, and, e) incorporating both ribosomal and protein–coding genes allows for higher certainty in assessing phylogenetic relationships.

Sequence Alignment and Phylogenetic Analyses

Each generated ITS and LSU sequence fragment was subjected to an individual blast search to verify its identity. Mcm7 sequences were only used from specimens which provided reliable ITS and/or LSU sequences. Sequences were assembled using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, Michigan), optimized by eye and manually corrected when necessary. Alignments of individual genes were created manually by eye in Sequencher 4.9 or using Muscle 3.7 (Edgar 2004) in Seaview 4.2 (Galtier et al. 1996). Individual gene datasets were then analyzed using Gblocks 0.91b (Castresana 2000) to identify and remove ambiguous regions from the alignment.

The Akaike Information Criterion (AIC) (Posada and Buckley 2004) as implemented in jModelTest 0.1.1 (Posada 2008) determined GTR+I+G as the best fit model of evolution for both maximum likelihood and Bayesian inference. Maximum likelihood analyses were performed using PhyML.
Morphology


Ascomata scattered to gregarious occurring in soil, 1–3.3 cm high, hymenium borne on variously-shaped fertile heads, head glabrous, spathulate to obovoid or subglobose with concolorous wavy lobes, pale cinnamon to olivaceous, darkening with age, 3–9 mm broad (Fig 1A, B), hymenium distinctly separated from stipe (Fig 1C), stipe straight or flexuous, terete, tapering towards base, ochraceous above becoming paler toward base, squamulose above, becoming glabrous at base, 0.7–2.4 cm high. Hyphae at center of stipe easily separated, often swollen at the septa, thin-walled and often branched. Hyphae near the periphery of the stipe thin-walled and tightly bundled. Paraphyses filiform, upper cells hyaline, with brownish guttules in lower cells, septate, sometimes branched at apex or base, curved at the apex, slightly longer than asci, 1–1.5 µm wide, expanding to 2–3 µm wide at apex. Asci cylindrical–clavate, with crosiers, inoperculate, apical ring euamyloid, deep blue in IKI, small, not occupying entire apex, 150–180 × 9.5–12.5 µm (Fig 1B), 8–spored, biseriate. Asco- spores fusiform to narrowly obclavate, rounded at apex, acute at base, hyaline, smooth, multi- guttulate, single–celled in ascus, becoming up to 5–septate when mature or old, 35–47 (–55) × 3.5–5.5 (–6) µm (Fig 1D).

Habitat: Growing among Sphagnum and Aulocornium palustre (Hedw.) Schwägr., often accompanying Geoglossum sphagnophilum Ehrenb. September–October.

Distribution: Known from Austria and France.

Anamorph: Unknown.

Material examined – France, Jura, Bellefontaine, September 2001, 1100 m, leg. J.M. Moingeon s.n., ILLS Acc. ANM463; ILLS Acc. ANM538; ILLS Acc. ANM540; October 2001, leg. J.M. Moingeon s.n., ILLS Acc. ANM549.

Phylogenetic analyses

Twenty-two taxa were included in the final analyses (Table 1). Mcm7 data for Microglossum olivaceum and Sarcoleotia globosa were not available. The final data matrix had an aligned length of 2720 base pairs, which was reduced to 2091 after the removal of 629 ambiguous characters by Gblocks. Of the 2091 characters used in the final analyses, 76 were...
constant, 819 were parsimony–uninformative, and 1196 were parsimony informative. The maximum likelihood tree produced from the combined ITS, LSU, and Mcm7 dataset is presented in Fig 2. The topology of Geoglossomycetes is congruent with those produced from similar analyses including Geoglossomycetes taxa (Schoch et al. 2009, Ohenoja et al. 2010, Wang et al. 2011). Two major clades are present and strongly supported in our analyses: the Leotiomycetes clade (BP=100%, PP=1.0) and the Geoglossomycetes clade (BP=100%, PP=1.0). Nothomitra cinnamomea was placed within Geoglossomycetes as a sister taxon to Sarcoleotia globosa with moderate support (BS=78%). Geoglossum occurred as a strongly supported monophyletic group (BP=100%, PP=1.0), whereas Trichoglossum was paraphyletic.

**Discussion**

Our analyses confirm Nothomitra cinnamomea as a strongly supported member of Geoglossomycetes, closely aligned with Sarcoleotia globosa as the most basal members of the class. Morphologically, S. globosa is rather similar to N. cinnamomea (Fig 3). Both species possess a distinct capitate hymenium that is clearly separated from the stipe when mature, but the margin of the hymenium is completely free in N. cinnamomea and completely inrolled in S. globosa. Both species also possess hyaline ascospores that develop 3-5 septa upon maturation. Lastly, both species are terrestrial and collection data suggests that an association with mosses exists in both species (Maas Geesteranus 1964, Schumacher and Silvertsen 1987). These morphological and ecological similarities support the close phylogenetic relationship of N. cinnamomea and S. globosa revealed by the molecular phylogeny (Fig 2).

Another morphological feature that links N. cinnamomea within Geoglossomycetes is that the hyphae at the axis of the stipe are not
Table 1 List of taxa, GenBank and herbarium accession numbers, collections numbers, and locality for specimens used in this study.

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<th>Name</th>
<th>Collection Number</th>
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<th>LSU</th>
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Agglutinated and easily separable, a character commonly seen in Geoglossomycetes. Maas Geesteranus (1964) cited this character in his original proposal to separate Nothomitra from Microglossum, and this character appears to be one of the few conserved characters throughout the class. As in previous molecular based phylogenies (Wang et al. 2006a and b, Schoch et al. 2009, Ohenoja et al. 2010), Microglossum olivaceum and Thuemenidium atropurpureum were shown to occur in the Leotiomycetes. Both Microglossum and Thuemenidium possess hyaline ascospores but this character is not sufficient to exclude these genera from Geoglossomycetes since several Geoglossum species possess hyaline ascospores. Microglossum can be delineated from Geoglossomycetes based on its ascomata that range from brightly colored to brown. Thuemenidium is a polyphyletic genus composed of at least two disparate species, T. arenarium, which belongs in Geoglosso
Fig 2 – Maximum likelihood phylogeny of Geoglossomycetes based on a combined dataset (2091 bp) of ITS, LSU, and Mcm7 DNA sequences representing 22 taxa using PhyML ((-ln)L score = 13700). Thickened branches indicate significant Bayesian posterior probabilities (≥95%); numbers refer to PhyML bootstrap support values ≥70% based on 1000 replicates. *Neurospora crassa* and the Leotiomycetes were used as outgroup taxa.

Fig 3 – *Sarcoleotia globosa*. Arrow indicates distinct separation of fertile head and stipe.
mycetes, and T. atropurpureum, shown by this study and Ohenoja et al. (2010) to belong in Leotiomycetes. Thuemenidium atropurpureum produces ascomata ranging from brown to purplish black, whereas T. arenarium does not possess any purplish coloration.

The Geoglossomycetes are an early-diverging lineage appearing on a long branch within the Ascomycota and further molecular research is needed in the group to construct a comprehensive phylogeny of the class. Several genera have historically been associated within this group which are now considered incertae sedis (e.g. Hemiglossum Pat., Leucoglossum Imai, and Maasoglossum Thind and Sharma), and representatives from these genera need to be examined using molecular phylogenies to fully understand their place within the Pezizomycotina. Moreover, several species complexes are likely present within the group and Australasian lineages appear to have origins entirely separate from Northern Hemisphere counterparts (Wang et al. 2011). Further molecular data are also needed to provide accurate reference sequences for environmental sampling as ongoing efforts in this field may shed some light on the enigmatic host associations within Geoglossomycetes.

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