
Inclusion of *Nothomitra* in Geoglossomycetes

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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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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 18th 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

(Guindon and Gascuel 2003) under the GTR substitution model with six rate classes and invariable sites optimized. A BioNJ starting tree was constructed and the best of nearest neighbor interchange (NNI) and subtree pruning and regrafting (SPR) tree improvement was implemented. Bootstrap support (Felsenstein 1985) (BS) was determined with 100 bootstrap replicates. Clades with $\geq 70\%$ BS were considered significant and highly supported (Hillis and Bull 1993).

Bayesian inference employing a Markov Chain Monte Carlo (MCMC) algorithm was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as an additional means of assessing branch support. The GTR+I+G model with six rate classes was employed. Four independent chains of MCMC were run for 10 million generations to insure that trees were not trapped in local optima. Clades with Bayesian posterior probability (BPP) $\geq 95\%$ were considered significant and highly supported (Alfaro et al. 2003).

The individual ITS, LSU, and Mcm7 datasets were examined for potential conflict before concatenation into a single dataset for total evidence analysis (Kluge 1989, Eernisse and Kluge 1993). The individual gene phylogenies were considered incongruent if clades with significant ML bootstrap and Bayesian posterior probability ($\geq 70\%$ BS or $\geq 95\%$ BPP) were conflicting in the individual tree topologies (Wiens 1998, Alfaro et al. 2003, Lutzoni et al. 2004). As no incongruencies were found among the three individual data sets, they were concatenated using Seaview 4.2 and subjected to phylogenetic analyses as above.

Results

Morphology

Nothomitra cinnamomea Maas Geest.,
Persoonia 3(1): 92, 1964.

= *Microglossum cinnamomeum* S.
Moingeon & J.M. Moingeon,
Miscellanea Mycologica 80–81:
31, 2004.

Type: Austria, Attergau, Fehra Moos, SW of St. Georgen, 29 September 1969, J.T. Palmer 11391. L 962.271-144.

Ascomata scattered to gregarious occurring in soil, 1–3.3 cm high, hymenium borne on variously-shaped fertile heads, head glabrous, spatulate 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 crociers, inoperculate, apical ring euamyloid, deep blue in IKI, small, not occupying entire apex, 150–180 \times 9.5–12.5 μm (Fig 1B), 8-spored, biseriate. Ascospores 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) \times 3.5–5.5 (–6) μm (Fig 1D).

Habitat: Growing among *Sphagnum* and *Aulacomnium 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



Figs 1 (A-D) – *Nothomitra cinnamomea*. **A** In situ photograph of ascomata. **B** Ascus, total magnification = 400X. **C** Close up of fertile tip, arrow denotes separation of head and stipe. **D** Ascospores illustrating variable septation, total magnification = 800X.

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 Leotiomyces 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.

Name	Collection Number	Herbarium #	ITS	LSU	Mcm7	Locality
<i>Bisporella citrine</i>	VPH s.n.	ILLS61033	JQ256414	JQ256432	JN672971	Champaign County, Illinois
<i>Cudoniella clavus</i>	ANM2087	ILLS60488	JQ256415	JN012006	JN672988	GSMNP, Tennessee
<i>Geoglossum barlae</i>	Moingeon s.n.	ILLS61034	JQ256416	JQ256433	JQ256444	France
<i>Geoglossum cookeanum</i>	ANM2257	ILLS61035	JQ256417	JQ256434	JQ256445	GSMNP, North Carolina
<i>Geoglossum difforme</i>	ANM2169	ILLS61036	JQ256418	JN673044	JN672990	Cades Cove, GSMNP, Tennessee
<i>Geoglossum fallax</i>	J. Gaisler s.n.	ILLS61037	JQ256419	JQ256435	JQ256446	Hamrstejn, Czech Republic
<i>Geoglossum glabrum</i>	ANM2267	ILLS61038	JQ256420	JQ256436	JQ256447	GSMNP, Tennessee
<i>Geoglossum simile</i>	ANM2171	ILLS61039	JQ256421	JQ256437	JQ256448	GSMNP, Tennessee
<i>Geoglossum umbratile</i>	CFR251108	ILLS61040	JQ256422	JQ256438	JQ256449	Kennemerland, Netherlands
<i>Graddononia coracina</i>	ANM2018	ILLS60491	JQ256423	JN012009	JN672993	GSMNP, Tennessee
<i>Hymenoscyphus fructigenus</i>	ASM10619	ILLS61041	JQ268558	JN673046	JN672997	Samara, Russia
<i>Microglossum olivaceum</i>	GenBank	N/A	AY789398	AY789397	N/A	N/A
<i>Neurospora crassa</i>	GenBank	N/A	JN198494	AF286411	XM958785	N/A
<i>Nothomitra cinnamomea</i>	Moingeon s.n.	ILLS61042	JQ256424	JQ256439	JQ256450	Bellefontaine, Jura, France
<i>Propolis versicolor</i>	ANM2050	ILLS60497	JQ256425	JN012015	JQ256451	GSMNP, North Carolina
<i>Sarcoleotia globosa</i>	GenBank	N/A	AY789300	AY789299	N/A	N/A
<i>Thuemenidium arenarium</i>	CFR181007	ILLS61043	JQ256426	JQ256440	JQ256452	Kennemerland, Netherlands
<i>Thuemenidium atropurpureum</i>	ASM4931	ILLS61044	JQ256427	JQ256441	JQ256453	Cortland County, New York
<i>Trichoglossum hirsutum</i>	J. Gaisler s.n.	ILLS61045	JQ256428	JQ256442	JQ256454	Hamrstejn, Czech Republic
<i>Trichoglossum octopartitum</i>	JPP10191	ILLS61046	JQ256429	JQ256443	JQ256455	Senavelle, France
<i>Trichoglossum walteri</i>	ANM2203	ILLS61047	JQ256430	JN673053	JN673022	GSMNP, North Carolina
<i>Vibrissia filisporia</i> f. <i>filisporia</i>	ANM2064	ILLS60499	JQ256431	JN012017	JN673023	GSMNP, North Carolina

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

mycetes, and *T. atropurpureum*, shown by this study and Ohenoja et al. (2010) to belong in Leotiomycetes. *Thuemenidium atropurpureum* produces ascospores 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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