

Molecular phylogeny of the fungus gnat family Mycetophilidae (Diptera, Mycetophiliformia)

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Abstract. A molecular phylogeny of the fungus gnat family Mycetophilidae based on the nuclear 18S, 28S, and the mitochondrial 16S rRNA genes is presented. The total alignment included 58 taxa and 1704 bp. The family was recovered as monophyletic in parsimony and Bayesian analyses. In the Bayesian analysis, Mycetophilinae and its two tribes, Mycetophilini and Exechiini, were monophyletic with good statistical support. The subfamily Mycomyinae was found consistently in a sister-group relationship to Mycetophilinae. Gnoristinae was rendered paraphyletic, subtending Mycomyinae and Mycetophilinae. Within Gnoristinae, the genera *Coelosia* Winnertz, *Boletina* Staeger, *Gnoriste* Meigen group with *Docosia* Winnertz, usually considered to be a member of Leiinae. No support was found for the monophyly of the subfamilies Sciophilinae and Leiinae.

Introduction

Fungus gnats (Mycetophilidae) constitute a group of moderately sized ‘lower’ Diptera found on all continents except Antarctica. Adult flies often live a secluded life in humid and shadowy forests, and their biology is still mainly unknown. Large numbers can be found in crevices along streams and brooks, next to uprooted and overhanging trees, and moist hollows. The larvae of the majority of species seemingly feed on sporophores of soft fungus, or on mycelium penetrating dead wood or other organic matter. Forest continuity has a strong impact on the species composition of fungus gnats (Økland, 1994, 1996; Økland *et al.*, 2005), and precipitation seems important in controlling abundance (Økland *et al.*, 2005).

According to Amorim & Rindal (2007), Mycetophilidae is one of two families in the superfamily Mycetophiloidea, being sister to the Lygistorrhinidae. Traditionally, Mycetophilidae has been divided into three subfamilies, namely Mycetophilinae, Sciophilinae and Manotinae (Edwards, 1925). Furthermore, Edwards (1925) divided Mycetophilinae into the two tribes Mycetophilini and Exechiini, and Sciophilinae into the tribes Sciophilini, Gnoristini, Leiini and Mycomyini. Although the four latter tribes were raised to the level of subfamilies (Tuomikoski, 1966; Hennig,

1973), the monophyly of some has been questioned by authors, including Väisänen (1986) and Söli (1997). Despite the lack of consensus concerning the monophyly of Sciophilinae, Gnoristinae, Leiinae and Mycomyinae, as a matter of convenience we prefer to use these names.

The first formal cladistic treatments of Mycetophilidae were conducted by Söli (1997) and Tozoni (1998), both based on morphology. Except for the support for the monophyly of Sciophilinae, the two authors reached quite different conclusions. Tozoni (1998) found support for all postulated subfamilies and tribes, whereas Söli (1997) did not, and tentatively recommended treating the entire group as one family with a modified tribal classification following Edwards (1925).

In his morphological analyses, Söli (1997) (Fig. 1) found strong support for a group of genera commonly included in the Sciophilinae. The other genera representing Gnoristinae, Leiinae, Mycomyinae and Mycetophilinae were found in a common clade. Although Mycomyinae and Mycetophilinae were assumed to be monophyletic, they were represented by only five out of a total of 39 genera, and nested in a larger clade.

More genera were included in Tozoni’s (1998) analysis (although several were derived only from literature) (Fig. 2). The following order of the subfamilies was proposed: (Sciophilinae (Gnoristinae (Mycomyinae (Leiinae (Allactoneurinae (Manotinae, Mycetophilinae)))))). The obvious discrepancies between these two studies clearly demonstrate the need for

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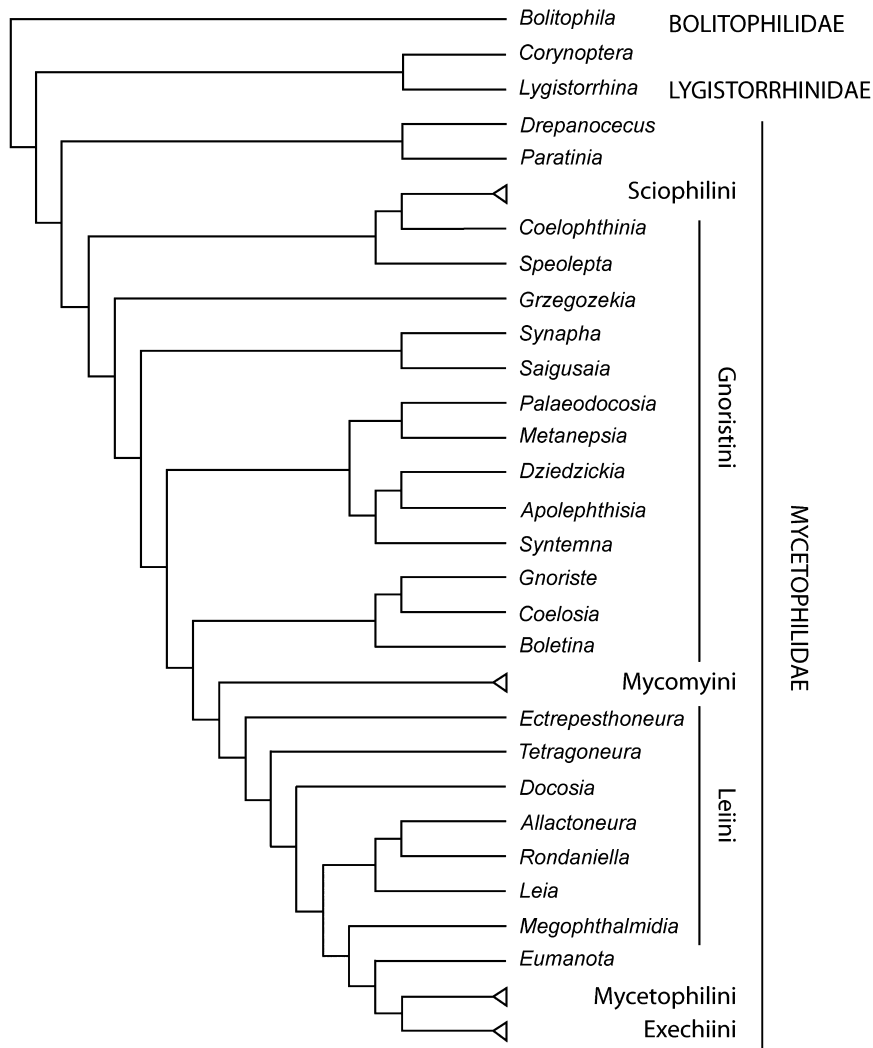


Fig. 1. Phylogenetic relationships of Mycetophilidae simplified from Söli (1997).

further studies in order to understand the character evolution and the phylogeny of the family Mycetophilidae better.

In a study of the phylogeny of Sciarioidea (=Mycetophiliformia) using molecular markers, Baxter (1999) analysed the mitochondrial 16S and 12S ribosomal RNA sequences, and in a second part focused on Mycetophilidae s.s. using only the 12S gene. Although Baxter's (1999) conclusions were based on few taxa, three main conclusions concerning the relationships within Mycetophilidae were deduced in addition to the monophyly of the family, namely: (i) Mycetophilinae and the tribes Mycetophilini and Exechiini were monophyletic, (ii) Mycetophilinae and Leiinae were not closely related and (iii) Leiinae and Gnoristinae were paraphyletic.

Here we present a molecular phylogeny for the fungus gnat family Mycetophilidae based on the nuclear 18S, 28S, and the mitochondrial 16S rRNA genes to test the validity of the traditionally recognized subfamilies, and their relationship to each other.

Materials and methods

Sampling and species identification

Forty-six species of different genera of Mycetophilidae and 12 outgroup taxa were included (Table 1). The genitalia of all species were dissected and are stored as reference material in the collections of the Natural History Museum, Oslo, Norway. The remaining parts of the specimens were used for DNA extraction.

DNA isolation, polymerase chain reaction amplification, sequencing and sequence alignment

DNA was extracted according to the protocol 'DNA purification from 50 to 100 mg fresh or frozen solid tissue' of the Puregene kit (Gentra Systems, Minneapolis, MN, U.S.A.). For a detailed description of polymerase chain

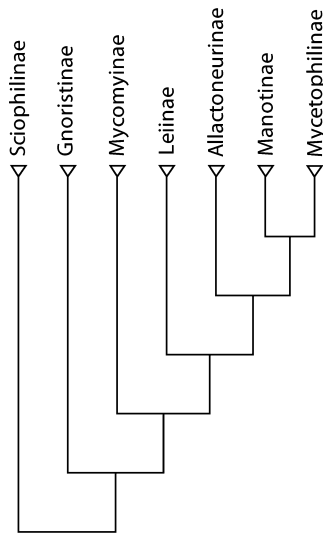


Fig. 2. Phylogenetic relationships of Mycetophilidae simplified from Tozoni (1998).

reaction primers and amplification conditions for amplifying the 18S, 28S and 16S genes, and sequencing procedures, see Rindal *et al.* (2007, in press).

Proofreading of the obtained nucleotide sequences and alignment used GENETOOLS 2.0 (Wishart & Fortin, 2001). Subsequently, the alignment was optimized by eye and adjusted based on published structures of the respective genes from *Apis mellifera* (Gillespie *et al.*, 2006) and *Drosophila melanogaster* (Cannone *et al.*, 2002). Variable regions in the 18S, 28S and 16S alignment, which were considered arbitrary due to a high number of indels, were omitted from subsequent analyses.

Phylogenetic reconstruction

The software TNT version 1.1. (Goloboff *et al.*, 2003, 2008) was used to construct the most-parsimonious cladograms, obtaining trees from random addition sequences with 1000 replicates, using all four tree-searching methods: sectorial search, with RSS and CSS options chosen; tree fusing with three rounds, the parsimony Ratchet and Drift. A bootstrap analysis was performed with 1000 replicates with ten replicates within, using the same settings as above.

Bayesian analysis was conducted with MRBAYES 3.04b (Ronquist & Huelsenbeck, 2003), as implemented at the Bioportal at the University of Oslo (http://www.bioportal.uio.no/applications/phylogenomic/mrbayes_info.php). MRMODELTEST (Nylander, 2004), a simplified version of MODELTEST 3.06 (Posada & Crandall, 1998), was used to estimate the best-fitting substitution model. The nucleotide substitution model chosen was the general time reversible model with gamma distributed rate heterogeneity and a significant proportion of invariable sites (GTR + I + G). Bayesian inference analyses were performed under 20 000 000 generations and four

Metropolis-coupled Markov chains, taking samples every 1000 generations, with the first 9 000 000 generations used as burn-in. From the resulting 110 000 trees, a posteriori probabilities for individual clades were assessed based on their observed frequencies.

Saturation plots (Fig. 3a–c) were made individually for the 18S, 28S and 16S genes, using *p*-distances plotted against GTR + I + G distances in accordance with Sullivan & Joyce (2005). *Cluzobra* Edwards was excluded from the 16S plot due to missing data.

Results

The saturation plots showed no indication of saturation for the 18S gene. By contrast, saturation of the mitochondrial 16S gene was obvious, whereas the 28S gene showed only moderate saturation (Fig. 3a–c).

The phylogenetic analysis using parsimony yielded 42 most-parsimonious trees. The consensus tree (Fig. 4) revealed the entire family Mycetophilidae to be monophyletic, although the bootstrap support was low. Sciophilinae, Leinae and Gnoristinae were not monophyletic in any of the trees. Mycomyinae, as represented by the two genera *Mycomya* Rondani and *Neoempheria* Osten Sacken, and Mycetophilinae were found to be monophyletic with high statistical support. Within Mycetophilinae, the tribe Mycetophilini was found to be monophyletic, but not Exechiini. Interestingly, Mycomyinae constituted a sister group to Mycetophilinae.

The Bayesian analysis gave a consensus tree (Fig. 5) with 93% posterior probability for the monophyly of Mycetophilidae. Mycetophilinae and its two tribes, Mycetophilini and Exechiini, were also recovered as monophyletic with high posterior probabilities. Also, in this analysis, the tribe Mycomyinae was found to be monophyletic (100%) in a sister-group relationship to Mycetophilinae. None of the other subfamilies were found to be monophyletic, but several genera commonly included within the same subfamily grouped together. Among Gnoristinae this holds for *Coelosia* Winnertz, *Gnoriste* Meigen, *Boletina* Staeger and *Paleodocosia* Meunier. This clade also includes *Docosia* Winnertz, which is commonly assigned to the tribe Leinae. Within Sciophilinae, *Megalopelma* Enderlein, *Monoclona* Mik, *Phthinia* Winnertz and *Allocotocera* Mik form a monophyletic clade.

The Leinae genus, *Tetragoneura* Winnertz, was found to be a sister group to all other Mycetophilidae. The three subfamilies Mycomyinae, Mycetophilinae and 'Gnoristinae' occur in a common clade supported with a posterior probability of 98% in the Bayesian analysis.

Discussion

The current study provides the most comprehensive molecular-derived phylogeny of the family Mycetophilidae to date.

Table 1. List of Mycetophilidae fungus gnat specimens used in this study.

Taxa	Collection number	GenBank accession numbers		
Mycetophilidae	NHM, Oslo	28S	18S	16S
<i>Anatella lenis</i>	NHM_MYC_ER_125	EU219582	DQ787911	DQ787936
<i>Allodia</i> sp.	NHM_MYC_ER_018	EU219584	DQ787912	DQ787937
<i>Allodiopsis rustica</i>	NHM_MYC_ER_079	EU219593	DQ787913	DQ787938
<i>Boraceomyia</i> sp.	NHM_MYC_ER_138	FJ171971	FJ171935	FJ172006
<i>Brachypeza bisignata</i>	NHM_MYC_ER_090	EU219596	DQ787919	DQ787944
<i>Brevicornu improvisum</i>	NHM_MYC_ER_028	EU219587	DQ787915	DQ787940
<i>Cordyla</i> sp.	NHM_MYC_ER_024	EU219586	DQ787904	DQ787929
<i>Exechia frigida</i>	NHM_MYC_ER_004	EU219575	DQ787906	DQ787931
<i>Exechiopsis sagittata</i>	NHM_MYC_ER_100	EU219577	DQ787908	DQ787933
<i>Notolopha cristata</i>	NHM_MYC_ER_093	EU219598	DQ787918	DQ787943
<i>Pseudobrachypeza helvetica</i>	NHM_MYC_ER_094	EU219599	DQ787920	DQ787945
<i>Pseudorymosia fovea</i>	NHM_MYC_ER_102	EU219578	DQ787910	DQ787935
<i>Rymosia</i> sp.	NHM_MYC_ER_003	EU219574	DQ787905	DQ787930
<i>Stigmatomeria crassicornis</i>	NHM_MYC_ER_082	EU219594	DQ787916	DQ787941
<i>Synplasta gracilis</i>	NHM_MYC_ER_083	EU219595	DQ787917	DQ787942
<i>Tarnania dziedzickii</i>	NHM_MYC_ER_098	EU219600	DQ787923	DQ787948
<i>Dynatosoma reciprocum</i>	NHM_MYC_ER_092	EU219597	DQ787903	DQ787928
<i>Epicypa aterrima</i>	NHM_MYC_ER_108	EU219579	EU219568	EU219603
<i>Macrobrachius</i> sp.	NHM_MYC_ER_122	EU219581	EU219570	EU219605
<i>Mycetophila fungorum</i>	NHM_MYC_ER_017	EU219583	DQ787902	DQ787927
<i>Phronia strenua</i>	NHM_MYC_ER_019	EU219585	EU219571	EU219606
<i>Platurocypta testata</i>	NHM_MYC_ER_049	EU219590	EU219567	EU219601
<i>Trichonta</i> sp.	NHM_MYC_ER_029	EU219588	EU219572	EU219607
<i>Zygomylia angusta</i>	NHM_MYC_ER_113	EU219580	EU219569	EU219604
<i>Boletina plana</i>	NHM_MYC_ER_047	EU219589	DQ787901	DQ787925
<i>Docosia gilvipes</i>	NHM_MYC_ER_072	EU219592	DQ787900	DQ787926
<i>Leia bilineata</i>	NHM_MYC_ER_066	EU219591	DQ787899	DQ787924
<i>Acomoptera difficilis</i>	NHM_MYC_ER_103	FJ171964	FJ171928	FJ172000
<i>Synapha vitripennis</i>	NHM_MYC_ER_104	FJ171965	FJ171929	FJ172001
<i>Cluzobra</i> sp.	NHM_MYC_ER_107	FJ171967	FJ171931	FJ172002
<i>Manota unifurcata</i>	NHM_MYC_ER_109	FJ171968	FJ171932	FJ172003
<i>Phthinia humilis</i>	NHM_MYC_ER_42	FJ171974	FJ171938	FJ172009
<i>Mycomya annulata</i>	NHM_MYC_ER_44	FJ171976	FJ171940	FJ172011
<i>Sytemna stylata</i>	NHM_MYC_ER_48	FJ171978	FJ171942	FJ172013
<i>Rondaniella dimidiata</i>	NHM_MYC_ER_54	FJ171980	FJ171944	FJ172015
<i>Grzegorzekia collaris</i>	NHM_MYC_ER_55	FJ171981	FJ171945	FJ172016
<i>Paleodocosia</i> sp.	NHM_MYC_ER_56	FJ171982	FJ171946	FJ172017
<i>Leptomorphus walkeri</i>	NHM_MYC_ER_61	FJ171985	FJ171949	FJ172020
<i>Gnoriste bilineata</i>	NHM_MYC_ER_68	FJ171989	FJ171953	FJ172024
<i>Coelosia tenella</i>	NHM_MYC_ER_71	FJ171991	FJ171955	FJ172026
<i>Tetragoneura sylvatica</i>	NHM_MYC_ER_74	FJ171993	FJ171957	FJ172028
<i>Monoclona rufilatera</i>	NHM_MYC_ER_75	FJ171994	FJ171958	FJ172029
<i>Megalopelma nigroclavatum</i>	NHM_MYC_ER_76	FJ171995	FJ171959	FJ172030
<i>Azana</i> sp.	NHM_MYC_ER_80	FJ171996	FJ171960	FJ172031
<i>Neoempheria pictipennis</i>	NHM_MYC_ER_85	FJ171997	FJ171961	FJ172032
<i>Speolepta leptogaster</i>	NHM_MYC_ER_86	FJ171998	FJ171962	FJ172033
<i>Allocotocera pulchella</i>	NHM_MYC_ER_88	FJ171999	FJ171963	FJ172034
Outgroup				
Ditomyiidae				
<i>Symmerus annulatus</i>	NHM_MYC_ER_137	FJ171970	FJ171934	FJ172005
Lygistorrhinidae				
<i>Lygistorrhina</i> sp.	NHM_MYC_ER_139	FJ171972	FJ171936	FJ172007
Keroplastidae				
<i>Macrocera</i> sp.	NHM_MYC_ER_59	FJ171983	FJ171947	FJ172018
<i>Urytalpa macrocera</i>	NHM_MYC_ER_70	FJ171990	FJ171954	FJ172025
<i>Pyratula zonata</i>	NHM_MYC_ER_111	FJ171969	FJ171933	FJ172004
<i>Orfelia fasciata</i>	NHM_MYC_ER_40	FJ171973	FJ171937	FJ172008

Table 1. Continued.

Taxa	Collection number	GenBank accession numbers		
<i>Isoneuromyia semirufa</i>	NHM_MYC_ER_51	FJ171979	FJ171943	FJ172014
<i>Neoplatyura flava</i>	NHM_MYC_ER_60	FJ171984	FJ171948	FJ172019
Bolitophilidae				
<i>Bolitophila sp.</i>	NHM_MYC_ER_64	FJ171986	FJ171950	FJ172021
<i>Bolitophila hybrida</i>	NHM_MYC_ER_105	FJ171966	FJ171930	FJ172002
Diadocidiidae				
<i>Diadocidia valida</i>	NHM_MYC_ER_65	FJ171987	FJ171951	FJ172022
<i>Diadocidia spinosula</i>	NHM_MYC_ER_43	FJ171975	FJ171939	FJ172010

Regarding the number of taxa included, the results can be compared with the two previous, morphology-based, cladistic analyses by Søli (1997) and Tozoni (1998). Of these, the results appear to be more in accordance with the former. The similarity is most striking in the lack of support for the monophyly of Gnoristinae and Leiinae. The only molecular phylogeny focusing on Mycetophilidae is that of Baxter (1999), who did not find support for any subfamily other than Mycetophilinae, much in accordance with these results.

Relationships of the subfamilies

Of the assumed subfamilies Sciophilinae, Gnoristinae, Leiinae, Mycetophilinae and Mycomyinae, only the latter two proved monophyletic in our study. In both analyses, these two subfamilies were found in a sister-group relationship. Interestingly, this is not in accordance with Søli (1997), who recovered Mycomyinae in a more basal position, or, alternatively, in a sister-group relationship to a clade consisting of

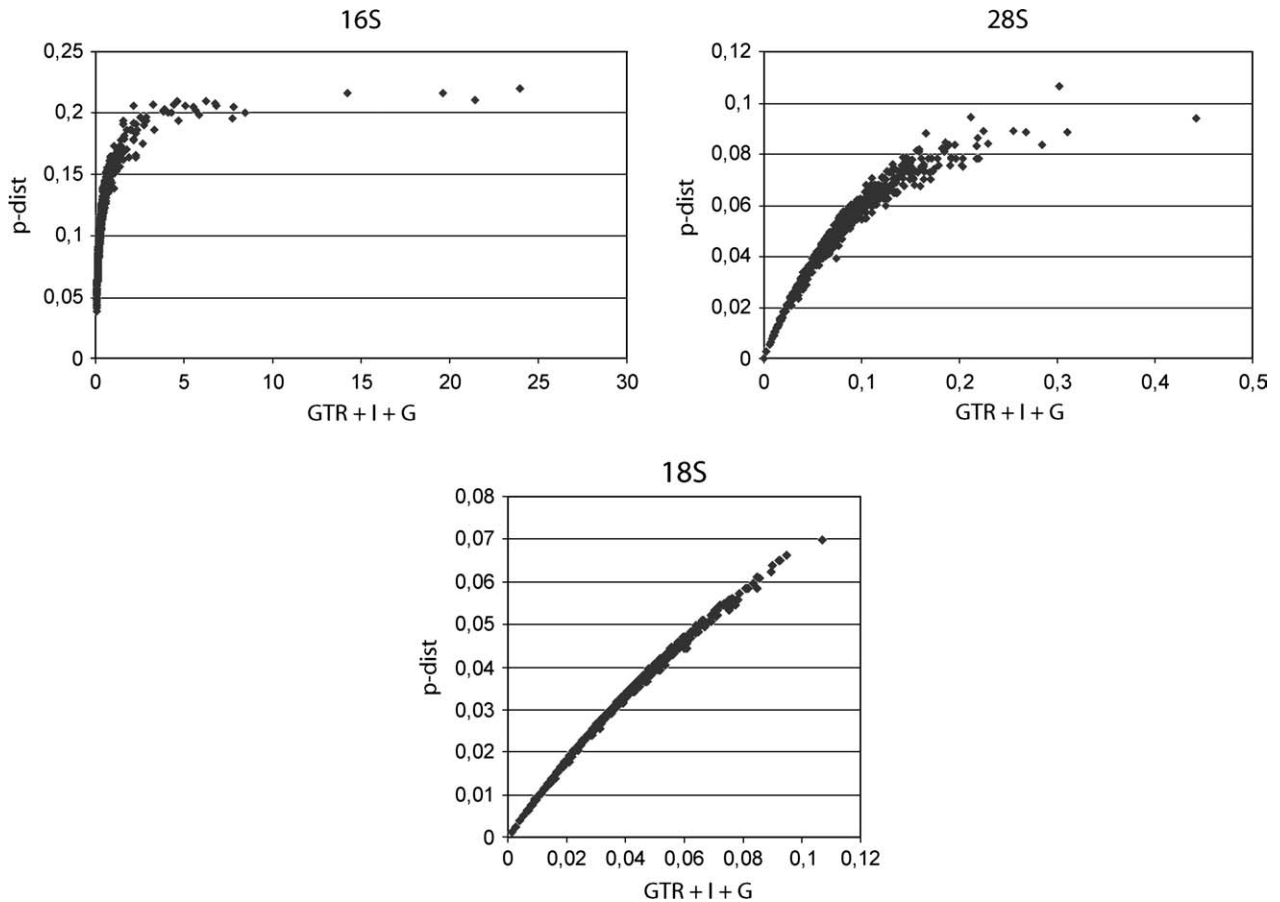


Fig. 3. Saturation plots for three molecular markers for 58 Mycetophilidae species, GTR + I + G distances were plotted against p -distances for: (a) 16S rDNA, (b) 28S rDNA, (c) 18S rDNA.

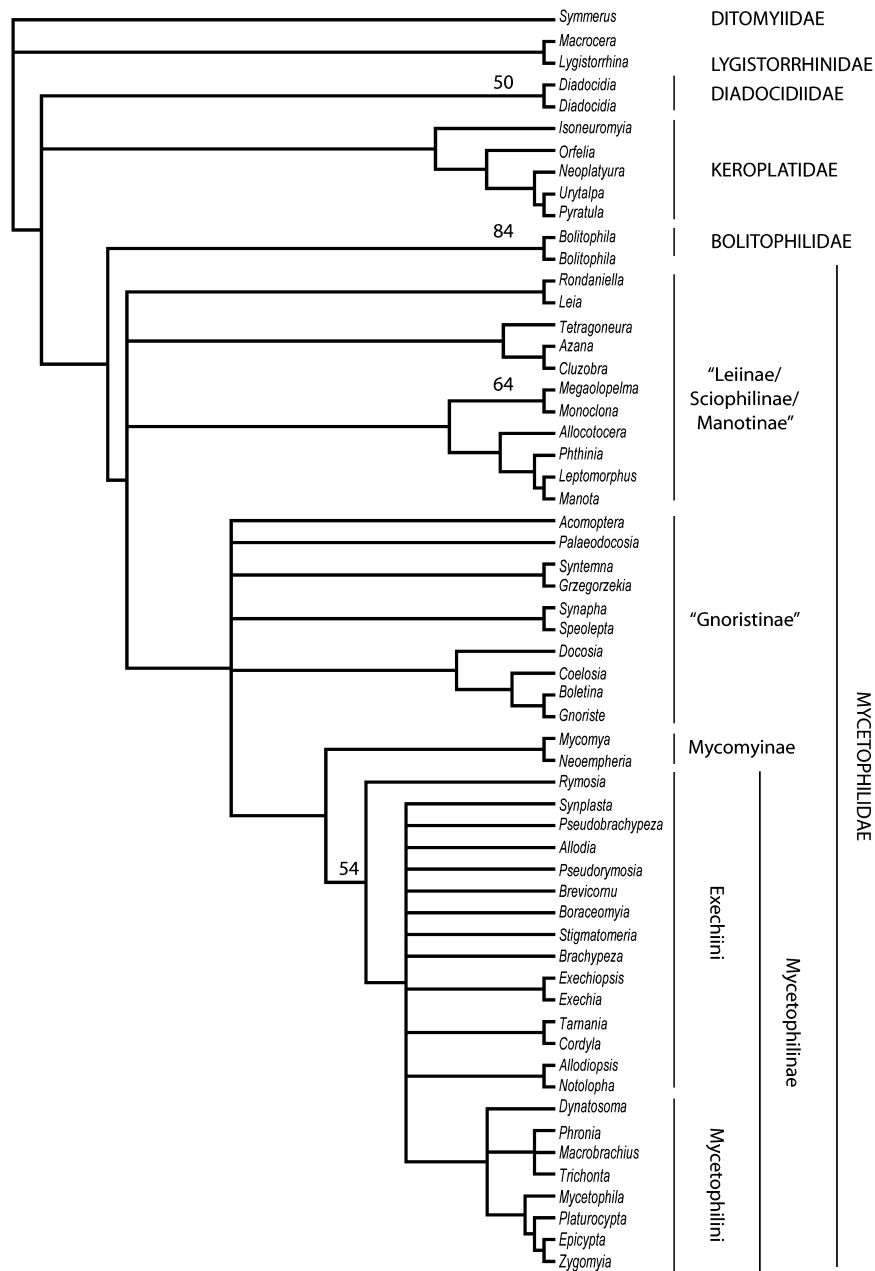


Fig. 4. Consensus of the 42 most-parsimonious trees of 2710 steps (retention index = 0.3769; consistency index = 0.3754) recovered for the fungus gnat subfamily Mycetophilinae based on the combined morphological and molecular dataset. Bootstrap values based on 1000 replicates that exceed 50 are indicated.

Mycetophilinae, *Eumanota* Edwards and Leiinae. A sister-group relationship as revealed on the basis of DNA sequence data, is also supported by two noteworthy, shared morphological traits: members of both clades have the tibial trichia arranged in rows and a reduced median ocellus. Both characters, however, are also found in taxa outside these two clades, e.g. in all Manotinae and in several Leiinae genera (see, e.g. Hippa *et al.*, 2004). Larval characters support this arrangement to some degree. The larvae of Mycetophilinae

share with Mycomyinae larvae some common traits in the shape of the mandibles, with wide and arched margins with massive teeth (Krivoshchina & Zaitzev, 2008). On the other hand, larvae of *Manota* Williston differ from the larvae of Mycetophilinae and Mycomyinae, with the stipes and lacinia fused into a single structure.

The members of the subfamily Gnoristinae were all found to be paraphyletic, in a clade also covering the two subfamilies Mycomyinae and Mycetophilinae (posterior

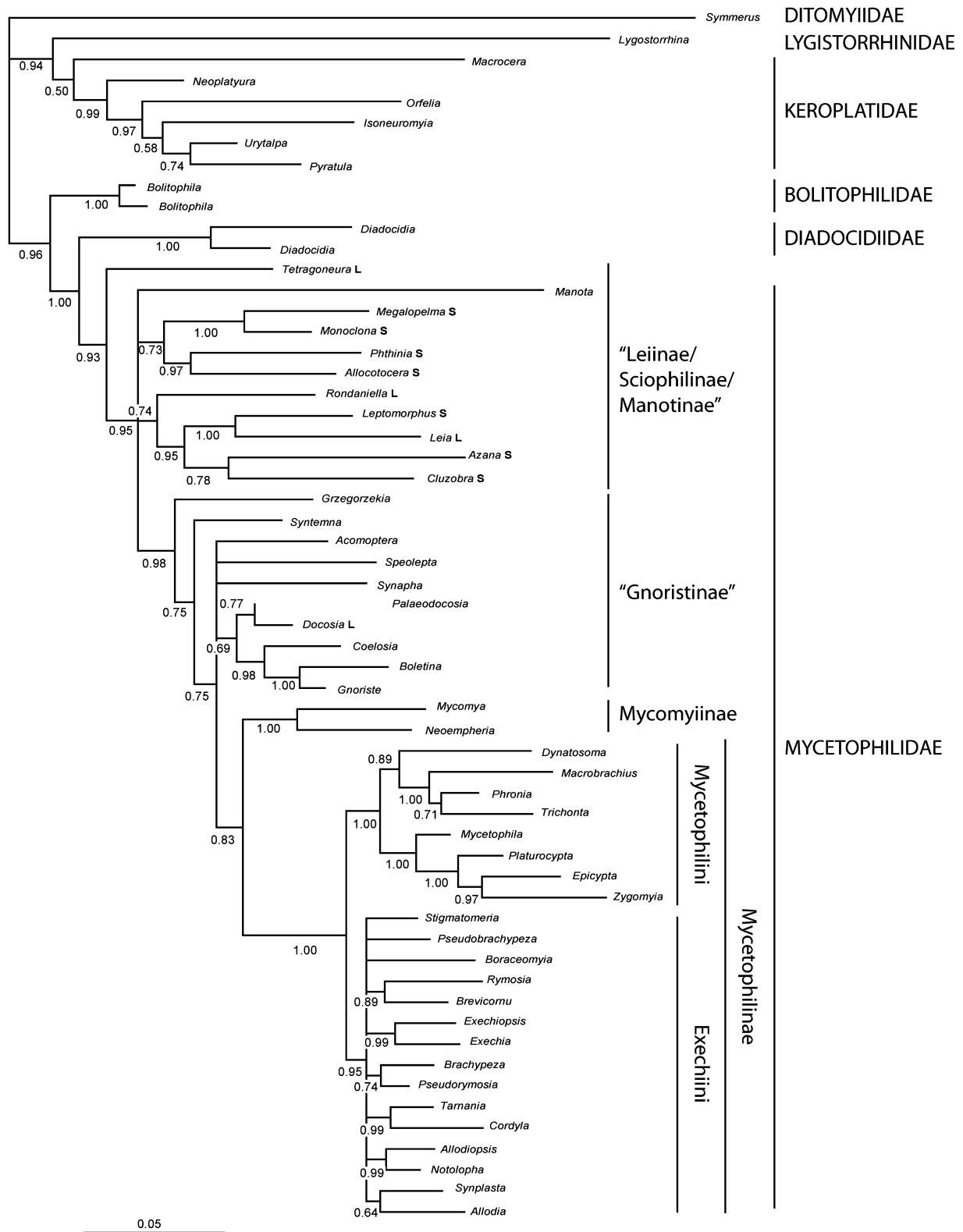


Fig. 5. Phylogenetic hypothesis of the fungus gnat subfamily Mycetophilidae as obtained with MRBAYES using the GTR + I + G model for the nuclear 18S, 28S and the mitochondrial 16S rDNA. Posterior probabilities exceeding 0.5 are indicated. L and S denote genera that are commonly ascribed to Leiinae and Sciophilinae, respectively.

probability of 98% in the Bayesian analysis). This novel arrangement deserves more attention, and demands a better understanding of the delineation of Gnoristinae.

The weak support for generic arrangements among the genera representing Sciophilinae and Leiinae is surprising, and in contrast to Söli (1997), who found very strong statistical support for Sciophilinae, excluding *Syntemma* Winnertz and *Paratinia* Mik, when using morphological characters.

Some notes on intergeneric relationships within the subfamilies

Mycetophilinae. The monophyly of the subfamily Mycetophilinae and its two tribes has been well documented recently by both molecular and morphological characters (Rindal & Söli, 2006; Rindal *et al.*, 2007, in press). This grouping was also recovered in our Bayesian analysis with high statistical support. The better resolution achieved within Mycetophilini than in Exechiini is also in accordance with the previous studies. It is noteworthy that Exechiini was not recovered as monophyletic in the parsimony analysis.

Mycomyiinae. It is debatable whether the monophyly of this subfamily is demonstrated in the present analysis, as it is represented only with two of 12 genera. There seems, however, to be a general agreement about the monophyly of this tribe, and a discussion of the characters delimiting it can be found in Väisänen (1984).

Gnoristinae. Interestingly, *Docosia*, normally included in Leiinae, is found among the Gnoristinae genera in both analyses. This genus takes a rather isolated position within Leiinae, and there are morphological characters supporting its position among Gnoristinae, such as the presence of setae behind the halter (Söli, 1997: character 58), shared by *Gnoriste*, *Syntemma*, *Speolepta* Edwards, *Synapha*, *Palaeodocosia* and *Boletina*; and the conspicuous outline of the cerci with rows of blunt megasetae. A very similar outline is found in most species of *Boletina*, and it is extremely well developed in *B. verticillata* (Stackelberg, 1943). It is noteworthy also that Baxter (1999; fig. 15) grouped *Docosia* with *Boletina* and *Gnoriste*.

Some other clades among the Gnoristinae deserve comment. The grouping of the three genera *Coelosia*, *Boletina* and *Gnoriste* in a common clade was also recovered by Söli (1997), but not by Tonzoni (1998), who recovered these genera in a larger clade together with other Gnoristinae genera.

The rather peculiar genus *Speolepta* with its apneustic larvae and troglophile life style was placed among Gnoristinae genera in our analyses. This genus was included tentatively in the Sciophilinae by Söli (1997), but this finding supports Edwards' (1925) original classification.

The analysis places *Syntemma* among Gnoristinae genera, which differs from Edwards' (1925) classification, but accords with Väisänen (1986) and Söli (1997).

Sciophilinae and Leiinae. Some Sciophilinae genera are grouped, but without any distinct patterns in the revealed trees. There are substantial differences in the position of these genera in the parsimony and Bayesian analyses. Our data cannot confirm monophyly. Leiinae as commonly recognized, constitutes a large and rather heterogenous assemblage of approximately 30 genera worldwide. Some of these genera are closely related and can be separated only on minor morphological features (see, e.g. Söli, 1996; Hippa *et al.*, 2004), whereas others have more in common with genera placed in the Gnoristinae.

Manotinae. The systematic position of *Manota* and its related genera (*Eumanota*, *Paramanota* and *Promanota*) has been discussed intensively (Tuomikoski, 1966; Hennig, 1973; Hippa *et al.*, 2004). Previous studies suggesting a close association to Leiinae (Zaitzev, 1990; Söli, 1997; Söli *et al.*, 2000; Hippa *et al.*, 2004) cannot be refuted by our results. The phylogenetic position, however, may also be due to the many indels that render it quite different from the other taxa, as reflected by the long branch in the Bayesian tree.

Conclusions

The number of taxa and genes sampled is always a concern in the design of phylogenetic studies. Here we have representatives of approximately one-third of the genera recognized presently in Mycetophilidae. Although this contributes significantly to a better understanding of the phylogenetic relationships within the fungus gnat family Mycetophilidae, many questions remain to be addressed. From the present state of knowledge, some provisional recommendations for the naming of different tribes and subfamilies within Mycetophilidae can be made. As there is good evidence for the monophyly of Mycomyiinae and Mycetophilinae, and for Exechiini and Mycetophilini, we see no reason to change their taxonomic rank as subfamilies and tribes. This leaves us with the question of how to treat the remaining 'subfamilies'. With the exception of some small, well-supported clades, these taxa are paraphyletic in relation to Mycetophilinae and Mycomyiinae. Nevertheless, we will not recommend creating new tribes or subfamilies, but keeping with the tradition and using the subfamily rank for Sciophilinae, Leiinae, Gnoristinae and Manotinae.

Acknowledgements

The study was funded by the Norwegian Research Council (NFR) and the National Centre of Biosystematics (NCB, Natural History Museum, University of Oslo). Thanks to Lars Ove Hansen for help with the collection of samples and advice regarding field work, and to Karsten Sund, Ole Lønnve, Øivind Gammelmo, Pierre Tripotin and Torbjørn Darup for help with sampling. TNT version 1.1 was made available with the sponsorship of the Willi Hennig Society.

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Accepted 20 January 2009

First published online 19 March 2009