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A mitochondrial 12S and 16S rRNA phylogeny of critical genera of Phoridae (Diptera) and related families of Aschiza

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Abstract

Phylogenetic analysis of mitochondrial 12S and 16S rRNA gene sequences supports the monophyly of the Phoridae. Within this family the Phorinae clade includes two aberrant termitophilous subfamilies, the Thaumatoxeninae and the Termitoxeniinae, which cluster with *Dohrniphora* and *Diplonevra*. These two genera include termitophiles and parasitoids of termites, so we hypothesize that these termitophilous phorids are a monophyletic group. While the data neither refute nor support the assumed monophyly of the Metopininae, the genera of this subfamily were not monophyletic in our analysis, but fell into two subclades that correspond with the tribes Metopinini and Gymnophorini.

Key words: Diptera, Aschiza, Phoridae, rRNA sequences, phylogeny, maximum likelihood, Bayesian phylogeny, mitochondria, mtDNA, 12S, 16S lsuRNA, ssuRNA

Introduction

The flies, midges, and gnats (Diptera) are a successful and widespread insect order that includes over 120 named families and over 140,000 named species. However, the majority of species still remain undescribed and unnamed. The Diptera exhibit a diverse range of habits, especially as larvae. Indeed, the range of habits is greater than that for any other order of invertebrates. This diversity means that many species are of major medical, veterinary, forensic or economic importance. Consequently, some families, such as the Culicidae (mosquitoes), have been intensively studied. Furthermore, the family Drosophilidae

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has become very well known through its use in laboratory studies in fields ranging from genetics to embryology.

The scuttle files (family Phoridae) are a diverse group within the Diptera which have been less-studied than many other dipteran families. Currently there are around 3400 named species of phorids, but estimated diversity for the family is in the range of 30,000 to 50,000 species (Brown 2004, Disney 1983, Gaston 1991). Phylogenetic relationships within the Phoridae are currently debated (Brown 1992, Disney 2003) The traditional arrangement into six subfamilies (e.g., Borgmeier 1968) is now considered unsatisfactory, and at least one subfamily has been abolished (Disney & Cumming 1992). Previous taxonomic studies of the phorids have used morphological characters (see above references and Yeates & Wiegmann 1999), but it is clear that some relationships may not be resolved using morphological characters alone (Jenner 2004), and that molecular data might help resolve the phylogeny of the group. In this paper we present mitochondrial ribosomal RNA sequence data with which we begin to establish a more robust classification of the Phoridae.

This study originated in an attempt to extract and analyze DNA from fossil insects preserved in amber. Included in this material were fossil scuttle flies (Diptera, Phoridae) from Dominican amber, thought at the time to be of Oligocene age but since re-assigned to Early-Middle Miocene (Iturralde-Vinent & MacPhee 1996). The Dominican ambers include 15 genera of Phoridae (Disney & Ross 1997, Brown 1999), two of which were included in the attempts to extract DNA. Brown (1999) added another eleven genera to this list. In order to evaluate the hypothesis that the genetic distance between two phorid genera today is greater than that between the same genera 15–20 million years ago, a series of modern specimens were compared by analyzing mitochondrial 12S and 16S rRNA gene sequences. However, all attempts to reproduce the previous claims regarding the extraction and analysis of DNA from amber-preserved insects failed and it was concluded that DNA does not survive over millions of years in amber (Austin *et al*. 1997). Nevertheless the data obtained from the modern specimens are still of considerable interest, as reported below. In particular, these data contribute to current debates regarding the realization of a phylogenetic classification of the genera of Phoridae (Brown 1992, Disney 2003).

Methods

Specimens: Samples included *Phora atra* and *Metopina pileata*, the type genera of the two major phorid subfamilies, Phorinae and Metopininae, and also *Conicera* Meigen and *Diplonevra* Lioy, which have been used as the type genera of proposed tribes or subfamilies. Representatives of the subfamilies Termitoxeniinae and Thaumatoxeninae were added, as the affinities of these flies have long been uncertain. Likewise, two further genera included were *Triphleba* Rondani, whose subfamily assignment is disputed and *Chonocephalus* Wandolleck whose assignment to the Metopininae is open to doubt. Flies

were collected using water traps or directly into 95% ethanol. Flies collected into water traps, even when later transferred to ethanol, did not yield amplifiable DNA. The species successfully analyzed are listed in Table 1. Where a species occurs twice it is because one male and one female were both sampled (labeled 1 and 2), and in the case of two of these, *Chonocephalus heymonsi* and *Termitophilomyia zimbraunsi*, the females were flightless and the males winged.

TABLE 1. Dipteran taxa used for this study. All sequences except *Drosophila melanogaster* and *D. yakuba* result from this study. A (2) adjacent to a species name indicates that two samples, one male and one female, were sequenced.

Species	Suborder	Family	16S GenBank acc. no.	12S GenBank acc. no.
Hilara maura (Fabricius)	Brachycera	Empididae	AF126346	AF126318
Cyclorrhapha:				
Lonchoptera lutea Panzer	Aschiza	Lonchopteridae	AF126438	AF126310
Opetia nigra Meigen	"	Opetiidae	AF126347	AF126319
Anevrina thoracica (Meigen)	"	Phoridae	AF126345	AF126317
Chonocephalus heymonsi Stobbe (2)	"	"	AF126341, AF126352	AF126313, AF126324
Clitelloxenia audreyae Disney	"	"	AF126339	AF126311
Conicera similis (Haliday)	"	"	AF126337	AF126309
Diplonevra mortimeri Disney	"	"	AF126344	AF126316
Diplonevra nitidula (Meigen)	"	"	AF126326	AF126298
Dohrniphora trigonae Disney	"	"	AF126343	AF126315
Megaselia aequalis (Wood)	"	"	AF126328	AF126300
Megaselia scalaris (Loew)	"	"	AF126330	AF126312
Metopina pileata Schmitz	"	"	AF126329	AF126301
Phora atra (Meigen)	"	"	AF126332	AF126304
Puliciphora borinquenensis Wheeler	"	"	AF126351	AF126323
Spiniphora punctipennis (Zetterstedt)	"	"	AF126342	AF126314
Termitophilomyia zimbraunsi Disney (2)	"	"	AF126349, AF126350	AF126321, AF126322
Thaumatoxena andreinii Silvestri	"	"	AF126353	AF126325
Triphleba distinguenda (Strobl) (2)	"	"	AF126327, AF126331	AF126299,AF126303
Triphleba nudipalpis (Becker)	"	"	AF126330	AF126302
Protoclythia modesta (Zetterstedt)	"	Platypezidae	AF126348	AF126320
Melanostoma scalare (Fabricius)	"	Syrphidae	AF126336	AF126308
Drosophila melanogaster Meigen	Schizophora	Drosophilidae	NC_001709	NC_001709
Drosophila subobscura Collin	"	"	AF126335	AF126307
Drosophila yakuba Burla	"	"	NC_001322	NC_001322
Hydrellia modesta Meigen	"	Ephydridae	AF126334	AF126306
Limnellia quadrata (Fallén)	"	"	AF126333	AF126305

Molecular analyses: Total DNA was extracted from air dried and crushed, whole or part specimens, using a modified version (Austin *et al.* 1997) of the Chelex method (Walsh *et al.* 1991). Segments of the mitochondrial 12SrRNA (ca. 375 base pairs) and 16SrRNA

(ca. 535 base pairs) genes were PCR amplified using primers 12Smb (5' CAGAGAGTGACGGGCGATTTGT, 14233) and 12Sma (5' CTGGGATTAGATACCCTGTTAT, 14588), and 16SRHTB (5' ACGCCGGTTTGAACTCAGATC, 12883) and LR-N-13398 (5' CGCCTGTTTAACAAAAACAT, 13398, (Kocher *et al*. 1989, Simon *et al*. 1994), respectively. Numbers refer to the position of the 3' nucleotide in the *Drosophila yakuba* complete mitochondrial genome (Clary & Wolstenholme 1985). PCR amplifications were carried out in 25µl or 50µl reaction volumes containing 2mM $MgCl_2$, 0.2mM of each dNTP, 0.5µM of each primer, 0.2 mg/ml BSA, 1U of Taq DNA polymerase (Promega) and 1x PCR buffer (Promega), in a Perkin Elmer PE9600 Thermocycler using 30–40 cycles of the following profile: 40 seconds denaturation at 94°C, 60 seconds annealing at 50°C or 55 °C, and 120 seconds extension at 72°C. PCR amplified DNA was electrophoresed in 1.4% agarose gels and purified using Qiaex II (Qiagen). Both strands were sequenced using the same primers as for the PCR with dye terminator automated sequencing chemistry (Amersham) and a 377 or 373 Automated DNA sequencer (ABI).

Phylogenetic analyses: Sequences were aligned using ClustalW (http://www.ebi.ac.uk) and the alignments adjusted manually using secondary structure models for the 12SrRNA gene (Hickson *et al*. 1996) and for the *Drosophila melanogaster* 16SrRNA gene (De Rijk *et al*. 1998). This alignment contained regions of ambiguity where gaps had been introduced. These regions were removed using Gblocks (Castresana 2000) with the default settings. The aligned 16S and 12S rRNA sequences were combined for the phylogenetic analyses. Flook & Rowell (1997) have shown that combining 12S and 16S rRNA sequences for intra-ordinal phylogenetic analyses of insects improves consistency.

We used a maximum likelihood (ML) method to estimate a phylogeny from the alignment as follows. First, we used the neighbor-joining method as implemented in PAUP* v.4.0b10 (Swofford 1998) with the default maximum likelihood distance parameters to generate a starting tree. We then used a likelihood ratio test (LRT) as implemented in Modeltest (Posada *et al*. 2000) to identify the GTR+I+Γ model as optimal for this dataset. Using this model, and the same tree, we estimated the likelihood when the number of rate categories varied between 1 and 8, and used a χ^2 test to determine when increasing the number of categories ceased to significantly improve the ML estimate. For these data 5 rate categories were optimal.

We then used MrBayes (Huelsenbeck & Ronquist 2001) to estimate phylogenies by Bayesian inference, with the same parameters (GTR+I+Γ model with 5 rate categories). Four chains were run with 200,000 generations, and the ML estimate and topology of every 100^{th} tree were stored. A graph of the likelihood values showed that these reached a plateau after approximately 40,000 generations, or 20% into the run. We used the last 1000 of the stored trees (the final 50%) to make a consensus tree. The frequency with which each branch of the tree is represented on the consensus tree represents a posterior probability of the likelihood of that branch. Note that such values, when calculated using Bayesian

inference, are reported to overestimate branch support in the tree (Simmons *et al*. 2004, Suzuki *et al.* 2002). We also identified the best tree found by MrBayes for use as a starting point for an iterative search of tree space.

In order to find the best estimate of phylogeny for any given data set we would like to calculate the ML value for every possible tree. For a dataset with 32 taxa this is not possible as it would be too computationally intensive. In order to optimize our estimate of the phylogeny for this dataset we used the best tree identified by MrBayes as the starting point for an iterative search using PAUP* as follows (Collins & Wiegmann 2002a, b; Telford *et al*. 2003). First, we used the nearest neighbor interchange (NNI) branch swapping algorithm to identify trees near the starting tree with "better" estimated ML values. Secondly, we estimated the variables in the GTR+I+Γ model (gamma shape parameter, proportion of invariant sites, GTR substitution-rate matrix, and nucleotide frequencies) for the tree found by the NNI search. We then used these values of the variables for a more thorough search of tree space using the tree bisection and reconnection (TBR) branch swapping algorithm. We recalculated the ML parameters for the TBR tree, and then repeated the NNI search, parameter recalculation, and TBR search steps until the likelihood score was stationary. We repeated this entire procedure using the original neighbor joining tree as the starting tree and reached the same final tree. This tree is shown in Figure 1.

Finally, in order to explore statistical support for this tree we performed a non-parametric bootstrap analysis with 500 replicates where the GTR+I+Γ model was used to generate a distance matrix, then an NJ tree for each replicate data set. A consensus tree was generated from these 500 trees. Branches supported in more than 50% of the bootstrap replicates are shown on the tree in Figure 1. Non-parametric bootstrapping is reported to be more conservative than Bayesian posterior probabilities (Simmons *et al*. 2004). The results from our analysis support this idea: we note that more branches are supported by Bayesian posterior probabilities than by bootstrapping, and for those branches supported by both the posterior probability is invariably higher than the bootstrap value. For this reason we considered only branches supported with high confidence (at least 60%) by both methods as well-supported.

Results and Discussion

The specimens successfully analyzed are listed in Table 1. The 12SrRNA and 16SrRNA alignments contained 362 and 474 sites, respectively. Within these 836 sites, 58 positions contained a gap in one or more taxa, 383 sites were constant and the remaining 395 sites were variable. After elimination of poorly aligned sites (all gaps and some nucleotides flanking those gaps) the final data set contained 734 aligned nucleotides. The nucleotide composition of these rRNA genes shows the strong AT bias typical of insects (Simon *et al*. 1994), with a mean AT content of 79.1% and 76.7% for the 12S and 16S genes, respectively.

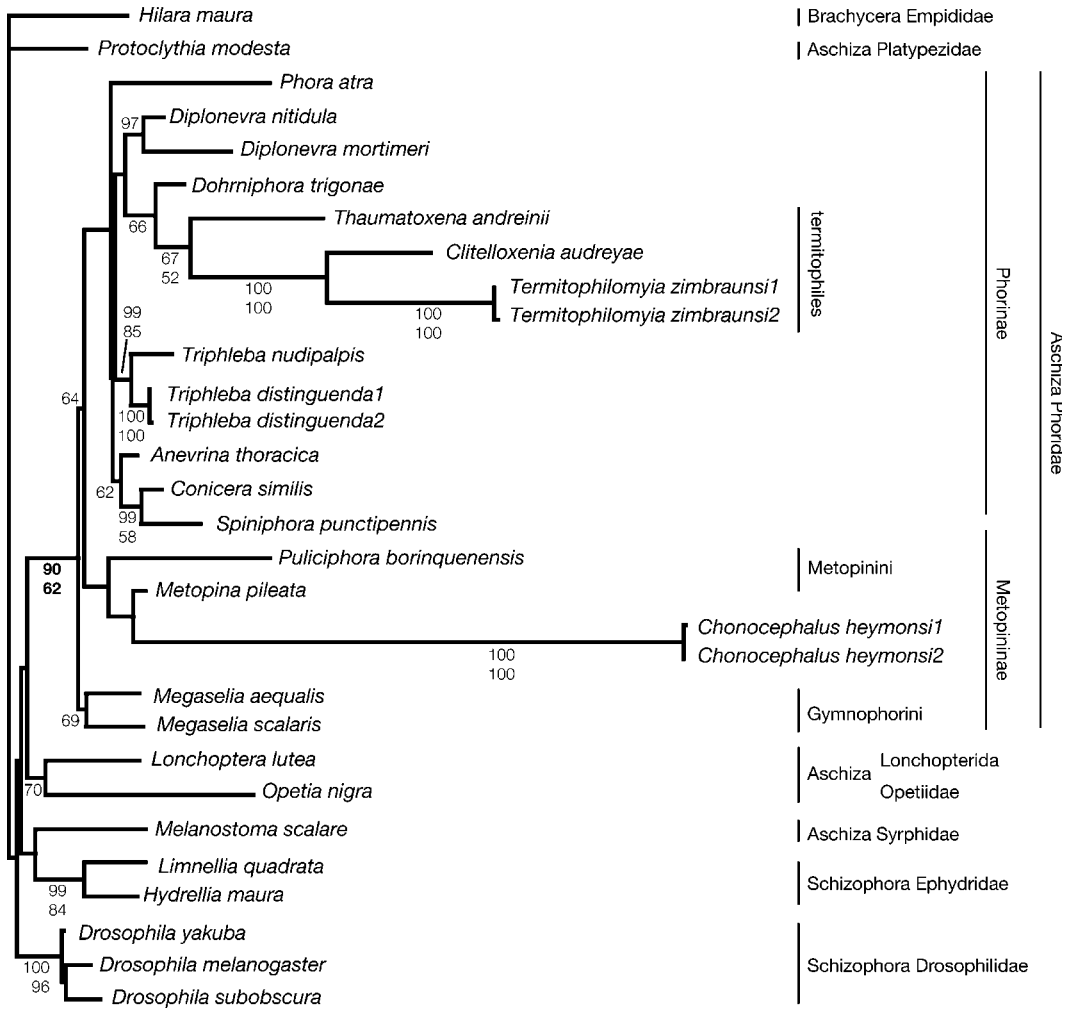

FIGURE 1. Maximum likelihood tree derived from analysis of concatenated dipteran 12S and 16S mitochondrial DNA sequences (-ln likelihood = 5910.51681, proportion of invariable sites 0.291, gamma shape parameter = 0.480). Taxa as in Table 1. The *Hilara maura* sequence was assigned as outgroup. The names of suborders, series, families, subfamilies and tribes are also indicated where they are relevant to the discussion in the text. Numbers refer to Bayesian posterior probabilities as percent (top) or percent support in a non-parametric bootstrap analysis by neighbor-joining of maximum likelihood distances (bottom). Where only one number occurs it refers to a Bayesian posterior probability: that branch received less than 50% support in the non-parametric bootstrap analysis. Support values for the branch leading to the Phoridae are in bold.

The maximum likelihood analysis produced a single tree characterized by many short internal branches and with two long-branched clades, the Termitoxeniinae and *Chonocephalus* (Fig. 1). Most branches of the tree are not well-supported by non-parametric bootstrap analysis or by Bayesian inference. This lack of resolution is perhaps due to the

high AT-bias in the sequences; this bias effectively reduces the number of character states from four to two. This result may also reflect a rapid divergence of phorid lineages during the evolutionary history of this taxon. We suggest that further work using different genes and more taxa might help better resolve relationships among the phorids.

Nevertheless, the relationships suggested by the tree in Fig. 1 are of considerable interest. The separation of the Ephydridae (Schizophora) from the Drosophilidae, and the grouping of the former with Lonchopteridae, Opetiidae, and Phoridae (Aschiza) is at variance with current classifications (Cumming *et al.* 1995, Zatwarnicki 1996, Collins & Wiegmann 2002b). Likewise the separation of the Lonchopteridae, Opetiidae, and Phoridae (Aschiza) from the Platypezidae (Aschiza) splits the Aschiza into two separate clades, contrary to current classifications.

To assess these results we performed a Shimodaira–Hasegawa test to compare the likelihoods of the tree shown in Figure 1 with the best trees found under two different null hypotheses; that the Aschiza are monophyletic and that the Schizophora are monophyletic (Shimodaira & Hasegawa 1999). The results are shown in Table 2. Neither hypothesis is rejected. The paraphyly of the Series Aschiza and Schizophora suggested by our results must therefore be considered a hypothesis in need of further testing rather than a conclusive result.

TABLE 2. Results from a Shimodaira–Hasegawa test in which the likelihood of the best tree found under the constraint shown is compared to the best tree found for the entire data set (Figure 1). The likelihood of the best tree under the constraint, the difference between that likelihood and the likelihood of the tree shown in Figure 1, and the results of the statistical test, shown as a probability (P), are presented. The P values shown, between 0.3 and 0.6, do not reject the null hypothesis trees, so we cannot say that the tree shown in Figure 1 is statistically better than any of the alternative topologies suggested by the constraints. For each constraint the best tree was found using PAUP* with the same iterative method used to find the best tree for the entire data set, but with the search restricted to only those trees that conformed to the constraint.

Constraint	-ln L	Difference –ln L	P
Best tree	5910.51681	-	-
Schizophora are monophyletic	5915.11348	4.59667	0.554
Metopinininae are monophyletic	5916.99524	6.47843	0.428
Aschiza are monophyletic	5919.28303	8.76623	0.337

The families Opetiidae (*Opetia*) and Platypezidae (*Protoclythia*) have usually been considered basal in the Aschiza. Historically the Opetiidae were included within the Platypezidae, but recent workers have separated the two families, considering them closely related members of the superfamily Platypezoidea, which also includes the Lonchopteridae (Cumming *et al*. 1995, Zatwarnicki 1996). Figure 1 breaks up the Platypezoidea by placing the Platypezidae at the base of the tree. However, as we cannot reject an alternative hypothesis of a monophyletic Aschiza we cannot say that our results conclusively support the break up of the Platypezoidea.

In a recent analysis of brachyceran relationships using 28S rDNA sequences Collins & Wiegmann (2002b) found very similar relationships among the Aschiza. In their analysis the Opetiidae and Lonchopteridae grouped together at the base of the Aschiza, with the Platypezidae and Phoridae branching subsequently. Our results differ in that the Platypezidae are more basal than the Opetiidae/Lonchopteridae clade.

The family Phoridae is supported by both Bayesian posterior probabilities and non-parametric bootstrapping. Traditionally the phorids used in our study were assigned to two clades, the Phorinae (*Phora-Spiniphora* on Figure 1) and the Metopininae (*Puliciphora-Chonocephalus* and *Megaselia* on Fig. 1). The Phorinae are monophyletic on this tree, but the branch leading to this clade has only weak statistical support. The Metopininae are paraphyletic on the tree in Figure 1, with a *Puliciphora-Metopina-Chonocephalus* clade grouping with the Phorinae and excluding the two *Megaselia* (tribe Gymnophorini). Again, we used a Shimodaira–Hasegawa test to evaluate the null hypothesis of a monophyletic Metopininae. The likelihood for this alternative tree was not significantly worse than the likelihood of the tree shown in Figure 1. Our results clearly support the monophyly of the Phoridae, but neither strongly support nor dispute the traditional division of the family into the clades Phorinae and Metopininae.

Chonocephalus groups with other members of the Metopinini, but as there is no support for the clade Metopininae (comprising the Metopinini and Gymnophorini) or any of the branches within the Metopinini except the branch joining the male and female *Chonocephalus*, this relationship must also be considered in need of further testing.

Figure 1 places the two aberrant termitophilous subfamilies (associated with the fungus gardens of the Macrotermitinae), namely the Thaumatoxeninae (represented by *Thaumatoxena*) and Termitoxeniinae (represented by the Oriental *Clitelloxenia* and the Afrotropical *Termitophilomyia*), as closely related subordinate groups of the Phorinae. This relationship could be the result of long branch attraction in the phylogenetic analysis, and should be tested further. However, this phenomenon is more often observed with maximum parsimony rather than maximum likelihood analyses (Swofford *et al*. 2001), and the association of both subfamilies with termite fungus gardens lends ecological support to the result shown, so we are inclined to believe the relationship as shown, and this leads to reconsideration of the taxonomy of the Thaumatoxeninae and Termitoxeniinae. Previous authors have treated these groups as separate families, and Rohdendorf (1974) even placed the Termitoxeniinae in a separate infraorder (Disney 1992, Disney & Cumming 1992). The case for treating these two clades as distinct subfamilies would now seem weakened. Perhaps their status would be better expressed at the level of tribes, or even a lower category still. Furthermore, Figure 1 suggests that both groups show affinity with *Dohrniphora*, a genus with numerous species whose larvae either inhabit the fungus gardens of termites of the subfamily Macrotermitinae or else parasitize their termite hosts (Disney 1994, Disney & Darlington 2000).

Figure 1 suggests that the Phorinae includes a number of distinct major clades, but the resolution is poor. It would seem wise, therefore, to refrain from reassessing the proposed tribes within the Phorinae until a consensus emerges as to the phylogenetic affinities of the constituent genera. Our analyses contribute little to the resolution of current debates regarding the inferred ground plan of the Phoridae (recently summarized by Disney 2003). However, our conclusions can be seen to have made a contribution with regard the affinities of some genera within the family and have especially clarified the affinities of the aberrant termitophilous Thaumatoxeninae and Termitoxeniinae in particular.

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