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Phylogeny of the Sciaroidea (Diptera) As Estimated from 16S and 12S Ribosomal RNA Sequences

by

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Abstract

Phylogeny of the Sciaroidea (Diptera) As Estimated from 16S and 12S Ribosomal RNA Sequences

by

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Doctor of Philosophy in Entomology

UNIVERSITY OF CALIFORNIA, BERKELEY

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The phylogeny of the superfamily Sciaroidea (Diptera) is estimated from 16S and 12S ribosomal RNA sequences in a series of analyses using various combinations of the sequence data. A historical overview of the classification and phylogeny of the taxon is provided and current hypotheses are discussed. Alignment of the 12S sequences was achieved by constructing models for the secondary structure of the RNA molecule. These models are included in the appendix.

Previous analyses of the Sciaroidea have assumed the Bibionidae to be the taxon's sister group. This assumption was tested by initial analyses in which the non-bibionomorph families Tipulidae , Culicidae, Simuliidae, Anisopodidae, Ragionidae, Empididae, Drosophilidae , and Calliphoridae were included. In these analyses the Bibionidae consistently emerged on the same clade as the sciaroid taxa and in the majority of cases formed the sister group of the Sciaroidea.

A second group of analyses based on the 12S data used the Bibionidae as the outgroup for an investigation of the relationships within the Sciaroidea. The Cecidomyiidae and Ditomyiidae emerge as the basal-most members of the superfamily with the Cecidomyiidae as the sister group of the remaining sciaroid taxa (the fungus-gnat families). The Sciaridae branch next from the main stem

of the phylogeny just above the Ditomyiidae, followed in order of sequence by the Keroplatidae, Diadocidiidae, Bolitophilidae, and Mycetophilidae (*sensu stricto*).

A third set of analyses using only 12S RNA sequences investigated the phylogenetic relationships within the Mycetophilidae using the Bolitophilidae as the outgroup. The Mycetophilidae has a three-clade structure with one clade consisting of the subfamily Mycetophilinae which further splits into two clades corresponding to the tribes Exechiini and Mycetophilini of present classifications. The Mycomyinae and Sciophilinae emerge together on a common clade, and the third clade includes taxa presently classified in the Gnoristinae and Leiini. The gnoristine-leiine clade splits into three subclades, one of which includes *Coelosia, Hadroneura, Synapha* (all Gnoristinae) and *Leia* (Leiinae); a second subclade consists of *Gnoriste* and *Boletina* (Gnoristinae) and *Docosia* (Leiinae): finally *Tetragoneura* and *Acompterella*, both genera presently included in the Leiinae, occupy a third subclade.

11/8/99

Chair Date

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INTRODUCTION

In recent years major efforts have been under way to reexamine and resolve basic phylogenetic relationships within the Diptera, most particularly those relationships affecting the higher classification, i.e., the relationships of infraorders to one another and, within infraorders, the relationships between superfamilies or families. For much of the latter part of this century, the phylogentic views of Hennig (1954, 1973,) have been the accepted dogma on dipteran phylogeny. Wood and Borkent's (1989) phylogenetic analysis of the "Nematocera" and their infraordinal revision based thereon has stimulated efforts to examine novel sources of data for phylogentically informative characters (Courtney 1991, Oosterbroek and Theowald 1991, Krzeminski 1992, Sinclair 1992, Oosterbroek 1995, Michelsen 1996, Friedrich and Tautz 1997). Although these studies have greatly advanced understanding of dipteran phylogeny, many areas are still poorly understood or lack convincing character support. One area much in need of further study is the phylogeny in the infraorder Bibionomorpha, and especially the relationships among the families of the Sciaroidea (=Mycetophiloidea). This superfamily, consisting of small terrestrial nematoceran flies commonly known as gall midges (family Cecidomyiidae) and fungus gnats (families Ditomyiidae, Sciaridae, Keroplatidae, Diadocidiidae, Bolitophilidae, Lygistorrhinidae, and the Mycetophilidae *sensu stricto*), encompasses the vast majority of species in the Bibionomorpha.

The Bibionomorpha, or at least a major portion thereof, has long been accepted as a monophyletic taxon, even though few convincing synapomorphies have been cited in support of this view. The inclusion in the infraorder of the families Anisopodidae, Scatopsidae, Axyiomyiidae, and Synneuridae has been

rejected by Wood and Borkent (1989) and Oosterbroek and Courtney (1995). Nonetheless, a "core group" consisting of the Pachyneuridae +Bibionidae+Sciaroidea (=Bibionomorpha *sensu stricto*) is still strongly felt to be monophyletic, despite the paucity of synapomorphies. The few studies that have touched on the phylogenetic relationships within the Bibionomorpha and/or the Sciaroidea have focused either on the basal branches in the infraorder (Amorim 1993) or have not included in the analysis as separate ingroup taxa most of the major sciaroid groups (Blaschke-Berthold 1994, Wood and Borkent 1989, Oosterbroek and Courtney 1995). These latter analyses have followed the traditional classification of recognizing only two families of fungus gnats, the Sciaridae and Mycetophilidae, including the Ditomyiidae, Keroplatidae, Diadocidiidae, Bolitophilidae, and Lygistorrhinidae in the Mycetophilidae as subfamilies, and treating this complex as one unit in the analysis, although Mycetophilidae in this broader sense is almost certainly paraphyletic, as even the authors acknowledge. Matile's analyses (1997, 1990b) are a notable exception, and represent the most comprehensive study of the Sciaroidea to date. They are, however, based on a limited number of characters.

A more taxonomically inclusive analysis, to complement as well as test Matile's phylogeny, is desirable, especially in light of recent suggestions by Wood and Borkent (1989) of a sister-group relationship between the Cecidomyiidae and Sciaridae based on the rather bizarre cytology found in these two groups (see White 1973 and Matuzzewski 1982 for an in-depth discussion and extensive bibliography). Wood and Borkent, followed by Oosterbroek and Courtney (1995), provisionally place this Cecidomyiidae + Sciaridae clade as the sister-group to the "Mycetophilidae", i.e. the rest of the Sciaroidea. Sciarid larvae, however, possess several synapomorphies in common with the "higher" fungus gnats, such as the broad, membraneous, one-segmented antennae and the rounded, serrate

maxilla (Madwar 1937, Plachter 1979), indicating that the Sciaridae are not the basal-most lineage in the "fungus gnat" clade. If this is so, Wood and Borkent's hypothesis implies that the gall midges are highly derived fungus gnats instead of being the sister-group to the rest of the Sciaroidea, as has been generally supposed. Matile's analysis (1997) does not support a sister-group relationship between the Cecidomyiidae and the Sciaridae.

In this study sequence data from the gene for mitochondrial 12S rRNA (small subunit) is used in a phylogenetic analyses of the Sciaroidea. This analysis is more inclusive than the above studies and, further, goes beyond the scope of these by also examining subfamilial relationships in the largest fungus gnat family, the Mycetophilidae *sensu stricto*. Before analyzing the relationships in the Sciaroidea, however, sequence data from both the 12S and 16S (mitochondrial, large subunit) rRNA genes are used, separately and together, to assess the appropriateness of using the Bibionidae as the outgroup for an analysis of the Sciaroidea, as was assumed by Matile and Blaschke-Berthold (1994). As mentioned above, few convincing characters support the monophyly of the Bibionomorpha s.s. This study will show that, of the dipteran families included in the analysis, the Bibionidae are phylogenetically closer to the Sciaroidea and are therefore a reasonable outgroup for the analysis of this group. The latter analysis is not intended to be a comprehensive study of the Bibionomorpha; some of the families that at one time or another have been included in the Bibionomorpha have not been included here. Therefore the results of this analysis do not preclude the possibility that one or more of the excluded families are closer to the Sciaroidea than is the Bibionidae. The scope and objectives of these analyses will be presented in greater detail after a historical overview of the systematics and evolution of the Sciaroidea.

Prior to this study, the 12S rRNA gene has not been used to examine phylogeny in the Diptera, although it has found widespread use in many other taxonomic groups, expecially among vertebrates. Phylogenetic analyses in the Diptera have been based mostly on morphological data. Molecular data, however, is starting to be more widely utilized. The ribosomal RNA genes in particular contain regions that evolve at slower rates than many other parts of the genome and therefore are more likely to preserve information for resolving the deeper branches in a phylogeny such as those pertaining to the sciaroid families. Particularly in cases where morphology has not resolved relationships, or where the relationships are either ambiguous or weakly supported, ribosomal RNA sequences, as true of sequences from other genes, are a potentially rich source for additional phylogenetically informative characters, albeit subject to many of the same pitfalls and drawbacks as morphological data. Despite their potential for resolving relationships between distantly related taxa, ribosomal RNA genes thus far have been used in the Diptera primarily to examine the closer relationships within genera or among closely related genera within families (Caccone et al. 1996, Miller et al. 1996, Russo et al. 1995, Polandakis and Solignac 1993, DeSalle 1992; Raich et al. 1993, Porter and Collins 1996, Tang et al. 1995, Xiong and Kocher 1993; McPheron and Han 1997, Vossbrinck and Friedman 1989); only two recent studies have utilized rRNA genes to examine higher level relationships (Pawlowski et al. 1996, Culicomorpha; Friedrich and Tautz 1997, dipteran infraorders, both studies based on 28S rRNA). 12S and 16S rRNA were selected for this study not only because of their sequence conservatism but also because sufficient DNA template for amplification of these genes is easily obtainable from very small tissue samples, thereby allowing preservation of the specimens from which the sequence data were procured.

HISTORICAL OVERVIEW: SYSTEMATICS AND PHYLOGENY OF THE SCIAROIDEA

The fungus gnats, the Sciaroidea minus the Cedidomyiidae, have long been known by the name Mycetophiloidea . The name Mycetophiloidea is based on *Mycetophilites* Newman, 1834. But, as discussed in Matile (1997), an earlier use of *Sciaraedes* by Billberg, 1820, was uncovered and applied by McAlpine et al. (1981), who also included the Cecidomyiidae in the superfamily. The priority clauses in the Code of Zoological Nomenclature mandate the use of the earlier name.

Differences in opinion among present systematists regarding the classification in the Sciaroidea mostly concern the ranking of more or less well-circumscribed fungus-gnat taxa[1]. Most European and South American specialists on the group recognize 7 families of fungus gnats (Ditomyiidae, Sciaridae, Keroplatidae, Diadocidiidae, Bolitophilidae, Lygistorrhinidae, and the Mycetophilidae) (Papavero 1977; Väisänen 1984; Matile 1997, 1990b, 1989,1981, and papers after 1979; Amorim 1992; 7-family system implied in Zaitsev 1994 and his other papers), with the recent addition of the Cecidomyiidae bringing the total number of families in the superfamily to 8. A few classifications recognize still more families (Soós and Papp 1986, Ostroverkhova 1979; Krivosheina et al. 1986; Krivosheina and Zaitsev 1982) by elevating also the Macrocerinae (family Keroplatidae) or Manotinae (Mycetophilidae) or both, and sometimes even the Sciophilinae (Mycetophilidae), to family rank. Some systematists (Oosterbroek and Courtney 1995; Colless and McAlpine 1991; Wood and Borkent 1989; Vockeroth

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[1] A few authors treat the Cecidomyiidae as several families in the superfamily Cecidomyioidea (=Itonidoidea)(Shchervakov et al. 1995; Kovalev 1987a; Hennig 1973,1954; Rohdendorf 1974, 1964). It is more typical to regard the gall midges as a single monophyletic family. A discussion of the phylogeny within the Cecidomyiidae is beyond the scope of this paper.

1981; Hutson et al. 1980; Colless and Liepa 1973; Colless 1970; Laffoon, 1965), mostly in English-speaking countries, recognize only two families of fungus gnats, the Sciaridae and the Mycetophilidae.

Regardless of the differences in ranking, the contemporary higher classifications of the fungus gnats are dependent on Edward's 1925 generic revision. Prior to Edwards, the most comprehensive revisionary work on fungus gnats was that of Winnertz (1863, 1867). Winnertz, primarily using characteristics of wing venation and trichiation, location of ocelli, and setation on legs, errected the Mycetobiinae, Bolitophilinae, Diadocidiinae, Keroplatinae, Macrocerinae, Sciophilinae and Mycetophilinae as divisions in the Mycetophilidae and separated the Sciaridae into its own family. In this, he was the first systematist to accord family rank to the Sciaridae. Most subsequent early systematists accepted Winnertz's classification, but varied in their treatment of the Sciaridae, some treating it as a family (Skuse 1889, 1891; Marshall 1895; Rübsaamen 1894, 1898; Meunier 1904; Lundström 1906; Johannsen 1909; Malloch 1917) while others including it in the Mycetophilidae as a subfamily (Theobald 1892; Lundbeck 1898; Williston 1908; Brunetti 1912; Johannsen 1910)[2]. A few systematists ignored Winnertz's system altogether while retaining the sciarids in the Mycetophilidae (van der Wulp 1877; Osten-Sacken 1878; Aldrich 1905), and some later workers also included *Pachyneura* Zetterstedt[3] under the subfamily Pachyneurinae. Enderlein (1911), in a rather remarkable display of phylogenetic thinking quite unusual for the pre-Hennigian era, saw in the complete eye bridge present in the Cecidomyiidae and the Sciaridae but lacking in other fungus gnats,

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[2] This list of citations is not exhaustive but includes the more important papers.

[3] *Pachyneura*, together with the monotypic genera *Pergratospes* Krivoshein and Mamaev and *Cramptonomyia* Alexander are presently classified in the bibionomorph family Pachyneuridae.

a derived trait phylogenetically uniting these two. He further postulated a sister-group relationship between the sciarids and the cecidomyiid subfamily Lestremiinae, and put these two taxa together in an expanded Sciaridae, which then formed the sister-group to the rest of the cecidomyiids. His concept of the Sciaridae, however, was universally rejected.

The only significant change to Winnert's classification prior to Edward's revision concerned the constitution of the subfamily "Mycetobiinae". Edwards (1916), after a detailed study of adult morphology, transferred *Mycetobia* Meigen and *Mesochria* to the Anisopodidae. He accepted Landrock's designation of Ditomyiinae for the remaining "mycetobiine" genera. Edwards' conclusions about the affinities of *Mycetobia* were later substantiated by Keilin's (1919) study of the larvae of *Mycetobia* and the two ditomyiine genera *Symmerus* Walker and *Ditomyia* Winnerz. Keilin further concluded that the ditomyiine larvae differed sufficiently from other fungus-gnat larvae to warrant the recognition of the Ditomyiinae as a seperate family. Edwards (1921) rejected this proposal since it would necessitate giving family status also to the Diadocidiinae, Bolitophilinae, and Keroplatinae, which in his opinion was not justified by adult morphology.

In his 1925 revision of the Mycetophilidae, Edwards recognized the same subfamilies as Winnertz, but included the Sciarinae and erected two new subfamilies, the Manotinae for *Allactoneura* Mik and *Manota* Williston and Lygistorrhininae for the genus *Lygistorrhina* Skuse (including *Probolaeus* Williston and the fossil *Palaeognoriste* Meunier), and excluded the Pachyneurinae, which he had earlier recognized as a separate family more closely related to the Anisopodidae. His revision thus recognized ten subfamilies: Ditomyiinae, Bolitophilinae, Diadocidiinae, Keroplatinae, Macrocerinae, Lygistorrhininae, Sciarinae, Manotinae, Sciophilinae, and Mycetophilinae. Additionally, Edwards substantially narrowed the concept of the Mycetophilinae by transfering to the

Sciophilinae all the genera in Johannsen's (1911) mycetophiline series 1, that is genera included in the Mycetophilinae in which the wing-membrane microtrichia and tibial setae are randomly arranged. In contrast, the genera retained by Edwards in the Mycetophilinae, corresponding to Johannsen's (1911, 1912) series 2, have wing michrotrichia and tibial setae arranged in straight rows. Edwards correctly recognized these traits as characters indicative of the monophyly--in his terminology, the "natural assemblage"--of the Mycetophilinae in this more restricted sense. Edwards' final contribution was to divide the two largest subfamilies, the Sciophilinae and Mycetophilinae, into tribes, the former into the four, the Mycomyini, Sciophilini, Gnoristini, and Leiini, and the latter into two, the Mycetophilini and Exechiini.

Edwards' classification was widely accepted by most dipterists working on the family (Tonnoir 1929; Tonnoir and Edwards 1927; Shaw 1935; Fisher 1937, 194; Fremann 1951). Some workers, however, continued to accord the sciarids family rank (Landrock 1927, 1940; Lengersdorf 1928, 1930; Frey 1942, 1948). Many authors following the two-family system indicated the close affinity of the two families by classifying them together in the superfamily Mycetophiloidea. Crampton (1925) suggested that the Cecidomyiidae and Mycetobiidae should be also included in the Mycetophiloidea; his proposal was not accepted by other authors. Madwar (1937), after a detailed study of larval morphology, gave the Ditomyiidae family rank, but retained the Sciarinae in the Mycetophilidae as a subfamily. Shaw (1948) concluded that the Sciarinae needed to be regarded as a separate family, since they were in his opinion more primitive than other mycetophilids in the shape of the katepisternum, the course of the mesosternal suture, and the presence of a midpleural pit. He reaffirmed this viewpoint in a subsequent paper (Shaw and Shaw 1951), but one year later (Shaw and Fisher 1952) changed his mind and again treated the sciarids as a mycetophilid subfamily.

Since the early 1960's the Sciaridae have been consistently treated as a family separate from the Mycetophilidae.[4]

In their 1951 paper, Shaw and Shaw proposed two new tribes in the Sciophilinae, Cycloneurini for *Cycloneura* Marshall and *Procycloneura* Edwards and the Allactoneurini for *Allactoneura* De Meijere. Previously, these the first two genera were included in the Leiini and the latter in the Manotinae. Later authors have generally suppressed these tribes and included all three genera in the Leini. The tribe Allactoneurini was reinstated for *Allactoneura* by Zaitsev (1981) and accepted by Matile (1988), but recently rejected by Söli (1996), who again included *Allactoneura* in the Leiini.

Hennig (1948, 1954), the first to propose a detailed higher classification of the Diptera on the basis of phylogenetic principles, assigned family rank to all of Edwards' subfamilies, placing them together along with the Sciaridae in the Sciaroidea (=Fungivoroidea[5], Mycetophiloidea). Similarly, he promoted the sciophiline and mycetophiline tribes of Edwards to subfamily rank in their

[4] One curious phenonomon likely connected with the taxonomic disassociation of the Sciaridae from the rest of the fungus gnats has been the almost total separation of the specialists working on the two groups. Before 1960, systematists who regarded the Sciaridae as a family rarely if at all involved themselves in the systematics of the Mycetophilidae. On the other hand, those who viewed the sciarids as a subfamily tended to regard the sciarids as within the scope of their studies. Since the 1960's, each of the two taxa have had their own group of specialists with very little crossover between the two. Tuomikoski is a notable exception.

[5] Hennig's early papers used names based on Meigen 1800. Meigen published generic names for a wide array of Diptera, including *Fungivora, Lycoria, and Itonida*, then renamed these genera *Mycetophila, Sciara, and Cecidomyia*, respectively in a later paper (Meigen 1803) . The 1803 names had enjoyed widespread usage long before the rediscovery of the earlier names. The confusion over the Meigen names was finally resolved by the International Commission of Zoological Nomenclature (Opinion 678) in 1963 which suppressed the usage of the 1800 names and all their derivatives.

respective "families". Hennig's classification was followed by Rodendorf (1961, 1964) and Tollet (1959).

Tuomikoski (1966b, 1966c), in his proposals for the classification of the Mycetophiloidea, accepted the family rank of the Ditomyiidae, Diadocidiidae, Bolitophilidae, Keroplatidae, and Sciaridae; he included the Macrocerinae and Lygistorrhinae of Edwards in the Keroplatidae and expanded the limits of the Mycetophilidae over that of Hennig to include the Sciophilinae, Mycetophilinae, and, provisionally, the Manotinae of Edwards. Mycetophilidae in this sense equates with Mycetophilinae *sensu* Malloch (1917). Hennig (1973) accepted this classification and further gave the sciophiline tribes of Edwards subfamily rank (Mycomyinae, Gnoristinae, Sciophilinae, and Leiinae), something hinted at in Tuomikoski (1966b, 1966c) but not explicitly adopted by him, and retained the Mycetophilinae of Edwards as a subfamily consisting of the tribes Exechiini and Mycetophilini. Thompson (1975), critical of Tuomikoski's placement of the Lygistorrhinidae in with the Keroplatidae, restored the group as a separate family.

Although the two-family classification has enjoyed broad acceptance throughout much of the latter half of the twentieth century and is still adhered to by many dipterists, most current specialists on the systematics of the group have adopted the seven-family system modified from Tuomikoski. This is the classification followed in this study. As mentioned above, the Cecidomyiidae is now included in the superfamily-- a suggestion earlier made by Crampton (1925)--and Sciaroidea is used to designate the group in place of the better known term Mycetophiloidea . The present classification of the Sciaroidea is summarized in table 1.

Family Cecidomyiidae	Family Mycetophilidae
Family Ditomyiidae	Subfamily Mycomyinae
Family Sciaridae	Subfamily Sciophilinae
Family Keroplatidae	Subfamily Gnoristinae
Family Diadocidiidae	Subfamily Leiinae
Family Bolitophilidae	Subfamily Manotinae
Family Lygistorrhinidae	Subfamily Mycetophilinae
	Tribe Exechiini
	Tribe Mycetophilini

Table 1. Classification of the Sciaroidea.

PHYLOGENY

One of the earliest presentations of a phylogeny including sciaroid taxa is that of Enderlein (1911). Although he offered little in the way of character support for most of the branches in his phylogeny (figure 1A), his work is significant in that he was the first to propose a close phylogenetic relationship between the Sciaridae and the Cecidomyiidae. His phylogeny consisted of two major branches, a Cecidomyiidae-Sciaridae-Scatopsidae-Bibionidae branch with the Bibionidae as it's basal-most member, and an opposing branch which included the rest of the fungus gnats plus the Pachyneuridae. Noting the similarity in wing venation, Enderlein considered the Sciaridae to be most closely related by descent to the gall midge subfamily Lestremiinae; he in fact included the latter with the Scaridae in his classification. The rest of the Cecidomyiidae were placed as the next closest phylogenetic relatives to the Lestremiinae + Scaridae, and in turn the Scaptopsidae and the Bibionidae were located more basally. Enderlein put much emphasis on the possession of a complete-eye bridge as a justification for the Scatopsidae + Cecidomyiidae + Sciaridae/Lestriminae branch. On the fungus-gnat branch, the Mycetophilidae were considered to be a rather primitive group

and the Ditomyiidae (included in his Mycetobiidae) to be derived. This is by and large the reverse of almost all later phylogenies.

Edwards (1925, 1926) rejected Enderlein's view, pointing out that according to natural history and anatomy of the larvae, the Lestremiinae are more allied with other Cecidomyiidae, and the Sciaridae have much in common with other fungus gnats, especially with the Mycetophilidae (his Mycetophilinae and Sciophilinae). The loss of spiracle 8 in the larvae of fungus gnats and sciarids and it's retention in the gall midges further argued against a close relationship between cecidomyiids and sciarids. Edwards recognized the loss of the 8th spiracle as a derived state. Given his acceptance of the irreversibility of evolution, the absence of spiracle 8 in the sciarids and many other fungus-gnat groups, and its presence in gall midges, including the Lestremiinae, clearly indicated to him that the sciarids were phylogenetically much closer to such fungus-gnats groups as the Mycetophilidae s.s. The gall midges belonged outside of this fungus-gnat branch. [6]

Edwards made few other phylogenetic statements. In his view the Diptera had split into three main branches by the time of the Jurassic with one of these branches consisting of the fungus gnats, gall midges, bibionids, scatopsids (Edwards 1926), taxa that in later authors' views comprised the Bibionomorpha. With regard to the phylogeny among the fungus gnats, Edwards (1925) regarded the Sciarids and the Leiinae as having a common ancestry, and that the Leiinae

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[6]Edwards was rather remarkable in that in his phylogenetic considerations he recognized derived characters, which he termed "coenogenetic", as indicators of a closer phylogenetic relationship. He also recognized that one or more of the related taxa possessing the archaic trait, his "palingenetic" characters, could be more phylogenetically related to the derived group than to any of the other "archaic" groups. His use of "coenogenetic" and "palingenetic" character states corresponds closely to Hennig's "apomorphic" and "symplesiomorphic" characters. This recognition on his part, however, goverened his notions on phylogeny but not his classification.

were likely the sister-group to the Mycetophilinae on the basis of general appearance but especially the close proximity of the lateral ocelli to the margin of the compound eye in both groups (Edwards 1925). Edwards considered the Gnoristinae, Leiinae, and Mycetophilinae each to be a natural group, i.e., monophyletic.

Shaw (1948) and Shaw and Shaw (1951), basing their conclusions on the structure of the thoracic sclerites, and Fisher (1937 and personal communication in Shaw 1948), using the structure of male genitalia, developed more extensive ideas about phylogeny of the fungus gnats. Shaw's and Fisher's ideas were in agreement except on the relationships of Ditomyiidae and the Sciaridae. Fisher placed the Ditomyiidae as the basal-most group in her phylogeny; Shaw regarded them as more advanced than some Keroplatidae. Shaw and Fisher also differed drastically in their views regarding the Sciaridae. Shaw thought that the Sciaridae were the most primitive fungus-gnat taxon; Fisher, on the other hand, thought they were highly derived, and regarded them as having been derived from the Leiinae. She differed from Edwards (1925) in regarding the Mycomyinae, and not the Leiinae, as the intermediate group between the Mycetophilinae and the rest of the "sciophiline" groups (Mycomyinae, Gnoristinae, and Sciophilinae s.s.). Shaw's and Fisher's phylogenetic views are summarized in the cladogram in figure 1B, drawn from an interpretation of their textual material.

Rodendorf (1946), approaching the problem of phylogeny from a paleontological viewpoint, produced a phylogeny of the Oligoneura. In his view the Pachyneuridae (including Anisopodidae in part) and the Bolitophilidae, both considered to be relict groups that had diverged in the late Triassic, were the basal-most branches (figure 2) in the phylogeny. The rest of the Sciaroidea along with two non-sciaroid taxa occupied a single clade. This large clade was subdivided into two more-or-less equally sized subclades, one of which included

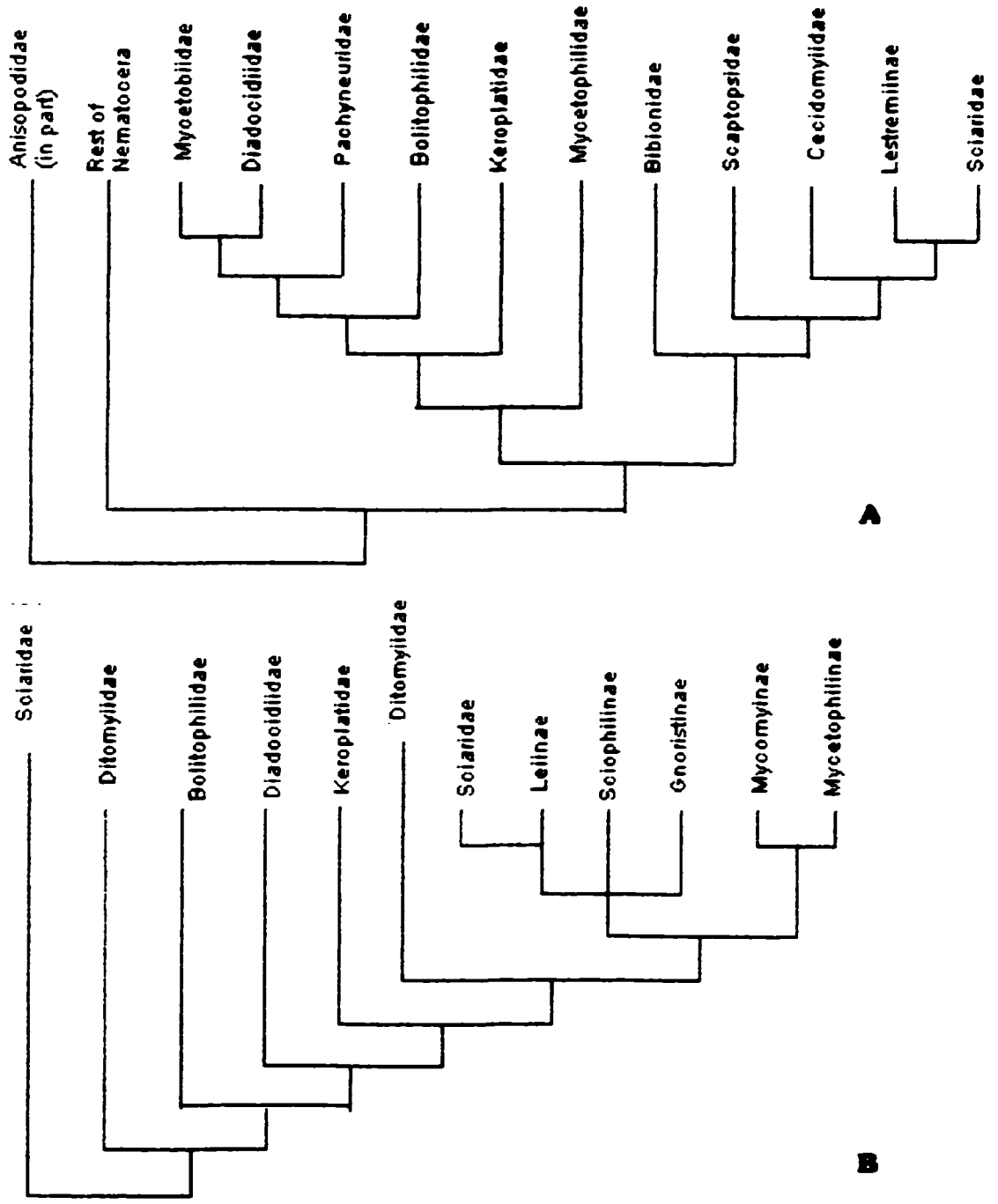

Figure 1. Early proposals for the phylogeny of the Sciaroidea. A. Enderlein's (1911) phylogeny (modified). Enderlein recognized two major branches in what roughly corresponds to the Bibionomorpha. The Pachyneuridae consisted of the genus *Pachyneura*, the Mycetobiidae consisted of the Ditomyiidae + Mycetobia, the latter is now included in the Anisopodidae. Enderlein united the Scatopsidae + Cecidomyiidae + Lestremiinae (Cecidomyiidae) + Sciaridae on the basis of the presence of a complete eye-bridge. B. Fungus gnat phylogeny according to Fisher and Shaw. Shaw and Fisher agreed on the relationships among fungus gnats except for the position of the Ditomyiidae and the Sciaridae. Shaw (shown in blue) believed the Ditomyiidae to be more "advanced" than some Keroplatidae and the Sciaridae to be the most primitive group of fungus gnats. Fisher (shown in red) beleived that the Ditomyiidae were the most basal group and that The Sciaridae had evolved from a leiine ancestor (indicated in the figure by the Sciaridae branch coming off at right angles from the Leiinae branch).

the Sciaridae, Lygistorrhinidae, Manotinae (as a family), Mycetophilidae and the family Allactoneuridae for the single genus *Allactoneura*, which, as discussed above, is usually included in the Leiinae. According to Rodendorf the Manotinae and Lygistorrhinidae had arisen rather recently from the Mycetophilidae. The second subclade diverged into two branches, one of which led to the Cecidomyiidae + Diadocidiidae, and the other of which included the two non-sciaroid taxa, Hesperininae (Bibionidae) and *Mycetobia* plus relatives (Anisopodidae), and the sciaroid Ditomyiidae and Keroplatidae. The rest of the bibionids and the Axymyiidae occupied a branch immediately basal to the sciaroids. Finally, the Scatopsidae + Synneuridae were shown as an early divergence splitting off between the Bolitophilidae and the bibionids/axymyiid branches.

Rodendorf's 1946 phylogeny was his most comprehensive with regard to the familial relationships in the Bibionomorpha. In later papers (Rodendorf 1961, 1962, 1964), his primary occupation was with the relationships between superfamilies and infraorders, and the phylogenies he produced rarely depicted family-level relationships. Nonetheless, judging from the text in his papers, his ideas regarding the family-level relationships in the Bibionomorpha did not deviate much from his 1946 conclusions. Recently, Courtney (1991) and Oosterbroek and Courtney (1995) included an interpretative cladogram (reproduced here in figure 3A) to illustrate Rodendorf's 1964 (English translation 1973) published phylogeny. Their figures, however, do not accurately reproduce Rodendorf's figure for the portion of the phylogeny within the Bibionomorpha. Nowhere in Rodendorf's figures are the Cecidomyiidae and Sciaridae to be found as sister-groups; it is also not in accord with his concepts to present the Bibionidae as the sister-group to the Scaptopsidae-Synneuridae +

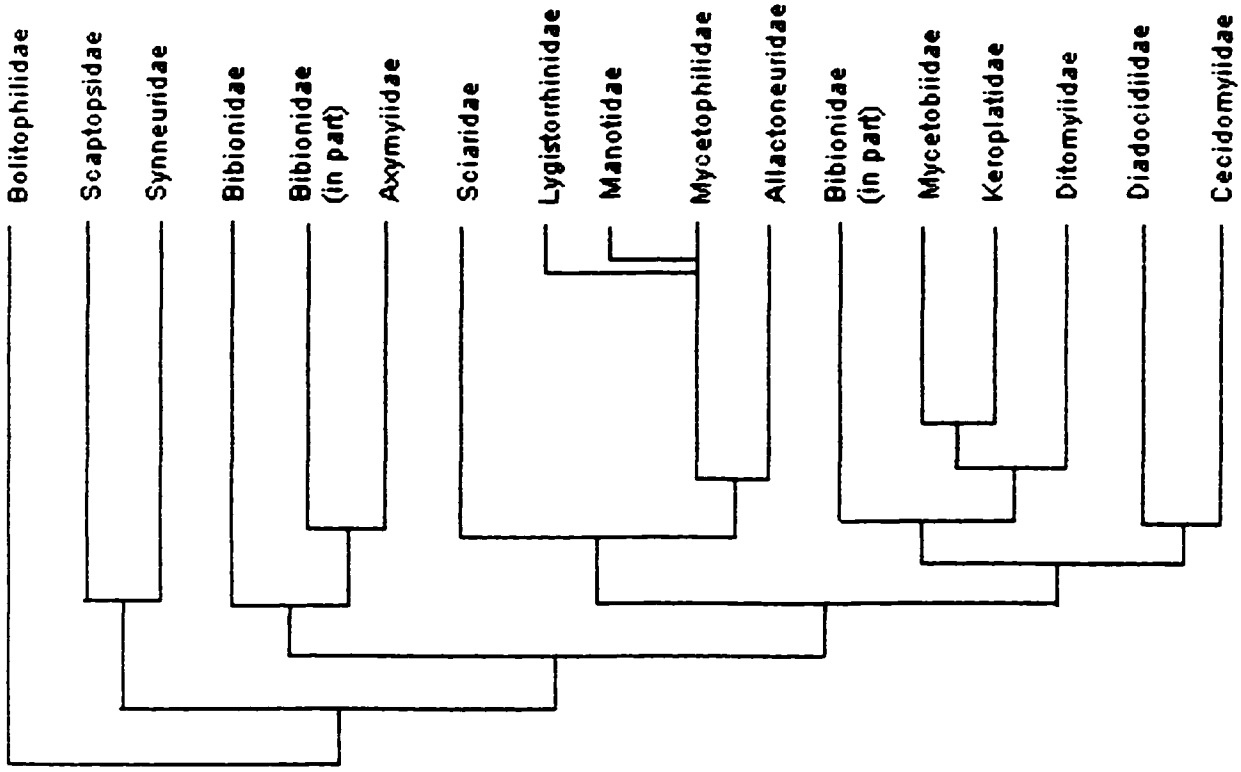

Figure 2. The phylogeny of the Oligoneura according to Rohdendorf's 1946 scheme (redrawn). Rohdendorf regarded the Bolitophilidae as as a relect group little modified from the forms ancestoral to the Oligoneura. The Sciaroidea and Bibionidae as treated in modern classifications appear polyphyletic in this scheme. The Bibionidae were recognized as three distinct families, each family corresponding to one of the bibionid branches shown in the figure. The Axyiomyiidae have arisen from a bibionid ancestor. The Lygistorrhinidae and Manotinae are shown as side branches of the main Mycetophilidae branch to indicate Rohdendorf's belief that these two groups have their evolutionary origin from within the Mycetophilidae.

Cecidomyiidae-Sciaridae nor to place the Mycetophilidae *sensu lato* basally to the Bibionidae as depicted in their figure. Oosterbroek and Courtney apparently included the Bolitophilidae with the Mycetophilidae *sensu lato*, something which Rodendorf did not do. This latter inclusion on their part may have led them to misinterpret Rodendorf in placing the Mycetophilidae s. l. as a basal bibionomorph group. It is difficult to translate Rodendorf's figures into a cladogram in such a way as to remain accurate to his concepts, since a cladogram generally assumes

that individual branches contain monophyletic taxa or at least taxa whose monophyly is tentatively assumed. Rodendorf's figures are not cladograms, but rather phylogenetic trees superimposed on a time scale in which the branches are represented by shaded areas of variable width intended to depict the relative species-richness of a taxon at any time period. Many of the taxa presented in his diagrams are clearly not implied to be monophyletic. A more accurate depiction, using conventions explained in the figure caption, of his 1964 work is shown in figure 3B. Rodendorf in 1964 believed that the Bibionomorpha had differentiated into three groups during the Triassic, the Bolitophilidea (Bolitophilidae), Rhyphidea (Anisopodidae and Cramptonomyiidae), and the Fungivoridea (Sciaroidea). The Triassic Fungivoridea[7] were not represented by any extant family. He merely recognized fossils from the Triassic as being close to the ancestral forms from which later groups of Fungivoridea evolved and thus included these ancestors in his Fungivoridea. Later in the Triassic the lineage leading to the Bibionidae separated from ancestral fungivorids, and later still, the Scatopsidae/Synneuridae lineage diverged from the ancestral stock. Aside from the Bolitophilidae, Rodendorf did not trace the origin of any extant fungus-gnat group back to a time before the divergence of the bibionid and scatopsid/ synneurid lineages. Although Rodendorf treated the Cecidomyiidae as a separate superfamily which had evolved from some fungivorid ancestor during the Cretaceous, he nowhere proposes a close relationship between the Cecidomyiidae and specifically the Sciaridae. In his 1964 work, Rodendorf did not discuss in detail the familial relationships within his Fungivoridea. Nonetheless he stated that the conclusions to his 1946 paper were still largely valid, and according to these the representation of his phylogeny to include a sister-group relationship between the Cecidomyiidae and Sciaridae is not justifiable. The only

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[7]See footnote 5, page 9.

interpretative differences between his 1946 and 1964 figures is that in the latter figure he extended the shaded area representing the Fungivoridea basally such that the Bibionidae and Scatopsidae/Synneuridae have their origins from ancestors included in the Fungivoridea, and secondly, he reversed the relative order divergence of the Bibionidae and Scatopsidae/Synneuridae from that in his 1946 figure.

It is perhaps not too surprising that the first modern phylogenies for the Diptera originated with Willi Hennig (1948, 1954, 1968, 1969, 1973), who also developed the phylogenetic argumentation that has come to dominate systematics in the latter half of this century. In his earliest work addressing dipteran phylogeny (table 2), Hennig (1948) divided the Nematocera into two "sections", for one of which he introduced the name Bibiomorpha, which he later (Hennig 1954) amended to Bibionomorpha. Within the Bibionomorpha, the Anisopodidae (including Pachyneuridae) was placed as the sister-group to the rest of the bibionomorphs. The latter consisted of two monophyletic clades, the Bibionidae + Scatopsidae (including Synneuridae) and the Sciariformia (= Fungivoriformia, Mycetophiliformia) . Two superfamilies were recognized in his Sciariformia, the Sciaroidea (=Fungivoroidea), the fungus gnats including the Sciaridae, and the Cecidomyiioidea (=Itonidoidea of Hennig, gall midges). Although he was uncertain of the relationships among families within the Sciaroidea, he did not question that the two superfamilies together constituted a monophyletic group. He suspected however, that the Sciaroidea could be paraphyletic; the monophyly of the Cecidomyiidae, on the other hand, was assured on the basis of the highly modified head capsule of the larvae.

Hennig's most comprehensive treatment of the phylogeny of the Bibionomorpha (fig 4), was developed in a later work (Hennig 1954), primarily from an extensive analysis of the wing venation of both fossil and recent forms.

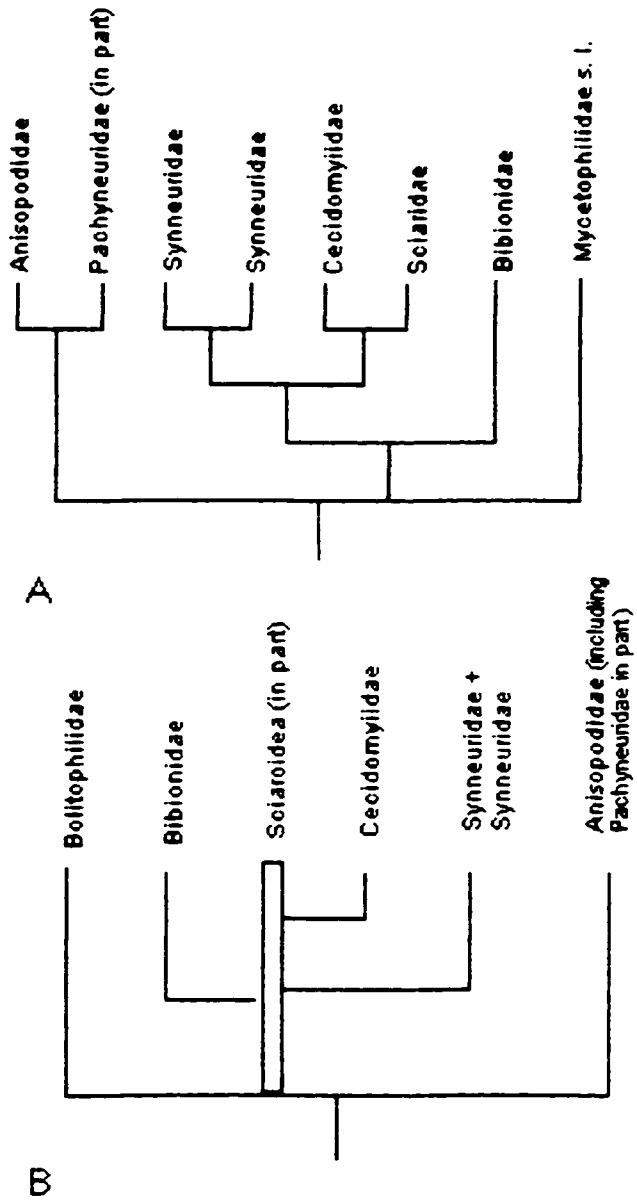


Figure 3. Cladogram intrerpretation of the phylogeny of the Bibionomorpha according to Rohdendorf (1964, 1974). A. Intrepretation of Rohdendorf's phylogeny by Oosterbroek and Courntey (1995). B. Author's intrepretation of the same phylogeny. Rohdendorf's figures are of phylogenetic trees in which some taxa have their origins from within other taxonomic groups. The cladogram in A implies relationships which do not accurately reflect Rohdendorf's actual views (see text for discussion). An attempt is made in B to represent Rohdendorf's view in the form of a cladogram which more closely corresponds to the content of his phylogenetic tree. Rohdendorf's superfamily Fungivoridea, shown here as a narrow rectangle (Sciaroidea in part), included several now extinct fossil families as well as all recent Sciaroidea with the exception of the Bolitophilidae, whose ancestors arose in the early Triassic around the same time as the ancestral forms of the Fungivoridea, and the Cecidomyiidae, whose evolutionary origin is from within the Fungivoridea. The Bibionidae and Scaptopsidae/Synneuridae likewise arose from early fungivorid forms. The Cecidomyiidae, Bibionidae, and Scaptopsidae/Synneuridae are show as lateral branches of the Fungivoridea to indicate their origin from within the latter taxon.

Section Bibionomorpha (as Bibiomorpha)
- subsection Anisopodiformia
  - family Anisopodidae (Phryneidae, including Pachyneuridae)
- subsection Bibioformia
  - family series Bibionidea
    - family Bibionidae
    - family Scatopsidae ?
  - family series Sciaridea (Mycetophilidea, Fungivoridea)
    - superfamily Sciaroidea (Mycetophiloidea, Fungivoroidea)
      - family Mycetophilidae (Fungivoridae)
      - family Sciophilidae
      - family Keroplatidae (Zelmiridae)
      - family Macroceridae
      - family Ditomyiidae
      - family Diadocidiidae
      - family Bolitophilidae
      - family Sciaridae (Lycoriidae)
      - family Manotidae
      - family Lygistorrhinidae
    - superfamily Cecidomyioidea (Itonidoidea)
      - family Lestremiidae
      - family Heteropezidae
      - family Cecidomyiidae (Itonididae)

Table 2. Hennig's 1948 classification of the Bibionomorpha drawing on larval and pupal characters. Taxonomic names are the presently accepted names. The names shown in parentheses, except for the Mycetophiloidea, are Meigen's 1800 names (now suppressed), used by Hennig. The designation of Sciaroidea for the superfamily has recently been shown to have priority over the name Mycetophiloidea.

As in his earlier work, Hennig regarded the Anisopodidae as the basal-most group in the Bibionomorpha due to the presence a discoidal cell and vein M_3, both of which have been lost in all other Bibionomorpha. Since these two venational attributes were also absent from *Mycetobia* and *Pachyneura* and their relatives, Hennig no longer regarded them as having affinities with other Anisopodidae but rather located them as basal lineages of the Sciariformia (Fungivoriformia). The Bibionidae, as in his earlier views, diverged between the Anisopodidae and the Sciariformia. The Scatopsidae and Syneuridae were, on the basis of rather weak

considerations, phylogetically associated with the Cecidomyiidae. Hennig rested the monophyly of the Sciariformia on the weakening of the base of the medial vein, which is somewhat chitinized in the Pachyneuridae but present in the more plesiomorphic fungus gnats merely as a chitinized fold. A well developed base of the medial vein appears to be present in the Bolitophilidae, Sciaridae, Mycetophilidae as well as the Cecidomyiidae, Scaptopsidae and Syneuridae. Hennig interpreted this vein, however, not as the base of the medial vein but as a longitudinalized tb crossvein [8] which had shifted into a horizontal position due to the displacement of the cubital fork toward the wing base. Earlier authors, according to Hennig, had mistakenly interpreted this vein as the base of medial vein. He derived further support for his interpretation from the fact that in all the aforementioned groups except the Bolitophilidae tb appears to be absent. This crossvein is widespread in the Nematocera and is present in the Keroplatidae, Ditomyiidae, and Diadocidiidae. The longitudinalization of tb clearly indicated to Hennig the monophyly of the Bolitophilidae + Sciaridae + Mycetophilidae .+ Sciophilidae (=Sciophilinae of Edwards). A similar but independent development had also occurred in the Cecidomyiiodea (Cecidomyiidae + Scaptopsidae+ Synneuridae). Hennig further postulated that in the ancestor of the Sciaridae + Mycetophilidae + Sciophilidae the m-cu crossvein connecting M_4 with CuA_1, still present in the Bolitophilidae, was obliterated with the transfer of M_4 onto the

[8]Some schemes for the nomenclature of veins in the Diptera regard the anterior branch of the cubital fork as a cubital vein, CuA_1 (Cu_1), which together with CuA_2 comprise the cubital fork (McAlpine et al. 1981, Vockeroth 1981, Väisänen 1984). With this interpretation the crossvein between the anterior branch of the cubital fork, and the medial vein is usually designated bm-cu or m-cu. More commonly, as in Hennig (1954, 1971), Colless and McAlpine (1991) and Matile (1987, 1990b) the anterior branch of the cubital fork is interpreted as M_4. In this scheme the above crossvein is usually called tb and the m-cu crossvein is therefore the oblique vein connecting M_4 to CuA. The latter nomenclature, following Matile (1987, 1990b), is used in this paper.

base of CuA, followed by a secondary elongation of the stem of the cubital fork. These changes in the wing base also affected the ta crossvein (r-m), resulting in the displacement of its posterior end toward the base of the wing. The Cecidomyiidae + Scatopsidae + Synneuridae was in turn the sister-group to Bolitophilidae+ Sciaridae + Mycetophilidae. The relationships of the remaining fungus-gnat families, the Ditomyiidae, Diadocidiidae, and Keroplatidae, in all of which the tb crossvein is more vertically oriented and crossvein-like, could not be resolved. Hennig was uncertain whether these groups arose on the branch between the Mycetobiidae and Cecidomyiidae or whether they originated basally on the Bolitophilidae + Sciaridae + Mycetophilidae branch or even if some of these families might be more closely related to the cecidomyioids. It should be noted that Hennig, like Fisher (cited in Shaw 1948) and Edwards (1925), envisioned a closer phylogenetic relationship between the Sciaridae and the Mycetophilidae *s.s.* than many other authors but, unlike Fisher and Edwards, he ruled out a close relationship between the Sciaridae and any particular group within the Mycetophilidae. The reduced venation of the Manotinae, which Hennig treated as a family, and Lygistorrhinidae did not allow for any definite conclusions to be drawn regarding their phylogenetic affinities.

Tuomikoski (1961) drew from the more diverse set of characters used by Keilen (1919) and Edwards (1926) in their studies of *Mycetobia* and relatives to argue against Hennig's placement of *Mycetobia* as the sister group to the Sciariformia. In agreement with Edwards, Tuomikoski pointed out apomorphies that indicated the phylogenetic affinities of *Mycetobia* lay more with the Anisopodidae, the latter which he excluded from the Bibionomorpha but, like Hennig, regarded as the sister group to the Bibionomorpha.

Tuomikoski (1966b, 1966c) also considered additional characters other than venation in an attempt to elucidate the possible phylogenetic affinities of

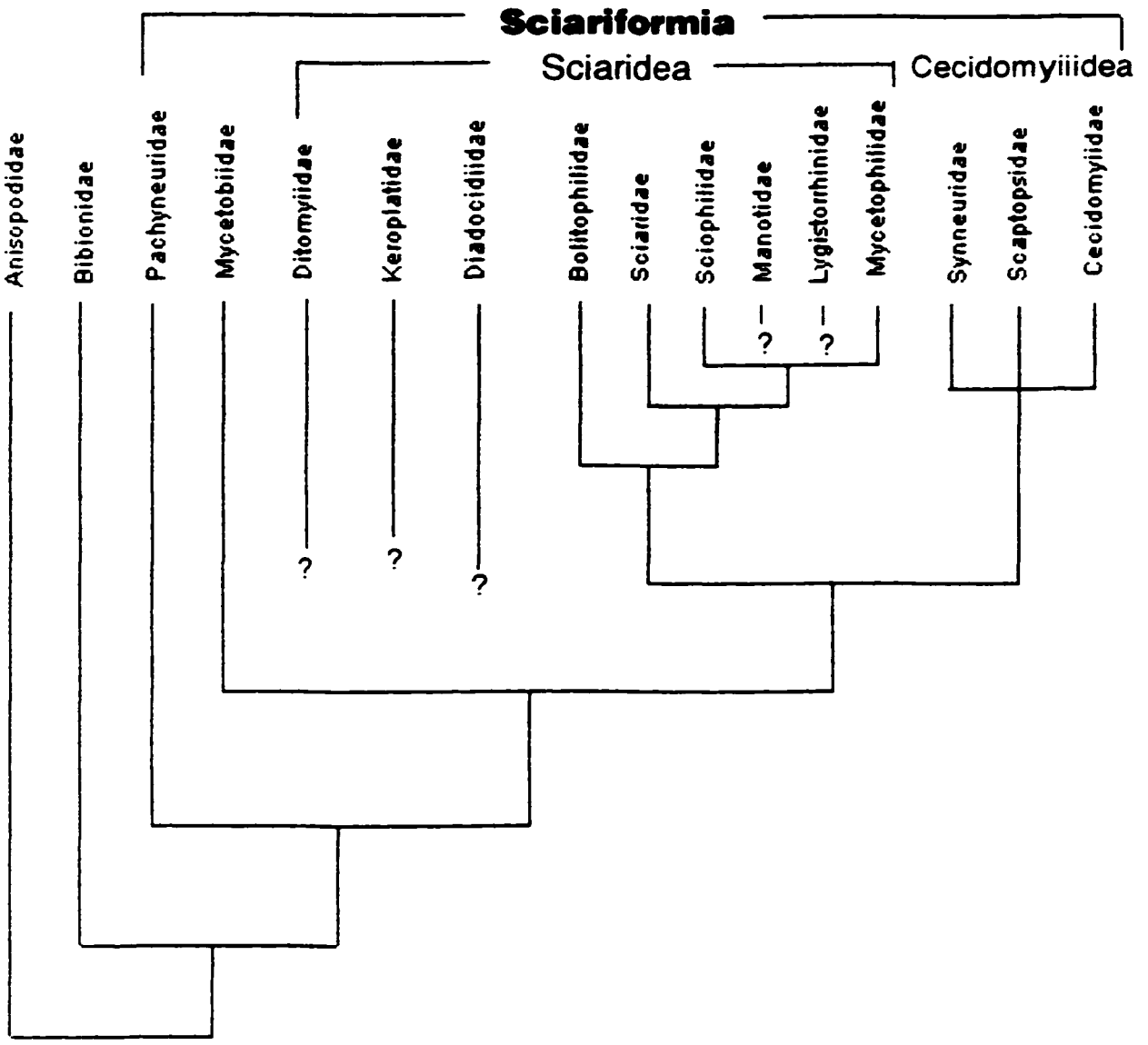


Figure 4. The phylogeny of the Bibionomorpha according to Hennig (1954). The unattached branches for the Ditomyiidae, Keroplatidae, Macroceridae and Diadocidiidae do not imply that Hennig viewed these groups as lying basal to the Cecidomyiidea + Bolitophilidae-Mycetophildae clade. He recognized these groups as ancient but left their placement near the base of the Mycetophilidea open. The relative lengths of branches are not proportional to time since separation.

the Manotinae and the Lygistorrhinidae. He could only state, however, that the Manotinae could at most be the sister group to the Leiinae, and that many of the similarities between the Manotinae and Leiinae were convergent. Tuomikoski recognized the narrow attachment of the abdomen to the thorax, in contrast to the

broader attachment found in most other Diptera including the Sciaridae, as a significant apomorphy for the Mycetophilidae + Keroplatidae. Since the narrow attachment in the Lygistorrhinidae is accompanied by other thoracic character states show similarities to those in macrocerine keroplatids, Tuomikoski concluded that the lygistorrhinids were highly apomorphic keroplatids. This conclusion was rejected by Thompson (1975), who argued that most of Tuomikoski's characters were either symplesiomorphic or rested on misinterpretations of character data. He left unspecified the phylogenetic affinities of the Lygistorrhinidae, noting only that the narrow attachment of the abdomen was a synapomorphy that the family shared with the Mycetophilidae and Keroplatidae.

Matile (1990b), in his recent phylogenetic analysis of the Sciaroidea, adopted Hennig's (1954) venational arguments and thus accepted the relationships Hennig had earlier established for the Bolitiphilidae, Sciaridae, and Mycetophilidae. The only improvement on Hennig that Matile made was in regarding the Lygistorrhinidae as the sister group of the Mycetophilidae, thus rendering the Sciaridae as the sister group of Lygistorrhinidae + Mycetophilidae (see above discussion on page 21). Matile regarded the narrow insertion of the abdomon to the thorax and the development of the laterotergite as synapomorphies for the Lygistorrhinidae + Mycetophilidae. The Diadocidiidae and Keroplatidae branched basal to the Bolitophilidae and the Ditomyiidae formed the basal-most lineage in Matile's (1990b) phylogeny. The Cecidomyiidae were not included in the first analysis and his phylogeny was constructed on the basis of only 12 characters. The Cecidomyiidae, however, were included in a subsequent study (Matile 1997) of the larval diet in the Sciaroidea, and additional characters were considered without affecting the topology of the earlier phylogeny. In the

latter study, the Cecidomyiidae occupied a branch between the Ditomyiidae and the Bibionidae. Matile's phylogenetic hypothesis is shown in figure 5B.

Considerably different results than those obtained by Matile were presented by Blaschke-Berthold (1994) in a phylogenetic analysis of the Bibionomorpha (figure 5A), the sciaroid branch in her phylogeny was based on an examination of 26 morphological characters. Although her phylogeny agreed with Matile's in placing the Cecidomyiidae as the basal-most lineage in the Sciaroidea, the Ditomyiidae, generally considered a rather plesiomorphic fungus-gnat group, occurred higher in the phylogeny as the sister group of the "Mycetophilidae", which in her delineation encompassed the Keroplatidae, Bolitophilidae, Lygistorrhinidae, and Mycetophilidae s.s. No attempt was made to resolve relationships among the latter taxa. The Diadocidiidae formed the sister group to the Ditomyiidae + "Mycetophilidae", and the Sciaridae was located between the Diadocidiidae and the Cecidomyiidae, though not in a sister-group relationship to the latter.

In contrast to the above studies which have mostly been concerned with the deeper phylogeny in the Sciaroidea, Söli (1997) examined 39 genera in a study confined to an analysis of the phylogeny within the Mycetophilidae s.s. There has long been uncertainty regarding the monophyly of Edward's (1925) sciophiline "tribes" (Manotinae, Leiinae, Mycomyinae, Sciophilinae s.s., Gnoristinae). Although the monophyly of the Manotinae and Mycomyinae is fairly certain, that of the other three subfamilies is in part questionable (Väisäinen 1986). In Söli's analysis, many of the genera cluster together in the same region of the tree in accord with traditional classification, but not necessarily as monophyletic groups (figure 16, page 86). The monophyly of the Mycetophilinae and Mycomyinae were supported, and the Manotinae, represented in the analysis by one genus, formed the sister group to the Mycetophilinae. Between the Manotinae and Mycomyinae

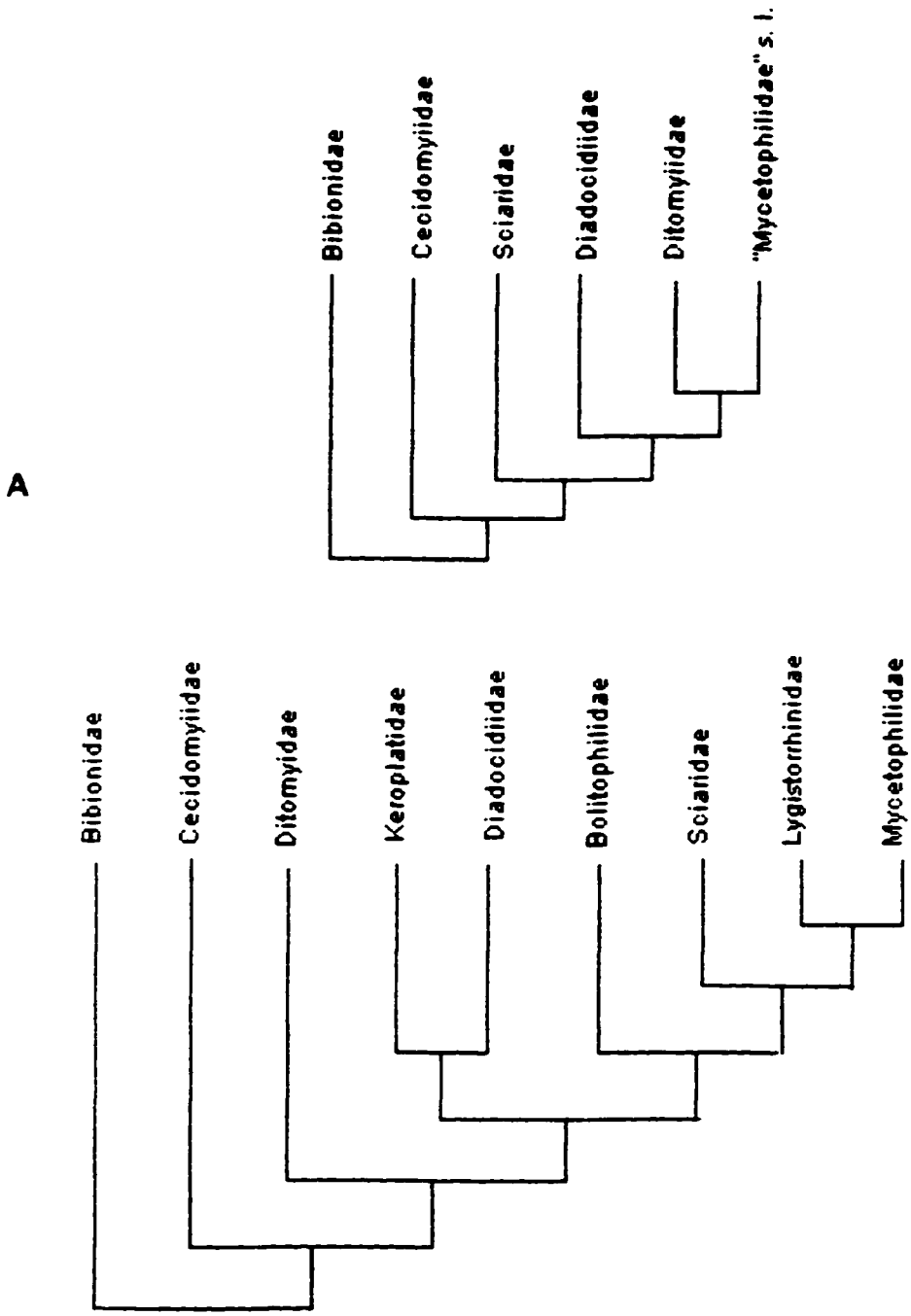


Figure 5. Recent hypotheses about the phylogeny in the Sciaroidea. A. Blaschke-Berthold (1994), redrawn. B. Matile (1990, 1997). Blaschke-Berthold only resolved basal nodes in the Sciariodea, treating the Keroplatidae, Bolitophilidae, Lygistorrhinidae, and Mycetophilidae as one taxonomic unit, the Mycetophilidae s. l.

were seven leiine genera all occupying their own individual branch. Most of the gnoristine genera occupied branches in the tree basal to the Mycomyinae, and the sciophilinae genera, ten in all, were located together on a single branch lying basal to the gnoristine genera. Soli's results will be discussed in more detail later in this paper.

In these more recent phylogenetic studies of the Sciaroidea, the position of the Sciaridae is the most controversial. Matile and Hennig placed the family close to the Mycetophilidae and far removed from the Cecidomyiidae, whereas Blaschke-Berthold located the family more basally between the Cecidomyiidae and the rest of the fungus gnat families, but not in a sister-group relationship with the Cecidomyiidae as proposed by Wood and Borkent (1989) and Oosterbroek (1995).

MONOPHYLY OF INDIVIDUAL TAXA

The monophyly of most of the families included in the Sciaroidea is not in major dispute. The Cecidomyiidae, the largest family in the Sciaroidea, is unquestionably monophyletic as is indicated by several apomorphies found in no other group of Diptera (Wood and Borkent 1989).

The monophyly of the Ditomyiidae has not been satisfactorily demonstrated. This is a small family circumscribed mostly by plesiomorphic character states and with only 9 relatively species-poor genera. Munroe (1974) found evidence of a sister-group relationship between *Symmerus* Walker and *Australosymmerus* Freeman, but his conclusion may have been based on a misinterpretation of the polarity of some of the character states he used (Blaschke-Berthold, 1994). Blaschke-Berthold (1994) listed 3 possible apomorphies for the family, one of which is a venational character (subcosta ending freely) not uncommonly encountered in other sciaroid groups, another a

larval character whose validity as a synapomorphy for the family needs to be verified by an examination of the tropical genera whose larvae are at present unknown.

The Sciaridae are morphologically a fairly cohesive group. Although this is a family in which essentially no phylogenetic studies have been done, a few convincing autapomorphies for the family are known. Undoubtedly, when more attention is turned to the phylogenetic relationships within the family, a greater number of characters in support of it's monophyly will be found. Matile (1990b) interpreted the complete eye-bridge as an apomorphy for the family. However, a complete eye-bridge, or at least an almost complete one, is found in some Manotinae and Ditomyiinae as well as in all but a few Cecidomyiidae. Blaschke-Berthold (1994) regarded possession of a complete eye-bridge as part of the groundplan of the Sciaroidea. Wood and Borkent (1989) listed as an apomorphy for the family the structure of the postgenal lobes in the larval head capsule which meet midventrally in two places to enclose a circular membranous area. A similar condition is also found in *Docosia* (Mycetophilidae, Leiinae) (Madwar 1937) where the condition is likely independently derived. Blaschke-Berthold gave two genitalic characteristics found thus far only in the Sciaridae. Finally, Steffan (1966) noted that the sternum and tergum of the first abdominal segment in adults are divided into an anterior and a posterior portion separated by a membranous area. The phylogenetic importance of this character as an apomorphy for the Sciaridae was pointed out by Matile (1990b).

The monophyly of the Keroplatidae has been well demonstrated by the extensive studies on the family by Matile (1990b, 1997).

The Diadocidiidae contains only one genus, *Diadocidia* Winnertz, with only 10 known species. The South American genus *Pterogymnus* Freeman was originally placed in the Diadocidiidae (Freeman 1951) but is known only from one

female specimen. The affinities of *Pterogymnus*, whether or not it belongs in the Diadocidiidae, are at present uncertain. The larvae of Diadocidia are unique in the Sciaroidea in having a propneustic tracheal system in contrast to the larvae in most other groups which are peripneustic (Cecidomyiidae, Ditomyiidae), hemipneustic (Sciaridae, Mycetophilidae), and apneustic (Keroplatidae). The larvae of the Lygistorrhinidae and of *Pterogymnus* are unknown.

The Lygistorrhinidae are a small family consisting of 1 fossil and 3 recent genera. The members of this family are very peculiar fungus gnats with reduced venation, large holoptic compound eyes and, except in *Seguyola* Matile, mouthparts developed into a long mosquito-like proboscis. The characteristics for the family have been discussed by Matile (1986, 1988, 1990a, 1990b), Thompson (1975, 1989) and Tuomikoski (1966b) The family is unquestionable monophyletic, its affinities to other fungus-gnat groups, however, remain enigmatic.

The Mycetophilidae is the largest family of fungus gnats in terms of number of species. Although the monophyly of this family has been widely accepted, very few apomorphies have been put forward in support of this view. Söli (1997) listed four apomorphies for the family: third palpomere in adult with sensilla on medial surface, crossvein tb long, abdominal sternites with one fold line, and anterior tibia with well-developed anteroapical depression. The long tb crossvein, replacing the stem of M, as discussed above, is an apomorphy, however, that arose deeper in the phylogeny. If Hennig's (1954) interpretation is correct, a longitudinalized tb crossvein is an apomorphy uniting Bolitophilidae, Sciaridae and Mycetophilidae. One or two fold lines on abdominal sternites is undoubtedly apomorphic since such fold lines are found nowhere else in the Sciaroidea except in the Mycetophilidae. However, abdominal fold lines are not found in any Mycomyinae (Väisänän 1984), they are also absent in some leiine and sciophiline

genera. The loss of fold lines, therefore, has occurred multiple times. This may not be so problematic an interpretation for the leiine and sciophiline taxa, but given the basal position of the Mycomyinae in Söli's Strict Consensus and the Majority Rule consensus trees, the absence in the Mycomyinae could well be symplesiomorphic. (The Mycomyinae were located substantially higher in his preferred parsimonious tree). The location and development of sensilla on the third palpomer is also variable and in Söli's study displayed many reversals within the family. Some sort of modified anteroapical area on the fore tibia, usually consisting of modified setae in a single row or a triangular dense brush of fine setae, is found in the Ditomyiidae and all other fungus-gnat families and therefore appears to be apomorphic for the fungus gnats; such a structure is not found in other Bibionomorpha including the Cecidomyiidae. Sometimes this tibial brush is located in a depression and sometimes not even within the same family, for example in the Sciaridae and Keroplatidae. It has been secondarily reduced or lost in some taxa, especially in leptomorphic forms such as *Bolitophila* and *Lygistorrhina*, two of the three taxa Söli used as outgroups for his analysis. In his third outgroup, *Corynoptera* Winnertz (Sciaridae), modified setae are present and, judging from the figures in Tuomikoski (1960), not always located in an obvious depression. It may have been better to have also included some keroplatid taxa in the outgroup or to have taken into account the distribution of character states throughout the Sciaridae.

Another possible apomorphy for the Mycetophilidae is the fact that R_4, when present, always terminates in R_1 (Hennig 1954). R_4 also terminates in R_1 in some Bolitophilidae and Keroplatidae, but here the character is clearly independently derived since many species in both families have R_4 terminating in the costal margin which is the plesiomorphic state in the Sciaroidea. Whether R_4 terminating in is an apomorphy for the Mycetophilidae, however, is debatable. R_4

is totally absent in the Lygistorrhinidae and the Sciaridae, two taxa placed close to the Mycetophilidae by both Hennig and Matile. The question arises then, if Hennig's and Matile's hypotheses are correct, whether capture of R_4 by R_1 occurred in the direct ancestor of the Mycetophilidae or in the ancestor of the Sciaridae + Lygistorrhinidae + Mycetophilidae or anywhere in between.

Within the Mycetophilidae, the Mycomyinae, Manotinae, and Mycetophilinae can be demonstrated to be monophyletic groupings. In the Mycomyinae the lateral ocelli are very close together in the middle of the frons and the middle ocellus is absent. Apomorphies in the shape of the head, palpi, orientation of the pleural suture have been cited (Tuomikoski 1966c) in support of the monophyly of the Manotinae. The Mycetophilinae have microtrichia on the wing membrane arranged in regular longitudinal rows, lateral ocelli located adjacent to the margin of the compound eye, and tibial setulae arranged in regular longitudinal rows. The arrangement of wing membrane microtrichia is quite unique in the Diptera and this character alone suffices to establish the monophyly of the subfamily. The lateral ocelli are located close to the eye margin also in many Leiinae, and tibial setulae are in regular longitudinal rows in the Mycomyinae, Manotinae, some Leiinae and in some Keroplatinae. Therefore the last two characters might prove to be apomorphies uniting the Mycetophilinae with one of the other mycetophilid taxa. (Väisänen 1984) suggested the regular arrangement of tibial setae as an apomorphy for Mycomyinae + Mycetophilinae, a proposal earlier advocated by Shaw (1948), Shaw and Shaw(1951) and Fisher (1937) on the basis of similarities in the structure of thoracic sclerites and male genital structures.

The monophyly of the remaining mycetophilid subfamilies, Leiinae, Sciophilinae, and Gnoristinae, is unclear. Although each of these subfamilies contain clusters of genera that are most likely monophyletic, their relationship to other such supergeneric taxa and to each other is poorly understood. There is

evidence that some genera traditionally included in the Gnoristinae and Leiinae may be phylogetically closer to genera now classified in totally different subfamilies (Väisänen 1984; Chandler 1980).

FOSSIL RECORD

Rodendorf (1964) described a number of fossil species from Issuk Kul in Russia, then dated as upper Triassic in age but now believed to be lower Jurassic, which he assigned to the Bibionomorpha. Specimens originally described by Rodendorf as belonging to his suborder Archidiptera have recently been shown to belong to the Tipulomorpha and Bibionomorpha (Krzeminski 1992). Fossils clearly belonging to now extinct taxa of bibionoids and sciaroids are known from the early Jurassic of Karatau (Rodendorf 1937, 1946, 1964). One family in particular, the Pleciofungivoridae, was a dominant group throughout the Jurassic of Russia and continued into the early Cretaceous before disappearing from the fossil record. The Pleciofungivoridae as well as the Mesosciophilidae, another extinct mesozoic group, resembled extant bolitophilid fungus gnats in many attributes. Fossil specimens of these groups posses elongated coxae and tibial spurs (1-2-2 formula), which are typical fungus gnat attributes , and venation similar to that found in the Bolitophilidae (Kovalev 1987). The phylogenetic position of these extinct groups in relation to recent taxa, however, is as yet uncertain (see Hennig 1954 and Matile 1981 for a discussion of their views on the relationships of these extinct taxa).

Although the above fossils are of Jurassic age, fossils from the upper Triassic have been described for the Bibionidae. Of other recent bibionomorph families, the Pachyneuridae are known from the upper Jurassic, the Cecidomyiidae from the upper Jurassic/lower Cretaceous, the Mycetophilidae from the lower Jurassic (Evenhuis 1994). The Keroplatidae (Matile 1981), Bolitophilidae (Kovalev

1986), and Sciaridae (I have examined an undescribed sciarid in Lebanese amber) are documented from the lower Cretaceous. Ditomyiidae, Diadocidiidae, and Lygistorrhinidae are known from Eocene (Meunier 1904). Fungus gnat inclusions, many of which are of species belonging to extant genera, are common in Baltic amber (Eocene/Oligocene) (Meunier 1904, Keilbach 1982, Spahr 1985). A fossil wing from lower Jurassic of England was described by Whalley (1995) as species possibly close to *Diadocidia* (Diadocidiidae). The resemblance of this fossil to Diadocidia, however, is superficial, the venation as illustrated has little in common with venation found in the Sciaroidea. Blagoderov (1995, 1997, 1998) has described of number of gnoristine, leiine, and sciophiline mycetophilids from the lower Cretaceous of Transbaikalia.

OBJECTIVES OF STUDY

Bibionomorpha

The Infraorder Bibionomorpha as first proposed by Hennig (1948) included such families as the Scaptopsidae, Synneuridae, Axymyiidae, and the Anisopodidae. Tuomikoski (1961) questioned the inclusion of the Anisopodidae in the Bibionomorpha, believing them to be quite removed, even as a sister group, from the Bibionomorpha. Wood and Borkent (1989), using mostly larval characters, concluded that the Anisopodidae, Scatopsidae, Synneuridae, and Perissomatidae evolved on a common branch along with the Psychodidae and Trichoceridae, and placed these families together in the infraorder Psychodomorpha. The Bibionomorpha, in Wood and Borkent's more restricted sense, therefore, consisted of only the families Pachyneuridae, Bibionidae and the superfamily Sciaroidea. Although they found no character support for the Bibionomorpha, they felt that the group was monophyletic. In Amorim's (1992) analysis of the Bibionomorpha, support was found for a Bibionidae + Sciaroidea clade; the Anisopodidae were the sistergroup of the Bibionidae + Sciaroidea, and

the pachyneurid taxa occurred on separate branches deeper in the tree. Only two venational characters, however, supported the Bibionidae + Sciaroidea clade. Oosterbroek and Courtney (1995) found four character states supporting an Axymyiidae + Bibionidae + Sciaroidea clade. Two of these characters, however, are homoplasious, and another involves, in my opinion, a rather unlikely reversal. Friedrich and Tautz (1997), using 28S rDNA sequences, found support for a Scaptopsidae + Anisopodidae + Bibionidae + Sciaroidea clade.

On the basis of Wood and Borkent's (1989) study, Matile (1990, 1997) and Blaschke-Berthold (1994) used the Bibionidae and Pachyneuridae as outgroups for their analysis. This choice of outgroup is undoubtedly appropriate, but as discussed above, character support for the Bibionidae + Sciaroidea clade , with the exception of the molecular data, is rather weak. Thus one of the objectives of this study is to test the appropriateness of using the Bibionidae as an outgroup for an analysis of the Sciaroidea. This is not intended to be a thorough analysis of the basal relationships in the Bibionomorpha. Due to a lack of material such relevant families as the Axiomyiidae and Pachyneuridae were not included. Although theoretically any group outside the Sciaroidea could serve as an outgroup for the analysis, the nature of the molecular data mandates the use of a phylogentically close outgroup. In both gene sequences, 12S and 16S, are found regions that are evolving at a much faster rate relative to other regions of the gene and therefore are likely to contribute a significant amount of phylogenetic noise resulting from the multiple substitutions at the sites in these regions. The resulting patterns would obliterate any sensible indication of phylogenetic relatedness in the case of remotely related taxa. The set I analyses, therefore, were undertaken to determine whether the Bibionidae are phylogenetically closer to the Sciaroidea than any other of the possible outgroups whose sequences were obtained for this study.

Sciaroidea

Although various hypotheses have been advanced for the relationships among sciaroid taxa, there are still many areas of uncertainty as well as conflict. This study will contribute toward a resolution of the following problems.

1. Position of the Sciaridae and its relationship to the Cecidomyiidae.

2. Position of the Bolitophilidae. Older workers generally regarded the Bolitophilidae as one of the more basal lineages, perhaps due in part to the presence of a three-segmented antennae in the larvae. A three-segmented antenna is also found in the Bibionidae and Ditomyiidae, and for that matter, appears to be the general condition in nematocerous Diptera. In all other fungus-gnat families, the antenna is reduced to a one-segmented broad membranous sensory structure found nowhere else in the Diptera, and therefore unquestionably apomorphic. The position given to the Bolitophilidae by Hennig (1954) and Matile (1990, 1997) requires either that the one-segmented antenna has evolved twice in the Sciaroidea or that the three-segmented condition in the Bolitophilidae is due to a reversal.

3. Monophyly of the Mycetophilidae. Although the monophyly of the Mycetophilidae is widely accepted, little in the way of character support can be offered. The criterion used by older authors to separate the Mycetophilidae (as the subfamily Mycetophilinae) from the other fungus-gnat taxa was the absence of the tb crossvein connecting the medial and cubital forks. If Hennig's (1954) hypothesis is accepted (see page 21), tb is present but longitudinalized, in which state it appears to be the base of M. This state, however, is not limited to the Mycetophilidae but is the condition of tb in the Bolitophilidae, Lygistorrhinidae, and Sciaridae among the fungus gnats. Convincing evidence for the monophyly of the Mycetophilidae is at present lacking.

4. The relationships among the "sciophiline" subfamilies in the Mycetophilidae. Although the taxa included in this study from the Leiinae, Sciophilinae, Mycomyinae, and Gnoristinae, are a limited representation of the diverse genera in these subfamilies, the molecular data nonetheless may provide a better indicator of relationships in and among these subfamilies. Thus far, morphological characters have not proven very satisfactory even in determining the limits of the Leiinae, Gnoristinae, and Sciophilinae.

Materials and Methods

Specimens

Nucleotide sequences for 12S rRNA were obtained from specimens representing 4 non-bibionomorph families and 8 families in the Bibionomorpha. The bibionomorph material included specimens from 3 genera in the Bibionidae and 29 genera (37 species) representative of the major taxa within the Sciaroidea (families, subfamilies). The Lygistorrhinidae and Manotinae (Mycetophilidae) were omitted from the study due to a lack of material or failure in attempts to extract DNA from the available specimens. For the same reason, usable sequences could be obtained from only one representative species from the Sciophilinae (Mycetophilidae). Additional sequences representing 5 other dipteran families, two nematoceran and three brachyceran, were obtained from GenBank. 16S sequences were also obtained for 8 of the above species, from the same individual specimens as used for 12S template, representing two non-bibionomorph and four bibionomorph families. Additional 16S sequences from two non-bibionomorph families were likewise obtained from GenBank. Sequences obtained from Genbank are listed with their accession numbers in Table 3.

The specimens used in this study were collected for the most part in the western United States within the past ten years and preserved in 95% ethanol. A

Culicidae	*Anopheles quadrimaculatus*	gb|L04272|MSQNCATR	both
	An. gambiae	gb|L20934|MSQMTCG	12S
Simuliidae	*Austrosimulium bancrofti*	gb|L02383|CXQMTRRSS	12S
	Simulium bivittatum	gb|U17727|SBU17727	16S
Drosophilidae	*Drosophila yakaba*	gb|X05915|MIDYTRN	both
	D.melanogaster	gb|U37541|DMU37541	12S
Muscidae	*Musca domestica*		12S
Calliphoridae	*Lucilia cuprina*	gb|AF086858|AF086858	16S

Table 3. 16S and 12S sequences obtained from GenBank. Third column lists the sequence accession numbers; the last column indicates which gene sequence. 12S, 16S or both, was obtained for the each species.

few sequences, however, were obtained from pinned specimens. Since the extraction methods used (see below) required the sacrifice of only a small amount of tissue, all sequences, other than those obtained from GenBank, can be tied to individual specimens whose identifiable remains have been retained as vouchers. All specimens will be deposited in the collection at the California Academy of Sciences, San Francisco, California, USA. The taxonomic information and collection data for specimens are given in table 4.

DNA Extraction

DNA was extracted from individual specimens by removing one or two legs or a small piece of flight muscle. The removed tissue was placed in a microcentrifuge tube containing 200-500 µl of 5% chelating solution of Chelex®-100 resin. The samples were incubated at 56° C for several hours, typically more than five. Incubation times less than five hours generally resulted in low PCR yields, possibly due to insufficient release of DNA from tissues. During incubation samples were rotated to keep the chelating beads in suspension. After incubation, samples were vortexed, heated at 95° for fifteen minutes, vortexed again, then centrifuged to pellet the chelating beads and tissue debris. The supernatant was then transferred to a clean 1.5 ml Eppendorf tube, and an aliquot

Table 4. Identity and collection data for specimens from which sequences were obtained for this study

Tipulidae	*Limonia* sp	CA San Mateo Co., Tarwater Creek, 27Jun1993, JE Baxter coll
	* *Tipula (Platytipula) ultimata*	KS Douglas Co., vicinity of Lawrence, 6Oct1994, G. Byers coll.
Ragionidae	* *Symphormyia*	CA San Mateo Co., Tarwater Creek, 17Jun1994, JE Baxter coll
Empididae	Empidid sp. 1	CA San Mateo Co., Tarwater Creek, 14Mar1994. JE. Baxter coll.
Anisopodidae	*Sylvicola* sp. 1	CA San Mateo Co., Tarwater Creek, 27Jun1993, JE Baxter coll
Bibionidae	*Dilophus* sp.	CA Alameda Co., Berkeley, UCB Campus, 30Aug1993. JE Baxter coll.
	Bibio sp	CA Alameda Co., 3Aug.1993, JE Baxter coll.
	**Penthetria heteropterna*	NY Hamiliton Co., Raquette Lake, 1-25Sept1984, Malaise trap, S. Teale coll.
Cecidiomyiidae	Cecidomyiid sp. A	Canada. B.C., near Squamish, 3Jun1994, JE Baxter coll.
	Cecidomyiid sp. B	Canada, B.C., near Squamish, 3Jun1994. JE Baxter coll.
Sciaridae	*Bradysia* sp A	CA Alpine Co., Carson Pass, 4Jul1993. JE Baxter coll.
	B	CA Alpine Co., Carson Pass, 4Jul1992. JE Baxter coll.
	Corynopterna sp C	CA San Mateo Co., Tarwater Creek, 12Mar1994, JE Baxter coll.
Ditomyiidae	*Ditomyia* sp.	CA Alameda Co, Berkeley, ex Polyporus sp., 11Jan1994, J. Powell coll.
Diadocidiidae	*Diadocidia* sp A	CA San Mateo Co., Tarwater Creek, 12Mar1994, JE Baxter
Keroplatidae	*Macrocera* sp. A	CA San Mateo Co., Tarwater Creek, 27Jun1993, JE Baxter coll
	Macrocera sp. B	Ca Plumas Co., Meadow Valley, 12Aug93, J. Powell coll.
	Urytalpa sp	CA El Dorado Co., 3 km E. Grizzly Flat, 4Jul1993, JE Baxter coll.
	Orphelia sp.	CA. Marin Co. China camp, 30Jul1994, J. Powell
	**Platyura* sp	CA Plumas Co., Greenville, 19May1982. JE Liebherr coll.
		CA Contra Costa Co., El Cerrito, 14Jun1982, J. Doyen coll

Table continued on next page

* Sequences were obtained from more than one specimen of this species.

Table 4 continued

Bolitophilidae	**Bolitophila* sp.	CA San Mateo Co., Tarwater Creek, 27Jun1993. JE Baxter coll.
		WA Callam Co., Elwha, June 1994 JE Baxter coll. (#2)
Mycetophilidae		
Mycomyiinae	*Mycomya* sp	CA Alpine Co., Carson Pass, 4Jul1993, JE. Baxter coll.
Sciophilinae	*Acnemia* sp.	CA El Dorado Co., Shingle Springs. 10Apr1993, JE Baxter coll.
Gnoristinae	*Boletina* sp. A1	CA Alpine Co., Carson Pass, 4Jul1992. JE Baxter coll.
	Boletina sp. 2	CA El Dorado Co., Leek Spring Valley, 4Jul1993, JE. Baxter coll.
	Boletina sp. 3	CA El Dorado Co., 2Apr1994, JE Baxter coll.
	Coelosia sp	OR Jackson Co., Rogue River, 29May1994, JE. Baxter coll.
	Gnoriste sp	WA Clallam Co., Elwha, June 1994
	Hadroneura oregona	CA El Dorado Co, N. side Leek Springs Valley, 4Jul1993, JE Baxter coll.
	Synapha 1	CA El Dorado Co., Sopiago Crk, 2 km NW Cooks Station, 19Jun1993, JE Baxter coll.
	Synapha 2	CA El Dorado Co., 3 km E. Grizzly Flat, 4Jul1993, JE Baxter coll.
Leiinae		
	Acompterella sp	CA San Mateo Co., Tarwater Creek, 12Mar1994
	Docosia sp (A1)	CA. Alpine Co. Carson Pass, 4Jul1992, JE Baxter
	Leia sp.	CA Alameda Co., Fremont, 13 March 1995. JE Baxter coll.
	Tetragoneura a1	CA San Mateo Co., Tarwater Creek, 17 June 1994, JE Baxter coll.
	Tetragoneura a2	
Mycetophilinae		
Mycetophilini	*Mycetophila fungorum*	CA. Alameda Co., Berkeley, UCB campus, ex Agrocybe, 7Mar1993, JE Baxter coll.
	Mycetophila paula 2	CA El Dorado Co., Grizzly Flat, 12Jun1992, JE Baxter coll.
	Mycetophila alea	CA Tehama Co, Deer Creek, 30Jul1992, JE Baxter coll.
	Mycetophila sp. 4	CA El Dorado Co., 3 km E. Grizzly Flat. 4Jul1993, JE Baxter coll.
	Phronia sp. 1	CA. San Mateo Co. Tarwater Creek, 27Jun1993. JE Baxter coll.
	Phronia sp. 2	CA. San Mateo Co. Tarwater Creek, 27Jun1993. JE Baxter coll.
	Dynatosoma sp.	CA San Mateo Co, Tarwater Creek, 17Jun1994, JE Baxter coll.
Exechiini	*Cordyla* sp. 1	CA. Tehama Co., Deer Creek, 30Jul1992. JE Baxter coll
	Cordyla sp. 2	CA. Tehama Co., Deer Creek, 30Jul1992. JE Baxter coll
	Rymosia sp.	CA El Dorado Co., Grizzly Flat, 12Jun1992, JE Baxter coll.
	Exechia sp	CA San Mateo Co, Tarwater Creek, 27Jun1993, JE Baxter coll.

of a 1M tris-HCl + 0.5mM EDTA solution was then added to the samples in a ratio of 1 part tris-EDTA per 25 parts extract. The purpose for the addition of the tris-EDTA solution was to lower the pH of the extract to between 7.2-8.0. Extracts were stored at 20° C and still yielded good results when used in PCR after up to 1.5 years of storage.

Amplification

The polymerase chain reaction was used to amplify an approximately 355 bp section of the gene for 12S rRNA using the primers SR-J-14233 (=12Sbi), 5'-AAGAGCGACGGGCGATGTGT-3', and SR-N-14588 (= 12Sai), 5'-AAAACTAGGATTAGATACCCTATT-3'. This portion of the gene encodes sequence for the third domain of the 12 rRNA molecule. For 16S sequences, the primers LR-J-12887, 5'-CCGGTTTGAACTCAGATCATGT-3', and LR-N-13398, 5'-CRCCTGTTTAWCAAAAACAT-3' . Both sets of primers were originally designed by Kocher (Kocher 1989) and modified for insects by Simon (Simon 1994). Redundant positions in the LR-N-13398 primer are modifications designed in this study. Numbers in the primer designation refer to the position of the 3' end of the primer sequence as found in *Drosophila yakuba* (Clary 1985).

Two to five µl of crude DNA extract (template) was added to PCR cocktail (0.4 µM of each primer, 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2mM MgCl2, 0.8 mM dNTP, 0.006% Gelatin) for a final volume of 50 µl. 2.5 U of taq polymerase was used per reaction. The PCR cocktail, including primers and DNA template, was preheated at 95° C for five minutes to denature template DNA before the addition of taq polymerase to the reaction tubes to initiate PCR. PCR was performed on a thermocycler using the following cycle parameters: a denaturation phase at 92° for 30 s, annealing phase at 53° for 30 s and an extension phase at 70° for 30 s for 35 cycles. The amplified DNA was purified by electrophoresis at 100V for 2.5 hours in a 1% low-melting point Agarose gel in TAE buffer; the purified DNA band

was excised with a clean, sterile razor blade, placed in a clean 1.5 ml Eppendorf tube and melted at 90° C in a sufficient volume of ddH_2O to yield a 0.6-4 nM DNA solution. This solution provided template for DNA sequencing.

DNA Sequencing

DNA sequencing was carried out using a double-stranded cycle sequencing system (Gibco BRL, Life Technologies, Inc.) per kit protocols. The same primers as were used in the amplification step were end-labeled with ^{32}P and employed for sequencing. Both the majority and minority (=Heavy and Light) strands were sequenced. It was usually not possible to read the 20-25 bases lying adjacent to the primer. Data for this part of the 12S sequence, however, could be obtained by reading the opposite strand. For the 16S analysis, sequence adjacent to the primers was not used.

Analysis

Alignment for the 16S sequences was achieved manually. Since the 12S data involved considerably more sequences, various computer programs were utilized, but this approach proved unsatisfactory. Alignment was finally achieved manually after constructing diagrams for the secondary structure of molecule from the sequence data, using the models in Hickman et al. (1996) as a guide.

Parsimony analyses were performed using PAUP 3.1.1 (Swofford 1993). The heuristic search method using the tree-bisection algorithm for branch swapping was used to find the shortest tree(s), then repeated to find trees one step longer. Bootstrapping was performed with 2,000 iterations. Data were treated as unordered and equal weights applied to all characters. Gaps in the alignment were treated as missing. Insertion/deletion events occurring in highly conserved regions, however, where placement of gaps were unambiguous and deemed phylogenetically informative, were included in the analyses by using the interleave option in the format command in PAUP and coding the

presence/absence of a gap numerically . Multiple consecutive gaps were treated as a single insertion/deletion event. Sequences with ambiguous alignment were included or excluded depending on the analysis.

The following sets of analyses were performed:

Set I: The first set of analyses was carried out to determine the appropriateness of using the Bibionidae as an outgroup for an analysis of the Sciaroidea as well as to assess the support, or lack thereof, provided by 12S and 16S gene sequences for the monophyly of that portion of the Bibionomorpha included in this study. Three pairs of analyses were performed using 1) 16S data only, 2) 12S data only, and 3) both 12S and 16S. Each pair consisted of a) an analysis including all of the aligned sequence and b) an analysis in which hypervariable (see page 45) or questionably aligned sequences were excluded. The Tipulidae, represented by sequences from two species, were used as the outgroup. The taxa included in this set of analyses are summarized in table 5.

Set II. Analyses of the Sciaroidea. A series of analyses using only 12S sequence data were performed with the three bibionid sequences serving as the initial outgroup. The analyses were divided into three subsets, with each subset consisting of three or four analyses. The first analysis in each subset was performed using all alignable positions, in the second analysis all hypervariable regions were excluded, and the third and fourth analysis performed included/excluded different combinations of hypervariable data. All sciaroid taxa were included in the first subset of analyses. In the second subset, the two basal sciaroid taxa obtained in the first subset analyses were selected as outgroups for a second round of analyses. The third subset of analyses included only taxa in the Mycetophilidae using the sister-group of the Mycetophilidae as determined from the previous subset analyses as the outgroup.

Table 5. Taxa included in the first set of analyses (Set I) to assess the monophyly of the Bibionomorpha.

16 S:		
	Tipulidae	*Tipula ultimata*
		Limonia sp.
	Culicidae	*Anopheles quidrimaculatus*
	Simuliidae	*Simulium bivittatum*
	Anisopodidae	*Sylvicola sp.*
	Drosophilidae	*Drosophilia yakuba*
	Calliphoridae	*Lucilia cuprina*
	Bibionidae	*Dilophus sp.*
	Cecidomyiidae	*Cecidomyiid sp. B*
	Ditomyiidae	*Ditomyia sp.*
	Keroplatidae	*Macrocera sp. 2*
		Orfelia sp.
		Urytalpa sp.
12S:		
	Tipulidae	*Tipula ultimata*
		Limonia sp.
	Culicidae	*Anopheles quidrimaculatus*
		An. gambiae
	Simuliidae	*Australosimulium bancrofti*
	Anisopodidae	*Sylvicola sp.*
	Drosophilidae	*Drosophilia yakuba*
		Drosophila melanogaster
	Ragionidae	*Symphormyia sp.*
	Empididae	*Empidid sp.*
	Calliphoridae	*Lucilia cuprina*
	Bibionidae	*Dilophus sp.*
		Bibio sp.
		Penthetria heteropterna
	Cecidomyiidae	*Cecidomyiid sp. A*
		Cecidomyiid sp. B
	Sciaridae	*Corynoptera sp.*
		Bradysia sp.
	Ditomyiidae	*Ditomyia sp.*
	Keroplatidae	*Macrocera sp. 2*
		Urytalpa sp.
12S + 16S		
	Tipulidae	*Tipula ultimata*
		Limonia sp.
	Culicidae	*Anopheles quidrimaculatus*
	Simuliidae	*Simulium bivittatum + Australosimulium bancrofti**
	Anisopodidae	*Sylvicola sp.*
	Drosophilidae	*Drosophilia yakuba*
	Calliphoridae	*Lucilia cuprina*
	Bibionidae	*Dilophus sp.*
	Cecidomyiidae	*Cecidomyiid sp. B*
	Ditomyiidae	*Ditomyia sp.*
	Keroplatidae	*Macrocera sp. 2*
		Urytalpa sp.
		*Platyrua sp. + Orfelia sp.**

* Composite sequence consisting of the 16S from first species and 12S from the second species. See text for discussion.

Results

ALIGNMENT

For the results of a phylogenetic analysis using molecular data to be meaningful there must be some certainty as to the positional homology of aligned sequences. Although the 16S and 12S rRNA genes contain highly conserved sequences which are easily aligned, the genes also contain many regions that have been evolving at a much faster rate. Frequently these more rapidly evolving regions have also experienced a number of deletion and/or insertion events which make an unambiguous alignment between distantly related taxa almost impossible. To compound the problem , the mitochondrial genome in insects has a strong A-T bias (Simon et al 1994) making it more difficult to distinguish between convergence and homology in rapidly evolving regions where the sequence mostly consists of just two bases. In the 16S and 12S genes the most variable regions are indeed A-T rich sequences that have experienced insertion/deletion events.

The alignment for the 16S and 12S ribosomal sequences are shown in appendix 1 and 2 respectively. The 16S sequence shown is that of the N (non-coding) strand. Position 1 in the alignment is located 23 bases from the 3' end of primer LR-N-13398; the final position (position 478) is located 33 bases in from the 3' end of primer LR-J-12887. The alignment was achieved by visual inspection without taking into consideration the secondary structure of the large ribosomal subunit molecule. Much of the sequence is sufficiently conserved to allow alignment without ambiguity. The regions between positions 18-32, 122-150, 218-275, 310-345, and 450-460, however, are A-T rich regions most of which also have insertion/deletions, and, therefore, alignments other than the one reproduced in appendix 1 are possible for these positions. In the Set I analyses,

to be discussed below, these ambiguously aligned regions, referred to in the text as hypervariable regions, were excluded or included depending on the analysis. When included, the alignment in appendix 1 was used; this alignment yielded 160 informative positions. Ninety of these informative positions, however, are located in the hypervariable A-T rich regions.

The alignment of the 12S sequences was more problematic than that of the 16S simply due to the larger number of taxa involved. Since the 12S sequences were used to evaluate both distant and close relationships, it was much more critical to obtain an unambiguous alignment in the variable regions because much of the phylogenetic information bearing on relationships between closely related taxa was more likely to be located in these regions. To this end the secondary structure of the `12S rRNA molecule was taken into account for the alignment.

Considerable work has been done to elucidate the secondary structure of the 12S rRNA molecule (Hickson et al. 1996, Simon et al. 1990, Simon et al. 1996, Kjer 1995) . According to these studies, the overall secondary structure is more conserved than the actual base sequence. Thus even in rapidly evolving regions of the molecule that have experienced numerous substitutions, the location of stems and loops do not seem to vary much across a wide spectrum of taxa, possibly due to the local constraints imposed on them by the molecule's functional role in the structure of the ribosome. Knowing which positions in the sequence data correspond to key stems and loops allow these regions to be aligned even when there may be little actual sequence similarity in these regions. Since stems consist of double-stranded regions held together by complimentary base pairing, the location of stems could be verified by the presence of complimentary sequences in those regions where a particular stem was expected to be found on the basis of it's location in related taxa. The model developed by Hickson et al. (1996) was utilized to construct secondary structural models for 15 of the

dipteran species used in this study. These models are shown in appendix 3. The numbers at either the beginning or end of selected stems give the position numbers in the aligned sequence (appendix 2) for the initial/terminal base pairs indicated. The numerical designations for stems follows that of Hickson et al. (1996). The location of the stems is indicated in the aligned sequences in appendix 2. Position 2 in the aligned sequence corresponds to the 3' end of primer SR-N-14588.

The length and locations of stems 32, 33, 34, 35, 36, 38, 40, 39, and 45 are well conserved . Stem 47, and to a lesser extent stem 48, are variable both in length and in base composition. Stem 42 and its loop is one of the most variable regions in the sequence. As pointed out by Hickson et al. (1996), it is not always true that the base composition in stems is more highly conserved over that in loops. This is born out by the results in this study. The GG motif in the single-stranded region between stem 33 and 34 is conserved across all taxa in this study; the base sequence in the loops between stems 47 and 33, 34 and 45, and the 3' half of the loop between 42 and 38 are likewise relatively well conserved, whereas stems 42, 47, and 48 are variable. Stem 36, even though nonvariable in terms of location and length, has a fair degree of sequence variation also. Most of the more important insertion/deletion events, however, have occurred in the single-stranded regions between stems 36 and 38, 40 and 39 and 45 and 47, and in the loop of stem 42. A 7-base long insertion not found in any of the other taxa occurs in the loop of stem 48 of *Tetragoneura* (Mycetophilidae, Leiinae). Both species of *Macrocera* (Keroplatidae) have a long insertion (11 and 20 bases in the respectively) between positions 51-71; this insertion is in a single-stranded region between stems 33 and 32 (the latter stem lies outside the area sequenced in this study).

In the following analyses the hypervariable/ambiguous regions were excluded from one or more of the analyses. These regions include positions 5-29; the loop between stems 36 and 38 (positions 163-173); the loop between stems 40 and 39 (positions 214-219); stem 42, its loop and some of the adjacent sequence (positions 226-249) and part of the loop between stems 45 and 47 (positions 313-318). These regions are referred to in the following as 12S hypervariable positions. Two other variable sections include the region extending from the beginning of the loop of stem 45 to the 3' end of stem 47 (positions 313-345), referred to in the following as stem 47, and the distal half of stem 48 and all of its loop (positions 369-389). The specific regions excluded for a particular analysis will be given below in conjunction with the discussion for that analysis.

The aligned 12S sequence yields 144 informative positions, 44 of which are in the hypervariable regions. Stem 47 includes 18 informative positions and stem 48 includes 11.

SET 1 ANALYSES

The first set of analyses were conducted to determine whether the molecular data provided any evidence for the monophyly of the Bibionomorpha represented by the Bibionidae and Sciaroidea and to assess the position of the Bibionidae with respect to the Sciaroidea. In this set of analyses only the taxa given in table 5 were included. This set consisted of four series of analyses: series A using only the 16S data, series B using both 16S and 12S sequence, series C using only 12S data and series D also based only on 12S data but extending the analysis to include additional sequence representation for all groups except the Anisopodidae. Series A included two analyses, the first of which was based on all of the sequence data, including hypervariable and ambiguously aligned regions; in the second analysis, all of the hypervariable and ambiguously aligned regions listed

above in the previous section were excluded. Series B consisted of six analyses: 1) all sequence data included, 2) the hypervariable regions of 16S excluded and all data from 12S included, 3) all sequence data for 16S included and all hypervariable 12S regions excluded, and 4) hypervariable regions for 16S and 12S both excluded, 5) the same as analysis 4 but with stem 48 included, and 6) the same as analysis 4 but with both stems 47 and 48 included. For all analyses in this set, positions 313-344 and 369-389 (stem 48 + loop) were part of the 12S exclusion set.

Series A: 16S only

When the 16S data alone was employed, with all positions included, PAUP yielded two most-parsimonious trees at 463 steps with a consistency index of .449, and 7 more trees one step longer (464 steps). In all nine of these trees the Bibionidae and Sciaroidea occupied single clade with the Culicomorpha (*Anopheles* and *Simulium*) and Brachycera occupying a second major clade. The position of *Sylvicola* was variable, in four of the nine trees, *Sylvicola* was the basal taxon in the Culicomorph-Brachyceran clade lying basally to the Culicomorpha. In the remaining trees, *Sylvicola* was the sister-group to the (Culicomorph + Brachycera) + Bibionomorpha. In none of the trees does *Sylvicola* appear on the Bibionomorpha branch. In the Culicomorph-Brachycera clade, *Drosophila* and *Lucilia* consistently occurred together with the Culicomorpha lying basally to these. Although the Culicomorpha was located basally to the Brachycera in all trees, the two culicomorph taxa did not occur together on a common branch in any of the trees, but rather *Anopheles* was situated basally to *Simulium*. The monophyly of the Culicomorpha, however, is well supported by morphology. Most of the variation between the nine trees was due to the arrangement of taxa in the Bibionomorpha clade. Tree number two from this analysis is shown in figure 6A. This tree is one of the two most parsimonious trees. The node between the outgroup (Tipulidae) and the rest of the Diptera is supported by character-state

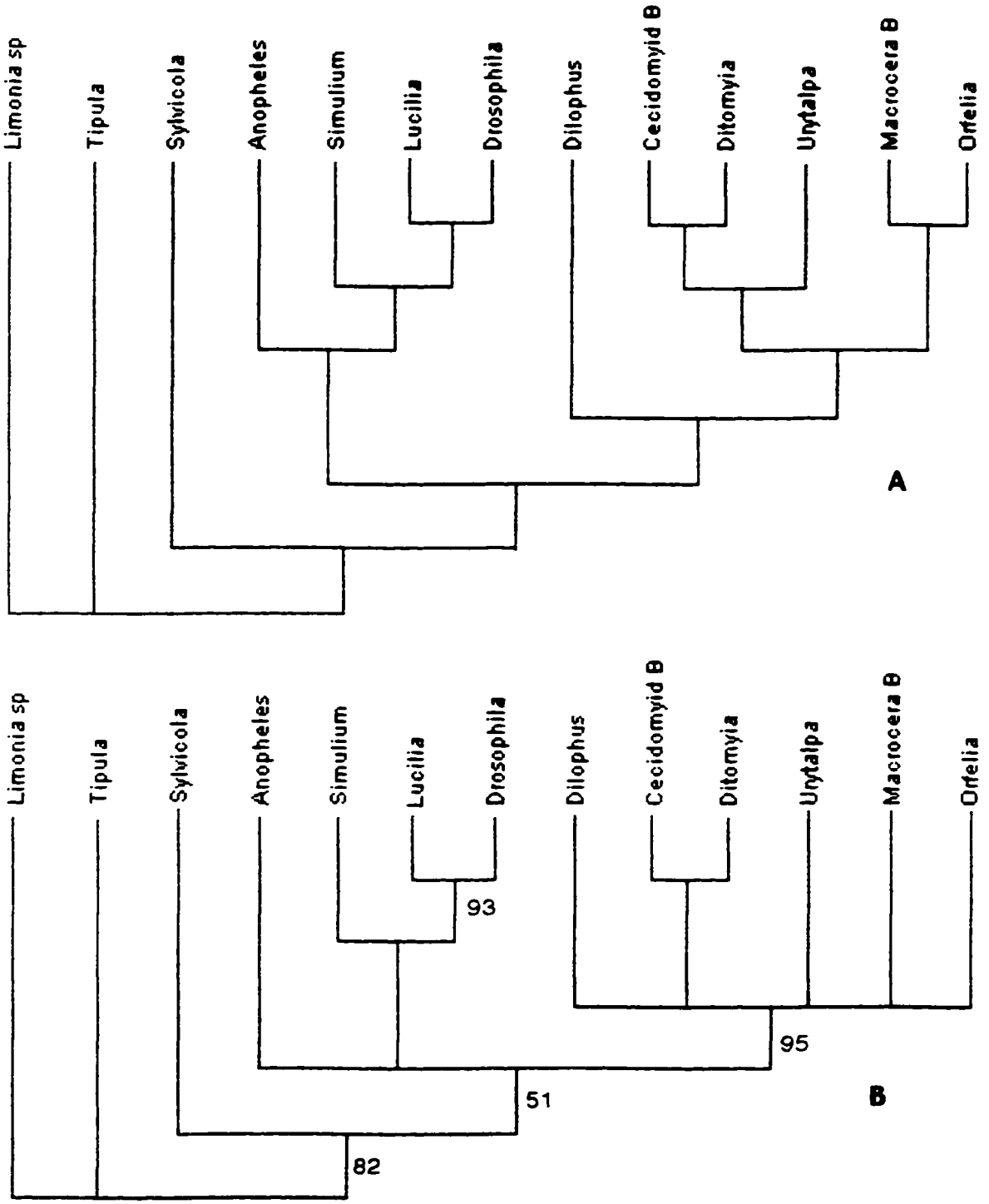


Figure 6. Set 1 analysis using 16S sequence with all positions included. A. One of the two most parsimonious trees obtained in the analysis. B. A consensus tree of the nine shortest trees with bootstrap percentages based on 2000 iterations. Even when ambiguous and hypervariable characters were included in the analysis, there is still strong support for the Bibionomorpha *sensu stricto*. The results of this analysis does not support a close association of the Anisopodidae , represented by *Syvicola*, with the Bibionomorpha.

changes at 18 positions, 8 of which are unambiguous, that is, occurring in all nine reconstructions. Only two of the unambiguous changes are non-homoplasious. Although 15 character-state changes support the basal position of *Sylvicola* in this tree, only 5 of these are unambiguous and all are homoplasious. The Bibionomorpha branch is supported by changes at 29 positions, 15 of which occur in all nine reconstructions. Six of these changes are unique and show no reversals or parallelisms on other branches of the tree. A consensus tree based on all nine trees is shown in figure 6B. Bootstrap percentages are given for key branches.

A little over one half of the informative positions in the 16S sequence are located in hypervariable regions (88 out of 160 positions). When these hypervariable regions were excluded, four trees were found at 181 steps with a consistency index of .514. PAUP found 18 additional trees one step longer. The four shortest trees had the same overall topology as the trees obtained in the previous analysis when all sites were included, except that *Sylvicola* was the basal taxon of the Culicomorph + Brachycera clade in all four trees. The two culicomorph species did not occupy a common branch but, as in the previous analysis, occurred adjacent to each other on separate branches. All of the topological variation between the four trees was due to arrangements within the Bibionomorpha. The Culicomorph + Brachycera clade, which in this analysis included *Sylvicola*, was supported by six character changes, four of which were unambiguous and two of which were unique. The Bibionomorpha clade was supported by eleven changes, all but one of which were unambiguous and four of which were unique. The strict consensus tree using the 22 shortest trees (181 and 182 steps) along with bootstrap percentages is shown in figure 7. Although the consensus well supports the Bibionomorpha clade, there is no resolution of taxa within the clade.

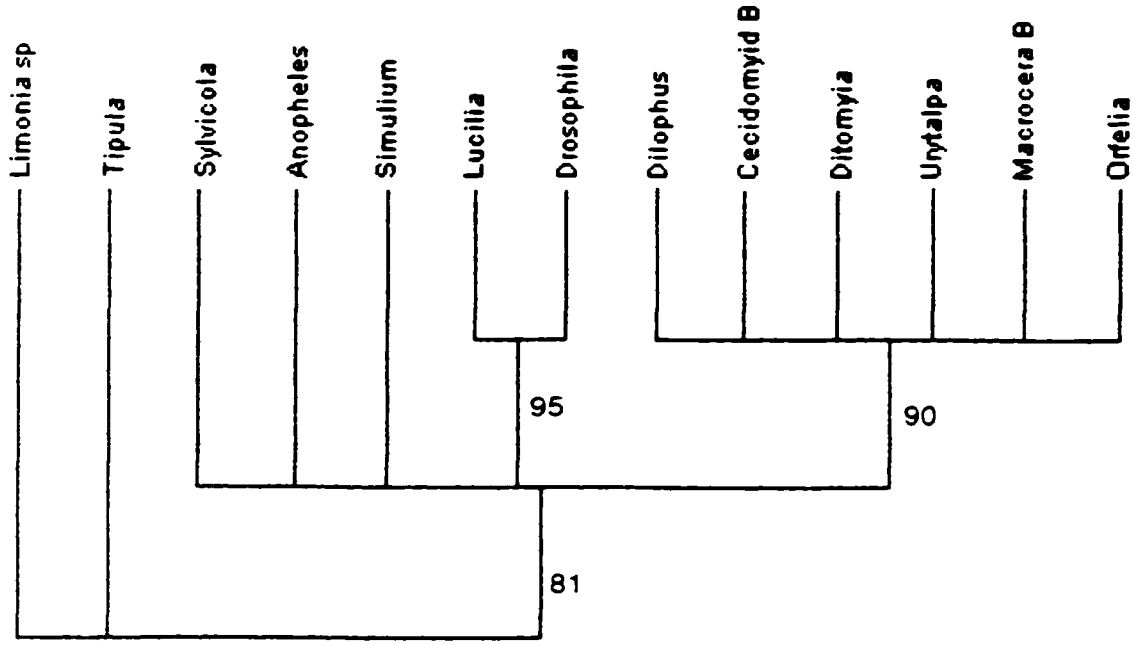


Figure 7. Set 1 analysis using 16S sequence with hypervariable positions excluded. The strict consensus tree was constructed from the 22 shortest trees. High bootstrap percentages strongly support the Bibionomorpha and the Brachycera.

Series B: 16S + 12S

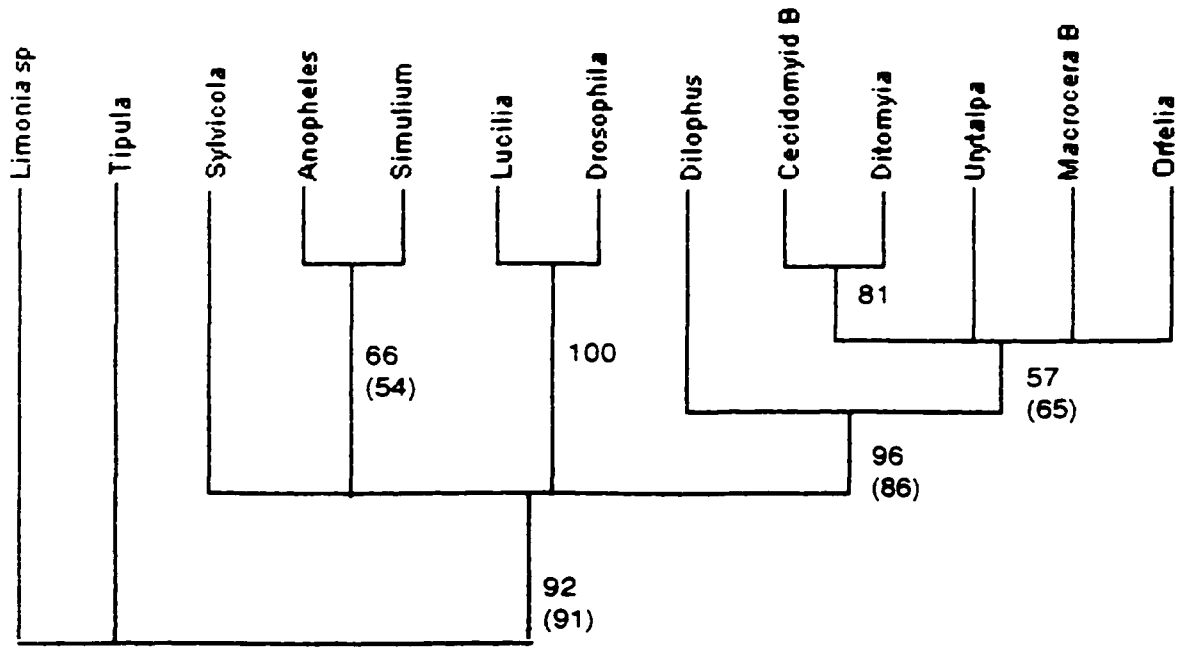
Analysis 1. The combined data sets for the 16S and 12S genes contain 303 informative sites. The inclusion of all positions yielded 3 trees at 903 steps with a consistency index of .443. One additional tree was found at 904 steps and 4 trees at 905 steps. All three of the shortest trees had the same overall topology, differing only in arrangements within the Sciaroidea. Two major clades were present, a Culicomorpha + Brachycera clade and a Bibionomorpha clade. The two culicomorph taxa occurred together on the same branch. The Anisopodidae (*Sylvicola* sp.), as in the previous analysis (A-1), occupied a basal position as the sistergroup of (Culicomorpha + Brachycera) + Bibionomorpha. Within the Bibionomorpha the Bibionidae (*Dilophus* sp.) consistently occurred as the sistergroup to the Sciaroidea. The ingroup taxa were separated from the outgroup (Tipulidae) by 43 changes, 22 of these involved unambiguous changes found in all

trees, four of these changes were non-homoplasious. Thirty-six changes occurred along the node between *Sylvicola* and the (Culicomorpha + Brachycera) + Bibionomorpha; 11 of these were unambiguous but only one was unique. The Bibionomorpha was well supported by 50 character changes, 20 of which were unambiguous and nine unique. The node separating the Sciaroidea and the Bibionidae had 35 changes, 14 unambiguous and one unique. The strict consensus of the three most parsimonious trees is shown in figure 8A. The consensus of the eight shortest trees, together with bootstrap percentages, is shown in figure 8B. A rather high bootstrap value of 81 percent was obtained for a Cecidomyiidae + Ditomyiidae relationship.

Analysis 2. When the hypervariable 16S positions were excluded and all 12S positions included, two trees were found at 620 steps with a consistency index of .458. Ten more trees were found at 621 steps and ten additional trees at 622 steps. The exclusion of the 16S hypervariable regions still yielded trees with the same general topology as in the previous analysis. In all of the twelve shortest trees the Bibionidae appeared as the sistergroup to the Sciaroidea. The topology of the consensus of the twelve shortest trees did not differ from the consensus tree in the previous analysis (figure 8B) except for the absence of a Cecidomyiidae + Ditomyia clade. The bootstrap values remained about the same as in the previous analysis: 54% for the Culicomorpha node, 86% for the Bibionomorpha node, and 65% on the node separating the Bibionidae (*Dilophus sp*) from the Sciaroidea.

Analysis 3. When only the hypervariable 12S positions (stem 48 and 47 inclusive) were excluded and all 16S positions included, one tree was found at 662 steps (consistency index=.452), three trees at 663. and thirteen at 665 steps. The consensus based on these sixteen trees was the same as the tree shown in figure 7 except for the presence of a Cecidomyiidae + Ditomyidae clade.

A



B

Figure 8. Set 1 analysis using 16S and 12S sequence with all positions included (series B, analysis 1). A. The strict consensus of the three shortest trees. B. The strict consensus of the eight shortest trees, with bootstrap percentages shown on supported nodes. The percentages in parentheses are those obtained when 16S hypervariable positions were excluded (series B, analysis 2). In the latter analysis the Ceciodmyiidae + Ditomyia clade collapses into the sciaroid polytomy.

The exclusion of the 12S variable data resulted in the collapse of the Bibionidae + Sciaroidea node present in the two previous analyses. The bootstrap values for supported nodes remained close to those obtained in the previous analysis. The consensus of the sixteen shortest trees for this analysis along with bootstrap values is shown in figure 9A.

Analysis 4. The exclusion of all hypervariable positions from both 12S (stem 48 and 47 also excluded) and 16S resulted in a larger number of trees but once again there were no significant changes in the overall topology. One tree was found at 385 steps with a consistency index of .488, and 18 more trees at 386 steps and 54 trees at 387. Despite the large number of trees that were obtained, a strict consensus of all 72 trees was identical to the consensus tree obtained in the previous analysis (figure 9A). The bootstrap value for the Bibionomorpha node, as in the previous analysis, was 99%..

Analysis 5. Stems 47 and 48 in the 12S sequence are A-T rich regions. Nonetheless, positional homology in these stems is not problematic. When the same analysis as above (hypervariable 12S and 16S excluded) was again performed but with the inclusion of stem 48, two trees were found at 423 steps (consistency index=.467), six more one step longer, and 12 two steps longer. In all of the eight shortest trees, *Sylvicola sp.* consistently occurred as the sistergroup of the Bibionomorpha, the Brachycera as the sistergroup of *Sylvicola* + Bibionomorpha, and the Culicomorpha occupied a branch between the Brachycera and the outgroup. The variation in topology between these trees was due to alternate arrangements within the Bibionomorpha. *Dilophus* occurred as the sistergroup of the Sciaroidea in four of the eight trees. The consensus of the eight shortest trees is shown in figure 9B . The basal nodes, however, collapsed with the addition of the twenty-two 425-step trees to the consensus, yielding a topology the same as in figure 9A but without support for the Culicomorpha. This

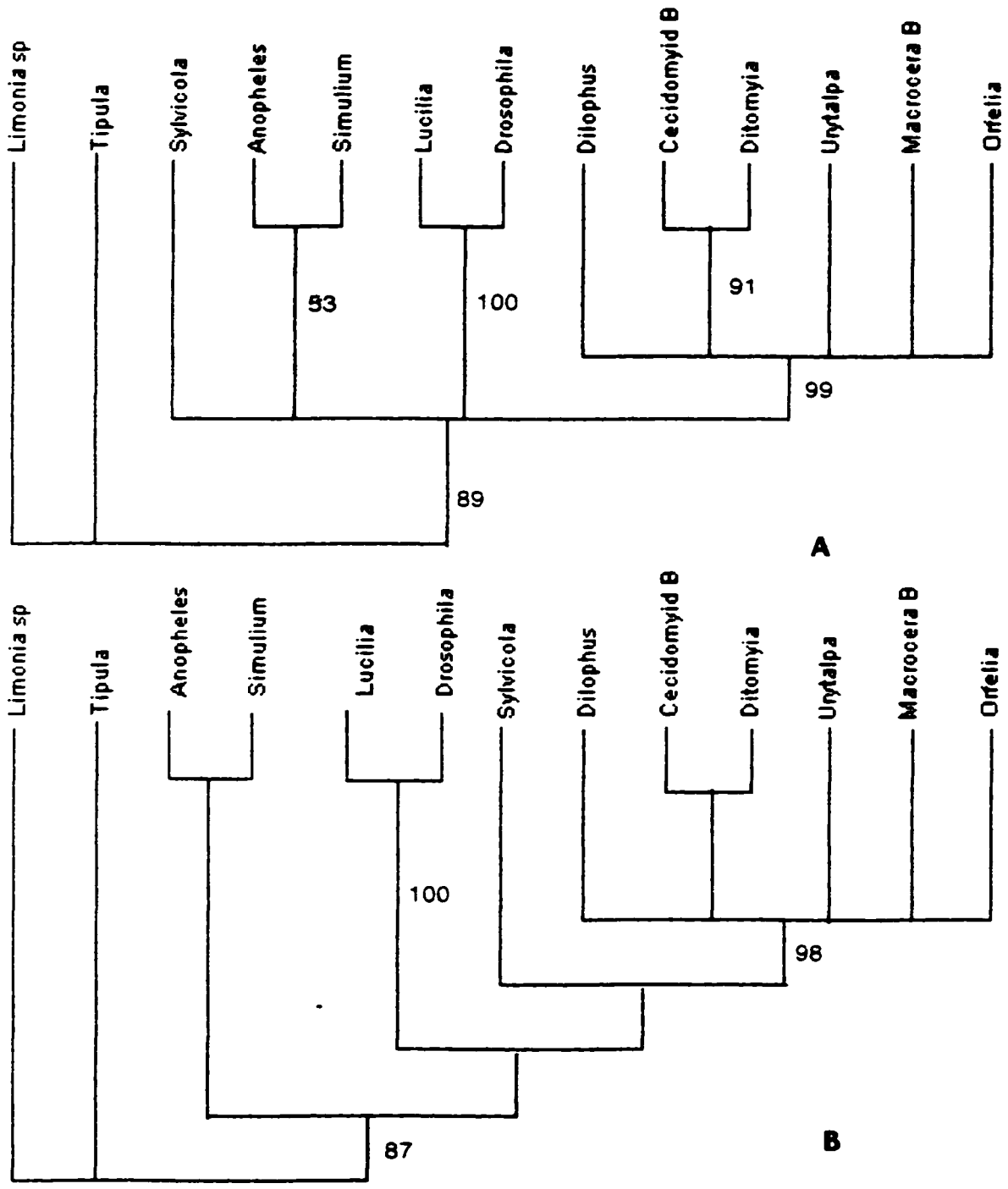


Figure 9. A. Consensus of the 16 shortest parsimony trees from set 1 analysis using 16S and 12S sequence with only the hpyervariable 12S positions excluded (series B, analysis 3). Bootstrap values are shown for supported nodes. The same consensus was also obtained when both the 12s and 16S hypervariable positions were excluded (series B, analysis 4). B. Consensus of the 8 shortest parsimony trees from the same data set as in A but with hypervariable 12S and 16S positions excluded and stem 48 (12S) included (series B, analysis 5). Bootstrap percentages for nodes other than those given in the figure were 50% or less.

was also the topology obtained in the bootstrap analysis (values of 97% for the Bibionomorpha and 51% for Cecidomyiidae + Ditomyiidae).

Analysis 6. The further addition of stem 47 to the analysis did not substantially alter the above outcome. The consensus of the six shortest trees (two at 487 and four at 488 steps) yielded the same topology as in figure 9B. The addition of 13 more trees obtained at 489 steps to the consensus gave the same topology as in figure 9A. In both of the shortest trees and in nine of the longer trees, Sylvicola occurred as the sistergroup to the Bibionomorpha and in seven of the trees as the basal branch of the Culicomorpha + Brachycera clade. Dilophus was the sistergroup of the Sciaroidea in both of the shortest trees and in six of the longer trees. The bootstrap values from this data set were not substantially different than those in the previous two analyses.

Series C: 12S only

In the third series of analyses, all 16S data was excluded and various combinations of 12S data were analyzed: 1) all positions included; 2) all hypervariable positions excluded, excluding also stem 47 and 48; 3) including stem 48 but excluding other hypervariable positions; and 4) including stems 47 and 48 but excluding other hypervariable positions.

In analysis 1, one tree was found at 427 steps (consistency index .443), three at 428 steps, and zero trees at 429 steps. The consensus for the four shortest trees along with bootstrap percentages for supported nodes are shown in figure 10A. In all four trees, *Sylvicola* occurred as the sistergroup to the Bibionomorpha and *Dilophus* as the sistergroup to the Sciaroidea. Although the consensus tree was fairly well resolved except among the sciaroid taxa, bootstrap percentages for many of the internal nodes were low. Although in the consensus tree *Sylvicola* occupied a sistergroup relationship to the Bibionidae + Sciaroidea, this

relationship occurred in only 62% of the bootstrap trees. A higher bootstrap value of 76% separated the Bibionidae from the Sciaroidea. The Cecidomyiidae and *Ditomyia*, as in many of the previous analyses, occurred together on one branch of a polytomy shared with all other sciaroid taxa. In general, the results of this analysis was similar to those obtained in series B analyses 5 and 6.

Analysis 2. Excluding the hypervariable 12S, stem 47, and stem 48 positions resulted in two trees at 187 steps (consistency index=.492) and fourteen more one step longer. Three of the 188-step trees could not be rooted such that the outgroup (Tipulidae) was monophyletic. The consensus of all sixteen shortest trees (figure 10B) was largely unresolved, with a basal polytomy consisting of *Limonia sp.*, *Tipula*, the Culicomorpha, *Sylvicola*, and the Bibionomorpha. In the bootstrap analysis the bibionomorph taxa occupied a common clade in 82% of the trees. The Culicomorpha had higher bootstrap support (81%) than was the case in the previous analyses. When the three trees incompatible with the monophyly of the Tipulidae were excluded, the topology of the resulting consensus tree was the same as that shown in figure 9A. This result was more resolved than, but fully compatible with, the consensus obtained in the previous analysis.

Analysis 3. When stem 48 was added back to the data set, two trees at 226 (C.I.=.465) and fifteen trees at 227 were found. Two of the 227-step trees were incompatible with the monophyly of the outgroup. The consensus of all seventeen trees is shown in figure 11. The topology is similar to the consensus in analysis 1. The Bibionidae, however, shares a polytomy with the sciaroid taxa, and the node between the outgroup and the Culicomorpha collapses due to the incompatibility of the two 227-step trees mentioned above. The inclusion of stem 48 did not much affect the bootstrap percentages at most nodes in relation to those in analysis 1, except for that for the Cecidomyiidae + *Ditomyia* node increased from

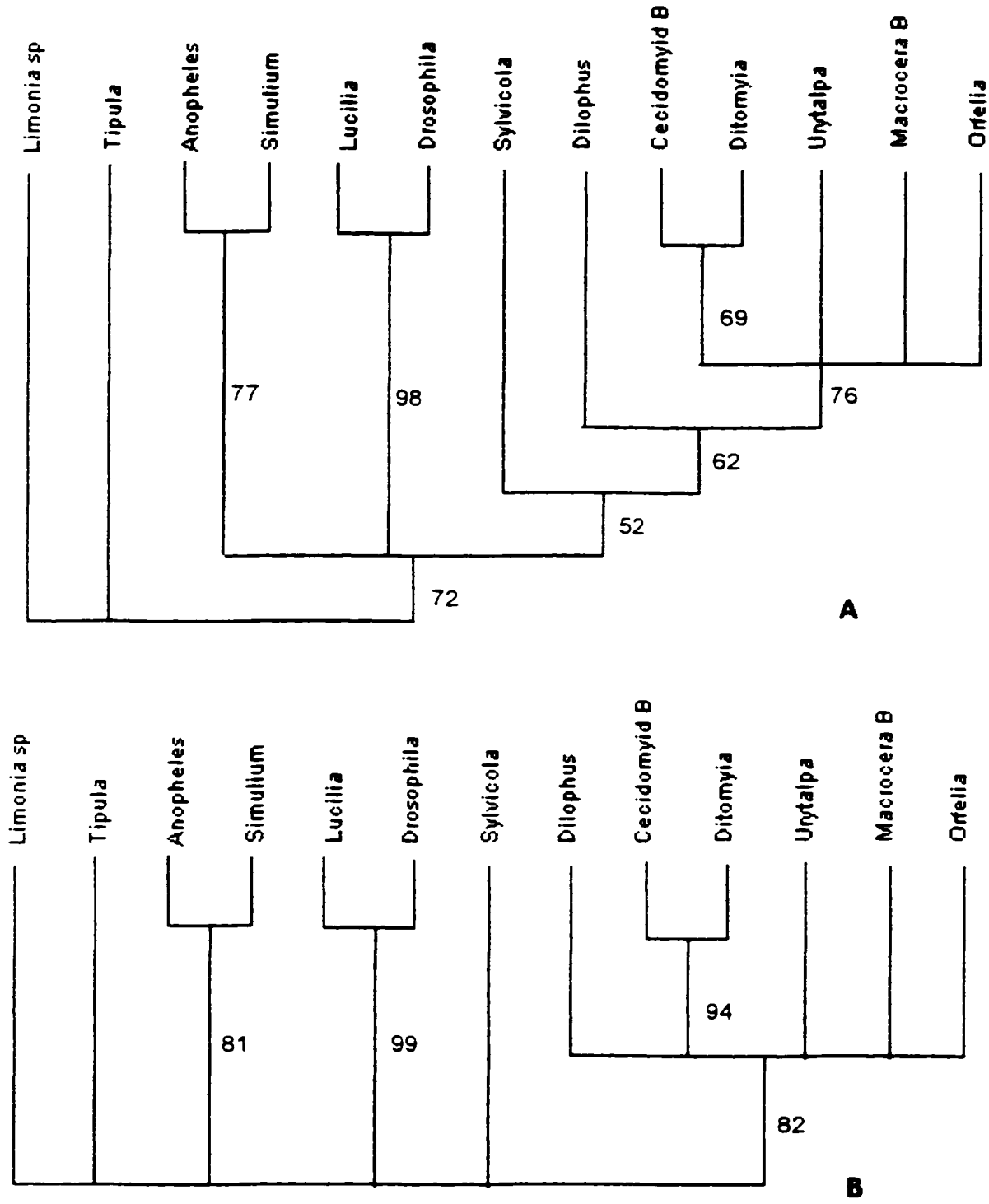


Figure10. Set 1 series C analysis using only 12S data set. A. Consensus of the 4 shortest trees when all positions were included (analysis 1). B. Consesnus of the 16 shortest trees when hypervariable, stem 47, and stem 48 positions were excluded (analysis 2). Bootstrap values, shown on supported nodes..

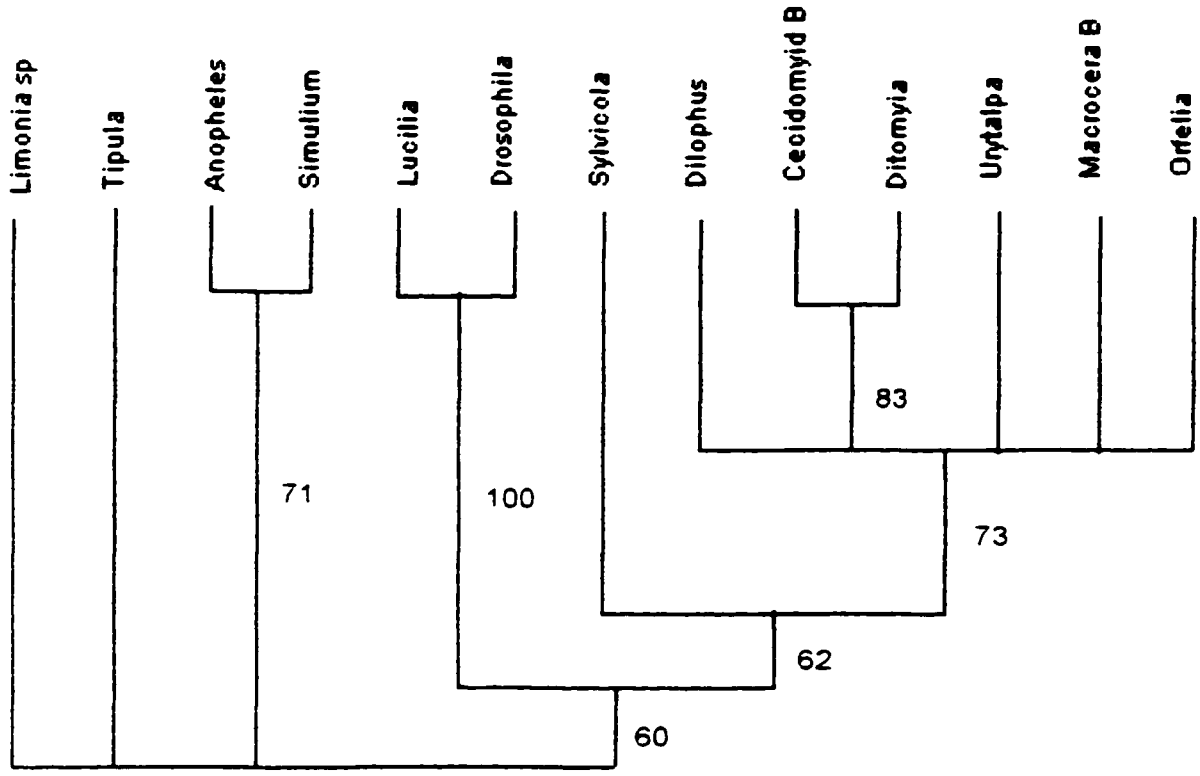


Figure11. Set 1 series C analysis using only 12S data set with hypervariable positions and stem 47 excluded and stem 48 included (analysis 3). Adding stem 47 to the data set (analysis 4) resulted in a resolution of the basal polytomy but otherwise yielded the same consensus.

69% to 83%.. The exclusion of the two incompatible 227-step trees from the consensus results in a tree identical to that shown in figure 9A.

Analysis 4. The addition of stem 47 to the data set resulted in three trees at 290 steps (C.I.=.455), four at 291, and three at 292. The topology of the consensus of all ten shortest trees is the same as that shown in figure 9B and differs from the consensus tree in the previous analysis (analysis 3) only in the displacement of the Culicomorpha basally to a position between the outgroup and the Brachycera.

Series D: 12S only with additional taxa

Since more sequences had been obtained for the 12S gene than for the 16S rRNA gene, the same analyses as in series C were conducted but with the addition of ten more taxa as follows: Culicomorpha, *Anopheles quadrimaculatata;* Brachycera: *Symphoromyia sp.* (Rhagionidae), unidentified empidid sp. (Empididae), *Drosophila melanogaster* (Drosophilidae), and *Musca domestica* (Muscidae); Bibiomorpha: *Bibio sp.* and *Penthetria heteropterna* (both Bibionidae), cecidomyiid sp. A (Cecidomyiidae), *Corynoptera sp.* and *Bradysia sp.* (the latter two Sciaridae). The same four analytical variations were performed: 1) all sites included, 2) hypervariable + stem 47 + stem 48 excluded, 3) stem 47 added back to data, and 4) stem 48 added to data with only hypervariable positions excluded.

Analysis 1. With no positions excluded the analysis found one tree at 766 (consistency index=.383) steps, shown in figure 12A, and 22 trees one step longer. The strict consensus and bootstraps trees (figure 12B) had the same general topology as the consensus trees in previous analyses involving 12S sequences. Once again, *Sylvicola* appears as the sistergroup to the Bibionomorpha. Within the Bibionomorpha the sciaroid taxa occurred in the same clade in 20 of the 23 shortest trees, usually forming a tritomy with *Penthetria* and *Dilophus + Bibio. Dilophus* and *Bibio* occurred together in 100% of the bootstrap trees and the Bibionidae *(Dilophus + Bibio + Penthetria)* in 44% .

Analysis 2. Excluding the variable A-T regions and stems 47 and 48, 16 trees were found at 315 steps (consistency index=.423) and 619 trees one step longer. The inclusion of additional taxa led to a much higher number of alternative trees. A strict consensus of all 345 trees was mostly unresolved. Nonetheless, a Culicomorpha clade was present in 94% of the trees, Brachycera without the Ragionidae occurred in 93%.. A Bibionomorpha clade was present in

all trees, finally, within the Bibionomorpha, a monophyletic Bibionidae occurred in 58% of the trees and occupied a sistergroup relationship to the Sciaroidea 40% of the time. A consensus of the 16 shortest trees was identical, except for arrangements within the Bibionomorpha, to the shortest tree found in the first analysis of this series (figure 12A). The exclusion of the variable regions resulted in the displacement of *Penthetria* into the Sciaroidea, with the latter in a sistergroup relationship to *Dilophus + Bibio* in 75% of the trees. A bootstrap analysis was not performed for this data.

Analysis 3. The reintroduction of stem 47 into the analysis yielded one tree at 425 steps (consistency index=.398) and 40 more at 426 steps. The consensus was identical to the consensus in shown in figure 12B with quite similar bootstrap values. A Bibionomorpha clade was present in all 41 and a monophyletic Bibionidae in 21 of the trees.

Analysis 4. The further addition of stem 48 yielded two trees at 497 (consistency index=.389) and 29 one step longer. The consensus of these trees is much like the one obtained in the first analysis (figure 12B), except for the collapse of the basal node into a polytomy with the outgroup. As in the previous analyses, a Bibionomorpha clade was present in all 31 of the shortest trees.

Summary of Set I Analyses

This set of analyses was undertaken to either confirm or refute the hypothesis of a close relationship between the Bibionidae and the Sciaroidea. I have taken a conservative approach in examining not only the shortest parsimony trees but also those within one or two steps of the shortest to ascertain which nodes appear to be reliably supported under a variety of data set combinations. The species representative of the infraorders Culicomorpha and Brachycera fairly consistently occurred together in their respective clades; the two lower Brachycerans, the Empididae and Rhagionidae, however, sometimes shifted onto

adjacent branches, but their positions could be determined only by 12S data alone. The Bibionomorpha was the most consistent clade occurring in this set of analyses, being present in every tree that was examined within one or two steps of the shortest possible tree in every series of analyses. Within the Bibionomorpha, the bibionid taxa occurred as the sistergroup to the Sciaroidea in well over 60% of the trees examined, mostly when 12S sequence was involved. The 16S alone, while supporting the Bibionomorpha, did not resolve the relationship between the Bibionidae and the Sciaroidea. The Anisopodidae (*Sylvicola*) occupied as the sistergroup to the Brachycera in only one tree in this set. In most of the trees generated with the inclusion of 12S sequence, *Syvicola* frequently occupied a position between a Culicomorpha + Brachycera clade and the Bibionomorpha as the sistergroup to the latter, and was displaced further down the phylogeny below the Culicomorpha + Brachycera branch only when 16S alone was used. When it did occur on the Culicomorpha + Brachycera branch, in all but one instance it was the basal-most branch of the clade.

The Bibionidae occurred as the sistergroup of the Sciaroidea in over half of the examined trees when the Tipulimorpha were used as the outgroup. However, when the same series of analyses were performed after eliminating the Tipulimorpha from the study moving the Culicomorpha to the outgroup, *Dilophus* appeared as the sistergroup of the Sciaroidea in all trees within two steps of the shortest under all combinations which included 12S sequence. Only in the analyses using 16S alone did a clear sistergroup relationship not emerge.

The inclusion of stems 47 and 48 (12S), although A-T rich regions, resulted in less ambiguity in the data as evinced by the much larger number of alternate trees obtained in analyses in which they were excluded.

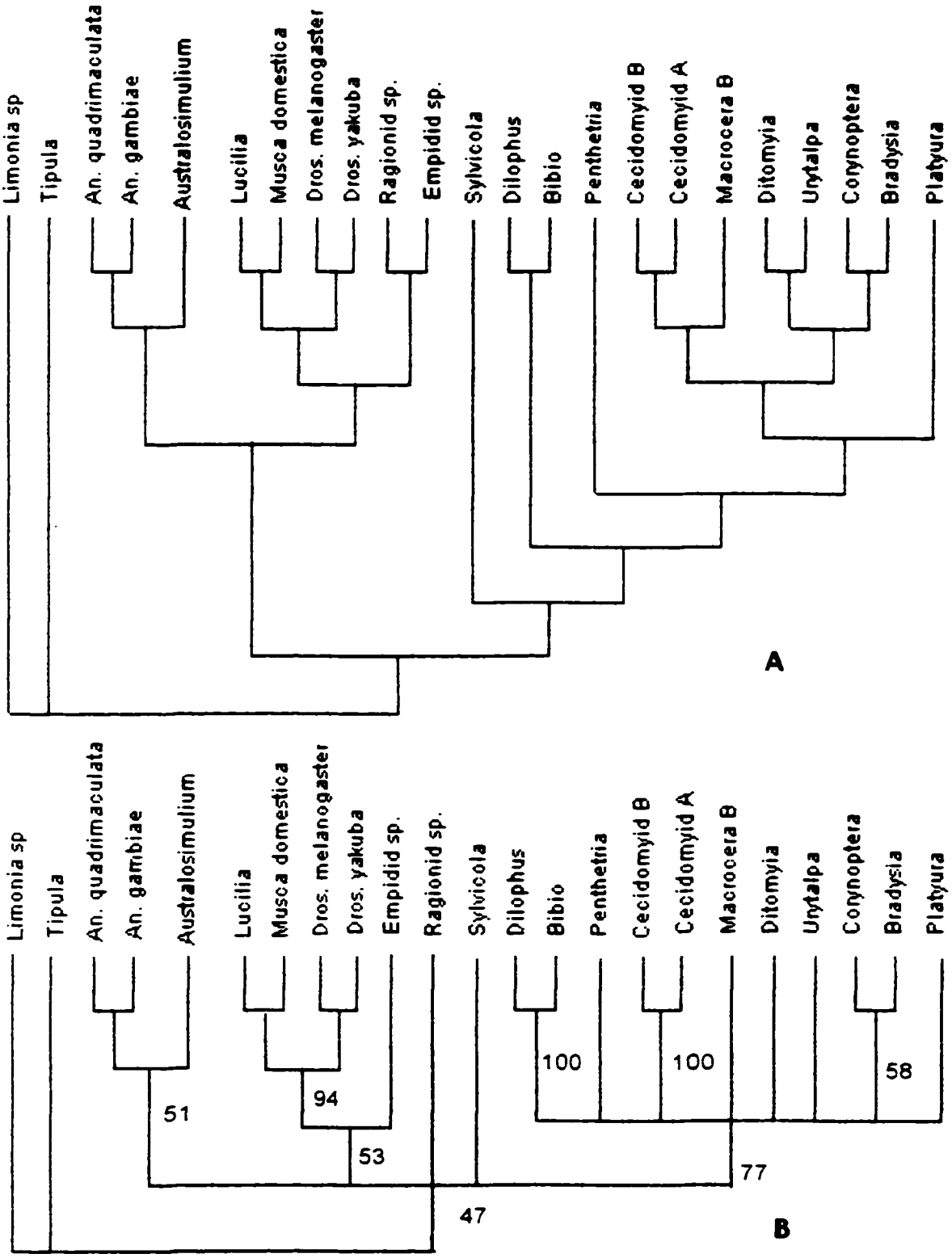


Figure12. Set 1 series D analysis using only 12S data set with additional taxa. A. Shortest obtained when all positions were included (analysis 1). B. Consesnus of the 23 shortest trees obtained in the same analysis as A. Bootstrap values, shown on supported nodes..

SET II ANALYSES

Introduction

In the second set of analyses the Bibionidae was used as the outgroup for the examination of the phylogeny in the Sciaroidea. This set of analyses involved many more sequences than the previous one, over half of which came from the large family Mycetophilidae. The inclusion of a greater number of sequences complicated the analysis for several reasons. The third domain of the 12S rRNA gene contains well-conserved regions as well as rapidly evolving ones. Simon et al. (1994) noted that the gene seems to be useful for the analysis of deeper branches in a phylogeny but works less well for intermediate-level relationships. Simon suggested that this may be due to the unalignability of the variable regions between distantly related taxa, which are then usually excluded from the analysis. This leaves the more stable positions, which are more likely to contain phylogenetically informative signals, to predominate in the analysis. On the other hand, the conserved regions are quite uniform and uninformative if the analysis is restricted to a group of closely related species in a single genus or in a few closely related genera; in this case the variable regions are both more alignable and less likely to have experienced multiple substitutions. Nonetheless, at this level, as was evident in this study, there is very little variation, and what variation is present is too often found to be in conflict with each other. For example, among the three species of *Boletina* in this study, only six positions are variable, all of which occur in A-T rich regions close to gaps and which are also quite variable in other genera. Even for closely related species, these positions are still likely to be homoplasious. At intermediate levels the variable regions contribute a substantial degree of homoplasious noise which often results in the clustering together of longer basal branches (long branch attraction) simply due

to the chance similarity generated by multiple substitutions at these more rapidly evolving positions.

A similar pattern was recognizable in this study. However, the stability of deeper branches, contrary to the explanation given by Simon et al. (1994), does not appear to be due solely to the exclusion of ambiguously aligned variable regions. In the set 1 analyses the inclusion of hypervariable regions had little effect on the topology of the consensus trees and, for that matter, except for relations in the Bibionomorpha, on the topology of the parsimony trees. A more probable explanation is that in most studies which are restricted to deeper branches, only a few very distantly related taxa generally are included in the study. Under such circumstances the more stable regions have a sufficient number of changes to carry a stronger phylogenetic signal than the noise generated by the hypervariable positions. This probably would not be the case if the number of taxa representing a given taxon were to be increased.

In the following examination of the Sciaroidea, all of the above contributed to the generation of a large number of alternate trees, indicating conflict in the data due to the rapidly evolving positions. Additionally, since there was greater sequence homology, especially for taxa in the Mycetophilidae, more positions, usually in variable regions, could be included. Despite the greater sequence homogeneity the number of informative positions increased from the 144 in the set 1 analyses to 198 because of the taxonomic representation, but the number drops to 179 when the Bibionidae are excluded.

Fewer analyses were performed in this set to avoid a priori assumptions about the data. Separate analyses using different combinations of data were not performed. The whole sequence, including the moderately variable regions in stem 47 and 48 as well as portions of stem 42 was included in all analyses with only the exclusion of ambiguously aligned positions confined to a narrow zone flanking

gaps. These excluded positions were 16-25, 52-73 (an insertion found only in *Macrocera*), 168-173, 206-208 (part of the loop of stem 40), 214-215, 239-247 (part of loop and 3' half of stem 42), 303-306 (insertion in the loop of stem 45), 381, 313-315, and 375-380 (a five base insert in *Tetragoneura*). These exclusion positions are referred to below as gap regions. Gaps were treated as missing.

The following groups of analyses were performed.

A. A global analysis including all sciaroid taxa with the Bibionidae as outgroup.

B. The same as in A but using the basal Sciaroid lineage as determined in part A as the outgroup.

C. An analysis of the Mycetophilidae using the its sistergroup as determined by the above analyses as outgroup.

Although the general results of these analyses will be reported here, in each case a number of separate analyses were carried out on subsets of the taxa in the data matrix. In general, the same sequence positions were employed in all analyses. Exceptions to this will be noted where appropriate.

Analysis A

A global analysis of the Sciariodea resulted in 14 equally trees at 821 steps (consistency index = .348). Over 1700 trees one step longer were obtained but were not examined. A strict consensus of the 14 short trees is shown in figure 13A. The root for the ingroup in these trees was rather unstable, in some cases the mere inclusion or exclusion of a few positions bordering excluded gap regions was enough to move the root toward the middle of the ingroup such that the Cecidomyiidae, Ditomyiidae, and Sciaridae slid onto a basal branch with the Cecidomyiidae occupying the terminal position. This effect is undoubtedly due to long-branch attraction since the alteration did not affect the position of these

taxa with respect to each other or to adjacent taxa in the unrooted network, the change was merely in where this network was rooted with respect to the Bibionidae. Furthermore, the clustering of these three families makes little morphological sense, as will be discussed further below. When all positions, regardless of the ambiguity in alignment, were included, the root of the tree was the same as that shown in figure 13A.

The basal-most clade emerging from this analysis consists of the Ditomyiidae and Cecidomyiidae. Because of the instability of the root of the tree, the clustering of these two families may be due to long-branch attraction and does not necessarily indicate a sistergroup relationship between them, the results of this analysis are not adequate to allow a confident assessment of their relationship. Be that as it may, both families nonetheless emerge as basal members of the Sciaroidea. *Urytalpa* does not occur together with the rest of the Keroplatidae but is located more basally. The evidence for the monophyly of the Keroplatidae, however, is well established (Matile 1990, 1998). The rest of the Keroplatidae are on a common branch as sistergroup to the Sciaridae. The Bolitophilidae occurs as the sistergroup of a monophyletic Mycetophilidae. The node between the Bolitophilidae and the Mycetophilidae is supported by only seven base changes. Within the Mycetophilidae, most of the species belonging to the Mycetophilinae occur on a single branch, the only aberrant mycetophiline not on this branch is *Rhymosia*, which occurs on a branch with *Mycomya* and *Acnemia* (Mycomyinae and Sciophilinae respectively). The central mycetophilid clade is occupied by taxa classified in the Gnoristinae (*Boletina, Coelosia, Synapha, Gnoriste,* and *Hadroneura*) and the Leiinae *(Leia, Acompterella, Tetragoneura, and Docosia).*

Analysis B

In the second analysis, the Bibionidae were eliminated and the basal two sciaroid families, Ditomyiidae and Cecidomyiidae, were transferred to the outgroup. This analysis found two trees at 703 steps (consistency index = .364) and at least 600 trees one step longer before the search was terminated. The consensus of the two shortest trees is shown in figure 14A. The effect of selecting an outgroup one branch up the tree and eliminating the Bibionidae primarily affected the basal part of the tree. The two sciarids are dislocated toward the base to a position immediately above the Ditomyiidae-Cecidomyiidae. Even though the sciarids do not turn out monophyletic in this analysis, they at least occur in the same vicinity of the tree adjacent to one another. As was discussed in the introduction, the Sciaridae are most likely monophyletic. *Urytalpa*, once again, does not cluster with the other keroplatids, but has moved up the tree to a position immediately above them. The upper branches of the tree were considerably affected by the change in outgroups. The root between *Urytalpa* and the Mycetophilidae + Diadocidiidae + Bolitophilidae clade shifted to a position well within the Mycetophilidae, resulting in a major inversion in their relationships in comparison to those obtained in the previous analysis and dislocates the Bolitophilidae and Diadocidiidae to a position inside the Mycetophilidae, an outcome that would be difficult to justify on the basis of morphology . Additional analyses in which taxa were excluded singly or in combinations suggested that it was the sequence for *Urytalpa* that was responsible for the inversion. When *Urytalpa* was excluded from the analysis, the topology in the upper portion of the consensus tree (Figure 14B), derived from 14 equally parsimonious trees, was identical to that obtained in the global analysis using the Bibionidae used as outgroup. The exclusion of taxa other than *Urytalpa*

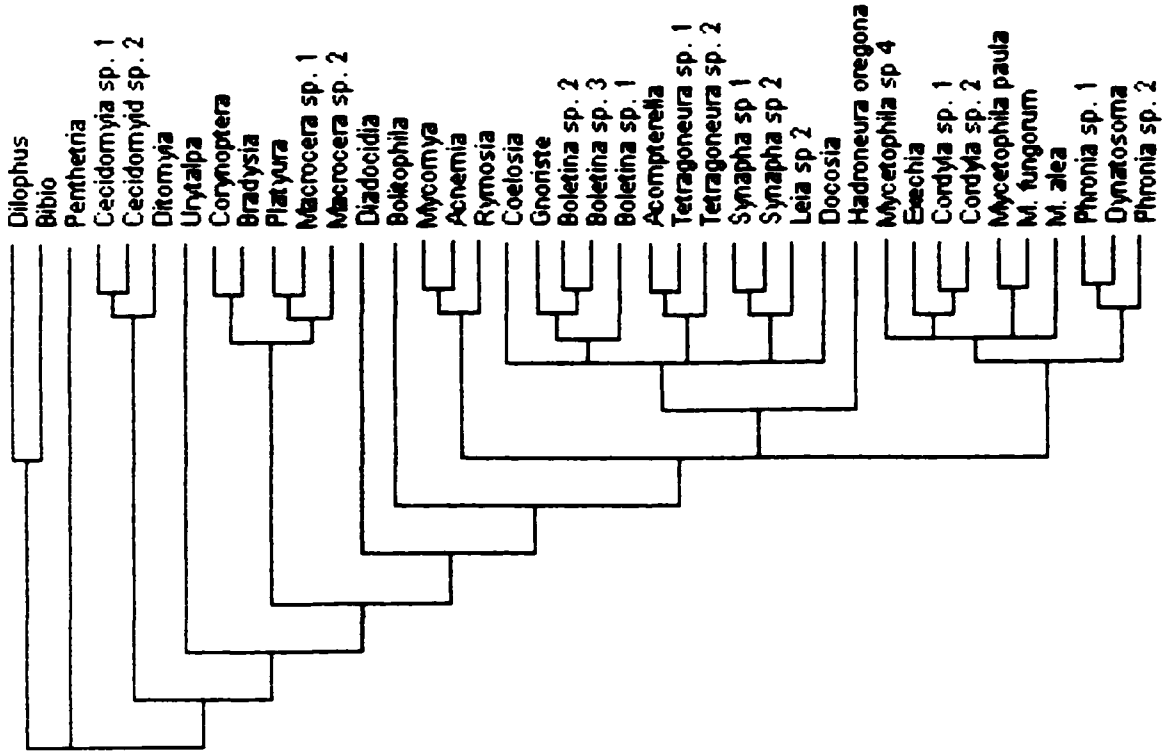


Figure 13. Phylogeny of the Sciaroidea. Strict consensus of the 14 equally parsimonious trees obtained in the second set of analyses using 12S rRNA sequences.

did not produce a similar inversion in the upper branches of the tree. The basal relationships remained the same whether or not *Urytalpa* was included, in fact, the same branching pattern was found at the base of all 600+ of the next-shortest trees. The large number of trees that was generated came mostly from rearrangements and alternate character interpretation among groups in the Mycetophilidae.

Although additional evidence would be needed to clarify the relationship of the Ditomyiidae to the rest of the Sciaroidea, the results obtained here lend some credence to the hypothesis that the Ditomyiidae are the sistergroup to the remaining fungus gnats and that the Cecidomyiidae are the sistergroup of the

Figure 14. Phylogeny of the Sciaroidea. Inner phylogeny using the Cecidomyiidae and Ditomyiidae as outgroups. A. Resulting phylogeny when *Urytalpa* was included resulting in a distortion of the relationships in the higher branches of the tree (see text). B. Same analysis excluding *Urytalpa*.

Ditomyiidae plus the other fungus gnats. If the Cecidomyiidae are transferred to the ingroup, the resulting tree contains a basal tritomy consisting of the Ditomyiidae, Cecidomyiidae, and the clade with the remaining Sciaroidea. On the other hand, when the Cecidomyiidae alone are used as the outgroup, this trichotomy is resolved with the Ditomyiidae in a sistergroup relation to the other fungus-gnat families.

Analysis C

In the previous two analyses, the Bolitophilidae emerged as the sistergroup of the Mycetophilidae, and the next family lower in the tree was the Diadocidiidae. Therefore these two families were used as the outgroups for an analysis of only the Mycetophilidae. Eliminating the influence of sequences from the lower portion of the phylogeny did not alter the general outcome for the Mycetophilidae obtained in the previous two analyses. Three clades are still evident: *Mycomya + Acnemia*, the Gnoristinae-Leiinae, and the Mycetophilinae. Surprisingly, PAUP uncovered only one tree, shown in figure 15, at 364 steps (consistency index=.442). Seventy-one additional trees were found one step longer. A strict consensus of all 72 trees had very little basal structure, but the following taxa were present in every tree: *Mycomya + Acnemia* ; *Synapha + Leia; Tetragoneura + Acompterella; Phronia* and *Dynatosoma;* and the Exechiini (*Exechia + Cordyla + Rymosia).* The three species of *Boletina* and *Gnoristina* (Gnoristinae) occurred together in all but three of the trees, and genera traditionally classified in the Leiinae and Gnoristinae occurred in a common clade in all but four of the trees.

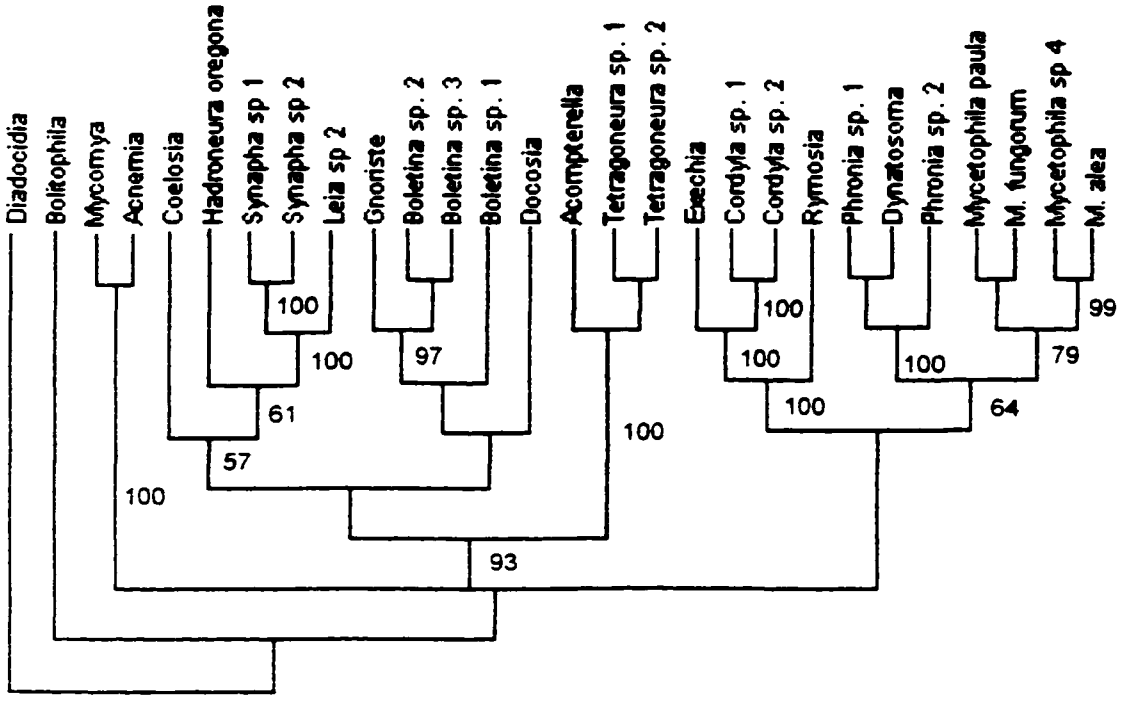


Figure 15. Phylogeny of the Mycetophilidae. The most parsimonious tree obtained when the analysis was confined to the Mycetophilidae using the Bolitophilidae and Diadociidae as outgroups. At the base of the Mycetophilidae is a tritomy whose three clades consist of taxa classified in the Mycomyinae and Sciophilinae *(Mycomya + Acnemia)*, the Gnoristinae and Leiinae (the large central clade), and the Mycetophilinae (the clade to the left). Numbers indicate the percentage of the shortest trees (n=72) in which the designated node occurs.

DISCUSSION

The results of the present study provide an independent test of the several morphologically derived hypotheses about the phylogeny of the Sciaroidea discussed earlier in this work. Although relationships outside the Bibionomorpha were not the object of this study, it is instructive to compare the results obtained in the Set I group of analyses, since these included a number of non-Bibionomorpha taxa, with other recent phylogenetic proposals for the Diptera. The 12S and 16S data, together and separately, reconstructed two

morphologically well-supported clades, the Culicomorpha and Brachycera. The basal members of both clades, however had a tendency in some of the alternate trees to slip down into an unresolved polytomy with adjacent clades or even to be transferred to the base of a neighboring clade. This was particularly the case for the Rhagiondae (Brachycera).

Of particular interest is the position of the Anisopodidae. This family is one of the families included in the Bibionomorpha by Hennig (1954, 1968, 1969, 1973) and whose position has continued to be controversial. On the basis of larval characters Wood and Borkent (1989) moved the Anisopodidae along with the Scatopsidae and Synneuridae to the Psychodimorpha. The Psychodimorpha was regarded by them as the sister group of the Ptychopteromorpha + Culicomorpha. Woodley (1989) further suggested that the Brachycera might be the sister group of some part of the Psychodomorpha such as the Anisopodidae. Sinclair (1992) concluded that the Psychodomorpha, including the Anisopodidae, was the sister group of the Brachycera. Oosterbroek and Courtney (1995) more specifically proposed the Anisopodidae as the sister group of the Brachycera. Neither the 12S nor the 16S data, however, provide much support for a close Anisopodidae-Brachycera relationship. The Anisopodidae and Brachycera were rarely found together in the same clade, and when they were, they were separated from each other by the Culicomorpha. The 12S rRNA data suggest that the Anisopodidae is closer to the Bibionomorpha than to the Brachycera. This agrees with the results obtained by Friedrich and Tautz (1997), who found strong support for an Anisopodidae-Bibionomorpha relationship from nuclear 28S rRNA.

Bibionomorpha

The molecular data strongly supported the monophyly of the Bibionomopha, or at least that portion of the Bibionomorpha consisting of the Bibionidae and Sciaroidea. The molecular evidence, moreover, is certainly more compelling than

the morphological evidence. Although they strongly felt that the Bibionomorpha, in their sense restricted to the Pachyneuridae + Bibionidae + Sciaroidea, was monophyletic, Wood and Borkent (1989) were unable to find convincing synapomorphies in support. Subsequent studies (Wood 1991, Amorim 1993, Blaschke-Berthold 1994, Oosterbroek and Courtney 1996) have found some morphological support for the Bibionomorpha, but most of this evidence needs further evaluation. Wood (1991) regarded a flattened aedeagus, in contrast to the tubular aedeagus widely found in the Diptera, as a synapomorphy of Bibionidae (in part) + Sciaroidea. Oosterbroek and Courtney (1995) adopted the same character but as a synapomorphy for the Sciaroidea. A tubular aedeagus is found in the Pachyneuridae and *Hesperinus* (Bibionidae) whereas, according to Wood, a flattened aedeagus appears to be the condition in the rest of the Bibionidae as well as in the Sciaridae and Cecidomyiidae. As a synapomorphy of the Bibionidae (*minus Hesperinus*) + Sciaroidea the shape of the aedeagus merits further study, especially in view of the variation found in some other fungus-gnat groups where it is not unusual to encounter a tubular aedeagus (Fisher 1939, personal observations).

Oosterbroek and Courtney (1996) located the Pachyneuridae between the Bibionidae and the Sciaroidea on the basis of two characters, the loss of a sperm pump in the males and non-superposed pupal leg sheaths. The first character is questionable, as acknowledged by the authors themselves, since some fungus gnats have a sperm pump (Matile 1990), the second involves a reversion to the plesiomorphic state.

Whether the Pachyneuridae or the Bibionidae, or for that matter the Hesperininae, which is sometimes treated as a family separate from the Bibionidae, is the sister group of the Sciaroidea is outside the scope of this study. But in view of the strong support for the Bibionidae + Sciaroidea clade present in

the 12S and 16S data, an extension of this study to include other relevant taxa such as the Pachyneuridae and Hesperininae, both taxa belonging to the Bibionomorpha in the strict sense, as well as the Scaptopsidae and Synneuridae, the latter two of which were included in the infraorder by Hennig, is desirable. The inclusion of the last two families is pertinent in view of the evidence from 28S rRNA (Friedrich and Tautz 1997) which clustered the Scatopsidae with other bibionomorph taxa.

Sciaroidea

As with the Bibionomorpha, few synapomorphies have been found in support of the monophyly of the Sciaroidea, perhaps because this is one of the lesser studied groups in the Diptera. Wood and Borkent (1989) cited the fusion of the posterior section of the larval cardo with the head capsule and the loss of the metathoracic spiracle in the larva as synapomorphies for the superfamily. The condition of the cardo in the Cecidomyiidae, however, is very poorly known. Wood and Borkent's assessment rested on a single species of gall midge illustrated in Petralia et al. (1979) . Before concluding that this condition is widespread, a greater number of species needs to be examined.

The distribution of functional spiracles in the larval tracheal system is compatible with a monophyletic Sciaroidea. The larvae of the Bibionidae and *Pachyneura* are holopneustic, provided with prothoracic, metathoracic, and eight abdominal spiracles. This is presumably the plesiomorphic state for the Diptera although it is found only in the Bibionidae and *Pachyneura.* In all families in the Sciaroidea the metathoracic spiracles have been lost in the fourth instar larva. If the Pachyneuridae are monophyletic, then the loss of the metathoracic spiracles in *Cramptonomyia* and *Pergratospes* [9] has occurred independently.

[9]A peripneustic tracheal system is also present in the Scatopsidae and Synneuridae. Hennig (1954) regarded these two families as close relatives of the

Another possible synapomorphy for the Sciaroidea is the reduction of the number of spermathecae in the female from the plesiomorphic number of three to two or fewer (Blaschke-Berthold 1994). Three spermathecae are present in the Bibionidae. The reduction in the number of spermathecae, however, has occurred independently many times in the Diptera.

The 12S and 16S sequences provide additional support for the monophyly of the Sciaroidea. This is especially true for the 12S gene. In the first set of analyses, the Bibionidae, represented by *Dilophus*, emerged as the sister group of a monophyletic Sciaroidea in only slightly more than half of the alternate trees examined, but in the extended analysis which included *Bibio* and *Penthetria*, a monophyletic Sciaroidea was present in all but a few of the trees. When this was not the case, the situation was due to the slippage of *Platyura* onto the Bibionidae clade. The rest of the sciaroid taxa remained together in all trees.

The inner phylogeny of the Sciaroidea as estimated from 12S rRNA (figure 14B) agrees in most features with the hypothesis presented by Matile (1990b, 1997) (figure 5B). The one major difference between the two phylogenies concerns the position of the Sciaridae, which Matile placed as the sister group of the Lygistorrhinidae + Mycetophilidae. The molecular data, however, point to a more basal origin for the Sciaridae.

The relationships of the Sciaridae have long been problematic. The family has been hypothesized (see introduction) to be the sister group to some portion of the Mycetophildae (Edwards 1925; Fisher 1937 and personal communication in Shaw 1948) (figure 1B), as the sister group of the Mycetophilidae (Rodendorf 1948, Hennig 1954) (figures 2 and 4)--Matile's hypothesis is a variation of this viewpoint-- or as the sister group of all the rest of the fungus gnats (Shaw 1948,

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Cecidomyiidae (see figure 4, p. 23). The absence of the metathoracic spiracles is definitely congruent with, even supportive of, this hypothesis.

Shaw and Shaw 1951, Blaschke-Berthold 1994)(figures 1B and 5A). The last hypothesis is untenable in view of several unique larval characters sciarids share with the higher fungus gnats. For example, the larval antenna is a broad, oval, one-segmented membranous structure quite unique in the Diptera. Among the fungus gnats, only the Ditomyiidae and Bolitophilidae posses a three-segmented cylindrical antenna (plesiomorphy). Additionally the larva of sciarids and other higher fungus gnats have lost the function of the spiracles on the eighth abdominal segment; the Cecidomyiidae and Ditomyiidae are the only sciaroids in which the 8th abdominal spiracles are still functional. The apneustic and propneustic conditions in the Keroplatidae and the Diadocidiidae respectively, are most likely derived from hemipneustic ancestors. In both families, non-functional spiracles are present. Finally, an convincing list of several other larval characters further argues against the Sciaridae as the sistergroup of the rest of the fungus gnats: the frontoclypeal apodeme extends to the posterior margin of the head capsule, the larval maxilla is flat and strongly sclerotized, the maxillary palps are reduced (Matile 1990, 1997), and the maxilla is strongly serrated along the anteromedial margin (Madwar 1937). These clearly apomorphic character states are found in the larvae of all Sciaroidea except the Ditomyiidae and Cecidomyiidae. These morphological characters therefore indicate that the Ditomyiidae lies more basally in the phylogeny than the Sciaridae. On this point the molecular evidence is in strong agreement.

In view of the above evidence, both morphological and molecular, the traditional two-family classification for the fungus gnats into the Mycetophilidae and the Sciaridae, which is still widely adhered to in some quarters, is phylogenetically untenable, since the Mycetophilidae in this broader sense is unquestionably paraphyletic. This comes as no surprise, since it has long been

suspected to be the case, but the additional support of the molecular data clearly substantiates this suspicion.

Although not the sister group to the rest of the fungus gnats, the Sciaridae also do not appear to be, according to the molecular evidence, the sistergroup of the Mycetophilidae as proposed by Hennig (1954). Hennig's hypothesis rested on his particular ideas about venetional evolution in the Sciaroidea[10]. In Hennig's interpretation, the Sciaridae and Mycetophilidae are united by two venational synapomorphies, the loss of m-cu and the longitudinalization of ta. This interpretation has been followed by Matile (1990b) with the interpolation of the Lygistorrhinidae, which Hennig viewed as a part of the Mycetophilidae, between the Sciaridae and Mycetophilidae (figure 5B).

The molecular data, however, places the Sciaridae between the Ditomyiidae and the Keroplatidae, two families which are the plesiomorphic with regard to tb (short and crossvein-like) and m-cu (present). The longitudinalization of ta and loss of m-cu has to be posited for the Cecidomyiidae as well. Thus these same complicated venational changes would have to have evolved at least three times independently. An alternative interpretation is that what appears to be the base of M in the Cecidomyiidae, Bolitophilidae, Sciaridae, and Mycetophilidae, actually is the base of M, and that it is tb that has been lost independently three times, just as earlier workers had supposed. The independent loss of a crossvein would seem more likely than the three-time independent occurrence of a rather complicated set of venational transformations. The absence of tb, furthermore, would have contributed to the smoothing out of m-cu such that its identity has been lost in the Sciaridae, Cecidomyiidae and Mycetophilidae. The "loss" of m-cu in the latter case depends on the loss of tb and therefore does not represent an independent transformation.

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[10]See discussion on page 21.

The position of the Sciaridae between the Ditomyiidae and Cecidomyiidae in this study is unique. No previous phylogenetic hypothesis has placed the family here. Although *Bradysia* and *Corynoptera* never occupied a common clade in any of the trees, they nonetheless always occurred adjacent to each other on the node between the Ditomyiidae and the Keroplatidae. Given the morphological evidence (discussed on page 28) there is no reason to doubt the monophyly of the Sciaridae. The fact that the 12S data does not unite them in a common clade can be easily attributed to the noise in the data generated by the more variable nucleotide positions.

Although the Sciaridae occur as a basal branch in the Sciaroidea, the 12S data does not support a sister group relationship between the family and the Cecidomyiidae as proposed by Wood and Borkent (1989). The primary argument for assuming a sister-group relationship between these two families has been the unusual cytology found in these two families. In both families, germ line cells contain extra chromosomes not found in somatic cells, and is unusual in involving unequal divisions in meiosis and chromosome eliminations from the somatic cell lineage in the early divisions in the embryo (White 1973, Matuzzewski 1982). The cytology in all "other" fungus gnats that have been examined thus far is orthodox (LeCalvez 1947). But this evaluation is based on an examination of only a few specimens in the Mycetophilidae. The cytology of basal fungus-gnat taxa closer to the sciarid and cecidomyiid branches in the phylogeny, such as that of the Ditomyiidae, Keroplatidae, Diadocidiidae, is unknown. Until the cytology of these other groups is known, the cytological evidence may be intriguing, but is far from being conclusive evidence of a Cecidomyiidae + Sciaridae clade.

Oosterbroek and Courtney (1995), citing the studies of Dallai et al. (1993), give the absence of the central microtubules in the sperm tail as another apomorphy for the Cecidomyiidae + Sciaridae. The data in Dallai et al. (1993) do

involve broader taxonomic representation than the cytological data in also including two species from the Keroplatidae. Subsequent studies (Dallai, R., B. A. Afzelius, and B. Mamaev, 1996; Dallai et al., 1996a, 1996b, 1997) have shown that a considerable amount of variation in axonemal structure is present in the Cecidomyiidae, some of which, like the cytology in this family, borders on the bizarre. Despite this variation, the absence of the central microtubules is apparently common in the family. The structure of the sperm tail among nematocerous groups, according to Dallai et al. (1993), is rather variable. Central microtubules are also lacking in the Psychotidae. In the Bibionidae only one central microtubule is present, as is also the case in the Culicidae. Given the variation in axoneme structure found in the Nematocera, it is desirable to know the axonemal structure in additional sciaroid taxa, particularly the Ditomyiidae, and to verify whether the lack of central microtubules is the general condition in the Sciaridae since only one species has thus far been examined. Should the central microtubules prove to be absent also in the Ditomyiidae, the possibility that the Cecidomyiidae, Ditomyiidae and Sciaridae evolved from a common ancestor distinct from that of the other sciaroids would need to be entertained. At present larval characters argue against such a relationship. Nonetheless, as noted above (page 66), the Cecidomyiidae, Ditomyiidae and Sciaridae did sometimes occupy a common basal clade in a few of the 12S trees (set IIA analyses). Although the clustering of these families together is very likely an artifact of long-branch attraction, the results from the 12S data for the root of the Sciaroidea with respect to the Bibionidae is not solid enough to definitively rule out a Cecidomyiidae + Ditomyiidae + Sciaridae clade. In any case, even in the few trees in which such a clade occurred, the Ditomyiidae were always located intermediately between the Cecidomyiidae and the Sciaridae.

At the moment the weight of evidence, molecular and morphological taken together, more strongly favors the hypothesis that the Cecidomyiidae are the sister group of a monophyletic taxon which includes all of the fungus-gnat families with the Ditomyiidae as the basal-most member of the fungus-gnat clade.

The position obtained from the 12S data for the Diadocidiidae and Keroplatidae does not differ extremely from that of Matile (1990b, 1997) (compare figs. 14 with figs. 5b). The 12S data, however, never places the Diadocidiidae and Keroplatidae on a common clade as in Matile, but rather located the Diadocidiidae between the Keroplatidae and the Bolitophilidae + Mycetophilidae. The node between the Keroplatidae and Diadocidiidae was supported by 9 base changes, 7 of which were unambiguous in all 14 of the shortest trees (with *Urytalpa* excluded). Matile (1990b) cites the loss of functional abdominal spiracles in the larvae as an apomorphy uniting the two families. The molecular phylogeny suggests that the abdominal spiracles were lost twice independently. However, none of the 7 unambiguous base changes on the node between the Diadocidiidae and the Keroplatidae are unique. Blaschke-Berthold (1994) (fig. 5A, p. 26) located the Diadocidiidae in a basal position below the Keroplatidae with the Ditomyiidae located between them. As discussed above, such a high position in the tree for the Ditomyiidae is not tenable on the basis of larval characters.

The removal of the Sciaridae in Hennig's and Matile's phylogenies to a more basal position leaves the Bolitophilidae as the sister group of the Mycetophilidae (+ Lygistorrhinidae). The 12S data is in agreement with this position. Although older workers often regarded the Bolitophilidae as a basal group (Rodendorf 1946, 1964; Shaw 1948; Shaw and Shaw 1951), their considerations were often based on vague attributes of primitiveness that they ascribed to the family. The presence

of a three-segmented antennae in the larvae would seem to place the family basally, since among the fungus gnats the only other family in which a three-segmented antennae is found is the Ditomyiidae. An antenna consisting of two or more segments is plesiomorphic in the Bibionomorpha, as evidenced by multi-segmented antennae in the Bibionidae and Pachyneuridae. The reduction of the antennae to a single-segmented broad oval membranous structure as found in the Sciaridae, Diadocidiidae, Keroplatidae and Mycetophilidae (the larvae of the Lygistorrhinidae are unknown) is quite unique in the Diptera and unlikely to have arisen more than once. The position of the Bolitophilidae above the Sciaridae, Diadocidiidae and Keroplatidae requires the reversion of the antenna to the plesiomorphic state in the family, as Matile (1990b) interpreted the situation. The results of the molecular analysis supports this interpretation. Additionally, one other clearly derived character, the semicircular serrated larval mandible, connects the Bolitophilidae to the Mycetophilidae as well.

The phylogenetic position of the Lygistorrhinidae remains enigmatic. Although regarded by Matile as a family separate from the Mycetophilidae, no compelling evidence clearly precludes the group from having its origin within the Mycetophilidae, as supposed by Hennig (1954). It is unfortunate that attempts to obtain amplifiable DNA from dried specimens of *Lygistorrhina sanctaecatharinae* for this study were unsuccessful. Due to the highly derived nature of so many morphological characters in this group, which leave no question of the family's monophyly but which obscure its phylogenetic affinities, molecular data may provide the only key to solving the family's position in the Sciaroidea.

Mycetophilidae

As noted in the introduction (page 29) the Mycetophilidae are felt to be monophyletic despite the lack of strong character support. If the

Lygistorrhinidae are retained as a family distinct from the Mycetophilidae, the loss of m-cu (or alternatively, the loss of ta, depending on one's interpretation- see discussion above on page 78) cannot be used as an synapomorphy for the family since the loss also pertains to the Lygistorrhinidae. A similar loss has also occurred elsewhere, once in the Sciaridae and once in the Cecidomyiidae. Other possible synapomorphies that have been proposed in support of the monophyly of the Mycetophilidae were discussed above (page 29-31) and will not be reiterated here.

The 12S data confirm a monophyletic Mycetophilidae, albeit not as compellingly as one would like. Only 6 and 7 character changes (set II B and set II C respectively) occur on the node between the Bolitophilidae and the Mycetophilidae. In contrast, the node between the Diadocidiidae and the Bolitophilidae is supported by 11 character changes. The sequence for *Bolitophila* sp. is very similar to those in the Mycetophilidae.

Knowledge on the relationships of genera and subfamilies within the Mycetophilidae are presently at a very rudimentary stage. Certain taxa are clearly monophyletic, for example the Mycetophilinae, Manotinae, and Mycomyinae. On the other hand, the taxonomic limits of the Gnoristinae and Leiinae are debatable as are their phylogenetic affinities. The presence of microtrichia on the wing membrane and the presence of setae on the mediotergite was used by Edwards (1925) to distinguish the Sciophilinae from the other tribes. Phenotypically, both with regard to adult and larval (when known) morphology, the Sciophilinae appear to be fairly cohesive. However, some genera, for example *Syntemna*, despite the presence of wing-membrane microtrichia, show affinities with one of the other subfamilies, in particular with the Gnoristinae. The presence and nature of fold lines on the abdominal sterna in adults and the presence of a "sensory" pit on the hind tibia of the males in some gnoristine genera appear to

align these taxa more with the Sciophilinae (Väisänen 1986) than with other Gnoristinae. This tibial organ is also present in *Ectrepesthoneura* (Leiinae).

The only study that has attempted a comprehensive analysis of the phylogeny in the Mycetophilidae is Söli's 1997 analysis which examined 99 morphological characters across 39 genera using the Bolitophilidae (*Bolitophila*), Sciaridae (*Corynoptera*), and Lygistorrhinidae as outgroups. A modified version of Söli's scrict consensis tree (his figure 44) based on 36 equally short trees is redrawn and shown in figure 16. According to his results, the Mycomyinae along with two gnoristine genera occupy the basal-most branch of his tree. Just above the mycomyine brach is a polytomy, one branch of which contains most of the sciophiline genera (11 of 13). Two other branches of this polytomy include *Paratina* (Sciaphilinae) + *Drepanocercus* (Gnoristinae) and *Grzegozekia* (Gnoristinae). The final branch consists of a major clade, the basal members of which are predominantly gnoristine taxa but which also includes *Syntemna* (Sciophilinae). Several leiine genera branch off the main stem just above the basal scattering of gnoristine taxa. Three leiine genera are monophyletic and form the sister group of the Manotinae. A monophyletic Mycetophilinae occupies a branch just below the Leiinae (part) + Manotinae clade.

Söli examined a greater number of genera than was used in the present study. The 12S sequences among mycetophilid taxa, unfortunately, proved to be very uniform except in the most highly variable A-T rich regions near gaps. Therefore the inclusion of additional taxa would only have increased the noise in the data without contributing much to a resolution of relationships. Future analysis of the Mycetophilidae will need to include not only data involving a greater number of sites but also genes which exhibit a greater amount of variation.

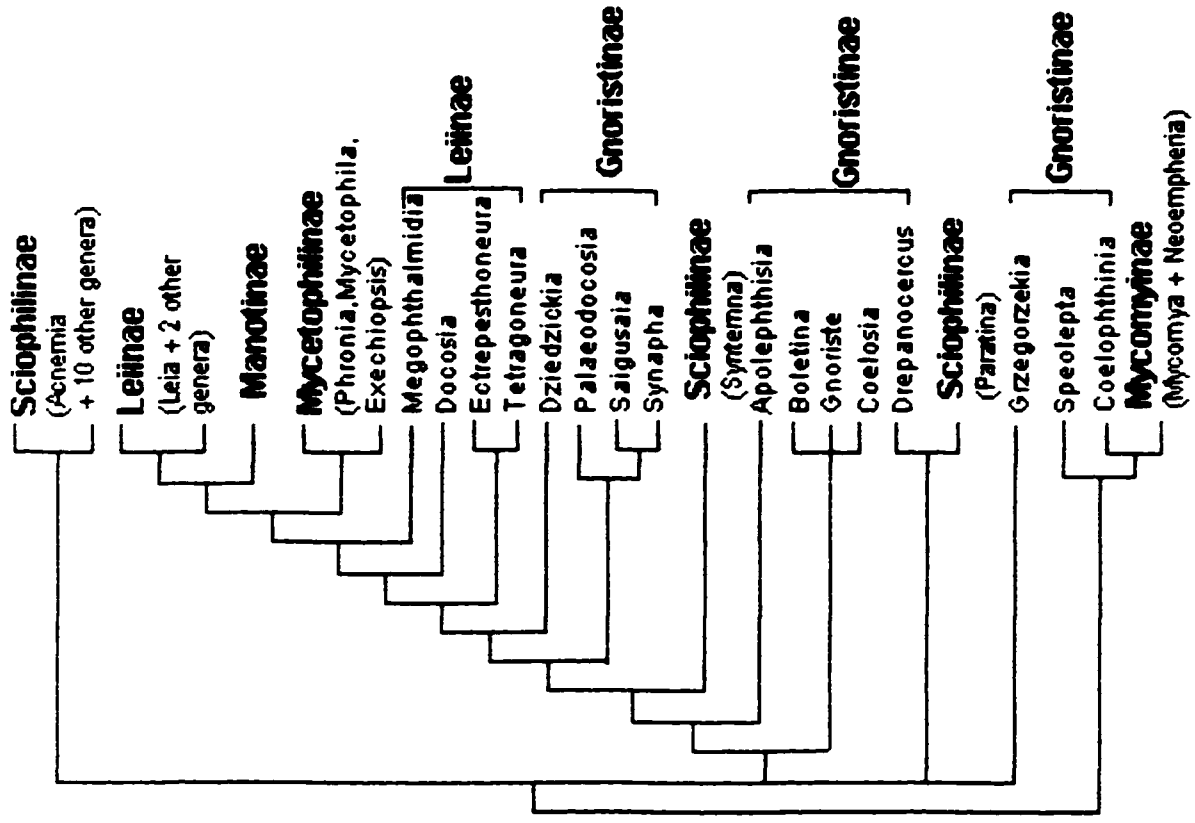

Figure 16. Phylogeny of the Mycetophilidae according to Söli (1997), redrawn and modified to show distribution of taxa by presently accepted subfamily divisions. The Sciophilinae, with the exception of two genera, form a cohesive clade (on the left side of the tree). The Leiinae and Gnoristinae are paraphyletic according to this analysis.

When those genera not included in the present study are removed from Söli's phylogeny, the resulting tree is that shown in figure 17. The molecular phylogeny of the Mycetophilidae (figure 15, page 72) differs in several respects from that obtained by Söli. First of all, the 12S phylogeny does not have the linear structure seen in figure 17; the Mycetophilidae are divided basally into three major clades whose relationships to each other are unresolved: a Mycomyinae + Sciophilinae clade, a monophyletic Mycetophilinae, and one clade including the rest of the mycetophilid taxa, namely all the Gnoristinae and Leiinae.

Both data sets are in agreement in placing the Mycomyinae and Sciophilinae (represented in this study by only *Acnemia*) in basal branches of the tree. The

molecular data, in fact, place the two subfamilies in the same clade. Too much emphasis, however, should not be given to this outcome since it rests on the inclusion of only one sequence from the Sciophilinae.

As in Söli's analysis, the Mycetophiline come out as monophyletic group, the root of which, however, lies much deeper in the tree. In relative agreement with Edwards (1925), Söli located a core portion of the Leiinae (*Leia* and relatives) + Manotinae as the sister group of the Mycetophilinae. The molecular evidence, however, does not support this; the leiine genera are dispersed throughout the gnoristine-leiine clade and none of them are ever in a sister relationship witht he Mycetophilinae. Söli examined only three mycetophiline genera--*Mycetophila* and *Phronia* (Mycetophilini) and *Exechiopsis* (Exechiini)--thus nothing can be said about the validity of the division of the Mycetophilinae into the tribes Mycetophilini and Exechiini. The 12S data, however, divided the Mycetophilinae clade into two subclades corresponding to the Mycetophilini and Exechiini respectively. The results here confirm Tuomikoski's (1966a) inclusion of *Cordyla* in the Exechiini, contrary to Edwards' (1925) placement of this genus in the Mycetophilini one the basis of the presence of anepisternal setae. Anepisternal setae, present in the Mycetophilini, are absent from other Exechiini. Tuomikoski regarded the loss of the sagittal furrow on the head of adults, present in other Mycetophilidae, as an apomorphy for the Exechiini. A saggital furrow is present in *Cordyla*.

The central clade in the 12S phylogeny, which was present in 93% of the 72 shortest trees, contains only gnoristine and leiine taxa and has in turn a three-branched structure. This gnoristine-leine clade shows little correspondence to anything in Söli's tree, except in the occurance of *Boletina* and *Gnoriste* together

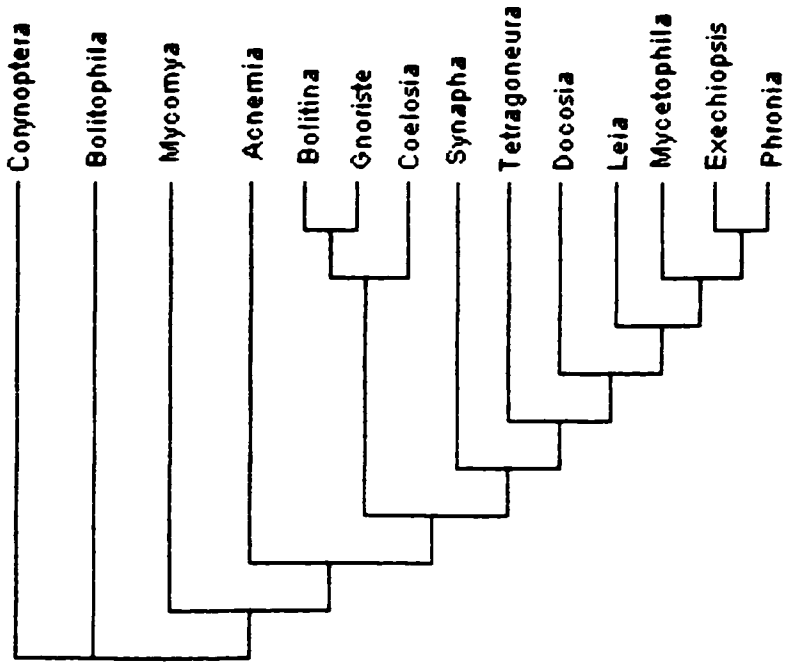

Figure 17. Phylogeny of the Mycetophilidae according to Söli (1997). redrawn and modified to show only the taxa included in the present study.

in a common clade. The molecular evidence, moreover, supporting the three-branched structure of this clade is rather weak. Of the three basal-most nodes in the clade, one is supported by 4 base changes, the other two by only two each. Even if these basal nodes were collapsed into a polytomy, the resulting unresolved tree would still show the Gnoristinae and Leiini to be not merely paraphyletic but polyphyletic. Even when a more distant outgroup is used (see figure 14B, analysis of the fungus gnats using the Cecidomyiidae and Ditomyiidae as outgroups), Leia (Leiinae) is more closely associated the Synapha (Gnoristinae) than it is with any other leiine taxon. Similarly *Docosia* (Leiinae) is more associated with *Boletina-Gnoriste* (Gnoristinae) than with other leiine taxa[11]. One surprising result is the lack of association between *Coelosia* and *Boletina-Gnoriste* to which the genus morphologically seems allied.

[11]Although *Docosia* is shown in figure 14B in an unresolved polytomy in the consensus tree, in the majority of alternate parsimony trees the genus occurred as the basal member of the *Boletina-Gnoriste* clade.

The contribution made by the molecular data toward a resolution the phylogenetic relationships within the Mycetophilidae at this point should be regarded as preliminary. A broader study utilizing a greater range of taxa than has been the case in this study is needed. The 12S gene, while useful for relationships deeper in the Sciaroidea, is simply too uniform within the Mycetophilidae to be of much use in resolving relationships among the large number of genera that exist in this family. This cautionary note aside, some conclusions, already touched on above, can be drawn form the molecular data:

1. The Mycetophilinae are monophyletic. This is in accord with morphological data. The molecular data, however, is ambiguous as to which group is the sister group of the Mycetophilinae. The weak support for basal nodes in the tree does not rule out Väisänen's (1986) hypothesis of a sister group relationship between the Mycomyinae and the Mycetophilinae, but then neither does it provide any support in its favor.

2. The molecular data does not support a close association of the Leiinae (specifically *Leia* and related genera) to the Mycetophilinae.

3. The Leiinae and Gnoristinae, as presently conceived, are not natural groups. Despite the great morphological variation in these two groups, morphology has yielded few clues as to their phylogeny. Many character states have apparently evolved independently several times among these taxa and exhibit at times little congruence with each other. It is particularly this area of the mycetophilid phylogeny that could be benefited most by additional molecular studies. Although the picture presented here may be tidier than that presented by Söli (figure 16) in that at least some support was found for a clade which included only the gnoristine and leiine genera, this could well change as more sciophiline taxa are added to the analysis.

4. Edwards in his 1925 revision of the Mycetophilidae (senso lato) treated the Mycomyinae, Sciophilinae, Gnoristinae, and Leiinae as tribes in a single subfamily Sciophilinae, which stood acoss from the Mycetophilinae. The results of the present study neither confirm nor reject the concept of Edward's Sciophilinae as a monophyletic group.

Classification considerations

The classification of the fungus gnats into several families, as adopted in this study, is at present the one used by workers on the family with few exceptions. Outside the circle of fungus-gnat systematists, however, the fungus gnats are treated as two families, Sciaridae and Mycetophilidae. Both morphological and molecular data show that the Sciaridae are more derived than the Ditomyiidae. Therefore in the two family scheme the Mycetophilidae is paraphyletic.

Three solutions to avoid a paraphyletic Mycetophilidae are 1) the multi-family classification, 2) inclusion of the Sciaridae as a subfamily in a single family Mycetophilidae, and 3) a three-family scheme recognizing the Ditomyiidae, Sciaridae and Mycetophilidae, the latter including the Diadocidiidae, Keroplatidae, Lygistorrhinidae, Bolitophilidae, and Mycetophilidae s.s. as subfamilies.

Those who advocate the multi-family classification usually cite the great age of fungus-gnat lineages in the fossil record as the reason for treating them as separate families (Väisänen 1984; Matile 1997, 1990b). At present there is no universally agreed to criteria for establishing taxonomic rank on the basis of the taxon's age. Some fungus gnat families show up in the fossil record before do any representatives of many mammalian orders. The choice of taxonomic rank is somewhat arbitrary. The two criteria that are, in my view, most important elimination of known paraphyletic taxa from the classification and the

maintenance taxonomic stability until compelling evidence mandates otherwise. On this point, the second alternative above certainly has precedence in the traditional classification of Edwards (1925). However, the Sciaridae have long since been recognized as a separate family. To now demote the group to subfamily status in a broader Mycetophilidae is not desirable and would not be accepted by those who work in the family.

The third alternative, the recognition of the Ditomyiidae as a family along with the Sciaridae, is a feasible solution, but this is not a scheme that has any present adherents. The multi-family classification, as adopted in this study, is, and has been for some time, widely accepted by the majority of fungus-gnat workers. The multi-family classification, therefore, is the preferrable solution.

The recognition of the Sciophilinae, Gnoristinae, Mycomyinae, and Leiinae as subfamilies rather than tribes of a single subfamily Sciophilinae, as originally established by Edwards (1925), is a question independent of the mulit-family classification. The ranking of these taxa is irrelevant as to whether one or several fungus-gnat families are recognized. (The Mycetophilinae, for example, has been retained as a subfamily in all classifications which divide the fungus gnats into several families). The major reason for rejecting the Sciophilinae sensu Edwards would be if this taxon were paraphyletic. It is strongly suspected to be so by most workers, and Soli's study indicates that it is, but more study in this area is definitely need. Until the relationships within and among the "sciophiline" taxa are known with more certainty, coservatism in keeping the traditional groupings is preferrable. Whether these groupings should be ranked as subfamilies or tribes probably is not too important at this point. Both rankings are recognized and reference to the taxa by tribal or subfamily designations is unambiguous, quite unlike the use of the family name Mycetophilidae, which has to be further qualified to indicate what taxa this name encompasses.

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Appendix 1: Aligned Sequences-16S rRNA

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                          10        20        30        40        50        60
                    ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia             ??????????????????????????????GGGTCG?GGTAT??TGACCGAGCAAAGGT
Tipula              GTCTAGCCTGCCCACTGAAAT??TATTTAAAGGGCCGCGGTATTTTGACCGTGCAAAGGT
Anopheles.quad      GTCTAGCCTGCCCACTGAAA?????TTTAAAGGGCCGCGGTATTTTGACCGTGCGAAGGT
Simulium            GTCTGACCTGCCCACTGAATTATATTTTGAAGGGCCGCAGTATTTTGACTGTGCAAAGGT
Lucilia             GTCTGACCTGCCCACTGAAAT??ATTTTAAATGGCCGCAGTATACTAACTGTGCAAAGGT
D. yakuba           GACTAACCTGCCCACTGAAAA???TTTTAAATGGCCGCAGTATTTTGACTGTGCAAAGGT
Sylvicola           ???????????CCACTGT?AA???ATATAAAGGGCCGCGGTATTTTGACCGTGCAAAGGT
Dilophus            ????????????????????????????????????????????????????C?CAAAGGT
Cecidomyid sp.B     ATTTATCCTGCCCAT??AATAAAATTT?AACGGCCGGCAGTATTTTGACTGTGCTAAGGT
Ditomyia            GTCTAACCTGCCCAT?GAAAAT???TTTAAAAGGCCGCAGTATTTTGACTGTGCAAAGGT
Urytalpa            GTCTAACCTGCCCATTGAAATA????T???AAGGCCGCAGTATTTTGACTGTGCAAAGGT
Macrocera sp. B     G?TTAACCTG?CCAT?GAAAT???T?TTAAAAGGCCGCAGTATTTTGACTGTGCAAAGGT
Orfelia             GTCTAATCTGCCCATTGAATAA?CAATTAAAAGGCTGCAGTATTTTGACTGTACAAAGGT

                          70        80        90        100       110       120
                    ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia             AGCAAAATCATTAGTTTTTTAATT?AAAGCTTGTAT?AATGG?TTGGACGAAGTATTGAC
Tipula              AGCATAATCATTAGTTTTTTAATTGGAAGCTTGTATGAATGG?TTGGACGAAATATTGAC
Anopheles.quad      AGCATAATCAATAGTCTTTTAATTGAAGGCTGGTATGAATGG?TTGAATGAGATATATAC
Simulium            AGCATAATCATTAGTCTTTTAATTGAAGGCTGGTATGAATGG?TTGGATGAGGTACAAGC
Lucilia             AGCATAATCATTAGTCTTTTAATTGAAGGCTGGTATGAATGG?TTGGACGAGATATTAAC
D. yakuba           AGCATAATCATTAGTCTTTTAATTGAAGGCTGGAATGAATGG?TTGGACGAAATATTAAC
Sylvicola           AGCATAGTCATTAGTCTTTTAATTGAAGGTAAGTATGAATGG?TTGGATGAAGCATTAAC
Dilophus            A?CATAATCATTAGTTTTT?AATTAAAAACTAGAATAAATAG?TTAAACAAAATATATAC
Cecidomyid sp.B     AGCATAATCATTAGATTTTTAATTGAGATCTGGAATGAATGG?ATGAATGAAATATAAAC
Ditomyia            AGCATAATCATTAGTTTTTTAATTGAAAACTTGTATGAATGG?TTGAACGAAATAAAAAC
Urytalpa            AGCATAATCATTAGTTTTTTAATTG??AACTTGTATGAATGG?TTGA?TGAGATATTAGC
Macrocera sp. B     AGCATAATCATTAGTTTTTTAATTGAAAGCTGGAATGAATGG?TTGAATGAAATATTAAC
Orfelia             AGCATAATCATTAGTTTTTTAATTGAAAGCTTGTATGAATGGATTGAATGAAATATAAAC

                          130       140       150       160       170       180
                    ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia             TGTC????????TCATAAAAATTTATTATTGAAATTAATTTTTTTGTTAAAAAGCAAAAA
Tipula              TGTC????????TTTATTAAATTTATAATAGAATTTAACTTTTTTGTTAAAAGGCAAAAA
Anopheles.quad      TGTT????????TTTTTAAAATTTTTA?TAGAACTTTATTTTTTAGTTAAAAAGCTAAAA
Simulium            TGTG????????TCATAAAAATTAATATTTGAATTTAACTTTTTAGTCAAAAGGCTAAAA
Lucilia             TGTT????????TCATAAAAATTTATAATAGAATTTTATTTTTTAGTCAAAAAGCTAAAA
D. yakuba           TGTT????????TCATTTAAATTTAAAATAGAATTTTATTTTTTAGTCAAAAAGCTAAAA
Sylvicola           TGTC????????TCATAAAAATTAAA?ATAGAATTTTATTTTTTAGTTAAAAAGCTAAAA
Dilophus            TGTTATATACTGTCTTATTTA??TAA?ATTAAATTTTATTTTTTAGTTAAAAA?CTAAA?
Cecidomyid sp.B     T?TT????????TTTAATTTAAATTA?TTGAAAATTTATTTTTTAGTTAAAAAGCTAAAT
Ditomyia            TGTC????????TTAAATAATTTTTA?TTTGAAGTTTACTTTTCAATTAAAAGGTTGAAA
Urytalpa            TGTT????????TCATTTAAATTTTA?ATTGAATTTAATTTTTTAGTTAAAAAGCTAAAA
Macrocera sp. B     TGAC????????TCATAAAAAATAGA?TTAGAATTTTATTTTTTAGTTAAAAAGCTAAAA
Orfelia             TGTT????????TCATAGAAATTTTA?ATAGAATTTAATTTTTTAGTTAAAAAGCTAAAA
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Appendix 1 (con't). Aligned Sequences - 16S rRNA.

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                          190        200        210        220        230        240
                 ....:....|....:....|....:....|....:....|....:....|....:....|....:....|
Limonia          TGAAATTAGAGGACGAGAAGACCCTATAGAGCTTTAT??AA?TTTTATTATATAATTTT?
Tipula           TTATTTTAAAGGACGAGAAGACCCCATAGAGCTTTAT??ATTTTTTATATTATAATTTAT
Anopheles.quad   TTTAATTAAAGGACGAGAAGACCCTATAGATCTTTAT???TTTTATAAATTATAAATTAT
Simulium         TTTAATTAAAAGACGAGAAGACCCTATAGAGCTTTAT???ATAATGATTATTTAATTTAT
Lucilia          TTTATTTAAAAGACGAGAAGACCCTATAAATCTTTAT???ATTTATATTATTATAATTTT
D. yakuba        TTAATTTAAAAGACGAGAAGACCCTATAAATCTTTAT???ATTTTATTTATTTTAATTAT
Sylvicola        TTTTATTAGAGGACGAGAAGACCCTATAGAG?TTTAT??AATTATTAA?ATAAAAATTAT
Dilophus         ?AATTTTNATGGACGAAAAGACCCTATAAAGTTTTATATAATTTTATTTATTTTAATTTT
Cecidomyid sp.B  TAATAAAATGGGACGAGAAGACCCTATAGAATTTTAT????AATTATTAAT?TAAAAATT
Ditomyia         TACTTTTATAGGACGAGAAGACCCTATAGATCTTTAT??TTAAATTTTAAAATAAATAAA
Urytalpa         TATTTTTAGAGGACGAGAAGACCCTATAAAGTTTAAT??AATTTAA??AAAATAATTTTT
Macrocera sp. B  TAATTTTAAAGGACGAGAAGACCCTATAGAGTTTTATAAATTTATTTTTAATTAATTTTT
Orfelia          TGAAATTAAAGGACGAGAAGACCCTATAAAGTTTTAT??ATTAATATTGTATTAATAAAT

                          250        260        270        280        290        300
                 ....:....|....:....|....:....|....:....|....:....|....:....|....:....|
Limonia          ATAGAATAATT?AAATTT??TATAAA?CAAATTATTTTGTTGGGGTGACAATAAGATTT?
Tipula           AAAGAATTATTTAAATTT??TAGTGTAAAAATTATTTTGTTGGGGTGACAATAAAATTT?
Anopheles.quad   AAAGAATTTTAAAATTTA??TATTTTAAATAAAATTTTACTGGGGTGGTATTAAAATTT?
Simulium         TAAGATTTATTAAATTTA??ATTATTTTATTATATTTTGTTGGGGTGACAATAAAATTT?
Lucilia          TTAGATTTTTTTTGTTAT??AATAATAGATTATATTTTATTGGGGTGATATTAAAATTT?
D. yakuba        AAAGATTAATTTAATTTT??AATAAATTAAAATATTTTATTGGGGTGATATTAAAATTT?
Sylvicola        AAAGAATATTTTAATTTT??TATTAAAATAATTAT????TTGGGGTGACAAAAAAATTT?
Dilophus         ATTTTATTGATTTTAGAT??TTTTTTTTATTTT?TAAA?T?AAAGTTTTATTTAATTTTT
Cecidomyid sp.B  TATT?TAATT?????????????TAAAAT???TATTTTATTGGGGAGATATTAAAATTT?
Ditomyia         TTTTATAATTAATTTATA??TTTTAACTTTTAAATTTTATTGGGGTGATA?AAAAATTT?
Urytalpa         TTGAATAATTT?ATATTT??TTTTTATTAAATTATTTTATTGGGGTGATAAT?AAATTT?
Macrocera sp. B  GTAATAATATTTTTATTAAATTATAAATAATTTATTTTATTGGGGAGATAATAAAATTT?
Orfelia          ATAATTTATTTTTTATTT??TTATTTTAGAAGTATTTTATTGGGGAGATAATAAAATTT?

                          310        320        330        340        350        360
                 ....:....|....:....|....:....|....:....|....:....|....:....|....:....|
Limonia          ?AAAAACTCTTATTATTTTA?AACATTGA?TTTATGAATA??TTTGATCCATTATTAATG
Tipula           ATTGAACTTTTATTATTTTATAACATTGA?TTTATGAATT??ATTGATCAGCTTTTATTG
Anopheles.quad   AATAAACTTTTATTTTTATTTAACATTGA?TTTATGAATT??TAAGATCCTGTATTATGG
Simulium         ATAAAACTTTTATTTTTATTTTACATTTA?TTTATGAATA??TATGATCCAGTTTTATTG
Lucilia          AATAAACTTTTAATTGTTTAAATCATTAA?TTTATGAATA??ATTGATCCGTTATTAGCG
D. yakuba        AAAAAACTTTTAATTTTAAAAAACATTAA?TTTATGAATA??ATTGATCCATTAATAATG
Sylvicola        AATAAACTTTTTTTTTATTT?AAACATTGAATTTATGAATT??AATGATCCAGTTTTATTG
Dilophus         AATTATCTTTTATATA???AATTCA??GA?G??????????????????????????????
Cecidomyid sp.B  ????AACTTTTATAATTTGATT?CATAAT?TATATG?TTAAAATTGATCTTTTATTATAG
Ditomyia         AAATAACTTTTTTAATTTATTAACAATTA?TAGTTGAATT??TATGATCCAATTTTATTG
Urytalpa         AAATAACTTTTATTTTTAATTTACATTGA?TTTA?GAATT??TATGATCCAATTTTATTG
Macrocera sp. B  AAAAAACTTTTAATAAAAATTTAACATAA?TAATTGA?TTAAT?TGATCCTA?CTTATAG
Orfelia          AAAAAACTTTTATGCTTAAA?AACATTGA?TTAATGAATTAATATGATCCTTTATTATGG
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Appendix 1 (con't). Aligned Sequences – 16S rRNA.

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                          370        380        390        400        410        420
                 ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia          ATTAAAAAAATTAAGTTACC?TAGGGATAACAG?CGTAATTTTTTTTAAGAGTTCTTATCG
Tipula           ATTAAAAGATTAAGTTACCTTGGGGATAACAG?CGTAATTTTTTTAAAGAGTTCATATCG
Anopheles.quad   ATTAAAAAATTAAGTTACCTTAGGGATAACAG?CGTAATTTTTTTAGAGAGTTCATATCG
Simulium         ATTATAAATTTAAGTTACCTTAGGGATAACAG?CGTAATTTTTTTTGAGAGTTCATATCG
Lucilia          ATTAAAAAAAACAAGTTACTTTAGGGATAACAG?CGTAATTTTTTTGGAGAGTTCATATCG
D. yakuba        ATTAAAAAATTAAGTTACTTTAGGGATAACAG?CGTAATTTTTTTGGAGAGTTCATATCG
Sylvicola        ATTAAAAAATTAAGTTACCTTAGGGATAACAGACGTAATGTTTTTTAAGAGTTCATATCG
Dilophus         ????????????????????????????????????????????????????????????
Cecidomyid sp.B  ATTAAAAAATTAAATTACCTTAGGGATAACAG?CATAATGATTTT?AA???TTTAAATTT
Ditomyia         ATTATTAGATTAAGTTACCTTAGGAATAACAG?CGTAATTTTTTTTGA?AGTTCAAATTT
Urytalpa         ATTAAAAAATTAAATTACTTTAGGGATAACAG?CATAATTTTTTTTT?AAGTTCATATTA
Macrocera sp. B  ATTAAAAATTT?AATTACCTTAGGGATAACAG?CGTAATTTTTTTTTGAAGTTCTTATTT
Orfelia          ATTAAAAAATTAAATTACTTTAGGGATAACAG?CGTTATTTTTTTTAAAAGTTCATATTT

                          430        440        450        460        470        480
                 ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia          ACAAAAAAGATTGCGACCTCGATGTTG?ATTAAGAATTATT??????????????????
Tipula           ACAAAAAAGATTGCGACCTCGATGTTGGATTAAGAG?TAATTTTAGGTGCAGAAGTTT
Anopheles.quad   ATAAAAAAGATTGCGACCTCGATGTTGGATTAAGAGTTATTTTTAGGTGTAGAAGTTT
Simulium         ACAAAAAAGATTGCGACCTCGATGTTGGATTAAGAG?TAATTTTGGGTGTAGAAGTTC
Lucilia          ATAAAAAAGATTGCGACCTCGATGTTGGATTAAGATATAATTTTAGGTGTAGCCGCTT
D. yakuba        ATAAAAAAGATTGCGACCTCGATGTTGGATTAAGATATAATTTTGGGTGTAGCCGTTC
Sylvicola        AAAAAAAAGATTGCGACCTCGATGTTGGATTAAGAATAAATTTTAGGTGCAGAAGTTT
Dilophu          ????????????????????????????????????????????????????????
Cecidomyid sp.B  A??AAATAGTTTATGACCTCGATGTTGGATT?AAA?TAATTTTTATACA?AGAAATAT
Ditomyia         ATAAAAAAGATTGCGACCTCGATGTTGGATTAAGAA?AATTTT?AGATGCAGAAGTTT
Urytalpa         ATAAAAAAGATTGTGACCTCGATGTTGGAT??????TTATTTT?AGGTG?????????
Macrocera sp. B  ATAAAAAAGATTGCGACCTCGATGTTGGATTAAAATT???TTTTAGGTGTAGAAGCTT
Orfelia          ATAATAAAGATTGCGACCTCGATGTTGGATTAAAAT?TAATTTTAGGTGCAGAAGTTT
```

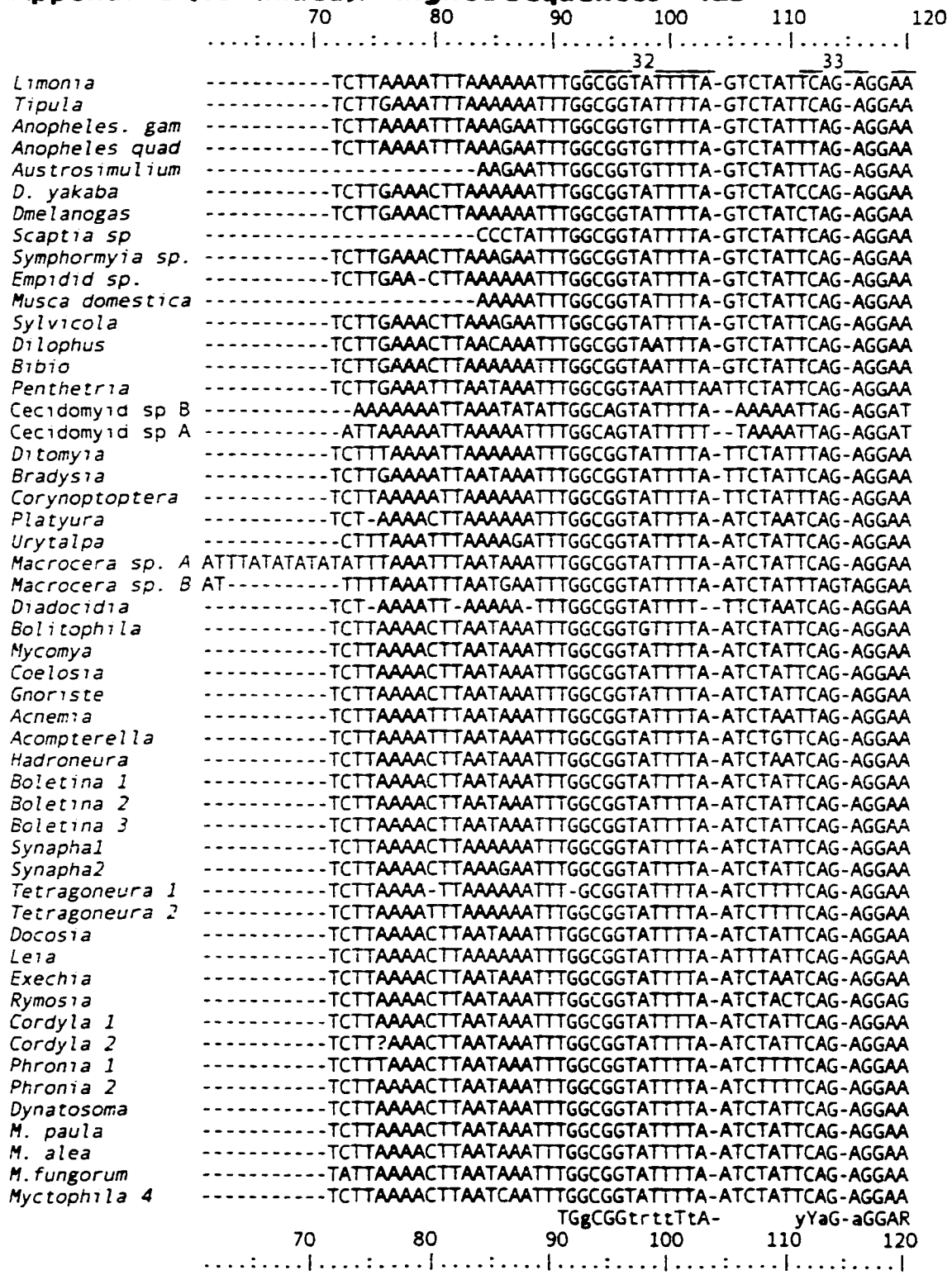
Appendix 2: Aligned Sequences--12S

Appendix 2. Aligned 12S sequence. The numbered bars at the top of the matrix shows the location of stem regions in the secondary structure of the 12S ribosomal RNA molecule. The bottom row gives the consensus for conserved regions taken from the structural model of Hickson et al. (1996).

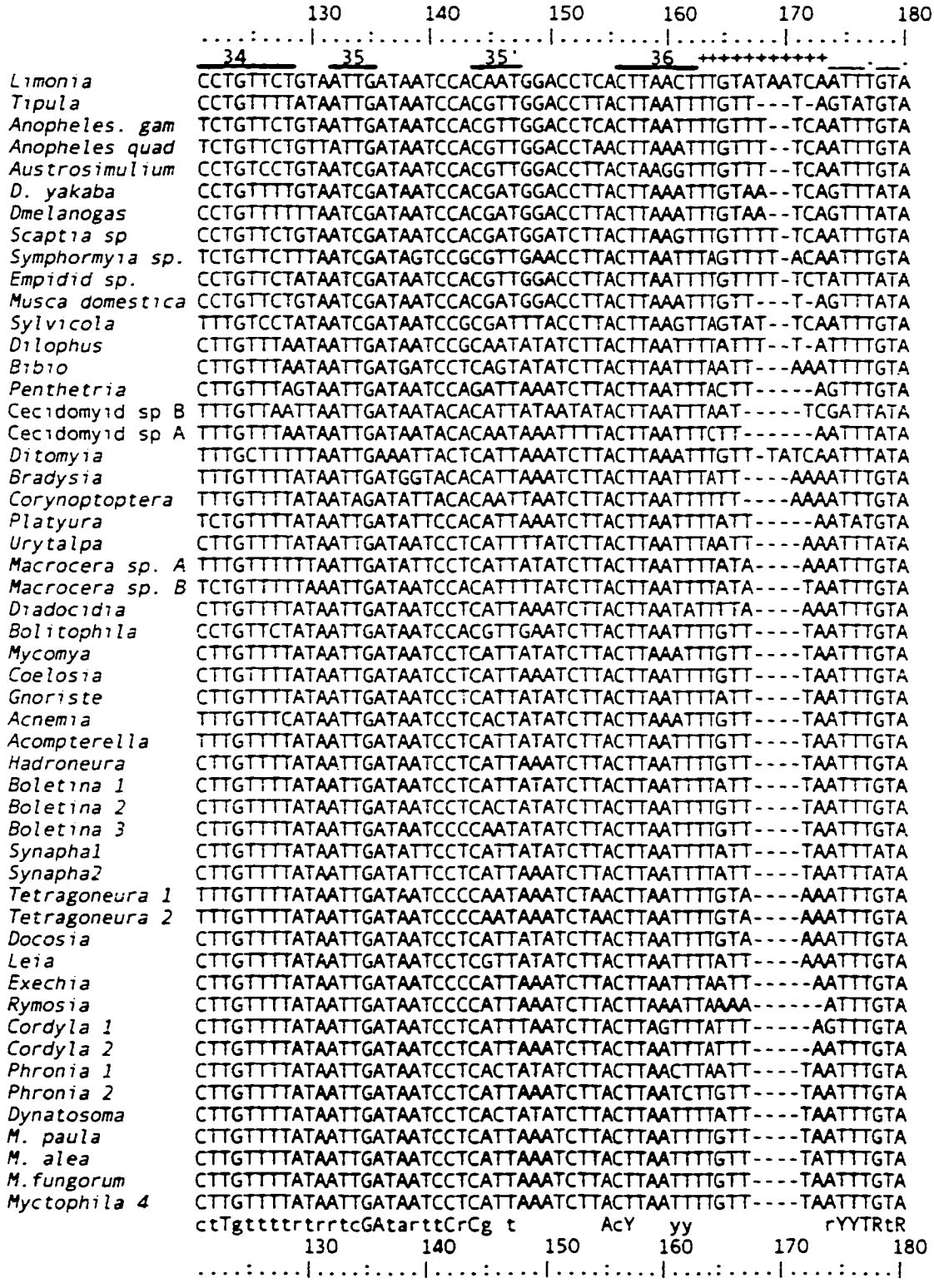
Appendix 2: Aligned Sequences--12S

```
                          10        20        30        40        50        60
                          ....:....|....:....|....:....|....:....|....:....|....:....|
Limonia                   TTATT-TAA--AATGTAAAATA---TAAACTAGAATAG-TAATAGTTATGA---------
Tipula                    TTATT-TAA--AATGTAAATTAA--TAA-TCAGAATAG-TATTAGTTATGT---------
Anopheles. gam            TTATT-AAA--ATTAAATATATAAGAATACTTAAGTAG-TATTAGTTATAT---------
Anopheles quad            TTATT-AAA--ATTAAATAATTAAAAATACTAAAGTAG-TATTAGTTATAT---------
Austrosimulium            TT----------------------------------------------------------
D. yakaba                 TTATT-TAA--AATGTAAA-TAA--ATTGCTAAAGTAG-TAATAGTTATGT---------
Dmelanogas                TTATT-TAA--AATGTAAA-TAA--ATTGCTAAAGTAG-TAATAGTTATGT---------
Scaptia sp                ----------------------------------------------------------
Symphormyia sp .TTATT-TAA--AATATATTTTTG--TTTATTTGAGTAG-TAATAGTTATGT---------
Empidid sp.               TTATT-TAA--AGTGTTTATGTAT-TTTACTAGAGTAG-TATTAGTTATGT---------
Musca domestica           ----------------------------------------------------------
Sylvicola                 TTATT-TAA--AAAGTAAGATTAA-TAAATTAGAGTAG-TAATAGTTATAG---------
Dilophus                  TTATA-AA---AATGGAAAATTTAAAATATTAAAGTAG-TAATAGAT-TAG---------
Bibio                     TTATA-AA---AATTTAAAATATAAAGTATTAGAGTAG-TAACAGAT-TAG---------
Penthetria                TTACT-TA--TAAAAGTAAAATAAAAATCTTAAAGTAG-TATTAGTT-TAT---------
Cecidomyid sp B           TTATT----------TTATTTAA--ATTATTAAATTAA-TATTTTT-ATAA---------
Cecidomyid sp A           TTATT-CAT--TTTACGATTTA---ATTTCTAAATTAG-TAATTAATATAA---------
Ditomyia                  TTATT-TTA--AATATAAATTAATTGTTACTAAATTAA-TAATAGTTATAT---------
Bradysia                  TTATT-TAG--AACGTAAATAAAT-AAAATTAGATTAT-TATTAGTTATGT---------
Corynoptoptera            TTATA--AA--AATATAATAAAAA-AAAAATAGATTAG-TACAAGATATAT---------
Platyura                  TTATT-TAA--AAATTAAATTTA--AAAATTAGAGTAG-TAATAGTTATGT---------
Urytalpa                  TTATT-TAA--AATATAAATAAA--AATATTAGATTAT-TAATAGATATAT---------
Macrocera sp. A           TTATT-CAA--TTAATAAAAAT---AAAATT-GAATAG-TAA-AATAATTTATATTAAAA
Macrocera sp. B           TTTAT-ATT--TAATTAGTTAAAA-ATAATTATATTAAATAA-AATAATATAAAATTAAT
Diadocidia                TTATA-AAT-AAAAGTAATAAAA--ATA-TTAGA-TAT-TAATAGT-A-G----------
Bolitophila               TTATT-TTA--AATGTAATTATTA-AAAATTAGAGTAG-TAACAGTTATAT---------
Mycomya                   TTATA-AAA--AAT-TA-TTAAAAATAAAATAGAGTAG-TAATAGTTATAT---------
Coelosia                  TTATT-AAA--AATGTAAATAAAT-AAAAATAGAGTAG-TAATAGTTATAT---------
Gnoriste                  TTATT-AAA--AATGTAAATTAAA-AAAAATAGAGTAG-TAATAGTTATAT---------
Acnemia                   TTATT-TAA--ATTGTAAATAAA--ATTTATAGAATAA-TAATAGTTATGT---------
Acompterella              TTATT-AGA--AATGTAAATTATT--AAAATAGACTAG-TATTAGATATAT---------
Hadroneura                TTATT-AAA--AATGTAAATAAAT-AAAAATAGAGTAG-TAGTAGTTATAT---------
Boletina 1                TTATT-AAA--AATGTAAATTTATAAAAAAATAGAGTAG-TAATAGTTATAT---------
Boletina 2                TTATT-AAA--AATGTAAATAAAT-AAAAATAGAGTAG-TAATAGTTATAT---------
Boletina 3                TTATT-AAA--AATGTAAATAAAT-AAAAATAGAGTAG-TAATAGTTATAT---------
Synapha1                  TTATAGAAA--ACAGTAATTAAAT-AAAAATAGGGTAG-TATTAGATATGT---------
Synapha2                  TTATAGAGA--AAAGTTAGATTAT-AAAAATAGGGTAG-TATTAGGTATAT---------
Tetragoneura 1            TTATT-AAA--AATGTTAATTAAAAAAAAAATAGATTAG-TATTAGGTATTT---------
Tetragoneura 2            TTATA-AAA--AATGTAA-TTAAAAAAAAATAGATTAG-TATTAGGTATTT---------
Docosia                   TTATT-AAA--AATGTAAATAAAT-AAAAATAGAGTAG-TATTAGTTATAT---------
Leia                      TTATT-TAA--AATGTAATTAAAA-AGAAATAGAGTAG-TAGTAGTTATAT---------
Exechia                   TTATA-AAA--AATGTAATTATA--AAAAATAGAGTAG-TAATAGTTATAT---------
Rymosia                   TTATA-AAA--ATTGTAATTTTAA-AAAGATAGAGTAG-TAATAGTTATAT---------
Cordyla 1                 TTATA-GAA--AATGTAAATTAAA-AAAAATAGAGTAG-TATTAGTTATGT---------
Cordyla 2                 TTATA-AAA--AATGTAAATTGA--AAAAATAGAGTAG-TATTAGTTATGT---------
Phronia 1                 TTATT-AAA--AATGTAAATATAAAAAAAAATAGAGTAA-TAATAGTTATAT---------
Phronia 2                 TTATT-TAA--AATGTAAATATAAAAAAAAATAGAGTAG-TAACAGTTATAT---------
Dynatosoma                TTATT-AAA--AATGTAAATATA--AAGGATAGGGTAG-TATTAGTTATAT---------
M. paula                  TTATA-AAA--AATGTAA-TTAAC-AATTAAAGAGTAG-TAATAGTTATAA---------
M. alea                   TTATA-AAA--AATGTAAATTAAA-AAATAAAGAGTAG-TAATAGTTATAT---------
M.fungorum                TTATA-A?---????TAAATAAA--AAGTAAAGAGTAG-TAATAGTTATAA---------
Myctophila 4              TTATA-AAA--AATGTAATTATT--AAATAAAGAGTAG-TAATAGTTATGT---------
                          10        20        30        40        50        60
                          ....:....|....:....|....:....|....:....|....:....|....:....|
```

Appendix 2 (continued): Aligned Sequences--12S



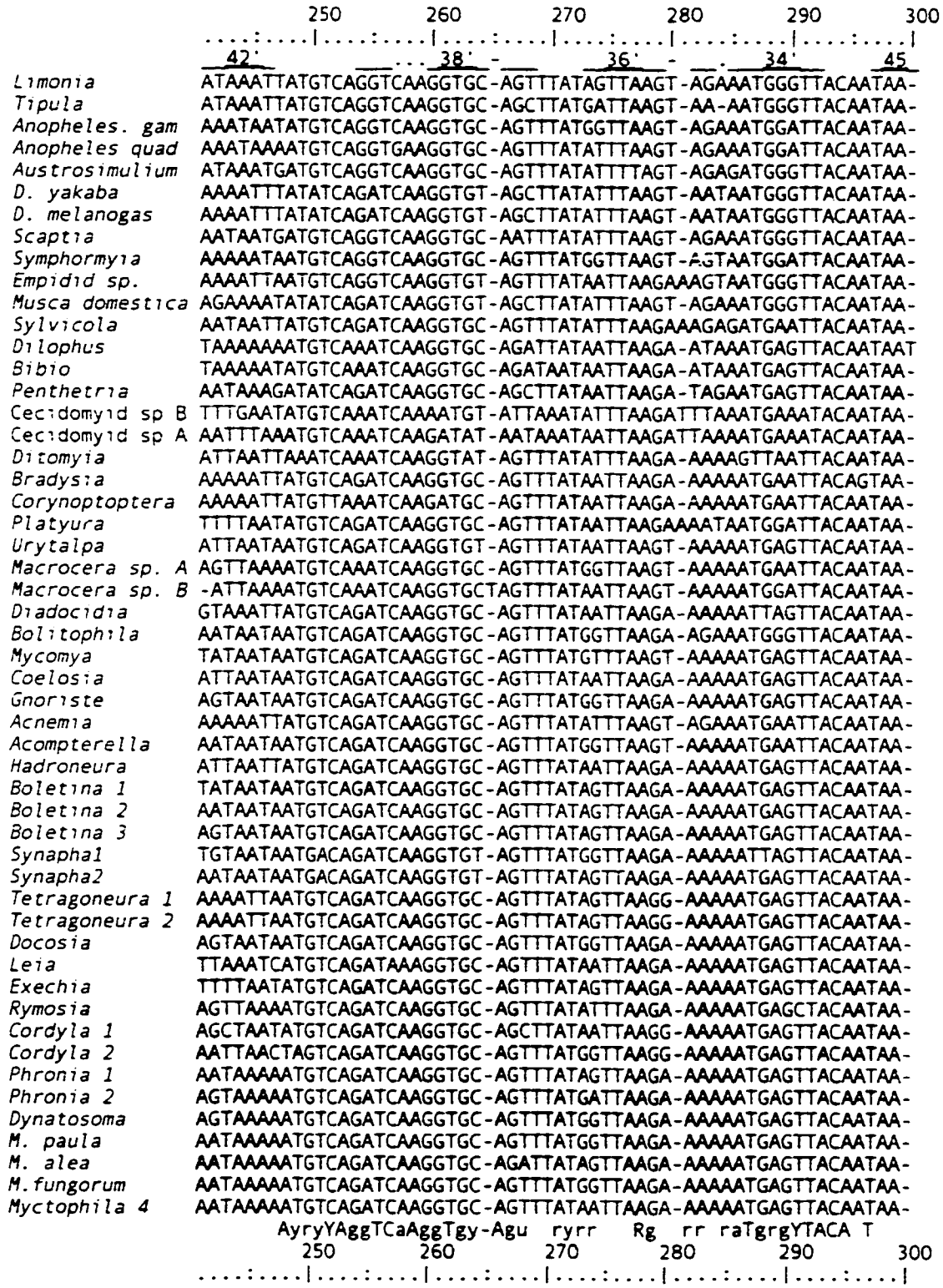
Appendix 2 (continued): Aligned Sequences--12S



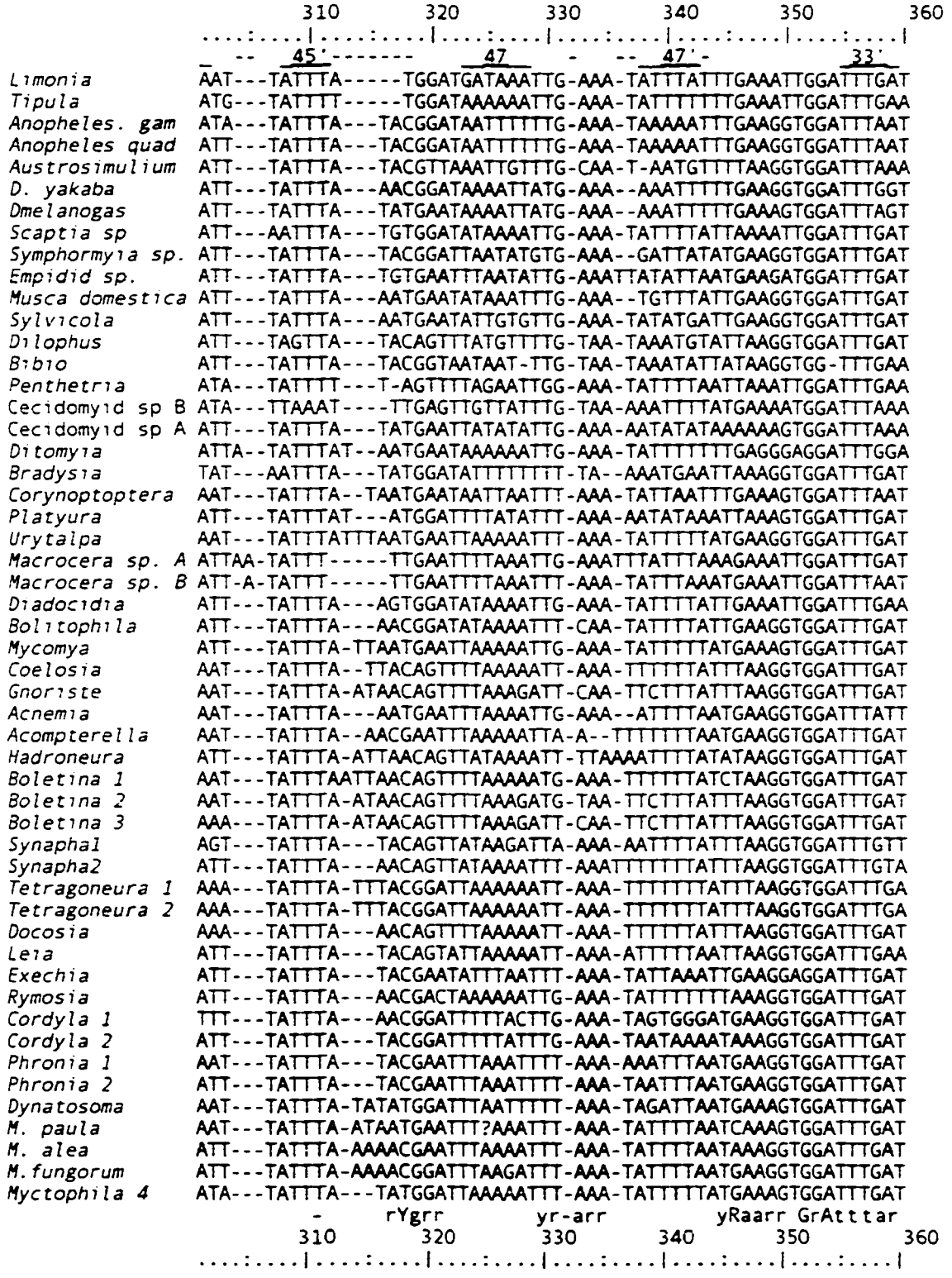
Appendix 2 (continued): Aligned Sequences--12S

```
                                190       200       210       220       230       240
                                ....:....|....:....|....:....|....:....|....:....|....:....|
                                ..38...__     ___39_140_-    40'-      39'    42__
Limonia                         TAT-CGCCGTCATCAGAA-TGTCTT-AAAAGAGTAAAAAATTTTCATAAATTAAAATT--
Tipula                          TAC-CGCCGTCATCAGAA-TGTCTT-AAAAAAG-AAATAATTTTCTAAAATTTTTAAT--
Anopheles. gam                  TAT-CGCCGTCATCAGAA-TATATT-ATAAGAT-TAATAATTTTCTTGATATTTCATT--
Anopheles quad                  TAT-CGCCGTCATCAGAA-TATATT-ATAAGAT-TAATAATTTTCA-AATATTTTATT--
Austrosimulium                  TAT-CGCCGTTATCAGAA-TATCTT-AAAAGAG-GAATAATTTTCTAAATTTAATAAT--
D. yakaba                       TAC-CGTCGTTATCAGAA-TATTTT-ATAAGAA-TAATAATATTCAATAATTTTAATA--
Dmelanogas                      TAC-CGTCGTTATCAGAA-TATTTT-ATAAGAA-TAATAATATTCAATAATTTTAATA--
Scaptia sp                      TAT-CGCCGTTATCAGAA-TATTTT-ATAAGAA-TAATAATTTTCTAAAATTTTAATT-A
Symphormyia sp.                 TAC-CGCCGTTATTAGAA-TATTTT-ATAAGAA-TAATAATTTTCTTTATTTTTAATT--
Empidid sp.                     TAC-CGCCGTTATTAGAA-TATTTT-ATAAGAAATAATAATTTTCAATATTTTTAATA--
Musca domestica                 TAC-CGTCGTTATTAGAAATATTTT-ATAAGAA-TGTTAATTTTCAAAATTTTATAAA--
Sylvicola                       TAC-CGTCGTCATCAGAA-TATTTT-AGAAAAATTTTTTAATTTTCTAACATATTTATAAT
Dilophus                        TAT-CGTTGTCTTTAGAA-TATTTT-AAAAAAA-TAATAATTTTCTTATTTTTTAATTTT
Bibio                           TAT-CATTGTCTTTAGAA-TATTTT-AATAAAT-TAATAATTTTCTTAAATTTAGATT--
Penthetria                      TAT-CGTCGTTACCAGAA-TTTCTT-GAAAAAG-AAATAATTTTCTAATATATTAATG--
Cecidomyid sp B                 TAT-TGTTGTTATAAAAT-AATTTT-TAAAAAA----C-ATTATTAAAATTATTAA----
Cecidomyid sp A                 TAT-CGTTGTCATAAAAA-AATTTT-ATAAAAA----T-ATTTTTAAAATAATTTATT--
Ditomyia                        TAC-CGTTGTTATAAGAA-AATATTTTTAAAAT-AAATAATTTTCAAATATAAAAATTA-
Bradysia                        TAT-CGTCGTCATAAGAA-TATCTT-AAAAAAG-GTTTAATATTCTAAAATTTTTAAT--
Corynoptoptera                  TAT-CGTTGTCATAAGAA-AATCTT-AAAAGAGAAGTTAATTTTCTAAAATTTTAGTT--
Platyura                        TAT-CGTCGTCATAAGAT-TATTTT-TAAAAAATAAATAATTTTCTTAATAAAAAAAT--
Urytalpa                        TAT-CGTTGTCATAAGAA-TATCTT-AAAAAAG-AAAAAATTTTCTAATTTAAAAATT--
Macrocera sp. A                 TACTCGTTGTCATAAGAA-TATCTT-A-ATAGG-AAATAATTTTCTTAATAATGAATT--
Macrocera sp. B                 TAT-CGTTGTCATAAAAA-TATCTT-ATTAGAGTAAAAAATTTTTTAATTATATATTT--
Diadocidia                      TAC-CGTCGTCATCAGAA-TATCTA-AAAAAAG-AAAAAATTTTCTAAAATTTTTATT--
Bolitophila                     TAC-CGTCGTCATCAGAA-TATCTT-ATAAGAG-AAAAAATTTTCTAAAATTTTAATT--
Mycomya                         TAC-CGTCGTCATAAGAA-TTTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTTTATT--
Coelosia                        TAC-CGTCGTCATAAGAA-TCTCTT-TAAAAAG-AAAAAATTTTCTAAAATTTTTATT--
Gnoriste                        TAC-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTTAATT--
Acnemia                         TAT-CGTCGTCATAAGAA-TATCTT-AAAAAAG-AAAAAATTTTCTAAAATTTATATT--
Acompterella                    TAT-CGTCGTCATAAGAA-TTTCTT-TATAAAG-AAAAAATTTTCTAAAATTTTTATT--
Hadroneura                      TAC-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATAAAAAT---
Boletina 1                      TAC-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTAAATT--
Boletina 2                      TAC-CGATGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTTTATT--
Boletina 3                      TAC-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTTAATT--
Synaphal                        TAT-CGTCGTCGTAAGAA-TTTTTTTAAAAAAGTTTAAAATTTTCTAAAATTTACATT--
Synapha2                        TAC-CGTCGTCATAAGAA-TTTTTTTAAAAAAG-GTTAAATTTTCTAAAATTTAGATT--
Tetragoneura 1                  TAT-CGTCGTCATAAGAA-TTTCTT-TATAAAGAAAAAAATTTTCTTAAGTTAAAATT--
Tetragoneura 2                  TAT-CGTCGTCATAAGAA-TTTCTT-TATAAAGAAAAAAATTTTCTTAAGTTAAAATT--
Docosia                         TAC-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATTTTAATT--
Leia                            TAT-CGTCGTCATAAGAA-TTTCTT-TAAAGAG-TAATAATTTTCTAAAATTTTTATT--
Exechia                         TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTCAATAATTAATT--
Rymosia                         TAC-CGTCGTCATAAGAA-T?TCTT-AAAAAAG-AAAAAATTTTCTTAATAATTTATT--
Cordyla 1                       TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAATAATTTTCTGGATAGCTTATT--
Cordyla 2                       TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAATAATTTTCTCGATAATTTATT--
Phronia 1                       TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCAAAAATAAAAAT---
Phronia 2                       TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCAAAAATATGAATT--
Dynatosoma                      TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAAATATTAATT--
M. paula                        TAC-CATCGTCATAAGAA-TCTCTT-TAAAAAG-AAAAAATTTTCTAAATTATTAATT--
M. alea                         TAT-CGTCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTTAATTATTAATT--
M.fungorum                      TAC-CATCGTCATAAGAA-TCTCTT-AAAAAAG-AAAAAATTTTCTAAATTATAAATT--
Myctophila 4                    TAC-CATCGTCATAAGAA-TCTCTT-TTAAAAG-AAAAAATTTTCTTAATTATTTATT--
                                TAc-crccgTc   Ar  - ryyyy-   rrrr-    rry   y  rr
                                         190       200       210       220       230       240
                                         ....:....|....:....|....:....|....:....|....:....|....:....|
```

Appendix 2 (continued): Aligned Sequences--12S



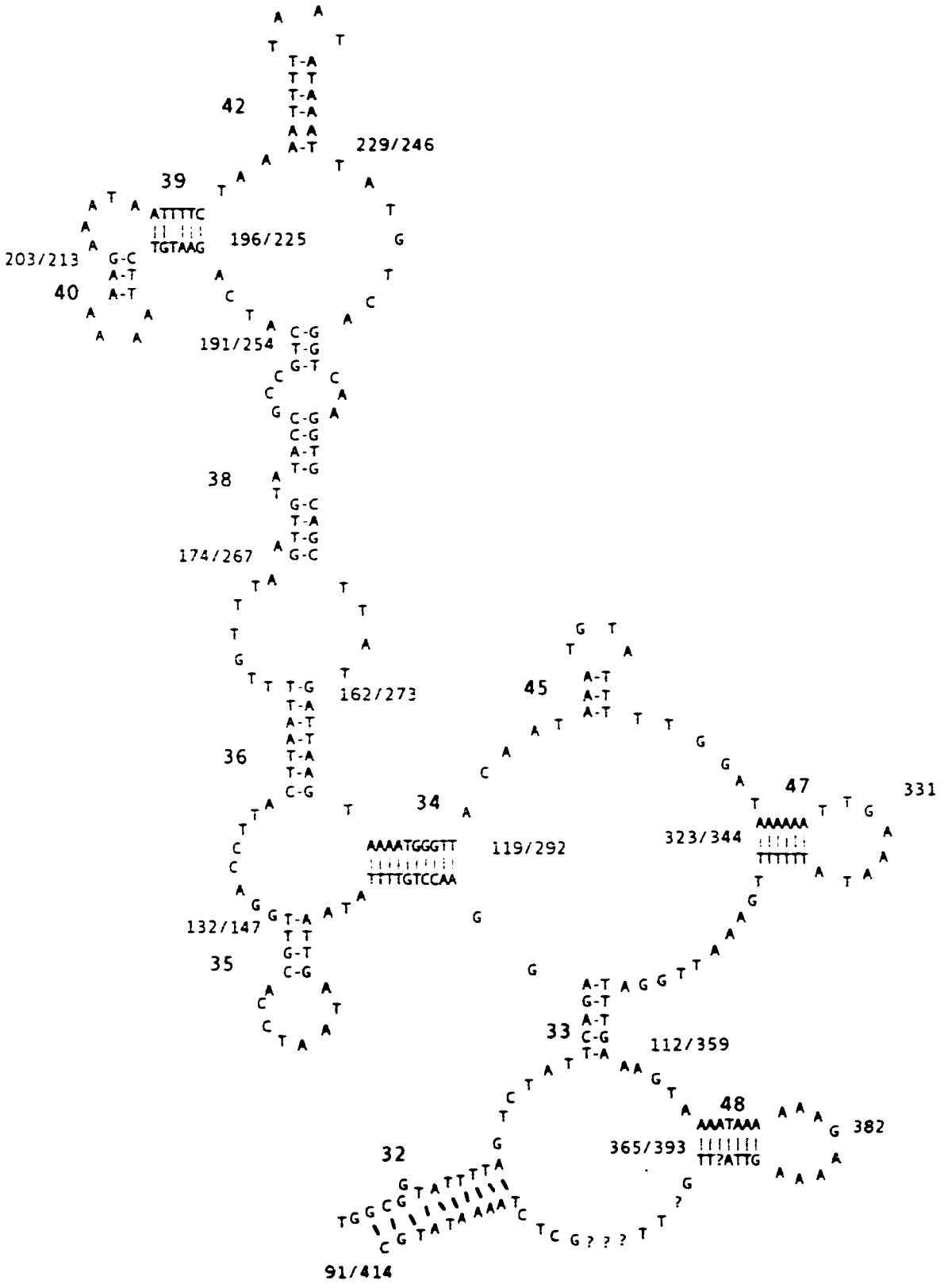
Appendix 2 (continued): Aligned Sequences--12S



Appendix 2 (continued): Aligned Sequences--12S

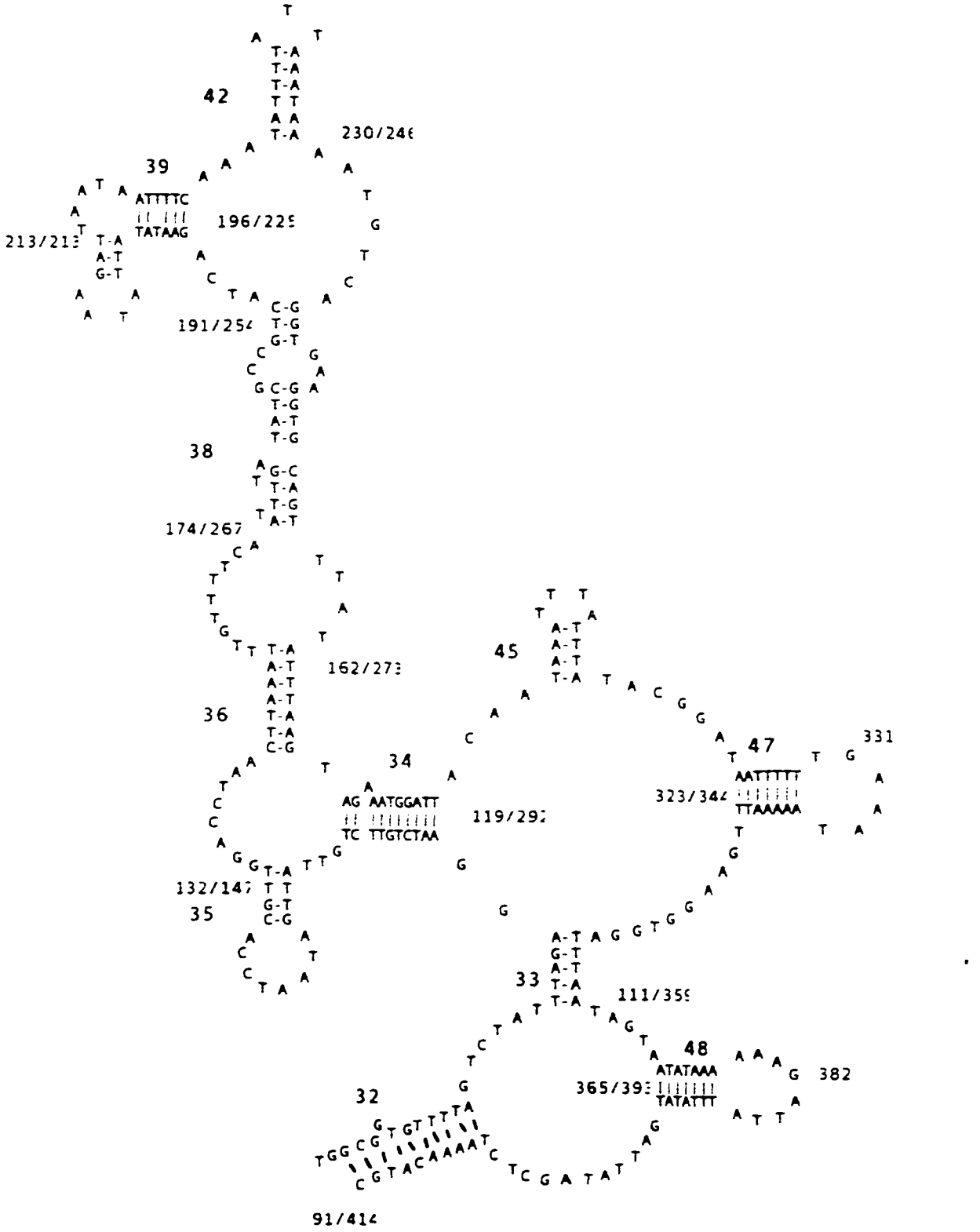
Appendix 3. Secondary structures of the 3rd domain of the 12S rRNA molecule for selected taxa.

A. *Tipula ultima* (Tipulidae)



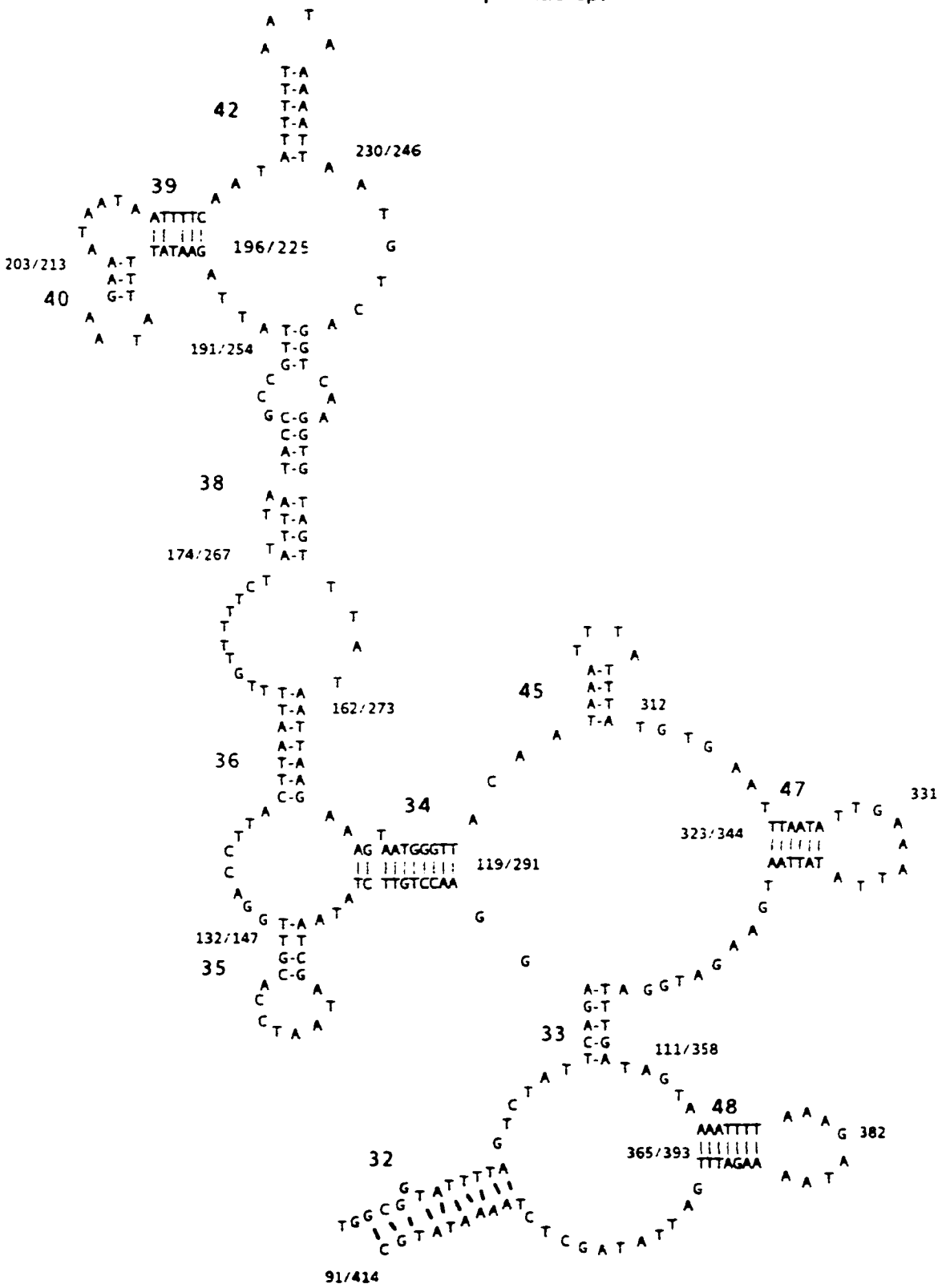
Appendix 3. **Secondary structures – 12S rRNA continued.**

B. *Anopheles quadrimaculata* (Culicidae)



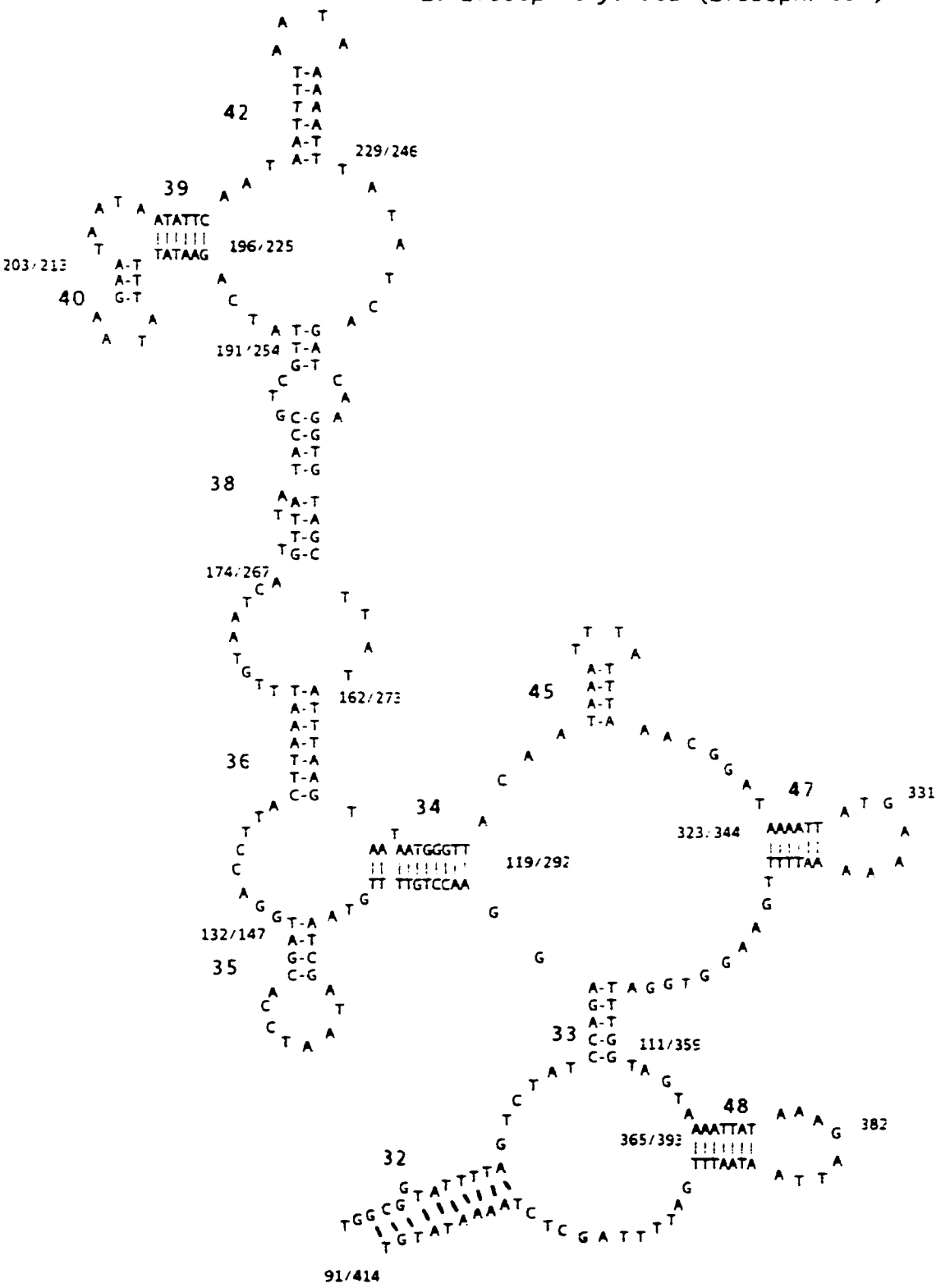
Appendix 3. Secondary structures – 12S rRNA continued.

C. Empididae sp.



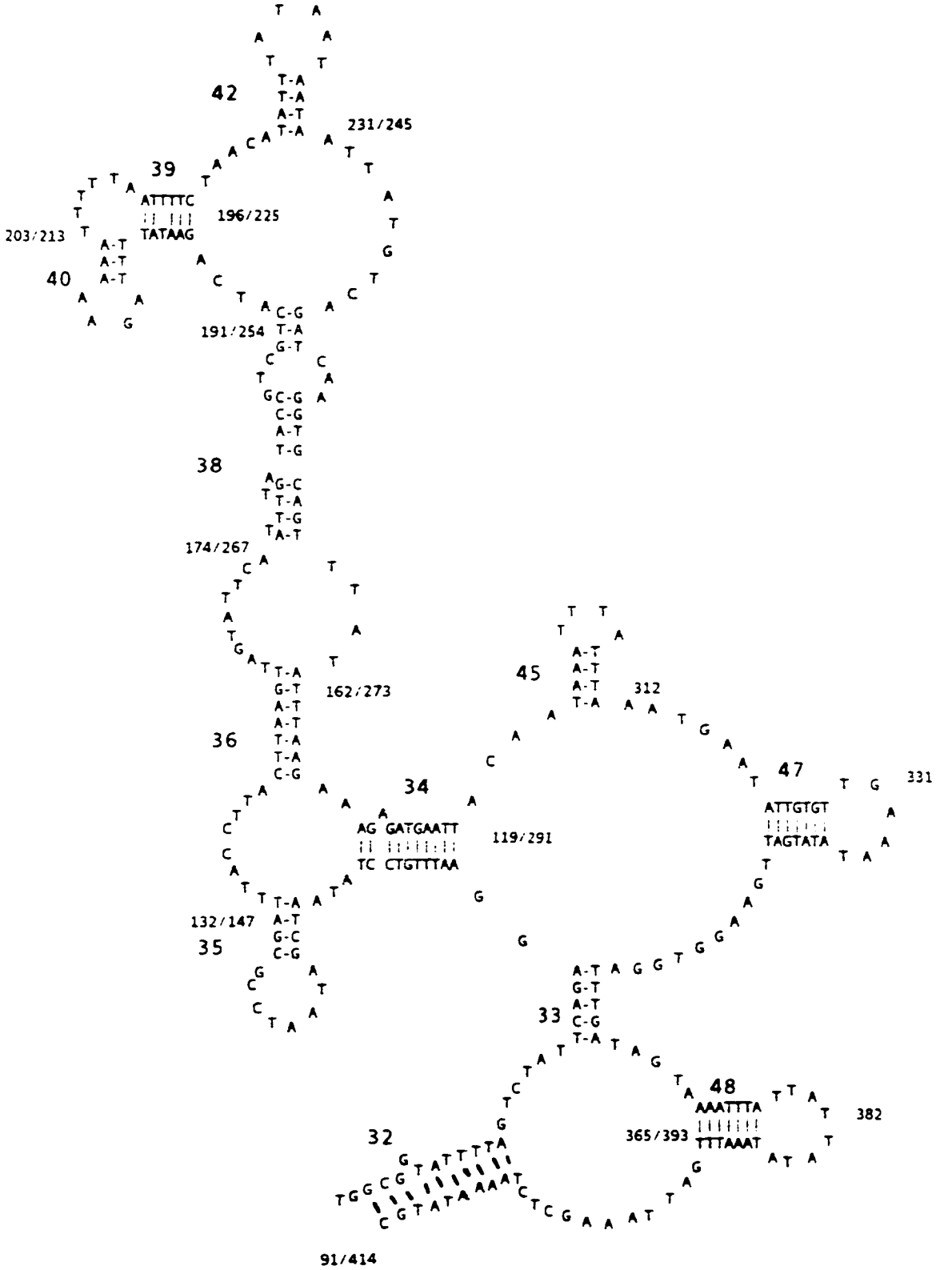
Appendix 3. Secondary structures – 12S rRNA continued.

D. *Drosophila yakaba* (Drosophilidae)



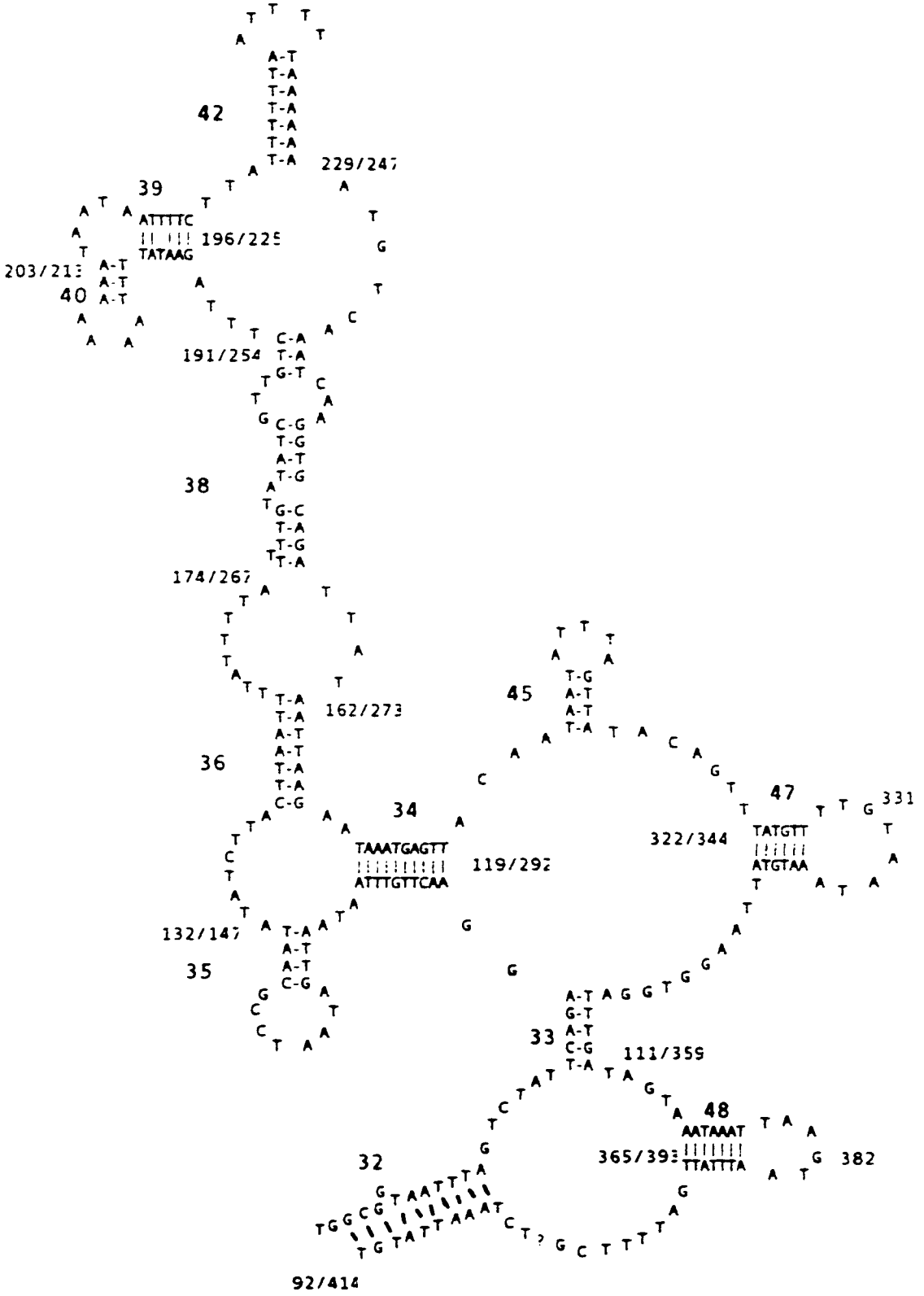
Appendix 3. Secondary structures – 12S rRNA continued.

E. *Sylvicola sp.* (Anisopodidae)



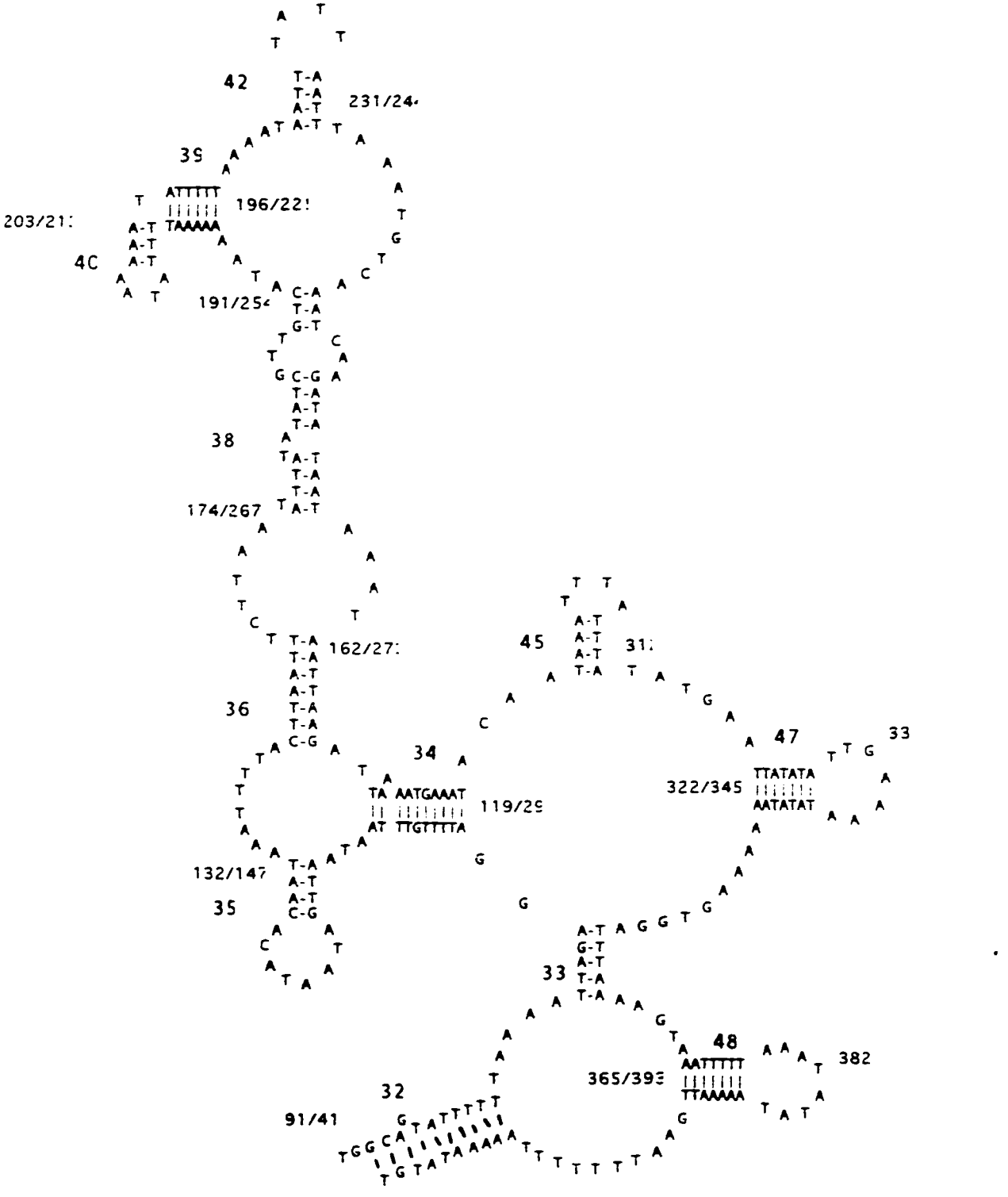
Appendix 3. Secondary structures – 12S rRNA continued.

F. *Dilophus sp.* (Bibionidae)



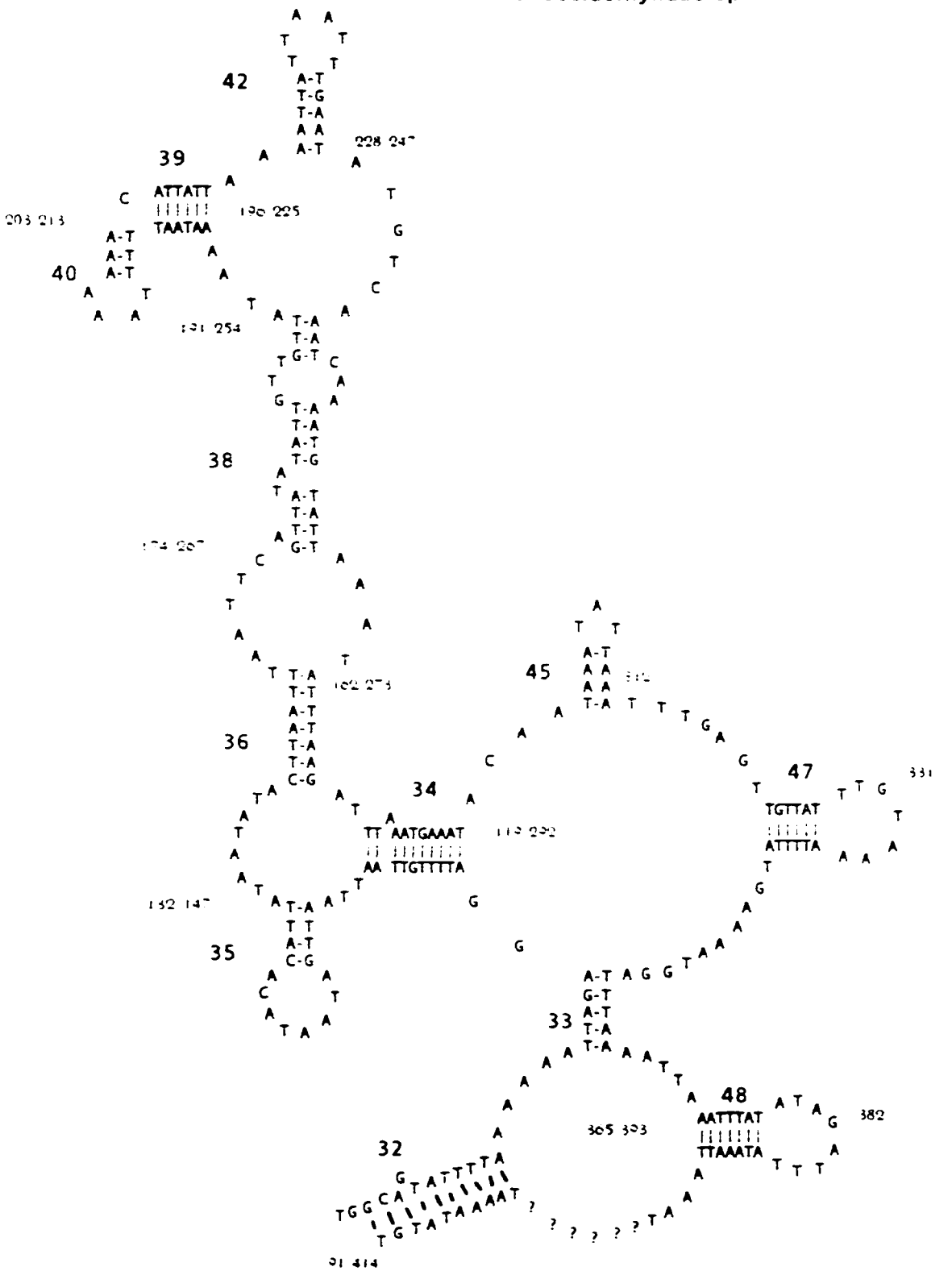
Appendix 3. **Secondary structures – 12S rRNA continued.**

G. Cecidomyiidae species A



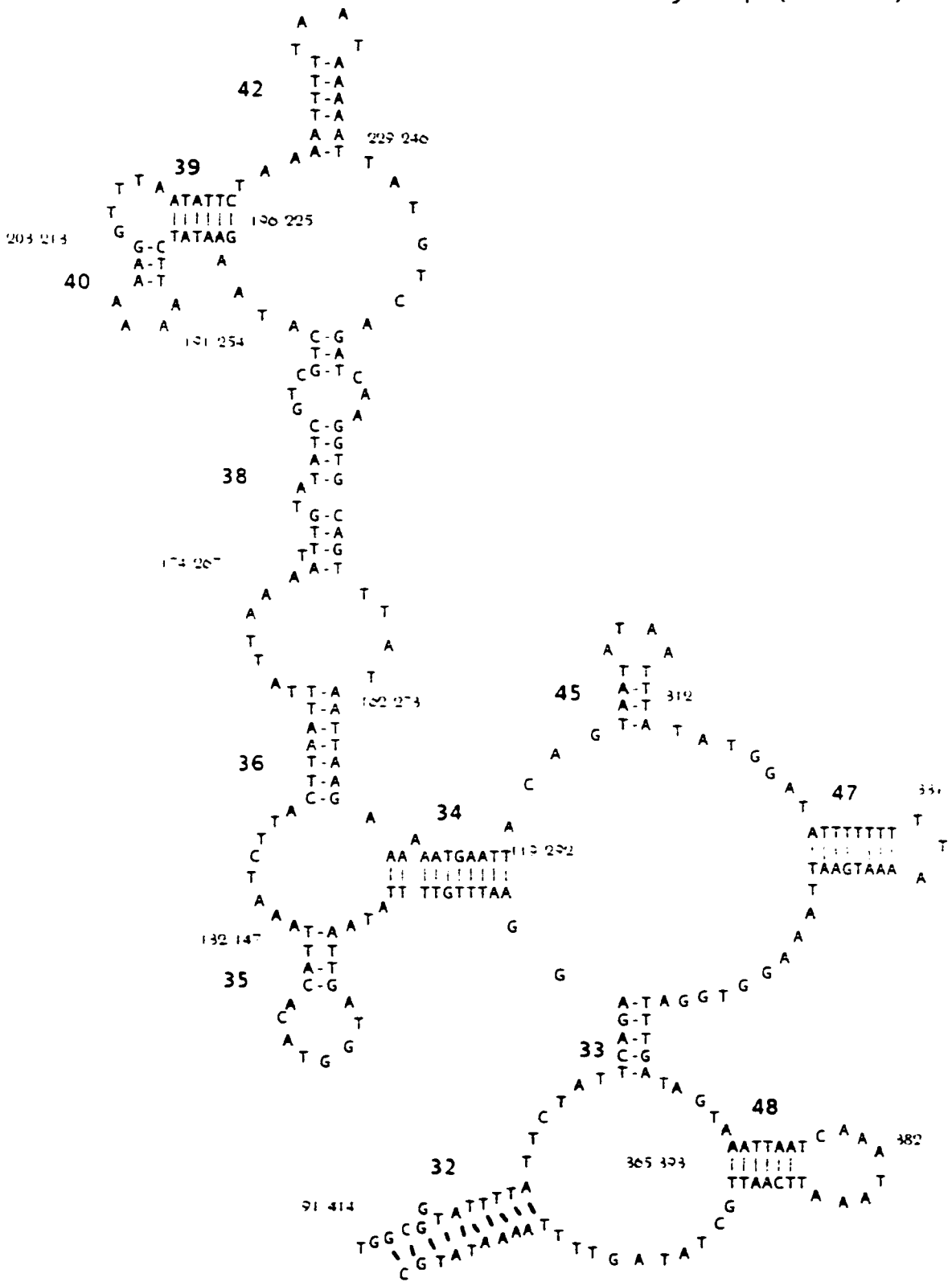
Appendix 3. Secondary structures – 12S rRNA continued.

H. Cecidomyiidae sp. B



Appendix 3. Secondary structures – 12S rRNA continued.

I. *Bradysia* sp. (Sciaridae)



Appendix 3. Secondary structures – 12S rRNA continued.

J. *Macrocera* sp. B (Keroplatidae)