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Phylogenetics and temporal diversification of the earliest true flies (Insecta: Diptera) based on multiple nuclear genes

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Abstract. Relationships among families of the lower Diptera (formerly suborder 'Nematocera') have been exceptionally difficult to resolve. Multiple hypotheses based on morphology have been proposed to identify the earliest lineages of flies and place the phylogenetic origin of the higher flies (Brachycera), but convincing support is limited. Here we resolve relationships among the major groups of lower Diptera using sequence data from four nuclear markers, including both ribosomal (28S rDNA) and protein-coding (CAD, TPI and PGD) genes. Our results support both novel and traditional arrangements. Most unexpectedly, the small, highly-specialized family Deuterophlebiidae appears to be sister to all remaining Diptera. Other results include the resolution of the traditional infra-orders Culicomorpha (including a novel superfamily Simulioidea = Thaumaleidae + Simuliidae), Tipulomorpha (Tipulidae sensu lato + Trichoceridae) and Bibionomorpha sensu lato. We find support for a limited Psychodomorpha (Blephariceridae, Tanyderidae and Psychodidae) and Ptychopteromorpha (Ptychopteridae), whereas the placement of several enigmatic families (Nymphomyiidae, Axymyiidae and Perissommatidae) remains ambiguous. According to genetic data, the infra-order Bibionomorpha is sister to the Brachycera. Much of the phylogenetic signal for major lineages was found in the 28S rDNA gene, whereas protein-coding genes performed variably at different levels. In addition to elucidating relationships, we also estimate the age of major lower dipteran clades, based on molecular divergence time estimates using relaxed-clock Bayesian methods and fossil calibration points.

Introduction

The nematocerous or lower Diptera are an ecologically and morphologically rich assemblage of true flies, encompassing approximately one-third of the order's extant diversity (~52 000 species in up to 40 families) (Yeates & Wiegmann, 1999, 2005; Amorim & Yeates, 2006; Evenhuis *et al*., 2007). Sharing many morphological characters with the earliest true flies (which arose during the late Permian or early Triassic; Shcherbakov *et al*., 1995; Krzeminski & Krzeminska, 2003; Blagoderov *et al*., 2007), extant lower Diptera occupy a great variety of ecological niches (e.g. aquatic, semi-aquatic and terrestrial habitats) and trophic levels (e.g. predators, saprophages, herbivores/fungivores, blood-feeders, pollinivores, parasitoids and parasites). Lower flies can be pests of agriculture (e.g. gall midges – Cecidomyiidae) (Barnes, 1946–1956) and many are important vectors of human and animal pathogens, including malaria, yellow fever, dengue (mosquitoes – Culicidae), leishmaniasis (sand flies – Psychodidae) and onchocerciasis (black flies – Simuliidae) (Mullen & Durden, 2002).

Historically, families of the lower Diptera were placed in the suborder Nematocera ('thread-horn flies'), distinguished from the suborder Brachycera ('short-horn flies') based largely on adult antennal structure (Yeates & Wiegmann, 1999, 2005). A number of other characters, including wing

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venation and larval head structure, also aid in distinguishing these two groups of flies. Although Brachycera is a firmly supported monophyletic clade (Yeates & Wiegmann, 1999, 2005; Yeates *et al*., 2007), evidence for nematoceran monophyly is lacking – the aforementioned characters representing plesiomorphies for the order. Current classifications of Diptera generally accept that Nematocera is paraphyletic and that the Brachycera originate from within this grade of lineages (Wood & Borkent, 1989; Oosterbroek & Courtney, 1995; Yeates & Wiegmann, 2005; Yeates *et al*., 2007). These lineages, traditionally referred to as infra-orders, have recently been elevated to suborders (Amorim & Yeates, 2006). It should be noted, however, that these groups have identical names (e.g. suborder Bibionomorpha = infra-order Bibionomorpha), and, thus, the issue is purely one of rank. For comparison's sake we have retained the term infra-order for these groups, despite our interest in furthering the subordinal concept.

Early classifications of Diptera express an array of hypotheses concerning lower flies, with conflicting arrangements attributed to the use of limited numbers and types of characters, phenetic and/or non-quantitative methods, and/or limited taxa (see reviews in Edwards, 1926; Wood & Borkent, 1989; Oosterbroek & Courtney, 1995). Alternative interpretations of ground plan character states and the difficulty in distinguishing homoplasy from homology across such a diverse morphological spectrum continue to hinder attempts to stabilize the phylogeny of lower flies. Recent attention has shifted to more quantitative methods for resolving the familial composition and interrelationships of the nematocerous infra-orders (Oosterbroek & Courtney, 1995; Friedrich & Tautz, 1997a). Nevertheless, phylogenetic controversies persist in this area of fly classification. This uncertainty seriously limits the accuracy and resolution of comparisons among major fly model organisms. In particular, resolving the phylogenetic sister group of the Brachycera from within the lower Diptera should aid in choosing phylogenetically appropriate (i.e. closely versus distantly related) taxa for comparisons to either lower dipteran (e.g. *Anopheles gambiae*, the malaria mosquito) or brachyceran (e.g. *Drosophila*) genomes, and on precisely calibrating these comparisons with rigorous age estimates.

Reconstructing the pattern and timing of fly diversification is a major goal of the current US National Science Foundation Assembling the Tree of Life (AToL) project on Diptera: FLYTREE (http://www.inhs.uiuc.edu/research/FLYTREE/), and of numerous research programmes in insect phylogenetics, paleontology and comparative genomics. Here, we report results of a FLYTREE project aimed at elucidating relationships among the earliest extant Diptera using evidence from multiple nuclear gene sequences. We sequenced one ribosomal (28S rDNA) and three protein-coding genes (CAD, TPI and PGD) from representatives of all major lineages of lower flies. Our goals are to (i) estimate a phylogeny for the earliest lineages within the Diptera, (ii) clarify the familial composition of, and relationships among, the lower dipteran infra-orders, (iii) identify the sister-group of the highly diverse clade Brachycera and (iv) use fossils and nucleotide sequence data to estimate divergence times to more firmly establish the temporal framework for the prodigious diversification of the earliest-branching lineages of true flies.

Previous phylogenetic hypotheses

Hennig (1973, 1981) was the first modern systematist to use explicit methods to resolve relationships within the lower Diptera (Meier, 2005). Based primarily on imaginal characters, Hennig recognized four main lineages (infraorders) of lower flies: Tipulomorpha, Psychodomorpha, Culicomorpha and Bibionomorpha (Fig. 1A). He considered Tipulomorpha (Tipulidae sensu lato and Trichoceridae; Fig. 1A: TP) to be the sole member of the Polyneura, a group distinguished from the remainder of the Diptera (Oligoneura) by the undifferentiated stalk and blade of the wing and the resultant presence of two anal veins reaching the wing margin (Oligoneura have only one anal vein reaching the wing margin). Hennig united several disparate families in the Psychodomorpha (Fig. 1A: PS) based on a single synapomorphy, the coalescence of the meron and the epimeron of the mesothorax. Hennig (1973) expressed doubt over the monophyly of this infra-order because of the high variability of this character within multiple families of lower Diptera. Unlike the Psychodomorpha, Hennig was able to identify a suite of synapomorphies for the Culicomorpha (Fig. 1 A:CU). The resulting family composition of this infra-order has been the most stable among all lower dipteran infra-orders (see below). Hennig placed the remaining nematocerous fly families in the Bibionomorpha (Fig. 1A: BB), based on the reduction of the costa along the posterior margin of the wing. He also proposed a somewhat tenuous relationship between this infra-order and the Brachycera, citing an enlargement of the second laterotergite and an undivided thoracic postphragma as possible synapomorphies.

In their landmark chapter in volume 3 of the *Manual of Nearctic Diptera*, Wood & Borkent (1989) were first to formally evaluate Hennig's interpretations of nematoceran relationships. Primarily using characters from larvae and pupae (but also including adult characters), they grouped the families of lower Diptera into seven infra-orders: Tipulomorpha, Blephariceromorpha, Axymyiomorpha, Bibionomorpha, Psychodomorpha, Ptychopteromorpha and Culicomorpha (Fig. 1B). Like Hennig before them, Wood & Borkent proposed a sister-group relationship between Tipulomorpha and all remaining Diptera; however, their definition of the Tipulomorpha differed from Hennig's in comprising only the family Tipulidae sensu lato (=Tipuloidea including Pediciidae, Limoniidae, Cylindrotomidae and Tipulidae sensu stricto; Oosterbroek & Theowald, 1991) (Fig. 1B:TP). Separation of Tipulidae from the rest of flies was based on one larval character, namely the condition and position of the mandibular prostheca. Wood & Borkent (1989) recognized the infra-order Blephariceromorpha for three torrenticolous families (Belphariceridae, Deuterophlebiidae and Nymphomyiidae) (Fig. 1B: BL) that were

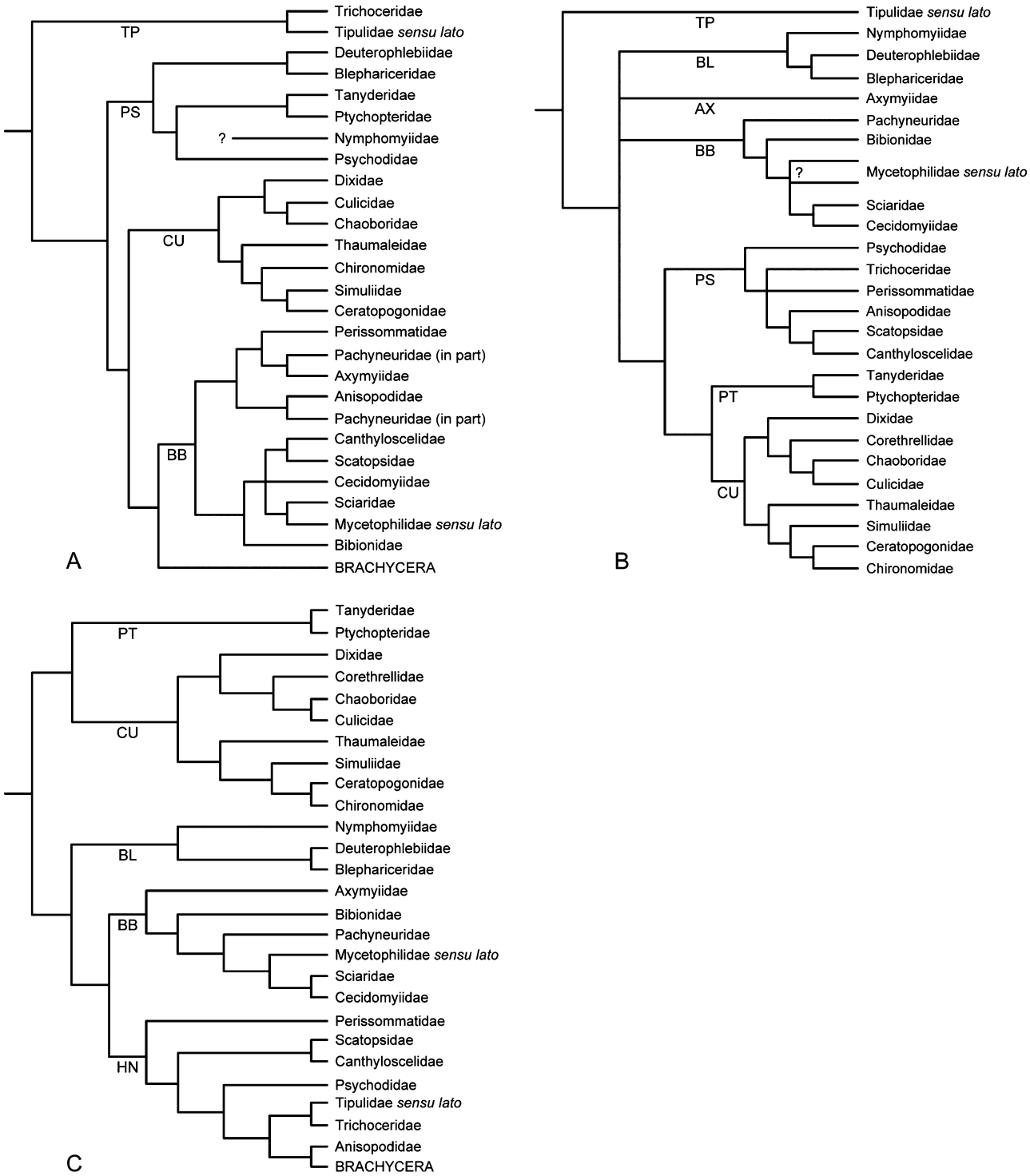

Fig. 1. Phylogenetic hypotheses of lower Diptera relationships from (A) Hennig (1973), (B) Wood & Borkent (1989), and (C) Oosterbroek & Courtney (1995). AX = Axymyiomorpha; BB = Bibionomorpha; BL = Blephariceromorpha; CU = Culicomorpha; HN = Higher Nematocera + Brachycera; PS = Psychodomorpha; PT = Ptychopteromorpha; TP = Tipulomorpha.

formerly placed in Hennig's Psychodomorpha. Although unsure of their placement of Nymphomyiidae in the Blephariceromorpha, they found strong evidence for a relationship between the other two families. In the absence of synapomorphies uniting the Axymyiidae with other flies, these workers erected the monotypic infra-order Axymyiomorpha

(Fig. 1B: AX). Adult axymyiids resemble some Bibionomorpha superficially in wing venation and general appearance, features that Hennig used to place them in that infra-order. Larval axymyiids, on the other hand, are highly autapomorphic and have several unusual characters and habits. The Bibionomorpha of Wood & Borkent (1989) contained only a portion of Hennig's original composition, including the Pachyneuridae, Bibionidae, Mycetophilidae (all three in the broad sense), Sciaridae and Cecidomyiidae (Fig. 1B: BB). They considered this arrangement to be phenetic, however, as they were unable to determine any synapomorphies for the infra-order. They united the last three families (Sciaroidea) with limited phylogenetic evidence. The composition of the Psychodomorpha was perhaps the most controversial result of Wood & Borkent's study: six families with highly divergent adult morphologies were placed in this infra-order (Psychodidae, Trichoceridae, Perissommatidae, Anisopodidae, Scatopsidae and Canthyloscelidae; Fig. 1B: PS) based on several larval synapomorphies. Wood & Borkent (1989) proposed the infra-order Ptychopteromorpha for two small families of flies, the Ptychopteridae and Tanyderidae (Fig. 1B: PT). This grouping was based on only one character, the ability of males to fold the last tarsomere onto the penultimate one. Although this character is not present in all Ptychopteridae, members lacking the character were thought to have secondarily lost it as a result of leg modification. A large proportion of the characters used by Wood & Borkent (1989) (29 of 83 total characters) were associated with the monophyly and relationships of the infra-order Culicomorpha. The composition and arrangement of this infra-order (Fig. 1B: CU) was unchanged from Hennig's concept except instead of Simuliidae, Ceratopogonidae was supported as sister to the Chironomidae. Although Wood & Borkent (1989) were not explicit about the placement of Brachycera within their cladogram, Sinclair (1992), continuing to study larval mouthparts, concluded that the Brachycera were closely related to the Psychodomorpha of the former authors. Detailed observations on the structures of larval Deuterophlebiidae and other Blephariceromorpha by Courtney (1990, 1991) resulted in the further resolution of relationships among the infra-orders of Wood & Borkent (1989), by suggesting a relationship between the Blephariceromorpha and either the Psychodomorpha alone or the clade Psychodomorpha + (Culicomorpha + Ptychopteromorpha).

Oosterbroek & Courtney (1995) were first to publish and explicitly analyse a comprehensive matrix of morphological characters from nematocerous Diptera, scoring 98 characters from larvae (57), pupae (6) and adults (35) for all families. Quantitative phylogenetic analysis resulted in a single most parsimonious tree containing five major groups of lower Diptera: Ptychopteromorpha, Culicomorpha, Blephariceromorpha, Bibionomorpha and a clade termed 'higher Nematocera + Brachycera' (Fig. 1C). The family composition and relationships within the Ptychopteromorpha, Culicomorpha and Blephariceromorpha were identical to those of Wood & Borkent, as was the sister-group relationship between Ptychopteromorpha and Culicomorpha. Several of Oosterbroek & Courtney's (1995) groups departed significantly from those of Wood & Borkent (1989), including the placement of the Axymyiidae in the Bibionomorpha (as sister to the remaining families; Fig. 1C: BB), and the resolution of a new clade containing the Psychodomorpha (of Wood & Borkent), Tipulidae and Brachycera (Fig. 1C: HN). This latter group was unconventional in two main respects: it showed a derived position for the Tipulidae (placed with the Trichoceridae) and resolved the Anisopodidae as the sister-group of the Brachycera.

Michelsen (1996) addressed the utility of prothoracic/cervical skeleto-musculature for the resolution of Dipteran relationships. He divided the order first into the 'polyneuran' and 'oligoneuran' families [compositionally different from those of Hennig (1973)], the former containing the Tipulidae sensu lato, Tanyderidae, Trichoceridae and Ptychopteridae, and the latter including all remaining Diptera. Further, Michelsen defined a clade of oligoneurans, termed 'Neodiptera', using several synapomorphies including the presence of the precervicale, episternal lobe and apomorphic muscle structures. This group included the Bibionomorpha sensu lato (i.e. Scatopsidae, Canthyloscelidae, Anisopodidae, Axymyiidae, Perissommatidae, Pachyneuridae, Bibionidae, Sciaridae and Cecidomyiidae) and the Brachycera.

The first intensive molecular analysis of the group was performed by Friedrich & Tautz (1997a), who analysed 488 sites of the 28S rDNA gene (159 of which were parsimony informative) from a small sample of lower Diptera (14 taxa in 12 families). The resulting tree strongly supported monophyly of the Diptera, Tipulidae sensu lato, Culicomorpha and Brachycera, as well as a Bibionomorpha sensu lato (Anisopodidae, Scatopsidae, Bibionidae, Cecidomyiidae, Mycetophilidae and Sciaridae). Relationships between the major groups, however, were not well supported, as too little variation was provided in this 28S fragment to establish with confidence an early branching arrangement for the order. In addition to these hypotheses, several recent workers (Shcherbakov *et al*., 1995; Krzeminski & Krzeminska, 2003; Blagoderov *et al*., 2007) have relied largely on wing venation to place both extant and fossil representatives of lower Diptera in a phylogenetic context.

Materials and methods

Taxon sampling

We sampled 64 ingroup taxa representing 26 lower dipteran families (Supporting Information ST1). For ease of comparison, family names and concepts used in this study follow Wood & Borkent (1989) and Oosterbroek & Courtney (1995) except for Synneuridae, for which we use the current valid name Canthyloscelidae (Evenhuis *et al*., 2007). Several non-traditional and/or recently elevated families are not included in this study, based mainly on unavailability of specimens, namely Rangomaramidae (Jaschhof & Didham, 2002) and Bolitophilidae of the Sciaroidea (=Mycetophiliformia) and Valeseguyidae (Amorim & Grimaldi, 2006) of

the Scatopsoidea. Three species of Brachycera were included as representatives of the lower Brachycera (*Exeretonevra*), lower Cyclorrhapha (*Lonchoptera*) and Calyptratae (*Cochliomyia*) (Supporting Information ST1). Non-dipteran outgroups include species from two scorpionfly families (Mecoptera: Meropeidae and Nannochoristidae) and a flea (Siphonaptera: Pulicidae) (Supporting Information ST1). Scorpionflies and fleas are considered close relatives of flies and are united in most insect classifications together with flies as the Antliophora (Kristensen, 1981; Wood & Borkent, 1989; Grimaldi & Engel, 2005; Sinclair *et al.*, 2007; but see Hünefeld & Beutel, 2005). Specimens were collected into 70–100% ethanol by the authors, or by contributors listed in the acknowledgements, and stored at −20°C. Except where otherwise stated (Supporting Information ST1), vouchers are deposited in the laboratory of B.M.W. at North Carolina State University and the NC State University Insect Museum.

Nucleotide sampling and laboratory procedures

Total genomic DNA was extracted from whole specimens or muscle tissue using either a standard phenol-chloroform procedure (stored in TE buffer; see Moulton & Wiegmann, 2004) or using the DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA). All genomic templates were stored at −80°C.

DNA amplifications were performed in 50 μL solutions made up of 35.8–37.8 μL ddH20 (depending on use of MgCl2), 5 μL of 10X PCR buffer (Takara Bio USA, Madison, WI), 2 μL of 25 mM MgCl2 (used when amplifying protein-coding genes), 1 μL of each primer (10 pmol/μL), 4 μL of 10 mM dNTPs, 0.25 μL of Taq polymerase (ExTaq, Takara Bio USA, Madison, WI) and 1 μL of template DNA. Approximately 3.8 kb of the 28S rRNA gene were amplified, via standard three-step polymerase chain reaction (PCR) (50°C annealing temperature; 30 cycles), in four sections using four primer pairs (Supporting Information ST2): rc28A (or rc28Ab)-28C, rc28B-28E, rc28D-28K and rc28Q-28Z (or 28Zc) (Supporting Information ST2). The first section contained one internal sequencing primer (28B), whereas sections two and three each contained two internal sequencing primers (28P & rc28P and 28H & rc28H, respectively). No internal sequencing primers were used within the rc28Q-28Z section. Two overlapping fragments from the carbamoylphosphate synthetase (CPS) region of the CAD (rudimentary) gene were sampled (Moulton & Wiegmann, 2004). These sections were amplified using primers 787F-1098R (fragment 4) and 1057F-1278R (fragment 5) (Supporting Information ST2). Sequenced products resulted in approximately 1400 base pairs(bp) of the gene. The phosphogluconate dehydrogenase (PGD) gene was amplified using a single pair of primers (PGD2F-PGD3R or PGD4R) (Supporting Information ST2) yielding approximately 800 bp. Roughly 500 bp of the triose phosphate isomerase (TPI) gene were amplified and sequenced using two primers, 111Fb-R275 (Supporting Information ST2). Both M13 tailed and un-tailed primers were available for amplification of CAD, PGD and TPI. Amplification of all protein-coding genes (CAD, PGD and TPI) used the following touchdown PCR programme: 4 min denaturation at 94°C followed by 5 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 2 min, 5 cycles of 94°C for 30 s, 51°C for 1 min, 72°C for 2 min and 36 cycles of 94°C for 30 s, 45°C for 20 s, 72°C for 2 min 30 s.

Amplification products and negative controls were identified on 1% low-melt, agarose gels. Bands of appropriate length were excised for purification. Genetic material was extracted from excisions using the QIAquick® Gel Extraction Kit (Qiagen Inc.). Sequencing was initiated using the BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA), and sequenced on either a Prism™ 377 automated DNA sequencer (PE Applied Biosystems) or at the North Carolina State University, Genome Research Laboratory (Raleigh, NC).

Sequence alignment and phylogenetic analyses

Editing and contig assembly of complementary sequence chromatograms were performed using SEQUENCHER 4.1 (Gene Codes Corp., Ann Arbor, MI). Sequences of 28S rDNA were aligned with reference to the secondary structure inferred for the mosquito, *Aedes albopictus* (Kjer *et al.*, 1994). Using this secondary structure-based alignment as a guide, hyper-variable regions containing confounding length variation and/or uncertain positional homology were excluded from analyses. Protein-coding genes were aligned manually with reference to the translated amino acid sequence in Se-Al (Rambaut, 1996) and introns and highly variable regions were excluded. The final nucleotide alignment, translations, secondary stucture model and phylogenetic data sets are available on the FLYTREE website, and are deposited in Treebase.

Phylogenetic data sets (gene partitions treated: individually, combined, with/without third codon positions and translated into amino acid sequences) were analysed using equally weighted parsimony methods in PAUP* 4.0 (Swofford, 2003) with gaps treated as missing data. Heuristic searches were performed (1000 random addition replicates) using the tree bisection-reconnection (TBR) branch-swapping algorithm. Bootstrap support values were obtained from 500 simple-addition replicates (TBR).

Maximum likelihood (ML) and Bayesian Markov chain Monte Carlo (MCMC) analyses were performed on the combined data set (28S, CAD, PGD and TPI excluding third codon positions) in GARLI 0.951-1 (Zwickl, 2006) and MrBayes 3.1.1 (Ronquist & Huelsenbeck, 2003), respectively. MODELTEST 3.7 (Posada & Crandall, 1998) was used to compare models of evolution for this data set. Based on the Akaike Information Criterion (AIC) and hierarchical likelihood ratio tests (hLRTs), the general time reversible model (GTR; four nucleotide frequency state parameters; six substitution rate parameters) with a proportion of invariable sites (I) and a gamma distribution for the remaining sites (Γ) was identified as the best model for 28S, CAD and PGD. The SYM + I + Γ model was chosen

as the best fitting model for TPI. In addition to using the default settings in GARLI for the likelihood tree search (which implements the GTR + I + Γ model), 500 ML bootstrap replicates were also performed. Bayesian MCMC searches were performed using four chains for 20 000 000 generations, sampling every 5000 generations. A burn-in of 9 000 000 generations (or 45% of the sampled trees) was chosen as a conservative value, despite the average standard deviation of splits converging and stabilizing on ~0.01 at 1 000 000 generations.

Divergence time analysis

To estimate divergence times for lower dipteran clades, we used the parametric Bayesian-relaxed clock approach implemented in the programs ESTBRANCHES and MULTIDIVTIME (Thorne & Kishino, 2002). In addition to priors on evolutionary rates, MULTIDIVTIME and ESTBRANCHES require an assumed phylogenetic topology, maximum and minimum root node age constraints and, ideally, several minimum-age clade constraints from fossils or other external evidence (Wiegmann *et al*., 2003; Rutschmann *et al*., 2007). For the topology, we used a consensus of the best supported nodes from both the parsimony and model-based analyses as the best estimate of relationships based on our current nucleotide data. Because 28S was the only marker sequenced across all taxa, only data from this gene were used in calculating divergence time estimates. The dipteran root node was given a max-min boundary of 270–240 million years ago (Ma) spanning the hypothesized age of origin for the order and its closest relatives (Grimaldi & Engel, 2005), and the estimated age of the oldest definitive fossil dipteran, *Grauvogelia arzvilleriana* (Anisian; ~240 Ma) (Krzeminski *et al*., 1994). Three minimum age constraints were also used based on fossil specimens that could be defensibly assigned to a monophyletic group found in the input tree. These included: 220 Ma for Tipulidae (*Architipula youngi*; Carnian-Norian) (Krzeminski, 1992a), 210 Ma for the Chironomidae (*Aenne triassica;* Rhaetian) (Krzeminski & Jarzembowski, 1999) and 180 MYA for Psychodidae + Tanyderidae (*Nannotanyderus krzeminskii;* Toarcian) (Ansorge, 1994). We followed the analytical procedure described in Rutschmann *et al*. (2007) and in the MULTIDIVTIME readme files, and ran the Markov chain for 1.1×10^6 cycles with samples collected every 100 cycles and discarded the first 100 000 cycles as burnin. We performed the MULTIDIVTIME analysis multiple times from different initial conditions to confirm convergence of the Markov chain on highly similar resulting time estimates and posterior intervals.

Results

Phylogenetic analyses

Exclusion of introns and hyper-variable regions resulted in a final multigene dataset of 5272 characters, of which 2501 are parsimony informative (Supporting Information ST3). A high percentage (54.3%) of informative sites are third codon positions of the protein-coding genes, as expected given the high variability and potential for saturation at this site over the deep divergences sampled here. The observed number of informative sites (and percentage of total) for each codon position are as follows: CAD nt1 = 215 (16.0%), nt2 = 127 (9.4%), nt3 = 432 (32.1%); PGD nt1 = 127 (16.4%), nt2 = 68 (5.0%), nt3 = 247 (31.8%); TPI nt1 = 86 (18.0%), nt2 = 59 (12.4%) and nt3 = 152 (31.9%) (Supporting Information ST3). Because of the high variability in nt3, our standard set of analyses was carried out on a dataset that excludes third codon positions (4405 characters/1670 parsimony informative). Mean uncorrected distances within the Diptera range from 10.4% (28S) to 29.7% (TPI), but when third positions were excluded this range narrowed. Most of the higher pairwise divergence values we observed are attributable to comparisons involving a few highly divergent, autapomorphic taxa, especially *Nymphomyia* and *Perrisomma*, or involve deeply diverging lineages (e.g. ingroup/outgroup comparisons). Base composition (A + T %) ranges from 49.8% to 58.9% (Supporting Information ST3). The conserved regions of 28S exhibit a higher proportion of A + T (54.5%; Supporting Information ST3) than was previously observed for non-dipterans (37%; Friedrich & Tautz, 1997b), but is on par with values for other fly groups (e.g. Tabanomorpha: 53.9%; Wiegmann *et al*., 2000). There is, however, significant heterogeneity of base composition among taxa for the three protein-coding genes when third positions were included, as well as when these are combined with 28S (Supporting Information ST3).

Parsimony analysis of the combined multigene dataset (excluding codon position three) yields two, minimum length trees (length = 11537; CI = 0.314; RI = 0.523; Supporting Information ST4). Monophyly of the Diptera is well supported [100% bootstrap support (BS)] (Fig. 2) and all families are recovered as monophyletic, with the exception of the Mycetophilidae, the monophyly of which has been extensively questioned (see below). The only topological difference between the two MP trees is the position of the Psychodomorpha, placed as sister to the Culicomorpha + Nymphomyiidae + Axymyiidae, or sister to a Brachycera + Bibionomorpha clade. Support values between major groupings (i.e. along the tree's backbone) are generally low. When third codon positions are included (not shown), the general topology of the consensus tree is unchanged, although statistical support for major clades is reduced, resolution is reduced (9 MP trees versus 2 MP trees) and the degree of homoplasy increases (CI = 0.218; RI = 0.379; Supporting Information ST4). Notable differences in the consensus tree (when third codon positions are included) are changes in the relationships within the Culicomorpha, a paraphyletic Psychodidae (containing Tanyderidae) and the inclusion of *Perissomma* in the Bibionomorpha (sister to *Pseudobrachypeza*). Separate parsimony analyses of individual genes (Supporting Information ST4) resulted in either well resolved but incongruent topologies or were largely unresolved.

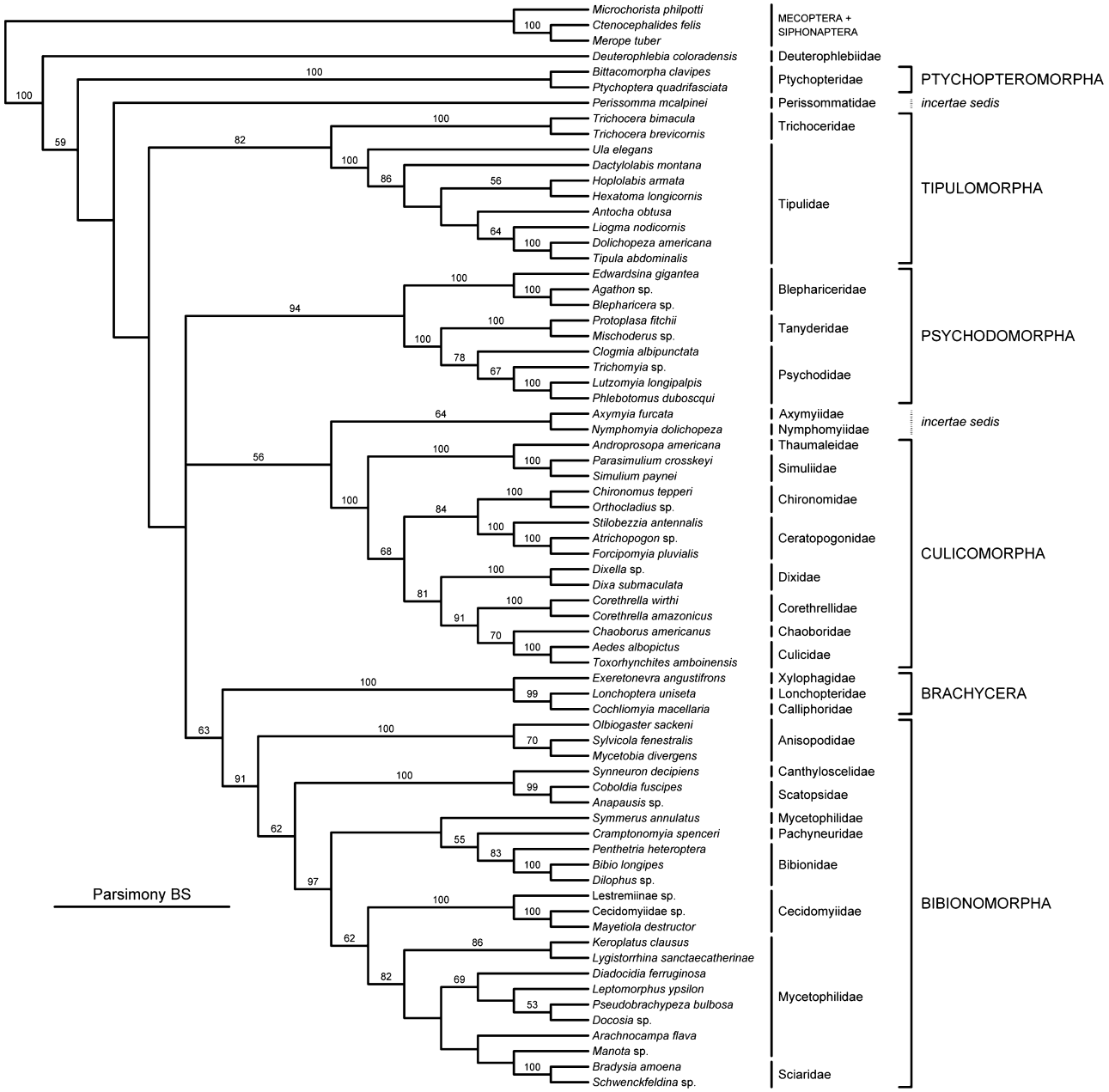

Fig. 2. Parsimony analysis of combined nuclear ribosomal (28S) and protein-coding (CAD, PGD and TPI) genes, with codon position three sites removed. Strict consensus of two most parsimonious trees (length = 11 537; CI = 0.314; RI = 0.523; RC = 0.164). Bootstrap (BS) values ≥50% shown above branches.

A sister-group relationship between the small, enigmatic family Deuterophlebiidae (mountain midges) and all remaining Diptera is weakly supported (59% BS). This is a novel placement for the family, although the archaic nature of deuterophlebiids was proposed by Rohdendorf (1974), albeit based primarily on the aberrancy of the family. The Ptychopteridae, representing a monotypic Ptychopteromorpha, and the Perissommatidae are placed topologically (although not statistically: <50% BS) as the next branching lineages, respectively (Fig. 2). The remaining families are grouped into several large clades (≥2 families), henceforth classified as infra-orders. In accord with Hennig (1973), the combined analysis supports a Tipulomorpha (82% BS) encompassing the sister taxa Trichoceridae and Tipulidae sensu lato. All remaining Diptera are placed in one of three clades forming an unresolved trichotomy:

Psychodomorpha, Culicomorpha + Nymphomyiidae + Axymyiidae and Brachycera + Bibionomorpha. The families Blephariceridae, Tanyderidae and Psychodidae are recovered as the infra-order Psychodomorpha with high support (94% BS). Although these taxa were in Hennig's (1973) original Psychodomorpha, other families he placed in the infra-order (e.g. Ptychopteridae and Deuterophlebiidae) are placed elsewhere by the molecular data. The traditional family composition of the Culicomorpha (Hennig, 1973; Wood & Borkent, 1989) is highly supported (100% BS) under parsimony, although relationships within the infra-order differ from previous morphological studies. Unlike these studies, the Thaumaleidae and Simuliidae are sisters to all remaining Culicomorpha, although support for this relationship is relatively low (68% BS). All other Culicomorpha fall into the traditional superfamilies Chironomoidea (Chironomidae and Ceratopogonidae) and Culicoidea (Dixidae, Corethrellidae, Chaoboridae and Culicidae) with moderate support (84% and 81% BS, respectively). A sister-group relationship (56% BS) between the Culicomorpha and two small aberrant families, Axymyiidae and Nymphomyiidae, is weakly supported. Although Nymphomyiidae have sometimes been considered closely related to the Culicomorpha (Courtney, 1994a; Sæther, 2000), Axymyiidae have not been associated with either Nymphomyiidae or Culicomorpha in past hypotheses. However, the effects of long-branch attraction cannot be ruled out, as shown by the alternative position of Nymphomyiidae in the reduced taxon analyses (only taxa with all gene partitions; Fig. 5). All remaining families of Diptera form a clade containing the Brachycera + Bibionomorpha (sensu Hennig, 1973). With the exception of the families Perissommatidae and Axymyiidae, this group is congruent with Michelsen's (1996) definition of the 'Neodiptera'. A broadly defined Bibionomorpha was supported with 91% BS. The Anisopodidae and Scatopsoidea (Scatopsidae + Canthyloscelidae) are among the earliest diverging bibionomorph lineages, whereas a strict Bibionomorpha (i.e. of Wood & Borkent, 1989) is well supported (97% BS). A monophyletic Sciaroidea is not recovered because of the placement of *Symmerus* as sister to the Bibionoidea (Pachyneuridae + Bibionidae sensu lato), rendering the former superfamily paraphyletic. Additionally, the Mycetophilidae sensu lato is rendered paraphyletic, containing within it the putative family Sciaridae. Historically, the monophyly of Mycetophilidae has not been clearly demonstrated, and the family has been divided into several families or has included the Sciaridae as a subfamily (Vockeroth, 1981; see review in Amorim & Rindal, 2007).

Results of model-based Bayesian MCMC and ML (Figs 3, 4) analyses are largely congruent with those obtained using parsimony. The Diptera are monophyletic [100% posterior probability (PP)/100% ML bootstrap (MLB)], with the Deuterophlebiidae placed as sister to all other flies (82% PP/61% MLB). The Ptychopteridae are resolved as the sister to the remaining Diptera (except Deuterophlebiidae), although with weak support (59% PP/<50% MLB). Support for the monophyly of Tipulomorpha remains high, with 100% PP and 88% MLB. Both the Culicomorpha and Psychodomorpha are well supported (100% PP/100% MLB and 100% PP/99% MLB, respectively) and both groups show the same internal topology as found in the parsimony analysis. In agreement with one of the possible arrangements under parsimony (although without the Perissommatidae), there is support (88% PP/53% MLB) for a relationship between the Psychodomorpha and the Perissommatidae + Brachycera + Bibionomorpha clade. Inclusion of the Perissommatidae in the latter group is well supported (100% PP/92% MLB) and contrasts with the more basal position of the family recovered in the parsimony analysis. Relationships within the Bibionomorpha + Brachycera remain stable when analysed under likelihood/Bayesian criteria, except for the branching pattern within the Mycetophilidae (excluding *Symmerus*) + Sciaridae clade. Bayesian MCMC analyses including third codon positions of the protein-coding genes differ little from the above results that exclude these sites. Major differences include low support (53% PP/<50% MLB) for the Tipulomorpha being sister to all Diptera except Deuterophlebiidae, a polytomy between Ptychopteromorpha, Culicomorpha + Axymyiidae + Nymphomyiidae, Psychodomorpha and Perissommatidae + Brachycera + Bibionomorpha, less resolution within the family Tipulidae, and a sister-group relationship between Anisopodidae and Scatopsoidea (82% PP/<50% MLB).

Divergence time analysis

Divergence time estimates based on our molecular data place the origin of crown group Diptera at approximately 267 Ma (CI = 260–269; Fig. 6), marking the split between Deuterophlebiidae and all remaining Diptera. The next four lineages (Tipulomorpha, Ptychopteridae, Culicomorpha + Axymyiidae + Nymphomyiidae and Psychodomorpha + Perissommatidae + Brachycera + Bibionomorpha), currently represented by an unresolved polytomy, are estimated to be almost contemporaneous with the earlier branching at 265 Ma (CI = 256-269; Fig. 6). The Tipulidae sensu lato, Trichoceridae, Ptychopteridae, Culicomorpha, Axymyiidae, Nymphomyiidae, Psychodomorpha and Neodiptera (excluding Axymyiidae) arose during the late Triassic, between 200 and 250 Ma (Fig. 6). By the Jurassic (145–200 Ma), all infra-orders and many of the nematocerous families were present, although a large proportion of extant bibionomorphan families had not arisen yet. Although fossil Sciaroidea (Mycetophilidae sensu lato and Sciaridae) are known from the lower Cretaceous (Blagoderov, 1997, 1998a, b), our estimates without fossil constraints infer these groups as younger in age. However, the confidence intervals presented for these lineages extend into the early Cretaceous (Fig. 6, Table 1). By the end of the Cretaceous (65 Ma) all major groups of extant lower Diptera were present.

Table 1. Divergence time estimates (millions of years ago (Ma)) and credibility/confidence intervals (CI) for nodes in Figure 6.

Node	Time	CI	Node	Time	CI
1	267	260–269	15	95.2	57.2–140
2	265	256–269	16	210	179–243
3	235	221–261	17	196	160–230
4	241	224–260	18	103	60.3–151
5	195	138–236	19	69.4	32.2–115
6	226	215–243	20	160	120–200
7	130	73.5–181	21	155	114–195
8	220	212–234	22	87.1	45.0–136
9	213	210–223	23	126	84.3–168
10	190	155–216	24	116	75.1–158
11	146	96.6–188	25	98.6	60.2–139
12	118	71.2–163	26	114	73.5–157
13	234	209–259	27	103	63.6–144
14	197	181–225	28	95.6	57.3–137

Discussion

Uncertainty over the higher-level, phylogenetic relationships among the lineages of lower Diptera has stimulated recent surveys of novel character systems and the application of modern methods to traditional evidence (Courtney & Oosterbroek, 1995; Michelsen, 1996; Friedrich & Tautz, 1997b; Yeates *et al*., 2007). Gene sequences are an increasingly important source of phylogenetic information for a wide range of insect groups (Caterino *et al*., 2000), but have not yet been thoroughly applied to the lower Diptera. Our analysis is the first to do so for a diverse sampling of lower dipteran flies.

Our results reveal both the promise and limitations of phylogenetic inferences from nucleotide data and highlight the difficulties involved in genetic sampling from hyperdiverse, ancient radiations such as in the earliest lineages of Diptera. Primer design, amplification, and sequencing are difficult for nuclear genes making it a challenge to target large gene regions and amplify across unpredictable introns. Nonetheless, the current data support traditionally recognized as well as completely novel hypotheses of relationships among early flies. The ribosomal gene (28S) contributes much of the information at the deeper levels of our trees – providing strong support for the monophyly of the order, and the monophyly and composition of the infra-orders Tipulomorpha, Psychodomorpha, Culicomorpha and Bibionomorpha (Fig. 5A; Supporting Information ST4). Perhaps more importantly, the current molecular data consistently place the Brachycera as sister to the Bibionomorpha (Figs 2–5).

Signal from the protein-coding genes sampled here (CAD, TPI and PGD) is either weak as a result of constraints on amino acid change, limited because of sequenced fragment size or appears saturated among the selected taxa. Phylogenies produced from these genes are incongruent with expected relationships (e.g. non-monophyly of firmly established families) from morphology or 28S, or lack sufficient resolution to support phylogenetic inferences (not shown). The relative contribution of each gene in the combined data topology can be assessed in Fig. 5, presenting results of the combined analysis of taxa for which all genes were sampled. Partitioned Bremer support shows that the two longest fragments (28S and CAD) provide much of the signal in the deeper nodes of the tree, while TPI and PGD provide either conflicting or limited support for terminal nodes (Fig. 5). Although not without problems (alignment issues, etc.), nuclear ribosomal genes remain a readily accessible and informative source of molecular evidence on deep relationships within insect orders (Danforth *et al*., 2005). Moreover, as genomic data become available across flies, it will be increasingly useful to combine multiple genes. Our results confirm the findings of many recent studies showing that protein-coding genes are highly unpredictable in evolutionary rate and levels of variability when applied to phylogenetic questions, but can add significantly to levels of resolution and support when combined with other genes or morphology (Danforth *et al*., 2005).

The earliest lineage of Diptera

Modern systematic analyses have shed light on the earliest lineages of many of the major holometabolous insect orders (reviewed in Beutel & Pohl, 2006). Still, defining the earliest lineages within the order Diptera has been notoriously difficult. Common candidates for the most plesiomorphic dipteran lineage include the Tipulomorpha (or at least the Tipulidae sensu lato) (Hennig, 1973; Wood & Borkent, 1989; Courtney, 1990, 1991; Sinclair, 1992; Grimaldi & Engel, 2005; Blagoderov *et al*., 2007), Nymphomyiidae (Rohdendorf, 1974; Hackman & Väisänen, 1982; Griffiths, 1990; Colless & McAlpine, 1991) or Diarchineura (extant families Tanyderidae and Psychodidae) (Krzeminski, 1992b; Krzeminski & Krzeminska, 2003), although some phylogenies show no clear progression from plesiomorphic to apomorphic clades (e.g. Oosterbroek & Courtney, 1995; Michelsen, 1996).

One striking result of our analyses is the placement of the family Deuterophlebiidae as sister group to all remaining Diptera. This small family (14 species; Courtney, 1990, 1994b), known only from the western Nearctic and eastern Palearctic, is among the most autapomorphic groups of Diptera in both adult and larval morphology. Larvae of these flies are restricted to cool, clean, swiftly flowing streams, where they attach to rocks using prolegs tipped with crochets. Adults are short-lived (lacking mouthparts and a complete digestive tract) and have several specialized features including a reduced wing venation, divided femora, extremely elongate fourth antennal flagellomere (male) and deciduous wings (female) (Courtney, 1990, 1991). The Deuterophlebiidae have long been associated with the Blephariceridae, sharing derived characters that are difficult to ignore (Wood & Borkent, 1989; Courtney, 1990, 1991; Oosterbroek & Courtney, 1995). Although these results are possibly a symptom of our gene selection (perhaps resulting in long-branch attraction) and/or taxon sampling, our current molecular data suggest that convergence has

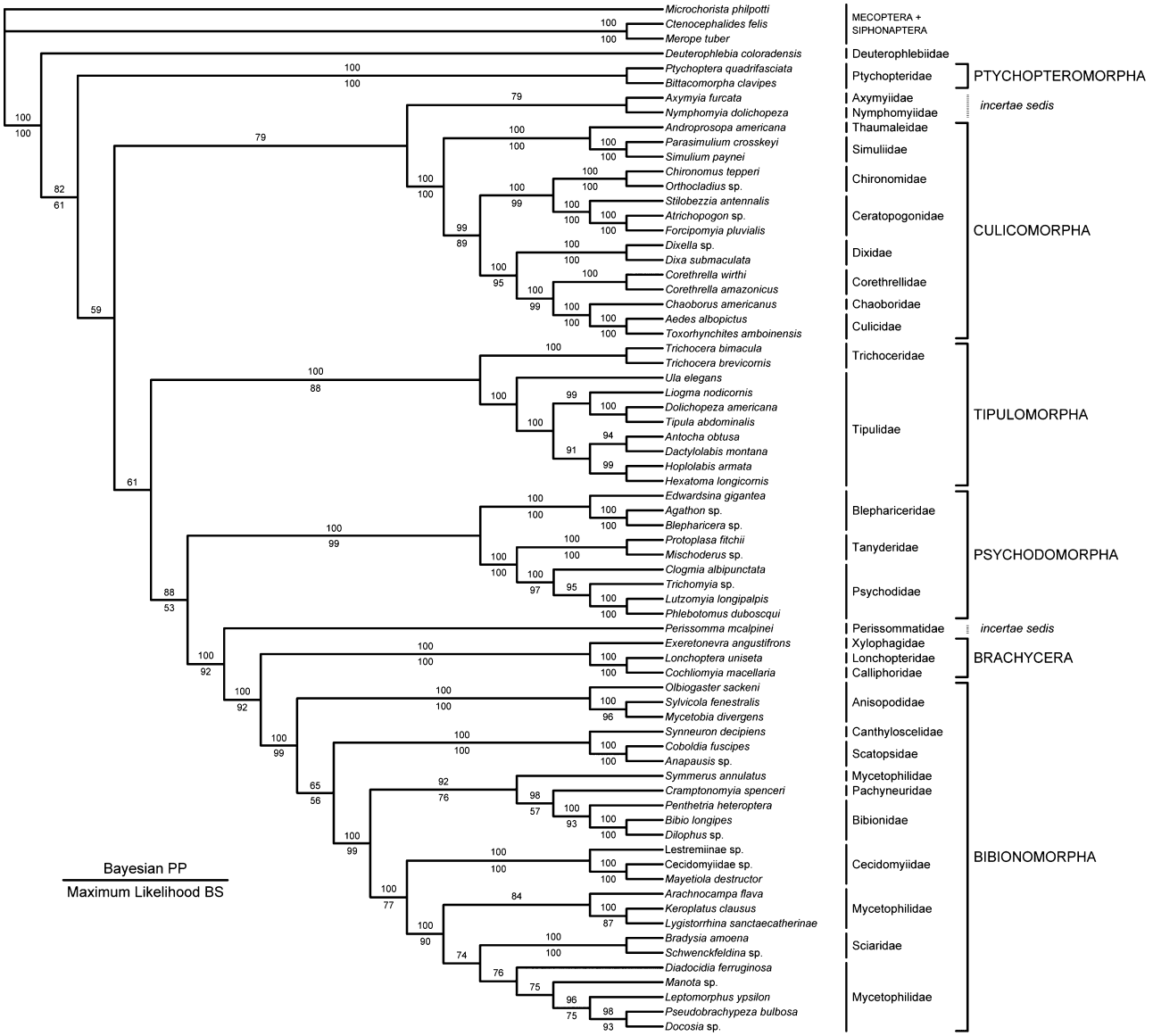

Fig. 3. Majority rule consensus of Bayesian Markov chain Monte Carlo (MCMC) (GTR + I + Γ model; four chains; 20 million generations). Support values above branches are posterior probabilities (PP) and below branches are maximum likelihood bootstrap (MLB) percentages.

occurred between the two families. Indeed, the extreme pressures exerted on these flies by their habitat (i.e. torrenticolous aquatic systems) may have lead to homoplasy, as many groups of insects have evolved specialized structures to cope with this kind of environment (Hora, 1930).

The evolutionary implications of an isolated, 'basal' origin for Deuterophlebiidae are unclear. The extreme morphology of these flies, especially the reduction or modification of structures, makes identifying ground plan characters for the Diptera difficult. Our data suggest that Deuterophlebiidae are extremely specialized, extant members of a relict lineage that diverged early in the history of Diptera. This result is based largely on a signal from 28S rDNA and, although the protein-coding genes were not decisive about the position of this family, identifying genes with a similar evolutionary history to 28S (e.g. with similar substitution rates) may strengthen our results. This novel hypothesis for Deuterophlebiidae will be tested with additional data currently being generated in the FLYTREE AToL project.

Ptychopteromorpha

Both Hennig (1973, 1981) and Wood & Borkent (1989) considered the Tanyderidae and Ptychopteridae to be sister taxa, the former placing these families in the superfamily Ptychopteroidea of the Psychodomorpha while the latter

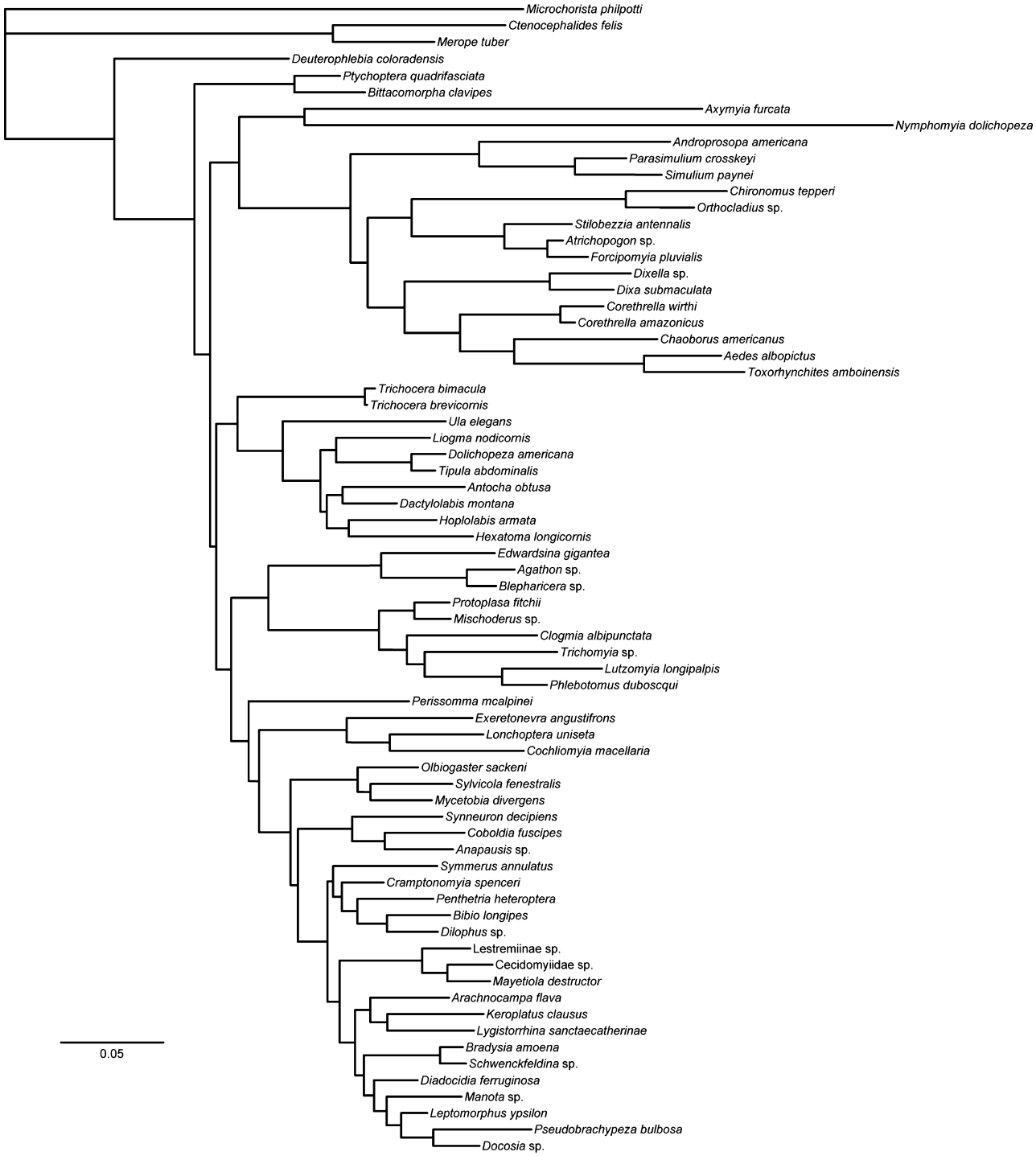

Fig. 4. Majority rule consensus tree for Bayesian Markov chain Monte Carlo (MCMC) runs showing branch lengths.

created the infra-order Ptychopteromorpha for them. Both hypotheses were based on a single character state (the folding condition of the last male tarsomere) with limited distribution in the Ptychopteridae. Oosterbroek & Courtney (1995) supported this infra-order, although all three additional larval synapomorphies they identified for the group (anal papillae non-retractable, five Malpighian tubules and Malpighian tubules ending in anal papillae) exhibit homoplasy. Furthermore, Wood & Borkent (1989) identified characters found in the Ptychopteridae and Culicomorpha

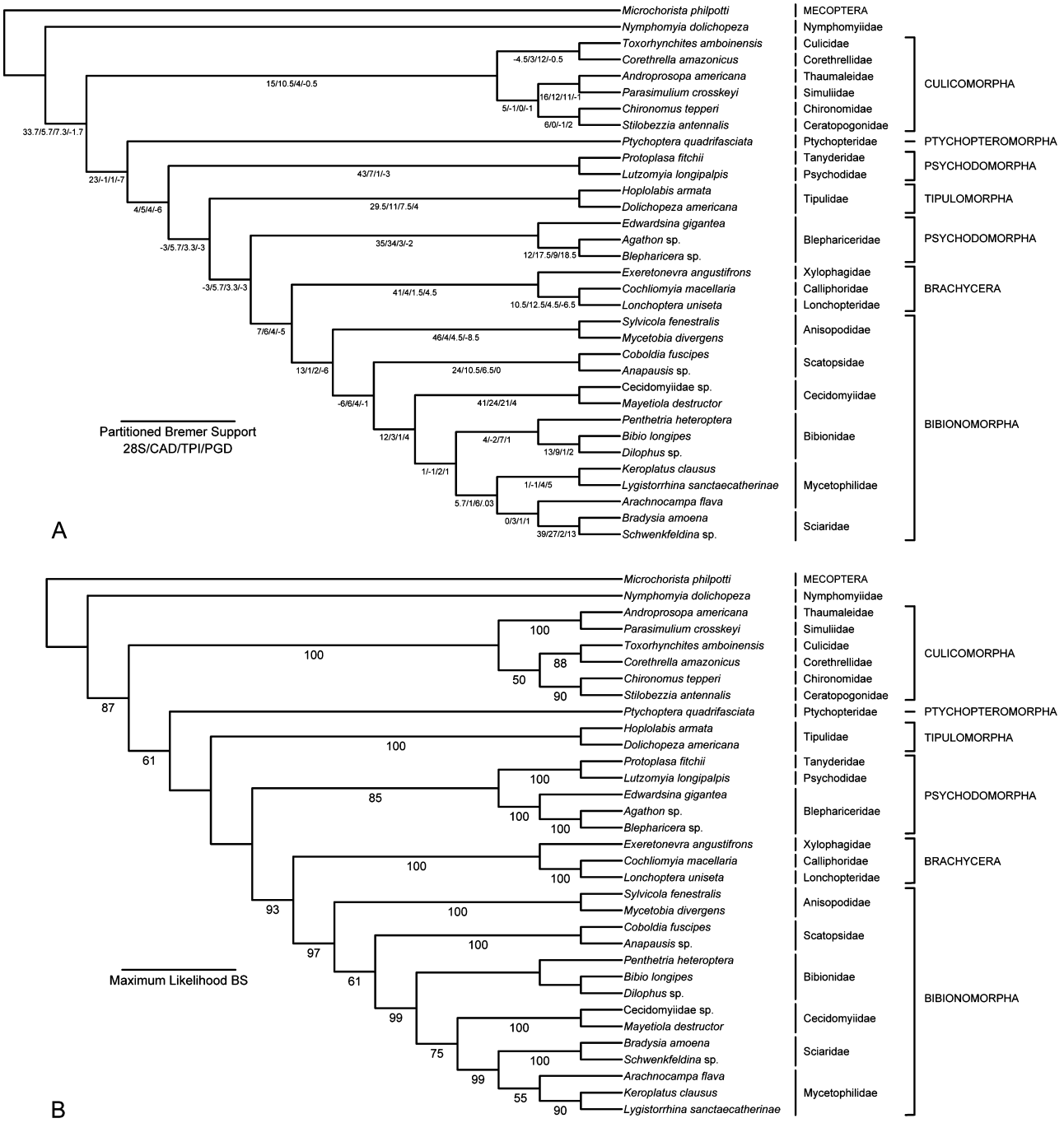

Fig. 5. Parsimony (A; length = 7049; CI = 0.420; RI = 0.444; RC = 0.186) and maximum likelihood (B; GTR + I + Γ) analyses of reduced data set (taxa with all genes); partitioned bremer support (PBS) (A; 28S/CAD/TPI/PGD) and maximum likelihood bootstrap values (B; ≥50%) shown below branches.

(invagination of premandible and presence of a dorsal mandibular comb), but not in the Tanyderidae, suggesting that the Ptychopteridae alone could be sister to the Culicomorpha. Wing vein characters from both fossil and extant taxa also conflict with the Ptychopteromorpha concept of Wood & Borkent (1989), instead supporting the Tanyderidae + Psychodidae and the Ptychopteridae + Culicomorpha (Shcherbakov *et al*., 1995; Krzeminski & Krzeminska, 2003).

Molecular evidence for a relationship between the Tanyderidae and Ptychopteridae is likewise lacking, and so Ptychopteridae appear to be the sole family in the

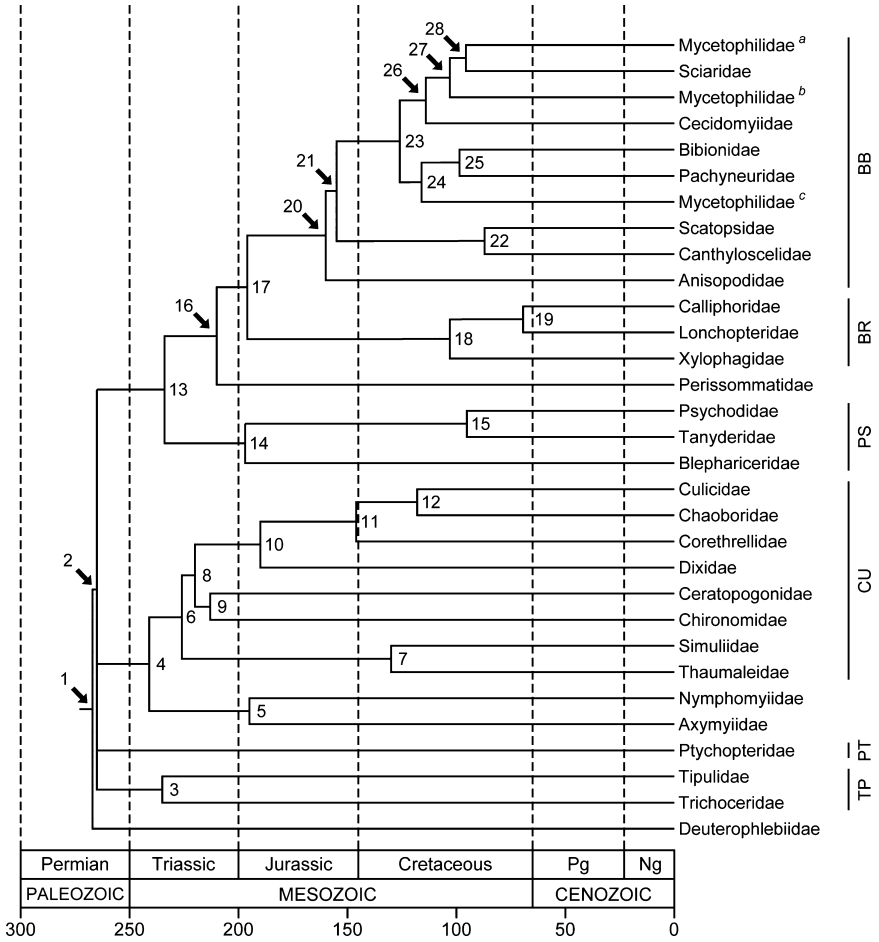

Fig. 6. Chronogram of the lower Diptera. Node ages and credibility/confidence intervals are presented in Table 1. Fossil calibration points (node; constraint age): *Grauvogelia arzvilleriana* (1; 240–270 Ma), *Architipula youngi* (3; 220 Ma), *Aenne triassica* (9; 210 Ma) *Nannotanyderus krzeminskii* (14; 180 Ma). BB = Bibionomorpha; BR = Brachycera; CU = Culicomorpha; PS = Psychodomorpha; PT = Ptychopteromorpha; TP = Tipulomorpha. [a]includes Diadocidiidae and Mycetophilidae *sensu stricto*; [b]includes Keroplatidae and Lygistorrhinidae; [c]includes Ditomyiidae.

Ptychopteromorpha. The position of this infra-order within the lower Diptera remains uncertain; the Ptychopteromorpha appear topologically as one of the earliest-branching lineages of the order, although statistical support based on parsimony and model-based analyses is low (<50% BS/59% PP/<50% MLB; Figs 2, 3). Thus, the Ptychopteridae, conservatively, may be considered an early-diverging independent lineage of flies (Figs 4–6). In all of our analyses, the Tanyderidae are strongly supported (100% BS/PP/MLB) as sister to the Psychodidae, with both placed in the Psychodomorpha sensu in this study (see below).

Tipulomorpha

The composition and placement of the infra-order Tipulomorpha has been contentious. Support for a sister-group relationship between Tipulidae sensu lato and Trichoceridae is largely dependent on the weight given to evidence from either larval or adult characters. Adult characters uniting these lineages include vein A_2 elongate and reaching the wing margin, vein R_2 (sometimes referred to as the r-r crossvein) ending in R_1, reduction of male cerci, female cerci with a single article and development of male terminalia from both imaginal discs and pupal ectoderm (Hennig, 1973, 1981; Dahl, 1980; Oosterbroek & Courtney, 1995). Nonetheless, larval Trichoceridae have some characters not found in the Tipulidae (see section on Psychodomorpha). These characters are variously present in all or part of Wood & Borkent's (1989) Psychodomorpha, or present in other nematocerous groups. One of these, a divided mandible, occurs in the hexatomine tipulids *Pilaria* and *Ulomorpha*, but is assumed to have an independent origin given the derived position of these closely-related genera within the Tipulidae (Oosterbroek & Theowald, 1991; Sinclair, 1992).

Molecular data provide support for a traditional Tipulomorpha containing the Tipulidae and Trichoceridae, an arrangement that is congruent with adult morphological synapomorphies more so than those of the larvae. The position of the Tipulomorpha within the Diptera remains equivocal, differing topologically under different analyses (compare Fig. 2 with Fig. 3). Thus, the Tipulomorpha do not represent the earliest branching infra-order (as in Hennig, 1973, 1981; Wood & Borkent, 1989; Sinclair, 1992), nor are they resolved as highly derived (as in Oosterbroek & Courtney, 1995).

Culicomorpha

The Culicomorpha contains most of the important haematophagous families in the lower Diptera, including serious vectors of human and animal diseases; as a result the families and relationships within this infra-order have been the most thoroughly studied. Both Hennig (1973) and Wood & Borkent (1989) found adult and larval characters to unite the members of this group, results that were confirmed analytically by Oosterbroek & Courtney (1995). Furthermore, all three studies divided the infra-order into the superfamilies Culicoidea (Dixidae, Corethrellidae, Chaoboridae and Culicidae) and Chironomoidea (Thaumaleidae, Simuliidae, Chironomidae and Ceratopogonidae). Although these groupings have remained relatively stable, other analyses of culicomorphan relationships lead to different hypotheses. Pawlowski *et al*. (1996) analysed data from the 28S ribosomal gene from 11 taxa representing all putative culicomorphan families. Their phylogeny differs from the two-superfamily concept pioneered by the previous authors, instead resolving the following relationships: {Chironomidae + [(Thaumaleidae + Simuliidae) + <Dixidae + {Ceratopogonidae + [Culicidae + (Corethrellidae + Chaoboridae)]}>]}. Miller *et al*. (1997) analysed sequence data from both the 18S and 5.8S ribosomal genes for exemplars of all families except Thaumaleidae. Their results are incongruent with previous studies, resulting in [(Simuliidae + Dixidae) <Ceratopogonidae + {Chironomidae + [Corethrellidae + (Chaoboridae + Culicidae)]}>]. In 2000, Sæther analysed 81 new and previously published morphological characters for the Culicomorpha, resulting in yet another phylogeny. Relationships among taxa were unstable under different weighting schemes, but the Thaumaleidae (or Thaumaleidae + Nymphomyiidae) were usually found to be sister to the remaining families, which were then either grouped into the Chironomoidea and Culicoidea of the previous authors, or arranged as {(Chironomidae + Simuliidae) + [Ceratopogonidae (Culicoidea)]}.

Our analyses resolved three main lineages within the Culicomorpha corresponding to the Culicoidea, a modified Chironomoidea and a proposed new superfamily, Simulioidea (Figs 2, 3). The Simulioidea is comprised of the families Thaumaleidae and Simuliidae and represents the sister-group of the remaining Culicomorpha. Although traditional hypotheses do not support a close relationship between these two families, molecular data provide consistent support for this relationship (Pawlowski *et al*., 1996; Moulton, 2000; Figs 2–5). As suggested by Pawlowski *et al*. (1996) and supported here, certain features of the Thaumaleidae and Simuliidae differ from other culicomorphans. For example, adults in these families are particularly robust in contrast to the delicate, midge-like forms of most Culicomorpha. Adult Thaumaleidae and Simuliidae also have short, stout antennae that are not particularly modified in the males (lacking an enlarged pedicel and plumose flagellum). Wood & Borkent (1989) interpreted these antennal characters to be lost in these two families, but according to our hypothesis their presence may be a synapomorphy for the Culicomorpha excluding the Simulioidea. Because the relationships based on molecular data among the remaining Culicomorpha were congruent with Wood & Borkent (1989) and Oosterbroek & Courtney's (1995) concepts, we accept character support identified for the Chironomidae + Ceratopogonidae and the Culicoidea.

Psychodomorpha

This infra-order traditionally has contained families that are difficult to place elsewhere. As the name suggests, the infra-order is defined according to the placement of the Psychodidae, a morphologically diverse family itself. Of all the infra-orders, Hennig (1973, 1981) was least sure of his concept of the Psychodomorpha, being reliant on a single character. Wood & Borkent (1989) were more confident about their Psychodomorpha, although it differed from most traditional hypotheses. They identified a suite of larval characters that supported the grouping of the Psychodidae with the Trichoceridae, Perissommatidae, Anisopodidae, Scatopsidae and Canthyloscelidae, including a conical labrum, the structure of the premandible, articulation of the torma with the dorsal labral sclerite, oblique to vertical orientation of the mandible, mandible divided and chelate, and a reduction of the cardo and maxillary palpus. Whether these structures are synapomorphic for the group has been questioned and, conversely, some of these characters have been viewed as plesiomorphic for the Diptera (Edwards, 1926; Anthon, 1943; Schremmer, 1951). States of these characters are distributed variously within the lower Diptera suggesting that they are either plesiomorphic (and lost multiple times) or are homoplasious (Griffiths, 1990; Courtney, 1990, 1991; Oosterbroek & Courtney, 1995).

Our molecular analyses present a fundamentally different view of the Psychodomorpha, supporting the relationship Blephariceridae + (Tanyderidae + Psychodidae) (Figs 2–5b). Although these families were placed in Hennig's Psychodomorpha, exclusion of other families that he included suggests that these two arrangements show that these two arrangements are incongruent. One possible character uniting these three families is the presence of mandibles in the adult. Within the lower Diptera, mandibulate adults only occur in the Culicomorpha, Blephariceridae, Psychodidae and Tanyderidae (Downes & Colless, 1967). As most adult

insects have mandibles, including Mecoptera (Kristensen, 1981), the outgroup of Diptera, this character may represent a symplesiomorphy. Thus, according to the phylogenies obtained from molecular data, mandibles were either retained in two lineages of lower flies (Psychodomorpha and Culicomorpha) or were retained once if we accept one parsimonious situation in which Psychodomorpha is sister to the Culicomorpha (Fig. 2). Support for a relationship between the Tanyderidae and Psychodidae is not completely unexpected – wing venation characters, including five radial veins reaching the wing margin, have been used to unite these taxa in the past (Diarchineura: Krzeminski, 1992b; Krzeminski & Krzeminska, 2003). In fact, fossil Tanyderidae and Psychodidae are very similar (Ansorge, 1994; Grimaldi & Engel, 2005), so much so that confusion of fossil taxa has occurred (Woodley, 2005). Support for a relationship between these two families is also found in results from analysis of 18S rDNA (Leathers & Judd, 2002).

Bibionomorpha

The infra-order Bibionomorpha has been difficult to define. Taxa traditionally placed here are generally similar in morphology, but cladistic support uniting these families is lacking. Hennig's (1973) classification placed several families together (Fig. 1A) whose adults shared resemblance and one questionable apomorphy, the reduction of the costal vein along the posterior wing margin. Amorim (1993) supported a similar clade containing the Axymyiomorpha (Axymyiidae, Perissommatidae and *Pachyneura*), Bibionomorpha (all other Pachyneuridae, Anisopodidae, Bibionidae and Mycetophiliformia = Sciaroidea) and the Brachycera. Although the families Scatopsidae and Canthyloscelidae were not included in his analysis, Amorim (1993) assumed they were part of the Mycetophiliformia, although later he considered them part of the Psychodomorpha (Amorim, 2000; Amorim & Rindal, 2007). Michelsen (1996) also supported Hennig's Bibionomorpha sensu lato (as the 'Neodiptera'), but included the Brachycera therein. Wood & Borkent, however, place the Anisopodidae, Perissommatidae, Scatopsidae and Canthyloscelidae in the Psychodomorpha and the Axymyiidae in its own infra-order. Although results from Oosterbroek & Courtney's (1995) study agreed with separating the former families from the Bibionomorpha, Axymyiidae was placed as sister to the remaining Bibionomorpha in their analysis.

Molecular data support Hennig's original hypothesis even with low taxonomic and genetic sampling (Friedrich & Tautz, 1997a; Figs 2–5). Exceptions are the positions found for the enigmatic families Perissommatidae and Axymyiidae, which, in the current analyses, remain ambiguous. Families of the Bibionomorpha are united by a loss of anal papillae (except for some Mycetophilidae sensu lato; Courtney, 1991), unique among lower Diptera and characteristic of the infra-order's terrestrial habits. The Anisopodidae and Scatopsoidea (Scatopsidae + Canthyloscelidae) appear to be sister to the rest of the infra-order, retaining larval characters that were interpreted as synapomorphic with other families (i.e. Psychodomorpha sensu Wood & Borkent, 1989). Although not sampled for genetic data, the famly Valeseguyidae presumably would appear as sister to the Canthyloscelidae + Scatopsidae (Amorim & Grimaldi, 2006). The Bibionomorpha sensu stricto (i.e. of Wood & Borkent) is well supported (97/100/99% MPB/PP/MLB) by molecular evidence, although relationships within this group are not in line with traditional hypotheses. Mycetophilidae is supported as polyphyletic here and agrees with previous hypotheses rejecting the monophyly of this family (Hennig, 1973; Vockeroth, 1981). *Symmerus*, although treated historically as a member of the Mycetophilidae (subfamily Ditomyiinae), is resolved in both parsimony and model-based analyses as sister to the Pachyneuridae + Bibionidae. Evidence for this relationship from larval morphology is substantial, including the shared presence of spiracles on abdominal segment VIII (found in Ditomyiinae, Pachyneuridae and Bibionidae; other Sciaroidea lack this spiracle) and several mouthpart characters (Madwar, 1937; Vockeroth, 1981). The other major lineage of Mycetophilidae sensu lato is paraphyletic with respect to the Sciaridae in both analyses, and should either be divided into separate families or the Sciaridae should be treated as a subfamily of the Mycetophilidae, excluding Ditomyiinae. Indeed, paraphyly of Mycetophilidae sensu lato, as a result of the inclusion of Sciaridae, has been found in recent morphological analyses of sciaroid relationships (Matile, 1990; Chandler, 2002; Hippa & Vilkamaa, 2005). Conversely, Amorim & Rindal (2007) found the Sciaridae to be the sister to all remaining Sciaroidea (=Mycetophiliformia) except Cecidomyiidae, and showed monophyly of both the new family Rangomaramidae and Mycetophilidae sensu lato. Our taxon sampling is far from complete for this particularly speciose group, and further molecular studies on the relationships within the Sciaroidea surely will advance the knowledge of this clade.

Position of the Brachycera

The phylogenetic position of the higher flies (Brachycera) has long been debated among systematists. Hennig (1973) preferred a relationship between the Bibionomorpha sensu lato and Brachycera based on two synapomorphies: enlargement of the second laterotergite (katatergite) and undivided thoracic postphragma. Sinclair (1992) placed the Brachycera as sister to the Psychodomorpha of Wood & Borkent (1989) based on characters of larval mouthparts, including the structure of the mandible. Woodley (1989), and subsequently Oosterbroek & Courtney (1995), supported a single psychodomorph family, Anisopodidae, as the sister taxon to the Brachycera, based on wing vein characters among others.

Our results are congruent with Hennig's (1973) hypothesis, supporting a sister-group relationship between the Brachycera and Bibionomorpha sensu lato (63/100/92% BS/PP/MLB; Figs 2–5). Rather than sister to the Brachycera alone, the Anisopodidae is reconstructed as sister to the remaining Bibionomorpha, thus possibly still close

morphologically to the stem group of the Brachycera + Bibionomorpha. This is the best-supported phylogenetic hypothesis for the placement of Brachycera to date, confirming the paraphyly of the lower Diptera or 'Nematocera', and suggesting that the subsequent diversification of Brachycera (to nearly 100 000 described species in over 120 families) began as a lineage with terrestrial larvae, closely related to the clade containing other terrestrial bibionomorphs.

Nymphomyiidae & Axymyiidae

Two families of lower Diptera have been particularly difficult to place in a phylogenetic context: Nymphomyiidae and Axymyiidae. Both families are small, restricted in distribution and exhibit morphological aberrancies (adult and/or larval). The family Nymphomyiidae comprises seven species of minute (<2 mm) flies in the genus *Nymphomyia* distributed in the eastern Nearctic and eastern/southeastern Palearctic (Courtney, 1994), and restricted mostly to moss-covered rocks in swiftly flowing, clean streams. The family has been placed alternately as sister to all Diptera (Rohdendorf, 1974; Hackman & Väisänen, 1982; Griffiths, 1990; Colless & McAlpine, 1991), in the Psychodomorpha (sensu Hennig, 1973), as sister to the Culicomorpha (Courtney, 1994; Sæther, 2000), or, more commonly, in the Belphariceromorpha (Wood & Borkent, 1989; Courtney, 1990, 1991; Oosterbroek & Courtney, 1995). Confusion about the placement of this family may be a result of neoteny obscuring characters of adults and larvae. Characters including non-fusion of abdominal ganglia in adult males and the prognathous pupa are unique among Diptera and have added to the idea of this family's isolation from other Diptera (Hackman & Väisänen, 1982; Wood & Borkent, 1989). Axymyiidae contains at present six described species found in the Nearctic (eastern and an undescribed western species) and Palearctic (eastern Europe and east Asia including Siberia and Japan) regions (Evenhuis *et al*., 2007). Larvae of Axymyiidae dwell in wood that is partially submerged in water and not yet fully decomposed. The diet of these flies is unknown and the mouthparts of the larvae appear unable to feed directly on the wood (Wood, 1981). Adult Axymyiidae appear similar to certain Bibionomorpha in wing venation and body shape, and the ecology of these flies is poorly understood. Often this family has been placed with the Bibionomorpha (Hennig, 1973; Oosterbroek & Courtney, 1995), but Wood & Borkent suggested it merits its own infra-order.

Results from the molecular data consistently place Nymphomyiidae and Axymyiidae together as sister to the Culicomorpha (Figs 2, 3). These results should be considered tentative because both taxa are extremely divergent in nucleotide sequence (long branched), a characteristic shared also with the Culicomorpha as a whole (Fig. 4). Affinities between long-branched taxa (long-branch attraction) have been identified previously as leading to erroneous relationships, particularly in parsimony analyses (Felsenstein, 1978). Evidence of this in the current study emerges from results if Axymyiidae are excluded (Fig. 5): resulting trees remain similar except for the Nymphomyiidae, which becomes the sister taxon to the remaining Diptera, possibly being attracted to the divergent outgroup taxa. When Nymphomyiidae are excluded, Axymyiidae remain in their original position (not shown), suggesting that the genetic sequence of *Axymyia* is particularly divergent. Thus, the current molecular data appear to be inadequate at resolving the relationships of these traditionally difficult families. Including additional genes and/or increasing the taxon sampling of these two families may alleviate possible long-branch symptoms and aid in identifying a plausible phylogenetic position for these enigmatic groups.

The history of Dipteran diversification

Diptera are among the oldest of the major holometabolous insect orders (Grimaldi & Engel, 2005). Mecopteroid precursors of the Diptera (families Permotipulidae and Robinjohniidae) appeared during the late Permian (260–250 Ma), and are represented by isolated wings or whole specimens with four wings (although reduction of the hind wings had occurred in some Permotipulidae) (Grimaldi & Engel, 2005; Blagoderov *et al*., 2007). The earliest true, two-winged flies appear in early Triassic deposits, and *Grauvogelia arzvilleriana* Krzesminski *et al*. appears to be the oldest (Shcherbakov *et al*., 1995; Krzeminski & Krzeminska, 2003; Blagoderov *et al*., 2007). Although Diptera are uncommon from early Triassic deposits, an increase in the diversity of dipteran fossils has been observed from the middle to late Triassic (230–210 Ma) (Blagoderov *et al*., 2007).

Our divergence time estimates are based on a single gene (28S) and a few fossil-based lineage age constraints. Data from 28S are the most complete in our study, and by limiting the influence of fossils on our estimates we can test the ages proposed in the fly paleontological literature more powerfully. Future studies that include more genes and additional fossils should improve estimates of the temporal diversification of the lower Diptera. However, current estimates, based on the data at hand, are congruent with many fossil ages and appear robust enough to make inferences about early fly history. Thus, divergence time estimates based on 28S place the earliest diversification of Diptera during the late Permian (267 Ma; Fig. 6). Early groups of flies appear to have radiated quickly during the middle Triassic, concordant with the abundance of fossil specimens discovered from that time. All major fly lineages are represented in the chronogram during this period (Fig. 6), including Deuterophlebiidae, Tipulomorpha, Ptychopteridae, Culicomorpha + Nymphomyiidae + Axymyiidae, Psychodomorpha and early 'Neodiptera' (excluding Axymyiidae). In fact, difficulties in attaining a stable phylogeny for early Diptera have been attributed to the explosive, rapid radiation of the order in the Triassic (Grimaldi & Engel, 2005; Blagoderov *et al*., 2007). Diversification of these groups continued through the Jurassic and by the end of the Cretaceous all modern families of lower Diptera existed.

Wing venation plays a vital role in phylogenetic studies of early Diptera because wings are frequently the only structures preserved as fossils. Two groups of extant flies are often said to be representatives of the earliest Diptera based on the retention of certain 'plesiomorphic' wing characters. These include the Tipulomorpha (Trichoceridae and Tipulidae) and the Diarchineura (Tanyderidae and Psychodidae) (Shcherbakov *et al.*, 1995; Krzeminski & Krzeminska, 2003; Blagoderov *et al.*, 2007). The Tipulomorpha have two complete anal veins, whereas most Diarchineura have five radial veins reaching the wing margin. Although both characters are unique to their respective groups, the question is which of these represents the more plesiomorphic condition? Although the oldest apparent dipteran, *Grauvogelia*, has characters congruent with the Diarchineura (Krzeminski & Krzeminska, 2003), other equally old taxa (i.e. *Archilimonia*) share characters with the Tipulomorpha (Shcherbakov *et al.*, 1995; Blagoderov *et al.*, 2007). Whether reduction of wing venation is derived phylogenetically within the lower Diptera has not been tested previously, and results from our analysis suggest that this trend is not the case. Indeed, our molecular analyses and dating (Fig. 6) show that even groups with fewer wing veins (i.e. Culicomorpha and Ptychopteridae) or even extremely reduced wing venations (i.e. Deuterophlebiidae) were present early in dipteran evolution, contemporary with the Tipulomorpha and Diarchineura. This perhaps reflects the early and rapid evolution of major dipteran clades, groups that may have evolved along an array of wing venation trajectories.

Early Diptera larvae were most likely associated with water. Immature Deuterophlebiidae, Nymphomyiidae, Culicomorpha, many Tipulidae and most Psychodomorpha are fully aquatic, whereas the Ptychopteridae and Axymyiidae are semi-aquatic. Larvae developing in these habitats feed largely on particulate matter (detritus), or graze/filter diatoms, algae or other aquatic plants (Labandeira, 2005). Although considered terrestrial, Perissommatidae appear to be partially adapted to liquid habitats (autodigested fungus and decaying matter in areas of heavy rainfall), even retaining anal papillae (Colless, 1962, 1969; Courtney, 1991). Major lineages of terrestrial Diptera, including the Bibionomorpha and Brachycera, did not originate until the late Triassic and early Jurassic (Fig. 6). Extant members of Anisopodidae, Scatopsidae and Canthyloscelidae, all early lineages of the Bibionomorpha, retain a saprophagous lifestyle that may have evolved from feeding in a liquid environment. More derived families of Bibionomorpha, which probably diversified during the early Cretaceous, feed on rotting wood or fungus as larvae, although one major group, Cecidomyiidae, has developed phytophagous/galling habits. The development of terrestrial larvae feeding on novel food sources may have been spurred by the rise of angiosperms in the Cretaceous.

Adult flies generally are restricted to a few trophic types, including nectivory, pollinivory, predation and haematophagy, although many are non-feeding or feed facultatively on nectar, plant sap or homopteran honey dew (Downes & Dahlem, 1987). Our phylogenetic evidence supports an ancestrally non-feeding adult, as is exhibited in Deuterophlebiidae, Ptychopteridae and most Tipulomorpha. It is also possible that haematophagy, as is the case for most Culicomorpha, is the ancestral adult feeding habit of flies. Haematophagy is present in at least a few members of all 'major' clades of flies (lower Diptera, lower Brachycera, Cyclorrhapha and Calyptratae), but it is most common in several lower dipteran clades (Culicomorpha and Psychodidae) and so it probably evolved sometime in the Triassic. Most dipteran radiations occurred before the rise of flowering plants in the Mid-Cretaceous (Labandeira, 2005), thus the array of extant flies that are specialized for taking nectar and pollen either were pre-adapted to flower feeding or these habits represent an early innovation that has proven to be exceptionally successful in allowing flies to utilize flowering plant resources throughout their history.

Supporting Information

Additional Supporting Information may be found in the Online version of this article from Wiley Interscience under DOI reference: doi: 10.1111/j.1365-3113.2008.00437.x

ST1 Taxa sampled, locations and GenBank accession numbers.

ST2 Oligonucleotide primer sequences used to amplify CAD, PGD, TPI and 28S.

ST3 Character attributes of each gene and partition.

ST4 Results from parsimony analyses for each gene partition and partitioned support for major clades. X = bootstrap support $\geq$50%; V = topological support only (<50% bootstrap).

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