A dated molecular phylogeny for the Chironomidae (Diptera)

PETER S. CRANSTON¹,², NATE B. HARDY¹,³ and GEOFFREY E. MORSE¹,⁴

¹Department of Entomology, University of California, Davis, CA, U.S.A., ²Evolution, Ecology and Genetics, Australian National University, Canberra, Australia, ³Queensland Primary Industries and Fisheries, Indooroopilly, Australia and ⁴University of San Diego, CA, U.S.A.

Abstract. We provide the first highly sampled phylogeny estimate for the dipteran family Chironomidae using molecular data from fragments of two ribosomal genes (18S and 28S), one nuclear protein-coding gene (CAD), and one mitochondrial protein-coding gene (COI), analysed using mixed-model Bayesian and maximum likelihood inference methods. The most recently described subfamilies Chilenomyiinae and Usambaromyiinae proved elusive, and are unsampled. We confirm monophyly of all sampled subfamilies except Prodiamesinae, which contains Propsilocerus Kieffer, previously in Orthocladiinae. The semifamily Chironomoinae is confirmed only if Telmatogenetinae is included, which is closer to Brundin’s original suggestion. Buchonomyiinae is excluded from Chironomoinae: it is a sister group to all remaining Chironomidae, conforming more to Murray and Ashe’s argumentation. Semifamily Tanypodoinae is a grade and unsupported as monophyletic: the austral Aphroteniinae alone is sister to all Chironomidae (less Buchonomyiinae). Podonominae is weakly supported as the next sister group, in contrast to some estimates that place this subfamily as sister group to Tanypodinae alone. In Diamesinae, the southern African Harrisonini is confirmed as a member, but embedded within austral tribe Heptagini, which is confirmed as sister to the undersampled Diamesini. Tribe Pentaneurini and ‘non-Pentaneurini’ taxa are reciprocally monophyletic in Tanypodinae. Recent molecular findings concerning Podonominae are substantiated, with a monophyletic tribe Podonomini, Boreoclini forming a grade and Lasiodiamesa Kieffer placed as sister to all other Podonominae, but with uncertainty. In Orthocladiinae, a postulated two-tribe system of Orthocladiini and Metriocnemini can be supported after exclusion of a Corynoneura group and a Brillia group, which is revealed as sister to Stictocladius Edwards. The marine Clunio Haliday and Thallassosmittia Strenzke & Remmert (given high rank in the past) are clearly embedded deep in Orthocladiinae. The finding of Shangomyia Sæther & Wang + Xyiaomyia Sæther & Wang as sister group to all other Chironominae justifies high rank, as their authors suggested. Pseudochironomini (untested by sampling shortfall) is sister to a monophyletic Tanytarsini (with a weakly supported inclusion of the enigmatic Nandeva Wiedenbrug, Reiss & Fittkau). The tribe Chironomini can be supported only by excluding Shangomyia + Xyiaomyia, and a postulated monophyletic clade comprising several taxa such as Microtendipes Kieffer, with six-segmented larval antennae and alternate Lauterborn organs, that is sister group to Pseudochironomini + Tanytarsini. The tempo of diversification of the family, deduced by divergence time analysis (BEAST), shows Permian origination with

Correspondence: Professor Peter S. Cranston, Evolution, Ecology and Genetics, Research School of Biology, Australian National University, Canberra 0200, Australia. E-mail: pscranston@gmail.com

© 2011 The Authors
Systematic Entomology © 2011 The Royal Entomological Society
subfamily stem-group origination from the mid–late Triassic to the early Cretaceous. Crown-group origination ranged from Podonominae on a short stem originating in the mid Jurassic to long-stemmed Aphroteniinae from the late Cretaceous. Node dates allow inference of some vicariance via Gondwanan fragmentation, including certain nodes involving southern Africa.

Introduction

The modern limits of the family Chironomidae have been recognized since Malloch (1917) and Edwards (1926, 1929) distinguished the ‘non-biting midges’ from the biting Ceratopogonidae. Listing the differences, Edwards (1926: 390) contrasted ceratopogonid mouthparts, complete with toothed mandibles and maxillary blades (= laciniae) with the reduced (‘non-biting’) chironomid mouthparts, stated to lack these structures. Although mandibulate chironomids exist (Cranston et al., 1987), the evolutionary significance of these mouthparts is controversial (Azar et al., 2008). However, in considering the relationships of female mandibulate chironomid Archaeochlus Brundin (Podonominae), several non-mouthpart morphological features proposed by Edwards (1926) were verified and extended by Cranston et al. (1987) to distinguish all chironomid taxa from all Ceratopogonidae. However, the long-assumed sister-group relationship of Chironomidae with the biting midges has been challenged by some morphological studies (including Hennig, 1973; Sæther, 1976, 2000a) and some molecular phylogenetic studies (e.g. Pawlowski et al., 1996; Miller et al., 1997), whereas other analyses of morphology (e.g. Wood & Borkent, 1989; Oosterbroek & Courtney, 1995) and molecules (Bertone et al., 2008; Cranston et al., 2010) infer they are sister groups.

Higher level internal relationships within Chironomidae

A first attempt to elucidate internal relationships within the Chironomidae (excluding the biting midges) was made by Goetghhebuer (1914), who included larval and pupal features in recognising two subfamilies: the Tanypodinae and Chironominae. The latter contained genera now placed in subfamilies Diamesinae, Prodiamesinae, Orthocladiinae and Chironominae. In modern terminology, recognition of a group centred on Chironomus (today’s subfamily Chironominae) arising from within an Orthocladius group (largely the currently understood subfamily Orthocladiinae) rendered it paraphyletic. The first formal fragmentation of this broadly circumscribed Orthocladiinae is attributed to Edwards (1929), who proposed subfamily status for the Diamesinae and the Clunioninae, a grouping of marine and intertidal midges. The next segregate, the subfamily Podonominae, was erected for a few northern hemisphere species with especially distinctive immature stages (Thienemann & Edwards, in Thienemann, 1937).

In distinguishing a major role for evolutionary novelty, the remarkably prescient Edwards (1926) undoubtedly was thinking in a ‘cladistic sense’. Ensuing changes in the classification of the Chironomidae increasingly involved explicit phylogenetics, following Hennig (1950). Thus in an early application of these phylogenetic methods, Strenzke (1960) confirmed Wirth’s (1949) view that the marine ‘clunionine’ midges were convergent, with one group being derived from within the Orthocladiinae, and with the other, centred on Telmatogenot Schiner, belonging to an unrelated ‘plesiomorphic’ grouping. This latter clade was treated by Brundin (1966) as being of elevated rank – the subfamily Telmatogenotinae. In the same work, Brundin’s Hennigian hierarchical argumentation justified the erection of a subfamily Aphroteniinae for some rare austral midges distinctive in all known life-history stages. The next subfamily to be proposed as new has had a chequered history: Buchonomya Fittkau (1955) was described first as a podonomine, but was suggested to be a plesiomorphic orthoclad by Brundin (1966), and then was maintained there until Brundin & Sæther (1978) argued for subfamily status (as Buchonomyiinae) based on phylogenetic reasoning. A similar rationale was applied by Sæther (1976) in proposing the new subfamily Prodiamesinae for anomalous genera treated previously as belonging either to Diamesinae or Orthocladiinae.

In contrast to the previous erection of new subfamilies based on some knowledge of the immature stages, the two subfamilies described most recently, Chilenomyiinae (Brundin, 1983) and Usambaromyiinae (Andersen & Sæther, 1994), were based on phylogenetic argumentation, but from adults alone from Chile and Tanzania, respectively. Thus arrived the modern complement of 11 extant subfamilies, many with hypothesized internal phylogenetic structure. Correct nomenclature and authorships are reported by Spies (2005).

Turning to putative relationships amongst the subfamilies, as noted above phylogenetic reasoning has accompanied each case since Hennig’s methods were adopted early by students of chironomids. Thus Brundin (1966) regarded the Tanypodinae as the ‘apomorphic sister group’ of Podonominae plus Aphroteniinae, with these three subfamilies combined forming the sister group of Chironominae plus a polytomy comprising Diamesinae, Telmatogenotinae and Orthocladiinae (Fig. 1A).

The reallocation of Telmatogenotinae was based largely on female genital features (Sæther, 1977), particularly a controversial interpretation of a plesiomorphic presence of gonostyly IX. A restricted grouping of Diamesinae [Prodiamesinae (Orthocladiinae + Chironominae), named as semifamily Chironomoidinae (Sæther, 1983) (Fig. 1B)] has been essentially unchallenged since. Sæther’s proposals for the remaining Chironomidae subfamilies have been less stable: a sister group,
the Tanypodinae, comprising a core grouping of Tanypodinae plus Podonominae and Aphroteniinae has been unchallenged, but contention concerns Telmatogenetinae, and more especially the placement of Buchonomyiinae. In recognising the Buchonomyiinae as a subfamily, Brundin & Sæther (1978) argued for it being sister group to, and included within, the Chironomoinae (Fig. 1C), with support adduced then and subsequently from the female genital morphology. Disputing this morphological interpretation, Murray & Ashe (1981, 1985) argued that character states had been misinterpreted, and challenged the contentious use of ‘underlying synapomorphies’ (non-universal character states treated a priori in analysis as if they were synapomorphies): they argued that Buchonomyiinae belonged in the Tanypodinae (Fig. 1D). The evidential basis for a placement of Telmatogenetinae as sister to the remaining Chironomidae (Sæther, 1977) has also been challenged, including by Ashe et al. (1987), but with restatement by Sæther (2000b) (Fig. 1E). The application of molecular data to these questions has been modest: Cranston et al. (2010) sampled the ‘Gondwanan taxa’ of Brundin (1966) in detail, and used exemplars of other subfamilies and two out-group families to provide a detailed phylogeny of the Podonominae. From this analysis, the subfamily relationships shown in Fig. 1F were derived.

Relationships within subfamilies of Chironomidae

Phylogenetically based internal relationships have been proposed for the Podonominae, Tanypodinae, Diamesinae, Orthocladiinae and Chironominae. Brundin’s (1966) proposal for two sister taxa of tribal rank, Podonomini and Boreochlini, was tested by Cranston et al. (2010), who found monophyly of the former and paraphyly of the latter. Postulated internal relationships in the Tanypodinae derive from Fittkau (1962), who erected tribes Anatopynini, Macropelopiini and Pentaneurini. In an unusual phenetic approach to chironomid classification, Roback & Moss (1978) added tribes Procladiini and Nataziini. Internal relationships in the Diamesinae derive from Brundin (1966), who proposed a system comprising Heptagyniae (austral tribes Parahyptagynia and Lobodiamesini), Diamesae (boreal tribes Diamesini and Protanytyni) and a tribe Harrisoni for the enigmatic monotypic South African Harrisonina Freeman.

The two largest (most speciose) subfamilies, the Chironominae and Orthocladiinae, have less well-understood internal structure, particularly the Orthocladiinae. For the Chironominae three tribes have been proposed: Chironomini, Tanytarsini and Pseudochironomini (Sæther, 1977). Although each can be defined in any life-history stage, the monophyly of the Pseudochironomini is not firmly established: it may be a grade or a clade. Morphology provides inadequate support for either hypothesis. Two adult-based Asian genera, Shangomyia Sæther & Wang and Xiaomyia Sæther & Wang, were suggested as deserving tribal rank (Sæther & Wang, 1993), although such status was not proposed formally. For the subfamily Orthocladiinae, monophyly cannot be established by any synapomorphies in any stage (Cranston, 1994). A putative tribal scheme of Orthocladiini and Metriocnemini of Brundin (1956), based on the genera Orthocladius Wulp and Metriocnemus Wulp, respectively, has not been recovered by numerical cladistic analyses (e.g. Rossaro, 1989; Sæther, 1989). Elevation of the Corynoneura group to tribal status as Corynoneurini (e.g. Oliver, 1971) based on morphology, but without phylogenetic analysis, has found limited acceptance.

The suite of characters available for morphological analyses in the Chironomidae is immense. The immature stages have been studied in minute morphological detail by freshwater biologists both for purposes of identification (e.g. larvae, Wiederholm, 1983; pupae, 1986) and for phylogenetic analyses. The traditionally studied adults also have the potential to provide hundreds of characters towards data matrices (e.g. Sæther, 1989). However, the derivation of robust estimates of phylogeny based on morphological studies is constrained by extensive homoplasy and difficulty in recognising characters that may provide unambiguous evidence of relationships. These recognized problems have led to weighting schemes of uncertain biological meaning and the use of ‘underlying synapomorphies’ alluded to above (Cranston & Humphries, 1988). In keeping with the pessimism of Scotland et al. (2003),

© 2011 The Authors
Systematic Entomology © 2011 The Royal Entomological Society, Systematic Entomology, 37, 172–188
we doubt the existence of many further morphological features that can test contentious relationships, despite the best possible morphological studies involving all life-history stages. Previous authors have shown the value of molecular data for revealing relationships in parts of the family (Cranston et al., 2002; Martin et al., 2002, 2007; Ekrem & Willassen, 2004; Ekrem et al., 2010). Here we derive a phylogeny reconstruction across a broadly sampled Chironomidae, using genetic data derived from both the mitochondrial and the nuclear genomes. We analyse genetic data alone, seeking to test with independent data sources the existing hypotheses derived from morphological data.

**Material and methods**

**Taxon sampling**

We sampled to seek representation in at least one life-history stage of each currently recognized subfamily and each postulated tribe therein. We sought multiple representations for speciose groups and at least one specimen from species-poor, but phylogenetically or biogeographically important, higher taxa. Many specimens derive from a collection made for a synchronous study of the subfamilies Podonominae, Aphroteniinae and austral Diamesinae, to test Brundin’s (1966) phylogenetic and biogeographic hypotheses (Cranston et al., 2010). Selected representative taxa are included in our current study.

Collection methods have favoured the freshwater aquatic taxa predominantly in the immature stages. Perhaps 95% of the described species are aquatic, with the remainder being poorly known soil dwellers in their immature stages. Marine and intertidal taxa were sought to test ideas concerning the polyphyly of the ‘Clunioninae’. We have sought important but geographically disparate taxa with some successes [Harrisonina Freeman in the Western Cape, South Africa; Buchonomynia in western Ireland; Xiaomyia and Shangomyia in south-west Asia (Thailand)]. Nevertheless, we acknowledge some failures, as we lack the significant monotypic subfamily Chilenomyiinae (despite much searching at and near the type localities), the East African monotypic Usambaromyiinae and the Holarctic diamesine tribe Boreoheptagini.

Field methods have emphasized the immature stages: by picking larvae directly from the substrate (hygropetric taxa), and by intercepting drifting larvae and pupae (and some drowned adults) from flowing water using 300-μm mesh drift nets. This technique, advocated by Thiennemann and used extensively by Brundin (Cranston, 2000), is especially valuable for lotic taxa. The ease of collection of a diversity of taxa as immature insects involves a trade-off against the difficulty in making identifications to species level, which remain highly reliant on adult male genitalia. We swept for adults in important sites. All life stages were sought to allow associations based on sequence identity. Repeated but geographically separated similar taxa were surveyed to assess cryptic diversity. The associated molecular life histories and/or revelation of some cryptic taxa using these data and techniques are discussed in Cranston (2009), Krosch et al. (2009), Cranston et al. (2010) and Krosch et al. (2011). We requested and received valuable assistance from colleagues, especially for missing northern hemisphere taxa (see Table S1). Eventually we culled the results to produce the final dataset (Table S1) by removing redundant and closely related cryptic taxa, so as to speed analyses.

All samples were field sorted, targeting the taxa of interest, under a binocular microscope, then preserved in 95% isopropanol and refrigerated in the dark as soon as was practicable until DNA extraction. From the outset in 1996, nearly all specimens sequenced were extracted from whole bodies (see below), with carcasses vouchedered as microscope slides using Euparal mountant or occasionally Hoyers (larvae). All critical specimens mounted initially in Hoyers have been remounted into Euparal. Vouchers, listed in Table S1, are labelled appropriately, and will be preserved in the slide collections of the Bohart Museum, University of California, Davis, CA, U.S.A.

Out-group selection was based on prevailing current ideas from morphological and molecular studies, using several species of Ceratopogonidae, a Thaumaleidae and a Simulidae, all members of the Culicomorpha (Bertone et al., 2008).

**DNA extraction, PCR amplification and sequencing**

Total genomic DNA was extracted from either whole specimens or from the abdomen using the Qiagen DNeasy Blood and Tissue kit and protocol provided by the manufacturer. Modifications to the protocol were as follows: (i) tissue was digested either for 3 h at 55°C or overnight at 37°C; (ii) after digestion with proteinase K, cuticles were removed and vouchedered; and (iii) the final elution volume was between 50 and 100 μL, depending on the size of the animal. Fragments of two ribosomal genes (18S and 28S), two sections of a nuclear protein-coding gene (CAD1 and CAD4) and one mitochondrial protein-coding gene (COI) were amplified. The primers used to amplify the four regions are shown in Table S2.

Reactions were performed in a total volume of 25 μL. Reactions contained 2 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 μM EDTA, 0.01 mM DTT, 0.5% Tween® 20, 0.5% Nonidet P-40, 50% Glycerol, 2.5 mM MgCl2, 0.2 mM of each dNTP, 0.64 μM of each primer, 0.5 U of Taq DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan) and 2 μL of DNA template. Amplifications for the COI region were performed in a thermocycler with an initial denaturation step of 94° C for 4 min, followed by 40 cycles of 94° C for 45 s, 45–65° C for 45 s, 72°C for 1 min, and one cycle at 72°C for 10 min. For the 18S and 28S gene regions a touchdown PCR amplification program was designed with an initial denaturation at 95°C for 3 min, with subsequent cycling as follows: in each cycle denaturation was performed at 95°C for 30 s, and elongation was performed at 72°C for 1 min. The annealing temperature of the reaction was decreased by 2°C every sixth cycle from 57°C to a touchdown at 47°C, at which temperature 30 cycles
were carried out. A final additional elongation step at 72°C for 10 min was also undertaken. Fragments of the carbamoyl-
phosphate synthase (CPSase) domain of CAD were amplified
with a different touchdown programme: initial denaturation
of 94°C for 1 min; seven cycles of denaturation at 94°C for 30 s,
annealing at 51°C for 1 min and elongation at 68°C for 1 min;
36 cycles of denaturation at 94°C for 30 s, annealing at 45°C
for 20 s and elongation at 68°C for 1 min 30 s; and final
extension at 68°C for 10 min. Amplification products were
purified using ExoSAP-IT® according to the manufacturer’s
instructions.

Direct sequencing of PCR products was performed using the
ABI Big Dye® Terminator 3.1 cycle sequencing kit following
the manufacturers instructions, and was carried out in an
ABI 3730 Capillary Electrophoresis Genetic analyser. Both
DNA strands were sequenced. Sequences were compiled and
dited using SEQUENCHER 4.2 (Gene Codes Corporation, Ann
Arbor, MI, U.S.A.) or BIOEDIT 3.0.9 (Hall, 1999). Sequences
were aligned using MUSCLE 3.6 (Edgar, 2004). Ambiguous
regions in the ribosomal alignments were excluded using the
GBlocks Server (Castresana, 2000; Talavera & Castresana,
2007), with all of the default selection criteria except
that gap positions were permitted in the selected blocks.
Alignments were concatenated, codon positions determined for
COI and CAD, and CAD introns delimited using MACCLADE
(Maddison & Maddison, 2002). Introns were excluded prior to
analysis.

Molecular phylogenetic analysis

Data were partitioned by locus and codon position in all analyses, and a separate general time reversible (GTR)
nucleotide substitution plus among-site rate variation model
was applied to each data partition. The data were analysed
under maximum likelihood (ML) using the sequential ver-
sion of RAxML 7.0.4 (Stamatakis, 2006). Five hundred non-
parametric bootstrap (BS) replicates were performed using
the GTR-CAT approximation. The data were also analysed with
Bayesian inference methods using MRBAYES 3.1.2 (Ronquist & Hueslenbeck, 2003). Four Markov
chain Monte Carlo (MCMC) chains were run for 5 million
generations, with trees sampled every 1000 generations. Sta-
tionarity was determined by examining traces of the likelihood
and individual parameter estimates: trees were sampled after
both runs converged, and successive iterations of MCMC failed
to improve the likelihood or alter parameter estimates. All trees
sampled before attaining stationarity were discarded.

We used BEAST 1.5.2 (Drummond & Rambaut, 2007)
to infer the joint posterior probability of phylogeny and
divergence times given a model of nucleotide substitution,
among-lineage rate variation, the phylogenetic branching
process and prior probability densities on specific node
ages. Sequence data were partitioned by locus and codon
position, and a separate HKY + Gamma model was applied
to each partition. The uncorrelated log-normal model for
among-lineage rate variation was used (Drummond et al.,
2006) in conjunction with a Yule model of the branching
process. Four nodes were calibrated with fossil data (see
below), following Cranston et al. (2010). The analysis was
run for 10 million generations, sampling trees every 10 000
generations after discarding samples from the first 1 million
generations. TRACER 1.4 (Rambaut & Drummond, 2007) was
used to analyse the BEAST log files, summarize posterior
distributions and estimate effective sample sizes (ESSs) for
each parameter. TREEANNOTATOR (within the BEAST package)
was used to analyse the sample of trees generated by BEAST
and draw 95% high posterior density (HPD) intervals for node
heights onto the ‘maximum credibility tree’ (the sampled tree
with the highest product of posterior probabilities) with median
node heights.

Fossil calibrations

Root. A soft maximum age constraint for Culicomorpha
is 295.4 Ma (http://www.fossilrecord.net), based on represen-
tatives of this lineage lacking in the rich insect fauna of
the Boskovice Furrow, Oboro, Moravia, Czech Republic, and
older deposits. The Oboro fauna is dated as early Sakmar-
ian (Zajic, 2000), which began 294.6 ± 0.8 Ma (Gradstein
et al., 2004). A hard minimum age constraint is 201 Ma, based
on the most ancient definitive Chironomidae, Aenne triassica
(Krzeminski & Jarzembowski, 1999), from sedimentary rocks
of the Rhaetian age (uppermost Triassic) limestone of England,
dated as 202 Ma ± 1 Myr (Benton & Donoghue, 2007). This
wing fossil is congeneric with a Lower Jurassic (Lower Toar-
cian, ca 185 Ma) wing fossil, Aenne liasia (Ansgore, 1999),
from Germany. For these taxa the subfamily Aenneinae was
erected by Ansorge (1999) based on features of the apical
wing venation (the wing bases of both were missing), includ-
ing the shape of r-r (R2), accredited as apomorphic, and with
purported plesiomorphic resemblances to Tanypodinae. Sub-
sequent authors since Krzeminski & Jarzembowski (1999),
including Grimaldi & Engel (2005), have accepted this sta-
tus as a likely sister group to all other extant subfamilies of
Chironomidae. The position of Cretaene Azar, Velte & Nel
either in an expanded Aenneinae sensu lato or a more derived
position (Azar et al., 2008) lends support to such a status for
Aenne.

In our study, in contrast to Cranston et al. (2010), the
minimum constraint for the root of Culicomorpha can be
derived from a fossil of an immature culicomorphan dipteran,
Anisindos crinitus (Lukashевич et al., 2010). The specimen,
from the Grès à Meules facies of the Grès-a-Voltaix Formation
in north-east France, dated as Lower Anisian, came from
the same deposits that contained the oldest definitive fossil
dipteran, Grawvogelia arcylleriana (Krzeminski et al., 1994),
thereby giving the same minimum age of 240.5 Ma for
Culicomorpha as was provided by the brachyceran, Gallia
alsatica Krzeminski & Krzeminska in Cranston et al. (2010).
For the root (node A), a normal prior was used, with a mean of 250 Ma and a standard deviation of 25 Myr, chosen such that 95% of the prior probability density was bounded by our constraints.

**Chironomidae.** The oldest definitive chironomid is *Aenne triassica*, from deposits 202 ± 1 Ma. An exponential prior was used (node B), with a zero offset of 201, and 95% of the prior probability density falling before our soft maximum age for Culicomorpha + Brachycera. Chironomid impression fossils from the Jurassic include a range of forms allocated to the extant subfamily Podonominae (e.g. Kalugina, 1985; Ansorge, 1996), based on the absence of wing vein R2+3 and the presence of cross veins r-m and tb (base of m-cu). Jurassic podonomin diversity is known from Grimmen, Germany, Lower Toarcian, 175.6–183.0 Ma (Ansorge, 1996) and Siberia (Kalugina, 1985). Although subfamily allocation can be confirmed based on preserved wings and some antennae, none can be allocated to extant genera.

The detailed preservation of many Chironomidae in different amber, commencing in the Cretaceous, has allowed the scrutiny of a range of morphological features, thereby enabling better emplacements into a modern taxonomic framework. The oldest amber investigated for chironomids comes from the Early Cretaceous of Lebanon, specifically Neocomian (Valanginian–Barremian)–Lower Aptian, dating to approximately 120 Ma. The first to be described from this amber was *Libanochlites neocomicus* Brundin, a female podonomin resembling extant *Paraboreochlus* Thienemann and *Boreochlus* Edwards, and certainly belonging to Brundin’s (1966) tribe Boreochlini. An undescribed male discovered by Schlee confirmed the tribal placement (Brundin, 1976): inclusion of the known female into a cladistic data matrix by Cranston & Edward (1998) confirmed the proximity to *Boreochlus*. Veltz et al. (2007) reported *L. neocomicus* from amber from Jezzine, Lebanon, and described males that clearly associate the taxon to the Tanypodinae (Azar et al., 2008). Notwithstanding the tibial combs, and because adult morphological discriminatory features are subtle, we place the taxon as a sister group to *Paraboreochlus* + *Boreochlus*. Cranston et al. (2010) experimented with inclusion and exclusion of *Libanochlites*: when included, an exponential prior was used with a zero offset of 100 and 95% of the prior density before our soft maximum constraint (parent node of P5). The first amber material of a podonomin associated unambiguously with a modern extant crown group of rank lower than tribe comes from Eocene amber, dated at 40 Ma in which the genera *Lasiodiamesa* Kieffer and *Paraboreochlus* can be recognized (Seredszus, 2003). An exponential prior (node G) with a zero offset and mean of 40 Ma was used.

**Results and discussion**

Exclusion of introns and hypervariable regions resulted in a final multifocus dataset of 3559 characters, of which 1924 were parsimony informative. Lengths by locus are: COI, 660; 28S, 437; 18S, 803; CAD, 1659. The positions of two taxa, *Beardius reissi* Jacobsen (Chironominae) and *Kaniwahaniiwamus* sp. (Orthocladiinae), were highly unstable in analyses, and were excluded as ‘rogue taxa’. A set of 197 species-level taxa remained, of which 143 were represented by all loci: five lacked COI data; seven lacked 28S data; 15 lacked 18S data; and 29 and 34 lacked CAD1 and CAD4, respectively (Table S1). Using simulations, O’Meara (2008) demonstrated that, in inferences based on supermatrices having taxa with missing data for one or more partitions, branch lengths are not biased if the partitions are analysed under separate models. Third codon positions accounted for 66% of parsimony-informative characters for COI sites and 49% for CAD. Mean base frequencies were A = 0.30341, C = 0.17961, G = 0.21243 and T = 0.30455, and were homogeneous across taxa ($\chi^2 P = 1.000$) in each partition except for third codon positions of COI and CAD ($P = 0.000$).

**Monophyly of Chironomidae in Culicomorpha**

As expected, the Chironomidae is confirmed as monophyletic (Fig. 2: node A; BS = 100; posterior probability, PP = 1). The expanded out-group compared with Cranston et al. (2010) shows a monophyletic Culicomorpha (BS = 100; PP = 1). Chironomidae are not sister group to Ceratopogonidae alone, with internal relationships postulated as Ceratopogonidae (Thaumaleidae + Simuliidae), with strong support. Inadequate sampling of genes and taxa in other Culicomorpha, and the revelation of long branch lengths, deter us from speculation concerning relationships. The postulated relationships are consistent with relationships derived from partial ribosomal RNA sequence data by Pawlowski et al. (1996), and from a study of ribosomal protein C-terminal extension by Fallon & Li (2007). The clade Thaumaleidae + Simuliidae has found support, including Moulton (2000), Grimald & Engel (2005) and Wiegmann et al. (2011). Although our hypothesis has not been recovered from any analysis based on combined morphology, support is available from culicomorphan pupal morphology (Borkent, 2010). The monophyly of superfamily Chironomoidea often failed to find support (e.g. Hennig, 1973; Sæther, 2000a, this study), and must now be considered as suspect.

**Monophyly and relationships among and within Chironomidae subfamilies**

(Fig. 2A–C)

Our study confirms the monophyly of all sampled subfamilies, excepting Prodiamesinae, which contains *Propsilocerus* Kieffer, previously in Orthocladiinae.

At the base of the family, the monogeneric Buchonomyiinae is supported as sister to the remaining extant sampled Chironominae (node B; BS = 100; PP = 1). Aphroteniinae branches at node C (BS < 70; PP = 1), Podonominae branches
to Aphroteniinae, Diamesinae, Orthocladiinae, Podonominae, Prodiamesinae, Tanypodinae, Telmatogoninae

Fig. 2. Bayesian tree. (a) Chironominae. Nodes are labelled as described in the text, with bootstrap support (BS, %) given above the branches and Bayesian posterior probabilities (PP) given below. Levels of support vary from BS = 100 and PP = 1 to BS < 70 and PP ≤ 0.9 (no support indicated on nodes).
Fig. 2. Bayesian tree. (b) Orthocladiinae, Prodiamesinae, Diamesinae and Telmatogetoninae. Nodes are labelled as described in the text, with bootstrap support (BS, %) given above the branches and Bayesian posterior probabilities (PP) given below. Levels of support vary from BS = 100 and PP = 1 to BS < 70 and PP ≤ 0.9 (no support indicated on nodes).
Fig. 2. Bayesian tree. (c) Tanypodinae, Podonominae, Aphroteniinae, and Buchonomyiinae and outgroups. Nodes are labelled as described in the text, with bootstrap support (BS, %) given above the branches and Bayesian posterior probabilities (PP) given below. Levels of support vary from BS = 100 and PP = 1 to BS < 70 and PP ≤ 0.9 (no support indicated on nodes).
Molecular phylogeny of Chironomidae (Diptera) at node D (without support) and Tanypodinae branches at node E (without support), successive sister groups, respectively, to the remaining taxa. These subfamilies, which comprise the core or totality of the semifamily Tanypodoinae, appear as a grade, although support for most nodes is disappointingly weak and the semifamily (excepting Buchonomyiinae) is not rejected. What may be considered as semifamily Chironomoinae is recovered (either node E if including Telmatogoniinae or node F if excluding Telmatogoniinae). Telmatogoniinae is hypothesized as sister to the remainder (node F, without support), with Diamesinae as the next sister (node H). At node H (BS = 70; PP < 0.9), Prodiamesinae is sister to Orthocladiinae + Chironominae (node I, without support). In the chronogram (Fig. 3), this relationship is altered, with Prodiamesinae as sister to Orthocladiinae (Fig. 3, node K), and these as sister to Chironominae.

In contrast to the weak or absent support for most stem nodes of the backbone structure, some crown subfamily and many internal relationships find stronger support. Thus Aphroteniinae (node Q; BS = 89; PP < 0.9) includes Aphroteniella Brundin (BS = 100; PP = 1). The reduced set of Podonominae terminals has an identical topology, but with lower support at deeper nodes, compared with Cranston et al. (2010). However, the monophyly of Archaeochlus Brundin (node P6; BS = 100; PP = 1) and Austrochus Cranston, Edward & Cook (node P7; BS = 100; PP = 1), and their mutual sister-group relations (PP = 1), remain strong. The monophyly of relationships of Parochlus Brundin and Podononus Brundin (nodes P1 and P2) have maximum support, and posterior probabilities of Podochlus Brundin and Podonomopsis Brundin (nodes P3 and P4) are significant at 0.95. In the diverse Tanypodinae (node L; BS < 70; PP = 1), the tribe Pentaneurini finds Bayesian support (node T1; BS < 70; PP = 1), with support for a monophyletic ‘non-Pentaneurine’ sister group (node T2, BS = 97; PP = 1) casting doubt on the validity of the existing tribal substructure in the Tanypodinae. The small marine intertidal or Hawaiian torrenticolous subfamily Telmatogoninae is strongly supported (BS = 100; PP = 1), as are the two sampled species of Telmatogon Schiner. Expansion of the sampling within Diamesinae compared with Cranston et al. (2010) suggests that the boreal Diamesa Meigen (our only representative of the essentially northern hemisphere tribe Diamesini) is sister to the austral representatives of Heptaginini and Harrisonini (node J; BS < 70; PP = 1), but these austral Heptagynini include the South African Harrisonina Free as sister to the neotropical Heptagynia Philippi (BS < 76; PP = 1). The ‘traditional’ subfamily Prodiamesinae is monophyletic (BS = 72; PP = 1), but Propsilocerus, previously considered an orthoclad is proposed as sister to this group (node O; BS = 95; PP = 1), and should thus be transferred from the Orthocladiolinae to a redefined Prodiamesinae. The subfamily Prodiamesinae is sister group either to Orthocladiinae + Chironominae (Fig. 2) or Orthocladiolinae alone (Fig. 3).

The recovery of Orthocladiinae (albeit without support, node N, and with the exception above) is unexpected given the historical scepticism based on an inability to find morphological synapomorphies in any life stage. Support is non-existent but the concept evidently is not refuted by our data. Some previous ideas concerning internal structure can be recovered: a monophyletic tribe Metriocnemini (following Metriocnemus Wulp) can be identified at node O1 (without support), tribe Orthocladiini (following Orthocladius Wulp) can be identified at node O2 (BS < 70; PP = 0.99) and tribe...
Corynoneurini can be identified at node O3 (BS = 100; PP = 1). A sister-group relationship between Orthocladiini and Corynoneurini finds Bayesian support of 0.99, and between these and Metriocnemini BS support of 100. Recognition of these three previously postulated tribes requires that the sister to all of them combined should be of equivalent rank. Although we refrain from formally naming a Brilliad group (node O4; BS < 70; PP = 1) and a Stictocladius group (node O5; BS = 100; PP = 1), proposed as sister groups to each others (but without support), until morphology is re-examined and sampling increased, we discuss some dating issues associated with these clades (below). Although in the past the marine orthocladi Clunio Haliday and Thalassomatiticia Strenzke & Remmert were given subfamily or tribal rank as Clunioninae/Clunionini, clearly they are embedded deep in tribe Metriocnemini of the Orthocladiinae, as proposed on Hennigian argumentation by Strenzke (1960). Another marine midge, Eremitoptera Kellogg, also once given high rank status, is an orthocladi, although distantly related to Clunio.

We recover a monophyletic Cardiocladius group as proposed by Sæther & Halvorsen (1981), although it is not associated with Corynoneura and relatives, but is proposed (without support) as sister to a clade centred on Limnophyes Eaton and comprising the newly revealed largely austral (Gondwanan) diversity of midges proposed by Krosch et al. (2011).

The long-established and uncontroversial subfamily Chironominae is monophyletic (node M; BS = 75; PP = 1), and is sister to the Orthocladiinae or Prodiamesinae + Orthocladiinae, without support. The first node (node C1; BS = 100; PP = 1), Shangomyia + Xyaomyia, is postulated in a deep position as sister to all remaining Chironominae, rather than as sister to tribe Chironomini implied by Sæther & Wang (1993). The hypothesized structure within the remainder of the subfamily contains previously recognized clades, some needing redefinition and others that are novel. Node C2 (BS = 75; PP = 1) separates a restricted tribe Chironomini that can be defined (node C3; BS < 70; PP = 1) to include Chironomus Meigen, and is sister group to a diverse cluster. The tribe Pseu-
dochironomini is untested, being represented only by Meigen, and is sister group to a diverse cluster. The tribe Pseu-
dochironomini is untested, being represented only by Meigen, and is sister group to a diverse cluster. The tribe Podonomini + Parakiefferiella, Parametriocnemus, Paratanytarsus, Parachironomus, Dicrotendipes, Kiefer-
feralis, Harrisius, Stenochironomus, Stictochironomus and Polypedilum.

The monophyly of Polypedilum (node C8; BS < 75; PP = 1), tested by six terminal species from at least three subgenera, is reassuring. The genus is very large, shows diverse morphology in each life-history stage and has proved difficult to define other than by multiple non-unique features. Not all possible sister taxa have been sampled, nor has the widest range of putative members been sampled, and its status and internal relationships remain to be strongly tested.

Although recognizing that our sampling of speciose genera in all cases was inadequate for rigorous testing, the following cases of non-monophyly are recognized: in Podonominae, Parochlus includes Zelandochus; in Tanypodinae, Zavrelimyia includes Paramerina and Ablabesmyia; and in Orthocladiinae, Stictocladius includes Lopescladius, Cricotopus includes Paratrichocladus, Ferringtonia includes Anzacladius and a Pseudosmittia, and Limnophyes includes Mesosmittia. In the Chironominae, only Endochironomus is rendered paraphyletic, by inclusion of Endotribelos. Amongst these results, Zelandochus embedded in Parochlus, proposed already on the basis of immature stage morphology (Boothroyd & Cranston, 1999), was confirmed from broadly sampled molecular data by Cranston et al. (2010). The paraphyletic relationships between...
tanypod pentaneurine genera Zavrelimyia and Paramerina is indicated by morphology, but the inclusion of the representative of Nilotanyus and the diverse Ablabesmyia is unexpected (S. McCluen, personal communication). Separation of the immature stages of Paratriechocladius from Cricotopus requires highly polythetic keying: despite Hirvenoja’s (1973) major study, their respective monophyly is not assured. The same applies to Endochironomus and Endotribelos (Grodhaus, 1987; Cranston et al., 1989), but much better taxon sampling is required for interpretation. Improved molecular sampling of more diverse austral Stictocladius taxa is ambivalent concerning the inclusion of Lopescladius (P.S. Cranston, personal observation), as indicated here. Wider sampling to target the ‘Gondwanan’ relatives of Ferringtonia and Anzacladius (Cranston, 2009) confirms suspicions of some non-monophyly (Krosch et al., 2011).

Selected morphological implications of the proposed phylogeny

Orthocladiinae/Prodiamesinae. The ‘near monophyly’ of the subfamily Orthocladiinae is somewhat surprising given the inability to find defining morphological autapomorphies in any life-history stage, with definitions based on ‘lack of’ character states of other groups and keys using polypletic couplets, with many exceptions. Even following the elevation of Diamesa and Prodiamesa groups, the remnant Orthocladiinae has been perceived as potentially paraphyletic. The postulated placement of Propsilocerus as sister to the Prodiamesinae (sampled by Monodiamesa Kieffer, Odontomesa Pagast and Prodiamesa Kieffer) can be reconciled on the basis of some morphology, such as the very large larval ventromental plates (though lacking any ‘beard’) and the complex and diverse volsellae in the male hypopygium. The pupa, with fringed anal lobe and several (more than three) macrosetae, resembles those of prodiamesines, but these features appear to be plesiomorphies, and exceptions abound. Notably the lack of wing vein M-Cu in Propsilocerus diminishes the ability to define Prodiamesinae, and exclusion from Orthocladiinae does not increase the diagnosability of the remainder of the subfamily. At least certain other genera, notably Diplocladius Kieffer and Abiskomyoia Edwards, must be sampled for their DNA before the status and composition of both subfamilies can be established.

Chironominae. Adult synapomorphies of Chironominae remain valid, including the lengthy tarsomere 1 of the foreleg versus the shorter tibia and the relatively immobile fusion of gonocoxite and gonostylus in the male genitalia. However, interpretation of the striated ventromental plate associated with silk extrusion, a strong larval synapomorphy, becomes more complicated with our results. The reduction or loss of this feature in the Stenochironomus complex is confirmed as secondary, based on the position in our analyses. However, the near-absent plates in larval Shangomyia is a different matter. A relationship of Shangomyia to the Stenochironomus complex was postulated by Cranston (2003), based on parsimony analysis of a morphological matrix including immature stages. Although all taxa share a larval wood-mining habit and some similar morphological modifications, no support for this (or any alternative) placement was obtained from features of the pupa and adult. Evidently the Stenochironomus complex is robustly monophyletic, as proposed by Borkent (1984), but it does not include Shangomyia. Larval wood mining is convergent: morphology including loss of ventromental plate striae must have driven the parsimony analysis, with pupae and adults uninformative and very divergent. This case of misleading larval morphology can be contrasted with deceptive adult morphology, especially of the male (e.g. as seen in some Podonominae; Cranston et al., 2010). In describing Shangomyia and Xiaomyia, Sæther & Wang (1993) list unusual adult features (lack of a dorsomedial eye extension, extended costa, Cu1 furcation near wing margin, all leg ratios >1.5, and reduction and fusion of the inferior volsella to the gonocoxite) as potential justification for a new tribe within Chironominae. Without explicit cladistic analysis, Sæther & Wang (1993) postulated Shangomyia + Xiaomyia as sister to tribe Chironomini, but our molecular data suggest a robust deeper position, as sister to all other Chironominae (node C8; BS = 100; PP = 1) justifying tribal, or possibly subfamily status (as Xiomyini or Xiomyiinae, respectively). However, it may be premature to formalize such a rank, as the position of Beardiass Reiss & Sublette is uncertain. This undoubtedly belongs to an early branch in the Chironominae group, but acted as an unstable ‘rogue’ taxon in our analysis. Andersen & Sæther (1996) suggested that synapomorphies were difficult to find, and that Beardiass was clearly ‘related closely to Paratendipes Kieffer’: this certainly was one (unsupported) position, but another subtended all sampled Chironominae (with PP = 1), and yet another had it lying within Orthocladiinae.

Tempo of diversification. (Fig. 3) The postulated origin of the stem Chironomidae in the Permain is earlier than, but within, the posterior probability of the 269 (308–231) Ma estimate of Cranston et al. (2010), and deviates significantly from the mid Triassic date estimated by Bertone et al. (2008). Our expanded out-group selection provides a different basal node that may drive the earlier date. Notably, rendering a Permain date improbable, no fossil Diptera have been reported earlier than the early-mid Triassic (ca 240 Ma; Lukashevich et al., 2010), despite several appropriate earlier deposits being studied. Nonetheless, it seems that morphological diversity of Culicomorpha was present already by the mid Triassic, including from variably preserved immature stages (Lukashevich et al., 2010).

Our chronogram estimates the date of the crown Chironomidae (node B) to the mid Triassic, which is roughly contemporaneous with the dating of the earliest Aeneinae fossil (Krzeminski & Jarzembowski, 1999), the presumptive sister to all extant taxa. The backbone of oldest subfamilies, the stem groups of Buchonomyiinae, Aphroteniinae and Podonomiae (nodes B, C and D) arise in the mid–late Triassic. The separation of stem Tanypodinae from stem Chironomonea (node E) is dated to the end of the Triassic.
The early-mid Jurassic saw the diversification of crown Podonominae (node G) contemporaneously with subfamily origin and diversification within Chironominae (nodes F, H, I, J and K). The three most diverse contemporary subfamilies arose at the end of the Jurassic/earliest Cretaceous, with the origin of crown groups of Tanypodinae (node L), Chironominae (node M) and Orthocladiinae (node N), with subsequent substantial diversification of each through the Cretaceous. Crown Prodiamesinae (with or without Propsiilocerus) (node O) and Telmatogotoninae (node P) originate in the early and mid Cretaceous, respectively. The Aphroteniinae, the last crown group to appear (after a long stem), is hypothesized to have originated in the Campanian of the late Cretaceous. By the end of the Mesozoic, many stem and several crown genera are present (e.g. Polypedilum (node C1)), and evidently much diversification has occurred prior to the Tertiary.

All nodes proposed as Mesozoic are slightly older than those dated in Cranston et al. (2010), but are based on more intense sampling outside their focal podonomine taxa. Nodal dates encompass in their posterior probabilities dates for recently reported new finds for Chironomidae, both as compression fossils and preserved in amber (e.g. Veltz et al., 2007; Jarzembowski et al., 2008; Azar & Nel, 2010). However, we cannot agree with these authors concerning the lack of evidence for Podonominae amongst the fossils considered. Admittedly stem Podonominae and Tanypodinae may be difficult to identify in the absence of immature stage synapomorphies, as stated by Veltz et al. (2007), but there are no strong reasons to accept reallocations including Libanochlites Brundin (contra Azar et al., 2008).

**Chironomidae biogeography and southern Africa**

Our dated phylogeny allows us to address issues concerning the biogeography associated with southern Africa in more detail than in Cranston et al. (2010). Ever since Freeman (1964) and Brundin (1966), the significance of southern African taxa with relationships to those on other austral land masses, has been recognized as providing early dates by reasoning based on the vicariance history of Gondwana (e.g. Cranston et al., 1987; Sæther & Ekrem, 2003). Eliminating recent intruders into Africa in cosmopolitan groups from consideration (Sæther & Ekrem, 2003), we have sampled and proposed dates for four significant events involving: the podonomines *Afrochlus + Archaeochlus*; the aphrotenine *Aphrotenia*; the diamesines *Heptagyiina* and *Harrisonina* Freeman; and an orthoclad *Elpiscladius* Harrison & Cranston. The first three were identified by Sammartin & Ronquist (2004) as conforming to the ‘classic’ southern Gondwanan pattern (SGP; their fig. 3A). Vicariance over time involving southern Africa and the remainder of Gondwana is thought to have begun at the opening of the South Atlantic, at 135 Ma, although the opening of the Somalian Basin at 121 Ma, separating southern Africa from Indo-Madagascar, and the opening of the North Atlantic at 110 Ma, separating southern Africa from northern South America, were required to complete the isolation of African biota from the other austral land masses (Sammartin & Ronquist, 2004). Although we have no evidence of these ‘Gondwanan midges’ remaining on the now-tropical land masses of India, Madagascar and northern South America, we cannot rule out extinction or primitive absence, and therefore the ‘time window’ for accepting a Gondwanan vicariant explanation for distribution patterns should be 110–135 Ma.

Examining first the disjunction between the Podonominae, the node separating southern African *Afrochlus + Archaeochlus* from the western/central Australian *Austrochlus*, is dated to 105 (76–139) Ma, strongly supporting the vicariance explanation proposed by Cranston et al. (1987). [Note that Brundin (1966) did not know of the Australian clade.] In contrast, dates associated with the Aphroteniinae and Diamesinae cases appear too young. The node separating the southern African *Aphrotenia tsitsikamae* Brundin from the Australian *Aphroteniella* is dated to 69 Ma, with no range calculated. The node separating southern African *Harrisonina* from South American *Heptagyiina* is similarly dated at 70 Ma, with a 95% PP of 49–93 Ma. It should be noted that the sampling around these latter two nodes is much less adequate than for the podonomine example, but it seems that Cretaceous dispersal is the most likely explanation for current distribution.

Turning to the fourth case, concerning the Brillia group of orthoclads, the basal node (Fig. 2, O4) separates the Australian/South American *Austrobrillia*, with a date of separation from the remainder of the group at 124 (105–143) Ma, and the next node, separating the South African *Elpiscladius* from the remaining sampled taxa (all non-Gondwanan) dated at 112 (92–130) Ma. The tight timing of these postulated divergences with tectonic events substantiate the morphological phylogeny and derived estimate of timing by Harrison & Cranston (2007).

In two of these cases, a dating of divergences of >100 Ma is associated with evidence for extremely protracted stasis in immature stage ecology. The larvae of *Afrochlus* and *Archaeochlus* in southern Africa and those of *Austrochlus* in western/central Australia are essentially identical, and are very unusual amongst the Chironomidae in developing almost exclusively in ephemeral water films formed following wetting by seasonal rains (Cranston et al., 1987; Edward, 1989). For the Brillia group, the early dating also supports the argument for very long-lasting ecological stasis in this clade as wood-mining larvae, and in keeping with morphological phylogenetic analysis (Cranston & McKie, 2006).

Some other biogeographic inferences derived from our molecular analyses involving younger nodes (i.e. Gondwanan, but excluding South Africa) are discussed in Cranston et al. (2010) and Krosch et al. (2011).

**Conclusions**

This extensive molecular sampling across the Chironomidae finds substantial congruence with previous ideas concerning relationships developed from morphological parsimony analyses, as was seen in the intensive study of the Podonominae (Cranston et al., 2010). Although support values vary.
across the tree, with little or no support for many ‘backbone’ nodes, all previously proposed subfamilies are recovered as monophyletic with one exception. Where internal relationships have been postulated before, many are substantiated and most different arrangements are uncontroversial with respect to morphological estimates. An exception is the robust finding that Buchonomyiinae forms the sister group to all remaining taxa, and Propsilocerus should be moved to Prodiamesinae from Orthocladiinae. Although the use of all semaphorins (life-history stages) surely aids in providing accurate phylogenetic estimates, our confirmation of the high rank of the adult-based genera Xiaomyia and Shangomyia demonstrates that even alone the adult can be reliable.

Our objective was to test existing ideas on the evolution of the Chironomidae with independent data from combined mitochondrial and nuclear sequences. Support for existing morphology-based phylogenies, and the ability to explain anomalies (by reciprocal illumination), suggests that a ‘total evidence’ unification of molecular and morphological data will not enhance our understanding of either source of data. Our results should not be taken as support for the primacy of molecular data in evolutionary studies: on the contrary, they show the value of morphological data derived from all stages. The undeniable advantage of molecular data is the ability to place an independent timeline on major evolutionary events.

In this regard, our proposed tempo concerning chironomid origination and subsequent diversification is largely in keeping with Brundin’s (1966) advocacy for a Mesozoic age, and is congruent with recent fossil finds. The Permian origination of the stem Chironomidae (and perhaps of Culicomorpha) is controversial and needs further study, perhaps with calibration from more reliably placed fossils. The problem here is that fossils earlier than about 100 Ma mostly comprise wings alone, and many other features are difficult or impossible to score. The increasing availability of fossils of immature stages of Culicomorpha from early in the Mesozoic may assist, but allocation to stems may continue to involve ‘similarity’ as we lack reliable synapomorphies for pupae. Inclusion in ongoing phylogenetic analyses of Diptera as a whole might test the placement of the family as sister to all other culicomorphs using more appropriate chironomid taxa (e.g. Buchonomyia, Aphrotenia and Archaeochlus) than a sole terminal, Chironomus.

Within the family, as is stated for all such studies, extended sampling and addition of new genes may be expected to provide better support for weak nodes and challenge controversial relationships. We recognize the need to sample the two unrepresented, geographically restricted subfamilies Chileno-myiiinae and Usambaromyiinae, Boreoheptagyia and more representatives of boreal tribe Diamesini in the Diamesinae, more taxa in non-Pentaneurine tanyods, and postulated early branches in Orthocladiinae (e.g. Diplocladius and Abiskomyia) and Chironominae (e.g. Beardiuous and Paratendipes, and relatives). Despite these acknowledged lacunae, we believe the extensive sampling over the past two decades has provided a geographically and taxonomically appropriate phylogenetic framework against which to test many proposals for the evolution of this diverse group of Endopterygota.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2011.00603.x

Table S1. List of taxa, codes, life stages, locations and GenBank accessions.

Table S2. Primers used for polymerase chain reaction amplification and sequencing.

Please note: Neither the Editors nor Wiley-Blackwell are responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Acknowledgements

This project was funded initially in the 1990s by the Australian Biological Resource Study, and subsequently (2000–2011) by the generous support given to the Entomology Department, University of California, Davis, by Evert Schlinger for the endowed chair in Systematic Entomology held by the first author, and in the late stages, by NSF award 0933218 ‘MIDGEPEET - A Collaborative Effort to Increase Taxonomic Expertise in Understudied Families of Nematocerous Diptera’ to J.K. Moulton, University of Tennessee. We acknowledge the generosity of the many colleagues that sought and provided specific taxa for this project – their names are in parentheses in Table S1. Major collection trips for this project were made with the assistance at various times of our colleagues Andrew Baker, Greg Courtney, Don Edward, Penny Gullan, Demian Kondo, Matt Krosch and Brendan McKie. Andy and Jen Cranston accompanied us on several early trips, without coercion. State and federal agencies gave permits more or less promptly, and we are grateful for the willingness and enthusiasm of all park rangers in providing local guidance and advice. Art Borkent kindly identified the out-group Ceratopogonidae, and has been a continuing discussant of many issues of morphology, phylogeny, fossils and the dating of the Chironomidae. Scott McCluen made the GenBank accessions – many thanks for the perseverance. We appreciate the reviews of Art Borkent, Torbjørn Ekrem, Nick Herold, Matt Krosch, Jon Martin and J.M. (Kevin) Moulton that assisted in the clarification of this article. The authors declare no conflicts of interest: the manuscript review was handled by editor Thomas Simonsen.

References


© 2011 The Authors


Accepted 24 August 2011
First published online 20 October 2011

© 2011 The Authors
Table S1. List of taxa, codes, life-stage, locations, GenBank accessions. * - excluded taxon

<table>
<thead>
<tr>
<th>GenBank Accession</th>
<th>Country</th>
<th>Region</th>
<th>Code</th>
<th>Full Taxon Name</th>
<th>Life-stage</th>
<th>Location Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ440707, HQ440868, HQ440557, HQ440415</td>
<td>South Africa</td>
<td>W. Cape, Betty’s Bay, Harold Port BG</td>
<td>28S COI</td>
<td>Aphrotenia tsitsikamae</td>
<td>New South Wales, Captain’s Flat, Molonglo R.</td>
<td>Aphroteniinae</td>
</tr>
<tr>
<td>HQ440708, HQ440869, HQ440558, HQ440416</td>
<td>USA</td>
<td>California, Madeline</td>
<td>18S</td>
<td>Aphrotenia tsitsikamae</td>
<td>Biodiversity</td>
<td>Aphroteniinae</td>
</tr>
<tr>
<td>HQ440703, HQ440877, HQ440555, HQ440422</td>
<td>USA</td>
<td>Massachusetts, Connecticut R. (St. Werle)</td>
<td>COI</td>
<td>Aphrotenia tsitsikamae</td>
<td>Biodiversity</td>
<td>Aphroteniinae</td>
</tr>
<tr>
<td>HQ440714, HQ440876, HQ440556, HQ440253</td>
<td>USA</td>
<td>Florida, Taylor Slough (Jacobsen)</td>
<td>28S</td>
<td>Aphrotenia tsitsikamae</td>
<td>Biodiversity</td>
<td>Aphroteniinae</td>
</tr>
<tr>
<td>HQ440719, HQ440883, HQ440572, HQ440428</td>
<td>USA</td>
<td>California, Ash Valley mud lake</td>
<td>COI</td>
<td>Aphrotenia tsitsikamae</td>
<td>Biodiversity</td>
<td>Aphroteniinae</td>
</tr>
<tr>
<td>HQ440721, HQ440885, HQ440565, HQ440423</td>
<td>Ireland</td>
<td>Killarney, R. Flesk</td>
<td>28S</td>
<td>Buchonomyia thienemanni</td>
<td>Biodiversity</td>
<td>Buchonomyiinae</td>
</tr>
<tr>
<td>HQ440724, HQ440888, HQ440574, HQ440430</td>
<td>Thailand</td>
<td>Chaiyaphum, Fad Tone Falls</td>
<td>28S</td>
<td>Buchonomyia thienemanni</td>
<td>Biodiversity</td>
<td>Buchonomyiinae</td>
</tr>
<tr>
<td>HQ440721, HQ440885, HQ440565, HQ440423</td>
<td>USA</td>
<td>California, S. Werle</td>
<td>28S</td>
<td>Buchonomyia thienemanni</td>
<td>Biodiversity</td>
<td>Buchonomyiinae</td>
</tr>
<tr>
<td>HQ440721, HQ440885, HQ440565, HQ440423</td>
<td>USA</td>
<td>California, S. Werle</td>
<td>28S</td>
<td>Buchonomyia thienemanni</td>
<td>Biodiversity</td>
<td>Buchonomyiinae</td>
</tr>
<tr>
<td>HQ440721, HQ440885, HQ440565, HQ440423</td>
<td>USA</td>
<td>California, S. Werle</td>
<td>28S</td>
<td>Buchonomyia thienemanni</td>
<td>Biodiversity</td>
<td>Buchonomyiinae</td>
</tr>
</tbody>
</table>

Cranston, P.S. et al., 2011 A dated molecular phylogeny for the Chironomidae. Systematic Entomology
<table>
<thead>
<tr>
<th>Species / Genus</th>
<th>Location</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naonella sp.</td>
<td>NZ07.4.2</td>
<td>L New Zealand South I., Tasman-Nelson, Kawatiri, Hope R.</td>
</tr>
<tr>
<td>Orthocladius (Eoorthocladius) latipes</td>
<td>CAM10</td>
<td>L USA California, Navarro R.</td>
</tr>
<tr>
<td>Paraneogeton sp.</td>
<td>CAS11</td>
<td>PI USA California, Navarro R.,</td>
</tr>
<tr>
<td>Paraparaleurodes sp. 1</td>
<td>Alr6</td>
<td>L South Africa W. Cape, Cape Peninsula (Krokoshoek)</td>
</tr>
<tr>
<td>Paraparaleurodes sp. 2</td>
<td>NSW2.2</td>
<td>L Australia New South Wales, Warumbungles,</td>
</tr>
<tr>
<td>Paraparaleurodes sp. 3</td>
<td>V505</td>
<td>L Australia Victoria, Buckland R.</td>
</tr>
<tr>
<td>Parameleocnemus sp. 1</td>
<td>V422</td>
<td>L Australia Victoria, Buckland R. F, U. Buckland R.</td>
</tr>
<tr>
<td>Parameleocnemus sp. 2</td>
<td>CAM05</td>
<td>L USA California, Mallard Redwoods State Forest,</td>
</tr>
<tr>
<td>Paranaleurodes acuminatus</td>
<td>CH19</td>
<td>L Chile U region, Antofagasta</td>
</tr>
<tr>
<td>Parachloristratius FN25.2</td>
<td>L Australia Queensland, Robson Ck.</td>
<td></td>
</tr>
<tr>
<td>Paraffemina pictipennis</td>
<td>NZ07.6.1</td>
<td>L New Zealand South I., Tasman-Nelson, w Lake Rotoroa</td>
</tr>
<tr>
<td>Propiogerrhes akamusi</td>
<td>PROK</td>
<td>L Japan lab culture</td>
</tr>
<tr>
<td>Pseudocladus (Allocladus) sp.</td>
<td>NCA8</td>
<td>L USA California, Castle Lake.</td>
</tr>
<tr>
<td>Pseudocladus (P.) sp.</td>
<td>NCA5</td>
<td>L USA Oregon, U. Klamath L.</td>
</tr>
<tr>
<td>Pseudosmittia sp.</td>
<td>Peru1</td>
<td>m Peru Poroc, Pusara</td>
</tr>
<tr>
<td>Rheocricotopus sp.</td>
<td>RHE3</td>
<td>L Australia Northern Territory, Macquarie Ck. (Hughes)</td>
</tr>
<tr>
<td>Stictocladius sp. 1</td>
<td>V420</td>
<td>L Australia Victoria, Buckland R. F, U. Buckland R.</td>
</tr>
<tr>
<td>Stictocladius picus</td>
<td>SP1</td>
<td>L Australia Victoria, Buckland R.</td>
</tr>
<tr>
<td>Synothocladius sp.</td>
<td>NCA7</td>
<td>L USA California, Castle Lake</td>
</tr>
<tr>
<td>Thienemanniella sp.</td>
<td>PH1</td>
<td>L Mexico Hidalgo State</td>
</tr>
<tr>
<td>Tonnoirocladius commensalis</td>
<td>NZTO</td>
<td>L New Zealand South I., Canterbury, Blackadder S.R.</td>
</tr>
<tr>
<td>Undet Orthocladiinae FNQ2</td>
<td>FNQ4.3</td>
<td>P Australia Queensland, Ngger R.</td>
</tr>
</tbody>
</table>

**PODIONINAE**

<table>
<thead>
<tr>
<th>Species / Genus</th>
<th>Location</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afrochlus hamsoni</td>
<td>ZAFR</td>
<td>P Zimbabwe Nipo, Kunwari</td>
</tr>
<tr>
<td>Archaeochlus bicirratus</td>
<td>ARBI</td>
<td>L South Africa Drakensberg Mts.</td>
</tr>
<tr>
<td>Archaeochlus sp. 1</td>
<td>NAMA</td>
<td>L Namibia Erongo</td>
</tr>
<tr>
<td>Austrochulus auruniti</td>
<td>AUBR</td>
<td>P Australia West Australia</td>
</tr>
<tr>
<td>Austrochulus neubelii</td>
<td>HUPE</td>
<td>L Australia Northern Territory, East Macquarie Range</td>
</tr>
<tr>
<td>Austrochulus paraparkinii</td>
<td>AUPB</td>
<td>P Australia West Australia</td>
</tr>
<tr>
<td>Boreothrocladius sp.</td>
<td>CABORL</td>
<td>L USA California, Placer, Bear Ck., above Truckee R. (Richards)</td>
</tr>
<tr>
<td>Gerus Chil</td>
<td>CHG 4.1</td>
<td>Pm Chile Neuquen Prov, Arroyo Quilanlahue</td>
</tr>
<tr>
<td>Lasiodiamesa sp.</td>
<td>L2</td>
<td>L Netherlands Overijssel (Verbek)</td>
</tr>
<tr>
<td>Paraboreothrocladius minutissimus</td>
<td>Pbm</td>
<td>M Germany Bavaria, Berchtesgaden (Stur)</td>
</tr>
<tr>
<td>Paracricotopus sp.</td>
<td>CARABP</td>
<td>P USA California, Plumas Co., Cow Ck.</td>
</tr>
<tr>
<td>Parochlus spinosus</td>
<td>NZ08 2P2</td>
<td>P New Zealand North I., Gullane, Mangawheru R</td>
</tr>
<tr>
<td>Parochlus antarcticus</td>
<td>NZ08 BP1</td>
<td>P New Zealand South I., Tasman-Nelson, Speargrass Ck.</td>
</tr>
<tr>
<td>Parochlus bassianus</td>
<td>TAS5</td>
<td>L Australia Tasmanina, Mt Field N.P. ex. Richea</td>
</tr>
<tr>
<td>Parochlus chiloensis</td>
<td>CH6.1 M</td>
<td>L Chile IX region, P.N. Vicente Perez Rosales, Petrohué</td>
</tr>
<tr>
<td>Parochlus emeryi</td>
<td>CARABP</td>
<td>L USA California, Plumas Co., Cow Ck. (Richards)</td>
</tr>
<tr>
<td>Parochlus steineri</td>
<td>PARS</td>
<td>M Antarctica South Georgia, Cooper Bay (Convey)</td>
</tr>
<tr>
<td>Parochlus trogonocerus</td>
<td>ARG07 2.6</td>
<td>L(P) Argentina Neuquen Prov, Arroyo Quilanlahue</td>
</tr>
<tr>
<td>Parochlus sp. 18</td>
<td>CHE 11</td>
<td>L Chile IX region, P.N. Vicente Perez Rosales, Petrohué</td>
</tr>
<tr>
<td>Podochlaus coquenius</td>
<td>ARG07 2.7</td>
<td>L Argentina Neuquen Prov, Arroyo Quilanlahue</td>
</tr>
<tr>
<td>Podochlaus tasmanianus</td>
<td>TAS11</td>
<td>PM Australia Tasmanina, Mt Field N.P. ex. Richea</td>
</tr>
<tr>
<td>Podonomus eurasi</td>
<td>AUPE</td>
<td>L Australia Australian Capital Territory, Brindabella</td>
</tr>
<tr>
<td>Podonomus decaminutus sp.</td>
<td>CH18</td>
<td>L Chile IX region, P.N. Vicente Perez Rosales, Petrohué</td>
</tr>
<tr>
<td>Podonomus orbicularus/Fittkaui</td>
<td>ARG07 2.2</td>
<td>PI Argentina Neuquen Prov, Arroyo Quilanlahue, below Xing</td>
</tr>
<tr>
<td>Trichotanypus posticalis</td>
<td>ZOC</td>
<td>L New Zealand South I., Fox Glacier</td>
</tr>
</tbody>
</table>

**PRODIAMESINAE**

<table>
<thead>
<tr>
<th>Species / Genus</th>
<th>Location</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monodiamesa sp.</td>
<td>PRO3</td>
<td>L USA Montana, Blackfoot Ck. (Ecoanalysts)</td>
</tr>
<tr>
<td>Odonotemesta sp.</td>
<td>PRO2</td>
<td>L USA Montana, Blackfoot Ck. (Ecoanalysts)</td>
</tr>
<tr>
<td>Paradamesa sp.</td>
<td>PRO5</td>
<td>L USA Idaho, Lindsay Creek (Ecoanalysts)</td>
</tr>
<tr>
<td>Taxon</td>
<td>Genus</td>
<td>Species</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td><strong>Tanypodinae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanypodina sp.</td>
<td>CAM02</td>
<td>L USA</td>
</tr>
<tr>
<td>Apsicliniomyia sp.</td>
<td>APS</td>
<td>L Australia</td>
</tr>
<tr>
<td>Apsicliniomyia sp.</td>
<td>AP5</td>
<td>L Victoria</td>
</tr>
<tr>
<td>Aludanius venustus</td>
<td>APS2</td>
<td>L USA</td>
</tr>
<tr>
<td>Australiomyia pronoptera</td>
<td>FNG10.12</td>
<td>L Australia</td>
</tr>
<tr>
<td>Brundinella sp.</td>
<td>AF1P1</td>
<td>L South Africa</td>
</tr>
<tr>
<td>Conchapeletopia sp.</td>
<td>CAM07</td>
<td>L USA</td>
</tr>
<tr>
<td>Gomphocera sp.</td>
<td>APA</td>
<td>L Australia</td>
</tr>
<tr>
<td>Gomphocera sp.</td>
<td>AP1</td>
<td>L Australia</td>
</tr>
<tr>
<td>Larsia sp.</td>
<td>TH2B</td>
<td>L Thailand</td>
</tr>
<tr>
<td>Larsia sp. 2</td>
<td>FNG7.21</td>
<td>L Australia</td>
</tr>
<tr>
<td>Nitconius sp.</td>
<td>AUN107</td>
<td>L Australia</td>
</tr>
<tr>
<td>Paramerina sp. 3</td>
<td>TH69</td>
<td>L Thailand</td>
</tr>
<tr>
<td>Paramerina sp. 4</td>
<td>N111</td>
<td>L Australia</td>
</tr>
<tr>
<td>Pentaneurini sp.</td>
<td>TRP</td>
<td>L USA</td>
</tr>
<tr>
<td>Pentaneurini genus A sp.</td>
<td>V214</td>
<td>L Australia</td>
</tr>
<tr>
<td>Pentaneurini undet sp.</td>
<td>Penta</td>
<td>missing</td>
</tr>
<tr>
<td><strong>Telmatogetoninae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telmatogegeton japonicus</td>
<td>WATL</td>
<td>in Australia</td>
</tr>
<tr>
<td>Telmatogegeton mcswaini</td>
<td>CATL</td>
<td>in USA</td>
</tr>
<tr>
<td>Thalassomyia frauenfeldi</td>
<td>THAL</td>
<td>in Italy</td>
</tr>
<tr>
<td><strong>Outgroups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forsimyia brevipennis</td>
<td>CER01</td>
<td>adult USA</td>
</tr>
<tr>
<td>Atrichopogon (s.l.) minitus</td>
<td>CER02</td>
<td>adult USA</td>
</tr>
<tr>
<td>Brachypogon (isoleuca) sp.</td>
<td>CER03</td>
<td>adult USA</td>
</tr>
<tr>
<td>Culicoides leechi</td>
<td>CER04</td>
<td>adult USA</td>
</tr>
<tr>
<td>Dasyplecta sp.</td>
<td>CERA1</td>
<td>adult USA</td>
</tr>
<tr>
<td>Simulidae inedit</td>
<td>SIM1</td>
<td>adult Thailand</td>
</tr>
<tr>
<td>Austrothaumalea sp.</td>
<td>Thaum</td>
<td>adult Chile</td>
</tr>
</tbody>
</table>
Cranston, P.S. et al., 2011. A dated molecular phylogeny for the Chironomidae (Diptera)
*Systematic Entomology*

**Table S2.** Primers used for PCR amplification and sequencing.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rDNA</td>
<td>18S_ai</td>
<td>CCT GAG AAA CGG CTA CCA CAT C</td>
<td>Whiting et al., 1997</td>
</tr>
<tr>
<td></td>
<td>18S_bi</td>
<td>GAG TCT CGT TCG TTA TCG GA</td>
<td>Whiting et al., 1997</td>
</tr>
<tr>
<td>28S rDNA</td>
<td>S3660</td>
<td>GAG AGT TMA ASA GTA CGT GAA AC</td>
<td>Morse and Normark 2006</td>
</tr>
<tr>
<td></td>
<td>A335</td>
<td>TCG GAA GGA ACC AGC TAC TA</td>
<td>Whiting et al., 1997</td>
</tr>
<tr>
<td>COI mtDNA</td>
<td>s2183</td>
<td>CAA CAT TTA TTT GGA TTT TTT G</td>
<td>Simon et al., 1994</td>
</tr>
<tr>
<td></td>
<td>a3014</td>
<td>TCC AAT GCA CTA ATC TGC CAT ATT A</td>
<td>Simon et al., 1994</td>
</tr>
<tr>
<td>CAD 1</td>
<td>54F</td>
<td>GTN GTN TTY CAR ACN GGN ATG GT</td>
<td>Moulton and Wiegmann, 2004</td>
</tr>
<tr>
<td></td>
<td>405R</td>
<td>GCN GTR TGY TCN GGR TGR AAY TG</td>
<td>Moulton and Wiegmann, 2004</td>
</tr>
<tr>
<td>CADIV</td>
<td>787F</td>
<td>GGD GTN ACN ACN GCN TGY TTY GAR CC</td>
<td>Moulton and Wiegmann, 2004</td>
</tr>
<tr>
<td></td>
<td>1098R</td>
<td>TTN GGN AGY TGN CCN CCC AT</td>
<td>Moulton and Wiegmann, 2004</td>
</tr>
</tbody>
</table>

Supporting Information References
Entomological Society of America, 87, 651–701.