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Phylogenetic relationships of leptophlebiid mayflies as inferred by *histone H3* and *28S ribosomal DNA*

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Abstract. Leptophlebiidae is among the largest and most diverse groups of extant mayflies (Ephemeroptera), but little is known of family-level phylogenetic relationships. Using two nuclear genes (the D2 + D3 region of *28S ribosomal DNA* and *histone H3*) and maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), we inferred the evolutionary relationships of 69 leptophlebiids sampled from six continents and representing 30 genera plus 11 taxa of uncertain taxonomic rank from Madagascar and Papua New Guinea. Although we did not recover monophyly of the Leptophlebiidae, monophyly of two of the three leptophlebiid subfamilies, Habrophlebiinae and Leptophlebiinae, was recovered with moderate to strong support in most analyses. The Atalophlebiinae was rendered paraphyletic as a result of the inclusion of members of Ephemerellidae or the Leptophlebiinae clade. For the species-rich Atalophlebiinae, four groups of taxa were recovered with moderate to strong branch support: (i) an endemic Malagasy clade, (ii) a Paleoaustral group, a pan-continental cluster with members drawn from across the southern hemisphere, (iii) a group, uniting fauna from North America, southeast Asia and Madagascar, which we call the *Choroterpes* group and (iv) a group uniting three New World genera, *Thraulodes*, *Farrodes* and *Traverella*. Knowledge of the phylogenetic relationships of the leptophlebiids will aid in future studies of morphological evolution and biogeographical patterns in this highly diverse and speciose family of mayflies.

Introduction

The Leptophlebiidae Banks or 'prong-gilled' mayflies are a cosmopolitan, speciose and morphologically diverse family. The oldest identified leptophlebiid fossil is *Aureophlebia sinitshenkovae* Peters & Peters from the Upper Cretaceous, dated to about 90 mya (Peters & Peters, 2000), and representatives of the modern subfamilies are documented from Baltic Amber, dated to about 50 mya (Hubbard & Savage, 1981). The Leptophlebiidae consists of approximately 110 genera and more than 600 described species, roughly a quarter of all currently recognized species of mayflies. In understudied regions like Madagascar, taxonomic work on leptophlebiids is expected to yield upwards of 15 genera and 100 species new to science (Benstead *et al.*, 2003). Leptophlebiids are thought to have undergone extensive adaptive radiation resulting in their present occupation of a myriad of different aquatic microhabitats (Tsui & Peters, 1975) and highly diverse gill morphologies. Historically, gill morphology has been linked to ecological factors (Peters *et al.*, 1964; Riek, 1973; Towns & Peters, 1996). Many specialized lineages within leptophlebiids have converged upon morphological features found in other mayfly families. These features include ventrally-positioned gills that form a disc used to adhere to rocks (in *Deleatidium* Eaton in New Zealand and in the heptageniid *Rhithrogena* Eaton), tusks used for burrowing (in *Jappa* Harker in Australia and most Ephemeroidea) and operculate gills (in *Adenophlebiodes* Ulmer and many genera of Pannota) (Peters & Edmunds, 1964; Landa *et al.*, 1980). A small-scale example of the pattern of dramatic specialization within Leptophlebiidae occurs in New Caledonia, where the native mayfly fauna is entirely composed of Leptophlebiidae with genera strikingly

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reminiscent of Heptageniidae, Baetidae, Ephemerellidae and Ephemeridae (Peters, 1980; Peters & Peters, 1980). Many watersheds in the southern hemisphere are dominated by leptophlebiids both in terms of taxonomic diversity and abundance measures and endemism is commonplace (Peters & Peters, 1980; Towns & Peters, 1996). In South America, it is estimated that 60% of genera and 80% of the species are endemic (Pescador *et al*., 2001).

Despite morphological diversity, leptophlebiids exhibit several distinguishing features in both nymphal and alate stages. For example, leptophlebiid nymphs exhibit a rake-like spine on the maxillae (Peters & Edmunds, 1970; Kluge, 2004), reduced canines and dentisetae (Kluge, 2004), aflagellate sperm (Soldan, 1979a; Gaino & Mazzini, 1991a, b) and a sperm pump (Grimm, 1977, 1985; Soldan, 1979b). Within leptophlebiids, three subfamilies are currently recognized: (i) the strictly northern hemisphere Leptophlebiinae Banks (Peters, 1980) containing six genera [*Paraleptophlebia* Lestage, *Leptophlebia* Westwood, *Habrophlebiodes* Ulmer, *Calliarcys* Eaton, *Gilliesia* Peters and Edmunds and *Dipterophlebiodes* (Demoulin)] and approximately 60 described species, (ii) the highly diverse and largely southern hemisphere Atalophlebiinae Peters (Peters, 1980) with more than 100 described genera and upwards of 500 species, and (iii) the Habrophlebiinae Kluge (Kluge, 1994), inclusive of *Habrophlebia* Eaton and *Habroleptoides* Schoenemund with 22 species distributed in the Holarctic (primarily in North America and Europe). Characters that define the three subfamilies include differences in setation in nymphal mouthparts and wing and genitalic characters in adults; Atalophlebiinae is also characterized by the unique presence of square-shaped eye facets in males (Peters & Gillies, 1995). Work to date on leptophlebiid phylogenetics has focused on investigations of region-specific faunas including the Eastern Hemisphere, Australia, South America and New Zealand (Peters & Edmunds, 1964, 1970, 1972; Towns & Peters, 1996; Christidis, 2001, 2005).

In this work, we use two nuclear markers [the D2 + D3 region of *28S ribosomal DNA* (28S) and *histone H3* (H3)] and a geographically broad sampling of taxa to generate a molecular phylogenetic hypothesis of relationships for this diverse family of mayflies. Using the resultant phylogeny, we test the monophyly of Leptophlebiidae and assess support for generic-level groupings both within and across biogeographic realms. In doing so, we illuminate the utility of molecular data for taxonomic and phylogenetic efforts currently underway for mayflies.

Materials and methods

Taxon sampling

Leptophlebiid specimens used in this study included samples from North America, South America, Europe, South Africa, Madagascar, Australia, New Zealand, Malaysia, Papua New Guinea and Japan for a total of 30 genera plus 11 taxa of uncertain taxonomic rank collected from Madagascar and Papua New Guinea (Supporting Information ST1). When available, multiple species from species-rich lineages were included, resulting in a total of 69 species of leptophlebiids. Voucher material from specimens used in DNA extraction was deposited in the University of Connecticut Museum, Storrs (UCMS).

Outgroup choice

Outside of the Leptophlebiidae, ten species representing eight mayfly families were included: Baetidae (two species), Heptageniidae (1), Siphlonuridae (1), Ameletidae (1) Isonychiidae (1), Ephemerellidae (2), Polymitarcyidae (1) and Ephemeridae (2) (Supporting Information ST1). Outgroup taxa were used to assess leptophlebiid monophyly and to root the tree. Several hypotheses currently exist for the sister group of the leptophlebiids. The burrowing mayflies [infraorder Scapphodonta (McCafferty, 2004)] have been repeatedly hypothesized to be sister to the Leptophlebiidae (Edmunds, 1962; McCafferty & Edmunds, 1979; Landa & Soldan, 1985; McCafferty, 1991; Tomka & Elpers, 1991) based on a combination of nymphal and adult characters. Alternatively, Riek (1973) and Kluge (1998) postulated an association of the leptophlebiids with the Ephemerellidae, Tricorythidae and the burrowing mayflies using nymphal and wing venation characters, respectively. Ogden & Whiting's (2005) molecular cladistic analysis of family level relationships across all mayflies supported a sister group relationship between Leptophlebiidae and a large assemblage including the Scapphodonta, Caenoidea and Ephemerelloidea, although their results using maximum likelihood analyses were largely incongruent with the parsimony-based topology. More recently, analyses using 18S rDNA (Sun *et al*., 2006) have proposed a group consisting of Scapphodonta + Caenidae as sister to the Leptophlebiidae. We included representatives of all lineages hypothesized to be sister to the Leptophlebiidae in order to accommodate this uncertainty. Based on the results of Ogden & Whiting (2005), trees were rooted by specifying Baetidae [*Centroptilum triangulifer* + baetid sp. (an unidentified baetid nymph)] as the outgroup in all analyses. Baetidae is also the only sampled lineage not previously hypothesized to be part of the sister group of Leptophlebiidae.

DNA extraction and gene amplification

DNA was extracted from mayfly thoracic or abdominal muscle of nymphal or adult specimens preserved in >85% ethanol with either a CTAB-based method, modified from Murray & Thompson (1980) or a Nucleospin kit (BD Biosciences/Clontech, San Jose, CA). The D2 + D3 expansion regions of the 28S gene were amplified using four primers (3665F, 4413R, 3549F and 4749R) for 28S (Gillespie *et al*., 2004, 2005); H3 was amplified using HexAF and HexAR (Svenson & Whiting, 2004) under standard PCR conditions. 28S amplification was optimized by the addition of 10%

DMSO to the PCR reactions. Reactions were electrophoresed on 1% agarose gels; amplicons of the appropriate size were excised and DNA extracted from the gel. Clean products were cycle-sequenced using ABI BigDye version 1.1 (Applied Biosystems, Foster City, CA) with the addition of 5% DMSO and electrophoresed on an ABI3100 automated DNA sequencer (Applied Biosystems). For several taxa in which chromatograms of H3 showed double peaks, bands of the appropriate size were cloned (TOPO 10 kit; Invitrogen, Inc., Carlsbad, CA) and multiple clones were sequenced. All sequences were deposited in GenBank (see Supporting Information for accession numbers).

Sequence alignment and phylogenetic analyses

Chromatograms were examined in Sequencher (Gene Codes Corp., Ann Arbor, MI) and double peaks for the H3 marker were coded using standard ambiguity codes. Edited sequences were imported into MacClade v4.06 (Maddison & Maddison, 2000). For H3, alignment was straight forward as amino acid sequence was absolutely conserved across taxa. The initial phenetic alignment of the 28S fragment was constructed using Clustal-X v.1.82 (Thompson *et al*., 1994) and subsequently adjusted using secondary structure information obtained via MFOLD (Zuker *et al*., 1999). The secondary structure of 28S rRNA was inferred computationally using sequences from eight taxa chosen to represent the breadth of geographic and subfamilial diversity in the leptophlebiids. MFOLD-inferred structures were inspected for consistency of stem and loop regions across taxa. Stem regions were verified by locating base-pairing 'doublets' which correspond to complementary regions of hydrogen bonding along the stems of ribosomal DNA. Using paup* v.4.0b10 (Swofford, 2003), base composition and general time reversible (GTR) pairwise distances were calculated for each gene.

Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). ModelTest v.3.7 (Posada & Crandall, 1998) was used to choose an appropriate model of molecular evolution for each data partition used in likelihood or Bayesian analyses. Best-fit models were selected using the Akaike information criterion (AIC). Analyses were performed on individual loci and on a concatenated dataset to assess the effects of the markers on topology resolution. MP analyses were performed in paup* using a heuristic searching algorithm seeded with trees obtained via stepwise addition, tree bisection and reconnection (TBR) branch swapping and 10 000 random addition replicates with a maximum of five trees held at each replicate. To assess sensitivity of our results to the treatment of gaps in the 28S alignment, we employed two coding schemes: gaps coded as missing data or as a fifth character state. Although coding gaps as a fifth state performs well in simulation studies (Ogden & Rosenberg, 2007; Simmons *et al*., 2007), this may be in large part because it effectively upweights insertion/deletion events relative to substitutions (Simmons *et al*., 2007). Ambiguous (double-peak) sites in H3 were treated as polymorphisms under the multistate character option in paup*, as they resulted from variation across alleles or loci encoding H3. Strict consensus trees were constructed from all equally-parsimonious trees and branch support was assessed with (i) non-parametric bootstrapping with 1000 random addition replicates/bootstrap (BS) replicate ($n = 100$ replicates) and TBR branch swapping using paup* and (ii) decay indices calculated using TreeRot v.2c (Sorenson, 1999).

For ML analyses, heuristic searches in paup* used starting trees from ten random addition replicates and TBR branch swapping with parameters fixed to the ML estimates derived in paup*. ML analyses were also conducted using garli (Zwickl, 2006) and seeded with a (i) random tree, (ii) neighbour-joining tree generated in paup* and (iii) MP tree generated in paup* to explore garli's sensitivity to starting conditions in heuristic searches (Zwickl, 2006). garli searches were set to terminate if 10 000 generations passed without an improvement in the lnL score, and analyses were conducted multiple times on the same dataset to ensure robust results. Branch support was assessed in garli with 100 BS replicates when the number of generations allowed to pass without score improvement decreased to 5000.

Partitioned Bayesian estimates of tree topology were conducted in MrBayes v.3.12 (Huelsenbeck & Ronquist, 2001). Partitioning of molecular datasets has been shown to be particularly useful when nucleotide sites experience very different substitution rates across genes and gene regions (Nylander *et al*., 2004). A total of four partitions were recognized: 28S sequences were delineated into stem and loop regions while H3 was split into 1st + 2nd codon positions separate from third codon positions. For 28S stems, the doublet model in MrBayes was invoked to allow paired sites to evolve non-independently. The rate matrices, shape parameter, proportion of invariant sites and base frequencies were unlinked prior to running Bayesian searches. The rate prior was set to 'variable' to account for differences in rates of substitutions across partitions (reflected in the parameter 'm'). The mean of the exponential prior placed on branch lengths was estimated empirically (Yang & Rannala, 2005) based upon ML estimates of tree length, such that $\lambda = 1/\text{mean branch length}$ [see (Marshall *et al*., 2006) for a discussion of branch length priors in Bayesian analyses]. The prior for α (rate heterogeneity) was assigned an exponential distribution with a mean of 1. Markov Chain Monte Carlo (MCMC) searches were run for 5 million generations with two independent runs to ensure convergence. 50% consensus trees were constructed from the sampled parameters after the first 10% of values (corresponding to the burnin) were discarded. Parameter files were examined in Tracer (Rambaut & Drummond, 2007) to ensure that searches had reached stationarity and parameter estimates included the ML estimate, an indication that an appropriate portion of parameter space was searched.

Results

Characteristics of amplified loci

Several characteristics of the amplified loci for both ingroup and outgroup taxa are reported in Supporting Information ST2 along with the models of molecular evolution chosen by ModelTest for each partition. Guanidine-cytosine (GC) content in H3 was highly variable, especially at third codon positions. Base polymorphisms in H3 occurred in 64% of the included taxa and at most consisted of up to 4.6% of sites (in *Neochoroterpes nanita*) but more frequently occurred at levels of <1% (in 78% of included taxa). When sequences of individual H3 clones were included in phylogenetic analyses, they clustered by taxon. Consequently, analyses were conducted using direct rather than cloned sequences.

Alignment of the 28S sequences produced gaps ranging from 8.3 to 17.8% of the total alignment length for ingroup taxa and 3.3–11.8% across outgroup taxa. Indels occurred primarily in loops corresponding to putative regions of expansion and contraction (Gillespie *et al*., 2005) but the majority of loop structures were unambiguously alignable across ingroup taxa owing to relatively high sequence conservation across these regions. MFOLD-inferred secondary structures for the eight focal taxa suggest that the D2 region (sites 1–614 in the aligned dataset) is composed of three major helices and 32 smaller helical regions (10, 7 and 15 smaller helices per major helix) while the D3 region folds into 13 small helices. In the outgroup taxa, a small number of 28S sites for which alignment between ingroup and outgroup taxa was ambiguous were coded as missing. We explored the effects of coding gaps as a fifth character state in MP analyses and recovered phylogenies that were highly similar to the MP, ML and BI analyses in which they were coded as missing data.

Support for phylogenetic relationships

In order to ascertain support for the phylogenetic relationships of leptophlebiids, we consider the best-supported phylogenetic hypotheses to be the product of combined 28S + H3 analyses. As is conventional, MP/ML BS values >70% (Hillis & Bull, 1993) and posterior probabilities (pp) > 0.95 are interpreted as strong support for clades, while support was considered moderate when MP/ML BS values fell between 50 and 70% and pp between 0.85 and 0.95. We consider weakly supported clades to be those that are recovered in analyses but received MP/ML BS and pp values below 50% and 0.85, respectively. We consider high support values corroborated across different phylogenetic inference methods as the most robust indication of well-supported clades.

Monophyly of Leptophlebiidae

Phylogenetic analyses did not return the Leptophlebiidae as a clade; instead, the combined dataset analyses consistently returned topologies with the two ephemerellid taxa nested within Leptophlebiidae. In the combined MP tree, the two ephemerellid taxa were resolved as sister to *Adenophlebia sylvatica* Crass, but the placement of the ephemerellids within Atalophlebiinae did not receive strong bootstrap support (<50%, Fig. 1). MP searches constrained to recover a monophyletic Leptophlebiidae found trees just one step longer than the MP trees from the unconstrained search. The combined BI analysis placed the ephemerellids as more closely related to the habrophlebiines and atalophlebiines than either of these was to the leptophlebiines. Although this clade received weak support (pp = 0.59, Fig. 2), the low support resulted from uncertainty about where within the Leptophlebiidae the Ephemerellidae were placed, rather than from conflicting signal for a monophyletic Leptophlebiidae. None of the trees in the Bayesian posterior distribution included a monophyletic Leptophlebiidae. In the combined ML tree, the two ephemerellids were placed in a polytomy containing all leptophlebiids except Habrophlebiinae (Fig. 3) with weak support (BS < 50%). Analyses of individual genes also never returned a monophyletic Leptophlebiidae; 28S topologies (Fig. 4) were largely congruent with those obtained in combined analyses (Figs 1–3) although none of the deep branches received substantial support in any of the single gene analyses (BS < 50%).

The inclusion of Ephemerellidae within Leptophlebiidae is unexpected based on previous hypotheses of relationships. Therefore, to more fully explore the effects of the Ephemerellidae on our results, we performed phylogenetic analyses including five additional taxa of ephemerellids obtained from GenBank [*Hyrtanella*: AY750012, *Ephemerella*: AY 749919, *Attenella*: AY749986, *Caudatella*: AY749962 and *Drunella*: AY749915 (Ogden & Whiting, 2005)] as well as analyses removing all ephemerellids from the alignment. MP and BI phylogenies using the expanded dataset did not remove the ephemerellids from the ingroup, instead rendering a polytomy including most of the Atalophlebiinae plus Ephemerellidae (MP) or else returning a topology indistinguishable from our original taxon sampling (BI) although not altering relationships among leptophlebiid taxa. Removal of all ephemerellids and reanalysis of our dataset returned an ingroup topology indistinguishable from our original analyses in both MP and BI approaches. Removal of the ephemerellid taxa coupled with similar topologies across analyses (i.e. Ephemerellidae associated with Leptophlebiidae) suggests that there is a phylogenetic signal in the dataset allying Ephemerellidae with Leptophlebiidae. Irrespective of the precise placement of Ephemerellidae, leptophlebiid relationships (i.e. Leptophlebiinae, Atalophlebiinae and Habrophlebiinae and groups therein) were consistently resolved across analytical methods and were not impacted by the inclusion or exclusion of the ephemerellid taxa.

The placement of other non-leptophlebiids was consistent with previous hypotheses based on both molecular and morphological data. We recovered a cluster uniting three species of burrowers with strong support, and a clade uniting *Ameletus*, *Siphlonurus* and *Isonychia* with moderate support.

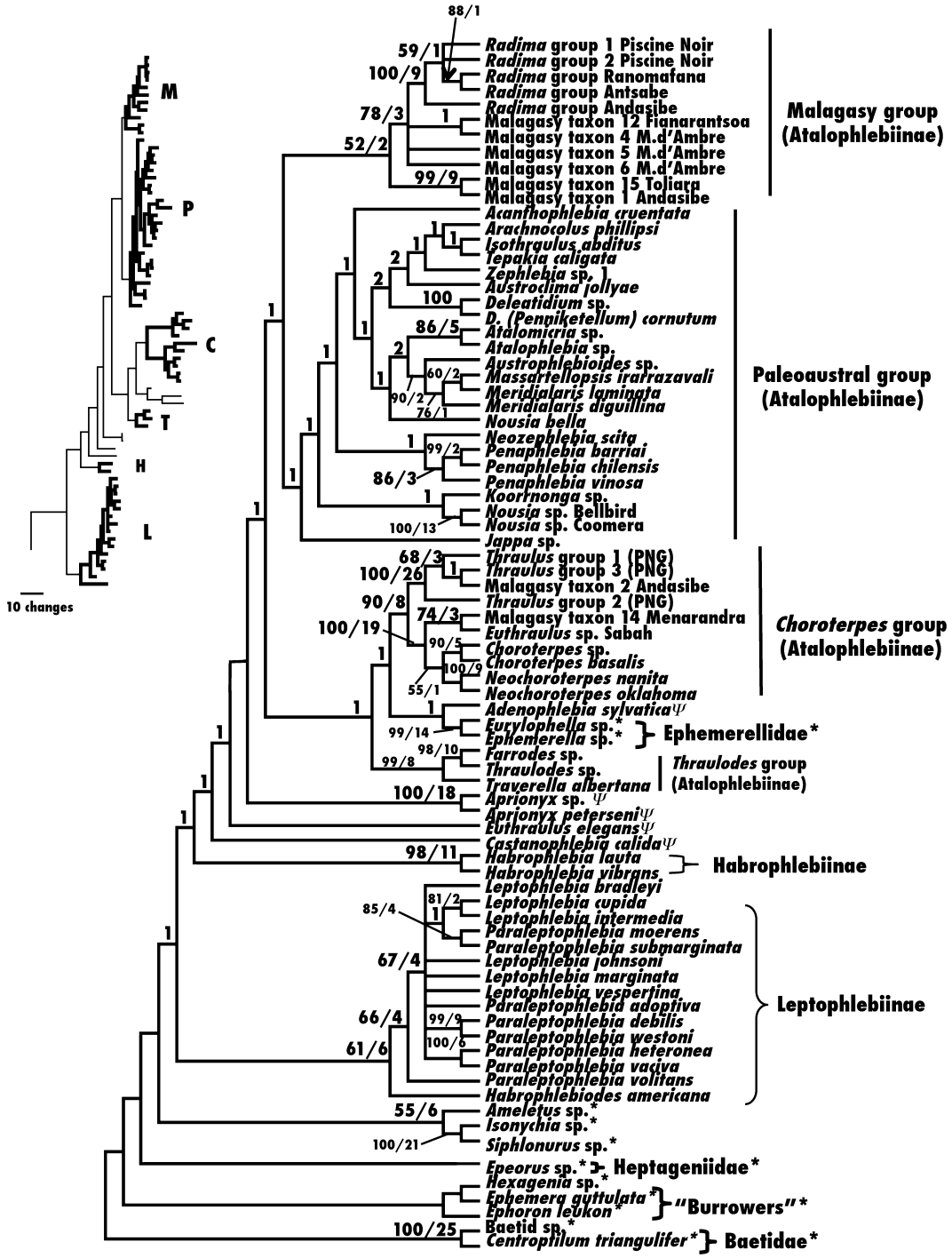

Fig. 1. Cladogram showing the strict consensus of 11 maximally parsimonious trees (MPTs) from the analysis of the H3 + 28S combined dataset (2431 steps, CI = 0.387, RI = 0.613). One MPT phylogram is shown to the left to illustrate branch lengths; outgroups (except Ephemerellidae) have been pruned from the inset phylogram for clarity. Parsimony bootstrap percentages above 50% (BS) and decay indices (DI) indicated on branches (BS/DI). Groups named as in the results. * denotes non-leptophlebiid taxa. ψ denotes atalophlebiine taxa that consistently fall outside of the four named groups.

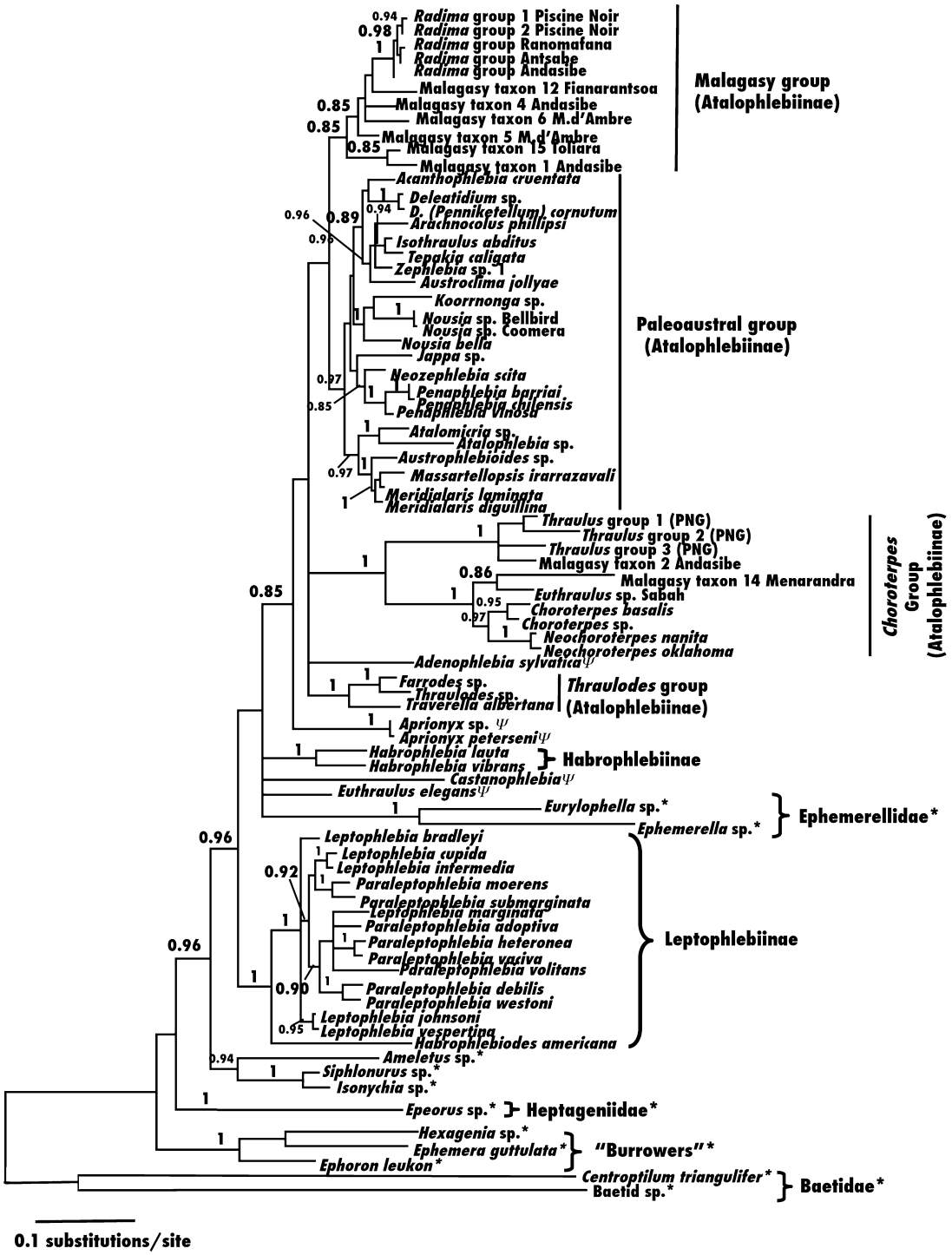

Fig. 2. Consensus phylogram resulting from partitioned Bayesian analysis of the combined H3 + 28S dataset (-LnL = 12617.31). Posterior probabilities greater than 0.85 indicated above branches. Groups named as in the results. Taxa labelled as in Fig. 1.

Subfamilial relationships within Leptophlebiidae

Leptophlebiinae, represented by eight species of *Paraleptophlebia* and six species of *Leptophlebia* and *Habrophlebiodes americana* was recovered with moderate to strong support across analyses (Figs 1–3). The two habrophlebiines, representing one of two genera, clustered with strong support in all analyses (Figs 1–3). Although support for the monophyly of Leptophlebiinae and Habrophlebiinae was strong, Atalophlebiinae was not recovered as monophyletic as a result of

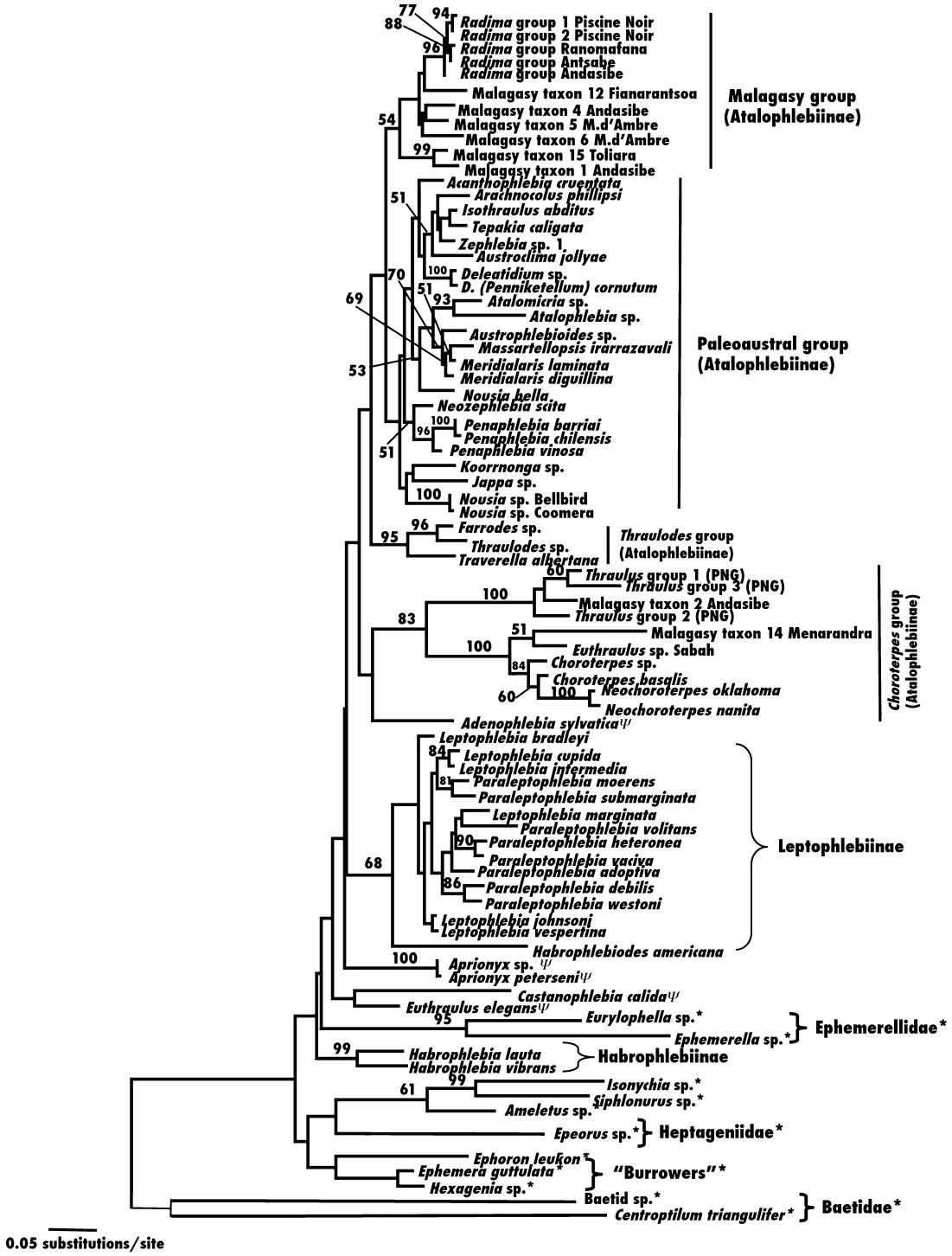

Fig. 3. Phylogram showing the single most likely tree (-LnL = 13016.31) resulting from analysis of the H3 + 28S combined dataset (PAUP*). Likelihood bootstrap percentages above 50% shown on branches. Taxa labelled as in Fig. 1.

the nesting of the ephemerellid taxa or the leptophlebiine clade within it, dependent upon analysis; a clade containing a large subset of the Atalophlebiinae received moderate support (pp = 0.85) in the Bayesian analysis.

None of the deeper level structure of leptophlebiid relationships at the level of the subfamilies received strong support in any analysis. Relationships among subfamilies were not well resolved: MP and BI combined analyses

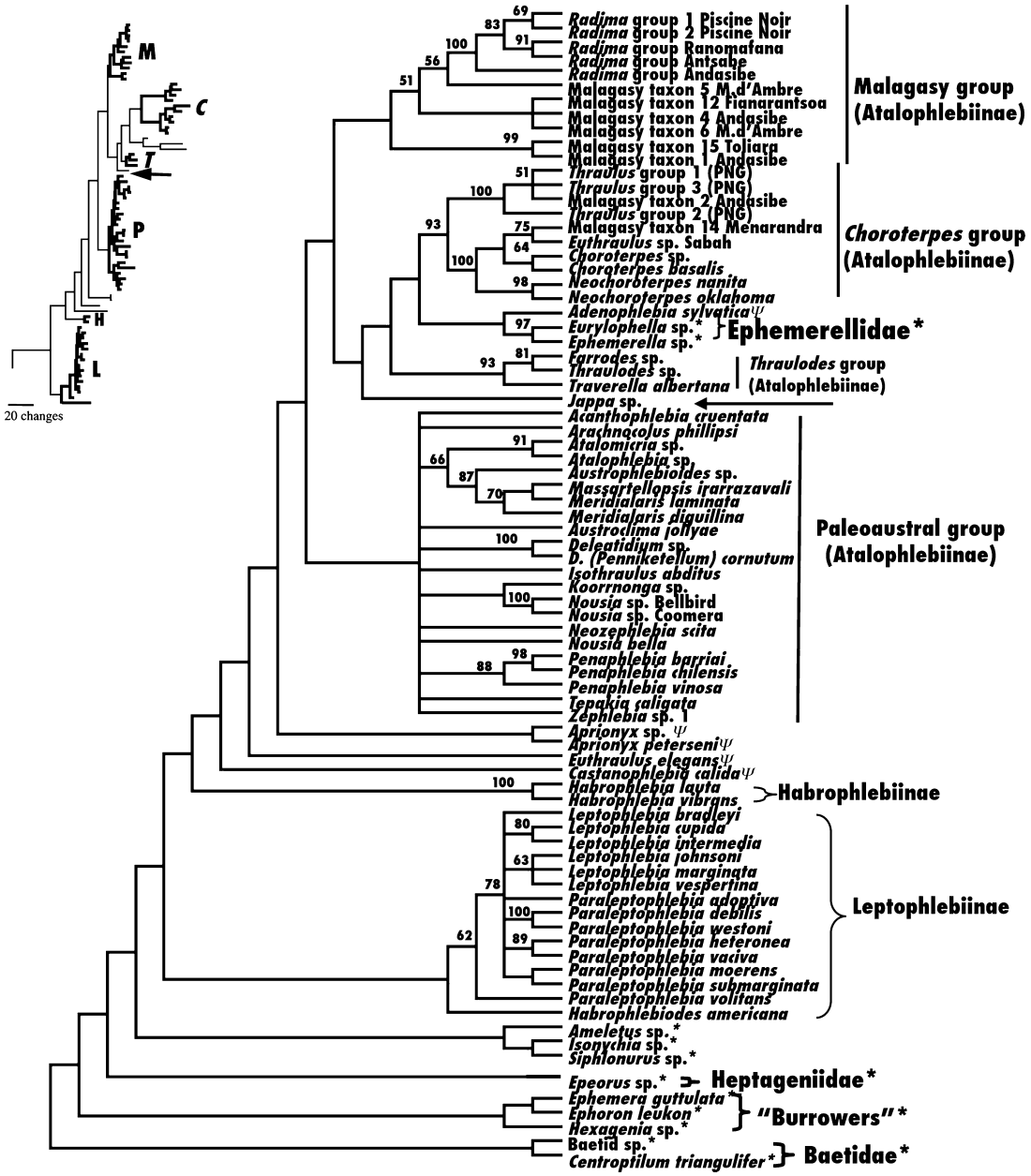

Fig. 4. Strict consensus of 80 MPTs (1859 steps, CI = 0.417, RI = 0.659) and one MPT phylogram (inset) from analysis of the 28S dataset; outgroups (except Ephemerellidae) have been pruned from the inset phylogram for clarity. Parsimony bootstrap percentages above 50% shown on branches. Arrows indicate the placement of *Jappa* sp. outside of the Paleoaustral group. Taxa labelled as in Fig. 1.

recovered a monophyletic Habrophlebiinae + Atalophlebiinae to the exclusion of Leptophlebiinae (Figs 1, 2), whereas ML recovered Leptophlebiinae as nested within Atalophlebiinae to the exclusion of Habrophlebiinae (Fig. 3).

Phylogenetic relationships within the Leptophlebiinae

Within the Leptophlebiinae, *Habrophlebiodes americana* was consistently resolved as sister to a clade containing all species of *Paraleptophlebia* and *Leptophlebia*. This cluster of *Paraleptophlebia* spp. and *Leptophlebia* spp. received moderate support (Figs 1–3); each of these genera was always rendered paraphyletic in all combined analyses (Figs 1–3). Consequently, the monophyly of each of these genera is not supported by this molecular dataset.

Phylogenetic relationships within the Atalophlebiinae

We consistently recovered four atalophlebiine subgroups that we call the Malagasy, Paleoaustral, *Choroterpes* and

Thraulodes groups and supported many relationships within these groups. Two of these subgroups (*Thraulodes* and *Choroterpes* groups) are presented for the first time in this work and are named on the basis of the oldest genus contained within the grouping. Relationships between these four subgroups were not well resolved, although the Malagasy and Paleoaustral groups were recovered as sister groups in all analyses (with weak to moderate support). These groups contained all atalophlebiine taxa sampled except for four genera from South Africa: *Aprionyx* (two spp.), *Adenophlebia sylvatica*, *Euthraulus elegans* and *Castanophlebia calida*. These four genera each had relatively long branches in all analyses and their phylogenetic placement was not well resolved (Figs 1–3). In addition, *Euthraulus* was not recovered as a monophyletic genus. *Euthraulus* sp. from Sabah was placed within the *Choroterpes* group while the placement of *Euthraulus elegans* from South Africa was unresolved (see above) but never closely linked with the *Choroterpes* group (Figs 1–3).

The Malagasy group, which includes eleven of the thirteen taxa sampled from Madagascar, received moderate support. Within this group, the *Radima* group includes a cluster of morphologically similar taxa that received strong support. Additionally, a strongly supported sister group relationship between two taxa not assigned to genus (Malagasy taxon 1 and Malagasy taxon 15) was consistently returned.

The Paleoaustral group includes 22 genera with an amphinotic distribution in southern South America, New Zealand and Australia. This group is strongly supported in BI analyses and was recovered with weak support in MP and ML analyses. Many relationships within this group are well resolved (Figs 1–3), and include several instances of transcontinentally distributed clades. For instance, *Austrophlebioides* (Harker) (Australia) is strongly supported to be sister to a clade containing the Chilean lineages *Massartellopsis irrarrazavali* Demoulin and two species of *Meridialaris* Peters and Edmunds from South America, and this clade is sister to the clade formed by *Atalomicria* Harker and *Atalophlebia* Eaton, two Australian endemics. The three species of *Nousia* Navas were not recovered as a monophyletic group in any analysis; instead, the Australian *Nousia* was suggested to be more closely related to *Koorrnonga* Campbell and Suter, an Australian endemic, than to the South American species *N. bella* Pescador and Peters (Figs 1–4). In a single instance, *Jappa*, an Australian endemic, was resolved as outside the Paleoaustral group (in the 28S MP topology, Fig. 4).

A clade containing *Choroterpes* spp., *Neochoroterpes* spp., *Euthraulus* and taxa from Papua New Guinea and Madagascar, which we call the *Choroterpes* group, received strong support across analyses. Within the *Choroterpes* group, three taxa from Papua New Guinea (*Thraulus* group 1, 2 and 3) and one from Madagascar (Malagasy taxon 2) form a strongly supported clade. Malagasy taxon 14 and *Euthraulus* sp. (Sabah) also cluster with moderate support, and they were resolved as sister to a strongly supported group containing *Choroterpes* and *Neochoroterpes*.

The *Thraulodes* group, restricted to the western hemisphere, includes *Thraulodes* Ulmer, *Traverella albertana* Edmunds and *Farrodes* Peters and received strong support in all analyses. Within this group, *Farrodes* sp. and *Thraulodes* sp. were strongly supported as most closely related.

Base composition bias in H3 and 28S

Base composition varied by data partition, and for the H3 marker the base composition bias varied across taxa, specifically at third codon positions. Enrichment of the GC content of the both markers was particularly increased in the third codon positions of H3 and in the stem regions of 28S (Supporting Information ST2). Analyses of H3 alone (phylogenies not shown) returned several anomalous relationships that were strongly rejected by the 28S and combined datasets, and appeared to be supported by convergent evolution of GC content. Accommodating changing base composition bias using LogDet distances (Lockhart *et al.*, 1994) and the minimum evolution (ME) method did not aid in the resolution of the groups contained therein for either gene (trees not shown). ME, MP, ML and BI phylogenies of H3 alone recovered only the Leptophlebiinae clade and some detail of fine-scale structure of relationships, much of which was shared across analyses (e.g. the three *Penaphlebia* spp. grouping together). LogDet ME trees for the 28S marker alone returned a topology with a monophyletic Leptophlebiidae, but excluding *H. americana*. In addition, 28S LogDet ME trees recovered the Paleoaustral group, the *Thraulodes* group and Habrophlebiinae but did not recover the Malagasy group, the *Choroterpes* group or Leptophlebiinae. The major changes in deep relationships that occurred when LogDet distances were used raises the possibility that changes in base composition may be obscuring some phylogenetic signal.

Discussion

Although the present analyses failed to support the monophyly of the Leptophlebiidae, six groups were consistently recovered that contained 65 of the 69 sampled leptophlebiids: Leptophlebiinae, Habrophlebiinae, and four subgroups in Atalophlebiinae: the *Choroterpes* group, *Thraulodes* group, Malagasy group and Paleoaustral group. Overall, these groups are in agreement with several traditional hypotheses of related clusters of genera within Leptophlebiidae based on morphology. Below, we discuss the relationship between the Ephemerellidae and Leptophlebiidae, the recovery of several clusters of genera in line with traditionally held ideas and occurrence of several novel groupings of taxa that are consistent with the morphological and distributional evidence.

Leptophlebiidae

The finding of a paraphyletic Leptophlebiidae is surprising and worthy of continued investigation. As unconstrained

MP searches found trees just one step longer than trees recovering a monophyletic Leptophlebiidae, MP results do not reject the hypothesis of a monophyletic Leptophlebiidae. On the other hand, none of the trees in the Bayesian posterior recovered a monophyletic Leptophlebiidae, strongly rejecting the hypothesis. Most previous analyses of molecular data that sampled multiple leptophlebiids also failed to find support for monophyly. The exception is the study of Ogden & Whiting (2005), who recovered a monophyletic Leptophlebiidae in their equally weighted MP analysis using POY (Phylogenetic Analysis of DNA and other Data using Dynamic Homology), which was sister to a large group containing both the burrowing mayflies and Ephemerellidae (in addition to other members of Pannota). However, as in this study, their ML analysis failed to support monophyly of the Leptophlebiidae, but in their case, this was as a result of an unresolved trichotomy between Paraleptophlebia, all other members of Leptophlebiidae ($n = 7$) and a large clade containing numerous families but excluding Ephemerellidae and the burrowing mayflies. Recently, Ogden *et al*. (2008) expanded and re-analyzed the 18S rDNA molecular dataset of Sun *et al*. (2006) and did not resolve a monophyletic Leptophlebiidae, although ephemerellid taxa were not the culprits in rendering Leptophlebiidae paraphyletic in this instance either. Taken together, molecular work to date is insufficient to resolve the question of leptophlebiid monophyly. Resolution of this question awaits an expanded dataset and further investigation of possible causes of homoplasy.

Our results suggest a close relationship between the Leptophlebiidae and Ephemerellidae. Historically, the Leptophlebiidae was placed within the superfamily Leptophlebiodea, along with Ephemerellidae and Tricorythidae (Edmunds & Traver, 1954). More recently, McCafferty & Edmunds (1979) placed the Leptophlebiidae and Ephemerellidae into two distinct suborders, Schistonota (Leptophlebiodea) and Pannota (Ephemerelloidea), yet most phylogenetic classification schemes have envisioned a close relationship between Leptophlebiidae, Ephemerellidae and Tricorythidae (Riek, 1973; Kluge, 1998; Ogden & Whiting, 2005). Edmunds & Traver (1954) were not explicit about which characters united the leptophlebiids with the ephemerellids and tricorythids, but Riek (1973) linked these families on the basis of whorls of hairs on the caudal filaments, a flattened nymphal habitus, reduced venation in the anal fan of the forewings and a posteriorly curved CuP vein in the forewings. Thus, the recovery of a close relationship between Ephemerellidae and Leptophlebiidae has some support from morphological data.

Leptophlebiinae

Monophyly of the Leptophlebiinae is supported by a suite of mouthpart characters and an elongate and deeply cleft ninth sternum in adult females (Peters & Edmunds, 1970; Peters, 1980; Kluge, 1994) relative to that of the atalophlebiines. However, Kluge (1994) contended that the original designation of Leptophlebiinae was in fact paraphyletic as a result of the inclusion of plesiomorphic characters. In our analyses, we included three out of the six genera in Leptophlebiinae and monophyly is moderately to strongly supported. Inclusion of the three other genera (*Gilliesia, Dipterophlebiodes* and *Calliarcys*) will be necessary to more rigorously test monophyly.

Within the leptophlebiines, a sister group relationship between *H. americana* and a clade containing *Paraleptophlebia* and *Leptophlebia* was consistently recovered. This association is supported by the structure of the styliger plate in adult females and the structure of maxillary brushes (Peters & Edmunds, 1970). *Paraleptophlebia* species and *H. americana* have very similar nymphal morphology, but *H. americana* is prognathous whereas *Paraleptophlebia* is hypognathous. Additionally, detail of forewing venation and the extent of development of the cubital region of the hind wings differ substantially between *H. americana* and *Paraleptophlebia* spp. (Peters & Edmunds, 1970). The genus *Habrophlebiodes* has a disjunct distribution, and is known from only four species in North America and an additional four in the Oriental realm. Whether or not these geographically disparate taxa share close evolutionary ties is of interest for future work.

Interestingly, the genera *Paraleptophlebia* and *Leptophlebia* are never resolved as distinct clades. Historically, the *Paraleptophlebia* + *Leptophlebia* complex has been posited as an ancient and highly plesiomorphic cluster within the leptophlebiines (Peters & Edmunds, 1970). In some treatments, the genus *Paraleptophlebia* has been assigned subgeneric ranking within *Leptophlebia*, owing to strong similarities in morphology among species in these groups (Kluge, 1997). *Leptophlebia* are differentiated from *Paraleptophlebia* based on the basal position of the CuP vein in the forewings as well as the fusion of the penes at the proximal-most point and the presence of long thin appendages off the apex of the penes (Burian, 2001). Nymphal characters distinguishing *Leptophlebia* and *Paraleptophlebia* centre on the size and shape of the abdominal gills (Burian, 2001), with *Leptophlebia* having bilamellate gills and *Paraleptophlebia* having deeply forked gills. However, variation in nymphal gills in these two genera has been documented dependant upon developmental stage and habitat characteristics (Burian, 2001). Although our data raise the possibility that species in the genus *Paraleptophlebia* should be transferred to *Leptophlebia*, we refrain from making formal taxonomic changes pending denser taxon sampling and the inclusion of more rapidly evolving molecular markers.

Habrophlebiinae

The two *Habrophlebia* species included in this study (Habrophlebiinae, in part) were strongly supported as sister taxa using molecular characters. These Holarctic taxa are united morphologically on the basis of several aspects of adult morphology: claw structure, the presence

of a costal projection and venational characteristics in the hind wings and structural detail of the subimaginal exuviae and a deeply cleft subanal plate in females (Kluge, 1994). Precedent for the Habrophlebiinae as a distinct group (preceding Kluge's work) is found in Peters & Edmunds (1970) phylogeny of eastern hemisphere leptophlebiids in their daughter line IA2, an association of *Habroleptoides* and *Habrophlebia* (along with the genus *Calliarcys*, a non-habrophlebiine) on the basis of a weakly developed costal projection off of the hind wings in combination with prognathous nymphs. As Habrophlebiinae also includes *Habroleptoides* (unsampled in this work and including 16 described species worldwide), a rigorous assessment of the strength of monophyly of Habrophlebiinae awaits inclusion of this genus.

Atalophlebiinae

Atalophlebiinae is united by the possession of square facets in the dorsal portion of the eyes of adult males (Peters & Gillies, 1995), a trait unique among hexapods, as well as by leg and styliger plate characters (Peters, 1980; Kluge, 1994) and a suite of nymphal mouthpart characters [e.g., patterning and arrangement of hairs and setae and shape/emargination of the labrum (Peters, 1980)]. While our molecular data failed to support the monophyly of Atalophlebiinae, this lack of support reflects uncertainty about relationships rather than support for an alternative hypothesis. However, molecular characters in the present study offer support for several groupings of genera within this subfamily, including the Malagasy, Paleoaustral, *Choroterpes* and *Thraulodes* groups.

Malagasy group

Within the Malagasy group, two separate clusters were resolved with strong support, despite only weak to moderate support for the entire group. The *Radima*-like group members (five *Radima* group specimens from Ranomafana, Andasibe, Antsabe and Piscine Noir regions of Madagascar) have very similar abdominal gill morphology but fewer denticles on the labrum and longer labial palps compared with described species of *Radima*. A strongly supported sister group relationship between Malagasy taxon 1 and Malagasy taxon 15, two species that differ greatly in mouthpart morphology, was also consistently returned.

Paleoaustral group

The leptophlebiid faunas of southern South America (SA), southern Africa (SAf), Madagascar, Australia (AU) and New Zealand (NZ) have historically been postulated to have close affinities (Penniket, 1961; Peters & Edmunds, 1964, 1972; Tsui & Peters, 1975; Pescador & Peters, 1980; Peters, 1980; Towns & Peters, 1980, 1996) and this association is supported by our molecular data. For taxa spanning AU, NZ and southern South America, Pescador & Peters (1980) delimited five lineages of related genera based on nymphal and adult characters: the *Meridialaris*, *Nousia*, *Hapsiphlebia* Peters and Edmunds, *Penaphlebia* and *Dactylophlebia* Pescador and Peters lineages, with each lineage composed of a cluster of Gondwanan genera. Two more recent studies (Christidis, 2001, 2005) explored the Australian-southern South American faunal connection with a morphology-based phylogenetic analysis using representative genera from both regions.

Christidis's work strongly supported the *Meridialaris* lineage of Pescador & Peters (1980) [=*Austrophlebioides* (AU), *Tillyardophlebia* Dean (AU), *Kirrara* Harker (AU), *Meridialaris* + *Massartellopsis* (SA), *Deleatidium* (NZ) and *Atalophlebioides* Phillips (NZ)]. Our molecular phylogeny provides moderate support for a clade containing three of the four genera sampled (*Austrophlebioides* + *Meridialaris* + *Massartellopsis*); however, the genus *Deleatidium* was never associated with the *Meridialaris* lineage using molecular characters (Figs 1–4) and instead was included in a large clade of New Zealand genera, suggestive of a more recent, intra-island diversification of the New Zealand atalophlebiines.

Christidis's morphological phylogenies (2001, 2005) also supported the *Nousia* lineage of Pescador & Peters (1980) (=*Nyungara* Dean, *Nousia* and *Koorrnonga*), a group that shares hind wing venation and the broad emargination of the labrum with denticles. In the present analyses, the genus *Nousia* was never resolved as monophyletic as a result of the inclusion of *Koorrnonga* sp., another member of the *Nousia* lineage of Pescador and Peters. Interestingly, the genus *Koorrnonga* was initially erected for several described species of *Nousia* and *Atalonella* Needham and Murphy (Campbell & Suter, 1988). Campbell & Suter (1988) also erected the subgenus *Australonousia* to include the Australian species of *Nousia*. Both studies are suggestive of the measure of distinctiveness between the South American and Australian members of *Nousia*.

The *Hapsiphlebia* lineage of Pescador & Peters (1980) contains the genera *Hapsiphlebia* (SA), *Atalophlebia* (AU), *Atalomicria* (AU), *Jappa* (AU), *Kalbaybaria* Campbell (AU), *Ulmerophlebia* Demoulin (AU), *Acanthophlebia* Towns (NZ) and *Aprionyx* (SAf). An alliance of these taxa was not recovered in any of our molecular analyses or in Christidis's (2005) work using morphological characters. Our topologies split members of the *Hapsiphlebia* lineage into three distinct lineages nested within the Paleoaustral group (Fig. 5). The sister group status of *Atalophlebia* sp. and *Atalomicria* sp. is strongly supported by molecular characters; this relationship was recovered but poorly supported in Christidis's (2005) morphological phylogeny. Several morphological traits, including the structure of mandibular denticles and possession of abdominal terga fringed with setae along their lateral edges (Pescador & Peters, 1980), unite these taxa.

The *Penaphlebia* lineage of Pescador & Peters (1980) is restricted to South America and Australia and includes three genera: *Penaphlebia*, *Massartella* and *Garinjuga*

Campbell and Suter. Our taxon sampling did not permit adequate testing of the validity of this lineage; however, *Neozephlebia* Penniket (a genus of uncertain affinity, once allied with the *Nousia* lineage) was consistently found to be sister to the *Penaphlebia* species included in the present study (Figs 1, 2, 4).

Austroclima Towns and Peters was the sole member of the *Dactylophlebia* lineage included; this taxon nested with other New Zealand endemics in a distinct cluster of related genera from New Zealand (Fig. 5). In Towns & Peters' (1996) phylogeny of the New Zealand Leptophlebiidae, an association between the genera *Isothraulus* Towns and Peters and *Tepakia* Towns and Peters (Lineage D) was proposed on the basis of elongate and tubular penes, and these genera were consistently resolved as sister taxa in the present analyses. This finding suggests that similarities in gill morphology of *Isothraulus abditus* and species in the genus *Thraulus* are likely to result from convergence, as *I. abditus* was consistently resolved as nested deeply within the Paleoaustral group.

Towns & Peters' (1996) Lineage C allies *Arachnocolus* Towns and Peters (NZ), *Zephlebia* Penniket (NZ), *Austronella* Towns (NZ), *Demoulinellus* Pescador and Peters (South America) and several New Caledonian genera. Our analyses included two New Zealand representatives, *Arachnocolus phillipsi* and *Zephlebia* sp., which formed a polytomy with a clade containing two other New Zealand species, *Isothraulus abditus* and *Tepakia caligata* (Lineage D) (Fig. 5). Our taxon sampling of New Zealand leptophlebiid genera included eight of the 12 described genera. All but *Neozephlebia scita* (Walker) form a clade in all analyses, but this clade only received support in the Bayesian analysis (pp = 0.89). Based upon morphological characters, the remaining four unsampled genera share a moderately cleft to entire ninth sternum in female adults and are thought to be deeply nested within this group (Towns & Peters, 1996). The grouping of the New Zealand taxa together as distinct from other members of the Paleoaustral group suggests that the New Zealand fauna has been evolving independently of other southern hemisphere faunas for longer than previously hypothesized (Fig. 5).

Pescador & Peters (1980) suggested that the closest biogeographical ties for southern hemisphere leptophlebiids were between southern South American and Australian taxa. Our results support the South America to Australia faunal connection in several instances. For example, the *Nousia* and *Meridialaris* lineages and the *Hapsiphlebia* + *Penaphlebia* cluster (Fig. 5) illustrate presumably ancient connections between the leptophlebiid faunas of Australia and South America.

Choroterpes group

There is historical precedence for the *Choroterpes* group that received strong support across analyses (Figs 1–3).

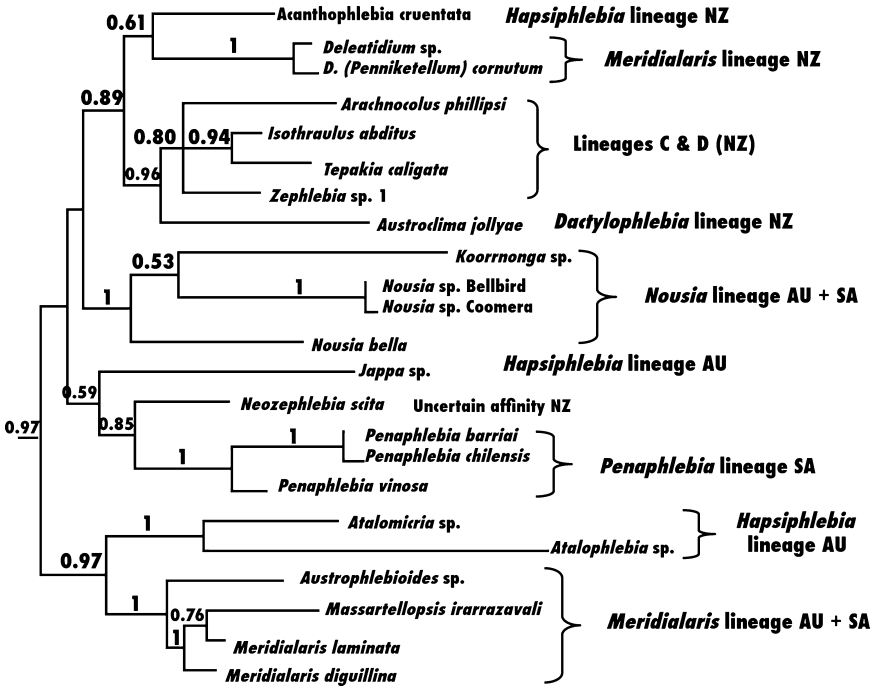

Fig. 5. Paleoaustral group, from Bayesian analysis shown in Fig. 2, with lineages marked after Peters & Pescador (1980) and Towns & Peters (1996). Values along branches refer to Bayesian posterior probabilities. *Neozephlebia scita* has unknown affinities, but has been linked alternately to the *Nousia* lineage or the fauna of New Caledonia which was not sampled in this study. AU = Australia, NZ = New Zealand, SA = South America.

First, the genera *Choroterpes*, *Neochoroterpes* and *Euthraulus* have been united under a variety of different scenarios since the mid 1970s (Gillies, 1957; Allen, 1974; Burian, 1995; Henry, 1995). In addition, the gills of *Choroterpes* spp. and *Euthraulus* spp. are very similar, so much so that Allen (1974) recognized *Euthraulus* as a subgenus of *Choroterpes*. Likewise, the genus *Neochoroterpes* was until recently recognized as a subgenus of *Choroterpes* (Henry, 1995). Gillies (1957) considered *Thraulus* and *Euthraulus* to be synonoyms on the basis of adult morphology in his work on specimens collected from East Africa and Asia; our molecular phylogenies support the distinctiveness of these genera. Peters *et al*. (1964) united members of this group, at the time known only from the Old World, on the basis of reduced venation in the wings, tubular and divided penes and mouthpart, gill and leg features. Since that time, several of these genera have been found to have cosmopolitan distributions [*e.g*., *Thraulus* (Grant & Peters, 1993)] and thus it seems likely that this is a large and geographically widespread clade. The *Choroterpes* group recognized here includes taxa from Papua New Guinea, Madagascar and North America. Two deeply differentiated and strongly supported groups were resolved in this clade: a strictly Old World group (Malagasy and Papua New Guinea) and a geographically widespread group comprising taxa from Madagascar, southeast Asia and North America. In terms of geographic sampling, it will be of interest to include the Chinese endemic *Cryptopenella* Gillies (a subgenus of *Choroterpes* with gills very reminiscent of *Euthraulus*) (Zhou, 2006) in future analyses.

Biogeographically, the alliance of leptophlebiids from Papua New Guinea with both Malagasy and African taxa is corroborated by work on Afrotropical baetids (Gillies, 1957; Monaghan *et al*., 2005). Several sister group relationships in the phylogeny of the Baetidae from this region were found to link Malagasy/South African and southern Asian genera, implicating cross-oceanic connections (Monaghan *et al*., 2005). In leptophlebiids, two groups [Malagasy taxon 14 + *Euthraulus* sp. (Sabah) and Malagasy taxon 2 + *Thraulus* group taxa] suggest links between southeast Asia and Madagascar, as opposed to a strictly mainland Africa connection for the Malagasy leptophlebiids. Malagasy taxon 14 has three-pronged abdominal gills reminiscent of that of *Euthraulus* spp. and *Choroterpes* spp. and is sister to *Euthraulus* sp. (Sabah) in all combined analyses (Figs 1–3).

Thraulodes group

The *Thraulodes* group (a cluster of *Thraulodes* sp., *Farrodes* sp. and *Traverella albertana*) received strong support across analyses, as did a sister group relationships between *Thraulodes* and *Farrodes* (Figs 1–3). Although not previously hypothesized based on morphology, this novel assemblage is strongly supported by the molecular data. This grouping is additionally supported by the similarity of maxillary palps in *Thraulodes* spp. and *Farrodes* spp. The genus *Thraulodes* has been allied with the *Meridialaris* lineage of Pescador & Peters (1980) on the basis of spines on the penes and setae on the foretibia (Flowers & Dominguez, 1991); however, phylogenetic placement of *Thraulodes* within the Leptophlebiidae has been clouded by the high degree of morphological variation reported for the genus (Allen & Brusca, 1978). Based upon morphological characters, the genus *Traverella* is thought to belong to the *Hermanella* complex, which has distant affinities to the *Meridialaris* lineage (Flowers & Dominguez, 1991). However, our molecular work does not recover a close relationship between the *Thraulodes* group and *Meridialaris* in any analyses. *Farrodes* has been hypothesized to be the sister lineage of several of the two-winged atalophlebiines of South America: *Homothraulus* Demoulin, *Simothraulopsis* Demoulin, *Perissophlebiodes* Savage and *Bessierus* Thomas and Orth (Dominguez, 1999); none of the two-winged atalophlebiines were sampled in our study.

The three genera in the *Thraulodes* group are restricted to the western hemisphere and are part of a fauna extending from North America to southern South America (McCafferty, 1998). Our current understanding of the relationships among Neotropical leptophlebiids is poor (Dominguez, 1999) and the taxon sampling for this region in the current work is limited. Thus, increased taxon sampling in the *Thraulodes* group, particularly taxa from northern South America, Central America and the West Indies is needed.

Atalophlebiine taxa of uncertain affinity

We sampled five southern African species of leptophlebiids in four genera, and while the two species of *Aprionyx* were strongly supported as sister taxa, the remaining genera were not inferred to be closely related to each other or to any of the other identified atalophlebiine subgroups (Figs 1–3). The deep divergences of the South African leptophlebiids, both from each other and from other included taxa, suggest the need for more work in this region. On the basis of morphology, *Castanophlebia* was hypothesized to comprise part of the deepest split within the Atalophlebiinae (along with the *Terpides* lineage, which includes *Terpides*, *Fittkaulus* and a number of undescribed genera from northern South America) (Peters, 1997). Our molecular data show a high level of divergence at the D2 + D3 region of 28S (11.7–24% between *Castanophlebia calida* and all other leptophlebiids, and including several indels that are autapomorphic with respect to all other included taxa), consistent with an ancient divergence *Castanophlebia* from other atalophlebiines. The *Terpides* lineage was unsampled in this study but is of interest for future work.

Adenophlebia and *Aprionyx* have been hypothesized to have affinities with the southern South American, Australian and New Zealand leptophlebiid faunas (Peters & Edmunds, 1964) on the basis of wing venation, fused penes, plate-like gills and mouthpart morphology. Based on morphology, these two genera have also been linked as sister taxa on the basis of maxillary characters (Peters & Edmunds, 1970). Our work supports the placement of these

genera within the Atalophlebiinae, but does not further resolve their relationships.

Conclusions

The molecular phylogeny of Leptophlebiidae presented herein expands our knowledge of the relationships of this speciose mayfly group across its global distribution, and highlights the need for additional work on this group. In particular, the question of whether or not Leptophlebiidae is monophyletic requires further exploration, with more extensive sampling of characters and taxa, including the Ephemerellidae (including *Melanemerella* sp.) and Leptophlebiidae (particularly Neotropical lineages) as well as other families of pannote mayflies closely related to Ephemerellidae.

Despite the question regarding the monophyly of Leptophlebiidae, many of the lower level groups recovered with this dataset are highly robust across analyses and are compatible with relationships hypothesized based on morphology. However, our molecular data also support some novel findings, including the paraphyletic genera *Paraleptophlebia* and *Leptophlebia*, a New Zealand clade distinct from other Paleoaustral leptophlebiids and a strongly supported sister group relationship between *Neozephlebia scita* (NZ) and *Penaphlebia* spp (SA). Additionally, striking convergence in form on a global scale is evidenced in the similarities among *Thraulus*-group (cosmopolitan distribution), *Traverella* (western hemisphere) and *Isothraulus abditus* (NZ), all with highly-fringed abdominal gills.

Several authors have remarked upon the homoplasious and convergent nature of several characters used to define taxonomic limits across mayflies; for example, features implicated in widespread convergence across taxa include wing venation, abdominal tubercules, egg morphology and abdominal gills (Sivaramakrishnan & Venkataraman, 1987; McCafferty & Wang, 1994; Wang *et al*., 1995; but see Peters & Edmunds, 1964; Wang & McCafferty, 1996). In light of a propensity for homoplasy in certain morphological characters in mayflies, continual and careful evaluation of the utility of included characters is warranted. One of the aims of phylogenetics is to identify the sets of characters that accurately circumscribe taxonomic groups, whereas at the same time unmasking characters that are homoplasious and misleading. Towards this goal, molecular characters offer an independent line of evidence to aid in distinguishing between homoplasious and highly informative morphological synapomorphies by which we can accurately diagnose evolving lineages of mayflies. However, molecular characters are also subject to homoplasy; thus, we highlight the fact that the novel relationships proposed here should not be accepted without additional corroboration.

A morphology-based phylogeny of the Leptophlebiidae would also be valuable for testing hypotheses of relationships derived from this particular set of molecular characters, and to date, no comprehensive investigation of relationships across the family has been published. Ultimately, expanded knowledge of leptophlebiid phylogenetics is likely to instigate a new crop of investigations of morphological evolution, phenotypic plasticity in mouthpart and gill morphology and biogeographical patterns in this cosmopolitan and highly diverse group of mayflies.

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.1365-3113.2008.00434.x

ST1 Taxa included in study.
ST2 Characteristics of amplified loci including number of characters and pairwise distances and gene content.

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