Phylogeny of the Polyneopterous Insects With Emphasis on Plecoptera: Molecular and Morpological Evidence

by

Matthew D. Terry

A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Integrative Biology

Brigham Young University

December 2003

GRADUATE COMMITTEE APPROVAL

of a dissertation submitted by

Matthew D. Terry

This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

_____	_____
Date	Michael F. Whiting, Chair
_____	_____
Date	Keith A. Crandall
_____	_____
Date	Richard W. Baumann
_____	_____
Date	C. Riley Nelson
_____	_____
Date	Joseph V. McHugh

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the dissertation of Matthew D. Terry in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

_____ _____

Date

Michael F. Whiting
Chair, Graduate Committee

Accepted for the Department

_____

Keith A. Crandall
Graduate Coordinator

Accepted for the College

_____

R. Kent Crookston
Dean, College of Integrative Biology

ABSTRACT

PHYLOGENY OF THE POLYNEOPTEROUS INSECTS WITH EMPHASIS ON PLECOPTERA: MOLECULAR AND MORPHOLOGICAL EVIDENCE

Matthew D. Terry

Department of Integrative Biology

Doctor of Philosophy

Polyneoptera is an assemblage of eleven insect orders comprising the "orthopteroid" insects. It includes familiar insects such as grasshoppers, roaches, termites, earwigs and preying mantises; as well as the more obscure web-spinners, angel insects and ice-crawlers. We present a phylogenetic analysis of the polyneopteran orders based on 18S rDNA, 28S rDNA, Histone 3, and a coded morphology matrix for an extensive sampling of taxa. We investigate the use of congruence between separate datasets as an *a priori* measure of alignment quality. Our results support the paraphyly of Polyneoptera, the monophyly of Dictyoptera, sister taxon relationships between Embiidina + Phasmatodea and Dermaptera + Zoraptera, and a relatively basal placement of Plecoptera. The analyses also support a sister taxon relationship between the newly described Mantophasmatodea and Grylloblattodea, a small order of cryophilic insects confined to the northwestern Americas and northeastern Asia. This placement coupled

with the morphological disparity of the two groups validates the creation of a new order for Mantophasmatodea. Our results also suggest the Direct Optimization (formerly Optimization Alignment) produces alignments that are more predictable across the parameter landscape than alignment via CLUSTAL X, as measured by congruence among independent data partitions.

Dense taxon sampling and phylogenetic analysis of six molecular markers (12S, 16S, 18S, 28S, COII, and H3) and morphological data for the order Plecoptera demonstrates that the subordinal groups Arctoperlaria and Antarctoperlaria are monophyletic. Euholognatha and Systellognatha are also monophyletic, with the exception of the genus *Megaleuctra* which is the basal lineage for the order and deserves recognition as a distinct family (Megaleuctridae). Notonemouridae is strongly supported as a monophyletic clade. Within the Systellognatha Styloperlidae is the basal lineage, followed by Peltoperlidae then Pteronarcyidae, and Perloidea is a strongly supported monophyletic group with Chloroperlidae as sister taxon to Perlidae + Perlodidae. The family Gripopterygidae is strongly supported as paraphyletic.

Many Plecoptera (stoneflies) exhibit a pre-mating communication known as "drumming." Species of the genus *Isogenoides* have complex drumming behavior in which (i) the male calls the female by tapping his abdomen against the substrate, (ii) the female answers with her own distinctive tapping, and (iii) the male responds with a confirmatory series of taps. These drumming patterns are specific to individual species and may vary within a species to form distinct dialects. Phylogenetic analysis for the genus based on six molecular markers (12S, 16S, 18S, 28S, COII, and H3) supports

Yugus as its nearest extant relative and *I. hansoni* as the basal lineage within the genus. Drumming behavioral characters appear to be largely incongruent with the phylogeny.

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Mantophasmatodea and Phylogeny of the Lower Neopterous Insects

Matthew D. Terry[1,2] and Michael F. Whiting[1]

[1]Department of Integrative Biology, Brigham Young University, Provo, UT 84602, USA

[2]Present Address: Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, Room 444, 1007 E. Lowell Street, Tucson, AZ 85721

Corresponding Author:

Matthew D. Terry

1007 E. Lowell St.

University of Arizona

Tucson, AZ 85721

(520) 422-2326 Fax: (520) 621-3709

email: matthew_terry@arizona.edu

Abstract. —Polyneoptera is a name sometimes applied to an assemblage of 11 insect orders comprising the lower neopterous or "orthopteroid" insects. These orders include familiar insects such as Orthoptera (grasshoppers), Blattodea (roaches), Isoptera (termites), (Mantodea) preying mantises, Dermaptera (earwigs), Phasmatodea (stick insects), Plecoptera (stoneflies); as well as the more obscure, Embiidina (web-spinners), Zoraptera (angel insects) and Grylloblattodea (ice-crawlers). Many of these insect orders exhibit a high degree of morphological specialization, a condition that has lead to multiple phylogenetic hypotheses and little consensus among investigators. We present a phylogenetic analysis of the polyneopteran orders, including the recently described Mantophasmatodea, based on 18S rDNA, 28S rDNA, Histone 3, and a coded morphology matrix for an extensive sampling of taxa. Extensive analyses utilizing different alignment methodologies and parameters values across a majority of possible ranges were employed to test for sensitivity of results to ribosomal alignment and to explore patterns across the theoretical alignment landscape. Our results support the paraphyly of Polyneoptera, the monophyly of Dictyoptera, sister taxon relationships between Embiidina + Phasmatodea and Dermaptera + Zoraptera, and a relatively basal placement of Plecoptera. The analyses also support a sister taxon relationship between the newly described Mantophasmatodea, which are endemic to arid portions of southern Africa, and Grylloblattodea, a small order of cryophilic insects confined to the northwestern Americas and northeastern Asia. This placement coupled with the morphological disparity of the two groups validates the creation of a new order for Mantophasmatodea.

All neopterous insects, those that can fold their wings, can be placed into one of three groups: Holometabola, insects that have complete metamorphosis; Paraneoptera, true bugs and their allies; or a basal assemblage sometimes called "Polyneoptera" or the "orthopteroid" insects (for the sake of clarity the term Polyneoptera will be used hereafter when referring to these orders collectively). There has been extensive discussion among entomologists regarding the monophyly of the polyneopteran lineages, a group that includes Blattodea (roaches), Isoptera (termites), Mantodea (preying mantises), Dermaptera (earwigs), Embiidina (web-spinners), Grylloblattodea (ice crawlers), Orthoptera (crickets and grasshoppers), Phasmatodea (stick insects), Plecoptera (stoneflies), Zoraptera (angel insects), and the recently described Mantophasmatodea (gladiators). These orders represent one of the largest and earliest insect radiations, yet there has been no clear answer regarding the monophyly of Polyneoptera and the phylogenetic relationships of the individual orders (for a summary see: (Kristensen, 1995). Hennig (1981) placed all of the polyneopteran orders except Plecoptera in a monophyletic group (Paurometabola), and considered Plecoptera as sister-taxon to the remainder of Neoptera. Boudreaux (1979) depicts a monophyletic Polyneoptera with Embiidina + Plecoptera as the basal lineage to the remaining orders; a group he calls "Orthopterodida." Kukalova-Peck (1991) presents a paraphyletic Polyneoptera divided into three major groups: the "Plecopteroid" orders, which consist of extant Plecoptera and extinct relatives; the "Orthopteroid" orders, which are comprised of Orthoptera, Phasmatodea, Embiidina, and extinct relatives; and the "Blattoid" orders which include the remaining orders and appear as sister-taxon to the higher insects (Paraneoptera + Endopterygota). Kristensen (1991) underscored the lack of consensus regarding the relationships among these orders in his summary of insect phylogeny, by representing Polyneoptera

as a nearly unresolved “comb”, with Dictyoptera (Blattodea + Mantodea + Isoptera) as the only supported monophyletic group. A recent analysis of insect phylogeny including 24 polyneopteran taxa and incorporating both morphological characters and multiple molecular markers (Wheeler et al., 2001) supports Polyneoptera as a monophyletic group, however relationships within Polyneoptera differ significantly from the previously cited studies.

Polyneopterous orders inhabit a wide range of ecological niches, exhibit incredible morphological diversity, and include many species which have significant impact on human activities (i.e.; grasshoppers, roaches, and termites). Entire orders within Polyneoptera have been reduced to a handful of species (Zoraptera, Grylloblattodea, and Mantophasmatodea), although amber and compression fossils demonstrate a much wider prehistoric distribution and hint at past diversity (Engel and Grimaldi, 2000; Rasnitsyn, 1976; Zompro et al., 2002). Polyneoptera represents nearly a third of extant ordinal diversity among insects, and insight into their evolutionary relationships would shed considerable light on the early radiation and diversification of insects, yet this has been hampered by the lack of consensus regarding relationships among these groups. The major reason for this disagreement is the lack of morphological synapomorphies between orders (Kristensen, 1991); a problem further exacerbated by the extinction of at least five major lineages (Grimaldi, 2001).

The recent discovery and description of Mantophasmatodea (gladiator insects), the first new insect order described in nearly a century, has elicited considerable excitement and controversy. Klass et al. (2002) state “mantophasmatodeans are phenetically ‘orthopteroid’ insects”, and list several morphological similarities that they share with other polyneopteran orders, including

Phasmatodea (stick insects), Orthoptera (grasshoppers), and Grylloblattodea (ice-crawlers). However, they do not propose any specific sister group for Mantophasmatodea and did not perform a phylogenetic analysis; and although the name of the new order suggests general similarity with both preying mantids ("Manto-") and stick insects ("-phasmatodea"), it is unclear whether mantophasmatodeans are closely related to either of these distinct orders. Mantophasmatodeans exhibit several characters similar to members of other polyneopteran insect orders; for instance, they have an enlarged arolium (a cushion-like pad between the tarsal claws) superficially similar to *Timema*, a basal genus of stick insects (Tilgner, 2002; Whiting et al., 2003). They also possess a "configuration of sclerites and lobes" in the proventriculus (a small region of the digestive tract) that is similar to Grylloblattodea, but also paralleled in other insects (Klass et al., 2002). A recent investigation detailing the sperm structure of *Mantophasmatodea zephyra* (Dallai et al., 2003) suggests similarities with Mantodea. Tilgner (2002) suggests that mantophasmatodeans may simply be "aberrant members of the order Orthoptera" and argues that characters used to exclude them from this group were misinterpreted, however he also failed to provide any formal phylogenetic analysis supporting this conclusion. The difficulty in placing Mantophasmatodea among insect orders highlights the confusion regarding the phylogenetic relationships among polyneopteran insects in general.

Klass et al. (2002) argue that mantophasmatodeans are deserving of ordinal status because they do not exhibit any morphological features that can unambiguously place them within a described order. However, rather than performing a standard phylogenetic analysis to place Mantophasmatodea among extant insect orders, Klass et al. argue that "the only feasible way to discuss the phylogenetic position of

Mantophasmatodea is by evaluating pertinent characters 'mentally'". This approach ignores recent strides in insect ordinal phylogeny which utilize formal analyses of morphological and molecular data (Beutel and Gorb, 2001; Wheeler et al., 2001), and we question the utility of mental analyses in modern phylogenetics. Thus we consider the new ordinal designation a hypothesis that has yet to be rigorously tested, and this work represents the first formal test of the validity of Mantophasmatodea.

Sensitivity analysis, in the context of phylogenetic studies, is the exploration of how variation in underlying assumptions can affect the outcome of analyses (Giribet, 2003) given the same initial data. Sensitivity analyses examining the effect of alignment parameters has become more and more common, particularly when dealing with ribosomal data (Giribet et al., 2002; Ogden and Whiting, 2003; Shull et al., 2001; Whiting et al., 2003). Results presented here represent a portion of a large sensitivity analysis focusing on alignment parameters and utilizing both the programs CLUSTAL X (Thompson et al., 1997) and POY (Gladstein and Wheeler, 2002). Results relevant to the phylogeny of the polyneopterous orders are presented here while specific details and patterns of the overall analysis can be found in Terry and Whiting (Terry and Whiting, Submitted-a).

MATERIALS AND METHODS

Taxon Sampling

To increase the accuracy of phylogenetic estimation we extensively sampled taxa (Zwickl and Hillis, 2002) by including exemplars of most major polyneopteran lineages representing the range of extant biological diversity. This includes 15 of 30 extant families for Orthoptera, six of nine for Blattodea, seven of eight for Isoptera, five of ten for Mantodea, eight of ten for Dermaptera, ten of ten for Phasmatodea, 16

of 16 for Plecoptera, ten of 11 for Embiidina, and one of one for both Grylloblattodea and Zoraptera, for a total of 79 of 116 described families, multiple exemplars from major subordinal groups (i.e.; Arctoperlaria, Antarctoperlaria, Ensifera, Caelifera), and two of three extant, described genera of Mantophasmatodea (*Sclerophasma* and *Tyrannophasma*). The excluded families are generally minor lineages, and this data set represents by far the most comprehensive treatment of polyneopteran relationships to date. Outgroup taxa include Archaeognatha (bristletails; one sp.), Zygentoma (silverfish; three spp.), Ephemeroptera (mayflies; three spp.), Odonata (dragonflies; four spp.), and exemplars of most holometabolan orders (16 taxa). See Appendix 1 for a complete list of all taxa included in this analysis. DNA sequences are deposited in GenBank under accession numbers #####-#### (to be provided upon acceptance).

DNA Extraction, Amplification and Sequencing

For larger specimens, a small portion of wing or leg muscle was dissected from the mesothorax. For smaller specimens, the thorax was cut lengthwise, any gut contamination was removed and the entire thorax was used. Individual vouchers as well as conspecific specimens for specimens collected in series are deposited in the Brigham Young University Insect Genomics Collection (IGC). Tissue dissected from specimens was subjected to either a phenol/ethanol extraction (Whiting et al., 1997) or extraction via Qiagen's® DNeasy™ tissue kit. Purified DNA was amplified for 18S, 28S, and H3 via polymerase chain reaction using previously published primers (see Appendix 2 for a complete list of primers) and amplification profiles (Colgan et al., 1998; Whiting, 2001; Whiting et al., 1997). Due to their large size, each of the ribosomal genes was amplified using three separate regions with sufficient overlap to insure continuity. These regions were approximately 1000, 800, and 600 nucleotides long for 18S and 1200, 600, and 1000 nucleotides long for 28S. Yield and potential

contamination were monitored by agarose gel electrophoresis. Target products were purified and cycle-sequenced using the ABI® dRhodamine cycle sequencing kit via flanking and, for long PCR products, internal primers. These sequencing reactions were then column purified and subjected to automated sequencing on ABI's® 377, 3100, or 3730xl automated sequencer. Complementary strands were independently sequenced and chromatographs were visually checked using Sequencher™ 4.1 (Sequencher, 2002).

Phylogenetic Analyses

Direct Optimization (DO)---For the ribosomal sequences, an initial alignment was performed using Sequencher™ 4.1 by manually aligning the conserved domains across all taxa. Sequences were then subdivided to facilitate finding an optimal solution during DO (Giribet, 2001). This yielded 26 and 28 separate sections for the 18S and 28S rDNA, respectively. Two small sections of 18S; corresponding to helices E21-3 and E21-4 of region V4 and a portion of helix 47 of region V9 (De Rijk et al., 1992); and five small sections of 28S; corresponding to portions of the D2 expansion region and the D5, D6 and D7b expansion regions, were judged non-homologous across taxa and excluded from the analysis. To better estimate polyneopteran relationships, portions of sequences for outgroup taxa that were judged to be unalignable relative to the polyneopteran taxa were excluded and treated as missing data. This was done for two regions of 18S; corresponding to portions of helix E10-1 of region V2 and helix 41 of region V7 (De Rijk et al., 1992); and five regions of 28S; corresponding to portions of the D2 expansion region and the D3, D4, and D7a expansion regions.

The morphology matrix from Wheeler et al. (2001) which was coded for all extant insect orders was stripped of characters uninformative for the taxa included in

this analysis. This resulted in a reduction from 275 to 125 characters, ten unordered and 115 ordered. Two unordered tarsal characters from Beutel and Gorb (2001) were also included. The matrix can be downloaded (in Winclada and MacClade format) from http://inbio.byu.edu/faculty/mfw2/whitinglab.

Analyses were performed for each individual data set (morphology, 18S, 28S, H3) and a combined total-evidence data set using the program POY version 2.0 on an IBM SP2 supercomputer. The ribosomal data sets were analyzed for 90 parameter combinations (see *ILD analysis* below). Protein reading frame was conserved across the H3 data set and it was designated as "pre-aligned" and analyzed for the ten transversion to transition parameter sets. For each analysis, POY was run in parallel across four nodes on the supercomputer. Each analysis produced a topology and implied alignments (for the ribosomal data) using the commands:

-fitchtrees -maxprocessors 8 -onan -onannum 1 -parallel -noleading -norandomizeoutgroup -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -random 10 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -numdriftchanges 30 -driftspr -numdriftspr 10 -drifttbr -numdrifttbr 10 -slop 10 -checkslop 10 –impliedalignment -molecularmatrix *.txt -seed -1

(* asterisk denotes filenames that varied between individual analyses)

Topologies generated from the most optimal parameter values as judged by the ILD metric were further searched by increasing the number of random additions to 200. The implied alignment for the parameter set with maximum congruence among data sets (1:1:1) can be downloaded at http://inbio.byu.edu/faculty/mfw2/whitinglab/.

Multiple Sequence Alignment via CLUSTAL X—To compare equivalent data sets the unalignable regions as described above were also excluded from the

CLUSTAL X alignment. Taxa missing data for specific regions were assigned a number of unknown character states ('N') equivalent to the number of character states of the taxon with the longest known sequence for that region and the ribosomal data sets were then concatenated. We examined the behaviour of CLUSTAL on 198 parameter combinations spanning the range of parameter values in CLUSTAL X using the command line file:

clustalx *.txt -batch -gapopen= -gapext=* -transweight=* -outfile=* -outorder=input -output=nexus -type=dna.

(*asterisks denote filenames or parameters that varied between individual alignments)

Combined total-evidence data sets were then assembled for each parameter combination using the pre-aligned morphology and H3 data sets and the CLUSTAL X aligned ribosomal data sets. Data sets constructed from the ribosomal alignments and combined total-evidence datasets were each analyzed in PAUP* with 50 random additions and tbr swapping.

All bootstrap support measures reported were performed with PAUP* using 1000 bootstrap replicates with 20 random additions per replicate. Bootstrap values for the DO analysis were done using the implied alignment generated by POY. Bremer support values were calculated using a modified PAUP block generated by TreeRot (Sorenson, 1999)with 50 random additions per constraint tree and using the implied alignment for the DO analysis.

Sensitivity Analysis

For the DO analysis nine gap-transversion ratios ranging from 0.5 to 100, and ten transversion-transition ratios ranging from 0.5 to infinity were sampled. Past studies demonstrate that the lower ratio values tend to produce more congruent results

(Giribet et al., 2001; Wheeler, 1995; Whiting et al., 2003) so this region of the search space was sampled more densely. Parameters for the CLUSTAL X analysis are somewhat limited by how they are implemented within the program. Portions of the theoretical search space as defined above are unavailable and specific parameter combinations are not directly comparable with DO (Ogden and Whiting, 2003). However, parameter combinations unavailable in CLUSTAL X are, for the most part, extreme values unlikely to yield reasonable alignments. CLUSTAL X allows the user to choose a "gap opening penalty" ranging from 0 to 100 and 19 values spanning this range were used in this analysis, with values between 0 and 20 sampled more densely. "Transition weight" can be set between 0 and 1, with 0 signifying a mismatch between transitions (high cost) and 1 signifying a match (no cost). We selected 11 values beginning at 0 and incrementally increasing by 0.1. The incongruence length metric (Mickevich and Farris, 1981) was computed for each parameter combination for both DO and parsimony analysis after CLUSTAL X alignment, by taking the difference between the length of the total tree, minus the sum of the lengths of the individual partitions (morphology, 18S, 28S, H3), divided by the length of the total tree. For DO the ILD metric was also calculated for the ribosomal data set and molecular data set for a subset of the parameter values to determine if the "pre-aligned" condition of the morphology and H3 data sets were skewing the ILD results. For a more complete description of the results from these analyses and a discussion comparing the performance of DO versus alignment via CLUSTAL X see Terry and Whiting (Submitted-a)

Additional Alignments and Analyses

Bayesian analysis of implied alignment (molecular data only)---The "implied alignment" generated via POY (Gladstein and Wheeler, 1999) was analyzed with

ModelTest (Posada and Crandall, 2001), a program which selects an evolutionary model best justified by the data. This data set (molecular data only) was then analyzed over 1,000,000 generations using four chains and a sampling frequency of 100 in MrBayes (Huelsenbeck and Ronquist, 2001). The first 50,000 generations were discarded as the "burn-in" period and the remaining generations were assembled into a majority rule topology.

Parsimony analysis of CLUSTAL X alignment---Alignments for each data partition were generated using CLUSTAL X (Thompson et al., 1997) under default parameters for nucleotide alignment (Gap Opening: 15.00, Gap Extension: 6.66, Delay Divergent Sequences: 30%, DNA Transition Weight: 0.50). These individual alignments were then assembled into a combined matrix and used for parsimony (PAUP*) and Bayesian (MrBayes) analyses. The parsimony analysis was performed using 200 random addition sequences incorporating TBR swapping. The resulting topology was subjected to 1000 bootstrap replicates with 25 random additions per replicate.

Bayesian analysis of CLUSTAL X alignment---The CLUSTAL X alignment from above was tested via ModelTest, and a Bayesian analysis was performed using the selected parameters. The matrix was analyzed over 1,000,000 generations using four chains and a sampling frequency of 100. The first 50,000 generations were discarded as the "burn-in" period and the remaining generations were assembled into a majority rule topology.

RESULTS

Sequencing

All amplified H3 sequences had a conserved reading frame, with the exception of one Diptera (*Dolichopeza subalbipes*) which has a 67 base pair insert that was

excised prior to analysis. The longest complete polyneopteran 18S sequence (*Grylloblatta sp.*) is 2125 base pairs in length with an average length of approximately 1900 base pairs. The longest complete polyneopteran 28S sequence is 2440 base pairs (also *Grylloblatta* sp.) with an average length of approximately 2300 base pairs.

DO Sensitivity Analysis

Of the 90 parameter sets investigated the set treating transitions, transversions, and gaps equally (1:1:1) yields the most congruent results, with an ILD value of 0.03503. The single optimal topology (Figure 1) had a length of 16535, a CI score of 0.417 and a RI score of 0.702, and was recovered for both the initial ILD search (10 random replicates) and the subsequent, more thorough search (200 random replicates). Both the CI and the RI were calculated from the implied alignment using PAUP*. The investigated parameter set with the least congruence between data partitions (ILD value of 0.12216) gives transversions a weight 4 times that of transitions and gaps a weight 100 times that of transversions. A complete listing of all ILD scores for the DO analysis can be found in Table 1.

CLUSTAL X Sensitivity Analysis

Of the 198 parameter sets investigated for the CLUSTAL analysis congruence between data sets was maximized with a gap opening cost of 50 and a transition weight of 0.9 (ILD value of 0.03059); the most incongruence was obtained when the parameter set had gap openings set to one and a transition weight of zero (ILD value of 0.10242; see Table 2 for complete list). The topology generated from the combined data sets using the CLUSTAL X parameters that maximized congruence appears in Figure 3. Eight most parsimonious trees were generated with a length of 22295, a CI of 0.302, and a RI of 0.652.

Additional Analyses

Using the implied alignment from POY, ModelTest selected model "GTR+I+Γ" as the most justified with the parameters: nst=6 rates=gamma shape=0.5406. For the CLUSTAL X alignment ModelTest selected GTR+I+Γ as the best justified model with parameters: nst=6 rates=gamma shape=0.5424. The majority rule topology recovered from Bayesian analyses with posterior probabilities as estimated by majority rule percentages above nodes appears in Figure 2. ModelTest selected the model "GTR+I+Γ" as the best justified for the CLUSTAL alignment with the parameters nst=6 rates=gamma shape=0.5424. The majority rule with posterior probabilities above the nodes appears in Figure 4.

DISCUSSION

Polyneopteran Relationships

This analysis represents the most comprehensive analysis to date for the relationships among major lineages of polyneopteran insects. Results from optimal alignments for all analyses (Figures 1-4) support a monophyletic Neoptera (folding-winged insects) and support Odonata (dragonflies) as its closest, extant relative, with the exception of the CLUSTAL/Bayesian analysis which supports two of the Zygentoma taxa as sister to Neoptera. All these analyses also support a monophyletic Holometabola. Polyneoptera is a monophyletic clade in the CLUSTAL/Parsimony analysis, but is paraphyletic in all other analyses. The exact nature of this paraphyly varies among the remaining analyses (Figures 1, 2, & 4). Blattodea is rendered paraphyletic by Isoptera in each of the analyses. The DO analyses and the implied alignment/Bayesian analysis support all other polyneopteran orders as monophyletic, however Zoraptera is paraphyletic in the CLUSTAL/Baysian analysis and Orthoptera, Isoptera, and Embiidina are paraphyletic in the CLUSTAL/Parsimony analysis.

Dictyoptera (Isoptera + Blattodea + Mantodea), the most universally accepted supra-ordinal grouping within Polyneoptera (Boudreaux, 1979; Kristensen, 1991; Snodgrass, 1935), is very strongly supported as monophyletic across all analyses, with Mantodea as the basal lineage in all but the CLUSTAL/Parsimony analysis. The paraphyly of Blattodea is supported by all analyses presented here, by previous molecular (Grandcolas and D'Haese, 2001; Lo et al., 2000) and behavioral data, and by paleontological evidence (Grimaldi, 2001). The preponderance of evidence confirms that termites are indeed "highly modified, social, myopic, wood-eating roaches" (Grimaldi, 2001), and molecular evidence supports this placement of termites relative to extant roaches. We suggest the recognition of Blattodea and Mantodea as valid orders and Isoptera as a sub-order within Blattodea. The term Blattaria, which is sometimes used interchangeably with Blattodea, is used more properly to refer to both extinct and extant roach lineages; a group which is most rendered paraphyletic by to both Isoptera and Mantodea (Grimaldi, 2001). All extant exemplars of the blattarian lineage are more commonly referred to as Dictyoptera.

The sister-relationship between Embiidina and Phasmatodea is well supported across multiple analyses and has been supported in other molecular analyses focusing on different groups, but including exemplars of both these orders (Terry and Whiting, Submitted-b; Thomas et al., 2000; Whiting et al., 2003). In the morphological data set used here Embiidina and Phasmatodea share several non-homoplasious characters including the presence of a secondary profurca-spinasternal muscle (Kristensen, 1975) and attachment of the first axillary sclerite close to the scutal margin (Kristensen, 1975). Both orders also have an aberrant primary flexor muscle and possess a secondary flexor muscle of the paraglossae (Kristensen, 1975; Rahle, 1970), a condition not seen in other orders. We have named the clade Embiidina +

Phasmatodea as “Eukinolabia” from the Greek words “eukinetos”, meaning agile, and “labia”, meaning lips; in reference to the shared unique musculature of the paraglossae, a portion of the insect labium.

Although placement of the clade Dermaptera + Zoraptera varies between methods of reconstruction and one zorapteran taxon tends to behave erratically in some analyses (see Figure 4), this relationship is prevalent throughout our analyses and well supported across data set partitions. All morphological characters included in this analysis and shared by Dermaptera and Zoraptera are homoplasious and sometimes very common across numerous polyneopteran orders (see morphological matrix); this is due to the highly derived status of Zoraptera, an order that has perhaps the most variation in placement across separate phylogenetic hypotheses (Kristensen, 1995; Rasnitsyn, 1998). Zoraptera and Dermaptera both possess single segmented cerci, which are small, oblong structures in nearly all Zoraptera (although one extant and one extinct species have single-segmented elongate cerci), and are highly modified as forcep-like structures in Dermaptera. For this reason we have chosen the term “Haplocercata”, from the Greek ‘haploos’ meaning simple and cercus, for this clade.

Among all polyneopteran orders, Plecoptera varies the most in its placement among separate analyses. It is the basal lineage for the largest assemblage of polyneopteran orders in the DO analysis (Figure 1), but is sister taxon to Haplocercata + Holometabola in both analyses utilizing Baysian methods (Figures 2 &4) and sister taxon to Haplocercata in the CLUSTAL/Parsimony analysis (Figure 3), although a single Orthoptera species nests within Haplocercata). The topology of basal nodes within Plecoptera in these analyses is quite different from that recovered in a recent

analysis including three additional molecular markers and a much better sampling of plecopteran taxa(Terry and Whiting, Submitted-b).

Hennig (1981) hypothesized an Orthoptera rendered paraphyletic by Phasmatodea, however these analyses, with the exception of CLUSTAL/Parsimony, support a monophyletic Orthoptera. Orthoptera also varies greatly in its placement among analyses, but is supported generally as most closely related to a group consisting of Eukinolabia, Lathonomeria, and Dictyoptera; although the monophyly and arrangement of this group varies among analyses.

This analysis demonstrates that Mantophasmatodea is neither the sister group of Mantodea nor Phasmatodea. These data very strongly support the sister taxon relationship of Grylloblattodea and Mantophasmatodea. This relationship is supported under multiple analytical methodologies (standard parsimony and Bayesian analyses), across multiple parameter sets for Direct Optimization, and under different alignment methodologies. Partitioned Bremer values (Baker and DeSalle, 1997) demonstrate support for this relationship among all data partitions of the combined analysis (18S, 28S, H3, and morphology) with a bootstrap value of 100%.

Grylloblattodea is a small, cryophilic order of insect (25 extant species, five genera) confined to northwestern North American and northeastern Asia. Extant representatives are wingless scavengers and/or predators (Storozhenko, 1979) adapted to cool and cold environments. The most diverse genus (*Grylloblatta*) is distributed throughout the mountains of Western Canada and the Northwestern United States and is adapted to the extreme temperatures associated with glaciers and ice caves. On the other hand, extant Mantophasmatodea are confined to the Karoo-Namib region of southern Africa (Picker et al., 2002), and are adapted to the hot, arid conditions prevalent in this area. Although the paleontological evidence indicates a much wider

historic distribution (Zompro et al., 2002), the fact that Mantophasmatodea remained undescribed for so long suggests that the limited records to date closely reflect the extent of their modern distribution. This disjunction of distributions and the small size of both groups suggest an ancient divergence with subsequent extinctions of intermediate lineages. This may be a common theme throughout Polyneoptera and could explain many of the difficulties regarding the phylogenetic reconstruction of relationships.

The goal of modern systematics is twofold; 1) to provide a biological "lingua franca" that facilitates exchange of information among researchers, and 2) to provide a hierarchical system that is meaningful in the context of our understanding of evolutionary history. This makes the field of systematics both rigid, as it must operate within a historic system generally accepted by the scientific community; and plastic, as new data can alter specific designations within that system. In this context phylogeny is a critical factor in determining the validity of a new ordinal designation. For instance, phylogenetic placement of Mantophasmatodea within a previously described order of insects, might indicate the secondary loss of morphological characters commonly used to unite that group, but would invalidate the erection of a new order merely to include a few newly discovered taxa. Placement as sister-taxon to an assemblage of two or more orders would require either recognition of a new order or a drastic revision of the currently accepted groups. And finally, placement of Mantophasmatodea as sister-taxon to a single order would require a judgment as to whether mantophasmatodeans should be included within that order as a basal lineage, or if they are somehow distinct enough to deserve ordinal recognition by themselves. This judgment would be affected by both the disparity (genetic, morphological,

ecological, etc.) between the two groups in question and the historic inertia behind the previously recognized group.

The results of these phylogenetic analyses, coupled with the ecological disparity between these two groups and the formal recognition of Grylloblattodea for nearly a century, strongly supports the validity of the "gladiator" as a new insect order. We have named the clade comprising Grylloblattodea + Mantophasmatodea as "Lathonomeria" derived from the Greek "lathos" (mistaken) and "noma" (name), meaning the "mistakenly named" group. This is in reference to the fact that mantophasmatodeans are neither mantids nor phasmids, just as grylloblattids are neither gryllids (crickets) nor blattids (roaches).

ACKNOWLEDGEMENTS

We thank E. Ross, J. Edgerly-Rooks, C. Nalepa, T. Miura, A. Mason, S. Clark, M. Picker, B. Stark, R. Baumann, I. Mclellan, T. Kishimoto, I. Sivec, J. Adis, O. Zompro, and E. Marais for providing specimens; and M. Gruwell, H. Ogden, G. Svenson, and K. Jarvis for assistance in generating sequence data. Analyses were performed at the Fulton Supercomputing Center at Brigham Young University with parallel software implementation by M. Clement and Q. Snell, based on code made freely available by W. Wheeler. This work was supported by NSF grants DEB-0206363 and DEB-9983195 with NSF REU supplements.

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Appendix 1. List of taxa included in this analysis (GenBank accession numbers to be provided upon acceptance). Higher level taxon names within quotation marks are of doubtful monophyly.

Order	Family	Genus	species	GenBank Accession #		
	"Polyneoptera"			18S rDNA	28S rDNA	Histone 3
"Blattodea"	Blaberidae	*Gromphadorhina*	*portentosa*	####	####	####
	Blattidae	*undet.*	*sp.*	####	####	####
	Blattidae	*undet.*	*sp.*	####	####	####
	Cryptocercidae	*Cryptocercus*	*punctulatus*	####	####	####
	Blattellidae	*Supella*	*longipalpa*	####	####	####
	Blattidae	*Blatta*	*orientalis*	####	####	####
Dermaptera	Labiduridae	*Doru*	*spiculiferum*	####	####	####
	Forficulidae	*Forficula*	*sp.*	####	####	####
	Forficulidae	*Eparchus*	*biroi*	####	####	####
	Chelisochidae	*Chelisoches*	*morio*	####	####	####
	Pygidicranidae	*Tagalina*	*sp.*	####	####	####
	Apachyidae	*Denroiketetes*	*novaguineae*	####	####	####
	Spongiphoridae	*Auchenomus*	*forcipatus*	####	####	####
	Chelisochidae	*undet.*	*sp.*	####	####	####
Embiidina	Oligotomidae	*Oligotoma*	*sp.*	####	####	####
	Teratembiidae	*Diradius*	*vandykei*	####	####	####
	Teratembiidae	*Diradius*	*vandykei*	####	####	####
	Embiidae	*Biguembia*	*multivenosa*	####	####	####
	Anisembiidae	*Brazilembia*	*beckeri*	####	####	####
	Embiidae	*Parahagdochir*	*minuta*	####	####	####
	Teratembiidae	*Teratembia*	*n. sp.*	####	####	####
	Australembiidae	*Australembia*	*nodosa*	####	####	####
	Clothodidae	*Antipaluria*	*urichi*	####	####	####
	Notoligotomidae	*Notoligotoma*	*sp.*	####	####	####
Grylloblattodea	Gryllblattidae	*Grylloblatta*	*campodeiformis*	####	####	####
	Gryllblattidae	*Grylloblatta*	*campodeiformis*	####	####	####
	Gryllblattidae	*Grylloblatta*	*n. species*	####	####	####
Isoptera	Rhinotermitidae	*undet.*	*sp.*	####	####	####
	Hodotermitidae	*undet.*	*sp.*	####	####	####
	Kalotermitidae	*Cryptotermes*	*sp.*	####	####	####
	Termitidae	*Nausititermes*	*sp.*	####	####	####
	Termitidae	*undet.*	*sp.*	####	####	####
	Termopsisdae	*Hodotermopsis*	*japonica*	####	####	####
	Mastotermitidae	*Mastotermes*	*darwinensis*	####	####	####
Mantodea	Mantidae	*Tenodera*	*aridifolia*	####	####	####
	Hymenopodidae	*Acromantis*	*sp.*	####	####	####
	Hymenopodidae	*Chrysomantis*	*sp.*	####	####	####
	Empusidae	*Gongylus*	*gongylodes*	####	####	####
	Mantidae	*Orthodera*	*novazeylandi*	####	####	####
Mantophasmatodea	Mantophasmatidae	*Sclerophasma*	*paresisensis*	####	####	####
	Mantophasmatidae	*Tyrannophasma*	*gladiator*	####	####	####
Orthoptera	Tettigoniidae	*Microcentrum*	*rhombifolium*	####	####	####
	Tetrigidae	*Paratettix*	*cucullatus*	####	####	####
	Tridactylidae	*Ellipes*	*minutus*	####	####	####
	Stenopelmatidae	*Stenopelmatus*	*fuscus*	####	####	####
	Romaleidae	*Romalea*	*sp.*	####	####	####
	Gryllidae	*Gryllus*	*assimilis*	####	####	####
	Rhapidiophoridae	*Ceuthophilus*	*utahensis*	####	####	####
	Acrididae	*Melanoplus*	*sp.*	####	####	####
	Tettigoniidae	*Pterophylla*	*camellifolia*	####	####	####
	Gryllotalpidae	*undet.*	*sp.*	####	####	####
	Haglidae	*Cyphoderris*	*monstrosa*	####	####	####
	Myrmecophilidae	*Myrmecophila*	*manni*	####	####	####
	Eumastacidae	*Morsea*	*californica*	####	####	####
	Gryllacrididae	*Camptonotus*	*carolinensis*	####	####	####

	Proscopiidae	*undet.*	*sp.*	####	####	####
Plecoptera	Nemouridae	*Malenka*	*californica*	####	####	####
	Leuctridae	*Paraleuctra*	*vershina*	####	####	####
	Taeniopterygidae	*Oemopteryx*	*vanduzeei*	####	####	####
	Pteronarcyidae	*Pteronarcys*	*californica*	####	####	####
	Perlidae	*Calineuria*	*californica*	####	####	####
	Capniidae	*Capnia*	*gracilaria*	####	####	####
	Gripopterygidae	*Trinotoperla*	*nivata*	####	####	####
	Notonemouridae	*Aphanicerca*	*capensis*	####	####	####
	Perlodidae	*Isoperla*	*davisi*	####	####	####
	Chloroperlidae	*Plumiperla*	*diversa*	####	####	####
	Peltoperlidae	*Tallaperla*	*lobata*	####	####	####
	Diamphipnoidae	*Diamphipnoa*	*virecentipennis*	####	####	####
	Austroperlidae	*Austroperla*	*cyrene*	####	####	####
	Eustheniidae	*Stenoperla*	*maclellani*	####	####	####
	Scopuridae	*Scopura*	*montana*	####	####	####
	Styloperlidae	*Cerconychia*	*sp.*	####	####	####
Phasmatodea	Heteronemiidae	*Diapheromera*	*femorata*	####	####	####
	Phasmatidae	*Eurycantha*	*coriacea*	####	####	####
	Bacillidae	*Heteropteryx*	*dilatata*	####	####	####
	Pseudophasmatidae	*Paraphasma*	*rufipes*	####	####	####
	Phyllidae	*Phyllium*	*bioculatum*	####	####	####
	Timematidae	*Timema*	*knulli*	####	####	####
	Heteronemiidae	*Sceptrophasma*	*langkawicensis*	####	####	####
	Heteronemiidae	*Neohirasea*	*maerens*	####	####	####
	Phasmatidae	*Baculum*	*thaii*	####	####	####
	Phasmatidae	*Lamponius*	*guerini*	####	####	####
Zoraptera	Zorotypidae	*Zorotypus*	*hubbardi*	####	####	####
	Zorotypidae	*Zorotypus*	*hubbardi*	####	####	####
	Zorotypidae	*Zorotypus*	*n. species*	####	####	####
		"Apterygota"				
Archaeognatha	Machilidae	*Machilis*	*sp.*	####	####	####
	Machilidae	*Machilis*	*sp.*	####	####	####
Zygentoma	Lepismatidae	*Thermobia*	*domestica*	####	####	####
	Noticoliidae	*Battigrassiella*	*wheeleri*	####	####	####
	Lepidotrichidae	*Tricholepidion*	*gertschii*	####	####	####
		"Paleoptera"				
Ephemeroptera	Ephemeridae	*Hexagenia*	*sp.*	####	####	####
	Leptophlebidae	*Paraleptophlebia*	*sp.*	####	####	####
	Baetiscidae	*Baetisca*	*sp.*	####	####	####
Odonata	Gomphidae	*Ophiogomphus*	*severus*	####	####	####
	Libellulidae	*Libellula*	*saturata*	####	####	####
	Coenagrionidae	*Hetaerina*	*americana*	####	####	####
	Ceonagrionidae	*Argia*	*vivida*	####	####	####
		Holometabola				
Coleoptera	Carabidae	*Omophron*	*sp.*	####	####	####
	Scarabaeidae	*Phyllophaga*	*sp.*	####	####	####
	Silvanidae	*Uleiota*	*sp.*	####	####	####
Diptera	Tipulidae	*Dolichopeza*	*subalbipes*	####	####	####
	Asilidae	*Mallophora*	*sp.*	####	####	####
Siphonaptera	Pulicidae	*Hoplopsyllus*	*anomalus*	####	####	####
Hymenoptera	Scoliidae	*Capmsomeris*	*sp.*	####	####	####
	Diprionidae	*Neodiprion*	*sp.*	####	####	####
Lepidoptera	Pieridae	*Anthocharis*	*sara*	####	####	####
	Cossidae	*Prionoxystus*	*robiniae*	####	####	####
Mecoptera	Boreidae	*Boreus*	*westwoodi*	####	####	####
	Panorpidae	*Panorpa*	*bicornuta*	####	####	####
Neuroptera	Sialidae	*Sialis*	*hamata*	####	####	####
	Hemerobiidae	*Hemerobius*	*sp.*	####	####	####
	Inocellidae	*Negha*	*meridionalis*	####	####	####
Trichoptera	Hydropsychidae	*Hydrospsyche*	*sp.*	####	####	####

Appendix 2. List of primers used to amplify molecular markers used in this analysis.

Gene	Primer Name	Sequence (5' - 3')	Length	Direction	Relative Position
18S	18S 1F	TACCTGGTTGATCCTGCCAGTAG	23	Forward	1
	18S ai	CCTGAGAAACGGCTACCACATC	22	Forward	2
	18S a0.7	ATTAAAGTTGTTGCGGTT	18	Forward	3
	18S a0.79	TTAGAGTGCTYAAAGC	16	Forward	4
	18S a1.0	GGTGAAATTCTTGGAYCGTC	20	Forward	5
	18S a2.0	ATGGTTGCAAAGCTGAAAC	19	Forward	6
	18S a3.5	TGGTGCATGGCCGYTCTTAGT	21	Forward	7
	18S 7F	GCAATAACAGGTCTGTGATGCCC	23	Forward	8
	18S 9R	GATCCTTCCGCAGGTTCACCTAC	23	Reverse	1
	18S 7R	GCATCACAGACCTGTTATTGC	21	Reverse	2
	18S bi	GAGTCTCGTTCGTTATCGGA	20	Reverse	3
	18S b0.5	GTTTCAGCTTTGCAACCAT	19	Reverse	4
	18S b2.5	TCTTTGGCAAATGCTTTCGC	20	Reverse	5
	18S b2.9	TATCTGATCGCCTTCGAACCTCT	23	Reverse	6
	18S b3.9	TGCTTTRAGCACTCTAA	17	Reverse	7
	18S b5.0	TAACCGCAACAACTTTAAT	19	Reverse	8
	18S b7.0	ATTTRCGYGCCTGCTGCCTTCCT	23	Reverse	9
28S	28s Rd 1a	CCCSCGTAAYTTAGGCATAT	20	Forward	1
	28s Rd 3a	AGTACGTGAAACCGTTCAGG	20	Forward	2
	28S A	GACCCGTCTTGAAGCACG	18	Forward	3
	28S Rd 4a	GGAGTCTAGCATGTGYGCAAGTC	23	Forward	4
	28S Rd 4.5a	AAGTTTCCCTCAGGATAGCTG	21	Forward	5
	28S Rd 4.8a	ACCTATTCTCAAACTTTAAATGG	23	Forward	6
	28S Rd 5a	GGYGTTGGTTGCTTAAGACAG	21	Forward	7
	28S Rd 6a	GGCGAAAGGGAATCYGGTTC	20	Forward	8
	28S Rd 7b1	GACTTCCCTTACCTACAT	18	Reverse	1
	28S Rd 6b	AACCRGATTCCCTTTCGCC	19	Reverse	2
	28S Rd 5b	CCACAGCGCCAGTTCTGCTTAC	22	Reverse	3
	28S B	TCGGAAGGAACCAGCTAC	18	Reverse	4
	28S C	ATAGTTCACCATCTYTCGGG	20	Reverse	5
	28S Rd 4b	CCTTGGTCCGTGTTTCAAGAC	21	Reverse	6
	28S Rd 3b	CCYTGAACGGTTTCACGTACT	21	Reverse	7
H3	H3 AF	ATGGCTCGTACCAAGCAGACVGC	23	Forward	
	Hex AF	ATGGCTCGTACCAAGCAGACGGC	23	Forward	
	H3 AR	ATATCCTTRGGCATRATRGTGAC	23	Reverse	
	Hex AR	ATATCCTTGGGCATGATGGTGAC	23	Reverse	

Table 1. Topology lengths and subsequent ILD values from DO analysis. Bold values represent global minimum ILD values. (Abbreviations: Tv = transversion, Ts = transition, morph. = morphology dataset, rib. = combine ribosomal dataset, mol. = combined molecular data set).

		Data Set									
Gap:Tv	Tv:Ts	morph.	28S	18S	rib.	rib. ILD	H3	mol.	mol. ILD	total	total ILD
0.5	0.5	238	18454	9651			5067			34816	0.04038
1	0.5	238	14505	7181			3468			26454	0.04015
2	0.5	238	18448	8416			3468			31907	0.04190
3	0.5	238	20938	9083			3468			35311	0.04486
4	0.5	238	23244	9671			3468			38455	0.04769
10	0.5	238	35384	12813			3468			55352	0.06231
20	0.5	238	54256	17526			3468			81888	0.07816
50	0.5	238	108609	31285			3468			158492	0.09396
100	0.5	238	197728	53577			3468			283770	0.10135
0.5	1	238	17043	8502			4596			31601	0.03867
1	1	238	11714	5298	17426	**0.02376**	2444	20163	**0.03506**	20409	**0.03503**
2	1	238	14644	5982	21198	0.02698	2444	23995	0.03855	24261	0.03928
3	1	238	16964	6571	24303	0.03160	2444	27130	0.04243	27410	0.04352
4	1	238	19430	7132	27285	0.02650	2444	30141	0.03766	30453	0.03970
10	1	238	31101	10100	43792	0.05917	2444	46499	0.06138	46958	0.06548
20	1	238	49741	14796			2444			72478	0.07256
50	1	238	104260	28822			2444			149534	0.09209
100	1	238	193383	49751			2444			277580	0.11443
0.5	2	238	13893	6479			3614			25111	0.03532
1	2	238	18466	7817	26999	0.02652	3614	31129	0.03958	31384	0.03980
2	2	238	23823	9166	33997	0.02965	3614	38195	0.04168	38486	0.04274
3	2	238	28389	10350	40116	0.03433	3614	44351	0.04505	44687	0.04690
4	2	238	32738	11341	45975	0.04124	3614	50170	0.04937	50488	0.05065
10	2	238	55443	17363	78650	0.07430	3614	83280	0.08237	83163	0.07822
20	2	238	92717	26705			3614			135590	0.09083
50	2	238	201120	53915			3614			291666	0.11239
100	2	238	381830	99067			3614			542762	0.10688
0.5	3	238	36885	16798			9481			66114	0.04102
1	3	238	24982	10257	36184	0.02612	4751	41597	0.03863	41873	0.03929
2	3	238	32785	12261	46577	0.03287	4751	52004	0.04244	52358	0.04437
3	3	238	39420	14074	55688	0.03940	4751	61111	0.04690	61473	0.04864
4	3	238	45936	15618	64375	0.04382	4751	69961	0.05226	70178	0.05180
10	3	238	80419	24804	113466	0.07265	4751	118677	0.07333	119829	0.08026
20	3	238	136299	38710			4751			198285	0.09223
50	3	238	298668	80489			4751			433227	0.11329
100	3	238	568333	147318			4751			811159	0.11159
0.5	4	238	22892	10266			5847			40970	0.04215
1	4	238	31389	12713	45259	0.02556	5847	52070	0.04073	52382	0.04190
2	4	238	41740	15321	59088	0.03430	5847	65818	0.04421	66150	0.04541
3	4	238	50768	17677	71073	0.03698	5847	77887	0.04616	78132	0.04610
4	4	238	59164	19764	82920	0.04814	5847	89576	0.05360	90218	0.05769
10	4	238	104864	31125	147986	0.08107	5847	154234	0.08038	155485	0.08625
20	4	238	179095	50496			5847			258662	0.08887
50	4	238	396597	105820			5847			567574	0.10408
100	4	238	753187	188234			5847			1079359	0.12216
0.5	10	238	48986	21309			12383			87052	0.04751
1	10	238	69772	27168	99834	0.02899	12383	114444	0.04475	114700	0.04480
2	10	238	95262	33794	133757	0.03515	12383	148159	0.04536	148561	0.04634
3	10	238	117160	39372	163685	0.04370	12383	178350	0.05290	178834	0.05413
4	10	238	138397	44708	192393	0.04828	12383	207547	0.05810	208066	0.05931
10	10	238	252027	73997	353512	0.07776	12383	368354	0.08130	367482	0.07847
20	10	238	435983	120499			12383			627388	0.09290
50	10	238	972439	253819			12383			1397900	0.11376

100	10	238	1879672	485520	12383	2628938	0.09552
0.5	20	238	92176	39675	23148	163306	0.04941
1	20	238	133157	51021	23148	218023	0.04797
2	20	238	183958	64378	23148	286421	0.05132
3	20	238	228360	75664	23148	345982	0.05368
4	20	238	268556	86543	23148	403947	0.06303
10	20	238	496955	145252	23148	722737	0.07907
20	20	238	858330	238855	23148	1235485	0.09301
50	20	238	1937117	515641	23148	2752190	0.10030
100	20	238	3758134	964116	23148	5307895	0.10593
0.5	50	238	221441	94788	55642	391329	0.04911
1	50	238	325274	123546	55642	529389	0.04664
2	50	238	449337	156489	55642	698099	0.05213
3	50	238	558362	182598	55642	847803	0.06011
4	50	238	663053	210047	55642	991255	0.06282
10	50	238	1226657	357391	55642	1796706	0.08726
20	50	238	2143954	575659	55642	3084376	0.10014
50	50	238	4834661	1271191	55642	6922670	0.10992
100	50	238	9323658	2401340	55642	13259818	0.11154
0.5	100	238	437154	186092	109508	773250	0.05206
1	100	238	643142	244696	109508	1047862	0.04798
2	100	238	894977	308566	109508	1386168	0.05258
3	100	238	1112212	364968	109508	1684113	0.05771
4	100	238	1320594	416714	109508	1978663	0.06651
10	100	238	2455429	711105	109508	3568587	0.08191
20	100	238	4275103	1176009	109508	6116514	0.09085
50	100	238	9653891	2515246	109508	13881738	0.11547
100	100	238	18832288	4843214	109508	26669629	0.10815
0.5	∞	238	8583	3615	2150	15408	0.05335
1	∞	238	6297	2363	1078	10503	0.05018
2	∞	238	8724	2921	1078	13777	0.05923
3	∞	238	10731	3374	1078	16543	0.06782
4	∞	238	12658	3839	1078	19143	0.06948
10	∞	238	23345	6414	1078	34089	0.08842
20	∞	238	40351	10334	1078	58624	0.11297
50	∞	238	90633	22099	1078	128617	0.11327
100	∞	238	178936	41704	1078	248784	0.10784

Table 2. Topology lengths and subsequent ILD values from CLUSTAL X alignments with PAUP* analyses.

		Data Set					
G.O.	T.S.W	18S	28S	H3	morph	total	ILD
1	0	5589	11154	2400	234	21588	0.10242
1	0.1	5513	10996	2400	234	21213	0.09758
1	0.2	5435	10920	2400	234	21045	0.09770
1	0.3	5427	10889	2400	234	21037	0.09921
1	0.4	5423	10804	2400	234	19980	0.05601
1	0.5	5365	10867	2400	234	19887	0.05134
1	0.6	5352	10761	2400	234	19660	0.04644
1	0.7	5358	10895	2400	234	19783	0.04529
1	0.8	5387	11072	2400	234	20049	0.04768
1	0.9	5498	11169	2400	234	20195	0.04427
1	1.0	5783	12175	2400	234	21697	0.05093
2	0	5488	10943	2400	234	20077	0.05041
2	0.1	5397	10858	2400	234	19840	0.04793
2	0.2	5348	10808	2400	234	19756	0.04890
2	0.3	5292	10701	2400	234	19550	0.04721
2	0.4	5289	10609	2400	234	19397	0.04459
2	0.5	5226	10623	2400	234	19315	0.04308
2	0.6	5249	10712	2400	234	19403	0.04164
2	0.7	5253	10788	2400	234	19464	0.04054
2	0.8	5293	11032	2400	234	19829	0.04388
2	0.9	5382	11120	2400	234	19963	0.04143
2	1.0	5625	11750	2400	234	20969	0.04578
4	0	5259	10824	2400	234	19552	0.04271
4	0.1	5239	10772	2400	234	19491	0.04340
4	0.2	5203	10753	2400	234	19440	0.04372
4	0.3	5146	10695	2400	234	19290	0.04225
4	0.4	5140	10768	2400	234	19353	0.04191
4	0.5	5192	10746	2400	234	19397	0.04253
4	0.6	5176	10828	2400	234	19456	0.04204
4	0.7	5157	10832	2400	234	19377	0.03891
4	0.8	5167	10905	2400	234	19493	0.04037
4	0.9	5119	11071	2400	234	19658	0.04243
4	1.0	5348	11450	2400	234	20258	0.04077
6	0	5239	10948	2400	234	19662	0.04277
6	0.1	5238	10965	2400	234	19733	0.04541
6	0.2	5227	10929	2400	234	19673	0.04488
6	0.3	5135	10844	2400	234	19442	0.04264
6	0.4	5236	10925	2400	234	19617	0.04190
6	0.5	5168	10918	2400	234	19522	0.04108
6	0.6	5218	10978	2400	234	19655	0.04197
6	0.7	5222	11020	2400	234	19702	0.04192
6	0.8	5259	11009	2400	234	19736	0.04226
6	0.9	5202	11077	2400	234	19746	0.04219
6	1.0	5413	11573	2400	234	20451	0.04063
8	0	5288	11051	2400	234	19806	0.04206
8	0.1	5251	11166	2400	234	19939	0.04454
8	0.2	5251	11125	2400	234	19932	0.04626
8	0.3	5288	11112	2400	234	19932	0.04505
8	0.4	5283	11175	2400	234	19987	0.04478
8	0.5	5252	11131	2400	234	19877	0.04327
8	0.6	5235	11105	2400	234	19807	0.04206
8	0.7	5301	11047	2400	234	19835	0.04300
8	0.8	5261	11204	2400	234	19968	0.04352
8	0.9	5173	11260	2400	234	19935	0.04354
8	1.0	5319	11617	2400	234	20440	0.04256
10	0	5416	11259	2400	234	20303	0.04896
20	0	5543	11671	2400	234	20855	0.04829
20	0.1	5503	11765	2400	234	20932	0.04921
20	0.2	5493	11725	2400	234	20853	0.04800
20	0.3	5423	11716	2400	234	20758	0.04745
20	0.4	5514	11686	2400	234	20796	0.04626
20	0.5	5469	11801	2400	234	20831	0.04450
20	0.6	5475	11876	2400	234	20968	0.04688
20	0.7	5500	11698	2400	234	20809	0.04695
20	0.8	5672	11913	2400	234	20967	0.03568
20	0.9	5303	12059	2400	234	20904	0.04344
20	1.0	5473	12010	2400	234	21199	0.05104
30	0	5653	11946	2400	234	21295	0.04987
30	0.1	5638	12096	2400	234	21504	0.05283
30	0.2	5596	12065	2400	234	21311	0.04767
30	0.3	5558	12257	2400	234	21556	0.05135
30	0.4	5620	12231	2400	234	21535	0.04876
30	0.5	5614	12216	2400	234	21485	0.04752
30	0.6	5619	12280	2400	234	21532	0.04640
30	0.7	5626	12286	2400	234	21566	0.04730
30	0.8	5666	12330	2400	234	21503	0.04060
30	0.9	5431	12542	2400	234	21568	0.04456
30	1.0	5607	12598	2400	234	22061	0.05539
40	0	5744	12349	2400	234	21766	0.04773
40	0.1	5753	12506	2400	234	21946	0.04798
40	0.2	5752	12517	2400	234	21990	0.04943
40	0.3	5754	12548	2400	234	22059	0.05091
40	0.4	5798	12662	2400	234	22188	0.04931
40	0.5	5780	12473	2400	234	21894	0.04599
40	0.6	5764	12558	2400	234	22001	0.04750
40	0.7	5743	12563	2400	234	21923	0.04484
40	0.8	5789	12573	2400	234	21957	0.04377
40	0.9	5541	12641	2400	234	21779	0.04422
40	1.0	5657	12802	2400	234	22101	0.04561
50	0	5850	12899	2400	234	22408	0.04574
50	0.1	5861	12833	2400	234	22347	0.04560
50	0.2	5855	12825	2400	234	22356	0.04661
50	0.3	5809	12897	2400	234	22359	0.04557
50	0.4	5855	12857	2400	234	22382	0.04629
50	0.5	5820	12893	2400	234	22400	0.04701
50	0.6	5804	12902	2400	234	22379	0.04643
50	0.7	5808	13012	2400	234	22541	0.04822
50	0.8	5853	12872	2400	234	22370	0.04519
50	0.9	5631	13348	2400	234	22295	**0.03059**
50	1.0	5761	13322	2400	234	22771	0.04629
60	0	5935	13206	2400	234	22867	0.04775
60	0.1	5950	13141	2400	234	22865	0.04986
60	0.2	5970	13145	2400	234	22893	0.04997
60	0.3	5921	13255	2400	234	22957	0.04996
60	0.4	5905	13259	2400	234	22942	0.04986
60	0.5	5859	13194	2400	234	22809	0.04919
60	0.6	5916	13125	2400	234	22772	0.04817
60	0.7	5948	13170	2400	234	22779	0.04509
60	0.8	5929	13274	2400	234	22898	0.04634
60	0.9	5743	13491	2400	234	22893	0.04477
60	1.0	5839	13778	2400	234	23461	0.05157
70	0	6092	13440	2400	234	23310	0.04908

10	0.1	5398	11289	2400	234	20280	0.04729		70	0.1	6148	13445	2400	234	23397	0.05001
10	0.2	5373	11302	2400	234	20224	0.04524		70	0.2	6108	13449	2400	234	23389	0.05122
10	0.3	5376	11331	2400	234	20250	0.04489		70	0.3	5940	13549	2400	234	23256	0.04872
10	0.4	5280	11327	2400	234	20167	0.04592		70	0.4	6038	13588	2400	234	23474	0.05172
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10	0.6	5407	11354	2400	234	20318	0.04543		70	0.6	5982	13840	2400	234	23639	0.05004
10	0.7	5373	11358	2400	234	20257	0.04403		70	0.7	6057	13775	2400	234	23621	0.04890
10	0.8	5408	11391	2400	234	20343	0.04473		70	0.8	6054	13825	2400	234	23662	0.04856
10	0.9	5224	11404	2400	234	20146	0.04388		70	0.9	5859	13961	2400	234	23478	0.04362
10	1.0	5322	11722	2400	234	20579	0.04378		80	1.0	6032	14315	2400	234	24183	0.04970
12	0	5467	11308	2400	234	20343	0.04591		80	0	6220	13587	2400	234	23582	0.04838
12	0.1	5386	11362	2400	234	20300	0.04522		80	0.1	6170	13566	2400	234	23520	0.04889
12	0.2	5432	11401	2400	234	20410	0.04620		80	0.2	6210	13630	2400	234	23640	0.04932
12	0.3	5385	11376	2400	234	20354	0.04712		80	0.3	6086	13631	2400	234	23437	0.04634
12	0.4	5314	11409	2400	234	20333	0.04800		80	0.4	6111	13693	2400	234	23570	0.04803
12	0.5	5455	11432	2400	234	20415	0.04379		80	0.5	6097	13878	2400	234	23798	0.04996
12	0.6	5468	11426	2400	234	20457	0.04541		80	0.6	6194	13857	2400	234	23965	0.05341
12	0.7	5447	11619	2400	234	20678	0.04730		80	0.7	6180	13860	2400	234	23830	0.04851
12	0.8	5413	11507	2400	234	20415	0.04217		80	0.8	6104	14039	2400	234	23972	0.04985
12	0.9	5219	11606	2400	234	20314	0.04209		80	0.9	5898	14086	2400	234	23678	0.04477
12	1.0	5318	11799	2400	234	20649	0.04349		80	1.0	6233	14647	2400	234	24714	0.04856
15	0	5392	11577	2400	234	20543	0.04576		90	0	6331	13822	2400	234	23972	0.04943
15	0.1	5376	11580	2400	234	20506	0.04467		90	0.1	6234	13828	2400	234	23822	0.04727
15	0.2	5460	11464	2400	234	20571	0.04924		90	0.2	6216	13836	2400	234	23793	0.04653
15	0.3	5425	11477	2400	234	20526	0.04823		90	0.3	6088	13832	2400	234	23613	0.04485
15	0.4	5489	11529	2400	234	20623	0.04708		90	0.4	6200	14086	2400	234	24194	0.05266
15	0.5	5463	11564	2400	234	20621	0.04655		90	0.5	6217	14084	2400	234	24219	0.05302
15	0.6	5474	11514	2400	234	20610	0.04794		90	0.6	6215	14105	2400	234	24253	0.05356
15	0.7	5452	11535	2400	234	20611	0.04803		90	0.7	6337	14341	2400	234	24508	0.04880
15	0.8	5434	11702	2400	234	20682	0.04410		90	0.8	6313	14699	2400	234	24838	0.04799
15	0.9	5223	11739	2400	234	20482	0.04326		90	0.9	6141	14998	2400	234	24850	0.04334
15	1.0	5328	11870	2400	234	20692	0.04156		90	1.0	6234	15146	2400	234	25171	0.04597
18	0	5519	11745	2400	234	20926	0.04913		100	0	6329	13876	2400	234	23944	0.04615
18	0.1	5496	11684	2400	234	20873	0.05074		100	0.1	6319	14189	2400	234	24434	0.05288
18	0.2	5505	11674	2400	234	20846	0.04955		100	0.2	6307	14134	2400	234	24294	0.05018
18	0.3	5440	11634	2400	234	20746	0.05003		100	0.3	6223	14076	2400	234	24161	0.05083
18	0.4	5495	11721	2400	234	20856	0.04824		100	0.4	6354	14333	2400	234	24590	0.05161
18	0.5	5500	11679	2400	234	20792	0.04709		100	0.5	6383	14481	2400	234	24718	0.04936
18	0.6	5483	11707	2400	234	20808	0.04729		100	0.6	6386	14757	2400	234	24960	0.04740
18	0.7	5500	11634	2400	234	20803	0.04975		100	0.7	6689	14978	2400	234	25544	0.04866
18	0.8	5530	11803	2400	234	20908	0.04501		100	0.8	6691	15506	2400	234	26251	0.05409
18	0.9	5290	11830	2400	234	20675	0.04455		100	0.9	6293	16034	2400	234	26237	0.04863
18	1.0	5447	12016	2400	234	21038	0.04473		100	1.0	6477	15422	2400	234	25784	0.04852

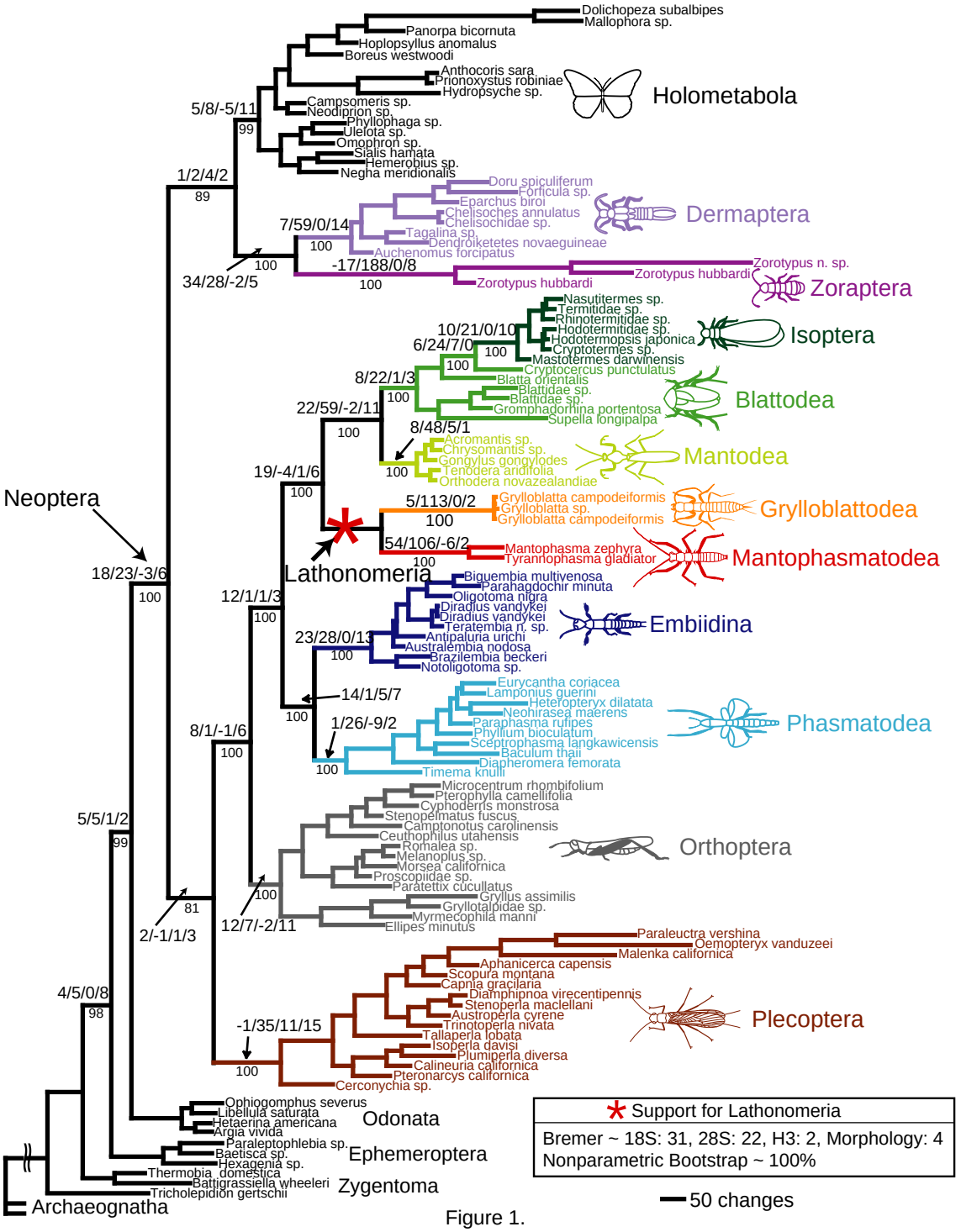
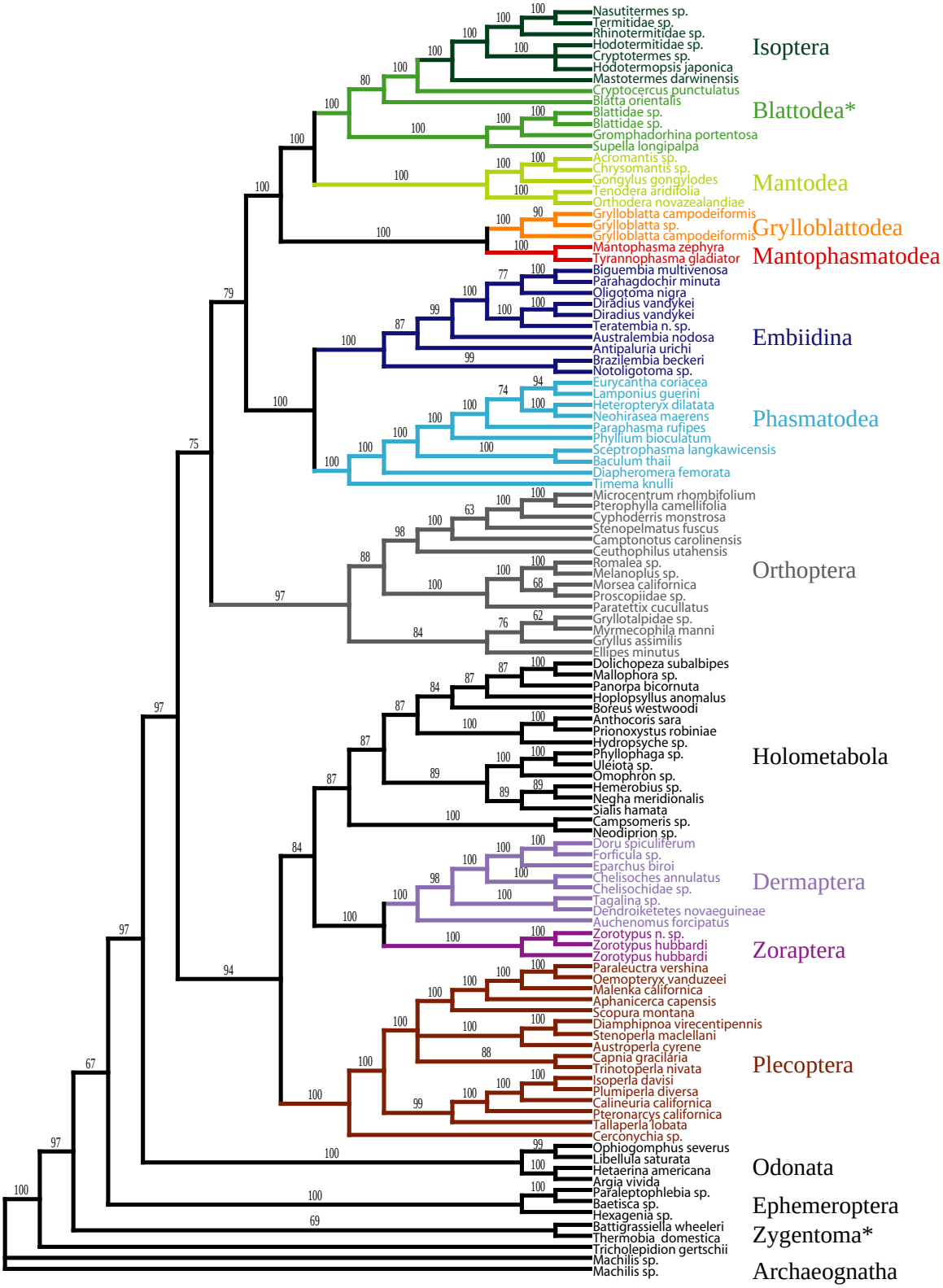

Dolichopeza subalbipes
Mallophora sp.
Panorpa bicornuta
Hoplopsyllus anomalus
Boreus westwoodi
Anthocoris sara
Prionoxystus robiniae
Hydropsyche sp.
Holometabola
5/8/-5/11
99
Campsomeris sp.
Neodiprion sp.
Phyllophaga sp.
Ulelota sp.
Omophron sp.
Sialis hamata
Hemerobius sp.
Negha meridionalis
1/2/4/2
89
Doru spiculiferum
Forficula sp.
Eparchus biroi
Chelisoches annulatus
Chelisochidae sp.
Dermaptera
7/59/0/14
100
Tagalina sp.
Dendroiketetes novaeguineae
Auchenomus forcipatus
100
34/28/-2/5
-17/188/0/8
100
Zorotypus n. sp.
Zorotypus hubbardi
Zorotypus hubbardi
Zoraptera
Nasutitermes sp.
Termitidae sp.
Rhinotermitidae sp.
Hodotermitidae sp.
Hodotermopsis japonica
Cryptotermes sp.
Isoptera
10/21/0/10
100
6/24/7/0
100
Mastotermes darwinensis
Cryptocercus punctulatus
Blatta orientalis
Blattidae sp.
Blattidae sp.
Gromphadorhina portentosa
Supella longipalpa
Blattodea
8/22/1/3
100
22/59/-2/11
100
8/48/5/1
100
Acromantis sp.
Chrysomantis sp.
Gongylus gongylodes
Tenodera aridifolia
Orthodera novazealandiae
Mantodea
19/-4/1/6
100
Neoptera
5/113/0/2
100
Grylloblatta campodeiformis
Grylloblatta sp.
Grylloblatta campodeiformis
Grylloblattodea
54/106/-6/2
100
Mantophasma zephyra
Tyrannophasma gladiator
Mantophasmatodea
18/23/-3/6
100
Lathonomeria
Biguembia multivenosa
Parahagdochir minuta
Oligotoma nigra
Diradius vandykei
Diradius vandykei
Teratembia n. sp.
Antipaluria urichi
Australembia nodosa
Brazilembia beckeri
Notoligotoma sp.
Embiidina
12/1/1/3
100
23/28/0/13
100
14/1/5/7
100
Eurycantha coriacea
Lamponius guerini
Heteropteryx dilatata
Neohirasea maerens
Paraphasma rufipes
Phyllium bioculatum
Sceptrophasma langkawicensis
Baculum thaii
Diapheromera femorata
Timema knulli
Phasmatodea
1/26/-9/2
100
8/1/-1/6
100
Microcentrum rhombifolium
Pterophylla camellifolia
Cyphoderris monstrosa
Stenopelmatus fuscus
Camptonotus carolinensis
Ceuthophilus utahensis
Romalea sp.
Melanoplus sp.
Morsea californica
Proscopiidae sp.
Paratettix cucullatus
Gryllus assimilis
Gryllotalpidae sp.
Myrmecophila manni
Ellipes minutus
Orthoptera
5/5/1/2
99
81
2/-1/1/3
12/7/-2/11
100
Paraleuctra vershina
Oemopteryx vanduzeei
Malenka californica
Aphanicerca capensis
Scopura montana
Capnia gracilaria
Diamphipnoa virecentipennis
Stenoperla maclellani
Austroperla cyrene
Trinotoperla nivata
Tallaperla lobata
Isoperla davisi
Plumiperla diversa
Calineuria californica
Pteronarcys californica
Cerconychia sp.
Plecoptera
4/5/0/8
98
-1/35/11/15
100
Ophiogomphus severus
Libellula saturata
Hetaerina americana
Argia vivida
Odonata
Paraleptophlebia sp.
Baetisca sp.
Hexagenia sp.
Ephemeroptera
Thermobia domestica
Battigrassiella wheeleri
Tricholepidion gertschii
Zygentoma
Archaeognatha
Support for Lathonomeria
Bremer ~ 18S: 31, 28S: 22, H3: 2, Morphology: 4
Nonparametric Bootstrap ~ 100%
50 changes

Figure 1.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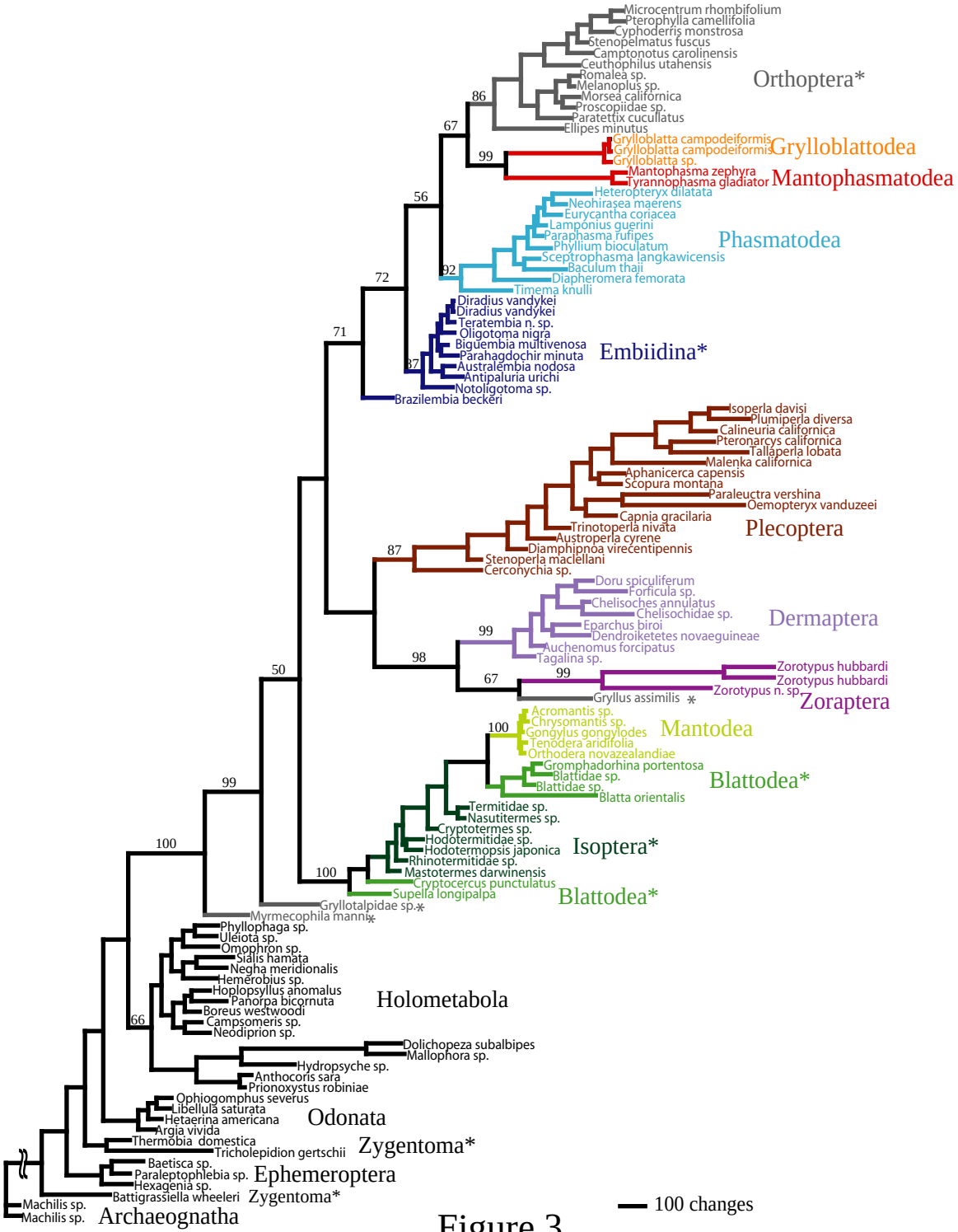
Rhinotermitidae sp.
Hodotermitidae sp.
Cryptotermes sp.
Hodotermopsis japonica
Mastotermes darwinensis
Isoptera
Cryptocercus punctulatus
Blatta orientalis
Blattidae sp.
Blattidae sp.
Gromphadorhina portentosa
Supella longipalpa
Blattodea*
Acromantis sp.
Chrysomantis sp.
Gongylus gongylodes
Tenodera aridifolia
Orthodera novazealandiae
Mantodea
Grylloblatta campodeiformis
Grylloblatta sp.
Grylloblatta campodeiformis
Grylloblattodea
Mantophasma zephyra
Tyrannophasma gladiator
Mantophasmatodea
Biguembia multivenosa
Parahagdochir minuta
Oligotoma nigra
Diradius vandykei
Diradius vandykei
Teratembia n. sp.
Australembia nodosa
Antipaluria urichi
Brazilembia beckeri
Notoligotoma sp.
Embiidina
Eurycantha coriacea
Lamponius guerini
Heteropteryx dilatata
Neohirasea maerens
Paraphasma rufipes
Phyllium bioculatum
Sceptrophasma langkawicensis
Baculum thaii
Diapheromera femorata
Timema knulli
Phasmatodea
Microcentrum rhombifolium
Pterophylla camellifolia
Cyphoderris monstrosa
Stenopelmatus fuscus
Camptonotus carolinensis
Ceuthophilus utahensis
Romalea sp.
Melanoplus sp.
Morsea californica
Proscopiidae sp.
Paratettix cucullatus
Gryllotalpidae sp.
Myrmecophila manni
Gryllus assimilis
Ellipes minutus
Orthoptera
Dolichopeza subalbipes
Mallophora sp.
Panorpa bicornuta
Hoplopsyllus anomalus
Boreus westwoodi
Anthocoris sara
Prionoxystus robiniae
Hydropsyche sp.
Phyllophaga sp.
Uleiota sp.
Omophron sp.
Hemerobius sp.
Negha meridionalis
Sialis hamata
Campsomeris sp.
Neodiprion sp.
Holometabola
Doru spiculiferum
Forficula sp.
Eparchus biroi
Chelisoches annulatus
Chelisochidae sp.
Tagalina sp.
Dendroiketetes novaeguineae
Auchenomus forcipatus
Dermaptera
Zorotypus n. sp.
Zorotypus hubbardi
Zorotypus hubbardi
Zoraptera
Paraleuctra vershina
Oemopteryx vanduzeei
Malenka californica
Aphanicerca capensis
Scopura montana
Diamphipnoa virecentipennis
Stenoperla maclellani
Austroperla cyrene
Capnia gracilaria
Trinotoperla nivata
Isoperla davisi
Plumiperla diversa
Calineuria californica
Pteronarcys californica
Tallaperla lobata
Cerconychia sp.
Plecoptera
Ophiogomphus severus
Libellula saturata
Hetaerina americana
Argia vivida
Odonata
Paraleptophlebia sp.
Baetisca sp.
Hexagenia sp.
Ephemeroptera
Battigrassiella wheeleri
Thermobia domestica
Zygentoma*
Tricholepidion gertschii
Machilis sp.
Machilis sp.
Archaeognatha

Figure 2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Pterophylla camellifolia
Cyphoderris monstrosa
Stenopelmatus fuscus
Camptonotus carolinensis
Ceuthophilus utahensis
Romalea sp.
Melanoplus sp.
Morsea californica
Proscopiidae sp.
Paratettix cucullatus
Ellipes minutus
Orthoptera*
Grylloblatta campodeiformis
Grylloblatta campodeiformis
Grylloblatta sp.
Grylloblattodea
Mantophasma zephyra
Tyrannophasma gladiator
Mantophasmatodea
Heteropteryx dilatata
Neohirasea maerens
Eurycantha coriacea
Lamponius guerini
Paraphasma rufipes
Phyllium bioculatum
Sceptrophasma langkawicensis
Baculum thaji
Diapheromera femorata
Timema knulli
Phasmatodea
Diradius vandykei
Diradius vandykei
Teratembia n. sp.
Oligotoma nigra
Biguembia multivenosa
Parahagdochir minuta
Australembia nodosa
Antipaluria urichi
Notoligotoma sp.
Brazilembia beckeri
Embiidina*
Isoperla davisi
Plumiperla diversa
Calineuria californica
Pteronarcys californica
Tallaperla lobata
Malenka californica
Aphanicerca capensis
Scopura montana
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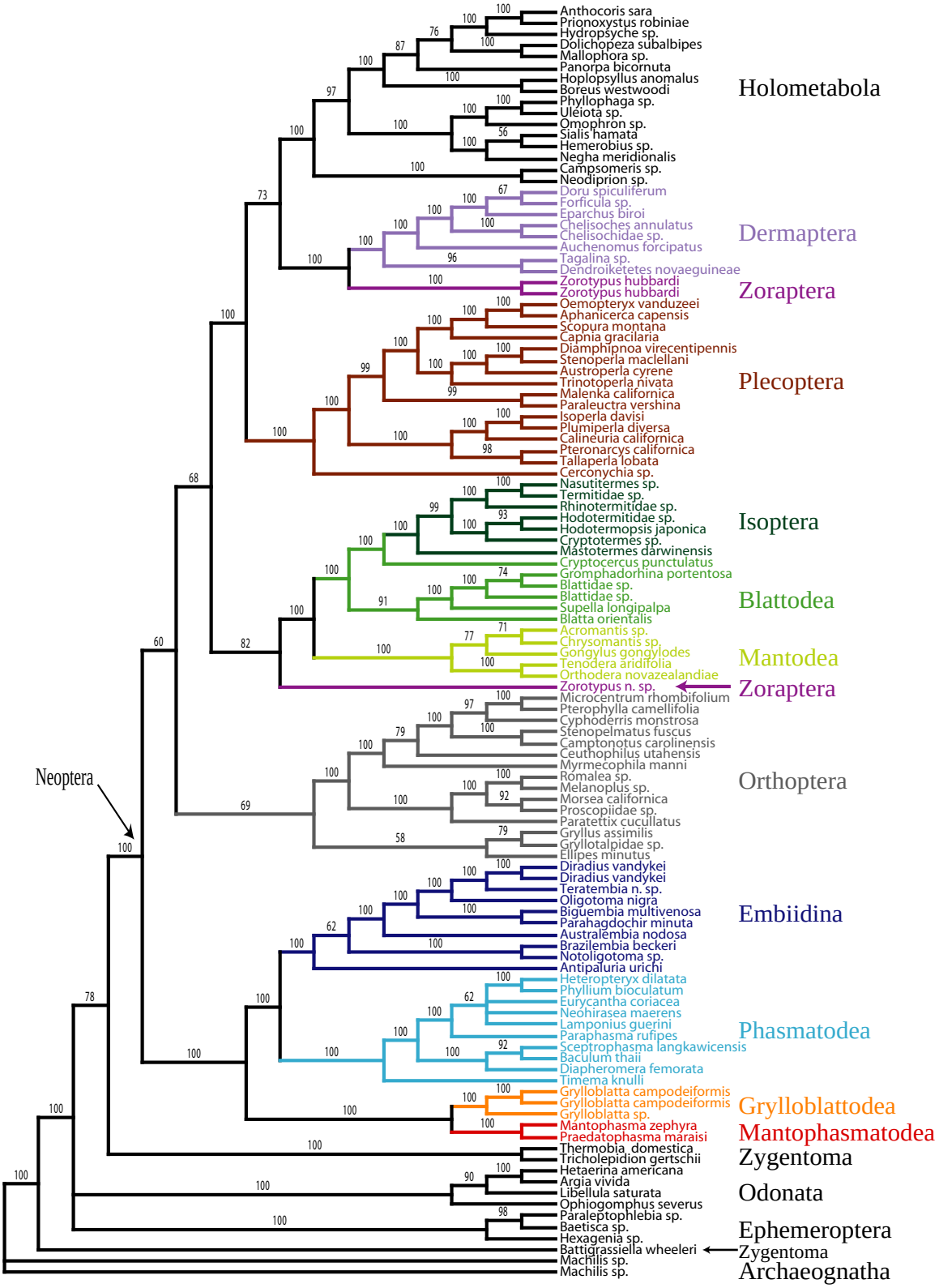
Oemopteryx vanduzeei
Capnia gracilaria
Trinotoperla nivata
Austroperla cyrene
Diamphipnoa virecentipennis
Stenoperla maclellani
Cerconychia sp.
Plecoptera
Doru spiculiferum
Forficula sp.
Chelisoches annulatus
Chelisochidae sp.
Eparchus biroi
Dendroiketetes novaeguineae
Auchenomus forcipatus
Tagalina sp.
Dermaptera
Zorotypus hubbardi
Zorotypus hubbardi
Zorotypus n. sp.
Gryllus assimilis *
Zoraptera
Acromantis sp.
Chrysomantis sp.
Gongylus gongylodes
Tenodera aridifolia
Orthodera novazealandiae
Mantodea
Gromphadorhina portentosa
Blattidae sp.
Blattidae sp.
Blatta orientalis
Blattodea*
Termitidae sp.
Nasutitermes sp.
Cryptotermes sp.
Hodotermitidae sp.
Hodotermopsis japonica
Rhinotermitidae sp.
Mastotermes darwinensis
Isoptera*
Cryptocercus punctulatus
Supella longipalpa
Blattodea*
Gryllotalpidae sp.*
Myrmecophila manni*
Phyllophaga sp.
Uleiota sp.
Omophron sp.
Sialis hamata
Negha meridionalis
Hemerobius sp.
Hoplopsyllus anomalus
Panorpa bicornuta
Boreus westwoodi
Campsomeris sp.
Neodiprion sp.
Holometabola
Dolichopeza subalbipes
Mallophora sp.
Hydropsyche sp.
Anthocoris sara
Prionoxystus robiniae
Ophiogomphus severus
Libellula saturata
Hetaerina americana
Argia vivida
Odonata
Thermobia domestica
Tricholepidion gertschii
Zygentoma*
Baetisca sp.
Paraleptophlebia sp.
Hexagenia sp.
Ephemeroptera
Battigrassiella wheeleri
Zygentoma*
Machilis sp.
Machilis sp.
Archaeognatha
86
67
99
56
72
92
71
87
87
99
98
99
67
50
100
100
99
100
66
100 changes

Figure 3.


Anthocoris sara
Prionoxystus robiniae
Hydropsyche sp.
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Mallophora sp.
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Hoplopsyllus anomalus
Boreus westwoodi
Phyllophaga sp.
Uleiota sp.
Omophron sp.
Sialis hamata
Hemerobius sp.
Negha meridionalis
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Chelisoches annulatus
Chelisochidae sp.
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Tagalina sp.
Dendroiketetes novaeguineae
Dermaptera
Zorotypus hubbardi
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Zoraptera
Oemopteryx vanduzeei
Aphanicerca capensis
Scopura montana
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Stenoperla maclellani
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Malenka californica
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Calineuria californica
Pteronarcys californica
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Rhinotermitidae sp.
Hodotermitidae sp.
Hodotermopsis japonica
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Mastotermes darwinensis
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Stenopelmatus fuscus
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Ceuthophilus utahensis
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Romalea sp.
Melanoplus sp.
Morsea californica
Proscopiidae sp.
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Gryllus assimilis
Gryllotalpidae sp.
Ellipes minutus
Orthoptera
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Diradius vandykei
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Teratembia n. sp.
Oligotoma nigra
Biguembia multivenosa
Parahagdochir minuta
Australembia nodosa
Brazilembia beckeri
Notoligotoma sp.
Antipaluria urichi
Embiidina
Heteropteryx dilatata
Phyllium bioculatum
Eurycantha coriacea
Neohirasea maerens
Lamponius guerini
Paraphasma rufipes
Sceptrophasma langkawicensis
Baculum thaii
Diapheromera femorata
Timema knulli
Phasmatodea
Grylloblatta campodeiformis
Grylloblatta campodeiformis
Grylloblatta sp.
Grylloblattodea
Mantophasma zephyra
Praedatophasma maraisi
Mantophasmatodea
Thermobia domestica
Tricholepidion gertschii
Zygentoma
Hetaerina americana
Argia vivida
Libellula saturata
Ophiogomphus severus
Odonata
Paraleptophlebia sp.
Baetisca sp.
Hexagenia sp.
Ephemeroptera
Battigrassiella wheeleri
Zygentoma
Machilis sp.
Machilis sp.
Archaeognatha

Figure 4.

Figure 1. Phylogeny of Polyneoptera and placement of Mantophasmatodea as sister group to Grylloblattodea (=Lathonomeria). Direct Optimization topology from a combined analysis of 18S rDNA, 28S rDNA, Histone 3, and morphology. Branch lengths were calculated in PAUP* under ACCTRAN optimization criteria using the implied alignment from POY. Numbers above ordinal and interordinal nodes represent partitioned Bremer support values for 18S/28/H3/Morphology calculated from the implied alignment. Numbers below these nodes represent non-parametric bootstrap support percentages for 1000 replicates. Tree length=16507 CI=0.417 RI=0.702.

Figure 2. Phylogeny of Polyneoptera with Bayesian analysis from implied alignment. Majority rule consensus of topologies generated via Mr. Bayes with 1,000,000 generations (first 50,000 discarded as "burn-in"), under the settings "lset nst=6 rates=gamma shape=0.5406". Numbers above nodes represent percentage of group inclusion among all topologies.

Figure 3. Parsimony analysis of CLUSTAL X alignment. Generated via PAUP* with 200 random additions and TBR swapping from CLUSTAL alignment under optimal parameter set as determined by ILD (Gap opening cost = 50, Transition Weight = 0.9). Gaps were treated as a "5^{th} position". Numbers above ordinal and interordinal nodes represent non-parametric bootstrap support percentages for 1000 replicates. Tree length=22295 CI=0.331 RI=0.661.

Figure 4. Baysian analysis of CLUSTAL X alignment. Majority rule consensus of topologies generated via MrBayes with 1,000,000 generations (first 50,000 discarded as "burn-in"), under the settings "lset nst=6 rates=gamma shape=0.5424". Numbers above nodes represent percentage of group inclusion among all topologies generated after burn-in period.

Comparison of two alignment techniques within a single data set: POY v. CLUSTAL

Matthew D. Terry[1,2] and Michael F. Whiting[1]

[1]Department of Integrative Biology, Brigham Young University, Provo, UT 84602,

USA

[2]Present Address: Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, Room 444, 1007 E. Lowell Street, Tucson, AZ 85721

Corresponding Author:

Matthew D. Terry

1007 E. Lowell St.

University of Arizona

Tucson, AZ 85721

(520) 422-2326 Fax: (520) 621-3709

email: matthew_terry@arizona.edu

Nuclear ribosomal DNA has become a common marker for inferring deep-level phylogenetic relationships, particularly among arthropods (Giribet et al., 2001; Jarman et al., 2000; Wheeler et al., 2001; Whiting, 2001b; Whiting et al., 1997). Because these markers contain both conserved and variable regions they can provide information across multiple levels of divergence, however, this regional divergence can render alignment of these sequences ambiguous (Wheeler, 1995). Multiple alignment strategies for non-protein coding sequences can be divided into two categories: methods that minimize a cost for the alignment based upon some quantifiable criterion and methods that are not specifically based on cost but rather on intuition (alignment "by eye") or by manually manipulating the alignment to match a structural model (manual alignment based on secondary structure: (Kjer et al., 2001); (Xia, 2000). Cost minimization methods of alignment are superior to manual methods in the same way that trees constructed via some optimality criterion are superior to trees constructed by hand: cost minimization allows for objective, reproducible, and quantifiable results, whereas manual methods are arbitrary and subject to the whims of the particular investigator. Consequently, this study will focus on two popular cost minimization methods (CLUSTAL and POY) and characterize their behaviour across a wide range of alignment parameter values. We use character congruence across a parameter landscape as a metric and sensitivity analysis as a method to better understand how these alignment strategies are influenced by parameter selection.

Cost minimization methods include progressive algorithmic alignment via computer programs such as MultAlign (Corpet, 1988) and CLUSTAL X (Thompson et al., 1997). These methods are algorithmic, rather than optimality based, sensu Swofford and Olsen (1990), in that they follow simple algorithms to arrive at the multiple alignment without regards to a global optima. CLUSTAL proceeds by (i)

calculating a distance matrix based on a pairwise comparison of all sequences, (ii) constructing a "guide tree" based on that distance matrix, and (iii) aligning sequences in the order specified by the topology of the guide tree (Thompson et al., 1994). This procedure generates an alignment based on overall similarity of sequences, and does not attempt to globally maximize any optimality criterion (for a complete description see Thompson et al., 1994). The advantage of this method is that it is relatively fast. The disadvantage is that it does not attempt to produce a globally optimal alignment, and hence it is very common for users to provide "manual adjustments" to the resulting CLUSTAL alignment (Crandall and Fitzpatrick, 1996; Renner and Won, 2001).

Alignments based on optimality criteria, including OMA (Reinert et al., 2000) and MALIGN (Wheeler and Gladstein, 1994) specifically attempt to find a globally optimal solution. Direct Optimization (formerly named "optimization alignment" and hereafter abbreviated as DO) using POY is somewhat different in that it treats multiple alignment as a dynamic statement of homology which changes under different topologies, and hence does not require a multiple alignment prior to tree reconstruction (Giribet et al., 2002; Gladstein and Wheeler, 2002). DO forgoes traditional multiple alignment by directly aligning and optimizing sequence data at every node of the topology and constructing hypothetical ancestral sequences for each node (Wheeler, 1996). Under DO, a cost is associated with each unique topology (using either a parsimony or maximum likelihood criterion), and each topology implies a different statement of character homology (Gladstein and Wheeler, 2002). Hence searching for the optimal alignment of sequences and optimal topology are treated simultaneously, rather than treating each as a distinct and unconnected analytical problem. The advantage of this method is that it allows the evaluation of

literally millions of topologies as alternate “guide trees,” is theoretically consistent in that the same criterion is used to construct the topology and sequence alignment, and it allows homology to be a dynamic rather than a static statement. The challenge lies in the complex computation associated with (1) evaluating all possible topologies and (2) evaluating all possible ways to optimize a set of sequences on any given topology. The search for the optimal solution under DO is thus a nested NP complete problem. POY employs heuristic searching methods such as tree ratchet, tree fusing, random replicates, and various swapping options that can ensure a rigorous search of tree space. Given an optimal solution, POY can generate an “implied alignment” similar to a standard multiple alignment, but with individual character states linking ancestor-descendent states (Wheeler, 2003a). While the “implied alignment” is not intended to be identical to a multiple alignment (Wheeler, 2003b), previous studies have indicated that DO produces a much more optimal multiple alignment than CLUSTAL under parsimony (Ogden and Whiting, 2003) and maximum likelihood (A. Whiting et al., in prep). Consequently, in this paper we are using DO as a tool for generating multiple alignments, and compare these alignments to those constructed by CLUSTAL.

All alignment methods based on optimality criteria must select specific parameter values in order to generate an optimal alignment. The most basic parameters are transition:transversion ratios and gap cost: nucleotide substitution ratios. What is not clear is how sensitive CLUSTAL and DO are to the selection of specific parameter values, and whether one alignment method is more sensitive than the other. Sensitivity analysis, in the context of phylogenetic studies, is the exploration of how variation in underlying assumptions can affect the outcome of analyses (Giribet, 2003). (Wheeler, 1995) This type of analysis can be performed as

an examination of the differences between methods of phylogenetic reconstruction (i.e., parsimony v. maximum likelihood) or the differences within methods (i.e., parsimony under a range of weighting schemes or maximum likelihood under a range of evolutionary models). This type of analysis has become more common recently, particularly in analyses using ribosomal data (Giribet et al., 2002; Ogden and Whiting, 2003; Shull et al., 2001; Whiting et al., 2003). This paper focuses on contrasting POY and CLUSTAL X across a broad parameter landscape for a complex ribosomal data set on insect ordinal phylogeny, with the goal of evaluating character congruence across these parameter landscapes for each alignment method.

METHODS

Analyses were performed using a data set consisting of 125 morphology characters and three genetic markers (18S rDNA, 28S rDNA, and Histone 3) for 113 insects. Sampling focused on the lower neopterous insect orders, but also included multiple outgroups; a complete characterization of the data set and phylogenetic results can be found in Terry and Whiting (Submitted).

All possible alignment parameters can be assessed via an *n*-dimensional space with each dimension defined by individual parameter ranges of interest (Wheeler, 1995). We explored the interaction of three parameters (gap cost, transversion cost, and transition cost) by defining a two-dimensional search space bounded on one side by the gap-transversion ratio and on the other by the transversion-transition ratio. The triangle inequality (Wheeler, 1993) sets the lower limit for both ratios at 0.5. Values below this number make it less costly to assume two transversions (i.e., T → A → C) rather than a single transition (i.e., T → C) or two indel events (i.e., T → gap → A) rather than a single base change (i.e., T → A). Ratios below 0.5 would thus create alignments that effectively eliminate one of the parameters of interest. There is no

theoretical upper bound for either the gap-transversion ratio or the transversion-transition ratio, therefore we selected arbitrarily high values for these ratios.

The incongruence length metric (ILD; (Mickevich and Farris, 1981) was used to assess character congruence across the parameter landscape. While the ILD has recently been challenged as a statistical test for determining data set heterogeneity (DeSalle and Brower, 1997; Dowton and Austin, 2002; Flynn and Nedbal, 1998; Messenger and McGuire, 1998; Yoder et al., 2001), in the context of character congruence it still remains an informative metric. The ILD was computed for each parameter combination for DO and CLUSTAL X alignment (Tables 1 and 2, respectively), by taking the difference between the length of the total tree, minus the sum of the lengths of the individual partitions (morphology, 18S, 28S, H3), divided by the length of the total tree. For CLUSTAL X, the ILD metric was computed from the lengths of the trees found for each partition and the total combined data under parsimony analysis with gaps treated as missing in PAUP* (Swofford, 2002) with TBR branch swapping and 50 random additions each. For DO the ILD metric was computed directly from the tree scores in two ways: (1) by comparing each partition versus the total data and (2) by comparing each ribosomal partition (18S and 28S) versus the total molecular data. The latter was done to determine if the "pre-aligned" condition of the morphology and H3 data sets were skewing the ILD results.

The range of possible parameter values for gap-transversion ratios and transition-transversion ratios available for CLUSTAL X are more narrow than in POY, and specific parameter values are not directly comparable between the two methods (Ogden and Whiting, 2003). Therefore, we attempted to cover a wide range of possible parameter values for both CLUSTAL X and POY in order to represent comparable parameter landscapes. For the DO analysis 9 gap-transversion ratios

ranging from 0.5 to 100, and 10 transversion-transition ratios ranging from 0.5 to infinity were sampled. Past studies demonstrate that the lower ratio values tend to produce more congruent results (Giribet et al., 2001; Wheeler, 1995; Whiting et al., 2003) so this region of the search space was sampled more densely. This sampling strategy produced 90 parameter combinations (Table 1). Analyses were performed for each individual data set (morphology, 18S, 28S, H3) and a combined total-evidence data set using the program POY version 2.0 on an IBM SP2 supercomputer. The ribosomal data sets were analyzed for all 90 parameter combinations. Thee H3 data set was designated as "pre-aligned" based on a conserved protein reading frame, lacked any indels, and thus was analyzed for only the 10 transversion to transition parameter sets. For each analysis, POY was run in parallel across 4 nodes on the supercomputer utilizing tree drifting and tree fusing with spr and tbr branch swapping and 10 random replicates using the commands:

-fitchtrees -maxprocessors 8 -onan -onannum 1 -parallel -noleading -norandomizeoutgroup -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -random 10 -stopat 25 -multirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -numdriftchanges 30 -driftspr -numdriftspr 10 -drifttbr -numdrifttbr 10 -slop 10 -checkslop 10 –impliedalignment -molecularmatrix *.txt -seed -1

(* asterisk denotes filenames that varied between individual analyses)

CLUSTAL X allows the user to choose a "gap opening penalty" ranging from 0 to 100 and 19 values spanning this range were used in this analysis, with values between 0 and 20 sampled more densely. "Transition weight" can be set between 0 and 1, with 0 signifying a mismatch between transitions (high cost) and 1 signifying a match (no cost). We selected 11 values beginning at 0 and incrementally increasing

by 0.1. This generated a total of 198 separate parameter sets (Table 2). CLUSTAL X also allows the user to designate a "gap extension penalty" which represents the cost of inserting a gap next to a previously inserted gap. For the purposes of this analysis this value was set at the default ratio (0.44 times the gap opening penalty) for all parameter sets.

RESULTS

Of the 90 parameter sets investigated using DO, the set treating transitions, transversions, and gaps equally (1:1:1) yielded the most congruent results, with an ILD value of 0.0350 (Table 1). These parameter values also maximized character congruence when only considering the ribosomal data, and produced a similar landscape (not shown). The parameter values with the least congruence between data partitions (ILD value of 0.1222) gives transversions a weight 4 times that of transitions and gaps a weight 100 times that of transversions. The standard deviation for all calculated congruence values is 0.0264 with a mean value of 0.0701. The sensitivity landscape generated from the DO analysis (Figure 1) is a nearly smooth topography with incongruence increasing more rapidly along the gap:transversion axis than the transversion:transition axis.

Of the 198 parameter sets investigated for the CLUSTAL analysis, character congruence between partitions was maximized with a gap opening cost of 50 and a transition weight of 0.9 (ILD value of 0.0306); the greatest incongruence was obtained when the parameter set had gap openings set to 1 and a transition weight of zero (ILD value of 0.1024). The CLUSTAL X default settings (Gap Opening = 15, DNA Transition Weight = 0.5) resulted in an ILD value of 0.0466. The standard deviation for all calculated congruence values is 0.0082 with a mean value of 0.0476.

The sensitivity landscape generated from this analysis (Figure 2) was much less smooth with bands of relatively congruent results when the gap open cost was low (between 2 and 8) and the transition weight high (between 0.8 and 0.9).

Discussion

Wheeler and Hayashi (1998) create an index of the ILD metric values for a single data set under various alignment parameters and argue that the parameter combination maximizing congruence should be preferentially chosen over others. The purpose of their analysis was to determine optimal analytical parameters, however, behavior of this measure across alignment space yields additional information about the predictability of alignment methods. Predictability, as applied to alignment, means that parameter sets located in close proximity to one another on the total landscape will have similar measures of congruence and that parameter sets most distant from the optimal set will be less congruent than more proximal sets. Such a pattern of predictability would allow researchers to make qualitative assessments of alignment parameters across separate analyses and would support the idea that sensitivity analysis is a valuable tool for finding analytical parameters that best explain phylogenetic data. Additionally, such a pattern if observed for a large number of data sets would suggest that future sensitivity analyses could be limited to exploration of a small portion of the possible parameter space, focusing on the area of maximal congruence, without compromising overall results.

For this data set congruence, as measured by the ILD, is more predictable over the entire parameter space under DO than under CLUSTAL X alignment followed by standard parsimony analysis (Figs. 1 and 2). This is most likely due to the fact that DO is operating under an optimality criterion while CLUSTAL X alignment is a

simple heuristic procedure based on a primary guide tree. This could also be the explanation for the extreme incongruence found in a small subset of the CLUSTAL X alignments, as the primary guide tree can vary as alignment parameters are altered, and one would expect some degree of unpredictability as small adjustments in parameter values could conceivably have drastic effects in the guide tree topology. Sensitivity analyses performed for other data sets using DO methods yield patterns nearly identical to those seen in this analysis, with maximally congruent parameter sets occurring when both gap:transversion cost and transversion:transition cost are low and incongruence more effected by increases in the former (Giribet, 2001; Phillips et al., 2000; Wheeler and Hayashi, 1998; Wheeler et al., 2001). Several of these studies also arrive at the same optimal parameter set (1:1:1) arrived at here (Giribet et al., 2001; Ogden and Whiting, 2003; Svenson and Whiting, 2004; Terry et al., Submitted; Whiting, 2001a; Whiting et al., 2003). There are no comparable studies exploring congruence across alignment parameters for CLUSTAL analyses, so the question of whether the results produced here are typical remains unanswered.

Although the range of ILD values obtained under DO analysis versus the CLUSTAL alignment are similar (0.0350 - 0.1222, and 0.0306 – 0.1024, respectively), the CLUSTAL values contain only four very high ILD measures (see Fig. 2) and have a much lower standard deviation (0.0082 vs. 0.0264 for DO analysis). The combination of low predictability (as defined above) and low standard deviation among ILD values for the CLUSTAL alignment leads to one of three conclusions. First, the data sets in question contain contradictory signals (high degree of ‘interdataset’ homoplasy) and, therefore congruence may be expected to be stochastic across the parameter space. However,, the relatively high predictability of DO congruence measures seems to argue against this conclusion. Second, over

portions of the parameter space congruence among data sets is fairly robust to variations in the alignment parameters and any one of a number of parameter combinations within this space could be selected. Or third, lack of predictability makes it impossible to objectively select alignment parameters and alternative, more internally predictable alignment procedures should be explored when dealing with ribosomal data.

Given the assumption that congruence among data sets is a valid criterion for judging the quality of alignments, it follows that parameter sets greatly violating the assumptions judged to be optimal should demonstrate much lower levels of congruence. This appears to be the case for DO analysis, but not for alignment by CLUSTAL X followed by standard parsimony analysis. DO sensitivity analysis for this data set and others demonstrates that congruence is maximized over a relatively small portion of the conceivable parameter space encompassing relatively small gap:change and transversion:transition ratios. These results suggest that future sensitivity analyses might effectively be limited to exploration of a small portion of the possible parameter space without compromising overall results. Further research addressing whether or not this is the case for analyses using data with taxa of various molecular diversity and different complements of included genetic markers may greatly reduce the time and resources necessary for individual sensitivity analyses.

ACKNOWLEDGEMENTS

Analyses were performed at the Fulton Supercomputing Center at Brigham Young University with parallel software implementation by M. Clement and Q. Snell, based

on code made freely available by W. Wheeler. This work was supported by NSF grants DEB-0206363 and DEB-9983195 with NSF REU supplements.

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Table 1. Topology lengths and subsequent ILD values from DO analysis. Bold values represent global minimum ILD values. (Abbreviations: Tv = transversion, Ts = transition, morph. = morphology dataset, rib. = combine ribosomal dataset, mol. = combined molecular data set).

		Data Set									
Gap:Tv	Tv:Ts	morph.	28S	18S	rib.	rib. ILD	H3	mol.	mol. ILD	total	total ILD
0.5	0.5	238	18454	9651			5067			34816	0.04038
1	0.5	238	14505	7181			3468			26454	0.04015
2	0.5	238	18448	8416			3468			31907	0.04190
3	0.5	238	20938	9083			3468			35311	0.04486
4	0.5	238	23244	9671			3468			38455	0.04769
10	0.5	238	35384	12813			3468			55352	0.06231
20	0.5	238	54256	17526			3468			81888	0.07816
50	0.5	238	108609	31285			3468			158492	0.09396
100	0.5	238	197728	53577			3468			283770	0.10135
0.5	1	238	17043	8502			4596			31601	0.03867
1	1	238	11714	5298	17426	**0.02376**	2444	20163	**0.03506**	20409	**0.03503**
2	1	238	14644	5982	21198	0.02698	2444	23995	0.03855	24261	0.03928
3	1	238	16964	6571	24303	0.03160	2444	27130	0.04243	27410	0.04352
4	1	238	19430	7132	27285	0.02650	2444	30141	0.03766	30453	0.03970
10	1	238	31101	10100	43792	0.05917	2444	46499	0.06138	46958	0.06548
20	1	238	49741	14796			2444			72478	0.07256
50	1	238	104260	28822			2444			149534	0.09209
100	1	238	193383	49751			2444			277580	0.11443
0.5	2	238	13893	6479			3614			25111	0.03532
1	2	238	18466	7817	26999	0.02652	3614	31129	0.03958	31384	0.03980
2	2	238	23823	9166	33997	0.02965	3614	38195	0.04168	38486	0.04274
3	2	238	28389	10350	40116	0.03433	3614	44351	0.04505	44687	0.04690
4	2	238	32738	11341	45975	0.04124	3614	50170	0.04937	50488	0.05065
10	2	238	55443	17363	78650	0.07430	3614	83280	0.08237	83163	0.07822
20	2	238	92717	26705			3614			135590	0.09083
50	2	238	201120	53915			3614			291666	0.11239
100	2	238	381830	99067			3614			542762	0.10688
0.5	3	238	36885	16798			9481			66114	0.04102
1	3	238	24982	10257	36184	0.02612	4751	41597	0.03863	41873	0.03929
2	3	238	32785	12261	46577	0.03287	4751	52004	0.04244	52358	0.04437
3	3	238	39420	14074	55688	0.03940	4751	61111	0.04690	61473	0.04864
4	3	238	45936	15618	64375	0.04382	4751	69961	0.05226	70178	0.05180
10	3	238	80419	24804	113466	0.07265	4751	118677	0.07333	119829	0.08026
20	3	238	136299	38710			4751			198285	0.09223
50	3	238	298668	80489			4751			433227	0.11329
100	3	238	568333	147318			4751			811159	0.11159
0.5	4	238	22892	10266			5847			40970	0.04215
1	4	238	31389	12713	45259	0.02556	5847	52070	0.04073	52382	0.04190
2	4	238	41740	15321	59088	0.03430	5847	65818	0.04421	66150	0.04541
3	4	238	50768	17677	71073	0.03698	5847	77887	0.04616	78132	0.04610
4	4	238	59164	19764	82920	0.04814	5847	89576	0.05360	90218	0.05769
10	4	238	104864	31125	147986	0.08107	5847	154234	0.08038	155485	0.08625
20	4	238	179095	50496			5847			258662	0.08887
50	4	238	396597	105820			5847			567574	0.10408
100	4	238	753187	188234			5847			1079359	0.12216
0.5	10	238	48986	21309			12383			87052	0.04751
1	10	238	69772	27168	99834	0.02899	12383	114444	0.04475	114700	0.04480
2	10	238	95262	33794	133757	0.03515	12383	148159	0.04536	148561	0.04634
3	10	238	117160	39372	163685	0.04370	12383	178350	0.05290	178834	0.05413
4	10	238	138397	44708	192393	0.04828	12383	207547	0.05810	208066	0.05931
10	10	238	252027	73997	353512	0.07776	12383	368354	0.08130	367482	0.07847
20	10	238	435983	120499			12383			627388	0.09290
50	10	238	972439	253819			12383			1397900	0.11376

100	10	238	1879672	485520	12383	2628938	0.09552
0.5	20	238	92176	39675	23148	163306	0.04941
1	20	238	133157	51021	23148	218023	0.04797
2	20	238	183958	64378	23148	286421	0.05132
3	20	238	228360	75664	23148	345982	0.05368
4	20	238	268556	86543	23148	403947	0.06303
10	20	238	496955	145252	23148	722737	0.07907
20	20	238	858330	238855	23148	1235485	0.09301
50	20	238	1937117	515641	23148	2752190	0.10030
100	20	238	3758134	964116	23148	5307895	0.10593
0.5	50	238	221441	94788	55642	391329	0.04911
1	50	238	325274	123546	55642	529389	0.04664
2	50	238	449337	156489	55642	698099	0.05213
3	50	238	558362	182598	55642	847803	0.06011
4	50	238	663053	210047	55642	991255	0.06282
10	50	238	1226657	357391	55642	1796706	0.08726
20	50	238	2143954	575659	55642	3084376	0.10014
50	50	238	4834661	1271191	55642	6922670	0.10992
100	50	238	9323658	2401340	55642	13259818	0.11154
0.5	100	238	437154	186092	109508	773250	0.05206
1	100	238	643142	244696	109508	1047862	0.04798
2	100	238	894977	308566	109508	1386168	0.05258
3	100	238	1112212	364968	109508	1684113	0.05771
4	100	238	1320594	416714	109508	1978663	0.06651
10	100	238	2455429	711105	109508	3568587	0.08191
20	100	238	4275103	1176009	109508	6116514	0.09085
50	100	238	9653891	2515246	109508	13881738	0.11547
100	100	238	18832288	4843214	109508	26669629	0.10815
0.5	∞	238	8583	3615	2150	15408	0.05335
1	∞	238	6297	2363	1078	10503	0.05018
2	∞	238	8724	2921	1078	13777	0.05923
3	∞	238	10731	3374	1078	16543	0.06782
4	∞	238	12658	3839	1078	19143	0.06948
10	∞	238	23345	6414	1078	34089	0.08842
20	∞	238	40351	10334	1078	58624	0.11297
50	∞	238	90633	22099	1078	128617	0.11327
100	∞	238	178936	41704	1078	248784	0.10784

Table 2. Topology lengths and subsequent ILD values from CLUSTAL X alignments with PAUP* analyses.

		Data Set					
G.O.	T.S.W	18S	28S	H3	morph	total	ILD
1	0	5589	11154	2400	234	21588	0.10242
1	0.1	5513	10996	2400	234	21213	0.09758
1	0.2	5435	10920	2400	234	21045	0.09770
1	0.3	5427	10889	2400	234	21037	0.09921
1	0.4	5423	10804	2400	234	19980	0.05601
1	0.5	5365	10867	2400	234	19887	0.05134
1	0.6	5352	10761	2400	234	19660	0.04644
1	0.7	5358	10895	2400	234	19783	0.04529
1	0.8	5387	11072	2400	234	20049	0.04768
1	0.9	5498	11169	2400	234	20195	0.04427
1	1.0	5783	12175	2400	234	21697	0.05093
2	0	5488	10943	2400	234	20077	0.05041
2	0.1	5397	10858	2400	234	19840	0.04793
2	0.2	5348	10808	2400	234	19756	0.04890
2	0.3	5292	10701	2400	234	19550	0.04721
2	0.4	5289	10609	2400	234	19397	0.04459
2	0.5	5226	10623	2400	234	19315	0.04308
2	0.6	5249	10712	2400	234	19403	0.04164
2	0.7	5253	10788	2400	234	19464	0.04054
2	0.8	5293	11032	2400	234	19829	0.04388
2	0.9	5382	11120	2400	234	19963	0.04143
2	1.0	5625	11750	2400	234	20969	0.04578
4	0	5259	10824	2400	234	19552	0.04271
4	0.1	5239	10772	2400	234	19491	0.04340
4	0.2	5203	10753	2400	234	19440	0.04372
4	0.3	5146	10695	2400	234	19290	0.04225
4	0.4	5140	10768	2400	234	19353	0.04191
4	0.5	5192	10746	2400	234	19397	0.04253
4	0.6	5176	10828	2400	234	19456	0.04204
4	0.7	5157	10832	2400	234	19377	0.03891
4	0.8	5167	10905	2400	234	19493	0.04037
4	0.9	5119	11071	2400	234	19658	0.04243
4	1.0	5348	11450	2400	234	20258	0.04077
6	0	5239	10948	2400	234	19662	0.04277
6	0.1	5238	10965	2400	234	19733	0.04541
6	0.2	5227	10929	2400	234	19673	0.04488
6	0.3	5135	10844	2400	234	19442	0.04264
6	0.4	5236	10925	2400	234	19617	0.04190
6	0.5	5168	10918	2400	234	19522	0.04108
6	0.6	5218	10978	2400	234	19655	0.04197
6	0.7	5222	11020	2400	234	19702	0.04192
6	0.8	5259	11009	2400	234	19736	0.04226
6	0.9	5202	11077	2400	234	19746	0.04219
6	1.0	5413	11573	2400	234	20451	0.04063
8	0	5288	11051	2400	234	19806	0.04206
8	0.1	5251	11166	2400	234	19939	0.04454
8	0.2	5251	11125	2400	234	19932	0.04626
8	0.3	5288	11112	2400	234	19932	0.04505
8	0.4	5283	11175	2400	234	19987	0.04478
8	0.5	5252	11131	2400	234	19877	0.04327
8	0.6	5235	11105	2400	234	19807	0.04206
8	0.7	5301	11047	2400	234	19835	0.04300
8	0.8	5261	11204	2400	234	19968	0.04352
8	0.9	5173	11260	2400	234	19935	0.04354
8	1.0	5319	11617	2400	234	20440	0.04256
10	0	5416	11259	2400	234	20303	0.04896
20	0	5543	11671	2400	234	20855	0.04829
20	0.1	5503	11765	2400	234	20932	0.04921
20	0.2	5493	11725	2400	234	20853	0.04800
20	0.3	5423	11716	2400	234	20758	0.04745
20	0.4	5514	11686	2400	234	20796	0.04626
20	0.5	5469	11801	2400	234	20831	0.04450
20	0.6	5475	11876	2400	234	20968	0.04688
20	0.7	5500	11698	2400	234	20809	0.04695
20	0.8	5672	11913	2400	234	20967	0.03568
20	0.9	5303	12059	2400	234	20904	0.04344
20	1.0	5473	12010	2400	234	21199	0.05104
30	0	5653	11946	2400	234	21295	0.04987
30	0.1	5638	12096	2400	234	21504	0.05283
30	0.2	5596	12065	2400	234	21311	0.04767
30	0.3	5558	12257	2400	234	21556	0.05135
30	0.4	5620	12231	2400	234	21535	0.04876
30	0.5	5614	12216	2400	234	21485	0.04752
30	0.6	5619	12280	2400	234	21532	0.04640
30	0.7	5626	12286	2400	234	21566	0.04730
30	0.8	5666	12330	2400	234	21503	0.04060
30	0.9	5431	12542	2400	234	21568	0.04456
30	1.0	5607	12598	2400	234	22061	0.05539
40	0	5744	12349	2400	234	21766	0.04773
40	0.1	5753	12506	2400	234	21946	0.04798
40	0.2	5752	12517	2400	234	21990	0.04943
40	0.3	5754	12548	2400	234	22059	0.05091
40	0.4	5798	12662	2400	234	22188	0.04931
40	0.5	5780	12473	2400	234	21894	0.04599
40	0.6	5764	12558	2400	234	22001	0.04750
40	0.7	5743	12563	2400	234	21923	0.04484
40	0.8	5789	12573	2400	234	21957	0.04377
40	0.9	5541	12641	2400	234	21779	0.04422
40	1.0	5657	12802	2400	234	22101	0.04561
50	0	5850	12899	2400	234	22408	0.04574
50	0.1	5861	12833	2400	234	22347	0.04560
50	0.2	5855	12825	2400	234	22356	0.04661
50	0.3	5809	12897	2400	234	22359	0.04557
50	0.4	5855	12857	2400	234	22382	0.04629
50	0.5	5820	12893	2400	234	22400	0.04701
50	0.6	5804	12902	2400	234	22379	0.04643
50	0.7	5808	13012	2400	234	22541	0.04822
50	0.8	5853	12872	2400	234	22370	0.04519
50	0.9	5631	13348	2400	234	22295	**0.03059**
50	1.0	5761	13322	2400	234	22771	0.04629
60	0	5935	13206	2400	234	22867	0.04775
60	0.1	5950	13141	2400	234	22865	0.04986
60	0.2	5970	13145	2400	234	22893	0.04997
60	0.3	5921	13255	2400	234	22957	0.04996
60	0.4	5905	13259	2400	234	22942	0.04986
60	0.5	5859	13194	2400	234	22809	0.04919
60	0.6	5916	13125	2400	234	22772	0.04817
60	0.7	5948	13170	2400	234	22779	0.04509
60	0.8	5929	13274	2400	234	22898	0.04634
60	0.9	5743	13491	2400	234	22893	0.04477
60	1.0	5839	13778	2400	234	23461	0.05157
70	0	6092	13440	2400	234	23310	0.04908

10	0.1	5398	11289	2400	234	20280	0.04729		70	0.1	6148	13445	2400	234	23397	0.05001
10	0.2	5373	11302	2400	234	20224	0.04524		70	0.2	6108	13449	2400	234	23389	0.05122
10	0.3	5376	11331	2400	234	20250	0.04489		70	0.3	5940	13549	2400	234	23256	0.04872
10	0.4	5280	11327	2400	234	20167	0.04592		70	0.4	6038	13588	2400	234	23474	0.05172
10	0.5	5400	11342	2400	234	20304	0.04571		70	0.5	6000	13779	2400	234	23604	0.05046
10	0.6	5407	11354	2400	234	20318	0.04543		70	0.6	5982	13840	2400	234	23639	0.05004
10	0.7	5373	11358	2400	234	20257	0.04403		70	0.7	6057	13775	2400	234	23621	0.04890
10	0.8	5408	11391	2400	234	20343	0.04473		70	0.8	6054	13825	2400	234	23662	0.04856
10	0.9	5224	11404	2400	234	20146	0.04388		70	0.9	5859	13961	2400	234	23478	0.04362
10	1.0	5322	11722	2400	234	20579	0.04378		80	1.0	6032	14315	2400	234	24183	0.04970
12	0	5467	11308	2400	234	20343	0.04591		80	0	6220	13587	2400	234	23582	0.04838
12	0.1	5386	11362	2400	234	20300	0.04522		80	0.1	6170	13566	2400	234	23520	0.04889
12	0.2	5432	11401	2400	234	20410	0.04620		80	0.2	6210	13630	2400	234	23640	0.04932
12	0.3	5385	11376	2400	234	20354	0.04712		80	0.3	6086	13631	2400	234	23437	0.04634
12	0.4	5314	11409	2400	234	20333	0.04800		80	0.4	6111	13693	2400	234	23570	0.04803
12	0.5	5455	11432	2400	234	20415	0.04379		80	0.5	6097	13878	2400	234	23798	0.04996
12	0.6	5468	11426	2400	234	20457	0.04541		80	0.6	6194	13857	2400	234	23965	0.05341
12	0.7	5447	11619	2400	234	20678	0.04730		80	0.7	6180	13860	2400	234	23830	0.04851
12	0.8	5413	11507	2400	234	20415	0.04217		80	0.8	6104	14039	2400	234	23972	0.04985
12	0.9	5219	11606	2400	234	20314	0.04209		80	0.9	5898	14086	2400	234	23678	0.04477
12	1.0	5318	11799	2400	234	20649	0.04349		80	1.0	6233	14647	2400	234	24714	0.04856
15	0	5392	11577	2400	234	20543	0.04576		90	0	6331	13822	2400	234	23972	0.04943
15	0.1	5376	11580	2400	234	20506	0.04467		90	0.1	6234	13828	2400	234	23822	0.04727
15	0.2	5460	11464	2400	234	20571	0.04924		90	0.2	6216	13836	2400	234	23793	0.04653
15	0.3	5425	11477	2400	234	20526	0.04823		90	0.3	6088	13832	2400	234	23613	0.04485
15	0.4	5489	11529	2400	234	20623	0.04708		90	0.4	6200	14086	2400	234	24194	0.05266
15	0.5	5463	11564	2400	234	20621	0.04655		90	0.5	6217	14084	2400	234	24219	0.05302
15	0.6	5474	11514	2400	234	20610	0.04794		90	0.6	6215	14105	2400	234	24253	0.05356
15	0.7	5452	11535	2400	234	20611	0.04803		90	0.7	6337	14341	2400	234	24508	0.04880
15	0.8	5434	11702	2400	234	20682	0.04410		90	0.8	6313	14699	2400	234	24838	0.04799
15	0.9	5223	11739	2400	234	20482	0.04326		90	0.9	6141	14998	2400	234	24850	0.04334
15	1.0	5328	11870	2400	234	20692	0.04156		90	1.0	6234	15146	2400	234	25171	0.04597
18	0	5519	11745	2400	234	20926	0.04913		100	0	6329	13876	2400	234	23944	0.04615
18	0.1	5496	11684	2400	234	20873	0.05074		100	0.1	6319	14189	2400	234	24434	0.05288
18	0.2	5505	11674	2400	234	20846	0.04955		100	0.2	6307	14134	2400	234	24294	0.05018
18	0.3	5440	11634	2400	234	20746	0.05003		100	0.3	6223	14076	2400	234	24161	0.05083
18	0.4	5495	11721	2400	234	20856	0.04824		100	0.4	6354	14333	2400	234	24590	0.05161
18	0.5	5500	11679	2400	234	20792	0.04709		100	0.5	6383	14481	2400	234	24718	0.04936
18	0.6	5483	11707	2400	234	20808	0.04729		100	0.6	6386	14757	2400	234	24960	0.04740
18	0.7	5500	11634	2400	234	20803	0.04975		100	0.7	6689	14978	2400	234	25544	0.04866
18	0.8	5530	11803	2400	234	20908	0.04501		100	0.8	6691	15506	2400	234	26251	0.05409
18	0.9	5290	11830	2400	234	20675	0.04455		100	0.9	6293	16034	2400	234	26237	0.04863
18	1.0	5447	12016	2400	234	21038	0.04473		100	1.0	6477	15422	2400	234	25784	0.04852

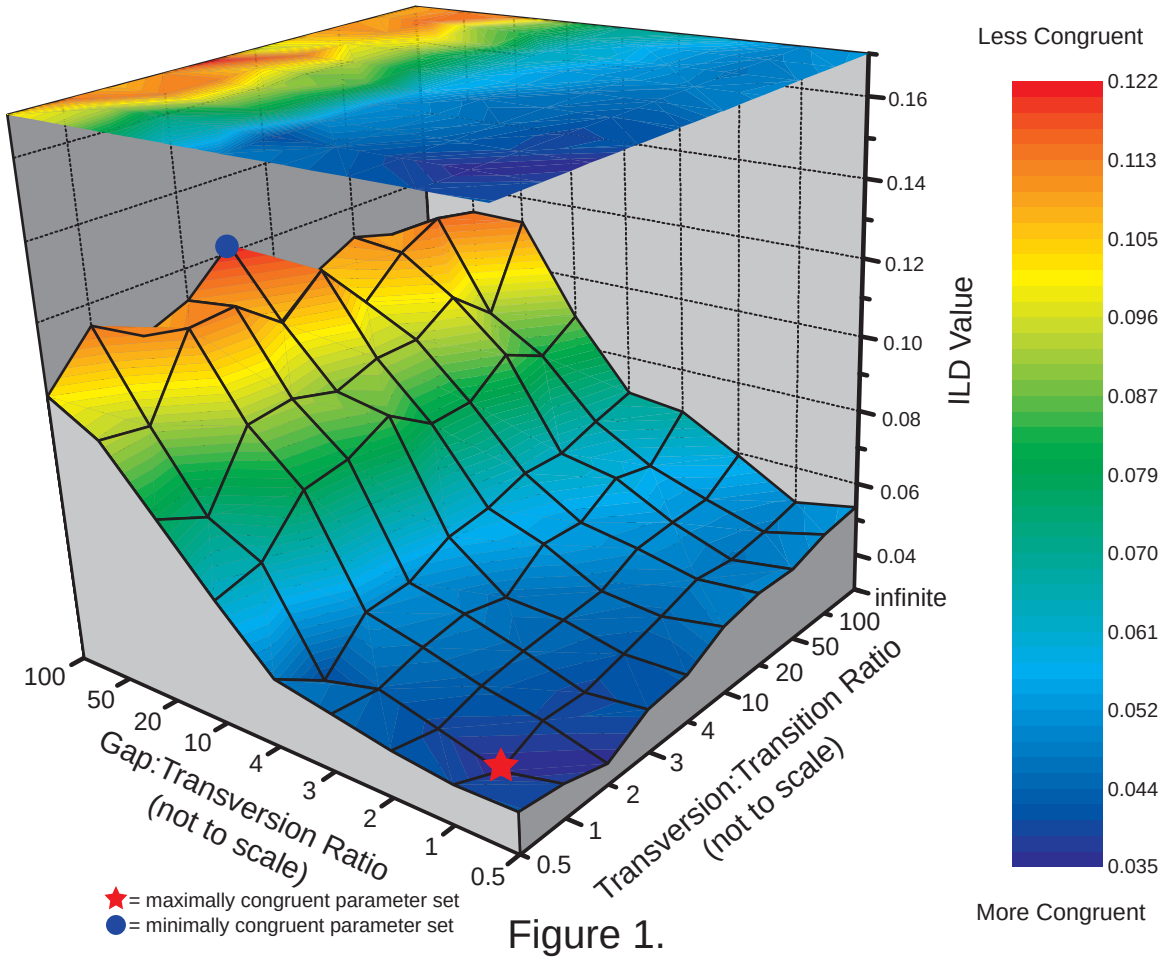
Less Congruent
0.122
0.113
0.105
0.096
0.087
0.079
0.070
0.061
0.052
0.044
0.035
More Congruent
ILD Value
0.16
0.14
0.12
0.10
0.08
0.06
0.04
100
50
20
10
4
3
2
1
0.5
Gap:Transversion Ratio
(not to scale)
0.5
1
2
3
4
10
20
50
100
infinite
Transversion:Transition Ratio
(not to scale)
= maximally congruent parameter set
= minimally congruent parameter set

Figure 1.

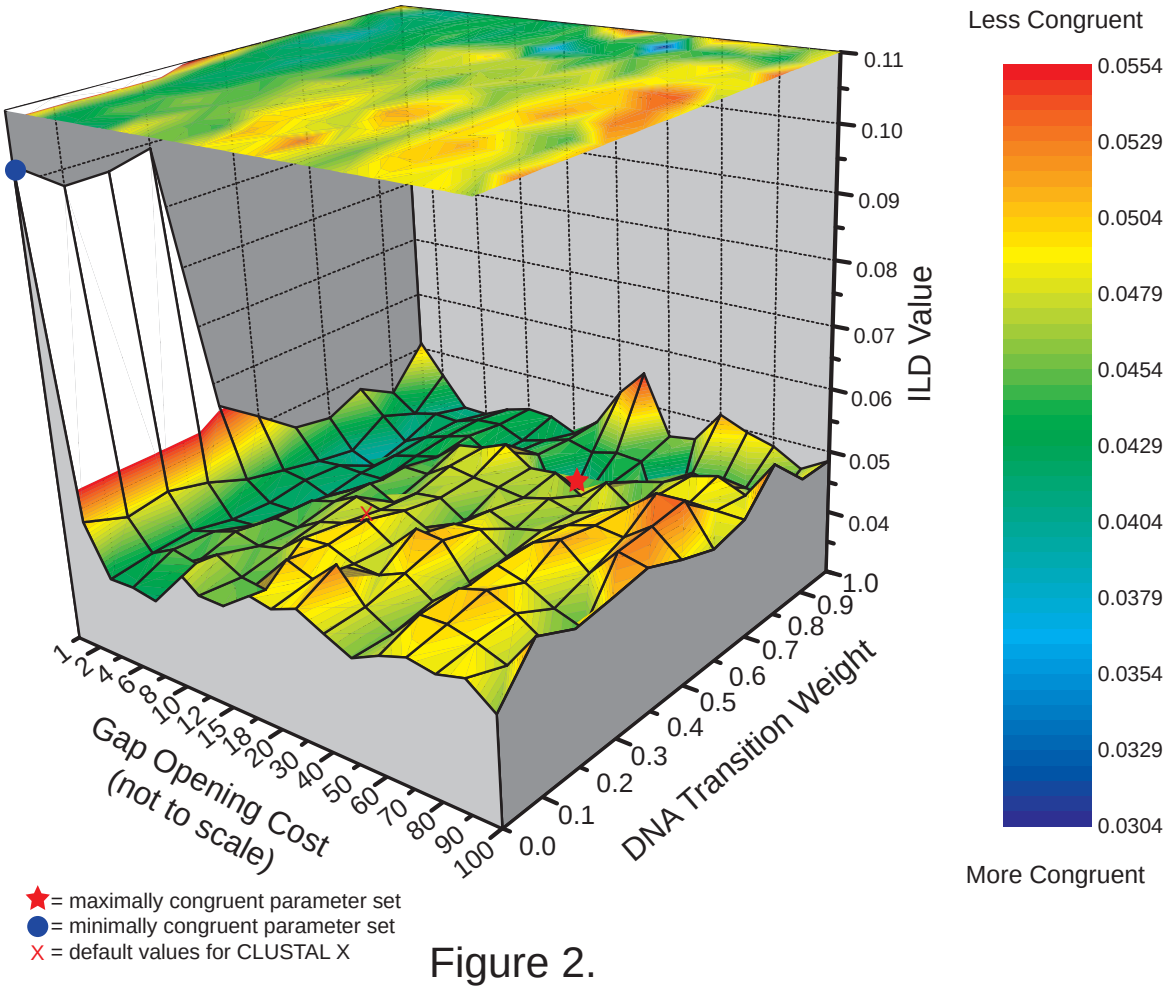
Less Congruent
0.0554
0.0529
0.0504
0.0479
0.0454
0.0429
0.0404
0.0379
0.0354
0.0329
0.0304
More Congruent
ILD Value
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
Gap Opening Cost
(not to scale)
1 2 4 6 8 10 12 15 18 20 30 40 50 60 70 80 90 100
DNA Transition Weight
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
= maximally congruent parameter set
= minimally congruent parameter set
X = default values for CLUSTAL X

Figure 2.

Figure 1. Contour map of ILD values showing levels of congruence between data sets across multiple cost parameter values for the DO analysis. Multiple analyses were conducted which varied the gap:transversion ratio from 0.5 - 100 and the transition:transversion ratio 0.5 – infinity across all data partitions. Congruence was measured using the ILD metric (Farris et al., 1995) with smaller numerical values representing more congruence. These results demonstrate that the data sets are maximally congruent when all parameters are set to unity.

Figure 2. Contour map of ILD values showing levels of congruence between data sets across multiple alignment parameter values for CLUSTAL X alignment followed by parsimony analysis in PAUP*. Analyses were conducted which varied the gap opening cost from 0 – 100 and the DNA transition weight from 0 – 1. Congruence was measured using the ILD metric (Farris et al., 1995) where smaller numerical values representing more congruence.

Phylogenetic Systematics of Plecoptera: Evidence from Morphology and Six Genes

Matthew D. Terry[1,2] and Michael F. Whiting[1]

1. Department of Integrative Biology, Brigham Young University, WIDB 401, Provo, UT 84602

2. Present Address: Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, Room 444, 1007 E. Lowell Street, Tucson, AZ 85721

Abstract—Phylogenetic relationships among stoneflies (Plecoptera) were reconstructed based on six molecular markers (12S, 16S, 18S, 28S, COII, and H3) and morphological data. Phylogenetic analysis demonstrates the monophyly of the subordinal designations Arctoperlaria and Antarctoperlaria, the superfamilial designations Systellognatha and Euholognatha are also supported as monophyletic by the combined data. Our analysis also supports the families Perlidae, Perlodidae, Peltoperlidae, Pteronarcyidae, Styloperlidae, Nemouridae, Notonemouridae, Capniidae, Taeniopterygidae, Eustheniidae, and Diamphipnoidae as monophyletic. Megaleuctra is the basal-most lineage within Plecoptera, thus rendering Leuctridae *sensu latu* paraphyletic, and we suggest recognition of the family Megaleuctridae. The families Chloroperlidae, Austroperlidae, and Gripopterygidae are not supported as monophyletic in this analysis, however, only Gripopterygidae is strongly supported as paraphyletic across multiple data partitions. Within the Systellognatha Styloperlidae is the basal lineage, followed by Peltoperlidae then Pteronarcyidae, and Perloidea is a strongly supported monophyletic group with Chloroperlidae as sister taxon to Perlidae + Perlodidae. Skimming behavior is a symplesiomorphy for Plecoptera, but the pre-mating behavior of drumming is a derived condition within Plecoptera.

Introduction

The order Plecoptera (commonly called stoneflies) is comprised of 16 families, a little over 2000 described species, and has representatives on every continent except Antarctica (Zwick, 1973). Plecopteran nymphs are entirely aquatic and, although a few

lineages have adapted to tropical and sub-tropical environments, the bulk of ordinal diversity occurs in the temperate regions of the world, particularly in habitats consisting of cool to cold rivers and streams with relatively high oxygen content. Many nymphs possess respiratory gills directly connected to the tracheal system and specialized osmoregulatory organs that enable them to take full advantage of their aquatic habitat. Plecoptera can be herbivorous, omnivorous, or carnivorous as nymphs and many do not feed as adults. They are also an important part of many aquatic ecosystems and can be used as bioindicators of pollution levels (U.S.EPA, 1990).

Plecoptera are traditionally associated with the lower Neoptera and often placed within Polyneoptera, a group comprised of ten other orders: Blattodea, Dermaptera, Embiidina, Grylloblattodea, Isoptera, Mantodea, Mantophasmatodea, Orthoptera, Phasmatodea, and Zoraptera. There has been little consensus regarding the placement of Plecoptera within this group (Kristensen, 1991; Wheeler et al., 2001). Hennig (1981) recognized all of the polyneopterous orders except Plecoptera as a monophyletic group; a group which he called Paurometabola. However, he was unable to place Plecoptera and left it as a lineage unconnected to the overall topology. Boudreaux (1979) placed Plecoptera (plus seemingly related extinct allies) within Polyneoptera and as the sister taxon to Embiidina. Kukalova-Peck (1991) depicts Polyneoptera as paraphyletic and places Plecoptera as sister taxon to the "Orthopteroid orders" (ie.; Polyneoptera), a group containing Orthoptera, Phasmatodea, Embiidina, and several extinct lineages. Kristensen (1991) leaves Polyneoptera (including Plecoptera) as a largely unresolved polytomy at the base of Neoptera. Wheeler et al. (2001) using both molecular and morphological data supported a monophyletic Polyneoptera and placed Plecoptera as sister taxon to

Embiidina. Most recently Terry and Whiting (Submitted), in a study focusing specifically on the phylogeny of Polyneoptera, supported a paraphyletic Polyneoptera. In this analysis Plecoptera was placed as the basal most lineage within a large assemblage including all polyneopterous orders except Dermaptera and Zoraptera.

The classification schemes of Plecoptera can be distilled into two main phylogenetic hypotheses. Klapalek (1909) divided Plecoptera into two suborders, Filipalpia and Setipalpia, with a third (Archiperlaria) added later by Illies (1965, see Figure 1a). Zwick (1973; 2000) supported a basal splitting of Plecoptera into the two main lineages of Arctoperlaria and Antarctoperlaria (Fig. 1b). The group Antarctoperlaria is supported by two leg muscle characters and the presence of floriform chloride cells (Zwick, 2000), while Arctoperlaria is supported by a single behavioral character (drumming). These names are derived from the nearly complete disjunction between Nortern and Southern Hemisphere Plecoptera taxa at the subordinal level, a condition unique among the insect orders. Arctoperlaria is divided into the groups Systellognatha and Euholognatha, with each group supported by several putative morphological synapomorphies. A recent analysis of a single molecular marker (approximately 1300 base pairs of 18S) and including 34 Plecoptera species from 32 genera and 15 families (Thomas et al., 2000) and 8 polyneopteran outgroups supported the monophyly of Systellognatha and the paraphyly of Arctoperlaria, Antarctoperlaria, and Euholognatha with the euholognathous orders as the first several basal lineages within Plecoptera. This study incorporated a broad, but limited sampling of Plecoptera taxa and utilized a single molecular marker (18S), and our goal is to provide a more comprehensive phylogeny of Plecoptera. Here we combine pertinent morphological

data compiled by Zwick (2000), a large complement of genetic markers, and by far the best sampling of Plecoptera taxa included in a phylogenetic analysis to produce a robust hypothesis regarding relationships within this order.

Materials and Methods

Taxon Sampling

The importance of dense taxon sampling in phylogenetic studies has been demonstrated by several studies (Poe and Swofford, 1999; Rannala et al., 1998; Zwickl and Hillis, 2002). We have sampled individuals representing every family, every subfamily except Microperlinae (Peltoperlidae), and 159 of approximately 250 extant genera, for a total of 173 ingroup plecopteran species. Genera missing from this analysis are mainly Palearctic in distribution, however, the Palearctic and Nearctic Plecoptera fauna are very similar (Zwick, 2000), so we do not anticipate that sampling these taxa is critical for understanding higher level relationships. This represents the most dense and thorough sampling of any insect order to date. In order to root the topology, we selected 10 outgroup taxa composed of exemplars of each of the remaining 10 polyneopteran orders. This resulted in a total sampling of 183 taxa that were sequenced for this analysis (Table 1). DNA sequences are deposited in GenBank under accession numbers #####-#### (see Appendix 1; to be provided upon acceptance).

Molecular Markers and Morphological Matrix

Molecular sequence data were generated for six genes: 12S and 16S mitochondrial rDNA, 18S and 28S nuclear rDNA, and Histone 3 (H3) and Cytochrome Oxidase II (COII) nuclear protein coding genes. This yielded a total unaligned data set of

approximately 6000 base pairs per taxon. These genes have proven informative in other studies dealing with deeper level arthropod phylogeny (Giribet et al., 2001; Jarman et al., 2000; Terry and Whiting, Submitted; Wheeler et al., 2001; Whiting, 2001b; Whiting et al., 1997) and show variable levels of divergence across the sampled taxa. The morphological matrix used in this analysis was initially developed by Zwick (1973). The original matrix is coded at the subfamily level and all species represented in this analysis were assigned the states appropriate to their family and subfamily, yielding a final matrix of 138 binary characters. Descriptions detailing the characters and states can be found in Zwick (2000).

DNA Extraction, Amplification and Sequencing

For larger specimens, a small portion of wing or leg muscle was dissected from the mesothorax. For smaller specimens, the thorax was cut lengthwise, any gut contamination was removed and the entire thorax was used. This tissue was then subjected to either a phenol/ethanol extraction (Whiting et al., 1997) or extraction via Qiagen's® DNeasy™ tissue kit. Purified DNA was amplified for molecular markers via polymerase chain reaction using previously published primers and amplification profiles (Colgan et al., 1998; Whiting, 2001b; Whiting et al., 1997). A list of primer sequences can be found in Appendix 2. Due to their large size, each of the nuclear ribosomal genes was amplified using three separate regions with sufficient overlap to insure continuity. These regions were approximately 1000, 800, and 600 nucleotides long for 18S and 1200, 600, and 1000 nucleotides long for 28S. Yield and potential contamination were monitored by agarose gel electrophoresis. Target products were purified and cycle-sequenced using the ABI® dRhodamine cycle sequencing kit via flanking and, for long

PCR products, internal primers. These sequencing reactions were then column purified and subjected to automated sequencing on ABI's® 377, 3100, or 3730xl automated sequencer. Complementary strands were independently sequenced and chromatographs were visually checked using Sequencher™ 4.1 (Sequencher, 2002).

Phylogenetic Analyses

The morphology matrix used for this analysis was taken from Zwick (2000), this is essentially the same matrix presented in Zwick (1973) and contains no homoplasy. It consists of 138 binary, polarized characters coded to the level of family and subfamily. Taxa were not re-examined and coded for this analysis, but were assigned character states congruent with their subfamilial designation. Outgroup taxa were assigned the plesiomorphic condition for all morphological characters.

Both protein coding genes (H3 and COII) had a conserved reading frame across the sample taxa rendering alignment unambiguous. Although total length varied slightly, the mitochondrial ribosomal genes both contained conserved, orthologous regions on either end of their sequence. For the nuclear ribosomal sequences, an initial alignment was performed using Sequencher™ 4.1 by manually aligning the conserved domains across all taxa. Sequences were then subdivided to facilitate finding an optimal solution (Giribet, 2001) during the procedure of direct optimization as implemented via POY (Wheeler et al., 2003). This yielded 8 sections for the 18S and 10 sections for the 28S rDNA. Direct optimization forgoes traditional multiple alignment by directly aligning and optimizing sequence data at every node of the topology and constructing hypothetical ancestral sequences for each node (Wheeler, 1996). A cost can be associated with each unique topology using either a parsimony or maximum likelihood criterion (Gladstein

and Wheeler, 2002). Evaluating the large number of possible trees is computationally intense, but can be accomplished via common algorithmic search strategies such as multiple random replicates, tree fusing, tree drifting, and branch swapping. Analyses for these data were performed for a combined data set (morphology, 12S, 16S, 18S, 28S, COII, and H3) using the program POY version 3.11 on an IBM SP2 supercomputer and an IBM 1350 Linux cluster located at the Fulton Supercomputing Center on the campus of Brigham Young University. All direct optimization analyses were performed using *Chelisoches* (Dermaptera) to root the topology. In total, 50 complete random replicates were performed using tree fusing and ratcheting with Tree-bisection-reconnection (TBR) and Subtree-pruning-regrafting (SPR) swapping on each replicate and parsimony as the optimality criterion using the command file:

-intermediate -outgroup DM013 -fitchtrees -numslaveprocesses 8 -onan -onannum 1 -parallel -noleading -norandomizeoutgroup -sprmaxtrees 1 -impliedalignment -tbrmaxtrees 1 -maxtrees 5 -holdmaxtrees 50 -slop 5 -checkslop 10 -buildspr -buildmaxtrees 2 -replicates 2 -stopat 25 -nomultirandom -treefuse -fuselimit 10 -fusemingroup 5 -fusemaxtrees 100 -ratchetspr 10 -ratchettbr 10 -checkslop 10 -molecularmatrix 111.txt -seed -1 > out.file 2> err.file

Due to the size of the data set these replicates were performed in batches of one and two replicates and the best overall topology (lowest cost) was retained. Due to the size of this data set partition Bremer support values were calculated in PAUP* version 4.0b10 (Swofford, 2002) using the implied alignment from POY and a modified command file from TreeRot (Sorenson, 1999) and 20 random additions for each constrained node. This method yields somewhat inflated values, but is a good measure of relative support. These

values were also normalized by dividing the total Bremer value for each partion by the number of informative characters for that partition, and then determining the percentage of support derived from these values. Bootstrap values were also calculated in PAUP* using the implied alignment under parsimony criteria with 1000 bootstrap replicates and 20 random additions per replicate and gaps treated as a 5^{th} state. This analysis was completed treating all changes equally (111) for both epistemological reasons supporting the equal treatment of all characters in a parsimony analysis (Grant and Kluge, 2003) and practical reasons (ie., the size of this data set makes sensitivity analyses extremely time consuming and computationally intense). Previous sensitivity analyses using portions of the data included here (Terry et al., Submitted; Terry and Whiting, Submitted), and those focusing on other groups, but using similar gene complements (Giribet et al., 2001; Ogden and Whiting, 2003; Svenson and Whiting, 2004; Whiting, 2001a; Whiting et al., 2003) have converged on the optimal parameter combination treating gaps, transversions, and transitions equally.

Alternate hypotheses were tested via both the Winning-sites test (Prager and Wilson, 1988) and the Wilcoxon ranked sums test (Templeton, 1983) in PAUP* using the implied alignment from POY. Constraint trees were constructed with MacClade (Maddison and Maddison, 2000) and implemented in PAUP* using 50 random additions for each constrained search. Hypotheses tested included: paraphyly of Antarctoperlaria, Arctoperlaria, Euholognatha, Systellognatha, and Notonemouridae; monophyly of Chloroperlidae, Gripopterygidae, Austroperlidae, Leuctridae, and Brachypterainae; and all families constrained to be simultaneously monophyletic considering Leuctridae *sensu latu* and Leuctridae without the inclusion of *Megaleuctra*.

RESULTS

Sequencing

All amplified COII and H3 sequences have a conserved reading frame with lengths of 720 and 376 nucleotides, respectively. The longest complete plecopteran 18S sequence (*Viehoperla ada*) is 2279 base pairs and average, unaligned length in Plecoptera is approximately 2050 base pairs. The longest complete plecopteran 28S sequence is 2728 base pairs (*Sierraperla cora*) and the average length is approximately 2300 base pairs. The longest complete plecopteran 12S sequence is 440 base pairs (*Malirekus iroquois*), and an average length of approximately 410 base pairs. The longest complete plecopteran 16S sequence is 570 base pairs (*Neoperla clymene*), and an average length of approximately 560 base pairs.

Phylogenetic Analysis

Analysis of the total combined data set yielded a single most parsimonious topology of length 50331. Partition Bremer support values over the entire topology derive 55% of their support from 28S, 26% from 18S, 7% from 12S, 6% from 16S, 3% from COII, 1% from morphology, and 1% from H3. When these percentages are normalized by the number of informative sites per partition 20% of Bremer support is derived from 12S, 19% from 28S, 16% from 18S, 16% from 16S, 10% from morphology, 9% from COII, and 9% from H3. A summary of the optimal topology is found in Figure 2.

Topological Results

All genera represented by multiple exemplars were supported as monophyletic, except *Isoperla*, which is rendered paraphyletic by the inclusion of *Clioperla clio.*

Multiple subfamilies were paraphyletic (see discussion below for detail). All families with the exceptions of Chloroperlidae, Leuctridae, Gripopterygidae, and Austroperlidae were supported as monophyletic clades. The suborders Arctoperlaria and Antarctoperlaria are supported as monophyletic, as are Systellognatha and Euholognatha. *Megaleuctra*, represented in this analysis by *M. kincaidi*, is sister taxon to the remainder of Plecoptera.

Alternate Hypothesis Testing

Nine of the 12 alternate hypotheses tested resulted in p-values of less than 0.0001. These included paraphyletic Antarctoperlaria, Arctoperlaria, Euholognatha, Systellognatha, and Notonemouridae; monophyly of Gripopterygidae, Leuctridae, and Brachypterainae; and complete monophyly at the family level. The Wilcoxon ranked sums test resulted in p-values of 0.0006 and 0.0378 when testing for monophyly of Chloroperlidae and Austroperlidae, repectively; and the Winning sites test resulted in p-values of 0.0009 and 0.0620 when testing for monophyly of these two families.

DISCUSSION

Subordinal Relationships

This analysis supports the monophyly of Archiperlaria and Setipalpia, however, Filipalpia is rendered paraphyletic by species placed within both of the other suborders. Relationships proposed by Zwick (1973; 2000) are much more congruent with this analysis than those of Illies (1965), in that Antarctoperlaria, Arctoperlaria, Systellognatha, and Euholognatha (without *Megaleuctra)* are supported as monophyletic clades.

Arctoperlaria is a diverse group and united in Zwick's system by the single putative synapomorphy of drumming. Drumming is a pre-mating behavior observed in many lineages in which the male taps his abdomen against the substrate creating a vibrational signal (Rupprecht, 1967). These signals are species specific (Stewart et al., 1988) and recognized by the female who answers with her own signal until the male finds her and initiates mating. Although this behavior is widespread throughout Arctoperlaria there are multiple lineages that apparently do not drum (Stewart et al., 1995). In the context of the phylogeny supported by this analysis this character is a synapomorphy for Arctoperlaria and, although the absence of drumming can be hard to demonstrate, numerous observations indicate a secondary loss of this behavior in multiple lineages (Maketon and Stewart, 1988; Stewart et al., 1995; Stewart et al., 1988).

Euholognathan taxa are united by three morphological characters: a soft egg chorion, crossing of the segmental nerves under longitudinal abdominal muscles, and a single (apparently fused) corpus allatum. The corpora allata are ectodermally derived glands (Snodgrass, 1935) that excrete juvenile hormone, a substance that plays an integral role in the molting cycle of insects (Wigglesworth, 1934). In most insects they form as two distinct bodies on either side of the posterior margin of the brain (Nabert, 1913), however, in Euholognatha they are fused into a single medial gland (Zwick, 1973). The corpora allata are separate, but closely adjacent in basal Systellognatha and one appears to have been lost in the genus *Microperla* (Uchida and Isobe, 1989). This analysis supports the fused corpus allatum and other morphological characters as synapomorphies for Euholognatha.

Familial Relationships

This analysis supports Nemouridae as sister taxon to Capniidae (Figure 4). This placement disagrees somewhat with previous morphological hypotheses (both Illies and Zwick place Leuctridae as its sister taxon), however, it is more incongruent with a recent molecular analysis (Thomas et al., 2000), which supports Nemouridae as sister to the remainder of Plecoptera. Both of the genera (*Nemoura* and *Amphinemura*) represented by multiple species are monophyletic. Subfamilies proposed by Baumann (1975) are not supported as monophyletic in this analysis, with represented Amphinemurinae (two Palearctic genera could not be sampled) forming the first few basal lineages within a derived clade of Nemourinae.

Within Capniidae *Mesocapnia*, the only capniid genus with multiple exemplars, is monophyletic and *Nemocapnia* is sister taxon to the remainder of the family. Although specific relationships vary, the basal lineages are essentially those proposed by Ross and Ricker (Ross and Ricker, 1971).

Two subfamilies of Taeniopterygidae are currently recognized (Zwick, 1973): Taeniopteryginae, which includes only the genus *Taeniopteryx*, and Brachypterainae, which includes the remaining genera. In this analysis Brachypterainae is rendered paraphyletic by *Taeniopteryx*. This placement is contradictory to morphological data and would necessitate the loss in *Taeniopteryx* of a suite of complex genitalic features(Zwick, 1973). Searches in PAUP* constrained for the monophyly of Brachypterainae result in the placement of *Taeniopteryx* as sister taxon to Brachypterainae, but are considerably longer (72 steps) than optimal trees and are statistical distinguishable by both the Wilcoxon and Winning sites tests (p-values < 0.0001). The monotypic family

Scopuridae is supported as sister taxon to Taeniopterygidae, and although there are no morphological characters uniting this group, Zwick (2000) places these families near each other as the two most basal lineages of Euholognatha.

This analysis supports Leuctridae *sensu stricta* (without *Megaleuctra*) as sister taxon to Notonemouridae. Zwick (2000) places Leuctridae as sister taxon to Capniidae, and their placement in our topology necessitate the hypothesis of convergent evolution in the reduction of the penis and subsequent modification of the inner paraproct lobes, and anterior separation of the female ovaries in these two families. The two genera represented by multiple species (*Leuctra* and *Paraleuctra*) are monophyletic. *Megaleuctra*, which has consistently given taxonomists problems and has been allied with Notonemouridae (Illies, 1967), is sister taxon to all other Plecoptera. The node uniting all of Plecoptera except *Megaleuctra* is relatively strongly supported and both the Wilcoxon ranked sums test and the Winning sites test rejected (p-value < 0.0001) the placement of *Megaleuctra* with other Leuctridae. We suggest that the genus *Megaleuctra* be placed in its own family, Megaleuctridae, and that further examination of this genus as possible sister taxon to all extant Plecoptera is in order.

Notonemouridae are supported as a monophyletic taxon and not a gradotaxon (sensu Zwick, 2000). All African genera of Notonemouridae included in this analysis form a monophyletic group, while the most basal clade is composed of species of New Zealand and South American origin. The remaining clade is made up of species from New Zealand, South America, and Australia. Both statistical tests performed with Notonemouridae constrained as paraphyletic rejected the possibility of paraphyly (p-value > 0.0001).

The monophyletic Antarctoperlaria have been divided into two main groups (Fig. 1). The first, Eusthenoidea, consists of two families: Diamphipnoidae + Eustheniidae (Eusthenioidea). This relationship has been recognized for some time (Illies' Archiperlaria, Fig. 1a) and is supported by two morphological characters (Zwick, 2000), and by this analysis (Fig. 3) Within Eustheniidae both subfamilies, Eustheniinae and Stenoperlinae (McLellan, 1996), are supported as monophyletic clades.

Austroperlidae + Gripopterygidae (Gripopterygoidea) has been proposed as a monophyletic group and is supported by three morphological characters (Zwick, 2000). In this analysis Austroperlidae is paraphyletic, however, topologies constrained for the monophyly of Austroperlidae are not significantly different than the optimal topology (p-value of 0.0620 for Winning sites test). Gripopterygidae is also paraphyletic and topologies constrained for its monophyly are 98 steps longer than the optimal tree and significantly different (p-value < 0.0001). The subfamilies Dinitoperlinae and Zelandoperlinae (McLellan, 1977) are monophyletic. The other three subfamilies as designated by McLellan (1977) are paraphyletic in this analysis. Taxon sampling, although extensive, is far from complete and several genera are missing (i.e.; *Apteryperla, Aucklandobius, Holcoperla, Megaleptoperla, Rakiuraperla, Rungaperla, Taraperla, Vesicaperla*), particularly from the subfamily Zelandoperlinae. Zwick (1973) depicted one subfamily of Gripopterygidae, Antarctoperlinae, with uncertain affinities and placed it midway between Austroperlidae and the remainder of Gripopterygidae. Most Gripopterygidae possess a rosette of terminal gill filaments and this is the only morphological character uniting the group. In the context of our phylogeny this becomes a symplesiomorphy for Antarctoperlaria with losses in several genera of Gripopterygidae

and the other three antarctoperlarian families. Gripopterygidae is a large, diverse family and its putative paraphyly needs to be further examined.

Systellognatha is well supported as a monophyletic group with Styloperlidae as sister taxon to the remaining families (Fig. 5). The next most basal branch within Systellognatha is Peltoperlidae, which is monophyletic, although the monogeneric subfamily Microperlinae was not represented in this analysis. Pteronarcyidae is sister taxon to the remaining Systellognatha and *Pteronarcys*, represented by three species, is monophyletic.

The remaining three families (Chloroperlidae, Perlodidae, and Perlidae) constitute Perloidea (Zwick, 2000), a clade which has long been recognized although previously named Subulipalpia (Zwick, 1973) or Setipalpia (Illies, 1965). Relationships of the three constituent taxa have, however, been unclear. This analysis supports the clade Perlidae + Perlodidae with Chloroperlidae as its sister taxon (Fig. 5). Chloroperlidae is a paraphyletic group with *Kathroperla* as sister taxon to the remaining Perloidea. The subfamily Chloroperlinae is rendered paraphyletic by Paraperlinae, and Paraperlinae is also paraphyletic (due to the placement of *Kathroperla*). Topologies constraining the monophyly of Chloroperlidae are 34 steps longer, and had significant p-values under both Wilcoxon ranked sums test (0.0006) and Winning sites test (0.0009).

Perlidae is strongly supported as monophyletic by both molecular and morphological data. In this analysis the larger subfamily Acroneuriinae is rendered paraphyletic by a monophyletic Perlinae. Interestingly, the monotypic *Claassenia*, which has been placed in its own tribe (Sivec et al., 1988) is sister taxon to Perlinae. A large group of Acroneuriinae genera are found throughout Central and South America, and are

the only Systellognatha (other than the genus *Neoperla* in sub-Saharan Africa) found in the Southern Hemisphere. These genera (represented in this analysis by *Iconeuria*, *Pictetoperla*, *Kempnyella*, and *Anacroneuria*) also form a monophyletic lineage.

Perlodidae is also strongly supported as monophyletic in this analysis (Fig. 5) despite the lack of any defining morphological synapomorphies (Zwick, 2000). The subfamily Perlodinae is paraphyletic and the subfamily Isoperlinae (represented in this analysis by multiple *Isoperla*) is rendered paraphyletic by the inclusion of *Clioperla*.

Plecoptera Biogeography

As mentioned above, one of the extraordinary features of Plecoptera distribution is the disjunction between Northern and Southern Hemisphere taxa. This pattern is unmatched among the Pterygota and reflected by the subordinal names Arctoperlaria and Antarctoperlaria. The only family found in both hemispheres is Perlidae with a closely related group of genera in South America and a single genus in southern Africa (Fig. 2). However, both of these groups are quite similar to Northern Hemisphere taxa and appear to be relatively recent invasions from the north (Illies, 1965; Stark and Gaufin, 1976). Four of the five families endemic to the Southern Hemisphere form the monophyletic group Antarctoperlaria, however, the fifth (Notonemouridae) is morphologically much more similar to taxa from the Northern Hemisphere. Its presence on all three major components of Gondwana has been difficult to reconcile with phylogenetic theories of plecopteran relationships. Zwick (2000), citing lack of detail in morphological knowledge regarding Notonemouridae, hypothesized they were a paraphyletic group that had colonized southern land masses multiple times.

This analysis supports the monophyly of Notonemouridae (Bremer value = 63, bootstrap = 100%, paraphyly rejected with a p-value < 0.0001) and suggests they are an ancient component of southern fauna. It is consistent with deep phylogenetic divisions within Plecoptera arising from the breakup of Pangea, as Zwick (2000) suggests. The presence of Notonemouridae on all southern land masses and their support as a monophyletic clade in this analysis seems to support an early invasion of Gondwanaland by notonemourid progenitors.

Plecoptera, Skimming and Insect Flight

Many Plecoptera skim on the surface of water, either incidentally or as a means of locomotion (Kramer and Marden, 1997; Marden and Kramer, 1994). This behavior appears to be somewhat widespread throughout Plecoptera (Thomas et al., 2000), however, it has yet to be fully characterized among many lineages. Marden and Kramer (1997) and later Thomas et al. (2000) hypothesized that this behavior is "an intermediate stage in the evolution of insect flight, which has perhaps been retained in certain modern stoneflies." Such a claim would imply that skimming should be a condition derived at the base of Plecoptera. The distribution of this character within Plecoptera on all published phylogenetic hypotheses, including this one, demonstrates that it is indeed a symplesiomorphy for the order and may well be an adaptation linked to their aquatic lifestyle. This has only anecdotal bearing on skimming as a precursor to full flight, however, as there is virtually universal support (a rare commodity in phylogenetic circles) for the basal position of Odonata and Ephemeroptera among the winged insects (Boudreaux, 1979; Hennig, 1981; Hovmöller et al., 2002; Kristensen, 1991; Ogden and Whiting, 2003; Terry and Whiting, Submitted; Wheeler et al., 2001), although the

arrangement of these two groups is still debatable. The hypothesis of skimming as a precursor to full flight necessitates the independent loss of this behavior in these aquatic lineages in addition to its loss among (the mostly terrestrial) “higher insects” (Will, 1995). Additionally the large size of many extinct early insects (Carpenter, 1992; Grimaldi, 2001) and the possibility that the earliest insects to evolve flight were not aquatic makes the skimming-to-flight theory problematic at best.

The monophyly of Plecoptera is unquestioned and well supported by all lines of evidence. Relationships of the other polyneopterous insect orders to Plecoptera are still not completely clear, although the most recent analysis including morphological and molecular data supports Plecoptera as a basal lineage within a large clade of the paraphyletic group Polyneoptera (Terry and Whiting, Submitted). Within Plecoptera the groups Arctoperlaria and Antarctoperlaria are monophyletic, as are Systellognatha and Euholognatha. *Megaleuctra* is sister taxon to the rest of Plecoptera and deserves recognition as its own family, Megaleuctridae. This analysis also renders Chloroperlidae, Austroperlidae, and Gripopterygidae paraphyletic. This paraphyly appears to be especially well supported for Gripopterygidae, as the remaining antarctoperlarians are well supported as a distinct lineage nested within that family, but need to be investigated more thoroughly before taxonomic revisions are warranted.

ACKNOWLEDGMENTS

A special thanks to Richard Baumann for verification and identification of most of the specimens used in this study. We also thank J. Sandberg, C. Nelson, K. Stewart, B. Stark, B. Kondratieff, P. Zwick, E. Ross, J. Edgerly-Rooks, T. Miura, M. Picker, R.

Fochetti, E. Tsyrlin, K. Miller, M. Losada, I. Mclellan, T. Kishimoto, I. Sivec, J. Adis, O. Zompro, and E. Marais for providing specimens; and M. Gruwell and H. Ogden. for assistance in generating sequence data. Analyses were performed at the Fulton Supercomputing Center at Brigham Young University with parallel software implementation by M. Clement and Q. Snell, based on code made freely available by W. Wheeler. This work was supported by NSF grants DEB-0206363 and DEB-9983195 with NSF REU supplements.

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Appendix 1. List of taxa included in this analysis with GenBank accession numbers for molecular markers. (Accession numbers to be provided upon acceptance)

Plecoptera								
Family	Genus	Species	12S	16S	18S	28S	COII	H3
Austroperlidae	*Acruroperla*	*atra*	###	###	###	###	###	###
	Austroperla	*cyrene*	###	###	###	###	###	###
	Crypturoperla	*paradoxa*	###	###	###	###	###	###
	Klapopteryx	*armillata*	###	###	###	###	###	###
	Penturoperla	*barbata*	###	###	###	###	###	###
	Tasmanoperla	*larvalis*	###	###	###	###	###	###
Capniidae	*Allocapnia*	*minima*	###	###	###	###	###	###
	Bolshecapnia	*sp.*	###	###	###	###	###	###
	Capnia	*gracilaria*	###	###	###	###	###	###
	Capnura	*wanica*	###	###	###	###	###	###
	Eucapnopsis	*brevicauda*	###	###	###	###	###	###
	Isocapnia	*hyalita*	###	###	###	###	###	###
	Mesocapnia	*sp.*	###	###	###	###	###	###
	Mesocapnia	*frisoni*	###	###	###	###	###	###
	Nemocapnia	*carolina*	###	###	###	###	###	###
	Paracapnia	*opis*	###	###	###	###	###	###
	Utacapnia	*logana*	###	###	###	###	###	###
Chloroperlidae	*Alloperla*	*severa*	###	###	###	###	###	###
	Chloroperla	*tripunctata*	###	###	###	###	###	###
	Haploperla	*brevis*	###	###	###	###	###	###
	Kathroperla	*perdita*	###	###	###	###	###	###
	Neaviperla	*forcipata*	###	###	###	###	###	###
	Paraperla	*frontalis*	###	###	###	###	###	###
	Plumiperla	*diversa*	###	###	###	###	###	###
	Sasquaperla	*hoopa*	###	###	###	###	###	###
	Siphonoperla	*torrentium*	###	###	###	###	###	###
	Suwallia	*teleckojensis*	###	###	###	###	###	###
	Sweltsa	*coloradensis*	###	###	###	###	###	###
	Triznaka	*pintada*	###	###	###	###	###	###
	Utaperla	*sopladora*	###	###	###	###	###	###
Diamphipnoidae	*Diamphipnoa*	*virecentpennis*	###	###	###	###	###	###
	Diamphipnopsis	*sp.*	###	###	###	###	###	###
Eustheniidae	*Cosmioperla*	*australis*	###	###	###	###	###	###
	Eusthenia	*costalis*	###	###	###	###	###	###
	Neuroperla	*schedingi*	###	###	###	###	###	###
	Neuroperlopsis	*patris*	###	###	###	###	###	###
	Stenoperla	*maclellani*	###	###	###	###	###	###
	Thaumatoperla	*sp.*	###	###	###	###	###	###
Gripopterygidae	*Acroperla*	*trivacauta*	###	###	###	###	###	###
	Antarctoperla	*sp.*	###	###	###	###	###	###
	Cardioperla	*lobata*	###	###	###	###	###	###
	Claudioperla	*sp.*	###	###	###	###	###	###
	Dinotoperla	*sp.*	###	###	###	###	###	###
	Dinotoperla	*serricauda*	###	###	###	###	###	###
	Eunotoperla	*sp.*	###	###	###	###	###	###
	Illiesoperla	*australis*	###	###	###	###	###	###
	Leptoperla	*sp.*	###	###	###	###	###	###
	Limnoperla	*sp.*	###	###	###	###	###	###
	Nesoperla	*fulvescens*	###	###	###	###	###	###
	Newmanoperla	*exigua*	###	###	###	###	###	###
	Notoperla	*sp.*	###	###	###	###	###	###
	Pelurgoperla	*personata*	###	###	###	###	###	###
	Rhithroperla	*sp.*	###	###	###	###	###	###
	Riekoperla	*triloba*	###	###	###	###	###	###
	Trinitoperla	*nivata*	###	###	###	###	###	###
	Zelandobius	*uniramus*	###	###	###	###	###	###
	Zelandoperla	*agnetis*	###	###	###	###	###	###
Leuctridae	*Calileuctra*	*ephemera*	###	###	###	###	###	###
	Despaxia	*augusta*	###	###	###	###	###	###

	Leuctra	*duplicata*	###	###	###	###	###	###
	Leuctra	*inermis*	###	###	###	###	###	###
	Megaleuctra	*kincaidi*	###	###	###	###	###	###
	Moselia	*infuscata*	###	###	###	###	###	###
	Paraleuctra	*vershina*	###	###	###	###	###	###
	Paraleuctra	*occidentalis*	###	###	###	###	###	###
	Perlomyia	*utahensis*	###	###	###	###	###	###
	Zealeuctra	*arnoldi*	###	###	###	###	###	###
Nemouridae	*Amphinemura*	*nigritta*	###	###	###	###	###	###
	Amphinemura	*sulcicollis*	###	###	###	###	###	###
	Lednia	*tumana*	###	###	###	###	###	###
	Malenka	*californica*	###	###	###	###	###	###
	Nanonemoura	*wahkeena*	###	###	###	###	###	###
	Nemoura	*trispinosa*	###	###	###	###	###	###
	Nemoura	*cinerea*	###	###	###	###	###	###
	Nemurella	*pictetii*	###	###	###	###	###	###
	Ostrocerca	*foersteri*	###	###	###	###	###	###
	Paranemoura	*claasseni*	###	###	###	###	###	###
	Podmosta	*decepta*	###	###	###	###	###	###
	Prostoia	*besametsa*	###	###	###	###	###	###
	Protonemura	*meyeri*	###	###	###	###	###	###
	Shipsa	*rotunda*	###	###	###	###	###	###
	Soyedina	*producta*	###	###	###	###	###	###
	Visoka	*cataractae*	###	###	###	###	###	###
	Zapada	*cinctipes*	###	###	###	###	###	###
Notonemouridae	*Afronemoura*	*amatolae*	###	###	###	###	###	###
	Apanicercopsis	*denticulata*	###	###	###	###	###	###
	Aphanicerca	*capensis*	###	###	###	###	###	###
	Aphanicercella	*flabellata*	###	###	###	###	###	###
	Austrocerca	*rieka*	###	###	###	###	###	###
	Austrocercella	*sp.*	###	###	###	###	###	###
	Austrocercoides	*zwicki*	###	###	###	###	###	###
	Austronemoura	*sp.*	###	###	###	###	###	###
	Cristaperla	*waharoa*	###	###	###	###	###	###
	Desmonemoura	*brevis*	###	###	###	###	###	###
	Halticoperla	*viridans*	###	###	###	###	###	###
	Kimminsoperla	*albomacula*	###	###	###	###	###	###
	Neofulla	*sp.*	###	###	###	###	###	###
	Neonemoura	*sp.*	###	###	###	###	###	###
	Notonemoura	*alisteri*	###	###	###	###	###	###
	Spaniocerca	*zwicki*	###	###	###	###	###	###
	Spaniocerca	*zelandica*	###	###	###	###	###	###
	Udamocercia	*sp.*	###	###	###	###	###	###
Perlodidae	*Baumanella*	*alameda*	###	###	###	###	###	###
	Calliperla	*luctuosa*	###	###	###	###	###	###
	Cascadoperla	*trictura*	###	###	###	###	###	###
	Chernokrilus	*misnomus*	###	###	###	###	###	###
	Clioperla	*clio*	###	###	###	###	###	###
	Cosumnoperla	*hypocrena*	###	###	###	###	###	###
	Cultus	*pilatus*	###	###	###	###	###	###
	Diura	*knowltoni*	###	###	###	###	###	###
	Frisonia	*picticeps*	###	###	###	###	###	###
	Helopicus	*bogaloosa*	###	###	###	###	###	###
	Hydroperla	*crosbyi*	###	###	###	###	###	###
	Isogenoides	*zionensis*	###	###	###	###	###	###
	Isogenoides	*hansoni*	###	###	###	###	###	###
	Isogenoides	*olivaceus*	###	###	###	###	###	###
	Isoperla	*davisi*	###	###	###	###	###	###
	Isoperla	*fulva*	###	###	###	###	###	###
	Isoperla	*phalerata*	###	###	###	###	###	###
	Isoperla	*oxylepis*	###	###	###	###	###	###
	Kogotus	*nonus*	###	###	###	###	###	###
	Malirekus	*iroquois*	###	###	###	###	###	###
	Megarcys	*signata*	###	###	###	###	###	###

	Oconoperla	*innubila*	###	###	###	###	###	###
	Osobenus	*yakimae*	###	###	###	###	###	###
	Perlinodes	*aurea*	###	###	###	###	###	###
	Perlodes	*dispar*	###	###	###	###	###	###
	Pictetiella	*expansa*	###	###	###	###	###	###
	Remenus	*bilobatus*	###	###	###	###	###	###
	Rickera	*sorpta*	###	###	###	###	###	###
	Salmoperla	*sylvanica*	###	###	###	###	###	###
	Setvena	*wahkeena*	###	###	###	###	###	###
	Skwala	*americana*	###	###	###	###	###	###
	Susulus	*venustus*	###	###	###	###	###	###
	Yugus	*bulbosus*	###	###	###	###	###	###
Perlidae	*Acroneuria*	*lycorias*	###	###	###	###	###	###
	Agnetina	*capitata*	###	###	###	###	###	###
	Anacroneuria	*sp.*	###	###	###	###	###	###
	Beloneuria	*stewarti*	###	###	###	###	###	###
	Calineuria	*californica*	###	###	###	###	###	###
	Claassenia	*sabulosa*	###	###	###	###	###	###
	Dinocras	*cephalotes*	###	###	###	###	###	###
	Doroneuria	*baumanni*	###	###	###	###	###	###
	Eccoptura	*xanthenes*	###	###	###	###	###	###
	Hansonoperla	*appalachia*	###	###	###	###	###	###
	Hesperoperla	*pacifica*	###	###	###	###	###	###
	Iconeuria	*sp.*	###	###	###	###	###	###
	Kempnyella	*sp.*	###	###	###	###	###	###
	Neoperla	*clymene*	###	###	###	###	###	###
	Paragnetina	*media*	###	###	###	###	###	###
	Perlesta	*decipiens*	###	###	###	###	###	###
	Perlinella	*drymo*	###	###	###	###	###	###
	Pictetoperla	*sp.*	###	###	###	###	###	###
Peltoperlidae	*Peltoperla*	*arcuata*	###	###	###	###	###	###
	Sierraperla	*cora*	###	###	###	###	###	###
	Soliperla	*campanula*	###	###	###	###	###	###
	Tallaperla	*lobata*	###	###	###	###	###	###
	Viehoperla	*ada*	###	###	###	###	###	###
	Yoraperla	*nigrisoma*	###	###	###	###	###	###
Styloperlidae	*Cerconychia*	*sp.*	###	###	###	###	###	###
	Styloperla	*wui*	###	###	###	###	###	###
Pteronarcyidae	*Pteronarcella*	*badia*	###	###	###	###	###	###
	Pteronarcys	*californica*	###	###	###	###	###	###
	Pteronarcys	*scotti*	###	###	###	###	###	###
	Pteronarcys	*sachalina*	###	###	###	###	###	###
Scopuridae	*Scopura*	*montana*	###	###	###	###	###	###
Taeniopterygidae	*Bolotoperla*	*rossi*	###	###	###	###	###	###
	Brachyptera	*seticornis*	###	###	###	###	###	###
	Doddsia	*occidentalis*	###	###	###	###	###	###
	Oemopteryx	*vanduzeei*	###	###	###	###	###	###
	Strophopteryx	*appalachia*	###	###	###	###	###	###
	Taenionema	*pallidum*	###	###	###	###	###	###
	Taeniopteryx	*nivalis*	###	###	###	###	###	###

Outgroups

Order	Genus	species	12S	16S	18S	28S	COII	H3
Blattodea	*Gromphadorhina*	*portentosa*	###	###	###	###	###	###
Dermaptera	*Celisoches*	*annulatus*	###	###	###	###	###	###
Embiidina	*Notoligotoma*	*sp.*	###	###	###	###	###	###
Grylloblattodea	*Galloisiana*	*nipponensis*	###	###	###	###	###	###
Isoptera	*Masotermes*	*darwinensis*	###	###	###	###	###	###
Mantodea	*Mantoida*	*sp.*	###	###	###	###	###	###
Mantophasmatodea	*Tyrannophasma*	*gladiator*	###	###	###	###	###	###
Orthoptera	*Ellipes*	*minutus*	###	###	###	###	###	###
Phasmatodea	*Timema*	*knulli*	###	###	###	###	###	###
Zoraptera	*Zorotypus*	*hubbardi*	###	###	###	###	###	###

Appendix 2. List of primers used in this analysis with sequence and relative position information.

Gene	Primer Name	Sequence (5' - 3')	Length	Direction	Relative Position
12S	12S ai	AAACTACGATTAGATACCCTATTAT	25	Forward	
12S	12S bi	AAGAGCGACGGGCGATGTGT	20	Reverse	
16S	16S A	CGCCTGTTTATCAAAAACAT	20	Forward	
16S	16S B	CTCCGGTTTGAACTCAGATCA	21	Reverse	
18S	18S 1F	TACCTGGTTGATCCTGCCAGTAG	23	Forward	1
18S	18S ai	CCTGAGAAACGGCTACCACATC	22	Forward	2
18S	18S a0.7	ATTAAAGTTGTTGCGGTT	18	Forward	3
18S	18S a0.79	TTAGAGTGCTYAAAGC	16	Forward	4
18S	18S a1.0	GGTGAAATTCTTGGAYCGTC	20	Forward	5
18S	18S a2.0	ATGGTTGCAAAGCTGAAAC	19	Forward	6
18S	18S a3.5	TGGTGCATGGCCGYTCTTAGT	21	Forward	7
18S	18S 7F	GCAATAACAGGTCTGTGATGCCC	23	Forward	8
18S	18S 9R	GATCCTTCCGCAGGTTCACCTAC	23	Reverse	1
18S	18S 7R	GCATCACAGACCTGTTATTGC	21	Reverse	2
18S	18S bi	GAGTCTCGTTCGTTATCGGA	20	Reverse	3
18S	18S b0.5	GTTTCAGCTTTGCAACCAT	19	Reverse	4
18S	18S b2.5	TCTTTGGCAAATGCTTTCGC	20	Reverse	5
18S	18S b2.9	TATCTGATCGCCTTCGAACCTCT	23	Reverse	6
18S	18S b3.9	TGCTTTRAGCACTCTAA	17	Reverse	7
18S	18S b5.0	TAACCGCAACAACTTTAAT	19	Reverse	8
18S	18S b7.0	ATTTRCGYGCCTGCTGCCTTCCT	23	Reverse	9
28S	28s Rd 1a	CCCSCGTAAYTTAGGCATAT	20	Forward	1
28S	28s Rd 3a	AGTACGTGAAACCGTTCAGG	20	Forward	2

28S	28S Rd 4.5a	AAGTTTCCCTCAGGATAGCTG	21	Forward	5
28S	28S Rd 4.8a	ACCTATTCTCAAACTTTAAATGG	23	Forward	6
28S	28S Rd 5a	GGYGTTGGTTGCTTAAGACAG	21	Forward	7
28S	28S Rd 6a	GGCGAAAGGGAATCYGGTTC	20	Forward	8
28S	28S Rd 7b1	GACTTCCCTTACCTACAT	18	Reverse	1
28S	28S Rd 6b	AACCRGATTCCCTTTCGCC	19	Reverse	2
28S	28S Rd 5b	CCACAGCGCCAGTTCTGCTTAC	22	Reverse	3
28S	28S B	TCGGAAGGAACCAGCTAC	18	Reverse	4
28S	28S C	ATAGTTCACCATCTYTCGGG	20	Reverse	5
28S	28S Rd 4b	CCTTGGTCCGTGTTTCAAGAC	21	Reverse	6
28S	28S Rd 3b	CCYTGAACGGTTTCACGTACT	21	Reverse	7
COII	COII 1a	TTAAGCTCCATATATAAAGGMTT	23	Forward	
COII	COII F-Leu	TCTAATATGGCAGATTAGTGC	21	Forward	
COII	COII 9b	GTACTTGCTTTCAGTCATCTWATG	24	Reverse	
COII	COII R-Lys	GAGACCAGTACTTGCTTTCAGTCATC	26	Reverse	
H3	H3 AF	ATGGCTCGTACCAAGCAGACVGC	23	Forward	
H3	Hex AF	ATGGCTCGTACCAAGCAGACGGC	23	Forward	
H3	H3 AR	ATATCCTTRGGCATRATRGTGAC	23	Reverse	
H3	Hex AR	ATATCCTTGGGCATGATGGTGAC	23	Reverse	

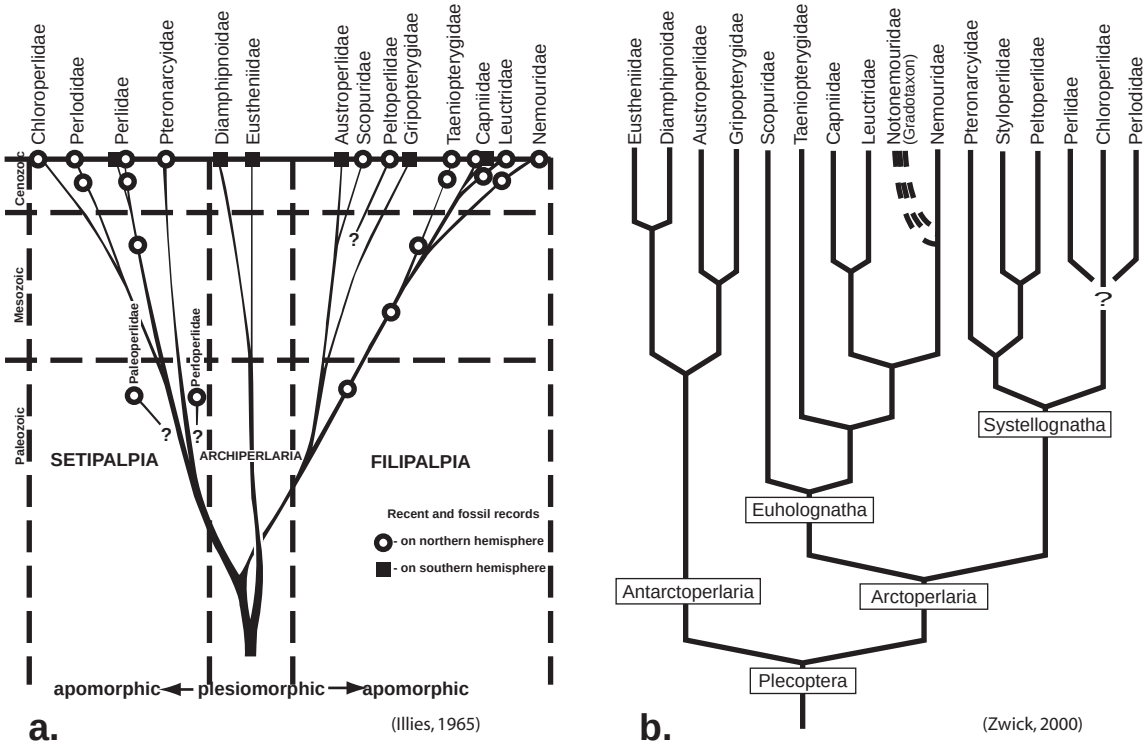
Chloroperlidae
Perlodidae
Perlidae
Pteronarcyidae
Diamphipnoidae
Eustheniidae
Austroperlidae
Scopuridae
Peltoperlidae
Gripopterygidae
Taeniopterygidae
Capniidae
Leuctridae
Nemouridae
Cenozoic
Mesozoic
Paleozoic
Paleoperlidae
Perlöperlidae
?
?
?
SETIPALPIA
ARCHIPERLARIA
FILIPALPIA
Recent and fossil records
- on northern hemisphere
- on southern hemisphere
apomorphic ← plesiomorphic → apomorphic
a.
(Illies, 1965)
Eustheniidae
Diamphipnoidae
Austroperlidae
Gripopterygidae
Scopuridae
Taeniopterygidae
Capniidae
Leuctridae
Notonemouridae
(Gradoiaxon)
Nemouridae
Pteronarcyidae
Styloperlidae
Peltoperlidae
Perlidae
Chloroperlidae
Perlodidae
?
Systellognatha
Euholognatha
Antarctoperlaria
Arctoperlaria
Plecoptera
b.
(Zwick, 2000)

Figure 1

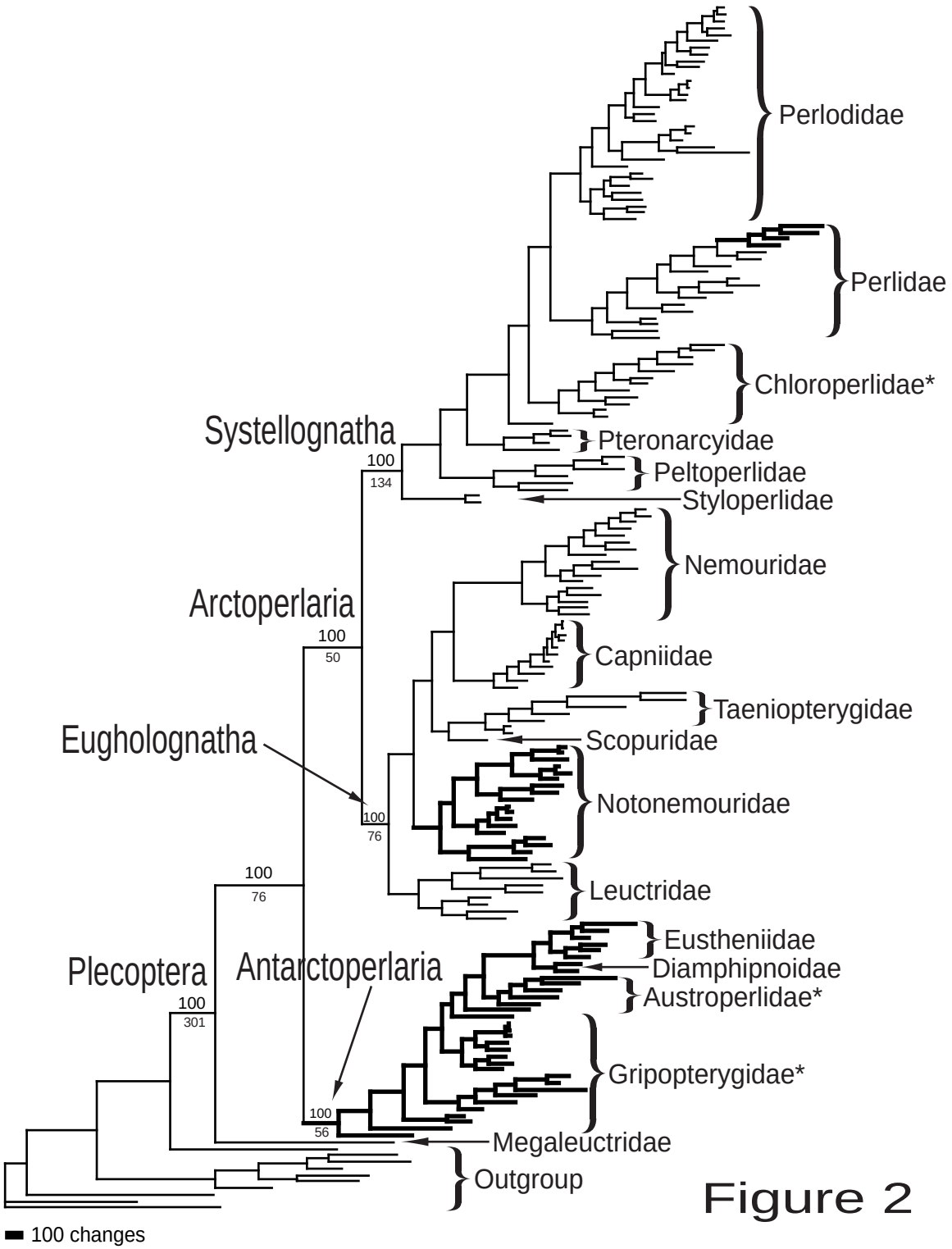
Perlodidae
Perlidae
Chloroperlidae*
Systellognatha
Pteronarcyidae
100
134
Peltoperlidae
Styloperlidae
Nemouridae
Arctoperlaria
100
50
Capniidae
Taeniopterygidae
Eughlognatha
Scopuridae
100
76
Notonemouridae
100
76
Leuctridae
Eustheniidae
Plecoptera
Antarctoperlaria
Diamphipnoidae
Austroperlidae*
100
301
Gripopterygidae*
100
56
Megaleuctridae
Outgroup
100 changes

Figure 2

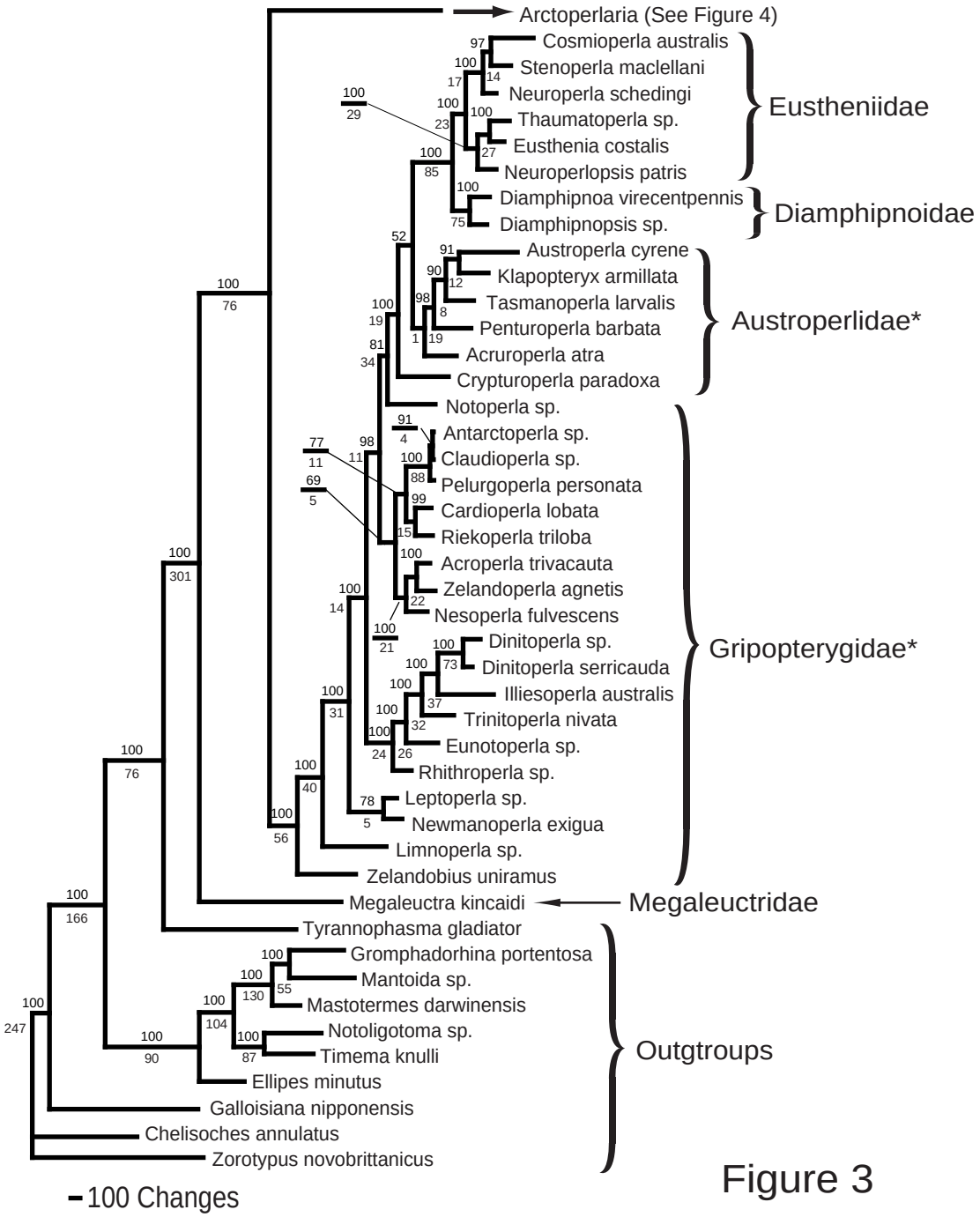
Arctoperlaria (See Figure 4)
Cosmioperla australis
Stenoperla maclellani
Neuroperla schedingi
Thaumatoperla sp.
Eusthenia costalis
Neuroperlopsis patris
Eustheniidae
Diamphipnoa virecentpennis
Diamphipnopsis sp.
Diamphipnoidae
Austroperla cyrene
Klapopteryx armillata
Tasmanoperla larvalis
Penturoperla barbata
Acruroperla atra
Crypturoperla paradoxa
Austroperlidae*
Notoperla sp.
Antarctoperla sp.
Claudioperla sp.
Pelurgoperla personata
Cardioperla lobata
Riekoperla triloba
Acroperla trivacauta
Zelandoperla agnetis
Nesoperla fulvescens
Dinitoperla sp.
Dinitoperla serricauda
Illiesoperla australis
Trinitoperla nivata
Eunotoperla sp.
Rhithroperla sp.
Leptoperla sp.
Newmanoperla exigua
Limnoperla sp.
Zelandobius uniramus
Gripopterygidae*
Megaleuctra kincaidi
Megaleuctridae
Tyrannophasma gladiator
Gromphadorhina portentosa
Mantoida sp.
Mastotermes darwinensis
Notoligotoma sp.
Timema knulli
Ellipes minutus
Galloisiana nipponensis
Chelisoches annulatus
Zorotypus novobrittanicus
Outgtroups
100 Changes

Figure 3

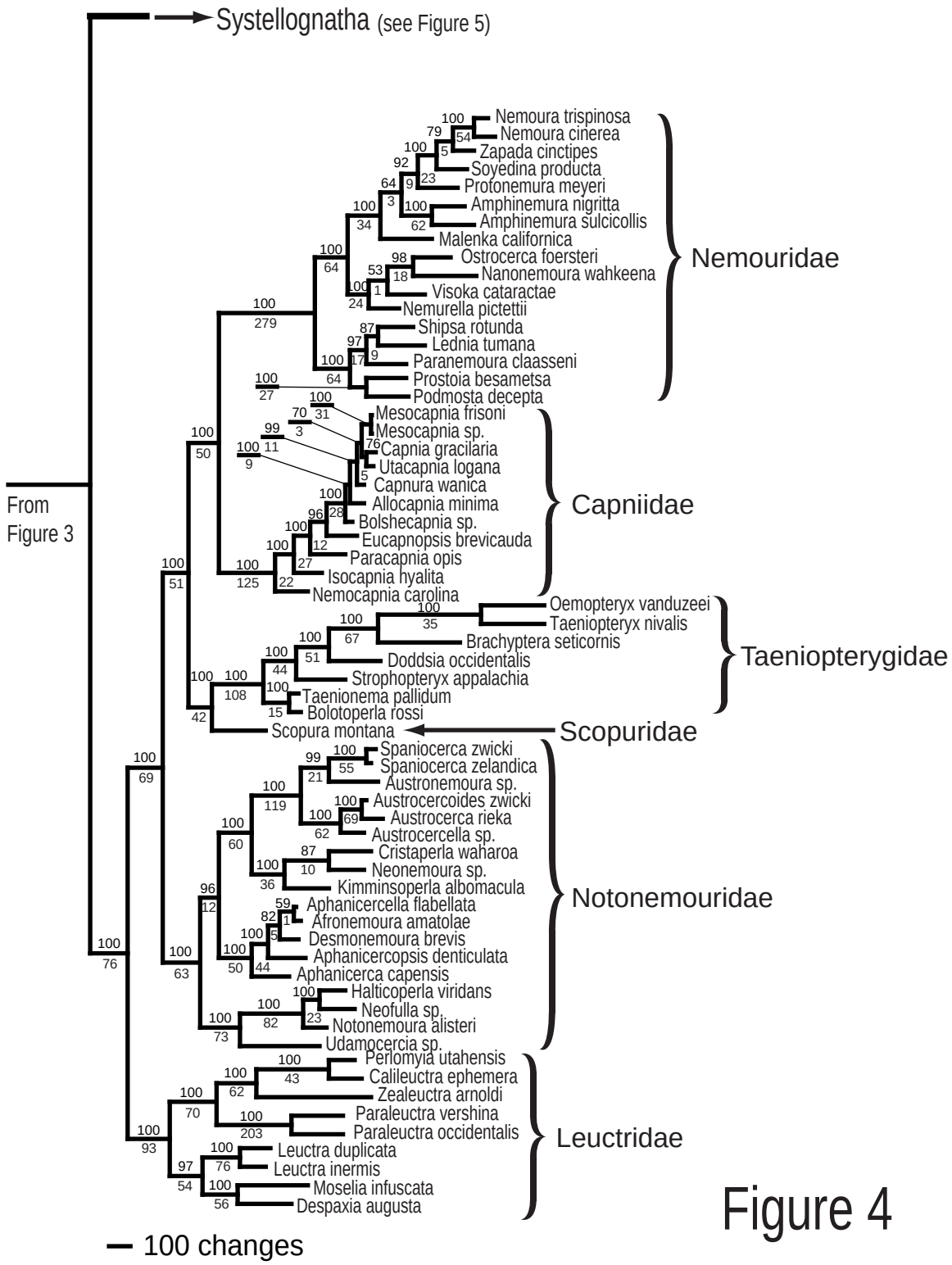
Systellognatha (see Figure 5)
From Figure 3
Nemoura trispinosa
Nemoura cinerea
Zapada cinctipes
Soyedina producta
Protonemura meyeri
Amphinemura nigritta
Amphinemura sulcicollis
Malenka californica
Ostrocerca foersteri
Nanonemoura wahkeena
Visoka cataractae
Nemurella pictettii
Shipsa rotunda
Lednia tumana
Paranemoura claasseni
Prostoia besametsa
Podmosta decepta
Nemouridae
Mesocapnia frisoni
Mesocapnia sp.
Capnia gracilaria
Utacapnia logana
Capnura wanica
Allocapnia minima
Bolshecapnia sp.
Eucapnopsis brevicauda
Paracapnia opis
Isocapnia hyalita
Nemocapnia carolina
Capniidae
Oemopteryx vanduzeei
Taeniopteryx nivalis
Brachyptera seticornis
Doddsia occidentalis
Strophopteryx appalachia
Taenionema pallidum
Bolotoperla rossi
Taeniopterygidae
Scopura montana
Scopuridae
Spaniocerca zwicki
Spaniocerca zelandica
Austronemoura sp.
Austrocercoides zwicki
Austrocerca rieka
Austrocercella sp.
Cristaperla waharoa
Neonemoura sp.
Kimminsoperla albomacula
Aphanicercella flabellata
Afronemoura amatolae
Desmonemoura brevis
Aphanicercopsis denticulata
Aphanicerca capensis
Halticoperla viridans
Neofulla sp.
Notonemoura alisteri
Udamocercia sp.
Notonemouridae
Perlomyia utahensis
Calileuctra ephemera
Zealeuctra arnoldi
Paraleuctra vershina
Paraleuctra occidentalis
Leuctra duplicata
Leuctra inermis
Moselia infuscata
Despaxia augusta
Leuctridae
100 changes

Figure 4

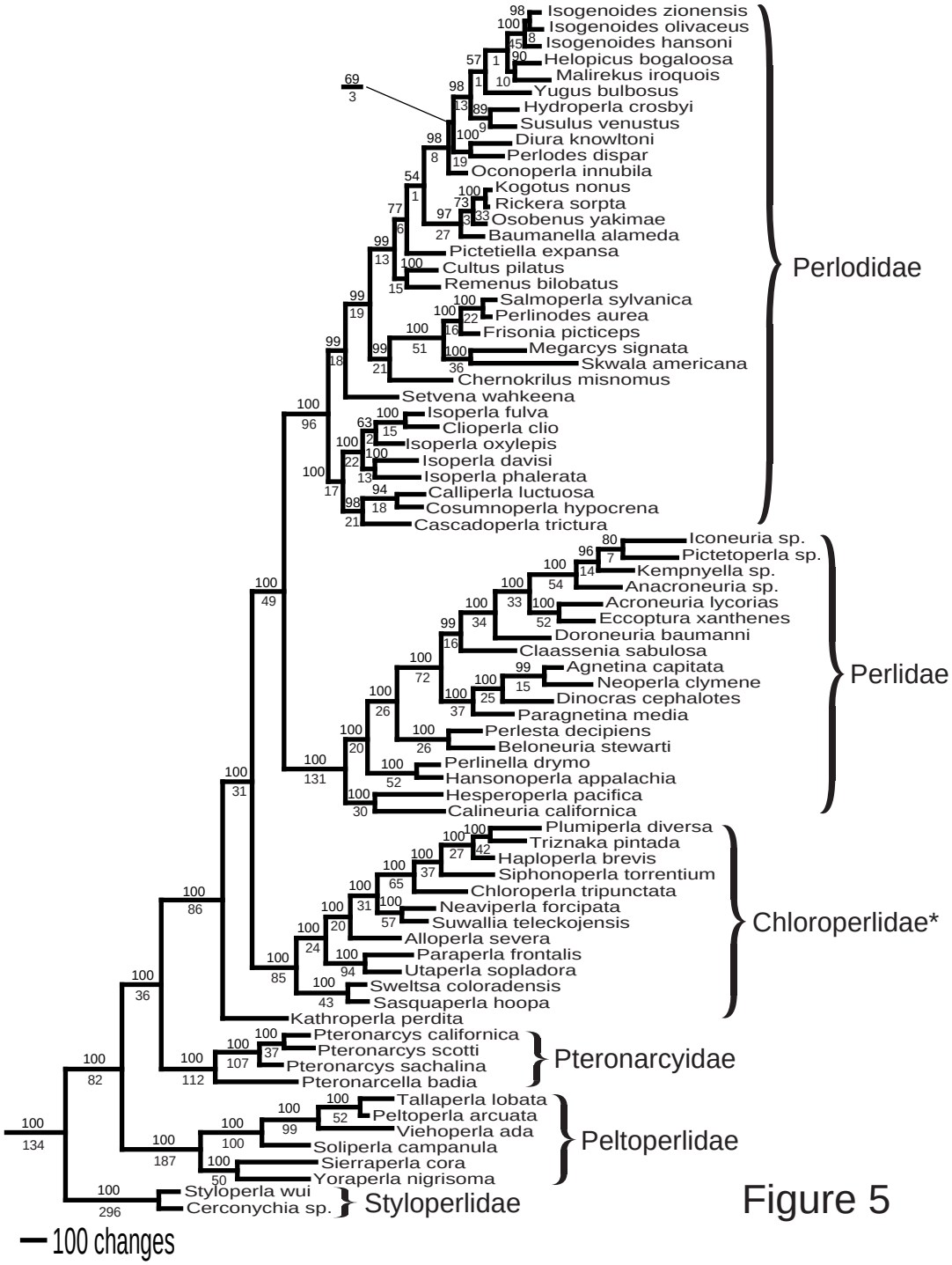
Isogenoides zionensis
Isogenoides olivaceus
Isogenoides hansoni
Helopicus bogaloosa
Malirekus iroquois
Yugus bulbosus
Hydroperla crosbyi
Susulus venustus
Diura knowltoni
Perlodes dispar
Oconoperla innubila
Kogotus nonus
Rickera sorpta
Osobenus yakimae
Baumanella alameda
Pictetiella expansa
Cultus pilatus
Remenus bilobatus
Salmoperla sylvanica
Perlinodes aurea
Frisonia picticeps
Megarcys signata
Skwala americana
Chernokrilus misnomus
Setvena wahkeena
Isoperla fulva
Clioperla clio
Isoperla oxylepis
Isoperla davisi
Isoperla phalerata
Calliperla luctuosa
Cosumnoperla hypocrena
Cascadoperla trictura
Perlodidae
Iconeuria sp.
Pictetoperla sp.
Kempnyella sp.
Anacroneuria sp.
Acroneuria lycorias
Eccoptura xanthenes
Doroneuria baumanni
Claassenia sabulosa
Agnetina capitata
Neoperla clymene
Dinocras cephalotes
Paragnetina media
Perlesta decipiens
Beloneuria stewarti
Perlinella drymo
Hansonoperla appalachia
Hesperoperla pacifica
Calineuria californica
Perlidae
Plumiperla diversa
Triznaka pintada
Haploperla brevis
Siphonoperla torrentium
Chloroperla tripunctata
Neaviperla forcipata
Suwallia teleckojensis
Alloperla severa
Paraperla frontalis
Utaperla sopladora
Sweltsa coloradensis
Sasquaperla hoopa
Kathroperla perdita
Chloroperlidae*
Pteronarcys californica
Pteronarcys scotti
Pteronarcys sachalina
Pteronarcella badia
Pteronarcyidae
Tallaperla lobata
Peltoperla arcuata
Viehoperla ada
Soliperla campanula
Sierraperla cora
Yoraperla nigrisoma
Peltoperlidae
Styloperla wui
Cerconychia sp.
Styloperlidae
100 changes

Figure 5

Figure 1. Previous hypotheses of Plecoptera phylogeny reproduced from a. Illies (1965) and b. Zwick (2000).

Figure 2. Single optimal topology (Length = 50331) for Plecoptera from direct optimization of total evidence (morphology, 12S, 16S, 18S, 28S, COII, H3) with major clades labeled. Taxonomic designations followed by asterisks were not supported as monophyletic. Numbers above nodes represent bootstrap values for 1000 replicates and numbers below the nodes Bremer values; both were calculated in PAUP* from the implied alignment. Clades in bold represent Plecoptera taxa endemic to the Southern Hemisphere. Portions of this topology appear in Figures 3-5 with each taxon labeled.

Figure 3. Detail of relationships for outgroup, *Megaluectra*, and arctoperlarian families from optimal topology in Figure 2. Taxonomic designations followed by asterisks are not supported as monophyletic. Numbers above individual nodes represent bootstrap values for 1000 replicates and numbers below the nodes Bremer values; both were calculated in PAUP* from the implied alignment.

Figure 4. Detail of relationships for Euholognatha from optimal topology in Figure 2. Numbers above individual nodes represent bootstrap values for 1000 replicates and numbers below the nodes Bremer values; both were calculated in PAUP* from the implied alignment.

Figure 5. Detail of relationships for Systellognatha from optimal topology in Figure 2. Taxonomic designations followed by asterisks are not supported as monophyletic. Numbers above individual nodes represent bootstrap values for 1000 replicates and numbers below the nodes Bremer values; both were calculated in PAUP* from the implied alignment.

PHYLOGENY OF THE GENUS *ISOGENOIDES* (PLECOPTERA: PERLODIDAE) AND THE EVOLUTION OF DRUMMING BEHAVIOR

Matthew D. Terry[1,3], John B. Sandberg[2], Kenneth W. Stewart[2], and Michael F. Whiting[1]

1. Department of Integrative Biology, Brigham Young University, WIDB 401, Provo, UT 84602

2. Department of Biological Science, University of North Texas, P.O. Box 305220, Denton, TX 76203

3. Present address: Department of Molecular and Cellular Biology, University of Arizona, Life Sciences South Building, Room 444, 1007 E. Lowell Street, Tucson, AZ 85721

Abstract—Many Plecoptera (stoneflies) exhibit a type of pre-mating communication known as “drumming.” Species of the genus *Isogenoides* have complex drumming behavior in which (i) the male calls the female by tapping his abdomen against the substrate, (ii) the female answers with her own distinctive tapping, and (iii) the male responds with a confirmatory series of taps. These drumming patterns are specific to individual species and may vary within a species to form distinct dialects. Phylogenetic analysis of the genus based on six molecular markers (12S, 16S, 18S, 28S, COII, H3) reveals evolutionary patterns in specific components of drumming. Pairwise partition homogeneity tests demonstrate that drumming characters, as a whole, are incongruent with the majority of molecular data partitions. This suggests a high level of evolutionary pressure on drumming, perhaps due to the pressure of sexual selection.

Representatives of the insect order Plecoptera, commonly called stoneflies, are entirely aquatic for most of their lifecycle. Juveniles generally inhabit cool to cold rivers and streams with relatively high concentrations of oxygen and play an important ecological role as either processors of plant material or predators of small invertebrates and also as a food resource for larger predators, particularly salmonid fishes. Adults emerge (often *en masse*), mate, and deposit eggs back into their aquatic environment within a relatively short amount of time.

Many stoneflies also exhibit the distinct pre-mating behavior of “drumming” (Maketon and Stewart 1988; Rupprecht 1967). In its simplest form drumming in

stoneflies begins with a male tapping the ventral side of its abdomen against the substrate; females available for mating answer with their own distinct pattern, which is produced in a similar manner; and this process is repeated until the male is able to locate the female and initiate mating. Drumming patterns are species specific (Stewart et al. 1988) and distinct dialects within a limited number of species have been recorded (Sandberg and Stewart 2003; Stewart et al. 1991; Stewart et al. 1982). The males of many stonefly species also possess specialized structures to facilitate both the production of their own drumming signal and the reception of the female answer (Stewart and Maketon 1991).

The genus *Isogenoides* (Plecoptera: Perlodidae) is composed of eight species endemic to North America. Although there is a clear disjunction between the three western and five eastern species within these two regions there is considerable overlap of species ranges. Among the stoneflies, *Isogenoides zionensis* exhibits some of the most complex drumming patterns. In addition to the generalized drumming behavior described above, the males of *Isogenoides* (with the apparent exception of *I. doratus*) respond to the female answer with a confirmatory response signal, completing a three-way communication composed of call/answer/response. Most species also use a pattern of sequenced drumming in which the male and female produce call and answer signals in rapid succession. The female of a single species (*I. zionensis)* even exhibits a complex wing-flutter response that has never been observed in other stoneflies. Although drumming behavior has been described for nearly 150 stonefly species, *Isogenoides* is the first completely characterized genus. *Isogenoides* is an ideal subject for the examination of drumming behavior in a phylogenetic context because of the small number of species

involved, their restricted distributions, and the range of complexity in observed drumming behavior.

Addressing the evolution of behavior from within a phylogenetic context can be difficult, especially when the behaviors in question may be subject to rapid evolution under the pressure of sexual selection (Kennedy et al. 1996; Ryan 1990). Various authors have put forth arguments supporting the exclusion of behavioral data as phylogenetic characters based on the difficulty of inferring primary homologies (Aronson 1981) or the overall lability of behavioral data (Greene and Burghardt 1978; Ryan 1996; Urbani 1989). Other studies have incorporated behavior as data in the inference of phylogenies of a wide range of organisms (Cannatella and Trueb 1988; Carpenter et al. 1992; Lanyon 1986) and argued against exclusion of behavioral data (Wenzel 1992; Wimberger and de Queiroz 1996). A review of phylogenetic studies incorporating behavioral data (de Queiroz and Wimberger 1993) concludes that behavioral data are no more homoplasious than morphological characters and consequently should not be excluded from phylogenetic analyses *a priori*. However, several data sets dealing specifically with the evolution of mate signaling demonstrate significant incongruence between this behavior and other potentially informative characters (Cannatella et al. 1998; Shaw 1996a). Of course there is also the issue of circularity. If the purpose of the research is to understand the evolution of a complex feature, then including that feature as part of the data set used for inferring the phylogeny may bias the generated phylogeny (Deleporte 1993). Here we attempt to determine if there is a phylogenetic pattern to drumming within the genus *Isogenoides*, and if so whether this pattern applies equally to all components of the drumming signals in both sexes. This investigation provides insight into the evolution of

complex behaviors associated with mate recognition and sexual selection, by analyzing their evolution in the context of a robust phylogenetic hypothesis generated from characters independent of the behavior.

MATERIALS AND METHODS

Behavioral Data Collection

Representatives of individual species were qualitatively collected either as newly emerged adults or as late instar nymphs from 1999-2002. Virgin adult males and females were held separately in manila-paper containers covered with clear plastic closures. These were placed in two separate, sound dampened sections of a portable, glass covered field-recording chamber described in Sandberg and Stewart (2003). All observations were made at comparable ambient temperature (20-28ºC) with indoor lighting ranging from 70-84 FTC under normal incandescent or florescent lights and outdoor settings under partially shaded sunlight near mountain streams. Stereo drumming signals were recorded with either a Sony® WALKMAN™ portable MiniDisc recorder (models MZ-R37 and MZ-R700) using Optimus® model 33-3013 omnidirectional microphones, or a Marantz® Cassette recorder (model PMD430) using Sony® Electret condenser microphones (model ECM-95S). Number of beats, beat interval, and trends within beats were visualized and measured via Ace of Wav (Polyhedric Software). Standard means and modes were calculated for each recorded component of the drumming complex (male call, female answer, and male response) using all observations (Table 1).

Taxon Sampling

Representatives of all eight described species of *Isogenoides* (*colubrinus* (Hagen), *doratus* (Frison), *elongatus (Hagen), frontalis* Newman, *hansoni* (Ricker), *olivaceus* (Walker), *varians* (Walsh), *zionensis* Hanson) were included in this analysis. A ninth species, *krumholzi*, has recently been synonymized with *doratus* (Sandberg and Stewart In prep.). Because there is no well-supported sister group for *Isogenoides*, eight outgroup taxa representing a wide range of Perlodidae diversity were also included in the analysis. Outgroups include *Diura knowltoni* (Frison), *Helopicus bogaloosa* Stark & Ray, *Hydroperla crosbyi* (Needham & Claassen), *Megarcys signata* (Hagen), *Pictetiella expansa* (Banks), *Setvena wahkeena* Stewart & Stanger, *Skwala americana* (Klapalek), and *Yugus bulbosus* (Frison). DNA sequences are deposited in GenBank under accession numbers #####-#### (to be provided upon acceptance). Voucher specimens and DNA templates are deposited at Brigham Young University, Insect Genomics Collection.

DNA Extraction, Amplification and Sequencing

A small portion of wing or leg muscle was dissected from the mesothorax and subjected to extraction via Qiagen's® DNeasy™ tissue kit. Purified DNA was amplified for 12S and 16S mitochondrial rDNA, 18S and 28S nuclear rDNA, Cytochrome Oxidase II and Histone 3 via polymerase chain reaction using previously published primers and amplification profiles (Colgan et al. 1998; Whiting 2001b; Whiting et al. 1997), and a complete list of these primers can be found in Appendix 1. Due to their large size, each of the nuclear ribosomal genes was amplified using three separate regions with sufficient overlap to insure continuity. These regions were approximately 1000, 800, and 600 nucleotides long for 18S and 1200, 600, and 1000 nucleotides long for 28S. Yield and potential contamination were monitored by agarose gel electrophoresis. Target products

were purified and cycle-sequenced using the ABI® dRhodamine cycle sequencing kit via flanking and, for long PCR products, internal primers. These sequencing reactions were then column purified and subjected to automated sequencing on ABI's® 3730xl automated sequencer. Complementary strands were independently sequenced and chromatographs were visually checked using Sequencher™ 4.1 (Sequencher 2002).

Phylogenetic Analyses

Analyses were performed for each individual data set (12S, 16S, 18S, 28S, COII, H3) and a combined total-evidence data set using the program POY version 3.0.11 (Wheeler et al. 2003) invoking parsimony as the optimality criterion. POY is a program that allows for simultaneous alignment and phylogenetic inference (Giribet 2001). This is performed by optimizing character states on ancestral nodes through a process called "Direct Optimization" (Wheeler 1996; Wheeler 2003). The ribosomal data sets were analyzed for 30 parameter combinations representing a large portion of the theoretical alignment landscape defined by axes representing gap:transversion cost and transversion:transition cost (Terry and Whiting Submitted-a; Wheeler 1995). As the protein reading frame was conserved across the COII and H3 data sets they were designated as "pre-aligned" and analyzed for the subset of parameter sets that varied transversion to transition weighting. For each analysis, twenty random replicates were performed utilizing tree fusing and ratcheting with spr and tbr swapping. The incongruence length metric (Mickevich and Farris 1981) was computed for each parameter combination by taking the difference between the length of the total tree, minus the sum of the lengths of the individual partitions (12S, 16S, 18S, 28S, COII, H3), divided by the length of the total tree (see Table 2). Bremer support values (i.e., Decay

Indices) were calculated in POY with a constraint file generated via the program "Jack2Hen." Partitioned Bremer support values were calculated in PAUP* (Swofford 2002) using a modified command block generated by TreeRot (Sorenson 1999) with 50 random additions per constraint tree and using the implied alignment generated via POY (Wheeler 2003). Non-parametric bootstrap percentages were calculated using PAUP* and the implied alignment from POY with 1000 bootstrap replicates and 20 random additions per replicate. The implied alignment formatted for PAUP* was also used to calculate consistency index (CI) and retention index (RI) values for the molecular data, and perform pairwise partition homogeneity tests (Farris et al. 1995; Farris et al. 1994) using individual genes and the behavioral data as partitions (results in Table 3). The latter test was performed to test the hypothesis of significant incongruence between the behavioral data and the molecular data. The homogeneity tests were performed with 1000 random addition replicates and a maximum of 100 trees held per replicate in PAUP*. An initial α-value of 0.05 was assumed for the partition homogeneity results and significance of individual values was determined via a sequential Bonferroni correction (Sokal and Rohlf 1981). CI and RI values for the drumming characters were calculated via MacClade (Maddison and Maddison 2000).

Drumming Character Coding and Mapping

Drumming was coded as 13 distinct components from all portions of the call/answer/response cycle using the calculated mode for each component (see Table 1). Number of beats, intrabeat interval, and interval description (increasing, decreasing, or complex) were coded for the male call, female answer, and male response. Characters

involving number of beats, interval between beats, and group count were treated as ordered characters. Intrabeat intervals, measured as a continuous value, were assigned discrete states according to their relative values; that is the character with the lowest interval was assigned character state "0", the next lowest "1", and so on. Although several methods for mapping continuous characters are available (Archie 1985; Farris 1970; Maddison 1991; Swofford and Maddison 1987), we have chosen to convert these into discrete characters. This allows us to preserve character state information that may be lost through processes such as gap-coding (Archie 1985), but still maintain the ability to map characters when some terminals are missing data (Maddison and Maddison 2000). Additional characters include: male response (present/absent), type of exchanges (sequenced/grouped/complex), mode number of male call groups, and female wing flutter (present/absent). Both the male response and female wing flutter characters are autapomorphic at the species level and number of male call groups is treated as an ordered character. Drumming behavior was recorded for two populations of *I. doratus*, *I. varians*, and *I. zionensis*. DNA was available for only one of each of these populations so, for the purpose of mapping characters, species were assumed to be monophyletic and additional terminals were added to the topology as sister taxa to the appropriate species. Individual characters were coded for these terminals as above. Mapping of characters was accomplished via MacClade 4.06 (Maddison and Maddison 2000) using the trace character function. Portions of the topology for which states could not be unambiguously assigned were designated as equivocal with all most parsimonious reconstructions represented in parentheses.

RESULTS

Sequencing

Amplified COII and H3 sequences have a conserved reading frame, yielding 648 and 374 base pairs, respectively. Average pairwise distance across *Isogenoides* is 0.1234 for COII and 0.0164 for H3. The longest complete 12S sequence is 411 base pairs (several taxa) and average complete length is 410 bp with and average pairwise distance across *Isogenoides* of 0.0152. The longest complete 16S sequence is 563 base pairs (several taxa) and average complete length is 562 bp with and average pairwise distance across *Isogenoides* of 00205. The longest complete 18S sequence is 2074 base pairs in length (three *Isogenoides* species) with an average length of approximately 2055 base pairs with and average pairwise distance across *Isogenoides* of 0.0039. The longest complete 28S sequence is 2462 base pairs (*Skwala americana*) with an average length of approximately 2430 base pairs with and average pairwise distance across *Isogenoides* of 0.0135.

Phylogenetic Analyses

Of the 30 parameter sets investigated the set treating transitions, transversions, and gaps equally (1:1:1) yields the most congruent results, with an ILD value of 0.011039. This result is parameter set is congruent with sensitivity analyses for other data sets (Giribet et al. 2001; Ogden and Whiting 2003; Svenson and Whiting 2004; Terry et al. Submitted; Terry and Whiting Submitted-b; Whiting 2001a; Whiting et al. 2003). The single optimal topology (Figure 1) has a length of 2627, a CI score of 0.698 and a RI score of 0.570. The parameter landscape generated from the ILD analysis is a nearly smooth topography

with congruence rising proportionate to the distance from the optimal parameter set (1:1:1). Partitioned Bremer support values reveal that approximately 72% of the overall signal across the topology is derived from the 28S data partition, 11% from 18S, 8% from 12S, 9 % from COII and less than 1% from the 16S and H3 partitions. When the partitioned bremer support is normalized by the number of informative characters per partition 27% is derived from the 28S data partition, 23% from 18S, 45% from 12S, 4% from COII and less than 1% from the 16S and H3 partitions. Pairwise partition homogeneity tests (Table 3) show significant incongruence between the drumming characters and four of the molecular markers (16S, 18S, 28S, and COII). None of the other possible pairwise comparisons showed any significant incongruence. The overall consistency and retention index values for all the drumming characters are 0.66 and 0.56, respectively; compared to a CI value of 0.70 and an RI value of 0.57 for the molecular data. The CI of individual drumming characters ranges from 0.50 to 1.0. Of the three major partitions (call/response/answer) the male response characters have the highest CI and RI values, 0.69 and 0.50.

DISCUSSION

Phylogeny and Biogeography of Isogenoides

This work stands as the first explicit phylogenetic hypothesis for the genus *Isogenoides* and the first analysis of plecopteran drumming behavior from a phylogenetic perspective. The genus *Yugus*, represented in this analysis by *Y. bulbosus*, is supported as sister group to *Isogenoides*. *Isogenoides hansoni* is sister group to the remaining Isogenoides species and *I. colubrinus* and *I. frontalis* are sister groups (Fig. 1).

Isogenoides elongatus and *I. zionensis* are strongly supported as sister taxa with a bootstrap value of 100% and a Bremer support value of 14, while the placement of *I. varians* and the grouping of *I. doratus* with *I. olivaceus* are only weakly supported (bootstrap values slightly over 50% and Bremer values of 1). The genus *Yugus* consists of two species confined to eastern North America; the genera *Hydroperla* and *Helopicus* are also absent from western North America. This outgroup distribution coupled with the basal placement of the eastern species *I. hansoni* is consistent with an Appalachian origin for *Isogenoides* with two subsequent dispersals to the Rocky Mountain region; one represented by *I. colubrinus*, and the second by the ancestor of *I. elongatus* + *I. zionensis* (Fig. 2). Overlapping and contiguous ranges of several species of *Isogenoides* and the presence of intraspecific variation in drumming hint at complexity not fully represented in this study. Such research, while beyond the scope of this work, would provide insight into the importance of character displacement (Dobzhansky 1940; Liou and Price 1994) and drumming as a mechanism of speciation in *Isogenoides*.

Evolution of Drumming Behavior

The drumming of *Isogenoides* is complex and varies greatly among species (see Figure 3 for a summary of drumming behaviors). Due to the potential for sexual selection pressure, lack of information regarding the evolution of drumming in Plecoptera, and other examples of pre-mating communication systems with high degrees of incongruence when compared to all potentially informative characters (Cannatella et al. 1998; Ryan 1990; Shaw 1996b) we chose to exclude drumming as characters for phylogenetic inference. Cannatella et al. (1998) suggest that, at least in leptodactylid frogs, “homologous similarity in calls of recently separated species is quickly lost as the

species diverge." Based on incongruence as measured by pairwise partition homogeneity tests (Table 3) this generalization appears to also apply to *Isogenoides*. We find that drumming is a complex behavior with many individual components that exhibit varying degrees of phylogenetic congruence.

Of the drumming characters included in this analysis, three (male response, male response interval description, and female wing flutter; characters 7, 10 and 13, respectively) are autapomorphic at the species level. Type of exchanges (character 11) is polymorphic across almost all of the taxa sampled and apparently monomorphic species may simply reflect missing observations. Mappings of the remaining nine characters, which provide a much more complex pattern, appear in Figure 4.

Male call patterns (Fig. 4a-c) have an overall CI of 0.60 and an RI of 0.58. Male call beat number (Fig. 4a) has a most parsimonious reconstruction (MPR) of six beats at the basal node and at most ancestral nodes. *Yugus*, supported as *Isogenoides* sister group in this analysis, also has a male call consisting of 6 beats. The greatest change for male call beat occurs at the node uniting *I. olivaceus* (7 beats/call) and *I. doratus* (3 beats/call). Male call beat interval (Fig. 4b) shows as largely similar pattern with the greatest change again occurring between *I. olivaceus* and *I. doratus*.

Female answer patterns (Fig. 4d-f) are considerably simpler and have a higher overall CI (0.67) than the male call. The greatest change for female answer beat number occurs again between *I. olivaceus* and *I. doratus*. An interesting correlation between the length of the male call and the female answer (Fig. 4b and 4e) occurs in *I. elongatus* and *I. zionensis*. These species appear as sister taxa in a relatively apical position on the tree and have the longest beat intervals, a feature especially pronounced in the male call.

Analysis of the male response signal is hampered somewhat by missing data (the putative loss of the male answer in *I. doratus* and no measurements for *I. hansoni*), however, it appears to be simpler than the male call. It has a maximum beat number of four as opposed to eight in the male call and only a single instance of decreasing intervals between successive beats, a feature which is much more complex in the male call (Fig. 4c). Also in contrast to the male call the longest beat intervals occur in more basal lineages (Fig. 4h).

CONCLUSIONS

Drumming characters in *Isogenoides* are, overall, incongruent with the phylogenetic history of this group, though individual components of drumming behavior show some patterns consistent with phylogeny, particularly at shallow nodes. This is consistent with the hypothesis of a high level of behavioral lability, perhaps due to the evolutionary pressure of sexual selection (Cannatella et al. 1998), and contrasts with other studies which found greater congruence between behavior and phylogeny (de Queiroz and Wimberger 1993; Kennedy et al. 1996). However, male call patterns exhibit the most complexity as measured by both the number and range of character states and the overall CI. This complexity needs to be further studied and characterized at the population level and in the context of distributional data for each *Isogenoides* species, in order to understand how these characters potentially influence the speciation and diversification of Isogenoides.

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Appendix 1. List of taxa included in this analysis (GenBank accession numbers to be provided upon acceptance).

	Molecular Markers					
	12S	16S	18S	28S	COII	H3
Isogenoides colubrinus	####	####	####	####	####	####
Isogenoides doratus	####	####	####	####	####	####
Isogenoides elongatus	####	####	####	####	####	####
Isogenoides frontalis	####	####	####	####	####	####
Isogenoides hansoni	####	####	####	####	####	####
Isogenoides olivaceus	####	####	####	####	####	####
Isogenoides varians	####	####	####	####	####	####
Isogenoides zionensis	####	####	####	####	####	####
Diura knowltoni	####	####	####	####	####	####
Helopicus bogaloosa	####	####	####	####	####	####
Hydroperla crosbyi	####	####	####	####	####	####
Megarcys signata	####	####	####	####	####	####
Pictetiella expansa	####	####	####	####	####	####
Setvena wahkeena	####	####	####	####	####	####
Skwala americana	####	####	####	####	####	####
Yugus bulbosus	####	####	####	####	####	####

Appendix 2. List of primers used in this analysis with sequence and relative position information.

Gene	Primer Name	Sequence (5' - 3')	Length	Direction	Relative Position
12S	12S ai	AAACTACGATTAGATACCCTATTAT	25	Forward	
12S	12S bi	AAGAGCGACGGGCGATGTGT	20	Reverse	
16S	16S A	CGCCTGTTTATCAAAAACAT	20	Forward	
16S	16S B	CTCCGGTTTGAACTCAGATCA	21	Reverse	
18S	18S 1F	TACCTGGTTGATCCTGCCAGTAG	23	Forward	1
18S	18S ai	CCTGAGAAACGGCTACCACATC	22	Forward	2
18S	18S a0.7	ATTAAAGTTGTTGCGGTT	18	Forward	3
18S	18S a0.79	TTAGAGTGCTYAAAGC	16	Forward	4
18S	18S a1.0	GGTGAAATTCTTGGAYCGTC	20	Forward	5
18S	18S a2.0	ATGGTTGCAAAGCTGAAAC	19	Forward	6
18S	18S a3.5	TGGTGCATGGCCGYTCTTAGT	21	Forward	7
18S	18S 7F	GCAATAACAGGTCTGTGATGCCC	23	Forward	8
18S	18S 9R	GATCCTTCCGCAGGTTCACCTAC	23	Reverse	1
18S	18S 7R	GCATCACAGACCTGTTATTGC	21	Reverse	2
18S	18S bi	GAGTCTCGTTCGTTATCGGA	20	Reverse	3
18S	18S b0.5	GTTTCAGCTTTGCAACCAT	19	Reverse	4
18S	18S b2.5	TCTTTGGCAAATGCTTTCGC	20	Reverse	5
18S	18S b2.9	TATCTGATCGCCTTCGAACCTCT	23	Reverse	6
18S	18S b3.9	TGCTTTRAGCACTCTAA	17	Reverse	7
18S	18S b5.0	TAACCGCAACAACTTTAAT	19	Reverse	8
18S	18S b7.0	ATTTRCGYGCCTGCTGCCTTCCT	23	Reverse	9
28S	28s Rd 1a	CCCSCGTAAYTTAGGCATAT	20	Forward	1
28S	28s Rd 3a	AGTACGTGAAACCGTTCAGG	20	Forward	2

28S	28S Rd 4.5a	AAGTTTCCCTCAGGATAGCTG	21	Forward	5
28S	28S Rd 4.8a	ACCTATTCTCAAACTTTAAATGG	23	Forward	6
28S	28S Rd 5a	GGYGTTGGTTGCTTAAGACAG	21	Forward	7
28S	28S Rd 6a	GGCGAAAGGGAATCYGGTTC	20	Forward	8
28S	28S Rd 7b1	GACTTCCCTTACCTACAT	18	Reverse	1
28S	28S Rd 6b	AACCRGATTCCCTTTCGCC	19	Reverse	2
28S	28S Rd 5b	CCACAGCGCCAGTTCTGCTTAC	22	Reverse	3
28S	28S B	TCGGAAGGAACCAGCTAC	18	Reverse	4
28S	28S C	ATAGTTCACCATCTYTCGGG	20	Reverse	5
28S	28S Rd 4b	CCTTGGTCCGTGTTTCAAGAC	21	Reverse	6
28S	28S Rd 3b	CCYTGAACGGTTTCACGTACT	21	Reverse	7
COII	COII 1a	TTAAGCTCCATATATAAAGGMTT	23	Forward	
COII	COII F-Leu	TCTAATATGGCAGATTAGTGC	21	Forward	
COII	COII 9b	GTACTTGCTTTCAGTCATCTWATG	24	Reverse	
COII	COII R-Lys	GAGACCAGTACTTGCTTTCAGTCATC	26	Reverse	
H3	H3 AF	ATGGCTCGTACCAAGCAGACVGC	23	Forward	
H3	Hex AF	ATGGCTCGTACCAAGCAGACGGC	23	Forward	
H3	H3 AR	ATATCCTTRGGCATRATRGTGAC	23	Reverse	
H3	Hex AR	ATATCCTTGGGCATGATGGTGAC	23	Reverse	

Table 1. Drumming characters for *Isogenoides* species. Char 1.= Number of beats in male call. 2.= Beat interval of male call (msec). 3.= Description of interval in male call. 4.= Number of beats in female answer. 5.= Beat interval of female answer (msec). 6.= Description of interval in female answer. 7.= Male response 8.= Number of beats in male response. 9.= Beat interval of male response (msec). 10.= Description of interval in male response. 11.= Type of exchanges (0 = sequenced, 1 = grouped, 2 = complex). 12.= Male call group count. 13.= Female wing flutter. Numbers in parentheses represents character states and question marks represent missing data. Characters 1, 2, 4, 5, 8, 9, and 12 were treated as ordered characters.

Species	**Char. 1**	**Char. 2**	**Char. 3**	**Char. 4**	**Char. 5**	**Char. 6**
I. colubrinus	5	65.0 (6)	increasing (0)	1	121.8	increasing (0)
I. doratus (a)	3	16.7 (1)	decreasing (1)	2	107.0 (4)	increasing (0)
I. doratus (b)	3	14.4 (0)	decreasing (1)	1	135.5	decreasing (1)
I. elongatus	6	221.1 (8)	increasing (0)	2	180.5 (7)	increasing (0)
I. frontalis	4	61.2 (5)	increasing (0)	2	93.8 (3)	decreasing (1)
I. hansoni	6	31.3 (3)	decreasing (1)	?	?	?
I. olivaceus	7	98.2 (7)	decreasing (1)	6	86.6 (2)	increasing (0)
I. varians (a)	6	26.7 (2)	increasing (0)	4	52.4 (0)	increasing (0)
I. varians (b)	8	36.9 (4)	increasing (0)	2	56.7 (1)	increasing (0)
I. zionensis (a)	4	364.3 (10)	both (2)	2	136.8 (6)	increasing (0)
I. zionensis (b)	4	344.8 (9)	both (2)	2	123.4 (5)	increasing (0)

Char. 7	**Char. 8**	**Char 9.**	**Char. 10**	**Char. 11**	**Char. 12**	**Char. 13**
present (0)	2	441.8 (6)	decreasing (1)	both (0&1)	2	absent (0)
absent (1)	?	?	?	grouped (1)	11	absent (0)
absent (1)	?	?	?	grouped (1)	6	absent (0)
present (0)	1	N/A	N/A	both (0&1)	1	absent (0)
present (0)	3	288.8 (4)	increasing (0)	both (0&1)	3	absent (0)
?	?	?	?	grouped (1)	3	?
present (0)	1	356.5 (5)	increasing (0)	both (0&1)*	1	absent (0)
present (0)	3	56.0 (0)	increasing (0)	both (0&1)	3	absent (0)
present (0)	3	73.4 (1)	increasing (0)	both (0&1)	3	absent (0)
present (0)	3	132.2 (2)	increasing (0)	both (0&1)	1	present (1)
present (0)	4	135.3 (3)	increasing (0)	complex (2)	2	present (1)

*** = only one grouped signal observed**

Table 2. ILD values from sensitivity analysis in POY. Columns represent the ratio of transversion cost to transition cost. Rows represent the ratio of gap to transversion cost. Values were calculated using the ILD Metric (Farris et al. 1995), lower numbers represent more congruence among individual data sets.

		Tv/Ts					
		0.5	1	2	3	4	infinite
Gap/Tv	0.5	0.012291	0.011106	0.01155	0.015087	0.014114	0.020054
	1	0.013844	0.011039	0.012913	0.015366	0.018642	0.028998
	2	0.018126	0.015656	0.015401	0.015183	0.01658	0.02783
	3	0.017714	0.019011	0.019571	0.020422	0.020506	0.02659
	4	0.017861	0.017638	0.016989	0.01665	0.017426	0.022512

Table 3. Results from pairwise partition homogeneity tests for drumming and individual molecular partitions. Values with asterisks are significantly incongruent at the P = 0.05 level after Bonferroni correction (Sokal and Rohlf 1981)

	drumming	12s	16s	18s	28s	coii
12s	0.1260	X	X	X	X	X
16s	0.0060*	0.9320	X	X	X	X
18s	0.0020*	0.1720	0.5270	X	X	X
28s	0.001*	0.5850	0.1100	0.1280	X	X
coii	0.004*	1.0000	1.0000	1.0000	0.1810	X
h3	0.2670	0.9770	1.0000	0.9750	0.7890	1.0000

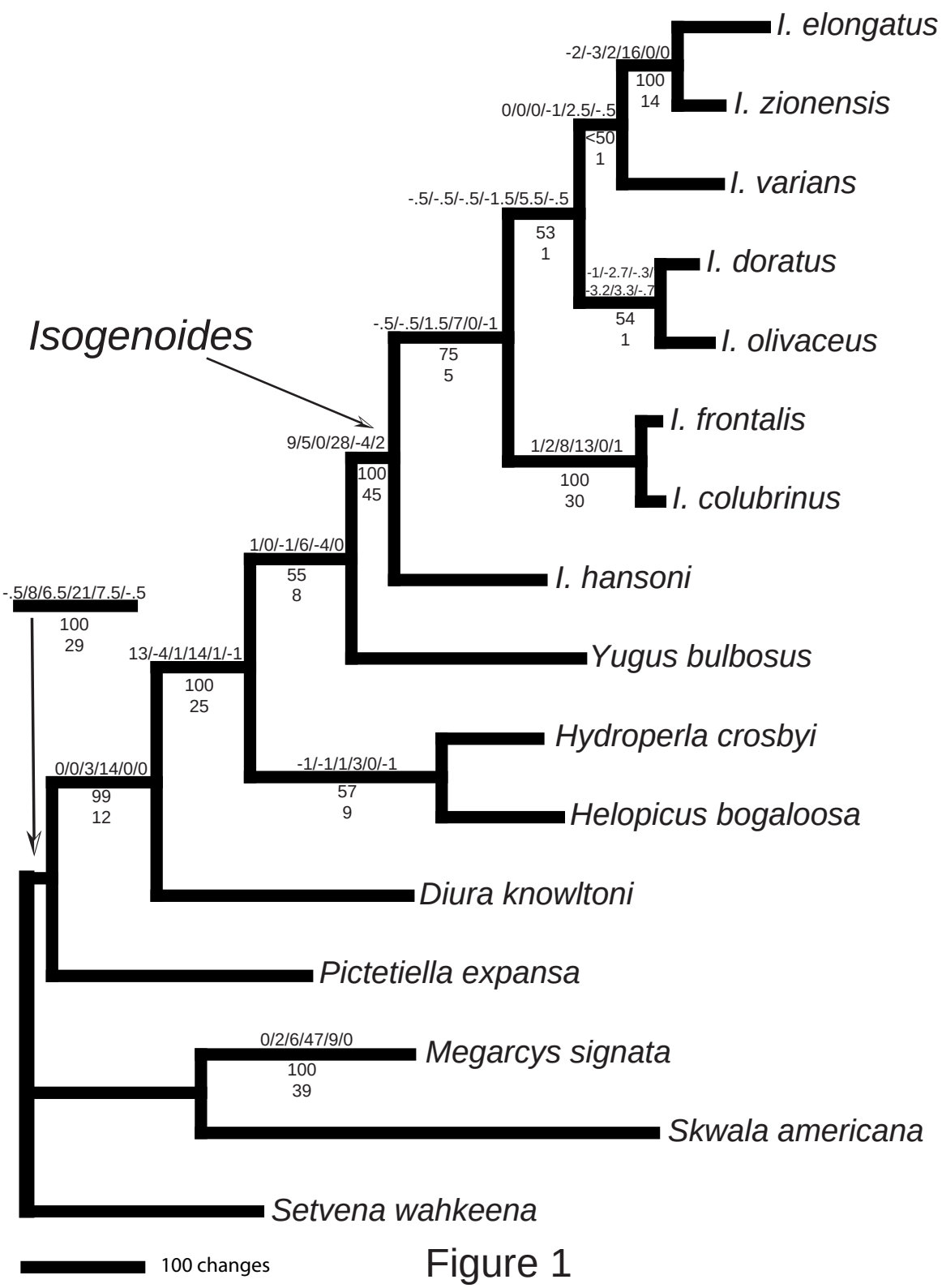

I. elongatus
-2/-3/2/16/0/0
100
14
I. zionensis
0/0/0/-1/2.5/-.5
<50
1
I. varians
-.5/-.5/-.5/-1.5/5.5/-.5
53
1
I. doratus
-1/-2.7/-.3/
-3.2/3.3/-.7
54
1
I. olivaceus
Isogenoides
-.5/-.5/1.5/7/0/-1
75
5
I. frontalis
1/2/8/13/0/1
100
30
I. colubrinus
9/5/0/28/-4/2
100
45
I. hansoni
1/0/-1/6/-4/0
55
8
-.5/8/6.5/21/7.5/-.5
100
29
Yugus bulbosus
13/-4/1/14/1/-1
100
25
Hydroperla crosbyi
-1/-1/1/3/0/-1
57
9
0/0/3/14/0/0
99
12
Helopicus bogaloosa
Diura knowltoni
Pictetiella expansa
0/2/6/47/9/0
100
39
Megarcys signata
Skwala americana
Setvena wahkeena
100 changes

Figure 1

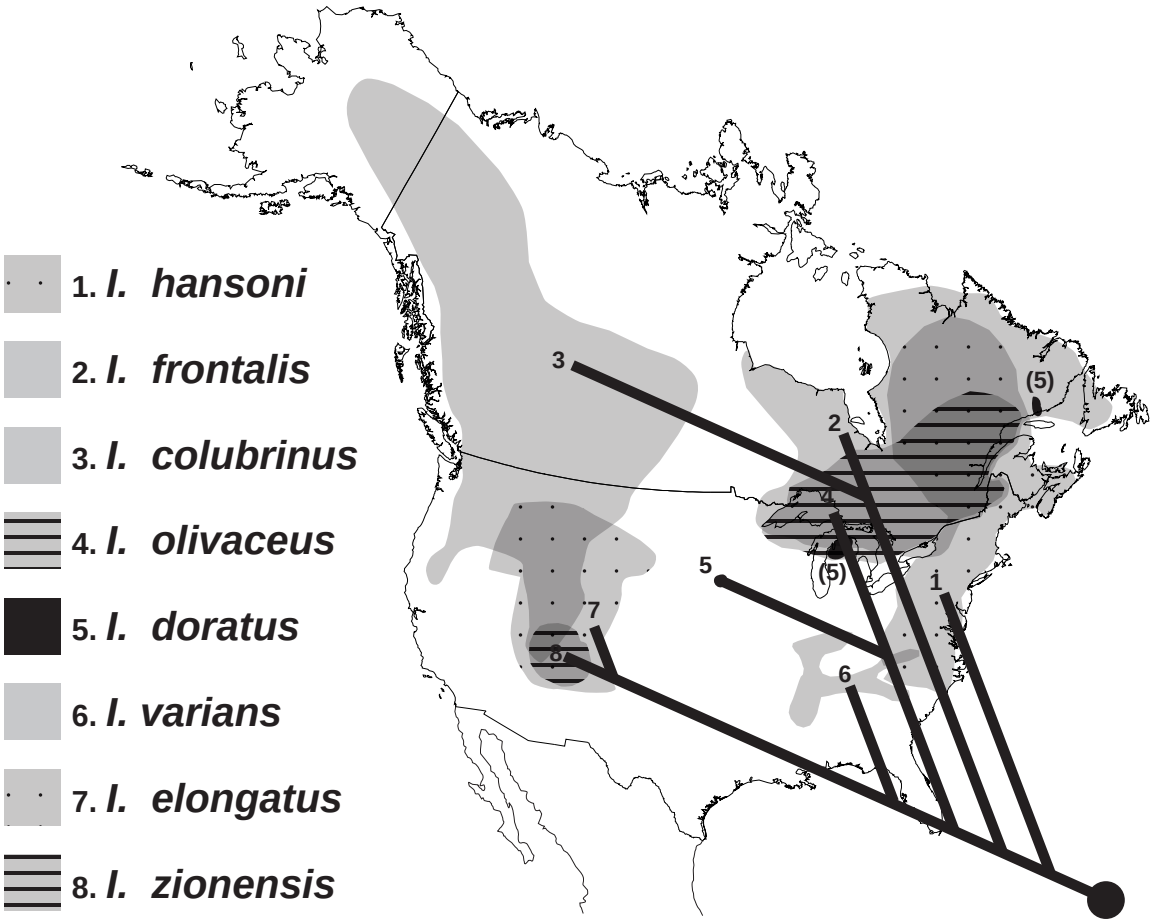
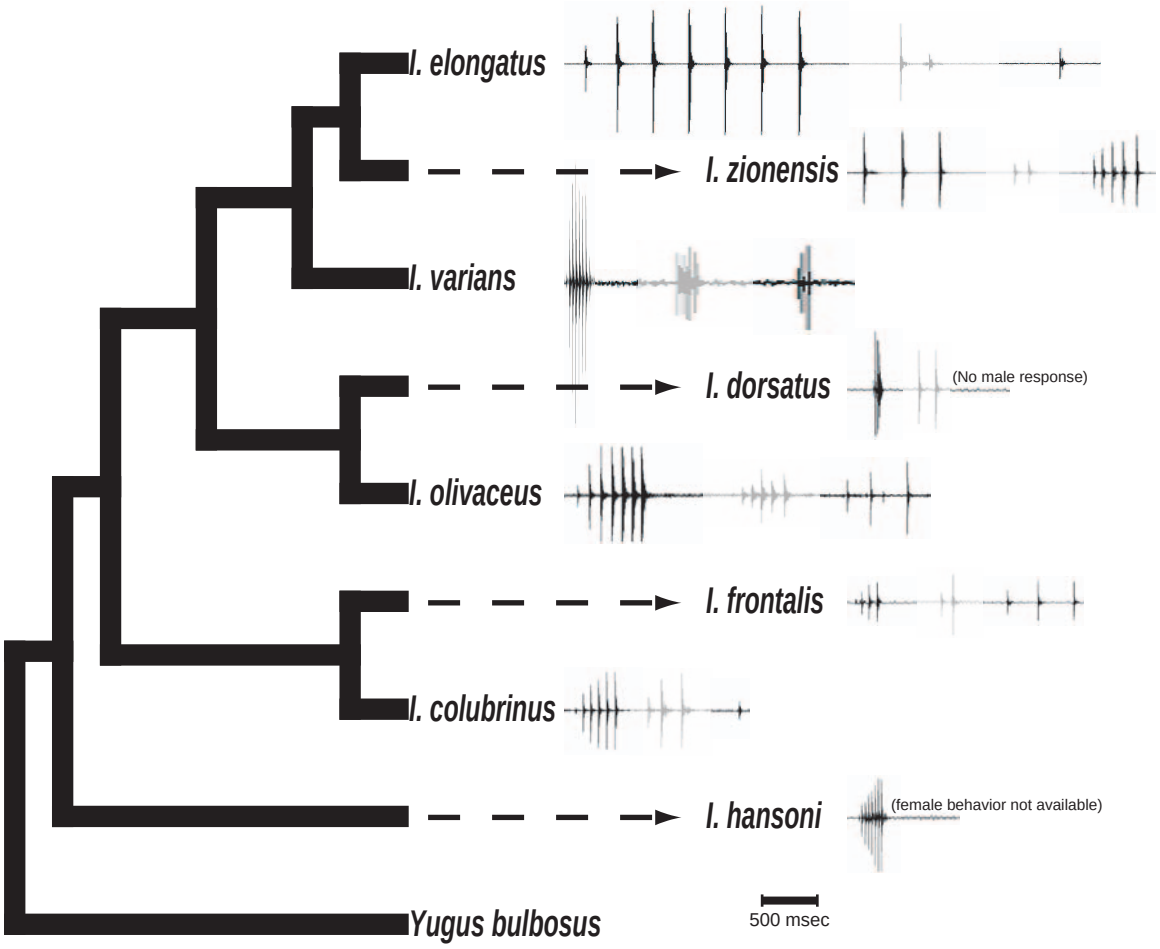

1. I. hansoni
2. I. frontalis
3. I. colubrinus
4. I. olivaceus
5. I. doratus
6. I. varians
7. I. elongatus
8. I. zionensis
3
2
(5)
4
(5)
5
1
7
8
6

Figure 2


I. elongatus
I. zionensis
I. varians
I. dorsatus
(No male response)
I. olivaceus
I. frontalis
I. colubrinus
I. hansoni
(female behavior not available)
Yugus bulbosus
500 msec


♂ call
♀ answer
♂ response

Figure 3

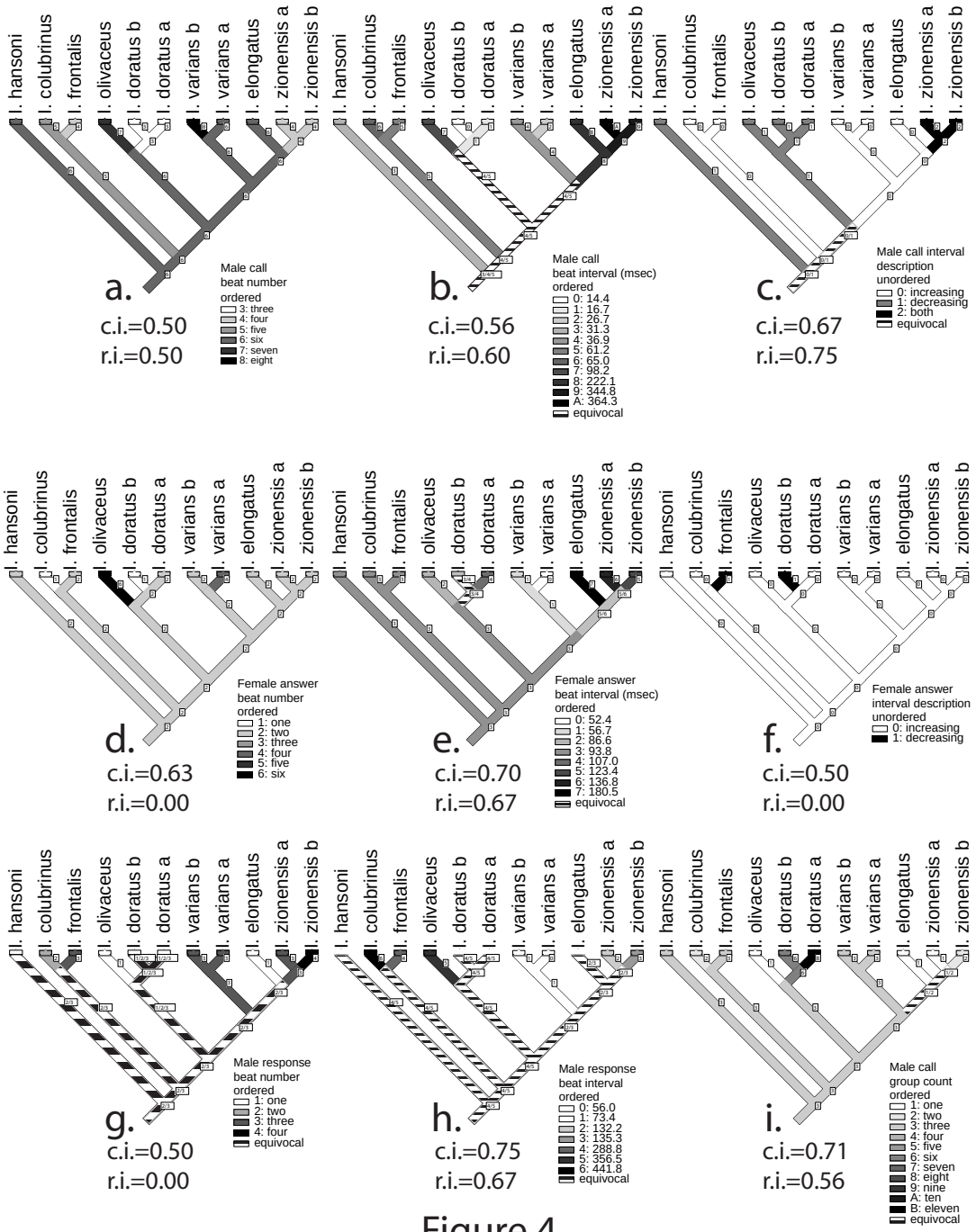

l. hansoni
l. colubrinus
l. frontalis
l. olivaceus
l. doratus b
l. doratus a
l. varians b
l. varians a
l. elongatus
l. zionensis a
l. zionensis b
a.
c.i.=0.50
r.i.=0.50
Male call beat number ordered
3: three
4: four
5: five
6: six
7: seven
8: eight
b.
c.i.=0.56
r.i.=0.60
Male call beat interval (msec) ordered
0: 14.4
1: 16.7
2: 26.7
3: 31.3
4: 36.9
5: 61.2
6: 65.0
7: 98.2
8: 222.1
9: 344.8
A: 364.3
equivocal
c.
c.i.=0.67
r.i.=0.75
Male call interval description unordered
0: increasing
1: decreasing
2: both
equivocal
d.
c.i.=0.63
r.i.=0.00
Female answer beat number ordered
1: one
2: two
3: three
4: four
5: five
6: six
e.
c.i.=0.70
r.i.=0.67
Female answer beat interval (msec) ordered
0: 52.4
1: 56.7
2: 86.6
3: 93.8
4: 107.0
5: 123.4
6: 136.8
7: 180.5
equivocal
f.
c.i.=0.50
r.i.=0.00
Female answer interval description unordered
0: increasing
1: decreasing
g.
c.i.=0.50
r.i.=0.00
Male response beat number ordered
1: one
2: two
3: three
4: four
equivocal
h.
c.i.=0.75
r.i.=0.67
Male response beat interval ordered
0: 56.0
1: 73.4
2: 122.2
3: 135.3
4: 288.8
5: 356.5
6: 441.8
equivocal
i.
c.i.=0.71
r.i.=0.56
Male call group count ordered
1: one
2: two
3: three
4: four
5: five
6: six
7: seven
8: eight
9: nine
A: ten
B: eleven
equivocal

Figure 4

Figure 1. Single most parsimonious topology from direct optimization analysis of molecular evidence for the genus *Isogenoides* and related Perlodidae. Numbers above individual nodes are partition bremer values for 12S/16S/18S/28S/COII/H3 calculated from implied alignment. Numbers directly below nodes are bootstrap values and numbers below that are bremer values calculated in POY. L= 2627, CI= 0.698, RI= 0.570.

Figure 2. Phylogeny of the genus *Isogenoides* with reference to geographical distribution. Numbers in parentheses are non-contiguous populations of species represented from other areas.

Figure 3. Summary of drumming patterns in the genus *Isogenoides*. For drumming graphs, X-axis represents time in milliseconds scaled to bar and Y-axis represents intensity of the recorded drumming signal.

Figure 4. Individual drumming characters mapped onto *Isogenoides* phylogeny. A. Number of beats in male call. B. Beat interval of male call. C. Description of interval in male call. D. Number of beats in female answer. E. Beat interval of female call. F. Description of interval in female call. G. Number of beats in male response. H. Beat interval of male response. I. Male call group count. Numbers on branches are MPR (most parsimonious reconstructions) of character states.