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Phylogeny of Trichoptera (Caddisflies): Characterization of Signal and Noise Within Multiple Datasets

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Abstract.—Trichoptera are holometabolous insects with aquatic larvae that, together with the Lepidoptera, make up the Amphiesmenoptera. Despite extensive previous morphological work, little phylogenetic agreement has been reached about the relationship among the three suborders—Annulipalpia, Spicipalpia, and Integripalpia—or about the monophyly of Spicipalpia. In an effort to resolve this conflict, we sequenced fragments of the large and small subunit nuclear ribosomal RNAs (1078 nt; D1, D3, V4-5), the nuclear elongation factor 1α gene (EF-1α; 1098 nt), and a fragment of mitochondrial cytochrome oxidase I (COI; 411 nt). Seventy adult and larval morphological characters were reanalyzed and added to molecular data in a combined analysis. We evaluated signal and homoplasy in each of the molecular datasets and attempted to rank the particular datasets according to how appropriate they were for inferring relationships among suborders. This evaluation included testing for conflict among datasets, comparing tree lengths among alternative hypotheses, measuring the left-skew of tree-length distributions from maximally divergent sets of taxa, evaluating the recovery of expected clades, visualizing whether or not substitutions were accumulating with time, and estimating nucleotide compositional bias.

Although all these measures cast doubt on the reliability of the deep-level signal coming from the nucleotides of the COI and EF-1α genes, these data could still be included in combined analyses without overturning the results from the most conservative marker, the rRNA. The different datasets were found to be evolving under extremely different rates.

A site-specific likelihood method for dealing with combined data with nonoverlapping parameters was proposed, and a similar weighting scheme under parsimony was evaluated.

Among our phylogenetic conclusions, we found Annulipalpia to be the most basal of the three suborders, with Spicipalpia and Integripalpia forming a clade. Monophyly of Annulipalpia and Integripalpia was confirmed, but the relationships among spicipalpians remain equivocal. [Annulipalpia; Bayesian inference; dataset combination; homoplasy; Integripalpia; Spicipalpia; pseudoreplicate reweighting.]

Trichoptera, or caddisflies, are an order of holometabolous insects with aquatic immature stages. They are integral components of almost all freshwater communities (Resh and Rosenberg, 1984). With a fauna of some 10,000 described extant species distributed among 45 families (Morse, 1997a), the order is diverse in terms of the microhabitats and trophic niches the species occupy (Mackay and Wiggins, 1979). Ecological diversity and general intolerance to pollution make the larvae excellent biological indicators of water quality (Rosenberg and Resh, 1993). The net-spinning and case-making behaviors of the larvae have long held the interest of biologists (Williams et al., 1987). Despite this interest in Trichoptera, a comprehensive analysis leading to a widely accepted phylogeny of higher-level relationships within the order has been elusive.

The purpose of this paper is, first and foremost, to provide a stable classification for the suborders of Trichoptera. Insights into caddisfly evolution gained by thorough phylogenetic analysis will provide a robust framework for examining biological attributes of general interest within the order, such as case- and retreat-making behavior (Sukatsheva, 1980; Weaver and Morse, 1986; Frania and Wiggins, 1997), egg-laying and oviposition behavior (Weaver, 1983), evolution of filter feeding (Alstad, 1982; Thorp, 1983; Thorp et al., 1986), pupation behavior (Wiggins and Wichard, 1989; Wichard and Klein, 1997), trophic relationships of larvae (Weaver and Morse, 1986), ancestral habitats (Shields, 1988; Kristensen, 1997), and biogeography (Gall, 1997; Novokshovov and Sukatcheva, 1997).

We collected sequence data from nuclear ribosomal RNA (rRNA), elongation factor 1alpha (EF-1α), and mitochondrial cytochrome oxidase I (COI), and we reevaluated a morphological dataset from Frania and Wiggins (1997). As in any study with data from multiple sources, we had to make

decisions about how the combined data would be treated. A secondary goal of the study was to evaluate the different sources of data so that we could perform an informed combined analysis and conclude with a limited set of hypotheses, from which the support for any particular set of relationships can be evaluated.

Dataset Combination

The debate over whether datasets should be combined or analyzed separately (e.g., Miyamoto, 1985; Kluge, 1989; Barrett et al., 1991; Bull et al., 1993; de Queiroz, 1993; Eernisse and Kluge, 1993; de Queiroz et al., 1995; Huelsenbeck et al., 1996; Springer et al., 1999) has centered around either philosophical objections to excluding data or theoretical explanations of why such exclusions would be a good idea, with examples supporting both sides. Less attention has been paid to whether or not different partitions could, in combination, answer questions in different parts of a tree (Sullivan, 1996; Wiens, 1998), or whether qualitative rankings of signal and noise could be used to filter through alternative hypotheses. Yet systematists who conduct preliminary analyses about the level of information in candidate genes and then carefully select their genes to match the divergence times of their questions implicitly expect different genes to be informative at different hierarchical levels. If different genes are informative at different levels, it is difficult to estimate relationships that span 200 million years with a single gene or to categorize any single gene as "good" or "bad". Here we evaluate datasets in a manner that allows a ranking of datasets according to localized phylogenetic utility.

Taxonomic History

The sister group relationship between Trichoptera and Lepidoptera, together comprising the Amphiesmeniptera, has long been recognized and is among the most strongly supported in entomology (Speyer, 1870, and others [see Betten, 1934, for discussion]; Hennig, 1981; Kristensen, 1991, 1997; Pashley et al., 1993; Chalwatzis et al., 1996; Whiting et al., 1997). Within Trichoptera, it is now accepted that the order contains two monophyletic suborders, Annulipalpia and Integripalpia, with a third suborder, Spicipalpia, whose monophyly is equivocal. Annulipalpian larvae make fixed retreats, from which they spin a silken net used to filter fine detritus or capture invertebrate prey. Integripalpian larvae make portable tube cases from which they feed in a variety of manners, most commonly as shredders or predators but also as scrapers, filterers, or herbivores, among others (Mackay and Wiggins, 1979). Spicipalpian larvae include the "free-living" predators (Rhyacophilidae and Hydrobiosidae); the "purse-case makers" (Hydroptilidae), which feed by piercing algal cells or by gathering fine detritus; and the "saddle-" or "tortoise-case makers" (Glossosomatidae), which are specialized for scraping periphyton off the upper surfaces of rocks. The Annulipalpia and Spicipalpia are primarily lotic, whereas the Integripalpia occur in both lotic and lentic habitats (Wiggins, 1996).

Ross (1956, 1964, 1967) provided the first modern phylogenetic hypotheses of subordinal and superfamily relationships, but earlier workers also constructed general classifications for the order, including Ulmer (1912), Martynov (1924), and Milne and Milne (1939). Ross' (1956, 1964, 1967) concept of Integripalpia contained two superfamilies, Limnephiloidea *sensu latu* (Integripalpia *s.s.*), and a paraphyletic "Rhyacophiloidea" (Fig. 1a). His hypothesis of the relationships among Rhyacophilidae (then including Hydrobiosinae), Glossosomatidae, Hydroptilidae, and the Limnephiloidea s.l. was based primarily on a presumed evolutionary transformation in larval case/pupal cocoon-making behavior. Recently, alternative morphologically based phylogenies have been proposed challenging Ross' view (summarized by Morse, 1997b). Weaver (1983, 1984, 1992a,b; Weaver and Morse, 1986) was the first to apply strict cladistic principles to caddisfly higher-level classification and examined about twice as many morphological characters as Ross (Fig. 1b). Wiggins and Wichard (1989; also Wichard, 1991; Wiggins, 1992; Wichard et al., 1993; Wichard and Klein, 1997) suggested that the closed, semipermeable cocoons of parchmentlike silk found in the spicipalpian families (limiting them to cold, well-oxygenated streams) represent the groundplan condition of the order and that the cocoons of permeable silk with ventilation openings found in the Annulipalpia and Integripalpia are uniquely derived (Fig. 1c). Ivanov (1997) challenged Weaver's

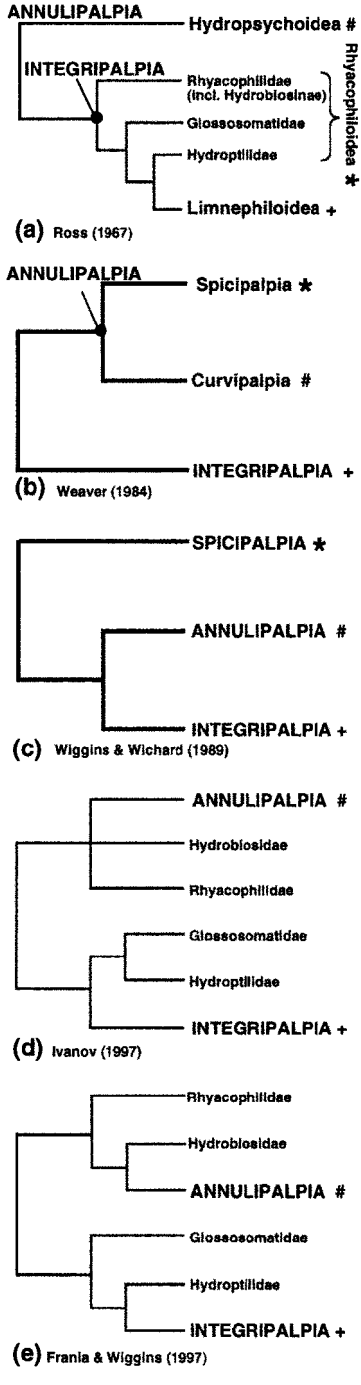

FIGURE 1. Five contemporary hypotheses of subordinal relationships of the Trichoptera. Equivalent taxonomic units are indicated by like symbols (e.g., Ross' Hydropsychoidea = Weaver's Curvipalpia = Wiggins and Wichard's Annulipalpia). (c) Representation of a phylogeny of pupation only (Wiggins, 1992). (e) Strict consensus of five trees (Frania and Wiggins, 1997: Figs. 24 and 25). Spicipalpia includes the families Rhyacophilidae, Hydrobiosidae, Glossosomatidae, and Hydroptilidae.

hypothesis of Spicipalpia monophyly, providing evidence that each of Weaver's four spicipalpian apomorphies were plesiomorphic or more generally distributed within Trichoptera (Fig. 1d). Most recently, Frania and Wiggins (1997) provided the first published analysis of Trichoptera relationships based on a computer- assisted search for most-parsimonious trees (using HENNIG86). Their analysis of 70 adult and immature characters resulted in five equally parsimonious trees. Their strict consensus supported monophyly of Annulipalpia and Integripalpia, but not of Spicipalpia (Fig. 1e).

In summary, at least five different hypotheses of basal Trichoptera relationships have been proposed or suggested (Fig. 1), differing in the placement and monophyly of Spicipalpia and its included families. Phylogenies challenging traditional classifications have also been proposed for family relationships within suborders (Fig. 2; see Morse, 1997b, for review).

Although the analysis of morphological characters has been extensive, consensus over relationships among suborders is at an impasse. However, molecular sequence data have not been examined, and only one of the published phylogenetic studies utilized automated searches for most-parsimonious trees (Frania and Wiggins, 1997). Additional data, rigorously analyzed, should provide a fresh perspective to help stabilize caddisfly classification.

MATERIALS AND METHODS

Laboratory Protocols

The use of freshly frozen specimens was preferred, but most taxa were available only as dried, pinned museum specimens. In both cases, for DNA extraction, a single leg was taken from larger specimens, or the head and thorax, including legs, from smaller specimens. The remainder of the specimen, including the wings and abdomen, the latter with its terminal genitalia, was vouchered. Bar-coded and standardized voucher specimen labels were applied to each specimen used in the study, and this information was entered into a Biota (Colwell, 1996) database maintained at the University of Minnesota Insect Collection, St. Paul (UMSP). Voucher specimens are deposited in UMSP and other institutions (see Appendix 2 at the *Systematic Biology* website,

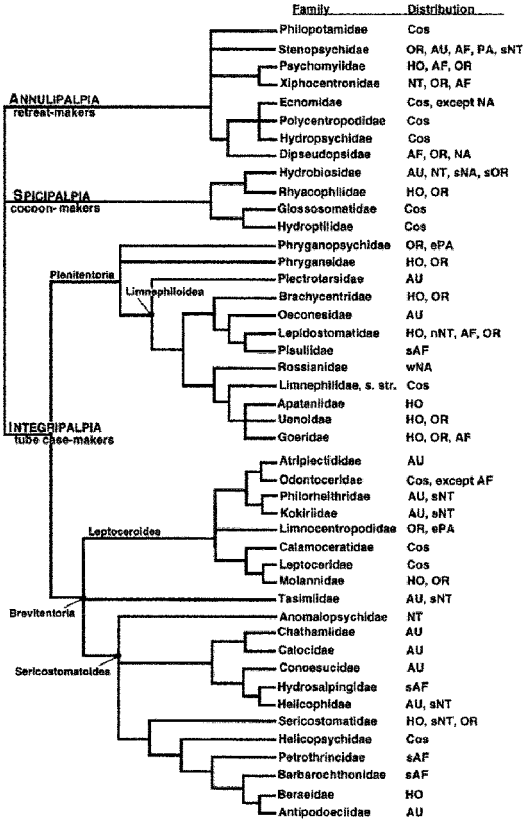

FIGURE 2. Composite phylogeny of family-group taxa of Trichoptera and general global distribution. Relationships based on Weaver (1983, 1984) and Weaver and Malicky (1994) for families of Annulipalpia; Weaver (1983, 1984) for families of Spicipalpia; Gall (1994, 1997) for families of Plenitentoria; Weaver (1983) and Weaver and Morse (1986) for families of Leptoceroidea; and de Moor (1993) for families of Sericostomatoidea. AU = Australasian, Cos = cosmopolitan, AF = Afrotropical, HO = Holarctic, NA = Nearctic, NT = Neotropical, OR = Oriental, PA = Palearctic, e = eastern, n = northern, s = southern, w = western.

www.utexas.edu/ftp/depts/systbiol). Specimens were identified to species, except for a few female or larval specimens, for which species determination was not possible. Samples were placed in labeled Eppendorf tubes and ground over liquid nitrogen, using microtissue grinders (Phoenix Research). DNA was extracted with sodium dodecyl sulfate, Proteinase-K, and phenol/chloroform, as described by Hillis and Davis (1986). Dried DNA pellets were resuspended in 50–250 μl of Tris–EDTA buffer. Most of this material was separated as a stock DNA collection and kept at −70°C. The rest was kept in a frostfree freezer for amplification by polymerase chain reaction (PCR). Samples were amplified on a thermal cycler using the reaction conditions described in Sambrook et al. (1989). Initially, primers were designed from GenBank, and additional primers were then designed from our own sequences. Primer sequences for the COI gene fragment were TAATTGGAGGATTTG GAAATG, paired with CCYGGTAAAATT AAAATATAAACTTC. The EF-1α fragment was amplified in three overlapping pieces, starting with ATCGAGAAGTTCGAGAAR GARGC paired with CCAYCCCTTRAAC CANGGCAT; TTGCACGGRGAYAACAT GTTRGA paired with either TTGAAAC CAACGTTRTCRCC or GAAATYTGRCC AGGRTGRTT; and ACYACTGAAGTNA ARTCNGT paired with GGGAAYTCCTG GAARGAYTC. The first variable region of the large subunit nuclear rRNA, D1, was amplified with GGAGGAAAAGAAAC TAACAAGGATT paired with CAACTTTC CCTTACGGTACT. The third variable region, D3, was amplified with ACCCGTCTTGAAA CACGGAC paired with either ATTCCCCT GACTTCGACCTGA or CTATCCTGAG GGAAACTTCGGA. The fourth through fifth variable region of the small subunit nuclear rRNA, V4–5, was amplified with either CAACTTTCCCTTACGGTACT or TGCG-GTTAAAAAGCTCGTAGT, paired with GC-CCTTCCGTCAATTCCTTTA. All primers are listed as 5′–3′. Some internal primers (not listed) were designed for individual taxa from the sequences we obtained by using the primers listed above. PCR conditions were 94°C, 30 s; 50°C, 30 s; and 72°C, 45 s for 35–45 cycles. Amplified DNA was separated on a 1.5% low-melting-point agarose gel (NuSieve 3:1; FMC Bioproducts). Bands of DNA were cut from the agarose gel, purified with GeneClean (Bio 101), and sequenced on an ABI 377 automated sequencer according to the manufacturer's recommendations (Applied Biosystems), except that we used one-half the recommended enzyme concentration in a reduced volume reaction. Sequences were completed in both strands and edited manually with the help of Sequence Navigator (Applied Biosystems). During editing of each strand, nucleotides that were readable but either showed irregular spacing between peaks or had some important competing background peak were coded with lowercase letters. These letters were converted to uppercase if the complementary

strand strongly confirmed them, were left as lowercase when both strands were lowercase, or were expressed as N's (nucleotide undetermined) when strands were contradictory.

Taxon Sampling

Initially, we sought to obtain two representative taxa from each family, each as distantly related as possible. In the absence of published phylogenetic hypotheses, we used subfamily designation or geographical distribution to select putative maximally distant representative taxa. Later, when it became apparent that sequences from Integripalpia were relatively similar to one another, whereas those from Annulipalpia and Spicipalpia were considerably more divergent, we increased representation from the latter groups and decreased representation from Integripalpia (see Appendix 2, *Systematic Biology* website).

Our dataset includes outgroup representatives from Mecoptera and Siphonaptera, as well as representatives of every lepidopteran suborder: Zeugloptera (Micropterigidae), Aglossata (Agathiphagidae), Heterobathmioidea (Heterobathmiidae), and Glossata (the vast majority of Lepidoptera). Ingroup taxa include all trichopteran families except Limnocentropodidae (Leptoceroidea); Petrothrincidae, Hydrosalpingidae, Barbarochthonidae, and Antipodoeciidae (Sericostomatoidea); and Pisuliidae and Rossianidae (Limnephiloidea). Missing taxa were either unavailable or we were unable to obtain PCR product from degraded specimens.

Genes Examined

We sequenced the first (D1: 334 nt) and third (D3: 233 nt) variable regions of the large subunit nuclear rRNA, the fourth through the fifth (V4–5: 511 nt) variable regions of the small subunit nuclear rRNA, and a fragment of the mitochondrial COI gene (441 nt) for most taxa. COI was sequenced to provide additional resolution within suborders. Initially, nearly the entire gene for EF-1α (1098 nt) was sequenced to obtain a second conservative marker (using either its nucleotides or its amino acids), independent of the rRNA genes. However, it became apparent from preliminary analysis that the EF-1α gene was less than ideal for the study of trichopteran suborders; third positions were excessively homoplastic, while few amino acids varied at all. Therefore, further sequencing of the EF-1α was limited to Annulipalpia because the EF-1α sequencing of this suborder was already nearly complete by the time of our preliminary analysis. Sequences were submitted to GenBank under accession numbers AF436131-AF436645 (see Appendix 2, *Systematic Biology* website).

Alignment

The COI gene was length invariant except for a single missing codon in Dipseudopsidae. The EF-1α gene lacked introns, did not vary in length, and alignment was trivial. We used MacClade 4.0 (Maddison and Maddison, 2000) to color-code sequences by translated amino acids to check for stop codons and proofread the edited sequences. When lowercase letters (see above section, Laboratory protocols) dictated amino acid changes, we returned to the original gels to confirm the nucleotide.

The rRNA was aligned manually with reference to secondary structure, and its notation follows that of Kjer et al. (1994) and Kjer (1995). Alignments followed the secondary structure models of Gutell et al. (1994), downloaded from the website http://www.rna.icmb.utexas.edu, and were modified where compensatory substitutions confirmed a customized secondary structural model for amphiesmenopteran rRNA. Regions that could not be aligned were excluded from the analysis. The criterion for data inclusion used secondary structure to identify the boundaries of length-heterogeneous regions. Nucleotides in variable-length regions between flanking hydrogen-bonded nucleotides were either excluded (Kjer, 1997) or recoded (see below). Three regions that could be unambiguously aligned among all Trichoptera were not alignable across outgroups. In these regions, the outgroup sequences were replaced with "?" (coded as missing data).

Character Coding

Nucleotides were treated as unordered characters with four alternative states. The coding of insertions and deletions is shown in Appendix 1. Typically, "indels," when considered at all, are treated by most investigators as a single kind of character; we,

however, do not consider all "indels" to be the same. Each length-heterogeneous region that contained phylogenetically informative insertions and/or deletions was evaluated separately and divided into one of three classes: insertions, deletions, or "indels". Insertions were defined as characters in which successive outgroups were all of identical length and lacked a nucleotide present in some or all of the ingroup (Trichoptera). The character state of the missing nucleotides was then defined as "ancestrally missing," coded with an asterisk, and defined in the "symbols" option in PAUP*. Using this same outgroup criterion, we defined deletions in the ingroup as missing data, because although "ancestrally missing" is a defined state, we could not define the state of a nucleotide that was lost. When we could not categorize a region as either an insertion or a deletion by outgroup comparison, we defined the region as an indel. Indel regions of variable length, even when these regions were not alignable across all taxa, often contained phylogenetic signal in some (if not all) lineages. Therefore, we eliminated the nucleotide characters from the analysis but coded each unique combination of nucleotides in these variable regions with a different symbol. PAUP* limits the number of character states, and for some indels we identified as many as the maximum of 32 states. Many of these states were synapomorphic for only a few taxa; even so, we preferred to retain some information rather than eliminate these regions altogether. In some length-heterogeneous regions, the number of nucleotides in the region was coded. The coding of each individual indel shown in Appendix 1 is fully explained on the *Systematic Biology* website. This method of coding indels was similar to Wheeler (1999) and Lutzoni et al. (2000), except that we did not use a step matrix.

Data Analysis

As a starting point, phylogenetic analysis was performed by way of character-based parsimony using PAUP* 4.0b3a (Swofford, 1999). Heuristic searches were implemented, with either 50 or 500 replicates of random-taxa additions (depending on tree island profiles). Support at each node in the cladogram was analyzed according to the decay index (Bremer, 1988; Donoghue et al., 1992) and nonparametric bootstrapping (Felsenstein, 1985). For bootstrapping, 500 pseudoreplicates were performed, each including 10 random addition searches. Homogeneity of base frequencies across taxa was evaluated with a chi-square test using PAUP*. When significant deviation from homogeneity of nucleotide composition was found, we performed Log-Det minimum evolution analyses (Lockhart et al., 1994). To further examine the possibility of nucleotide compositional effects, we constructed a "GC tree" using a matrix of Euclidean distances between nucleotide frequencies for each pair of taxa (Lockhart et al., 1994:formula 4), and compared it with the parsimony tree with consensus procedures.

We also used maximum likelihood as an optimality criterion. Model and parameters were selected after running MODELTEST 3.04–3.06 (Posada and Crandall, 1998) and using the Aikaike information criterion (AIC; Akaike, 1974). For all analyses, MODELTEST dictated use of a general time reversible (GTR) model with a gamma correction (Yang, 1993, 1994a,b, 1996) for among-site rate variation and invariant sites (Gu et al. 1995); nucleotide frequencies were estimated from the data.

Bayesian inferences were used to estimate phylogeny and branch support under likelihood, using the program MrBAYES 2.0 (Huelsenbeck, 2000). For each analysis, two Markov chains were run, with 480,000 cycles for each chain. Trees were saved to a file every 400 cycles, and the first 200 of these trees were discarded. This left us with two tree files, each containing 1,000 trees, which we then pooled. A majority-rule consensus of these 2,000 trees was then used to generate approximations of the posterior probability of each clade. We used a starting tree generated from a neighbor-joining analysis, having selected the model and parameters by MODELTEST.

Hypothesis Testing

We used the constraint option in PAUP* to examine the differences among alternative trees generated from other data partitions and optimality criteria. Following the recommendations of Swofford et al. (1996) and Goldman et al. (2000) in restricting the use of the Templeton test, only a priori alternative trees were compared with one another under parsimony with a

two-tailed Wilcoxon-ranked sum test (Templeton, 1983; Larson, 1994). The trees generated from the dataset under evaluation were excluded from the test because these trees cannot be assumed to fit the null hypothesis that they are no better than the other trees under comparison (Swofford et al., 1996; Goldman et al., 2000). So that conflict would be measured only among subordinal hypotheses and not among family relationships within suborders, taxa considered in the constraints were confined to orders, the suborders Annulipalpia and Integripalpia, and the families Hydroptilidae, Glossosomatidae, Rhyacophilidae, and Hydrobiosidae. Essentially, we treated the Trichoptera as a six-taxon group, resolving these six taxa as published by other authors, or as our analyses resolved them, but leaving the relationships within Annulipalpia and Integripalpia unconstrained. For example, even though Weaver and Morse (1986) presented relationships among annulipalpian and integripalpian families, for our evaluation of conflict, we were interested only in the relationships among the suborders. Accordingly, we defined our constraints as follows: "Constraints Weaver_and_Morse = ((Siphonaptera) (Mecoptera) ((Lepidoptera) (Integripalpia) ((((Hydrobiosidae) (Rhyacophilidae)) ((Glossosomatidae) (Hydroptilidae))) (Annulipalpia)))))." Our constraints can be found at the bottom of our NEXUS files, available on the *Systematic Biology* website. In addition to constraints used for hypothesis testing, some of our phylogenetic analyses included constraints. Constraints in the EF-1α data were implemented because one-third of the data was missing for *Micropteryx*, *Pseudostenophylax*, *Monocosmoecus*, *Lype*, *Gumaga*, and *Marilia*. Constraints in the amino acids–only analyses were necessary because unconstrained analyses required searching through hundreds of thousands of equally parsimonious trees, preventing the completion of searches. Constraints are indicated on our figures.

Data Combination and Signal Evaluation

We considered four independent datasets: the nuclear rRNAs, the nuclear EF-1α, the mitochondrial COI, and morphology, the latter as presented by Frania and Wiggins (1997). Each dataset was first analyzed separately and then combined with the others. We evaluated the potential for excessive homoplasy in three ways. First, we examined the left skew of tree distributions from exhaustive searches of five, six, seven, and eight taxa. Taxa were drawn according to a structured random selection. One outgroup, *Merope tuber* (Mecoptera), was fixed in each set. A second outgroup for each set, either *Catocola* or *Agathiphaga* (Lepidoptera), was selected by coin toss. Finally, one species each from Annulipalpia, Spicipalpia, and Integripalpia was selected by lot from a pool of taxa for which sequence data from all three molecular datasets were complete. Identical sets of taxa were used for both rRNA and COI datasets, but for EF-1α, for which fewer Integripalpia with complete data were available, we substituted taxa when necessary. Ten such sets of taxa were constructed. Separate exhaustive searches were completed on each set of taxa for the rRNA, EF-1α, and COI data, and the g1 statistic was recorded and compared to the significance levels presented by Hillis and Huelsenbeck (1992). For a sixth taxon, an additional annulipalpian was added and the analyses were repeated. If the first annulipalpian selected was in either the Philopotamidae or Stenopsychidae families, then the second was selected from another family (to ensure that signal was not being confined to closely related taxa). This division corresponds to currently recognized superfamily designations in the suborder (families with and without ocelli). The seventh taxon was an additional spicipalpian from a family different than the first. Finally, the eighth taxon was a second integripalpian. If the first integripalpian was from Plenitentoria, then a brevitentorian taxon was selected as the second, and vice versa. We were fortunate with Trichoptera, because both the ingroup and the outgroups can be separated into well-defined groups, and we could be relatively certain that our sampling plan for these groups provided maximally separated sets of taxa.

Signal was also evaluated by assessing whether or not trees from the g1 statistic searches (above) recovered highly corroborated clades. Three clades, highly corroborated as monophyletic by morphological and molecular evidence, were identified: Trichoptera, Annulipalpia, and Integripalpia. Failure of the structured random taxon subsets to recover these clades renders

the data suspect, especially if excessive homoplasy in the data is indicated by other means.

Finally, we attempted to visualize homoplasy in the data with the construction of a tree that spanned Trichoptera diversity and its outgroups and represented a reasonable estimate of phylogeny (Fig. 3). Uncorrected mean percent nucleotide differences were calculated and placed on the nodes of this tree. Although a clocklike rate was not assumed, we did assume that ancestors precede their descendants, and if true, then mean pairwise differences should increase with time unless the positions free to change have already changed. The following tree was considered highly corroborated: (Mecoptera, Diptera (Lepidoptera ((Annulipalpia) (Integripalpia)))). No Spicipalpia were included because there is not consensus in the literature on their monophyly or on their placement relative to Annulipalpia or Integripalpia, and because their inclusion was not necessary for evaluating whether or not divergence was increasing with time. We then plotted the observed mean uncorrected distances against the corrected distances estimated by using models and parameters selected from the MODELTEST analysis, marking points on the nodes from the "highly corroborated tree" (above). Because character-based analyses may not be subject to "saturation" as we are measuring it here, we evaluated patristic distances (total number of changes separating taxa) on a combined data tree (including all taxa) for each individual dataset and traced the mean of these values through our "highly corroborated tree."

These analyses were designed to evaluate datasets under parsimony. Even if we question the utility of a dataset for a distance analysis or an equally weighted parsimony analysis, some conservative signal could be hidden within the noise of the quickly evolving characters. For this reason, we evaluated "noisy" datasets under maximum likelihood, LogDet-corrected distances (Lockhart et al., 1994), and differentially weighted parsimony, because having the ability to accommodate among-site rate-variation, branch-length heterogeneity, unequal nucleotide frequencies, and differences among substitution frequencies, we might recover a reasonable estimate of phylogeny from even an apparently saturated dataset.

Morphology

The morphological data used in our analysis was as presented by Frania and Wiggins (1997), with some exceptions. First, Frania and Wiggins conducted only two heuristic searches on the combined larval and adult character sets, and they suspected the existence of additional tree islands because they found additional trees in their second search. We conducted 50 random addition searches of the morphological data with no limit to the number of trees evaluated. Second, Frania and Wiggins assigned plesiomorphic states to outgroup taxa, even when no homolog existed in the outgroup. We coded these characters in our dataset as "?", rather than "0". Finally, for the analysis of the morphological data alone, we used Frania and Wiggins' character polarity assignments because we felt that the differences in our approach warranted a new analysis that was as close as possible to the original dataset. In the combined data analysis, however, the morphological characters were considered unordered because additional molecular characters should provide alternative potential polarities. In our constrained analysis of alternative hypotheses, the "Frania and Wiggins" hypothesis corresponds to our analysis of their characters (Fig. 7A, not Fig. 1D). Composite taxa in the combined analysis were avoided whenever possible, but some taxa included in Frania and Wiggins (1997) were not available to us, and taxa that we included as composites (one taxon coded for morphology, combined with DNA sequences from another) are described in Appendix 2 (see the *Systematic Biology* website, where they are marked with an asterisk in the far right column). We also performed an unpolarized analysis, using differentially weighted parsimony (described below) on the morphological data.

Combined Analysis

In determining potential combinations used in a combined analysis, one criterion we used to avoid dataset combination was reciprocal rejection according to the Wilcoxon signed rank test (dataset A rejects the hypothesis from dataset B and vice versa). Another question we addressed was, "Does adding noisy data bring us a more accurate hypothesis?" Of course, this is complicated with the problem of how "accurate" is defined.

While we cannot predetermine an "accurate" hypothesis, we can evaluate signal in each of the partitions. An ideal combination would be a slowly evolving dataset to provide signal at the base of the tree while more rapidly evolving genes resolve the tips of the tree. If dataset A were found to be more appropriate for the resolution of relationships among suborders, and dataset B were less appropriate, then dataset B would be added to a combined analysis only if it did not overturn results from dataset A. Similarly, if an uncorrected (equally weighted parsimony) combined analysis revealed deep phylogenetic patterns shared with the partitioned "noisy" datasets, and a reasonable correction of the combined analysis recovered a deep history shared with the more conservative partitioned dataset, then the "corrected" hypothesis would be favored. Similar to this approach, others have constrained nodes that have been estimated through characters or combined analyses that have been deemed reliable (i.e., Moritz et al., 1992; Ballard et al., 1998).

The combined data included a subset of taxa, starting with those used by Frania and Wiggins (1997), and adding sequence data for which the majority of sequences were complete. Characters in this combined analysis included D1, D3, V4–5 rRNA, EF-1α nucleotides and amino acids, COI nucleotides and amino acids, and morphology. Justification for including both nucleotides and amino acids in a combined analysis was discussed by Agosti et al. (1996), Benabib et al. (1997), and Flores-Villela et al. (2000). Unordered data were both equally and differentially weighted in combined analyses.

In order to perform a combined analysis that would accommodate the extreme differences in substitution rates among the datasets, as well as among-site rate variation within the datasets, we utilized "pseudoreplicate reweighting" described by Kjer et al. (in press). Briefly, this is a weighting scheme that generates 1,000 trees from a fast heuristic bootstrap analysis of the combined data. Each character was then reweighted according to the highest rescaled consistency index from any of the 1,000 trees. Because each of these trees comes from a different pseudoreplicate of original data, and since a strict consensus is an unresolved polytomy (only Trichoptera was recovered), we predict that this weighting scheme will not be as subject to the circularity imposed by the original tree (Cunningham, 1997) as is successive weighting (Farris, 1969). However, missing data will inflate the weights (Archie, 1989), and smaller datasets will be underrepresented in combined analyses. Generation of the 1,000 trees for the pseudoreplicate reweighting of the morphological data included only the unpolarized morphological data rather than the combined data.

We also performed a likelihood analysis on the same set of taxa, using Bayesian inferences, on the combined nucleotide data. Bull et al. (1993) and Sullivan (1996) present contrasting conclusions about the combinability of datasets when different partitions evolve under different models. We agree with both studies, the differences between them depending on whether the parameters of separate datasets are sufficiently overlapping or not (Sullivan, 1996). We measured datasets with Bayesian estimates of parameters, with 95% confidence intervals, to see if they were closer to nonoverlapping (Bull et al., 1993) than complementary (Sullivan, 1996). Given excessive differences in parameter estimates among genes, we devised a method for assigning individual characters to site-specific rate classes from the pseudoreplicate reweighting scheme described above and have attempted to accommodate the criticism of site-specific rate models summarized by Buckley et al. (2001). We took the 1,000 bootstrap trees from the combined data and reweighted them according to the "best consistency index," with a base weight of 5. Each character was then assigned an integer from 1 to 5 as a result, and the characters were then sorted into character partitions according to their number. Bayesian analysis was performed with a GTR model, with the five site-specific rate-classes. We hope the inaccurate assumption that all members of an a priori assigned class (such as third-codon positions or COI nucleotides) evolve at an identical rate is more accurately reflected in this method, where individual rate classes were not assigned a priori.

RESULTS

Ranking Conserved Signal

All three of our tests for signal at the level of subordinal relationships indicated

that COI nucleotides and EF-1α nucleotides were less appropriate for the estimation of relationships among suborders than was the rRNA dataset at this level. As indicated in Table 1, the distribution of tree lengths for the rRNA data has a significant left skew in all analyses, whereas the COI nucleotides have a significant left skew in only 30% of the five-taxon (suborder) analyses. Similarly, EF-1α nucleotides failed to show significant left skew in all 10 of the five-taxon datasets. By examining the recovery of expected clades, the rRNA data recovered a monophyletic Trichoptera in 100% of analyses, a monophyletic Annulipalpia in 100% of analyses, and a monophyletic Integripalpia in 85% of analyses (Table 1). The COI nucleotides recovered the monophyly of Trichoptera, Annulipalpia, and Integripalpia in 40%, 35%, and 20% of the analyses, respectively (Table 1). EF-1α nucleotides recovered monophyletic Trichoptera, Annulipalpia, and Integripalpia in 70%, 90%, and 20% of the analyses, respectively (Table 1).

Figures 3 and 4 confirm the left skew analysis. These figures convey similar information and are meant to be compared with one another. For example, we suggest selecting one of the terminal nodes on Figure 3 and tracing the values of a particular gene as you go "back in time." Then find the same starting point on Figure 4 and trace from nodes 1 to 6. Points should move up and to the right. At node 4 (Figs. 3 and 4A), where pathways converge at the level of subordinal relationships, the rRNA data increased in mean uncorrected differences, whereas neither the COI nucleotides nor the EF-1α nucleotides increased in divergence from node 4 to node 5. Patristic distances accumulated in all datasets up through Trichoptera (Fig. 3, node 4), then decreased in both EF-1α and COI between Trichoptera and Lepidoptera (node 5), and then increased again between Amphiesmenoptera and the most distant outgroups (node 6). None of the datasets met the criterion of reciprocal conflict according to the Templeton test (Table 2; Templeton, 1983).

Summarizing the analyses of estimated homoplasy and signal strength, we found the rRNA data to be most appropriate for estimating relationships among suborders, but the signal from the rRNA within suborders is weak. We have little confidence in COI or EF-1α for the recovery of the deepest nodes, but increasing patristic distances (increasing at least within Trichoptera), combined with heavy taxon sampling and appropriate correction, may still yield useful phylogenetic information from these noisy datasets. However, the trees we present from individual partitions are meant to be viewed as explorations of the data rather than competing hypotheses.

Phylogenetic Analyses

Results from our equally weighted parsimony analysis of the COI and EF-1α nucleotide datasets corroborated our analysis of signal because even with all taxa included, they do not show the monophyly of groups such as Lepidoptera or Trichoptera. Hypotheses generated from equally weighted parsimony analyses of both COI and EF-1α nucleotide datasets were rejected under appropriately applied Templeton tests (Table 2). To save space in publication, equally weighted parsimony trees from these datasets, along with our other trees and executable NEXUS files, are included in the TREEBASE (herbaria.harvard.edu/treebase) websites but are not presented here. We concentrated parsimony analyses on the rRNA and combined data, while attempting to estimate phylogeny from the noisy datasets through maximum likelihood.

The result of the equally weighted parsimony analysis of the rRNA data is shown in Figure 5. This analysis recovered a monophyletic Annulipalpia and Spicipalpia, the latter allied with the Integripalpia. Plenitentoria and Brevitentoria (Leptoceroidea + Sericostomatoidea) were also monophyletic (Weaver and Morse, 1986). However, Plenitentoria was not identical to the taxon defined by Weaver and Morse (1986) because Kokiriidae was included within it in the rRNA analysis; Kokiriidae (as Plectrotarsidae) was included within Leptoceroidea in the phylogeny presented by Weaver and Morse (1986).

The tree presented in Figure 5 represents the first of our estimates of subordinal relationships within Trichoptera. Bootstrap values are given on Figure 5, but some nodes differed; the bootstrap analysis of the rRNA data showed a paraphyletic Spicipalpia, with Hydrobiosidae as the sister taxon to a monophyletic Integripalpia, and arctopsychines as the sister taxon to the rest of the hydropsychids. Several well-established families do

TABLE 1. Results of measurements of left-skew in tree distributions (Hillis and Huelsenbeck, 1992). The top row labels 10 separate analyses, each made up of sets of 5, 6, 7, and 8 taxa, which were successively added. Lists of these taxa, included in each set are indicated below the column number. In the column at the far left, below the list of taxa are the numerals 5, 6, 7, and 8 in the upper left corner of each box; these indicate the number of taxa in the analyses following to the right (see text for description of how these taxa were selected). "# Char." refers to the number of informative characters in the dataset, given the taxa for each analysis (listed above). "g1" refers to the statistic from Hillis and Huelsenbeck (1992) that describes the shape of the histogram of all tree lengths. A single asterisk following the g1 statistic indicates significance at $p < 0.05$. Double asterisks indicate significance at $p < 0.01$. Analyses determined to contain significant left skew in tree distributions are in bold. The question "Monophyletic?" refers to whether or not each analysis recovered a monophyletic test group: Y = yes, N = no, S = in some of the multiple shortest trees. The order of these questions is always the same. The test groups are: first letter = monophyletic Trichoptera, second letter = monophyletic Annulipalpia, third letter monophyletic Integripalpia. For the purposes of evaluating signal, we considered only the monophyly of Trichoptera, Annulipalpia, and Integripalpia to be expected. These tests indicated that the COI and EF-1α nucleotide data do not provide reliable information with respect the relationships among suborders.

Analysis	1	2	3	4	5	6	7
Outgroup	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*
Lepidoptera	*Agathaphaga*	*Agathaphaga*	*Agathaphaga*	*Catocola*	*Catocola*	*Agathaphaga*	*Catocola*
Spicipalia #1	*Oxethira*	*Agapetus*	*Atopsyche*	*Atopsyche*	*Oxethira*	*Rhyacophila*	*Oxethira*
Annulipalpia #1	*Arctopsyche*	*Phylocentropus*	*Stenopsyche*	*Xiphocentron*	*Xiphocentron*	*Ecnomus*	*Diplectrona*
Integripalpia #1	*Phryganopsyche*	*Caenota*	*Limnephilus*	*Phryganea*	*Psuedostenophylax*	*Trichovespula*	*Limnephilus*
Annulipalpia #2	*Phylocentropus*	*Arctopsyche*	*Xiphocentron*	*Hydropsyche*	*Dolophilodes*	*Arctopsyche*	*Psychomyia*
Spicipalpia #2	*Protoptila*	*Atopsyche*	*Oxethira*	*Rhyacophila*	*Palaeagapetus*	*Palaeagapetus*	*Glossosoma*
Integripalpia #2	*Psilotreta*	*Phryganopsyche*	*Helicopsyche*	*Caenota*	*Contulma*	*Phryganea*	*Caenota*
COI							
5 # char.	161	**227**	169	162	182	**159**	178
G1	−.232	**−.986****	−.399	+.897	−.279	**−.806***	−.166
Monophyletic?	Y	**Y**	S	N	N	**Y**	N
6 # Char.	230	232	181	171	197	166	**187**
G1	−.424	−.556	−.483	−.022	−.170	−.255	**−.735****
Monophyletic?	YY	YY	YN	NN	NN	SN	**NN**
7 # Char.	**243**	**244**	198	185	199	177	**194**
G1	**−.542****	**−.523****	−.383	−.196	+.187	−.401	**−.424***
Monophyletic?	**SS**	**YY**	SS	NN	NN	YY	**NN**
8 # Char.	**249**	**246**	209	195	206	190	206
G1	**−.483****	**−.400****	−.285	−.021	−.127	−.065	+.128
Monophyletic?	**YYN**	**YYN**	NNN	NNN	NNY	YYY	NNN
RNA							
5 # Char.	**314**	**293**	**296**	**300**	**310**	**308**	**307**
G1	**−1.401****	**−1.412****	**−1.430****	**−1.353****	**−1.412****	**−1.388****	**−1.361****
Monophyletic?	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
6 # Char.	**317**	**307**	**309**	**320**	**317**	**316**	**312**
G1	**−1.735****	**−1.785****	**−1.702****	**−1.555****	**−1.538****	**−1.732****	**−1.742****
Monophyletic?	**YY**	**YY**	**YY**	**YY**	**YY**	**YY**	**YY**

TABLE 1. (*Continued*) Results of measurements of left-skew in tree distributions (Hillis and Huelsenbeck, 1992). The top row labels 10 separate analyses, each made up of sets of 5, 6, 7, and 8 taxa, which were successively added. Lists of these taxa, included in each set are indicated below the column number. In the column at the far left, below the list of taxa are the numerals 5, 6, 7, and 8 in the upper left corner of each box; these indicate the number of taxa in the analyses following to the right (see text for description of how these taxa were selected). "# Char." refers to the number of informative characters in the dataset, given the taxa for each analysis (listed above). "g1" refers to the statistic from Hillis and Huelsenbeck (1992) that describes the shape of the histogram of all tree lengths. A single asterisk following the g1 statistic indicates significance at $p < 0.05$. Double asterisks indicate significance at $p < 0.01$. Analyses determined to contain significant left skew in tree distributions are in bold. The question "Monophyletic?" refers to whether or not each analysis recovered a monophyletic test group: Y = yes, N = no, S = in some of the multiple shortest trees. The order of these questions is always the same. The test groups are: first letter = monophyletic Trichoptera, second letter = monophyletic Annulipalpia, third letter monophyletic Integripalpia. For the purposes of evaluating signal, we considered only the monophyly of Trichoptera, Annulipalpia, and Integripalpia to be expected. These tests indicated that the COI and EF-1α nucleotide data do not provide reliable information with respect the relationships among suborders.

Analysis	1	2	3	4	5	6	7
7 # Char.	**332**	**311**	**325**	**323**	**324**	**322**	**323**
G1	**−1.988****	**−2.023****	**−1.889****	**−1.631****	**−1.732****	**−1.819****	**−1.814***
Monophyletic?	**YY**	**YY**	**YY**	**YY**	**YY**	**YY**	**YY**
8 # Char.	**341**	**320**	**341**	**330**	**334**	**324**	**330**
G1	**−1.969****	**−1.948**	**−1.935****	**−1.564****	**−1.878****	**−1.881****	**−1.790****
Monophyletic?	**YYY**	**YYY**	**YYN**	**YYY**	**YYY**	**YYY**	**YYY**
EF-1 alpha							
Outgroup	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*	*Merope*
Lepidoptera	*Catocola*	*Catocola*	*Catocola*	*Catocola*	*Catocola*	*Catocola*	*Catocola*
Spicipalia #1	*Anchitrichia*	*Agapetus*	*Atopsyche*	*Atopsyche*	*Brysopterix*	*Rhyacophila*	*Anchitrichi*
Annulipalpia #1	*Arctopsyche*	*Phylocentropus*	*Stenopsyche*	*Xiphocentron*	*Xiphocentron*	*Ecnomus*	*Diplectrona*
Integripalpia #1	*Oeconesus*	*Olinga*	*Limnephilus*	*Oeconesus*	*Limnephilus*	*Gumaga*	*Limnephilu*
Annulipalpia #2	*Phylocentropus*	*Arctopsyche*	*Xiphocentron*	*Hydropsyche*	*Dolophilodes*	*Arctopsyche*	*Psychomyia*
Spicipalia #2	*Anagapetus*	*Atopsyche*	*Anchitrichia*	*Rhyacophila*	*Rhyacophila*	*Brysopterix*	*Glossosoma*
Integripalpia #2	*Marillia*	*Oeconesus*	*Helicopsyche*	*Pycnocentrodes*	*Chathamiidae*	*Oeconesus*	*Olinga*
5 # Char.	312	318	298	322	309	398	284
G1	−.533	−.486	+.012	+.059	−.417	+.065	−.894
Monophyletic?	Y	N	Y	Y	Y	N	Y
6 # Char.	346	**339**	**328**	348	323	**352**	**298**
G1	−.505	**−.936***	**−.845***	−.003	−.334	**−.820***	**−.845***
Monophyletic?	YN	**NY**	**YY**	YY	YN	**YY**	**YY**
7 # Char.	**359**	**361**	**344**	381	367	**367**	**311**
G1	**−.591****	**−.744****	**−.765****	−.237	−.296	**−.754****	**−.532****
Monophyletic?	**YY**	**NY**	**YY**	YY	NN	**YY**	**YY**
8 # char.	**369**	**376**	**364**	400	**391**	**380**	**349**
G1	**−.378***	**−.677****	**−.642****	−.302	**−.572****	**−.840****	**−.542****
Monophyletic?	**YNN**	**YYY**	**YYN**	YYN	**YYN**	**YYN**	**NYN**

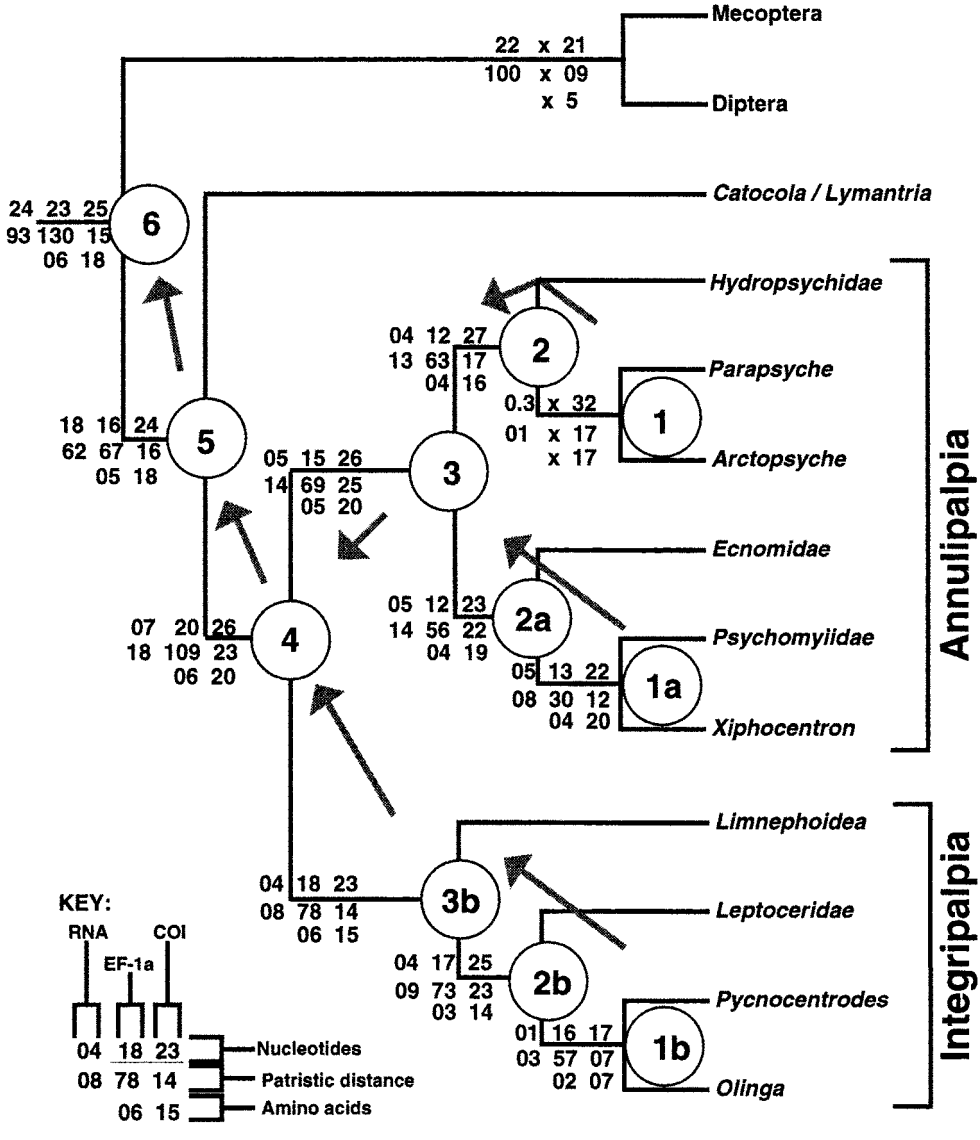

FIGURE 3. Subset of taxa whose relationships are highly corroborated by multiple datasets. Numerals at the internodes represent mean pairwise uncorrected percent differences and patristic distances. These values are spatially organized as shown in the key at lower left. The large numerals inside the circles are for reference to pathways in Figure 4, the arrows indicating the direction the eye should follow. The x's represent missing values for EF-1α, given the lack of data for Mecoptera and *Arctopsyche*.

not emerge as monophyletic in Figure 5, including the polyphyletic Philopotamidae and Glossosomatidae and the paraphyletic Hydrobiosidae. Similarly, *Psuedoneureclipsis* did not group with other Polycentropodidae, but its placement within Polycentropodidae has been questioned recently (Li et al., 2001).

The polyphyly of the Philopotamidae is surprising. The monophyly of the family, including *Wormaldia*, is supported by several morphological synapomorphies, especially the uniquely modified, T-shaped, membranous larval labrum (Wiggins, 1996). Frania and Wiggins (1997) recovered a monophyletic Philopotamidae but did not include *Wormaldia* in their taxon set. When the rRNA sequence data analysis are constrained to a monophyletic Philopotamidae, the tree

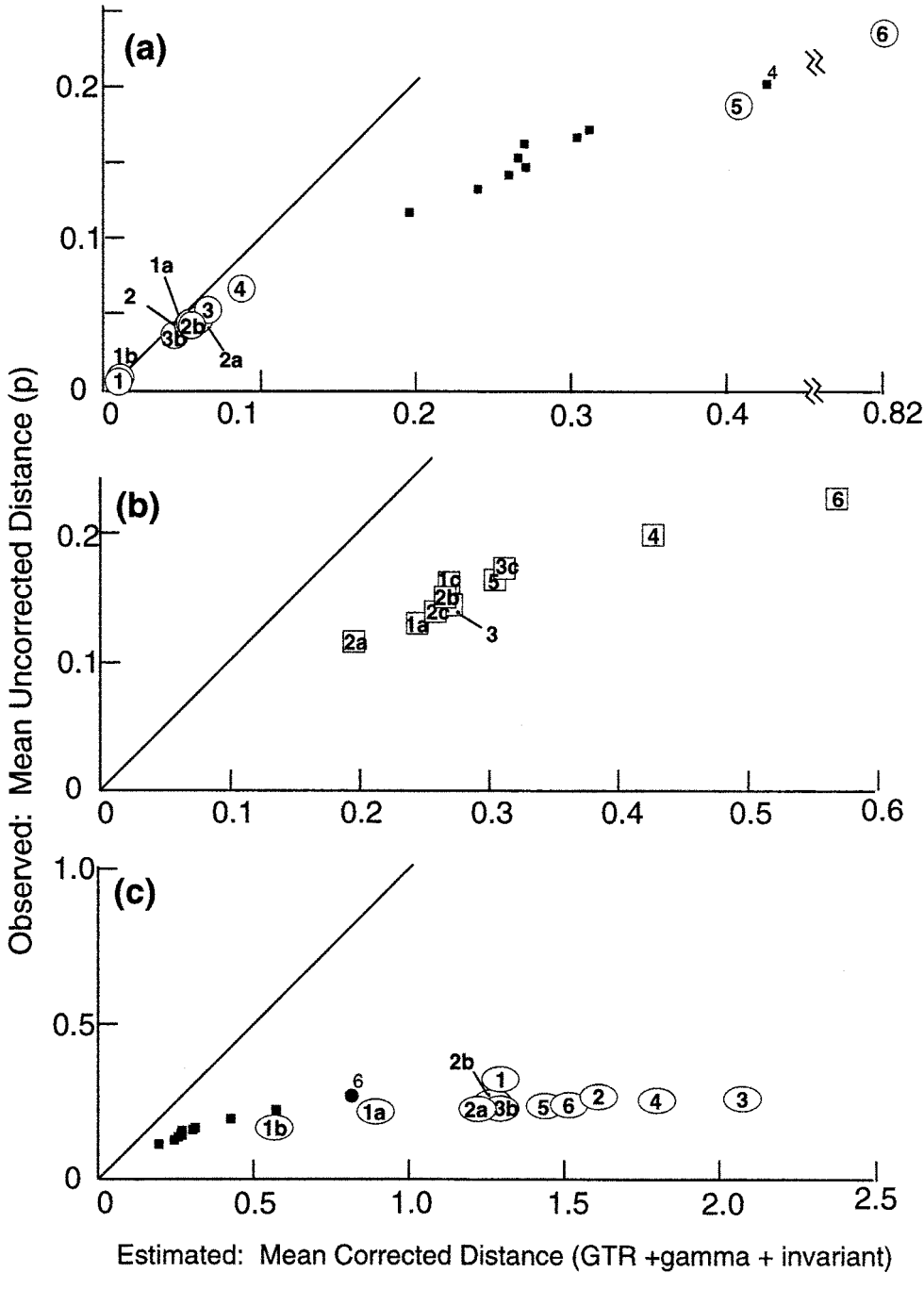

FIGURE 4. Graphical representation of the accumulation of substitutions at successively deeper nodes in the tree presented in Figure 3. Points are plotted as mean uncorrected distances versus mean corrected distances, the diagonal line representing a 1:1 relationship. (a) rRNA, in circles; (b) EF-1α, in squares; (c) COI, in ovals. Small filled squares in (a) and (c) represent the points for EF-1α, indicating the differences in scale between panels. The small filled square in (a) that is near the circled 5 represents the square marked 4 from (b) (the square marked 6 from [b] is not shown in [a]). Similarly, the small filled circle in (c) represents node 6 (the circled 6) from the rRNA for reference.

TABLE 2. Results of the Wilcoxon ranked sum test of alternative hypotheses with multiple datasets. Alternative hypotheses are listed across the top row, by dataset from this paper, or by author from previously published hypotheses (Fig. 1). The fit of alternative hypotheses is tested on the datasets listed in the far left column. Values in parentheses represent *a-posteriori* hypotheses generated from the dataset under evaluation, and were therefore not included in the tests. The top number listed in each box is the length of the most parsimonious tree(s), constrained to the various hypotheses. The bottom number is the highest p value given for the Wilcoxon ranked sum test for any of the *a-priori* constrained trees. Significance is indicated by * if $p < 0.05$ and ** if $p < 0.01$. Shortest solutions and analyses determined to contain significant conflict are in bold.

Hypothesis	RNA	RNA ML	EF-1a Parsimony	EF-1a ML	EF-1a LogDet	EF-1a AA	COI Parsimony	COI ML	COI AA	Ross	Weaver & Morse	Wiggins & Wichard	Frania & Wiggins	Ivanov
Dataset	**(2276)**	(2285)	**2463**	2293	2293	2289	**2317**	2293	2291	2287	2293	**2287**	2297	2296
RNA			**<.0001****	.300	.123	.542	**.001****	.104	.452	.481	.176	**1.000**	.067	.170
EF-1a	4226	4225	**(4184)**	(4217)	(4213)	4226	**4266**	4244	**4222**	4223	4225	4227	4232	4223
nucleotides	.724	.729					**.032***	.248	**1.000**	.812	.795	.717	.461	.989
EF-1a amino	**338**	340	(339)	(342)	(339)	**338**	349	343	342	340	339	339	342	341
acids	**1.000**	.686					.066	.282	.400	.644	.822	.782	.449	.479
COI	3365	3392	3384	3383	3380	3386	**(3354)**	(3377)	(3375)	3379	3386	**3359**	3382	3382
nucleotides	.812	.064	.249	.132	.153	.124				.164	.151	**1.000**	.179	.142
COI	603	610	608	606	606	608	(610)	(610)	**(600)**	607	608	**600**	605	606
amino acids	.816	.222	.252	.491	.610	.215				.564	.244	**1.000**	.628	.508
Morphology	353	356	360	**365**	358	**350**	**364**	354	358	(344)	(357)	(349)	**(342)**	(358)
ordered	.433	.264	.071	**.017***	.106	**1.000**	**.036***	.272	.245					
Morphology	301	301	308	309	306	**298**	313	309	307	(295)	(303)	(301)	**(295)**	(297)
unordered	.616	.616	.110	.118	.169	**1.000**	.053	.115	.239					

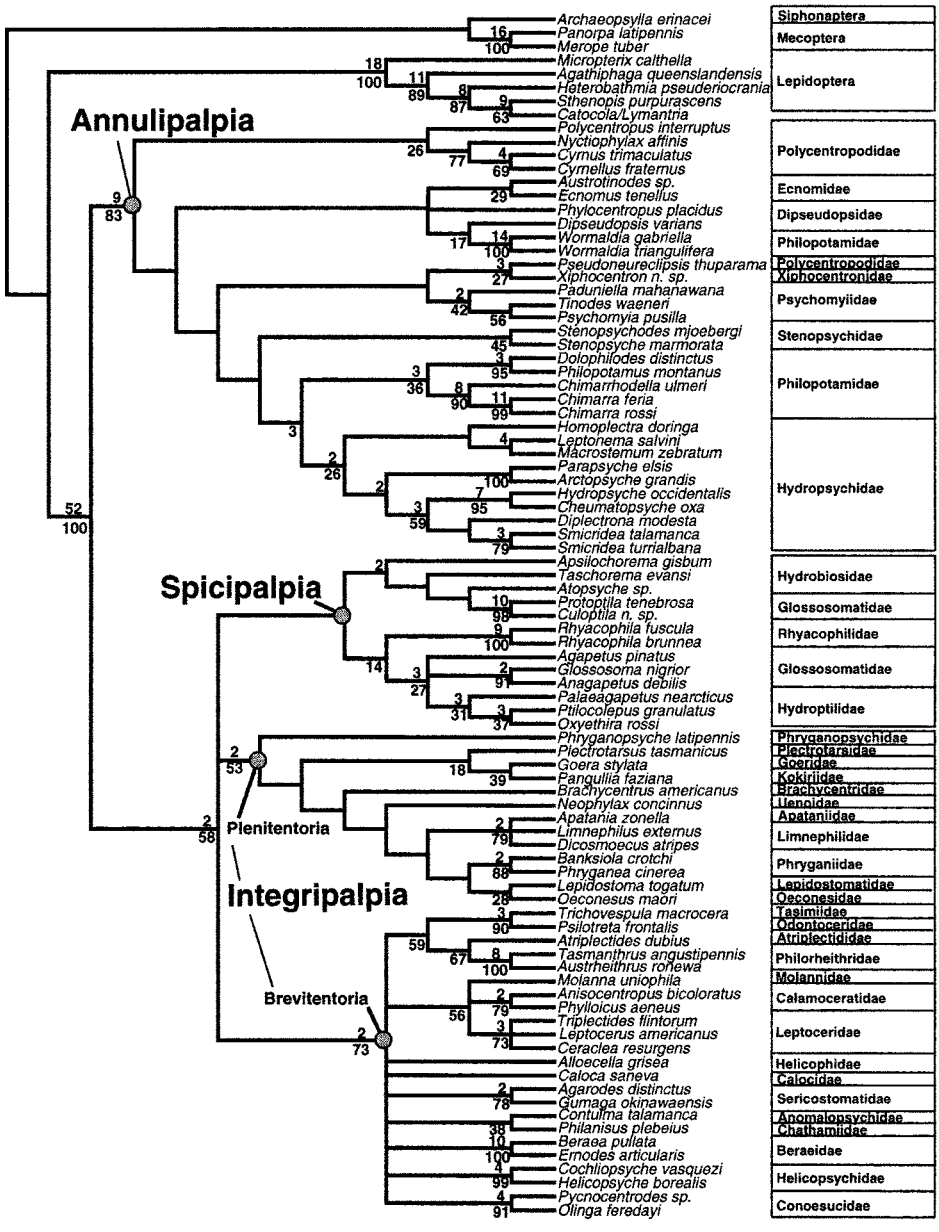

FIGURE 5. Results of an unconstrained, equally weighted parsimony analysis of rRNA data. This tree represents a strict consensus of 558 trees of 2,276 steps. Numerals above the internodes represent decay indices; those below the internodes represent bootstrap proportions. Internodes without numbers represent decay indices of 1 or bootstrap proportions not recovered in the bootstrap analysis.

length is increased by a single step. Finally, other molecular datasets (see below) recover a monophyletic Philopotamidae in unconstrained analyses. If *Wormaldia* is indeed misplaced in Figure 5, its misplacement has potentially severe consequences to the accuracy of inferred relationships within the rest of Annulipalpia. For example, if *Wormaldia* is in reality a member of the Philopotamidae, where does *Dipseudopsis* then belong? Is *Dipseudopsis* a member of a clade that includes Ecnomidae, or is it allied with Philopotamidae? If close to Philopotamidae, would *Dipseudopsis* then drag the Ecnomidae with it, to an affiliation with the other philopotamids? A single misplaced taxon in

a tree potentially can affect the reconstructed ancestral states at a large number of nodes and render the tree suspect in the neighborhood of the error.

The parsimony analysis of the rRNA (Fig. 5) has the Protoptilinae more closely related to Hydrobiosidae than to other Glossosomatidae. The polyphyly of Glossosomatidae is also difficult to accept. Glossosomatid monoplyly is supported by unambiguous synapomorphies, including unique larval morphology and case-construction behavior (Wiggins, 1996). The morphological analysis indicates that the protoptiline glossosomatids ally with the other Glossosomatidae, as they do in combined analyses. The rRNA sequences of the Protoptilinae, here represented by *Protoptila* and *Culoptila*, are among the most autapomorphic in of all Trichoptera, and their placement in Figure 5 could result from an accelerated substitution rate for these taxa. In addition, if the protoptilines are in the wrong place in Figure 5, then the paraphyly of the Hydrobiosidae may not be supported.

The hypothesis generated from a maximum likelihood analysis of the rRNA dataset is shown in Figure 6. Likelihood parameters are shown in Table 3. Empirical base frequencies of the rRNA data were 24%, 24%, 31%, and 21% A, C, G, and T, respectively. Although the likelihood-generated hypothesis agreed with the parsimony analysis in placing the Spicipalpia in a clade with Integripalpia, both groups emerged as polyphyletic in the likelihood analysis, with *Culoptila* (Glossosomatidae) nested within Integripalpia. This placement is weakly supported, and Integripalpia monophyly is being found in 6% of the Bayesian trees and protoptiline (*Protoptila* + *Culoptila*) monophyly in 16% of the Bayesian trees. One of the advantages of a Bayesian analysis is that alternative clades can be evaluated, and although 6% and 16% are not indications of strong support, the trees that include a monophyletic Protoptilinae and Integripalpia are among the set of "good" trees. Protoptilines are found to be monophyletic in both the COI nucleotide- and amino acid-derived trees.

An unconstrained equally weighted parsimony tree (not shown) constructed only from insertion, deletion, and "indel" characters that were excluded from the likelihood analysis, resulted in a strict consensus tree that included (Outgroup ((Lepidoptera) ((Annulipalpia) ("Spicipalpia" "Integripalpia")))). Other features of this tree included a monophyletic Philopotamidae. The protoptiline glossosomatids were monophyletic but still separated from the other Glossosomatidae. Kokiriidae again grouped inside a monophyletic Plenitentoria, and Xiphocentronidae was the sister taxon of a monophyletic Psychomyiidae.

Figure 7 represents the hypotheses generated from the morphological characters of Frania and Wiggins (1997), except that states not present in the outgroup were coded as missing data ("?") instead of plesiomorphic

TABLE 3. Likelihood parameters, given as means, with standard deviation and 95% confidence intervals, generated by the Bayesian analysis. The third through the eighth columns (labeled A–C through G–T) indicate estimated values from the general time reversible R-matrices, normalized to the RNA. "Inv" and "Alpha" refer to the estimated proportion of invariable sites and the gamma shape parameter, respectively.

		A–C	A–G	A–T	C–G	C–T	G–T	Inv	Alpha
RNA	mean	1.171	3.596	2.815	.768	7.003	1.000	.424	.326
	s.d.	0.046	0.215	0.268	0.044	1.731	0.000	.0002	.0012
	95%ci	.775–1.554	2.619–4.427	1.999–3.896	0.563–1.145	5.574–10.039	1.000	.393–.449	.291–.398
EF-1α	mean	3.728	11.405	3.739	5.803	19.655	1.553	.474	.8571
	s.d.	.336	3.600	.499	1.190	9.034	0.000	.0003	.0038
	95%ci	2.292–4.191	8.692–15.006	2.636–5.067	4.251–8.168	15.07–25.776	1.553	.4422–.5047	.742–.985
COI	mean	1.386	11.455	.3262	9.168	71.548	2.462	.209	.2143
	s.d.	.1531	2.002	.0037	2.886	615.16	0.000	.0095	.0031
	95%ci	0.4569–2.6119	7.649–16.250	.1553–.5219	4.653–14.697	24.59–134.7	2.462	.075–.339	.1546–.2777
Combined	mean	3.362	8.085	9.133	3.8324	13.197	1.654		
	s.d.	.0561	.3087	.3663	0.0733	.6499	0.000		
	95%ci	2.850–4.051	6.897–9.699	7.890–10.961	3.2359–4.5676	11.402–15.490	1.654		

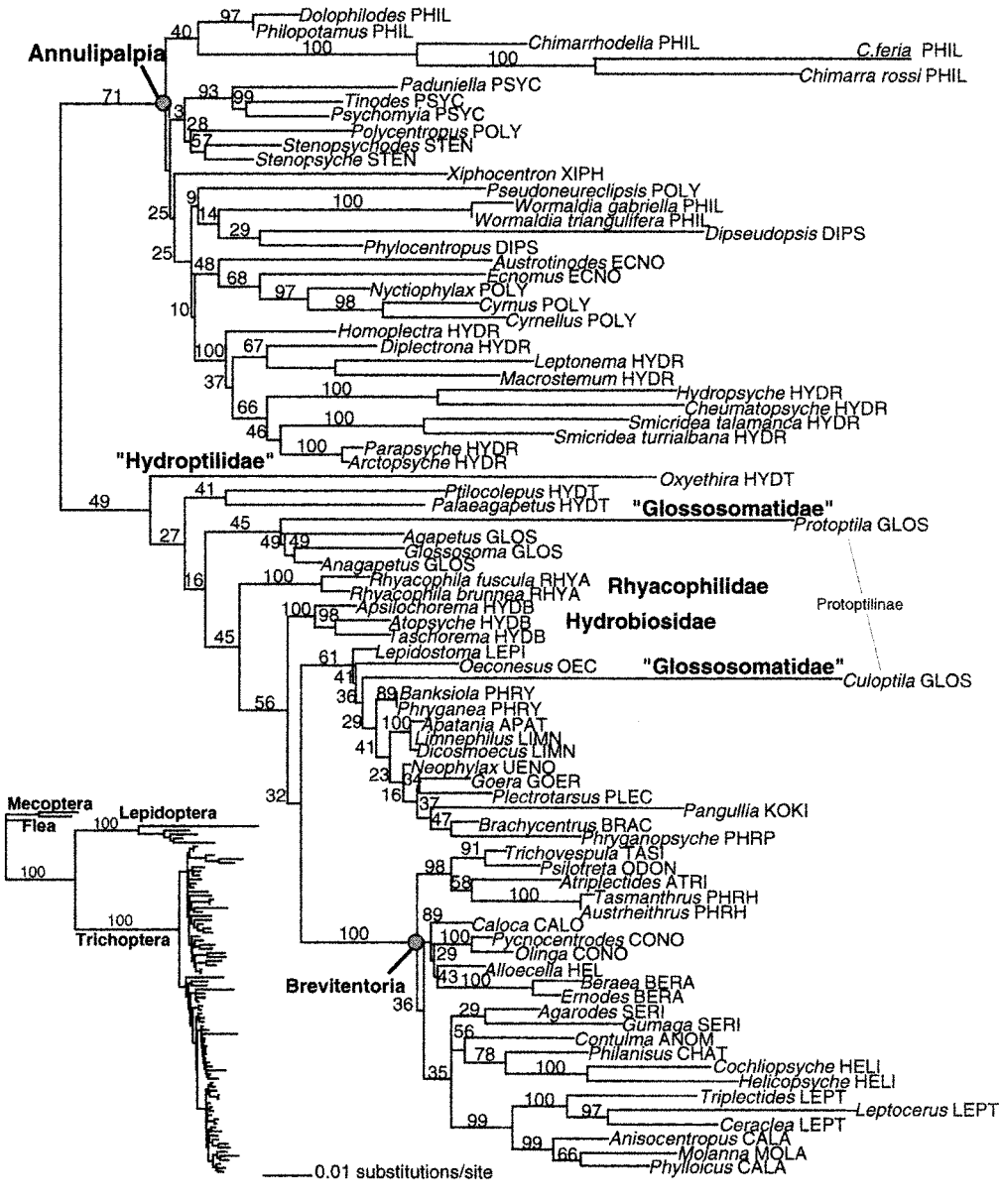

FIGURE 6. Phylogram resulting from an unconstrained Bayesian maximum likelihood analysis of rRNA data. This is a majority-rule consensus of the final 1,000 of 1,200 trees from each of two Monte Carlo Markov chains. A GTR model with a gamma correction for among-site rate variation and invariant sites was used. The likelihood score was –11,327.0. Species are labeled as genera, with capital letter mnemonic abbreviations that refer to families, but the taxa are identical to those used in Figure 5. Numerals on the nodes are percent recoveries from the consensus and can be translated as estimated posterior probabilities. In most cases, values are placed in the middle of the internode; sometimes, for considerations of space, the value is placed above and to the right of the internode or directly to the left. The insert in the lower left is included to show the scale of the phylogram among Trichoptera and its outgroups; including outgroups with the ingroup at the same scale as used for the main figure would require a figure spanning five pages.

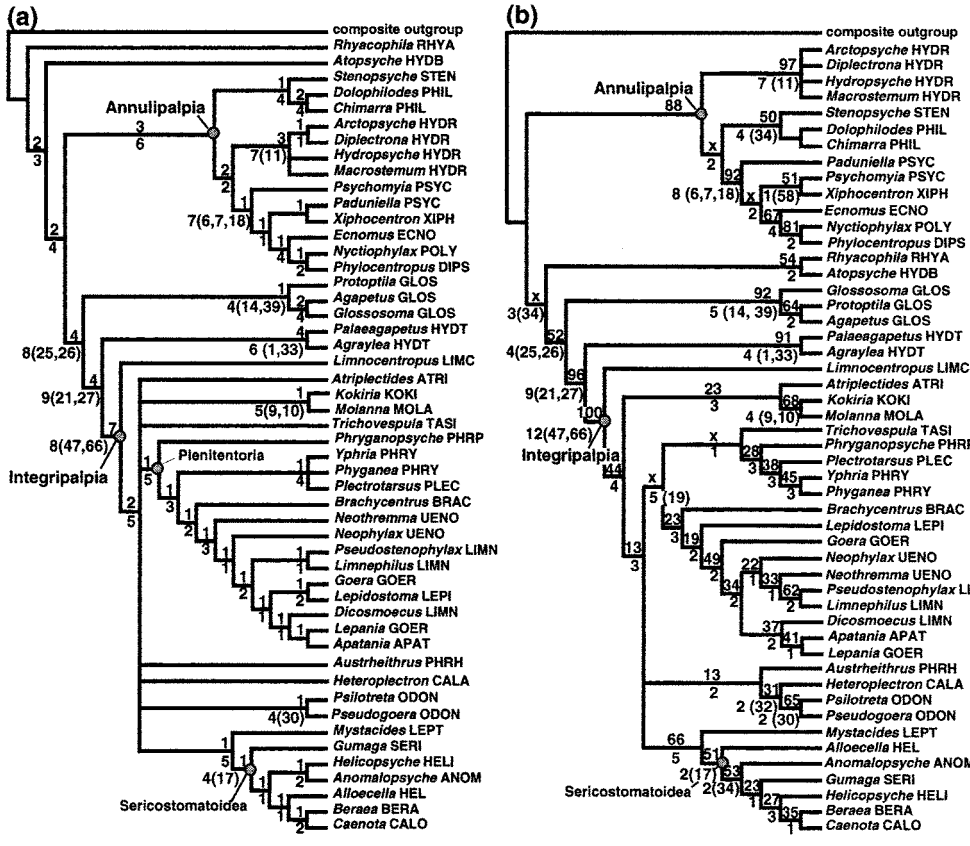

FIGURE 7. (a) Strict consensus of 18 trees (tree length = 354) from a reanalysis of the morphological characters presented by Frania and Wiggins (1997). Capital letter mnemonic abbreviations refer to families. Values above the internodes are decay indices, and below the internodes outside the parentheses are the number of characters that support each node. Characters that support the node without homoplasy are placed in parentheses below the node and numbered as in Frania and Wiggins (1997). (b) Hypothesis generated from the morphological data after pseudoreplicate reweighting. Values above the nodes indicate bootstrap support, while the numerals below the internodes are as in panel a. x indicates the node was not supported in a bootstrap analysis.

("0"). The decision to code character states this way made a difference in the analysis (Frania and Wiggins, 1997). When we treated the morphological data exactly as Frania and Wiggins did, we recovered 23 trees of 358 steps, instead of 5 trees recovered by Frania and Wiggins (1997), but the topology of the strict consensus was virtually the same as theirs, differing only by a collapse in resolving *Atriplectides* with the kokoriid plus molannid clade and a slight difference with the resolution of *Lepidostoma* (not shown). Our analysis, with the unknown outgroup states coded as missing data, recovered a monophyletic Annulipalpia and Integripalpia, with a polyphyletic (Fig. 7A) or paraphyletic (Fig. 7B) "Spicipalpia." The protoptiline glossosomatids grouped with the other glossosomatid. Plenitentoria was monophyletic but excluded Kokiriidae, as in Weaver and Morse (1986). The Leptoceroidea and Sericostomatoidea (Fig. 7, bottom) showed little resolution, and what resolution was obtained was only weakly supported and is uncorroborated by our data. Within the Plenitentoria, Phryganeidae and Phryganopsychidae were relatively basal, in agreement with Gall (1994), who used these taxa as outgroups in a study of the Limnephiloidea. Both the rRNA parsimony tree and the equally weighted morphology tree placed Phryganopsychidae as the most basal of the Plenitentoria. Our reanalysis of Frania and Wiggin's data supported their conclusion that Limnocentropodidae is the sister taxon to the rest of Integripalpia, but we could not confirm this with an independent dataset because we were unable to obtain

DNA sequence data from Limnocentropodidae during our study. A subsequent analysis did not support Frania and Wiggin's (1997) placement of Limnocentropodidae (Kjer et al., in press). When subjected to pseudoreplicate reweighting, using 1,000 trees generated from a fast heuristic bootstrap analysis of unordered morphological data, we find the relationships shown in Figure 7B. According to this analysis, Frania and Wiggin's larval character 34 (see also Ross, 1967) best defines higher groups without homoplasy in Trichoptera; the outgroup and Hydropsychoidea share two small sclerites on abdominal tergum IX with at least one long seta arising from each (Frania and Wiggins, 1997). Tergum IX is membranous in the Philopotamoidea and lacks conspicuous setae. "Spicipalpia," Plenitentoria, Leptoceroidia, and *Allocella* have a single large sclerite on tergum IX, with setae, whereas the nonhelicophid sericostomatoids have a membranous ninth tergite but retain long setae.

It is informative to examine the differences between Figures 7A and 7B. Figure 7B shows that when characters are differentially weighted, the topology changes with respect to the relationship among suborders. Yet bootstrap analyses of the differentially weighted data do not support the new relationships but, in fact, support a monophyletic *Rhyacophila* and *Atopsyche* as a sister taxon to Annulipalpia in 47% of the trees (not shown). This is not surprising, given that the weighting scheme favors the few (one) nonhomoplastic characters that may not always be sampled in a bootstrap analysis. Interestingly, when the equally weighted, unpolarized morphological data are subject to a bootstrap analysis, the topology in Figure 7B is supported! What this shows is that the hypotheses of relationships among suborders from morphological data are not stable to analysis assumptions, changing among many possibilities with every perturbation. This is important, because we argue that noisy datasets do not pose conflict with conservative datasets at the base of the tree, but the morphological dataset is not a noisy dataset.

Three trees from the EF-1α data are shown in Figure 8. The trees are remarkably similar, especially considering the estimates of homoplasy and considering that the unconstrained parsimony tree (TREEBASE website) contained *Micropteryx* (Lepidoptera) inside the Annulipalpia as well as Diptera inside the Integripalpia. Both nucleotide analyses recover a monophyletic Plenitentoria, Brevitentoria, and Sericostomatoidea, and Bayesian estimates of posterior probabilities are generally high. Likelihood parameters are shown in Table 3. Empirical base frequencies of the EF-1α data were 25%, 30%, 26%, and 19% A, C, G, and T, respectively. We consider nodes that are shared between nucleotide and amino acid trees to indicate relative confidence that those nodes are representative of the "gene tree" (not to be confused with independent congruence or "accuracy"). The amino acid tree recovers Brevitentoria and Sericostomatoidea. Dipseudopsidae and Stenopsychidae group together in all three analyses. The grouping of the predatory spicipalpians (Hydrobiosidae and Rhyacophilidae) and the resolution within both Philopotamidae and Integripalpia are shared between nucleotide trees and the amino acid tree. However, we cannot overstate these results, both because of the constraints we imposed and because of the alternative hypotheses with respect to the placement of Hydroptilidae.

The chi-square tests showed that nucleotide composition for individual taxa, when compared with mean values, did not deviate significantly from expected values for either the rRNA data or the COI data but did differ significantly for the EF-1α sequences. These differences remained when the outgroup was eliminated and each of the suborders was examined individually. This result dictated the analysis of EF-1α under a LogDet model (Lockhart et al., 1994), the only method we are aware of that accommodates among-taxon nucleotide compositional heterogeneity (Fig. 8B). To examine the possibility of nucleotide compositional effects on the EF-1α data, we constructed a "GC tree" (Lockhart et al., 1994). The GC tree was 544 steps longer than the parsimony tree (4,719 vs. 4,175), and a strict consensus of both unconstrained analyses was unresolved. However, after application of a few constraints, the tree length differences between the GC tree and the parsimony tree dropped to 254 steps (4,466 vs. 4,219), and some suspicious groupings were shared between both analyses. A parenthetical strict consensus of the GC tree and the parsimony tree, with constraints shown in

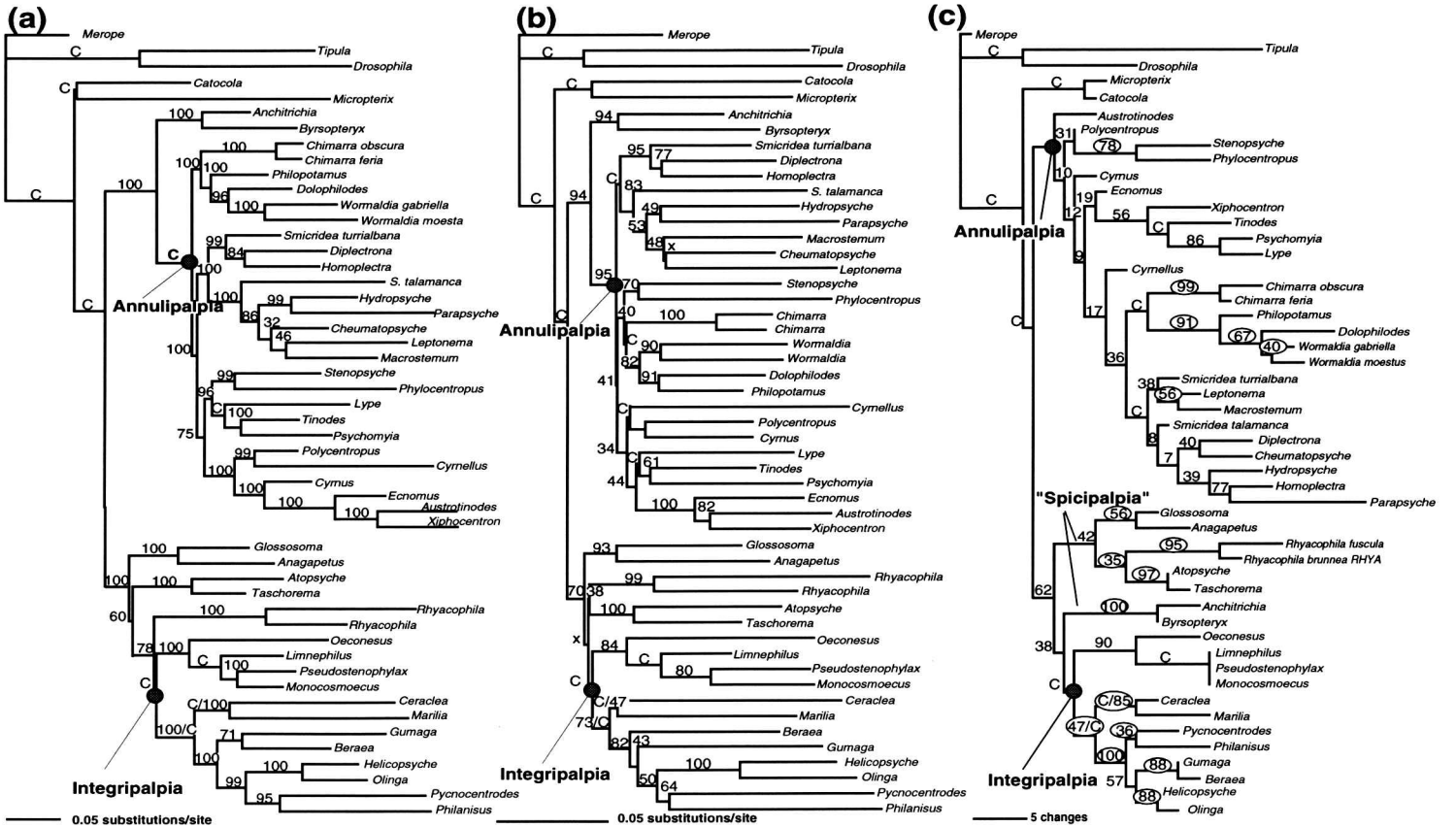

FIGURE 8. Phylogenetic hypotheses resulting from analysis of the EF-1α gene. C represents constrained nodes. (a) Hypothesis from a Bayesian maximum likelihood analysis of the nucleotides. The likelihood score was −18,050.3. Numerals above the nodes are estimated posterior probabilities. (b) Results from a minimum evolution heuristic search using the LogDet correction. Numerals above the nodes are bootstrap values. x represents lack of support by bootstrap. (c) Hypothesis from an equally weighted parsimony bootstrap analysis of amino acids. (In this analysis, no more than 100 trees were saved per replicate.) Ovals around bootstrap values represent unconstrained nodes on the amino acid tree that are also present in one or both of the nucleotide trees. The bootstrap values separated by a slash (e.g., C/85) represent bootstrap values with alternative constraints: Either *Ceraclea* was constrained with *Marilia*, or Brevitentoria was constrained.

brackets, is [*Merope*[Diptera] [[Lepidoptera] [Glossosomatidae Hydroptilidae Hydrobiosidae (*Rhyacophila*) [[Limnephilidae] (*Oeconesus Gumaga Beraea Helicopsyche Olinga Marilia* ((*Pycnocentrodes Philanisus*)*Ceraclea*))] [*Stenopsyche Polycentropus Cyrnellus Phylocentropus* [*Cheumatopsyche Macrostemum* ((*Diplectrona Homoplectra*) *Smicridea turrialbana*) (*Hydropsyche Leptonema* (*Smicridea talamanca Parapsyche*))] [Philopotamidae] [Psychomyiidae] (*Cyrnus Austrotinodes Ecnomus Xiphocentron*)]]]]. Parentheses show the taxa that share similar nucleotide compositions. Phylogenetically related taxa that share near identical sequences will, of course, share nearly identical nucleotide compositions, but more distantly related taxa may also group according to nucleotide compositional similarity. Suspicious groups shared between the GC tree and the parsimony tree involve the resolution within Hydropsychidae, and the grouping of *Cyrnus* with *Xiphocentron* and the ecnomids, resulting in the paraphyly of Polycentropodidae, and the grouping of *Pycnocentrodes* with *Philanisus*, resulting in the polyphyly of Conoesucidae (see Figs. 5 and 6). The LogDet tree (Fig. 8B) did not alter these "suspicious" relationships, but the amino acid tree (Fig. 8C) contradicts them all except (*Pycnocentrodes* + *Philanisus*). An Adams consensus (Adams, 1972; not shown) of the unconstrained GC tree and the unconstrained parsimony tree includes both *Micropteryx* (Lepidoptera) and Hydroptilidae nested within Annulipalpia.

Two trees generated from analyses of the COI data are shown in Figure 9. Empirical base frequencies of the COI data were 30%, 19%, 13%, and 38% A, C, G, and T, respectively. Most of the agreement between the COI nucleotide tree and the COI amino acid tree is confined to the more apical

FIGURE 9. Phylogenetic hypotheses resulting from analysis of the COI gene fragment. C represents constrained nodes. (a) Majority rule consensus from a Bayesian maximum likelihood analysis of the nucleotides (GTR + gamma + invariant sites). The likelihood score was −12,715.4. Numerals above the internodes represent posterior probabilities. (b) Hypothesis from an equally weighted parsimony bootstrap analysis of amino acids. Numerals above the internodes represent bootstrap proportions. Ovals around these values represent unconstrained nodes on the amino acid tree that are also present in the nucleotide tree.

nodes, although both recover the monophyly of Annulipalpia and Integripalpia. Note the striking similarity of the two trees (Fig. 9A and B) concerning the higher-level relationships within Annulipalpia. Both analyses subdivide Annulipalpia into Hydropsychidae, Philopotamoidea (Stenopsychidae + Philopotamidae), and a third clade containing the other annulipalpian families.

With the exception of basal placement of the Hydrobiosidae in both COI trees, for the most basal nodes, trees from nucleotides and amino acids were different from one another for the most basal nodes for both the EF-1α gene (Fig. 8) and the COI gene fragment (Fig. 9). Given that the amino acid and nucleotide data from any gene must share a common history, lack of agreement must represent a problem with the data or the assumptions behind some or all analyses. With the substitution rate profiles shown in Figures 3 and 4, we do not expect strong, concordant support for the most basal nodes, and we suspect that the lack of congruence reflects homoplasy in both genes (Table 1; Figs. 3 and 4).

We explored Wilcoxon signed rank tests a priori (Templeton, 1983) to evaluate the fit of a priori trees generated from other data in this study as well as those from previously published hypotheses on each of our datasets (Table 2). In general, noisy datasets accept alternative hypotheses without a large increase in tree length. Swofford et al. (1996) and Goldman et al. (2000) argued that the Kishino–Hasegawa test (Hasegawa and Kishino, 1989; Kishino and Hasegawa, 1989) is inappropriate for comparison of phylogenies that are not defined a priori and stated that this problem extends to the Templeton test as it is usually run: comparing the best trees generated from a particular dataset with other suboptimal trees. By using the "salvage" option described in Goldman et al. (2000) for inappropriately applied two-tailed Templeton tests, the EF-1α nucleotide dataset significantly rejects a tree generated from its own amino acids (not shown); this dataset would thus seem to be in conflict with itself. Because alternative hypotheses constrained on the EF-1α nucleotide data result in tree-length additions of from 38 to 82 steps, we suspect that the homoplasy in the EF-1α nucleotides is not randomly distributed but rather has some pattern. This nonrandom homoplasy could be coming from the among-taxon nucleotide compositional heterogeneity observed in the data, and our "GC-tree" (Lockhart et al., 1994) lends some support of this speculation. However, we cannot claim that these findings demonstrate compositional effects, because the "suspicious" groups were also found in the LogDet tree (Fig. 9B).

Complicating a combined likelihood analysis, we find the datasets to be evolving at excessively different rates; in most cases, 95% confidence intervals on parameter estimates are nonoverlapping (Table 3). The tree depicted in Figure 10 is a result of the combined likelihood analysis of the nucleotide data, using a site-specific rate method designed to compensate for heterogeneity among sites, both within and across datasets. Figure 11 shows the results of an equally weighted parsimony bootstrap analysis that includes all of the data. Congruence among datasets is also shown in Figure 11. The differences between the equally weighted and pseudoreplicate reweighted trees are minor enough to be summarized in the text rather than presented in a separate figure and are designated with x's on Figure 11. The pseudoreplicate reweighted tree placed *Diplectrona* at the base of Hydropsychidae, with *Arctopsyche* next, and *Macrostemum* and *Hydropsyche* together with a 97% bootstrap. Written parenthetically, with bootstrap values included, the other differences in relationships within Annulipalpia from the pseudoreplicate reweighted tree were Phylocentropus 39(89((*Paduniella* 51(*Lype, Psychomyia*)) 69((*Xiphocentron* 69(*Nyctiophylax, Ecnomus*))))). Within the Plenitentoria, *Pangulia* moved from its relatively apical position to the sister taxon of the rest of the clade, supported by a bootstrap value of 69. All other differences were extremely weakly supported; *Pseudoeconesus* was the next most basal taxon in the rest of Plenitentoria, after which the *Brachycentrus/Phryganopsyche* clade was the sister taxon to the rest of the limnephiloids. *Goera* switches place with the uenoids. In the Brevitentoria, *Psilotreta* and *Atriplectides* switched places, and *Allocella* and *Philanisus* moved from their position in Figure 11 to the base of the sericostomatoids, with *Allocella* being most basal (see morphology, character 34; Fig. 7B).

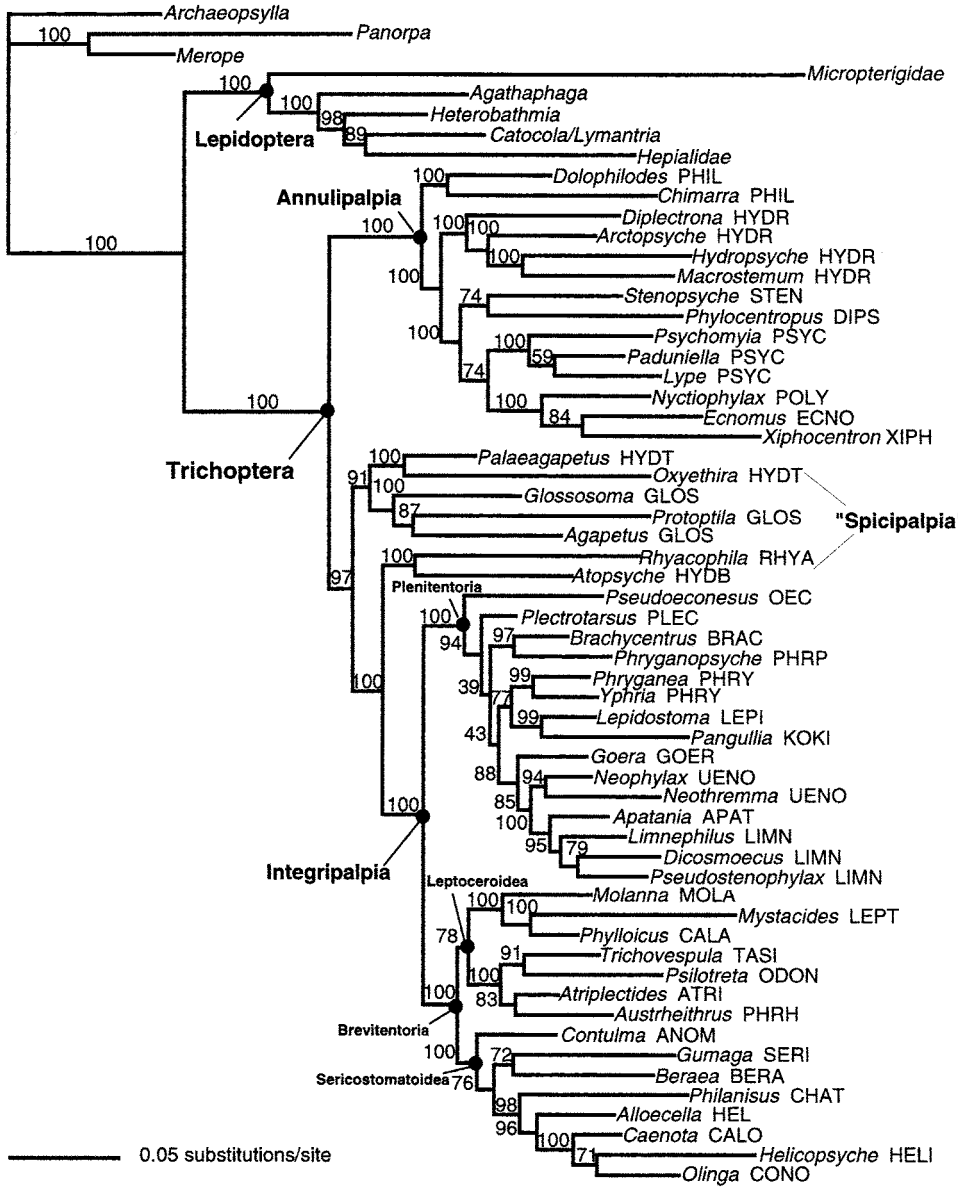

FIGURE 10. Results of the Bayesian combined nucleotide data. The likelihood score was −29,954.3. Estimated relative rates for the five character partitions were 1, 4.823; 2, 3.072; 3, 1.546; 4, 1.324; and 5, 0.091, with 207, 330, 188, 84, and 1,784 characters in each respective class. Numerals above the internodes represent estimated posterior probabilities.

DISCUSSION

Trichoptera Classification

Summarizing our molecular and combined hypotheses (Figs. 10 and 11), we conclude that Annulipalpia is the most basal suborder, with Spicipalpia and Integripalpia forming a clade, thus helping to resolve one of the major disputes about evolutionary relationships within the order Trichoptera. Although the support for this relationship is modest under equally weighted parsimony, with a decay index of 1, it is consistent with the rRNA data (parsimony, likelihood, and gap character analyses), the differentially weighted parsimony analysis of the morphology (Fig. 7B), and the EF-1α amino acid data (Fig. 8C). Our analyses of homoplasy

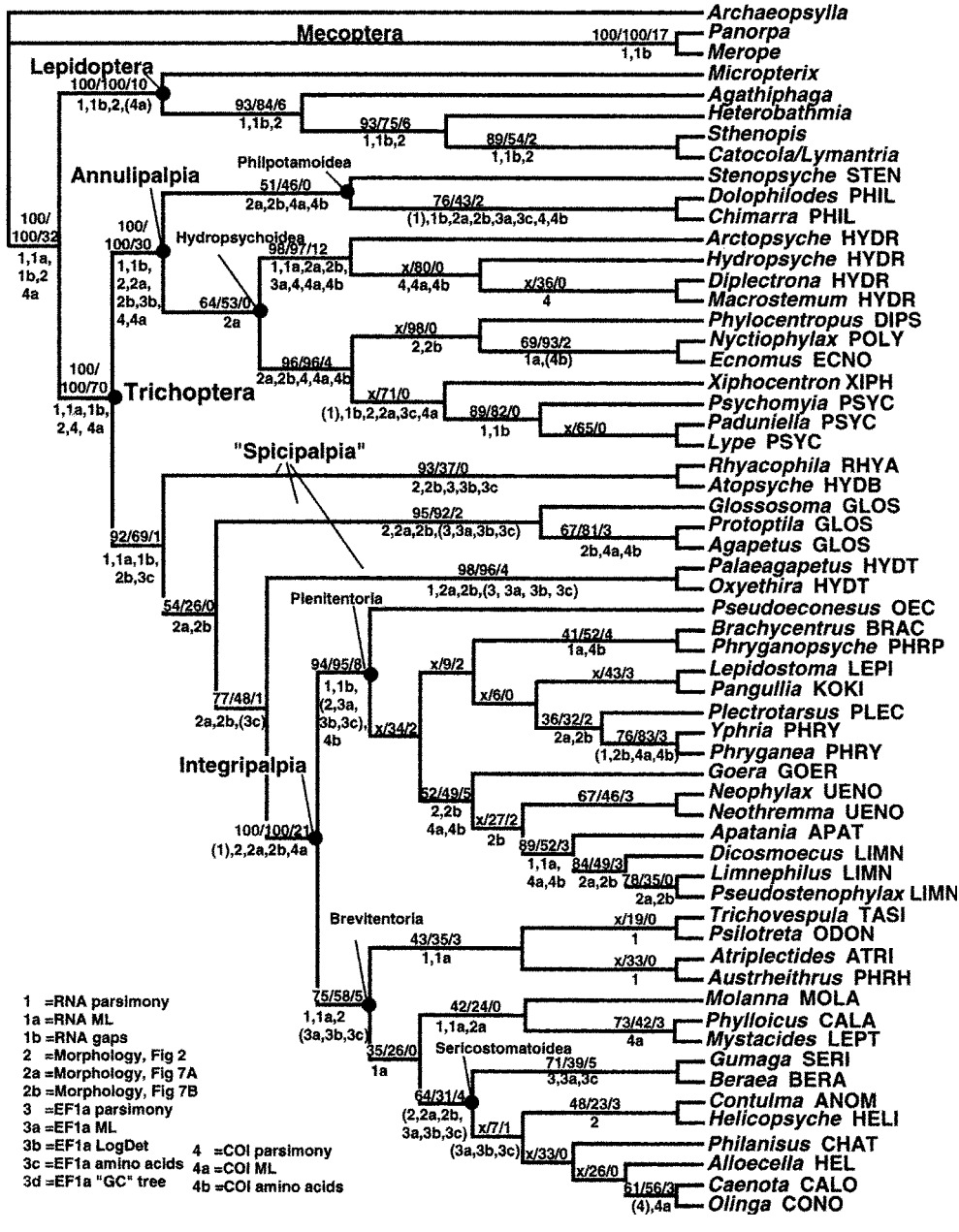

FIGURE 11. Tree from the combined equally weighted parsimony bootstrap analysis. Characters in this analysis included D1, D3, and V4–5 rRNA; complete EF-1α amino acids; COI nucleotides; COI amino acids; and morphology. Tree length = 5,241. Numerals above the internodes are bootstrap values from the differentially weighted analysis, followed by bootstrap values from this analysis, followed by decay indices from this analysis. Nodes in conflict between differentially weighted and equally weighted analyses are marked with an x. Numerals below the internodes refer to support by separate analyses of different datasets, according to the key (lower left), with parentheses included to draw attention to the differences among taxa in the different analyses. Note that some decay indices = 0 in the bootstrap tree, indicating nodes that collapsed in the strict consensus tree (the strict consensus tree can be reconstructed in this figure by collapsing those nodes).

plays a qualitative role in evaluating support, because the combined analyses agree with the most appropriate of the molecular datasets for inferring relationships among basal Trichoptera clades. Another qualitative vote of confidence for a node in question concerns whether or not support increases with analyses designed to reduce the impact of homoplastic characters. Support for the node linking the spicipalpians with the Integripalpia increases both with likelihood (97% posterior probability) and with differential weighting, where bootstrap support increases from 69 to 92 (Fig. 11). Alternative analyses support either a monophyletic arrangement or any of a variety of paraphyletic or polyphyletic arrangements of Spicipalpia, with no consistent pattern except that the relative relationships among the Spicipalpia, when considered unrooted, are remarkably consistent, with Hydrobiosidae and Rhyacophilidae next to one another, as are Glossosomatidae and Hydroptilidae. Although monophyly of Spicipalpia is not consistently supported, it does emerge from the analyses as a possibility (Fig. 5). We find this suggestive enough to retain spicipalpian monophyly as a suboptimal but viable hypothesis. Our hypothesis of relationships is not in strong conflict with the morphological data of Frania and Wiggins (1997), and the inclusion of these data actually adds support to our overall conclusions. In a general sense, the phylogeny lends support to one conclusion of Frania and Wiggins (1997), namely, that the "Spicipalpia" retain characters primitive for the order. According to our analysis, however, this results not from the basal position of the suborder in the overall phylogeny, but rather from its basal position in one of two major clades for the order, where some primitive characters might be expected to be retained. If Spicipalpia is monophyletic, then equally parsimonious solutions exist for the evolution of various key innovations, including cocoon-making and case-making behaviors. Both Annulipalpia and Integripalpia are found to be monophyletic, with relatively high bootstrap support, relatively high decay indices, and support from multiple independent datasets (Fig. 11). Clearly, characters shared between Annulipalpia and Integripalpia cannot be invoked to have a common origin, except with the possibility that the character state was primitive for the order and lost in the Spicipalpia. Shared primitive characters would be hard to reconcile if Spicipalpia is paraphyletic.

At the level of superfamily, both equally weighted morphology (Fig. 7A) and the combined analysis (Fig. 11) supported monophyly of both the Hydropsychoidea and the Philopotamoidea within Annulipalpia. Support for most alternative arrangements shown in Figures 5, 6, 7B, 8, and 9 is extremely weak, but Figure 10 shows that none of the Bayesian trees recover these superfamilies because of the placement of *Stenopsyche* with *Phylocentropus*—a clade supported strongly but exclusively in the analysis of the EF-1α (Fig. 8). We could not place the Pseudoneureclipsinae; rRNA places it either with Xiphocentronidae (Fig. 5) or with *Wormaldia* plus Dipseudopsidae (Fig. 6), whereas COI places it (*Antilopsyche*) with the polycentropodids and the ecnomids (Fig. 9). We suspect that the grouping of *Cyrnus* (Polycentropodidae) and the ecnomids with Xiphocentronidae in the EF-1α analysis (Fig. 8) was due to nucleotide compositional similarity and that the EF-1α played a relatively large role in the combined nucleotide analysis (Fig. 10); this suspicion is corroborated by other data (see Figs. 2, 5, 6, 8C, 9B, and 11, all of which place Xiphocentronidae with Psychomyiidae). Within the Integripalpia, we find strong support from multiple datasets for the division of the group into two infraorders: Plenitentoria and Brevitentoria, essentially as it has been divided, except that *Pangullia* (Kokiriidae) is now placed in the Plenitentoria. From our review of the data, the inclusion of *Pangullia* (Kokiriidae) is probably not an error. We checked the voucher specimen for accurate identification, and multiple molecular markers support placing *Pangullia* within Plenitentoria. However, the addition of another kokiriid taxon, particularly *Kokiria*, the nominate genus, would add strength to this argument. Relationships within Plenitentoria, however, are extremely weakly supported, with almost no agreement among alternative analyses, except for the grouping of Limnephilidae *sensu latu*: Uenoidae, Goeridae, Apataniidae, and Limnephilidae, with Apataniidae as the sister taxon to a monophyletic Limnephilidae *s.s.* The monophyly of Leptoceroidea was not supported in most analyses, but the nodes that contradict the monophyly of this clade are extremely weak, and our Bayesian likelihood (Fig. 11)

analysis does recover Leptoceroidea at 78%. Further analysis will be needed to evaluate relationships among trichopteran families, and we are working on expanding the dataset to address these questions.

Empirical Observations from the Analyses

"Saturation," as generally discussed, is a distance-based concept, with saturation curves derived from the pairwise comparison of sequences. For character-based phylogeny methods (e.g., parsimony or likelihood), "saturated" data may still provide meaningful phylogenetic information, even when sequences no longer show an increase in distance with increasing divergence. This is because homoplasy, when occurring in different parts of the tree, can be efficiently isolated with extensive taxon sampling (Swofford et al., 1996). Empirical evidence (Hillis, 1998) and simulation studies (Graybeal, 1998; Yang and Goldman, 1997) show that adding taxa can greatly improve the accuracy of character-based methods. Broughton et al. (2000) show that the removal of presumably noisy partitions (third positions or transitions) is inadvisable because even when homoplastic, these characters may not be problematic, given increasing patristic distances, and these noisy partitions tend to contribute a large number of characters to the analysis. Despite our utilization of saturation curves in this paper, we agree with results that indicate saturation can be compensated for with taxon sampling. However, we caution against an overextention of these findings to the assumption that extensive taxon sampling will always lead to accurate phylogenetic hypotheses, especially at the deepest nodes. Empirical examples cannot constitute proof that, for every set of taxa, sampling can always fix the problem. Having unambiguous signal is preferable to relying on the chance that the right taxa still exist to be sampled and that homoplasy will be distributed in the right places. The simulations mentioned (e.g., Yang and Goldman, 1997; Graybeal, 1998) were done with model parameters set constant across lineages, demonstrating that given the model, taxon sampling can help. However, the most serious problems in phylogenetic analyses of biological data occur when noise is *not* homogeneously distributed across taxa, and simulations may not address the effects of nonrandom noise on biological datasets. With "good" data, we expect a left skew to the tree distributions from five taxa; if there is not, then at best, we have good reason to hope that taxon sampling will sort out the homoplasy. At worst, we are left with four conflicting datasets with no way to rank them.

Some criterion had to be set for dataset combinations of analysis and inclusion for publication. A simple option would be that of a single "total evidence" analysis (Kluge, 1989), and we agree that this is probably the most objective approach. However, our goal was not to maximize objectivity at all costs, but rather to estimate phylogeny as objectively as we could without sacrificing our ability to make decisions, perhaps even subjective ones, based on observation. Although we combined all data, it would be a misinterpretation to assume that our analyses of saturation supports always combining data. Rather, we felt that we could rank our hypotheses, even if only qualitatively, and sort out conflict from noise. Now that "tests" used to indicate whether or not data should be combined (e.g., Templeton, 1983; ILD tests, Farris et al., 1995) have come under criticism (Dolphin et al., 2000; Goldman et al., 2000; Yoder et al., 2001), we feel it is increasingly important to examine data in a variety of ways and with respect for organismal expertise. We would have felt justified avoiding the addition of EF-1α or COI, simply because our measures of excessive homoplasy in these markers, but we were not forced to make that decision. In the end, we did combine all of the data, and the addition of even our noisiest data resulted in the hypotheses of subordinal relationships that we consider our best estimate (Figs. 10 and 11). We found we could include all of our data in a combined analysis without overturning the results gained from our most conservative marker, the rRNA.

The combination of approaches for evaluating signal offered a consistent picture of whether or not signal was located primarily within suborders or among higher clades. Figures 3 and 4 can be examined with reference to the numbers below the internodes in Figure 11, indicating which dataset was most responsible for the recovery of specific nodes. The g1 statistics and the recovery of expected clades in the five- to eight- taxon analysis all gave the same answers, and Figures 3 and 4 agree with the information from

Table 1. Since Hillis and Huelsenbeck (1992) showed that significant signal can come from any single clade in a dataset, even when signal is randomized among all other taxa, we find the g1 statistic to be of little value in proving signal exists but meaningful in casting suspicion on a dataset where signal may have broken down. This becomes important when attempting to reconcile "conflicting" hypotheses from different datasets because without some means of filtering the data, one would often either have to collapse a best estimate to an unresolved strict consensus or allow saturated data to determine the tree.

That the COI seemed to have outperformed the EF-1α within the Annulipalpia, as judged by the combined analysis, seems incongruous with the results shown in Figures 3 and 4 and Table 1. This outcome could have simply been due to greater taxon sampling in COI, but we also note that the accumulation of differences in EF-1α amino acids is surprisingly flat throughout the tree, perhaps indicating functional constraints. Amino acid sequences that are highly constrained could be saturated at even very low mean percent differences if the few positions permitted to vary actually toggle freely among a few similar amino acids (Simon et al., 1994). If we accept that nonconstrained nucleotides become relatively rapidly homoplastic in any sequence, then deeper-level signal in any protein-coding dataset is always going to occur against a background of inherently noisy silent sites (mostly third-codon positions; but see Broughton et al., 2000). The relevant issue then, in the search for appropriate protein-coding genes for recovering ancient relationships, is the proportion of amino acids free to vary; EF-1α sites were estimated to have 51% of its sites invariant. Perhaps counterintuitively, to be useful in a phylogenetic analysis at the level we are considering, a protein would need an even more rapid rate of amino acid divergence than that observed in the EF-1α gene, to give conservative, nonsilent substitutions a chance to accumulate. In other words, a "conservative-looking" gene, with highly constrained first- and second-codon positions, may be less appropriate for deep-level phylogenetic analysis than a "noisy-looking gene," the underlying signal of which is found in the variation occurring at nonsilent codon positions, given the likelihood that the majority of third positions in both genes provide noise. Additionally, even the amino acid substitutions that do occur in a too-conservative gene may contribute noise if the positions free to vary alternate between two or three structurally similar amino acids for which the precise character state is evolutionarily unconstrained, or nearly so.

We believe that the coding of insertions and deletions is important. Swofford (1993) recommended that "indels" be treated as missing data in the nucleotide sequence block and then coded in a presence/absence matrix at the end of the data file; Kjer (1995), Crandall (1996), and others have followed this advice. Wheeler (1999) and Lutzoni et al. (2000) proposed coding indels separately (even when they could not be aligned) as multistate characters and then running these characters through a step matrix. The rules we used to evaluate insertions and deletions shown in Appendix 1 are discussed at length in the *Systematic Biology* website. The widely used term "indel" may mistakenly imply an inherent difficulty in distinguishing between insertions and deletions in molecular data or at least indicate that a distinction between insertions and deletions is not important enough to use a separate term for these different events. We found that the polarity for most insertions and deletions could be unambiguously determined by the traditional examination of successive outgroups, as widely practiced by morphological systematists. Our coding of insertions, deletions, and "indels" was admittedly elaborate, but no more so than the character coding performed by morphologists. In our opinion, these characters should each be treated individually and described to the best ability of the investigator. Because of the lack of models for the evolution of insertions and deletions at the time of this analysis, these characters were left uncoded in the likelihood analysis (see Lewis, 2001). Because some of our analyses were conducted without the insertion/deletion characters, those uncomfortable with the complexity of the coding (or more comfortable with an explicitly model-based analysis) will find likelihood trees that still place Integripalpia and Spicipalpia together. Complex coding of insertions and deletions has the disadvantage that one cannot compare the performance of likelihood to that of parsimony, because the datasets are different. Although

we agree that likelihood remains a statistically superior criterion in many respects, we believe that parsimony's advantage has been its ability to combine data from multiple sources, such as morphology, insertions, deletions, and molecules evolving under exceedingly different conditions.

Pseudoreplicate reweighting performed well in resolving many more nodes than did the equally weighted analysis (note the nodes with a decay index of zero in Fig. 11); moreover, enough nodes differed among the equally and differentially weighted analyses to reassure that the procedure was not entirely circular. However, at least within Annulipalpia, we prefer the results from the equally weighted analysis. With the morphology, pseudoreplicate reweighting yielded hypothesized relationships among Annulipalpia, Integripalpia, and the four spicipalpian families that were identical to our combined analysis (Fig. 11); the characters responsible for this change are apparent on Figure 7.

We present a rRNA dataset that seemed to be evolving at an appropriate rate for the suborder study, a previously published morphological dataset that also resolved suborders but in an alternative way when data were equally weighted, and two datasets, the COI and the EF-1α, that taken alone, were practically useless for estimating relationships among suborders. Bootstrap values and decay indices from individual uncorrected analyses were weak, but it is expected that different sources of data to provide information at different levels in the tree. Corroboration from independent sources comes only from differentially weighted parsimony of the morphological data and from the EF-1α amino acids. However, these datasets, along with the rRNA, are the most slowly evolving datasets. We do not find "conflict," among trees, but rather find their differences to be easily explained by the properties of the data.

We are not entirely satisfied with any of the trees generated from single datasets. Despite the problems with the COI and the EF-1α, no informed trichopterist would take the relationships among annulipalpian families generated from the rRNA data (Figs. 5 and 6) seriously. The COI data provides a reasonable estimate of relationships within Annulipalpia (see all the 4's showing agreement with the COI data in Fig. 11). And we have converged with some level of confidence on the relationships among taxa in the "backbone" of the trees presented in Figures 10 and 11. Although we have presented many trees, all except Figures 7, 10, and 11 simply represent explorations of the data. There is a danger in presenting such a wide variety of analyses, selecting to present some and ignore others. The alternatives would be to perform and present either hundreds of arbitrary trees under every conceivable method, or to limit the analyses to a single optimality criterion, subjectively favored on the basis of philosophy (e.g., parsimony or likelihood). The guide in presentation must be the data, rather than a favored hypothesis, although the data did lead us to a favored hypothesis. Subjectively, we find Figure 11 to represent our best current estimate of phylogenetic relationships among Trichoptera suborders and major family-level taxa. Although our preferred hypothesis comes from equally weighted parsimony, this statement should not be mis-cited as a triumph for parsimony, in view of its performance in estimating relationships with the nucleotides from COI and EF-1α. To evaluate this performance, note that relatively few nodes in the combined analysis were recovered from either of these noisy markers (Fig. 11; methods 3 and 4). Both parsimony and likelihood analyses complemented one another. The agreement between Figures 10 and 11 represents a more conservative estimate of phylogeny, with the differences between them dependent on the relative contributions of the different datasets: Figure 11 favors the morphological characters with respect to the Spicipalpia and the Philopotamoidea, while Figure 10 (lacking morphology, gaps, and amino acids) looks more like the RNA tree with respect to the spicipalpians, with the COI and EF-1α playing a larger role in dictating relationships within Annulipalpia. We are currently involved in collecting additional data to estimate the phylogenetic relationships among families within Annulipalpia, Plenitentoria, Leptoceroidea, and Sericostomatoidea. It is our prediction that the nodes shared between Figures 10 and 11 will not change with the ongoing analyses. It was not our intention to prove that "saturated" data should never (or always) be used; rather, our analyses of homoplasy guided us in making analytical decisions that led to a credible hypothesis.

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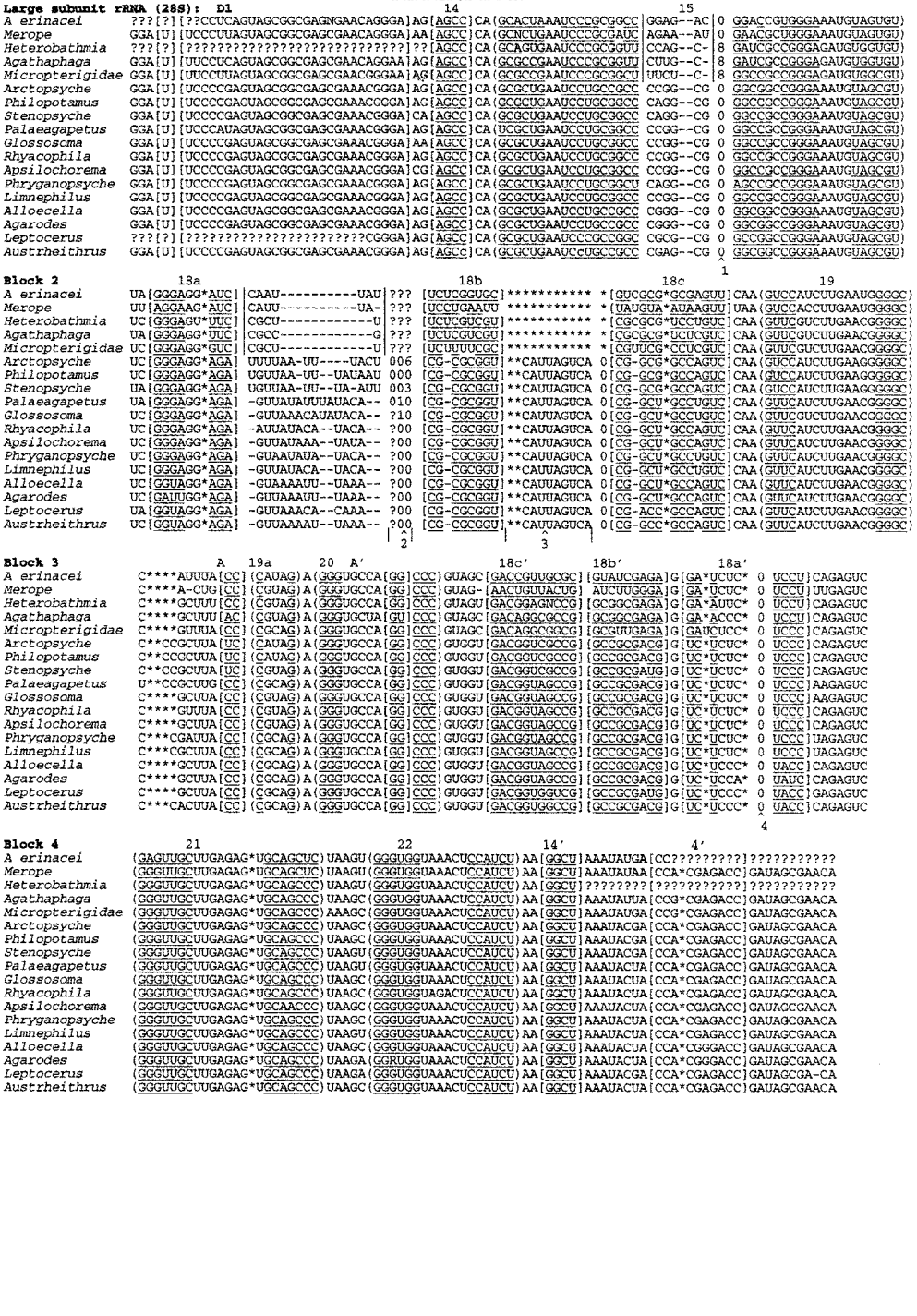
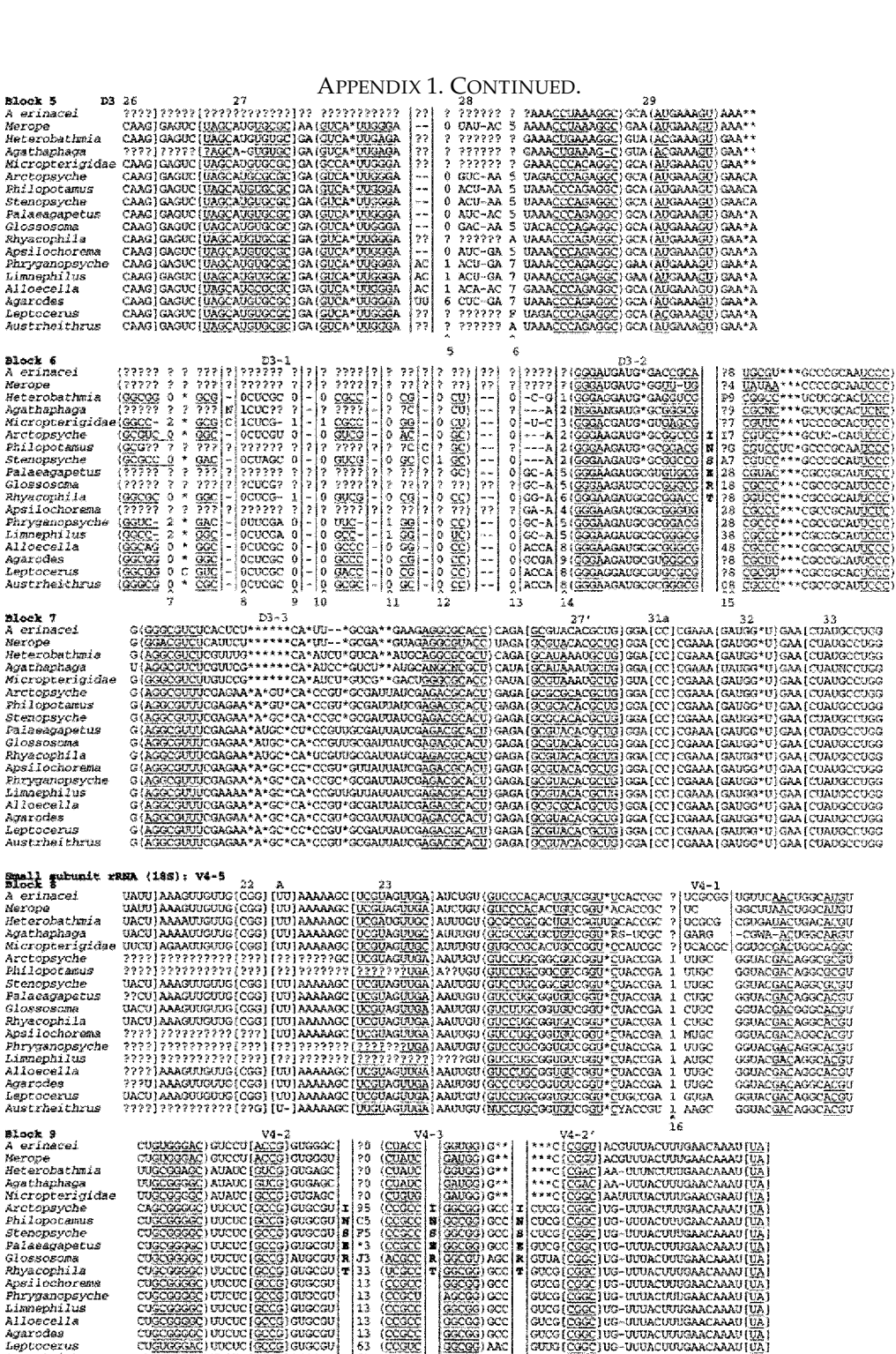
APPENDIX 1. ALIGNMENT OF SELECTED TAXA

Secondary structural symbols are presented as in Kjer et al. (1994). Nucleotides flanked by straight lines have been excluded from the analysis, as described in the "explanation of Appendix 1" on the *Systematic Biology* website. Numerals above the blocks number the stems as in Larsen (1992) for the large subunit rRNA and in van de Peer et al. (1993) for the small subunit rRNA. Numerals below the data blocks refer to alignment sites discussed on the *Systematic Biology* website.

APPENDIX 1.



APPENDIX 1. CONTINUED.



APPENDIX 1. CONTINUED.