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Aligned 18S for Zoraptera (Insecta): Phylogenetic position and molecular evolution

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Abstract

The order Zoraptera (angel insects) is one of the least known insect groups, containing only 32 extant species. The phylogenetic position of Zoraptera is poorly understood, but it is generally thought to be closely related to either Paraneoptera (hemipteroid orders: booklice, lice, thrips, and bugs), Dictyoptera (blattoid orders: cockroaches, termites, and mantis), or Embioptera (web spinners). We inferred the phylogenetic position of Zoraptera by analyzing nuclear 18S rDNA sequences, which we aligned according to a secondary structure model. Maximum likelihood and Bayesian analyses both supported a close relationship between Zoraptera and Dictyoptera with relatively high posterior probability. The 18S sequences of Zoraptera exhibited several unusual properties: (1) a dramatically increased substitution rate, which resulted in very long branches; (2) long insertions at helix E23; and (3) modifications of secondary structures at helices 12 and 18.

Keywords: Zoraptera; 18S rDNA; Secondary structure based alignment; Phylogeny; Molecular evolution

1. Introduction

Zoraptera (angel insects) is one of the least diverse and poorly known insect orders. To date, only 38 species (which includes six fossil species) are described, and all extant species are classified under a single genus, *Zorotypus* (Engel and Grimaldi, 2002). Some other genera have been proposed for extant species (Chao and Chen, 2000; Kukalová-Peck and Peck, 1993), but more the conservative taxonomic system is adopted here, as suggested by Engel and Grimaldi (2000) and New (2000). All species of Zoraptera live under the bark of rotting wood (Smithers, 1991).

Based on morphological characters, the order Zoraptera is thought to be closely related to either Paraneoptera (= hemipteroid orders: bugs, thrips, booklice, and lice: Hennig, 1981; Kristensen, 1975, 1981; Wheeler et al., 2001), Dictyoptera (= blattoid orders: cockroaches, termites, and mantis: Boudreaux, 1979; Kukalová-Peck and Peck, 1993; Smithers, 1991) or Embioptera (= web spinners: Engel and Grimaldi, 2000; Minet and Bourgoin, 1986). Combined morphological and molecular analysis by Wheeler et al. (2001) supported a close relationship between Zoraptera and Dictyoptera. However, separate analysis of molecular data (18S rDNA) placed Zoraptera as the sister taxon of Psocodea (booklice and parasitic lice), conflicting with combined tree (Wheeler et al., 2001).

Separate analyses of 18S data (Wheeler et al., 2001) resulted in tree with a very unconventional placement of some insect orders (e.g., Diplura and Grylloblattodea were imbedded within Holometabola). Wheeler et al. (2001) used direct optimization of morphological and molecular data, which minimizes incongruence between two data partitions. Kjer (2004) pointed out that, when

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support from molecular data for nodes is small, conclusions from molecular data by direct optimization would be highly dependent on some combination of (1) morphological data, (2) noise from the homoplastic data, and (3) arbitrarily optimized homology of unalignable data (see also Kjer, 1995 and Simmons, 2004).

To address the problems of direct optimization, Kjer (2004) conducted phylogenetic analyses of insect orders based on 18S sequences aligned manually according to secondary structure. The resulting tree matched traditional insect classification reasonably well. However, Kjer's (2004) study lacked a sequence of Zoraptera and thus could not address the phylogenetic position of this order. Part of the reason for the exclusion of Zoraptera by Kjer was that he concluded that the 18S of Zoraptera presented in Wheeler et al. (2001) was either contaminated in part by mite (Acari) DNA sequences, because of a homologous unique sequence shared by the Zoraptera and mites, or that if the zoraptteran sequence was not a contaminant, it was highly autapomorphic and problematic.

Thus, a more detailed molecular test of the phylogenetic position of Zoraptera is needed. The 18S rDNA gene has played an important role in resolving the deep phylogeny of insects (Campbell et al., 1995; Johnson et al., 2004; Kjer, 2004; Whiting et al., 1997). However, a correct 18S sequence of Zoraptera may not be available to date. In the present study, we amplified and analyzed the 18S rDNA of Zoraptera using samples collected in the USA, Malaysia, and Vietnam. These sequences of Zoraptera plus additional sequences of Blattodea (cockroaches), Phasmatodea (stick insects), Embioptera, and Paraneoptera were aligned with the 18S data provided by Kjer (2004). We address two questions: (1) is the 18S sequence of Zoraptera used by Wheeler et al. (2001) really a contaminant and (2) what is the closest relative of Zoraptera?

2. Materials and methods

We sequenced four species of Zoraptera, *Zorotypus hubbardi* from the USA, *Z.* sp.MY1 and *Z.* sp.MY2 from Malaysia, and *Z.* sp.VN from Vietnam (the latter three species are currently being described). Methods of total DNA extraction and 18S amplification and sequencing followed Johnson et al. (2004). Primer sets used were Ns1-Ns2a (Barker et al., 2003), 18Sai-18Sbi (Whiting et al., 1997), and Ns5aP2-Ns8P (Johnson et al., 2004). The 18S sequence of *Z. snyderi* was obtained from GenBank and was only used to check whether the 18S sequence of the species was contaminant or not. The sequence was not used for phylogenetic analyses because only a short piece of the 18S sequence was available for this species. Additional 18S sequences of Blattodea, Phasmatodea, Embioptera, Psocodea, Thysanoptera, and Hemiptera were obtained from GenBank (Appendix A). These sequences were manually aligned to the data matrix provided by Kjer (2004) according to the secondary structure model presented on his website. When we detected a modification of the secondary structure in the new sequences, the secondary structure of the region was estimated using GeneBee (Brodsky et al., 1995). Except for these additional samples, the taxon set was largely unchanged from Kjer (2004). However, we replaced sequences of Ectopsocidae Gen. sp. and *Pthirus pubis* with *Ectopsocus perkinsi* and *Pedicinus* sp., respectively, because only a short piece of 18S sequence was available for the former two species (Appendix A). Unalignable regions were excluded from the analyses, and the exclusion set followed Kjer (2004). Aligned data is available at http://insect3.agr.hokudai.ac.jp/psoco-web/data/.

Preliminary parsimony (MP) and neighbor-joining (NJ) analyses using PAUP* (Swofford, 2002) placed Zoraptera as the sister taxon of Diptera (flies). Diptera is a holometabolus order (insects with pupal stage) whereas Zoraptera is hemimetabolous (insects without pupal stage), so this result seems unlikely. As mentioned below, the basal branch leading to Zoraptera was very long as was the case for Diptera, and thus this result appeared to be an artifact of long-branch attraction (Felsenstein, 1978). Kjer (2004) also suggested that long-branch attraction was problematic for his MP analysis, with Diptera grouping outside of insect, as the sister taxon of Crustacea. In contrast to MP, the Bayesian tree recovered by Kjer (2004) was more reasonable. Likelihood analysis is thought to be less affected by long-branch attraction (Huelsenbeck, 1997; Huelsenbeck and Hillis, 1993). Therefore, we conducted further phylogenetic analyses using maximum likelihood (ML) in PAUP* (Swofford, 2002) and Bayesian ML in MrBayes (Huelsenbeck and Ronquist, 2001). The simplest model for ML analyses was determined by a hierarchic likelihood ratio test using Modeltest (Posada and Crandall, 1998). The GTR + I + G model was selected (unequal base frequencies: A = 0.2496, C = 0.2210, G = 0.2781, T = 0.2513; six substitution categories: A-C = 1.5445, A-G = 3.5713, A-T = 1.5224, C-G = 0.7884, C-T = 5.0195, G-T = 1; gamma distributions shape parameter = 0.6195; proportion of invariant sites = 0.1861). For ML analysis, the NJ tree was used as a starting tree and TBR branch swapping option was selected. For Bayesian analysis, we ran four chains for 10 million generations, and the tree was sampled every 1000 generations. By analyzing the change in likelihood score during the chain using Tracer (Rambaut and Drummond, 2004), we identified a suitable burn-in of 600,000 generations (Fig. 5). Therefore, the first 600 trees were excluded as burn-in, and we computed a 50% majority consensus tree of the remaining 9400

Table 1
Parameters from the Bayesian likelihood analysis under GTR + I + G model

	Kjer (2004)	Present
r(A ⟺ C)	1.52 ± 0.16	1.56 ± 0.27
r(A ⟺ G)	3.45 ± 0.27	3.57 ± 0.49
r(A ⟺ T)	1.23 ± 0.11	1.49 ± 0.22
r(C ⟺ G)	0.75 ± 0.08	0.69 ± 0.12
r(C ⟺ T)	5.17 ± 0.49	5.27 ± 0.67
r(G ⟺ T)	1.00 ± 0.00	1.00 ± 0.00
Alpha	0.58 ± 0.04	0.60 ± 0.05
Pinvar	0.17 ± 0.02	0.16 ± 0.04

Parameters obtained by Kjer (2004) are also indicated for comparison. Following abbreviations are used: r, substitution rates between the listed nucleotides; alpha, shape parameter of the gamma distribution; and pinvar, proportion of invariable sites.

trees to estimate posterior probabilities of branches in the tree Table 1.

For ML bootstrapping, the NJ tree was used as a starting tree, and the NNI branch swapping option was selected with 100 replicates. TBR branch swapping was not performed because it was computationally infeasible. However, as mentioned above, the tree obtained by NJ method was problematic, and preliminary analysis indicated that NNI branch swapping was not sufficient to escape from long-branch attraction caused by a number of problematic taxa: Diplura + Protura (Entognatha), Zoraptera (''Hemimetabola''), and Diptera (Holometabola) (Fig. 1). Therefore, to avoid long-branch attraction of these distantly related orders, monophyly of Insecta, Neoptera, and Holometabola were given as three constraints for ML bootstrapping. Monophyly of those higher level groups have previously received very strong support from morphological and molecular studies and are not controversial (Kjer, 2004; Kristensen, 1975, 1981; Wheeler et al., 2001). No constraints were given for ML and Bayesian tree searches.

3. Results

3.1. Sequences and data evaluation

We successfully amplified and sequenced the 18S rDNA gene from four species of Zoraptera. As mentioned below, the 18S of Zoraptera had large insertions (E23 sensu Wuyts et al., 2000) and modifications of secondary structure. However, all four *Zorotypus* 18S sequences obtained here, as well as sequences of *Z. hubbardi* obtained by Vawter (1991) and *Z. snyderi* obtained by Wheeler et al. (2001), could be readily aligned according to the secondary structure model for insect 18S (Kjer, 2004). As mentioned by Kjer (2004), our preliminary MP and NJ analyses (trees not shown) indicated that the 18S of *Z. hubbardi* analyzed by Vawter (1991) was not close to the other *Zorotypus* sequences, but was imbedded within an odonate (dragonflies) clade composed of the genera *Leucorrhinia*, *Sympetrum*, and *Celithemis* (Fig. 1). However, the 18S of *Z. snyderi* analyzed by Wheeler et al. (2001) was very similar to all the *Zorotypus* sequences obtained in the present study. For example, using MP and NJ analyses based only on the middle segment of 18S available for *Z. snyderi* (i.e., no missing data), a sister group relationship between *Z. snyderi* and *Z. hubbardi* and monophyly of Zoraptera were always recovered with 100% bootstrap support (trees not shown). The sequence AAAACTTA CCCGGCC, which appeared in the 18S of *Z. snyderi* near helix 36 and was considered by Kjer (2004) to be evidence of acarine contamination, was also detected in the newly sequenced samples, although the underlined bases were not A, T, and C but A,T, and A in other zorapterans (Fig. 4). As mentioned by Kjer (2004), when this sequence and the neighboring region was subjected to a BLAST search, the 18S of some Arachnida and Annelida were returned as the top three matches (September 23, 2004: Fig. 4). However, when the middle portion of these 18S sequences were aligned to the data set and analyzed by MP and NJ methods (trees not shown), the arachnid and annelid sequences were distant from Zoraptera and placed near the root of the tree.

In addition to this unusual short fragment, other unique characteristics were observed in the 18S sequences of Zoraptera. For example, helix 18 of Zoraptera could not be aligned to the other insect sequences, although the region was otherwise very conservative throughout insects. Analysis of secondary structure indicated that the shape of helix 18 in Zoraptera differed from that of other insects by having a longer stem and a very small hairpin loop (Fig. 3). Modifications of secondary structures were also identified in helix 12. Although the region was well aligned throughout insects including Zoraptera, the estimated secondary structures of helix 12 in Zoraptera were greatly modified from other insects (Fig. 2). Rather long insertions, ranging about 90–160 bp, were observed between helices E23-2 and E23-8 in Zoraptera. Such insertions were not observed in any other polyneopteran (orthopteroid insects: i.e., Neoptera excluding Paraneoptera and Holometabola) nor holometabolous orders, but were observed in some species of Paraneoptera (e.g., *Pedicinus* sp. had an 800 bp insertion). A very large insertion at E23 has also been reported for holometabolous Strepsiptera (twisted wings) by Gillespie et al. (in press), but sequences from this order are not analyzed in our study. Finally, Zoraptera was on a very long branch in the ML tree (Fig. 1), indicating an accelerated substitution rate of the 18S of Zoraptera.

3.2. Phylogenetic analyses

The trees obtained from our data set (Fig. 1) were generally in agreement with the tree obtained by Kjer

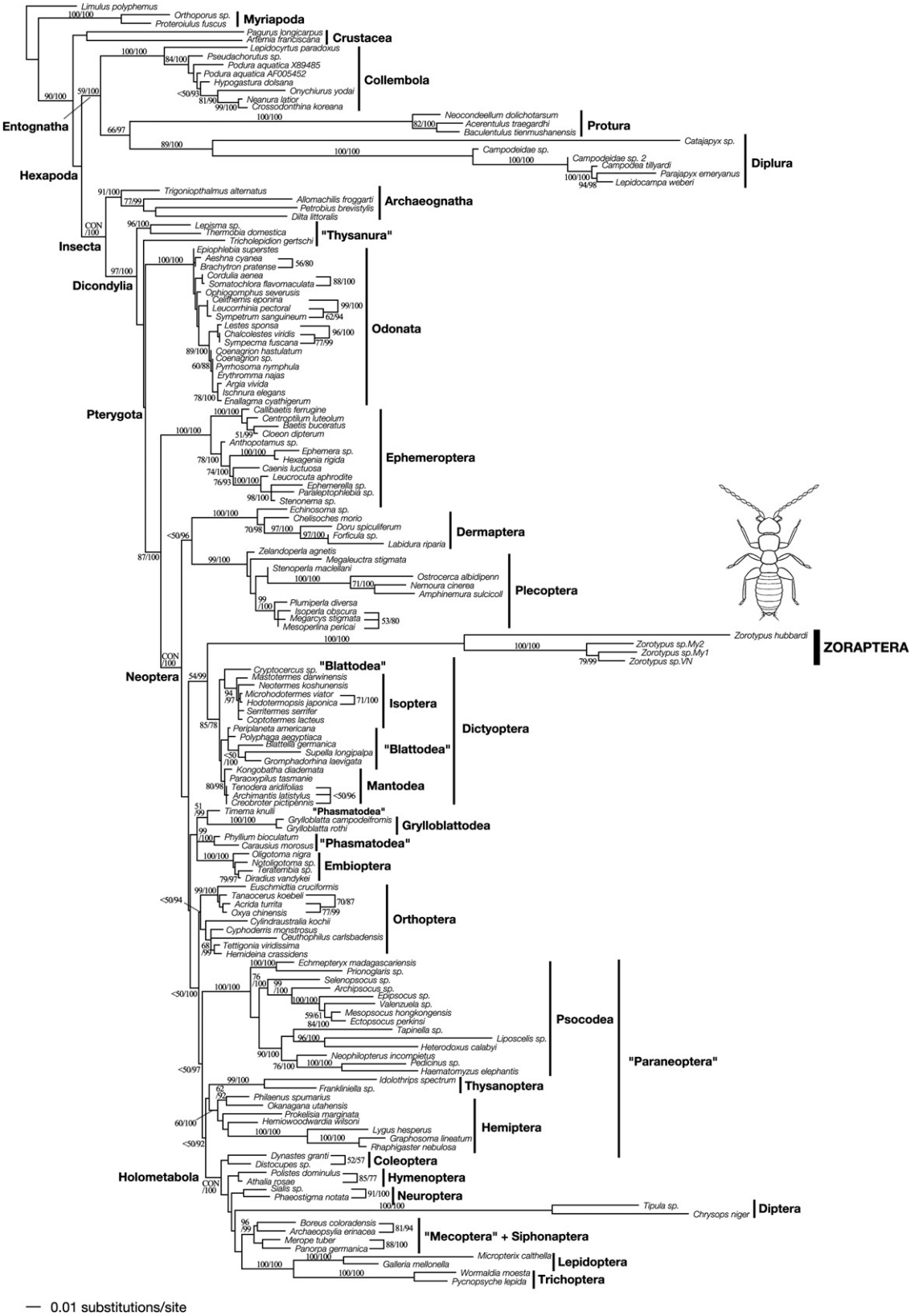

Fig. 1. Tree obtained by ML analysis of the 18S rDNA data ($-\ln L = 32543.23325$). The tree is rooted on *Limulus polyphemus* (horseshoe crab). Branch lengths are proportional to ML estimated branch lengths. The numbers associated with the nodes are bootstrap values or posterior probabilities obtained by ML/Bayes analyses. Bootstrap values higher than 50% and/or Bayesian posterior probabilities higher than 90% are indicated. Monophyly of Insecta, Neoptera and Holometabola are constrained for ML bootstrapping (indicated by CON). No constraints are given for tree searches.

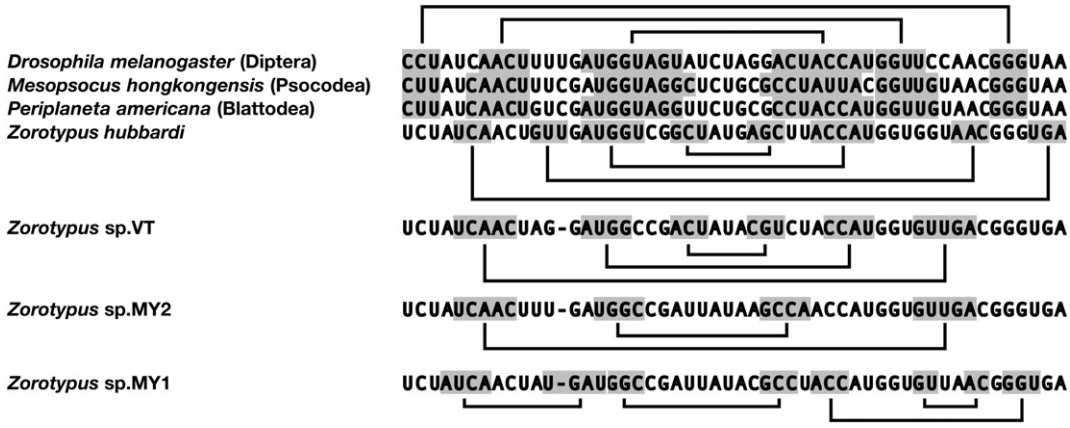

Fig. 2. Estimated secondary structure of helix 12 for selected samples. Stems are shaded, and complementary regions are connected by solid line.

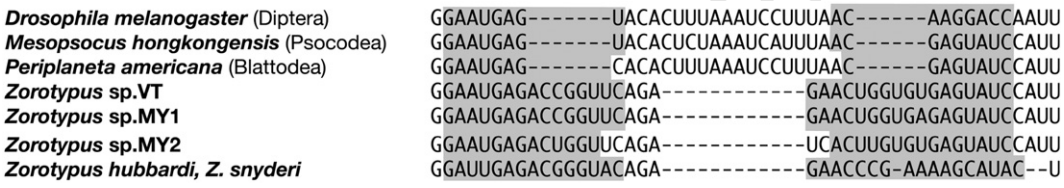

Fig. 3. Estimated secondary structure of helix 18 for selected samples. Stems are shaded. Secondary structure model follows Gutell (1993, 1994) and Gillespie et al. (in press). Their model employs some non-canonical pairs (such as G-A pairs), but Kjer (2004) did not follow Gutell model and employ canonical pairs only (A-T, G-C).

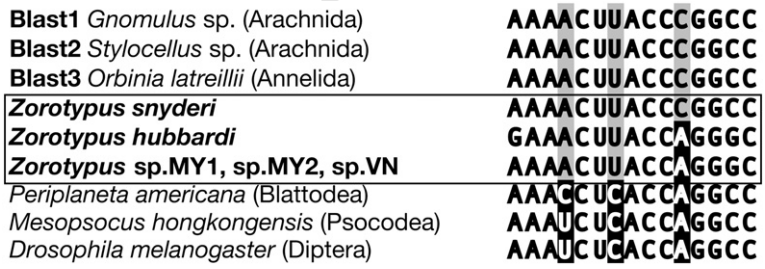

Fig. 4. Sequences near helix 36 for selected samples. Based on the character states at highlighted positions, Kjer (2004) concluded that the 18S sequence of *Z. snyderi* was an acarine contaminant. When this and neighboring regions of *Z. snyderi* were subjected to a BLAST search, the top three sequences (harvestmen spiders and an annelid) were identified as the closest matches.

(2004), which showed high congruence with the traditional classification of insect orders. However, four results from our analyses differed from Kjer's (2004) tree (outlined below).

(1) Monophyly of Pterygota (winged insects) was recovered by our ML analyses; in contrast, the tree obtained by Kjer (2004) placed wingless apterygote *Tricholepidon gertschi* (Thysanura: silver fish) as a sister taxon of Odonata (dragon flies). The ML bootstrap value for monophyly of Pterygota was low (<50%), and the present Bayesian consensus placed *T. gertschi* as a sister of Odonata with 54% posterior probability.

(2) Monophyly of Paraneoptera was not recovered by either ML or Bayesian analyses. Monophyly of Paraneoptera was recovered by Kjer (2004), but with low posterior probabilities (64–87%). The present results indicated a sister group relationship between Holometabola and Hemiptera + Thysanoptera. The posterior probability for non-monophyly of Paraneoptera was 92%, but the ML bootstrap value was lower than 50%.

(3) A close relationships between Holometabola and Paraneoptera and between Orthoptera (grasshoppers) and Paraneoptera + Holometabola were recovered by the present analyses as well as by Kjer (2004). Although the posterior probabilities

for these relationships obtained by Kjer (2004) were low (48–63% for Holometabola + Paraneoptera and 38–76% for Holometabola + Paraneoptera + Orthoptera), results from the present Bayesian analysis provided relatively strong support for these clades (97 and 100% posterior probability, respectively). In contrast, ML bootstrap support for the clades was lower than 50%.

(4) Two orders, Zoraptera and Thysanoptera (thrips), were not analyzed by Kjer (2004) but are newly added by our analyses. Zoraptera was always placed as the sister taxon of Dictyoptera (54% bootstrap and 99% posterior probability). Thysanoptera was placed as a sister taxon of Hemiptera (bugs, aphids, cicadas, etc.) which suggested monophyletic Condylognatha (Yoshizawa and Saigusa, 2001). However, support for the clade was low (<50% ML bootstrap and 72% Bayesian posterior probability).

4. Discussion

4.1. 18S of Zoraptera

Kjer (2004) suggested that the 18S sequence of *Zorotypus snyderi* analyzed by Wheeler et al. (2001) might be an acarine contaminant, at least in part. However, new obtained 18S sequences for four species of Zoraptera show a close match with the 18S of *Z. snyderi*. MP and NJ analyses based on the smaller fragment available for previously published sequences indicate that the 18S sequences from five zorapteran species compose a monophyletic group (100% bootstrap supports), and they are divided into two well supported clades: Oriental (*Z.* sp.MY1, *Z.* sp.MY2 and *Z.* sp.VN: 100% support) and North American species (*Z. snyderi* and *Z. hubbardi*: 100% support). In addition to the close match of the nucleotide sequences, all zorapteran sequences including *Z. snyderi* have indels at the same position of helix 18 (Fig. 3).

It is very unlikely that extractions from five species extracted in three different laboratories contain the same contaminant (*Z. snyderi* at lab of Wheeler and colleagues, USA, *Z. hubbardi* at Illinois Natural History Survey, USA and *Z.* sp.MY1, *Z.* sp.MY2 and *Z.* sp.VN at Hokkaido University, Japan). In addition, one of three base positions (Fig. 4), which was thought to be evidence of acarine contaminant by Kjer (2004), is variable within Zoraptera, and the nucleotide in that position in some zorapterans agrees with that of the other insects. The phylogenetic trees based on these 18S sequences placed Zoraptera within Neoptera, which is reasonable in light of morphological evidence (e.g., Kristensen, 1975, 1981). The intra-ordinal relationships of Zoraptera based on these sequences are also very reasonable, agreeing with morphological observations (the three Oriental species have an ovoid coil on the phallosome, which is lacking in the New World species; Engel and Grimaldi, 2000; New, 1978, 2000; Yoshizawa, pers. obs.). Therefore, we conclude that the zorapteran 18S sequence analyzed by Wheeler et al. (2001) is not a contaminant, and that the fragment near helix 36 in the 18S of Zoraptera is highly variable, which causes convergence with the acarine sequences, a possibility also considered by Kjer (2004).

In addition to the region near helix 36 (Fig. 4), the 18S of Zoraptera shows other unique characteristics, including an accelerated substitution rate (Fig. 1), modification of secondary structures (Figs. 2 and 3), and long insertions. These phenomena are uniquely and uniformly observed in all the 18S sequences of Zoraptera, and thus a correlated origin of these phenomena is likely. A correlation of unique molecular evolutionary trends is detected in the mitochondrial genomes of lice, which includes accelerated substitution rates, modifications of rRNA secondary structures, long insertions/deletions, increased GC contents, and genome rearrangements (Johnson et al., 2003; Page et al., 2002; Shao et al., 2003; Yoshizawa and Johnson, 2003). However, the forces that cause these trends in molecular evolution is less understood.

4.2. Phylogenetic analyses

Kjer (2004) showed that 18S sequences aligned according to a secondary structure model provide reasonable results for the phylogeny of insects. The results obtained by the present analyses are basically in agreement with Kjer (2004), so here we focus only on some novel findings or incongruence between our trees and Kjer (2004).

In the present analyses, a sister group relationship between Zoraptera and Dictyoptera is recovered by both ML and Bayesian methods. Zoraptera + Dictyoptera received 99% posterior probability. Morphologically, Zoraptera and Dictyoptera share a reduced pterothoracic phragmata and dorsolongitudinal muscles (Boudreaux, 1979) and a derived wing venation (Kukalová-Peck and Peck, 1993). Therefore, there is also some morphological support for this placement of Zoraptera.

Additional sequences of 18S for Thysanoptera are newly available for our broader study (Johnson et al., 2004). Morphologically, a close relationship between Thysanoptera and Hemiptera has been suggested (Kristensen, 1975, 1981; Yoshizawa and Saigusa, 2001, 2003). In contrast, the combined data set produced by direct optimization (Wheeler et al., 2001) recovered a sister relationship between Thysanoptera and Psocodea. Wheeler et al. (2001) mentioned that

there were no morphological apomorphies supporting Thysanoptera + Psocodea, but that the result had strong molecular support (10 transitions and 4 transversions in 18S). The 18S alignment analyzed here recovers a sister relationship between Thysanoptera and Hemiptera, whereas a sister relationship between Thysanoptera and Psocodea receives only 1% bootstrap support and 0.8% posterior probability. In addition, a sister relationship between Thysanoptera and Psocodea is not recovered by MP analysis. Therefore, it is evident that 18S has little phylogenetic signal supporting Thysanoptera + Psocodea, and the present results are congruent with morphological data.

While several of our results agree with morphological characters, incongruence between the present results and morphological characters occurs with respect to Paraneoptera. Monophyly of Paraneoptera is strongly supported by morphological autapomorphies such as modified mouth parts, derived wing base structures, a single abdominal ganglion, and the absence of cerci (Kristensen, 1975, 1981; Yoshizawa and Saigusa, 2001, 2003). However, the present results suggest a paraphyletic grade of Paraneoptera (i.e., Hemiptera + Thysanoptera sister to Holometabola) with posterior probability 92%. ML bootstrap support for the non-monophyly of Paraneoptera is very low (<50%). There is no morphological evidence published supporting Holometabola + (Hemiptera + Thysanoptera). Therefore, further evidence is needed to resolve whether Paraneoptera is monophyletic.

A close relationship between Orthoptera and Paraneoptera + Holometabola was recovered by our analyses as well as by Kjer (2004). However, this relationship is also unexpected from the morphological point of view. Although the support for this relationship obtained by Kjer (2004) was very weak (38–76% posterior probability), the present Bayesian analysis provides strong support for the clade (100% posterior probability). However, ML bootstrap support for the clade is very low (<50%). As far as we are aware, no one has ever suggested a close relationship between Orthoptera and Paraneoptera + Holometabola based on morphology.

Remarkable differences between bootstrap support and posterior probability are frequent at deep and short nodes as mentioned above. Recent analyses of empirical and simulated data sets revealed that posterior probability is excessively high and can provide erroneous conclusions more often (Cummings et al., 2003; Erixon et al., 2003; Simmons et al., 2004). Inflation of posterior probability is known to be especially frequent for short nodes (Alfaro et al., 2003; Lewis et al., in press). Nodes supporting Condylognatha + Holometabola and Orthoptera + Paraneoptera + Holometabola are very short, and bootstrap supports for these clades are very low (<50%) compared to high posterior probabilities (>92%). Therefore, further morphological and molecular data sets are required to test these clades. ML bootstrap support for Zoraptera + Dictyoptera is also relatively low (54%) in comparison to a high posterior probability (99%). However, the basal node supporting this sister relationship is not short, and is almost as long as, or even longer than the basal nodes of very well supported groups, such as Archaeognatha (91% bs, 100% pp), Neoptera (constrained for ML bootstrap, 100% pp), Holometabola (constrained for ML bootstrap, 100% pp) and Mecoptera + Siphonaptera (96% bs and 99% pp). Therefore, a different explanation may be required for the low ML bootstrap support for Zoraptera + Dictyoptera clade in comparison to a high posterior probability. One possible explanation is that NNI branch swapping was used for ML bootstrapping. As mentioned previously, a neighbor-joining starting tree for ML estimation was problematic especially for the placement of Zoraptera, and NNI branch swapping was not sufficient to escape from long-branch attraction problems. Because more thorough searches should more readily converge on the most likely tree, bootstrap support for some clades should be improved by the use of TRB branch swapping for ML bootstrapping. However, TBR is computationally infeasible for the present data set and processor power available.

The present result is based only on a single gene and thus represents gene tree. In addition, unusual characteristics of the zorapteran 18S sequences might be problematic for resolving the placement of this order. Thus, although the present result provides a very reasonable phylogenetic tree for insect orders and relatively strong support for a Zoraptera + Dictyoptera clade, it will be important to test the present results with additional morphological and molecular data.

4.3. Concluding comment

The 18S sequences aligned according to secondary structure model provide very reasonable insect phylogeny. It is especially notable that both Kjer (2004) and present analyses provided well resolved and very reasonable phylogenetic hypotheses among deep hexapod lineage (e.g., monophyly of Hexapoda), even though previous analyses of molecular data failed to provide a reasonable result (Bitsch et al., 2004). Samples of Zoraptera are newly analyzed, and a close relationship between Zoraptera and Dictyoptera is recovered by molecular data for the first time. In addition, examination of "molecular morphology" indicate unique evolutionary trends in the zorapteran 18S. Such interesting findings have also been provided for some insect ribosomal RNA by examinations of secondary structure (Ouvrard et al., 2000; Page et al., 2002; Yoshizawa and Johnson, 2003). Secondary structure-based manual alignment is valuable for both phylogenetic analyses and examinations of molecular morphology (Gillespie et al., in press; Kjer, 2004).

5. Uncited reference

Brodsky et al. (1992).

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Appendix A.

Additional taxa included in the present study. Species not listed here are from Kjer (2004). *Ectopsocus perkinsi* [*Ectopsocus* sp. of Johnson et al. (2004): re-identification based on DNA voucher by KY] and *Pedicinus* sp. are replacement samples of Ectopsocidae Gen. sp. and *Pediculus humanus* of Kjer (2004), respectively. *Zorotypus snyderi* and *Drosophila melanogaster* were included only for the analysis of 18S secondary structure and/or preliminary phylogenetic inference.

Order	Family	Species	GenBank accession number
Zoraptera	Zorotypidae	*Zorotypus snyderi*	AF372432
Zoraptera	Zorotypidae	*Zorotypus hubbardi*	DQ013288
Zoraptera	Zorotypidae	*Zorotypus* sp.MY1	DQ013289
Zoraptera	Zorotypidae	*Zorotypus* sp.MY2	DQ013291
Zoraptera	Zorotypidae	*Zorotypus* sp.VN	DQ013290
Phasmatodea	Timematidae	*Timema knulli*	AF423806
Blattodea	Polyphagidae	*Polyphaga aegyptiaca*	AF220575
Blattodea	Blattellidae	*Blattella germanica*	AF220573
Blattodea	Blattellidae	*Supella longipalpa*	AY491149
Blattodea	Blaberidae	*Gromphadrhina laevigata*	AY210820
Embioptera	Notoligotomidae	*Notoligotoma* sp.	AY338693
Psocodea	Prionoglarididae	*Prionoglaris* sp.	AY630456
Psocodea	Lepidopsocidae	*Echmepteryx madagascariensis*	AY630447
Psocodea	Troctopsocidae	*Selenopsocus* sp.	AY630457
Psocodea	Archipsocidae	*Archipsocus* sp.	AY630479
Psocodea	Epipsocidae	*Epipsocus* sp.	AY630539
Psocodea	Ectopsocidae	*Ectopsocus perkinsi*	AY630510
Psocodea	Mesopsocidae	*Mesopsocus hongkongensis*	AY630516
Psocodea	Pachytroctidae	*Tapinella* sp.	AY630466
Psocodea	Pedicinidae	*Pedicinus* sp.	AY077777
Thysanoptera	Thripidae	*Frankliniella* sp.	AY630445
Thysanoptera	Phlaeothripidae	*Idolothrips spectrum*	AY630443
Diptera	Drosophilidae	*Drosophila melanogaster*	M21017

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