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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.

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**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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55 support from molecular data for nodes is small, conclu-  
56 sions from molecular data by direct optimization would  
57 be highly dependent on some combination of (1) mor-  
58 phological data, (2) noise from the homoplastic data,  
59 and (3) arbitrarily optimized homology of unalignable  
60 data (see also Kjer, 1995 and Simmons, 2004).

61 To address the problems of direct optimization, Kjer  
62 (2004) conducted phylogenetic analyses of insect orders  
63 based on 18S sequences aligned manually according to  
64 secondary structure. The resulting tree matched tradi-  
65 tional insect classification reasonably well. However,  
66 Kjer's (2004) study lacked a sequence of Zoraptera  
67 and thus could not address the phylogenetic position  
68 of this order. Part of the reason for the exclusion of  
69 Zoraptera by Kjer was that he concluded that the 18S  
70 of Zoraptera presented in Wheeler et al. (2001) was  
71 either contaminated in part by mite (Acari) DNA  
72 sequences, because of a homologous unique sequence  
73 shared by the Zoraptera and mites, or that if the zorapt-  
74 eran sequence was not a contaminant, it was highly  
75 autapomorphic and problematic.

76 Thus, a more detailed molecular test of the phyloge-  
77 netic position of Zoraptera is needed. The 18S rDNA  
78 gene has played an important role in resolving the deep  
79 phylogeny of insects (Campbell et al., 1995; Johnson  
80 et al., 2004; Kjer, 2004; Whiting et al., 1997). However,  
81 a correct 18S sequence of Zoraptera may not be avail-  
82 able to date. In the present study, we amplified and ana-  
83 lyzed the 18S rDNA of Zoraptera using samples  
84 collected in the USA, Malaysia, and Vietnam. These  
85 sequences of Zoraptera plus additional sequences of  
86 Blattodea (cockroaches), Phasmatodea (stick insects),  
87 Embioptera, and Paraneoptera were aligned with the  
88 18S data provided by Kjer (2004). We address two ques-  
89 tions: (1) is the 18S sequence of Zoraptera used by  
90 Wheeler et al. (2001) really a contaminant and (2) what  
91 is the closest relative of Zoraptera?

## 92 2. Materials and methods

93 We sequenced four species of Zoraptera, *Zorotypus*  
94 *hubbardi* from the USA, *Z. sp.MY1* and *Z. sp.MY2*  
95 from Malaysia, and *Z. sp.VN* from Vietnam (the latter  
96 three species are currently being described). Methods  
97 of total DNA extraction and 18S amplification and  
98 sequencing followed Johnson et al. (2004). Primer sets  
99 used were Ns1-Ns2a (Barker et al., 2003), 18Sai-18Sbi  
100 (Whiting et al., 1997), and Ns5aP2-Ns8P (Johnson  
101 et al., 2004). The 18S sequence of *Z. snyderi* was ob-  
102 tained from GenBank and was only used to check  
103 whether the 18S sequence of the species was contami-  
104 nant or not. The sequence was not used for phylogenetic  
105 analyses because only a short piece of the 18S sequence  
106 was available for this species. Additional 18S sequences  
107 of Blattodea, Phasmatodea, Embioptera, Psocodea,

Thysanoptera, and Hemiptera were obtained from Gen- 108  
Bank (Appendix A). These sequences were manually 109  
aligned to the data matrix provided by Kjer (2004) 110  
according to the secondary structure model presented 111  
on his website. When we detected a modification of 112  
the secondary structure in the new sequences, the sec- 113  
ondary structure of the region was estimated using 114  
GeneBee (Brodsky et al., 1995). Except for these addi- 115  
tional samples, the taxon set was largely unchanged 116  
from Kjer (2004). However, we replaced sequences of 117  
Ectopsocidae Gen. sp. and *Pthirus pubis* with *Ectopsocus* 118  
*perkinsi* and *Pedicinus* sp., respectively, because only a 119  
short piece of 18S sequence was available for the former 120  
two species (Appendix A). Unalignable regions were 121  
excluded from the analyses, and the exclusion set 122  
followed Kjer (2004). Aligned data is available at 123  
<http://insect3.agr.hokudai.ac.jp/psoco-web/data/>. 124

125 Preliminary parsimony (MP) and neighbor-joining  
126 (NJ) analyses using PAUP\* (Swofford, 2002) placed  
127 Zoraptera as the sister taxon of Diptera (flies). Diptera  
128 is a holometabolus order (insects with pupal stage)  
129 whereas Zoraptera is hemimetabolous (insects without  
130 pupal stage), so this result seems unlikely. As men-  
131 tioned below, the basal branch leading to Zoraptera  
132 was very long as was the case for Diptera, and thus  
133 this result appeared to be an artifact of long-branch  
134 attraction (Felsenstein, 1978). Kjer (2004) also suggest-  
135 ed that long-branch attraction was problematic for his  
136 MP analysis, with Diptera grouping outside of insect,  
137 as the sister taxon of Crustacea. In contrast to MP,  
138 the Bayesian tree recovered by Kjer (2004) was more  
139 reasonable. Likelihood analysis is thought to be less  
140 affected by long-branch attraction (Huelsenbeck,  
141 1997; Huelsenbeck and Hillis, 1993). Therefore, we  
142 conducted further phylogenetic analyses using maxi-  
143 mum likelihood (ML) in PAUP\* (Swofford, 2002)  
144 and Bayesian ML in MrBayes (Huelsenbeck and  
145 Ronquist, 2001). The simplest model for ML analyses  
146 was determined by a hierarchic likelihood ratio test  
147 using Modeltest (Posada and Crandall, 1998). The  
148 GTR + I + G model was selected (unequal base fre-  
149 quencies: A = 0.2496, C = 0.2210, G = 0.2781,  
150 T = 0.2513; six substitution categories: A-C = 1.5445,  
151 A-G = 3.5713, A-T = 1.5224, C-G = 0.7884, C-T =  
152 5.0195, G-T = 1; gamma distributions shape param-  
153 eter = 0.6195; proportion of invariant sites = 0.1861).  
154 For ML analysis, the NJ tree was used as a starting  
155 tree and TBR branch swapping option was selected.  
156 For Bayesian analysis, we ran four chains for 10 mil-  
157 lion generations, and the tree was sampled every 1000  
158 generations. By analyzing the change in likelihood  
159 score during the chain using Tracer (Rambaut and  
160 Drummond, 2004), we identified a suitable burn-in of  
161 600,000 generations (Fig. 5). Therefore, the first 600  
162 trees were excluded as burn-in, and we computed a  
163 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.

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

166 For ML bootstrapping, the NJ tree was used as a  
167 starting tree, and the NNI branch swapping option was  
168 selected with 100 replicates. TBR branch swapping was  
169 not performed because it was computationally infeasible.  
170 However, as mentioned above, the tree obtained by NJ  
171 method was problematic, and preliminary analysis indi-  
172 cated that NNI branch swapping was not sufficient to es-  
173 cape from long-branch attraction caused by a number of  
174 problematic taxa: Diplura + Protura (Entognatha),  
175 Zoraptera (“Hemimetabola”), and Diptera (Holometab-  
176 bola) (Fig. 1). Therefore, to avoid long-branch attraction  
177 of these distantly related orders, monophyly of Insecta,  
178 Neoptera, and Holometabola were given as three con-  
179 straints for ML bootstrapping. Monophyly of those  
180 higher level groups have previously received very strong  
181 support from morphological and molecular studies and  
182 are not controversial (Kjer, 2004; Kristensen, 1975,  
183 1981; Wheeler et al., 2001). No constraints were given  
184 for ML and Bayesian tree searches.

185 **3. Results**

186 *3.1. Sequences and data evaluation*

187 We successfully amplified and sequenced the 18S  
188 rDNA gene from four species of Zoraptera. As men-  
189 tioned below, the 18S of Zoraptera had large insertions  
190 (E23 sensu Wuyts et al., 2000) and modifications of  
191 secondary structure. However, all four *Zorotypus* 18S  
192 sequences obtained here, as well as sequences of  
193 *Z. hubbardi* obtained by Vawter (1991) and *Z. snyderi*  
194 obtained by Wheeler et al. (2001), could be readily  
195 aligned according to the secondary structure model for  
196 insect 18S (Kjer, 2004). As mentioned by Kjer (2004),  
197 our preliminary MP and NJ analyses (trees not shown)  
198 indicated that the 18S of *Z. hubbardi* analyzed by  
199 Vawter (1991) was not close to the other *Zorotypus*

sequences, but was imbedded within an odonate (drag- 200  
onflies) clade composed of the genera *Leucorrhinia*, 201  
*Sympetrum*, and *Celithemis* (Fig. 1). However, the 18S 202  
of *Z. snyderi* analyzed by Wheeler et al. (2001) was very 203  
similar to all the *Zorotypus* sequences obtained in the 204  
present study. For example, using MP and NJ analyses 205  
based only on the middle segment of 18S available for *Z.* 206  
*snyderi* (i.e., no missing data), a sister group relationship 207  
between *Z. snyderi* and *Z. hubbardi* and monophyly of 208  
Zoraptera were always recovered with 100% bootstrap 209  
support (trees not shown). The sequence AAAACTTA 210  
CCCGGCC, which appeared in the 18S of *Z. snyderi* 211  
near helix 36 and was considered by Kjer (2004) to be 212  
evidence of acarine contamination, was also detected 213  
in the newly sequenced samples, although the underlined 214  
bases were not A, T, and C but A,T, and A in other 215  
zorapterans (Fig. 4). As mentioned by Kjer (2004), when 216  
this sequence and the neighboring region was subjected 217  
to a BLAST search, the 18S of some Arachnida and 218  
Annelida were returned as the top three matches (Sep- 219  
tember 23, 2004: Fig. 4). However, when the middle por- 220  
tion of these 18S sequences were aligned to the data set 221  
and analyzed by MP and NJ methods (trees not shown), 222  
the arachnid and annelid sequences were distant from 223  
Zoraptera and placed near the root of the tree. 224

In addition to this unusual short fragment, other un- 225  
ique characteristics were observed in the 18S sequences 226  
of Zoraptera. For example, helix 18 of Zoraptera could 227  
not be aligned to the other insect sequences, although 228  
the region was otherwise very conservative throughout 229  
insects. Analysis of secondary structure indicated that 230  
the shape of helix 18 in Zoraptera differed from that of 231  
other insects by having a longer stem and a very small 232  
hairpin loop (Fig. 3). Modifications of secondary struc- 233  
tures were also identified in helix 12. Although the region 234  
was well aligned throughout insects including Zoraptera,  
the estimated secondary structures of helix 12 in Zorap- 235  
tera were greatly modified from other insects (Fig. 2). 236  
Rather long insertions, ranging about 90–160 bp, were 237  
observed between helices E23-2 and E23-8 in Zoraptera. 238  
Such insertions were not observed in any other polyne- 239  
opteran (orthopteroid insects: i.e., Neoptera excluding 240  
Paraneoptera and Holometabola) nor holometabolous 241  
orders, but were observed in some species of Paraneop- 242  
tera (e.g., *Pedicinus* sp. had an 800 bp insertion). A very 243  
large insertion at E23 has also been reported for holome- 244  
tabolous Strepsiptera (twisted wings) by Gillespie et al. 245  
(in press), but sequences from this order are not analyzed 246  
in our study. Finally, Zoraptera was on a very long 247  
branch in the ML tree (Fig. 1), indicating an accelerated 248  
substitution rate of the 18S of Zoraptera. 249  
250

251 *3.2. Phylogenetic analyses*

The trees obtained from our data set (Fig. 1) were 252  
generally in agreement with the tree obtained by Kjer 253

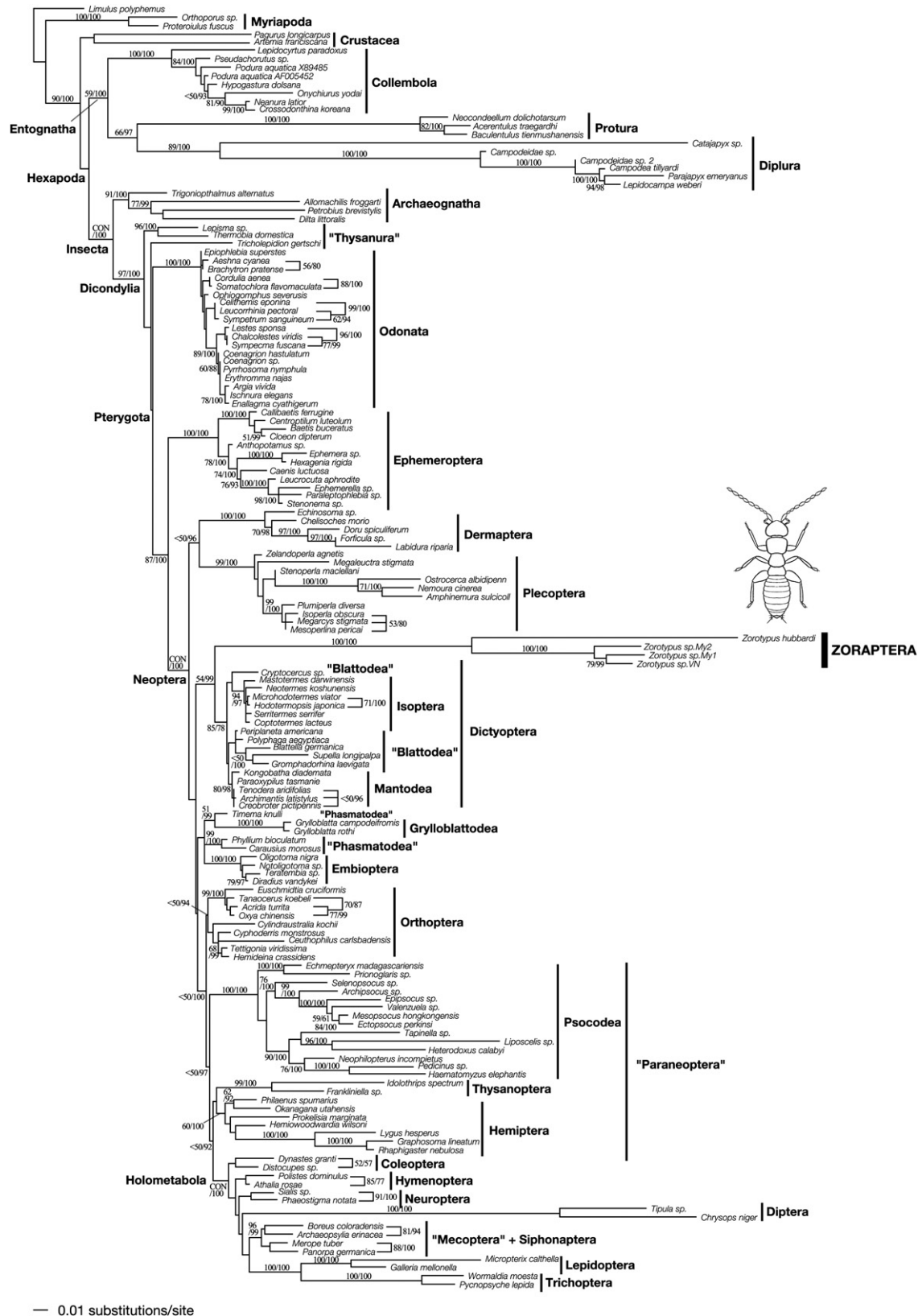


Fig. 1. Tree obtained by ML analysis of the 18S rDNA data ( $-\ln L = 32543.23325$ ). The tree is rooted on *Limulus polyphemus* (horseshoe crab). Branch lengths are proportional to ML estimated branch lengths. The numbers associated with the nodes are bootstrap values or posterior probabilities obtained 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.

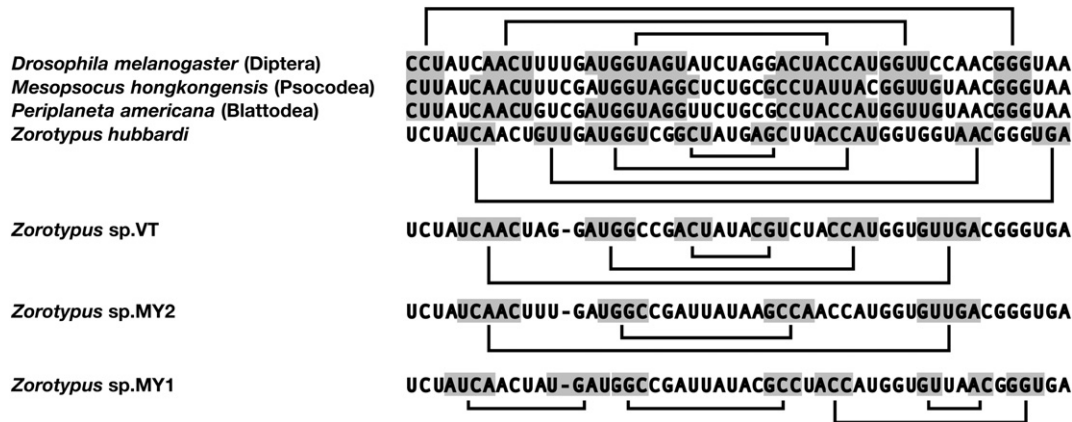


Fig. 2. Estimated secondary structure of helix 12 for selected samples. Stems are shaded, and complementary regions are connected by solid line.

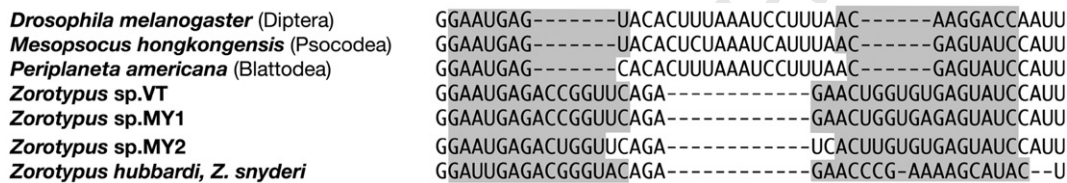


Fig. 3. Estimated secondary structure of helix 18 for selected samples. Stems are shaded. Secondary structure model follows Gutell (1993, 1994) and Gillespie et al. (in press). Their model employs some non-canonical pairs (such as G-A pairs), but Kjer (2004) did not follow Gutell model and employ canonical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

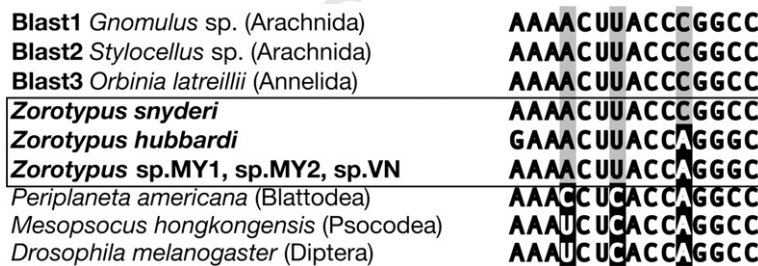


Fig. 4. Sequences near helix 36 for selected samples. Based on the character states at highlighted positions, Kjer (2004) concluded that the 18S sequence of *Z. snyderi* was an acarine contaminant. When this and neighboring regions of *Z. snyderi* were subjected to a BLAST search, the top three sequences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

254 (2004), which showed high congruence with the tradi- 267  
 255 tional classification of insect orders. However, four re- 268  
 256 sults from our analyses differed from Kjer's (2004) tree 269  
 257 (outlined below). 270

258 (1) Monophyly of Pterygota (winged insects) was 271  
 259 recovered by our ML analyses; in contrast, the tree 272  
 260 obtained by Kjer (2004) placed wingless aptery- 273  
 261 gote *Tricholepidon gertschi* (Thysanura: silver fish) 274  
 262 as a sister taxon of Odonata (dragon flies). The 275  
 263 ML bootstrap value for monophyly of Pterygota 276  
 264 was low (<50%), and the present Bayesian consen- 277  
 265 sus placed *T. gertschi* as a sister of Odonata with 278  
 266 54% posterior probability. 279

(2) Monophyly of Paraneoptera was not recovered by 267  
 either ML or Bayesian analyses. Monophyly of 268  
 Paraneoptera was recovered by Kjer (2004), but 269  
 with low posterior probabilities (64–87%). The 270  
 present results indicated a sister group relationship 271  
 between Holometabola and Hemiptera + Thysa- 272  
 noptera. The posterior probability for non-mono- 273  
 phyly of Paraneoptera was 92%, but the ML 274  
 bootstrap value was lower than 50%. 275  
 (3) A close relationships between Holometabola and 276  
 Paraneoptera and between Orthoptera (grasshop- 277  
 pers) and Paraneoptera + Holometabola were 278  
 recovered by the present analyses as well as by 279  
 Kjer (2004). Although the posterior probabilities 280

281 for these relationships obtained by Kjer (2004)  
 282 were low (48–63% for Holometabola + Paraneop-  
 283 tera and 38–76% for Holometabola + Paraneop-  
 284 tera + Orthoptera), results from the present  
 285 Bayesian analysis provided relatively strong sup-  
 286 port for these clades (97 and 100% posterior prob-  
 287 ability, respectively). In contrast, ML bootstrap  
 288 support for the clades was lower than 50%.

289 (4) Two orders, Zoraptera and Thysanoptera (thrips),  
 290 were not analyzed by Kjer (2004) but are newly  
 291 added by our analyses. Zoraptera was always  
 292 placed as the sister taxon of Dictyoptera (54%  
 293 bootstrap and 99% posterior probability). Thysa-  
 294 noptera was placed as a sister taxon of Hemiptera  
 295 (bugs, aphids, cicadas, etc.) which suggested  
 296 monophyletic Condylgnatha (Yoshizawa and  
 297 Saigusa, 2001). However, support for the clade  
 298 was low (<50% ML bootstrap and 72% Bayesian  
 299 posterior probability).

300

## 301 4. Discussion

### 302 4.1. 18S of Zoraptera

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

319 It is very unlikely that extractions from five species  
 320 extracted in three different laboratories contain the same  
 321 contaminant (*Z. snyderi* at lab of Wheeler and col-  
 322 leagues, USA, *Z. hubbardi* at Illinois Natural History  
 323 Survey, USA and *Z. sp.MY1*, *Z. sp.MY2* and *Z. sp.VN*  
 324 at Hokkaido University, Japan). In addition, one of  
 325 three base positions (Fig. 4), which was thought to be  
 326 evidence of acarine contaminant by Kjer (2004), is var-  
 327 iable within Zoraptera, and the nucleotide in that posi-  
 328 tion in some zorapterans agrees with that of the other  
 329 insects. The phylogenetic trees based on these 18S  
 330 sequences placed Zoraptera within Neoptera, which is  
 331 reasonable in light of morphological evidence (e.g.,  
 332 Kristensen, 1975, 1981). The intra-ordinal relationships

of Zoraptera based on these sequences are also very rea- 333  
 sonable, agreeing with morphological observations (the 334  
 three Oriental species have an ovoid coil on the phallo- 335  
 some, which is lacking in the New World species; Engel 336  
 and Grimaldi, 2000; New, 1978, 2000; Yoshizawa, pers. 337  
 obs.). Therefore, we conclude that the zorapteran 18S 338  
 sequence analyzed by Wheeler et al. (2001) is not a con- 339  
 taminant, and that the fragment near helix 36 in the 18S 340  
 of Zoraptera is highly variable, which causes conver- 341  
 gence with the acarine sequences, a possibility also 342  
 considered by Kjer (2004). 343

In addition to the region near helix 36 (Fig. 4), the 344  
 18S of Zoraptera shows other unique characteristics, 345  
 including an accelerated substitution rate (Fig. 1), 346  
 modification of secondary structures (Figs. 2 and 3), 347  
 and long insertions. These phenomena are uniquely 348  
 and uniformly observed in all the 18S sequences of 349  
 Zoraptera, and thus a correlated origin of these phe- 350  
 nomena is likely. A correlation of unique molecular evo- 351  
 lutionary trends is detected in the mitochondrial 352  
 genomes of lice, which includes accelerated substitution 353  
 rates, modifications of rRNA secondary structures, long 354  
 insertions/deletions, increased GC contents, and genome 355  
 rearrangements (Johnson et al., 2003; Page et al., 356  
 2002; Shao et al., 2003; Yoshizawa and Johnson, 357  
 2003). However, the forces that cause these trends in 358  
 molecular evolution is less understood. 359

### 4.2. Phylogenetic analyses 360

Kjer (2004) showed that 18S sequences aligned 361  
 according to a secondary structure model provide rea- 362  
 sonable results for the phylogeny of insects. The results 363  
 obtained by the present analyses are basically in agree- 364  
 ment with Kjer (2004), so here we focus only on some 365  
 novel findings or incongruence between our trees and 366  
 Kjer (2004). 367

In the present analyses, a sister group relationship 368  
 between Zoraptera and Dictyoptera is recovered by 369  
 both ML and Bayesian methods. Zoraptera + Dictyop- 370  
 tera received 99% posterior probability. Morphologi- 371  
 cally, Zoraptera and Dictyoptera share a reduced 372  
 pterothoracic phragmata and dorsolongitudinal mus- 373  
 cles (Boudreaux, 1979) and a derived wing venation 374  
 (Kukalová-Peck and Peck, 1993). Therefore, there is 375  
 also some morphological support for this placement 376  
 of Zoraptera. 377

Additional sequences of 18S for Thysanoptera are 378  
 newly available for our broader study (Johnson 379  
 et al., 2004). Morphologically, a close relationship be- 380  
 tween Thysanoptera and Hemiptera has been suggest- 381  
 ed (Kristensen, 1975, 1981; Yoshizawa and Saigusa, 382  
 2001, 2003). In contrast, the combined data set pro- 383  
 duced by direct optimization (Wheeler et al., 2001) 384  
 recovered a sister relationship between Thysanoptera 385  
 and Psocodea. Wheeler et al. (2001) mentioned that 386

387 there were no morphological apomorphies supporting  
 388 Thysanoptera + Psocodea, but that the result had  
 389 strong molecular support (10 transitions and 4 trans-  
 390 versions in 18S). The 18S alignment analyzed here  
 391 recovers a sister relationship between Thysanoptera  
 392 and Hemiptera, whereas a sister relationship between  
 393 Thysanoptera and Psocodea receives only 1% boot-  
 394 strap support and 0.8% posterior probability. In addi-  
 395 tion, a sister relationship between Thysanoptera and  
 396 Psocodea is not recovered by MP analysis. Therefore,  
 397 it is evident that 18S has little phylogenetic signal  
 398 supporting Thysanoptera + Psocodea, and the present  
 399 results are congruent with morphological data.

400 While several of our results agree with morphological  
 401 characters, incongruence between the present results and  
 402 morphological characters occurs with respect to Para-  
 403 neoptera. Monophyly of Paraneoptera is strongly sup-  
 404 ported by morphological autapomorphies such as  
 405 modified mouth parts, derived wing base structures, a  
 406 single abdominal ganglion, and the absence of cerci  
 407 (Kristensen, 1975, 1981; Yoshizawa and Saigusa, 2001,  
 408 2003). However, the present results suggest a paraphy-  
 409 letic grade of Paraneoptera (i.e., Hemiptera + Thysa-  
 410 noptera sister to Holometabola) with posterior  
 411 probability 92%. ML bootstrap support for the non-  
 412 monophyly of Paraneoptera is very low (<50%). There  
 413 is no morphological evidence published supporting  
 414 Holometabola + (Hemiptera + Thysanoptera). There-  
 415 fore, further evidence is needed to resolve whether  
 416 Paraneoptera is monophyletic.

417 A close relationship between Orthoptera and  
 418 Paraneoptera + Holometabola was recovered by our  
 419 analyses as well as by Kjer (2004). However, this rela-  
 420 tionship is also unexpected from the morphological point  
 421 of view. Although the support for this relationship ob-  
 422 tained by Kjer (2004) was very weak (38–76% posterior  
 423 probability), the present Bayesian analysis provides  
 424 strong support for the clade (100% posterior probabili-  
 425 ty). However, ML bootstrap support for the clade is very  
 426 low (<50%). As far as we are aware, no one has ever sug-  
 427 gested a close relationship between Orthoptera and Para-  
 428 neoptera + Holometabola based on morphology.

429 Remarkable differences between bootstrap support  
 430 and posterior probability are frequent at deep and short  
 431 nodes as mentioned above. Recent analyses of empirical  
 432 and simulated data sets revealed that posterior probabili-  
 433 ty is excessively high and can provide erroneous conclu-  
 434 sions more often (Cummings et al., 2003; Erixon et al.,  
 435 2003; Simmons et al., 2004). Inflation of posterior prob-  
 436 ability is known to be especially frequent for short nodes  
 437 (Alfaro et al., 2003; Lewis et al., in press). Nodes sup-  
 438 porting Condylgnatha + Holometabola and Orthop-  
 439 tera + Paraneoptera + Holometabola are very short,  
 440 and bootstrap supports for these clades are very low  
 441 (<50%) compared to high posterior probabilities  
 442 (>92%). Therefore, further morphological and molecu-

lar data sets are required to test these clades. ML boot-  
 strap 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 sup-  
 ported 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 re-  
 quired for the low ML bootstrap support for Zorapter-  
 a + 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 sup-  
 port 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 charac-  
 teristics of the zorapteran 18S sequences might be prob-  
 lematic 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 phyloge-  
 ny. It is especially notable that both Kjer (2004) and pres-  
 ent 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 new-  
 ly analyzed, and a close relationship between Zoraptera  
 and Dictyoptera is recovered by molecular data for the  
 first time. In addition, examination of “molecular mor-  
 phology” indicate unique evolutionary trends in the  
 zorapteran 18S. Such interesting findings have also been  
 provided for some insect ribosomal RNA by examina-  
 tions 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).

497 **5. Uncited reference**

498 Brodsky et al. (1992).

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

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