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

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9 Abstract

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

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

21 1. Introduction

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

33 Based on morphological characters, the order Zorap-
34 tera is thought to be closely related to either Paraneop-
35 tera (= hemipteroid orders: bugs, thrips, booklice, and

lice: Hennig, 1981; Kristensen, 1975, 1981; Wheeler 36
et al., 2001), Dictyoptera (= blattoid orders: cockroach- 37
es, termites, and mantis: Boudreaux, 1979; Kukalová- 38
Peck and Peck, 1993; Smithers, 1991) or Embioptera 39
(= web spinners: Engel and Grimaldi, 2000; Minet and 40
Bourgoin, 1986). Combined morphological and molecu- 41
lar analysis by Wheeler et al. (2001) supported a close 42
relationship between Zoraptera and Dictyoptera. How- 43
ever, separate analysis of molecular data (18S rDNA) 44
placed Zoraptera as the sister taxon of Psocodea (book- 45
lice and parasitic lice), conflicting with combined tree 46
(Wheeler et al., 2001). 47

Separate analyses of 18S data (Wheeler et al., 2001) 48
resulted in tree with a very unconventional placement 49
of some insect orders (e.g., Diplura and Grylloblattodea 50
were imbedded within Holometabola). Wheeler et al. 51
(2001) used direct optimization of morphological and 52
molecular data, which minimizes incongruence between 53
two data partitions. Kjer (2004) pointed out that, when 54

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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*
perkinsi and *Pedicinus* sp., respectively, because only a 118
short piece of 18S sequence was available for the former 119
two species (Appendix A). Unalignable regions were 120
excluded from the analyses, and the exclusion set 121
followed Kjer (2004). Aligned data is available at 122
<http://insect3.agr.hokudai.ac.jp/psoco-web/data/>. 123
124

Preliminary parsimony (MP) and neighbor-joining 125
(NJ) analyses using PAUP* (Swofford, 2002) placed 126
Zoraptera as the sister taxon of Diptera (flies). Diptera 127
is a holometabolus order (insects with pupal stage) 128
whereas Zoraptera is hemimetabolous (insects without 129
pupal stage), so this result seems unlikely. As men- 130
tioned below, the basal branch leading to Zoraptera 131
was very long as was the case for Diptera, and thus 132
this result appeared to be an artifact of long-branch 133
attraction (Felsenstein, 1978). Kjer (2004) also suggest- 134
ed that long-branch attraction was problematic for his 135
MP analysis, with Diptera grouping outside of insect, 136
as the sister taxon of Crustacea. In contrast to MP, 137
the Bayesian tree recovered by Kjer (2004) was more 138
reasonable. Likelihood analysis is thought to be less 139
affected by long-branch attraction (Huelsenbeck, 140
1997; Huelsenbeck and Hillis, 1993). Therefore, we 141
conducted further phylogenetic analyses using maxi- 142
mum likelihood (ML) in PAUP* (Swofford, 2002) 143
and Bayesian ML in MrBayes (Huelsenbeck and 144
Ronquist, 2001). The simplest model for ML analyses 145
was determined by a hierarchic likelihood ratio test 146
using Modeltest (Posada and Crandall, 1998). The 147
GTR + I + G model was selected (unequal base fre- 148
quencies: A = 0.2496, C = 0.2210, G = 0.2781, 149
T = 0.2513; six substitution categories: A-C = 1.5445, 150
A-G = 3.5713, A-T = 1.5224, C-G = 0.7884, C-T = 151
5.0195, G-T = 1; gamma distributions shape param- 152
eter = 0.6195; proportion of invariant sites = 0.1861). 153
For ML analysis, the NJ tree was used as a starting 154
tree and TBR branch swapping option was selected. 155
For Bayesian analysis, we ran four chains for 10 mil- 156
lion generations, and the tree was sampled every 1000 157
generations. By analyzing the change in likelihood 158
score during the chain using Tracer (Rambaut and 159
Drummond, 2004), we identified a suitable burn-in of 160
600,000 generations (Fig. 5). Therefore, the first 600 161
trees were excluded as burn-in, and we computed a 162
50% majority consensus tree of the remaining 9400 163

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,
235 the estimated secondary structures of helix 12 in Zorap-
236 tera were greatly modified from other insects (Fig. 2).
237 Rather long insertions, ranging about 90–160 bp, were
238 observed between helices E23-2 and E23-8 in Zoraptera.
239 Such insertions were not observed in any other polyne-
240 opteran (orthopteroid insects: i.e., Neoptera excluding
241 Paraneoptera and Holometabola) nor holometabolous
242 orders, but were observed in some species of Paraneop-
243 tera (e.g., *Pedicinus* sp. had an 800 bp insertion). A very
244 large insertion at E23 has also been reported for holome-
245 tabolous Strepsiptera (twisted wings) by Gillespie et al.
246 (in press), but sequences from this order are not analyzed
247 in our study. Finally, Zoraptera was on a very long
248 branch in the ML tree (Fig. 1), indicating an accelerated
249 substitution rate of the 18S of Zoraptera. 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

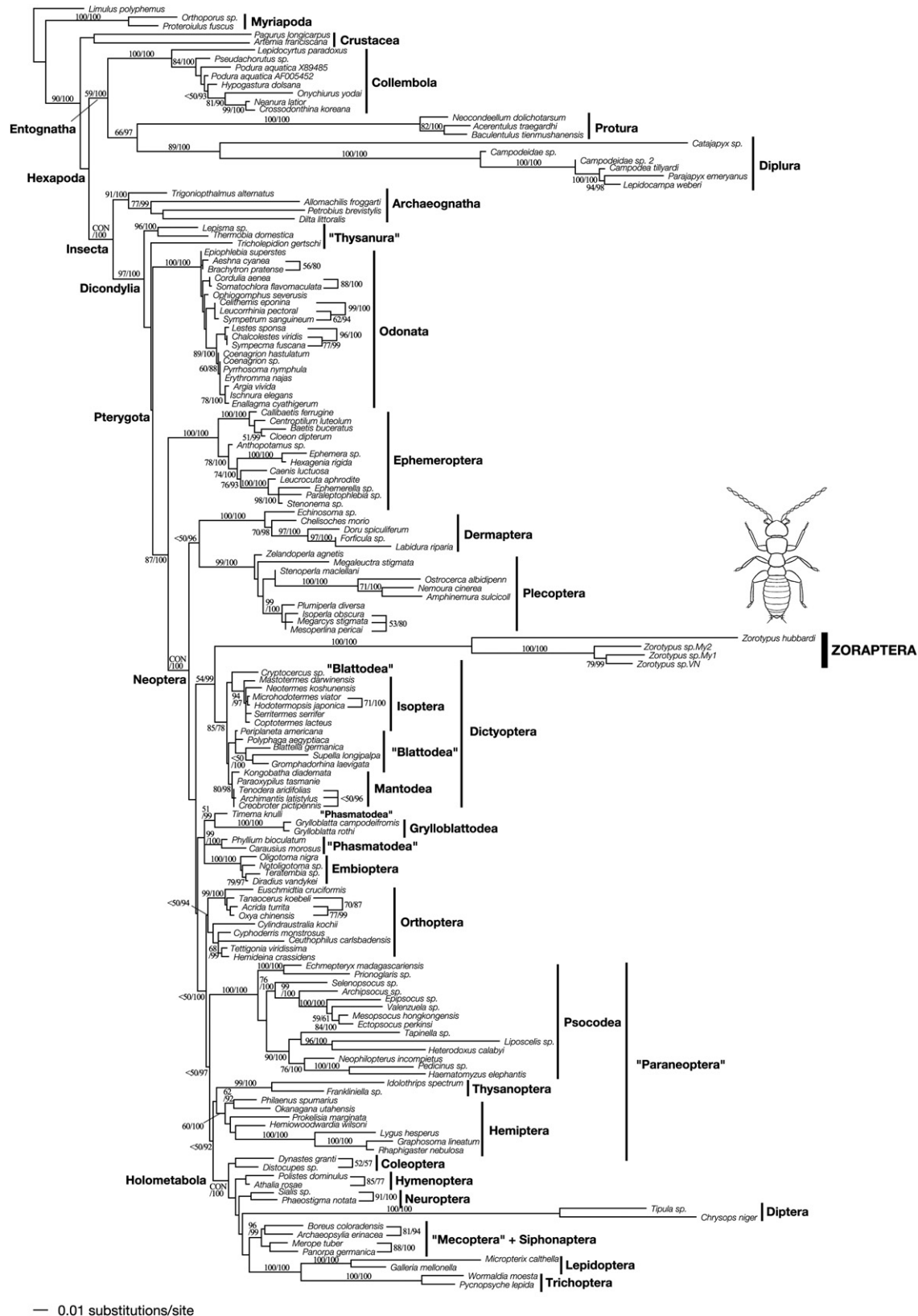


Fig. 1. Tree obtained by ML analysis of the 18S rDNA data ($-\ln L = 32543.23325$). The tree is rooted on *Limulus polyphemus* (horseshoe crab). Branch lengths are proportional to ML estimated branch lengths. The numbers associated with the nodes are bootstrap values or posterior probabilities obtained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

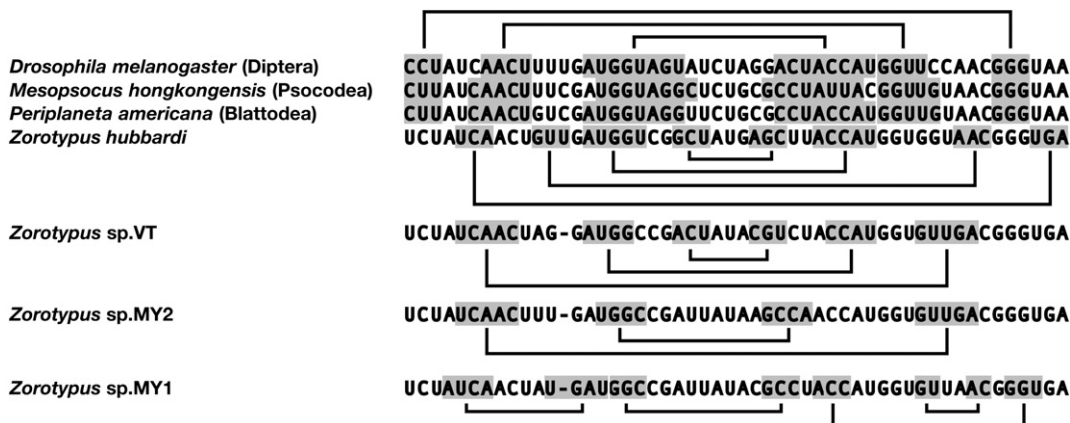


Fig. 2. Estimated secondary structure of helix 12 for selected samples. Stems are shaded, and complementary regions are connected by solid line.

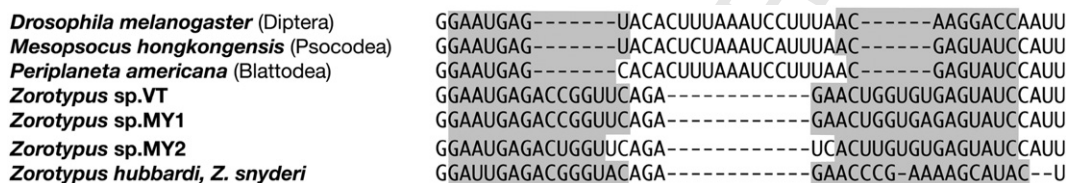


Fig. 3. Estimated secondary structure of helix 18 for selected samples. Stems are shaded. Secondary structure model follows Gutell (1993, 1994) and Gillespie et al. (in press). Their model employs some non-canonical pairs (such as G-A pairs), but Kjer (2004) did not follow Gutell model and employ canonical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

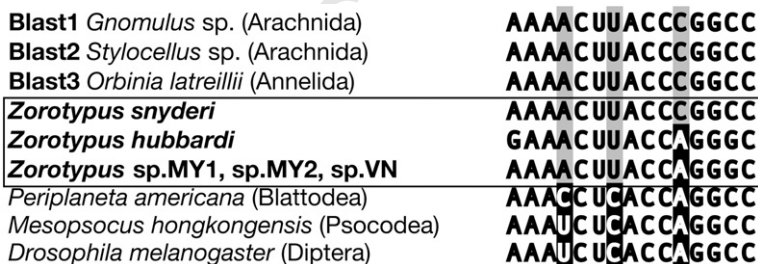


Fig. 4. Sequences near helix 36 for selected samples. Based on the character states at highlighted positions, Kjer (2004) concluded that the 18S sequence of *Z. snyderi* was an acarine contaminant. When this and neighboring regions of *Z. snyderi* were subjected to a BLAST search, the top three sequences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