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Molecular Systematics of Tanaidacea (Crustacea: Peracarida) Based on 18S Sequence Data, with an Amendment of Suborder/Superfamily-level Classification

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Phylogenetic relationships within Tanaidacea were analyzed based on sequence data for the 18S rRNA gene. Our results strongly supported a monophyletic group composed of Neotanaidae, Tanaoidea, and Paratanaoidea, with the first two taxa forming a clade. These results contradict three previously suggested hypotheses of relationships. Based on the molecular results, and considering morphological similarities/differences between Neotanaidomorpha and Tanaidomorpha, we demoted Suborder Neotanaidomorpha to Superfamily Neotanaoidea within Tanaidomorpha; with this change, the classification of extant tanaidaceans becomes a two-suborder, four-superfamily system. This revision required revision of the diagnoses for Tanaidomorpha and its three superfamilies. The results for Apsedumorpha were ambiguous: this taxon was monophyletic in the maximum likelihood and Bayesian analyses, but paraphyletic in the maximum parsimony and minimum evolution analyses.

Key words: Tanaidaceae, Neotanaidae, 18S rRNA, phylogenetic relationship, suborder/superfamily-level classification

INTRODUCTION

Order Tanaidacea is a group of aquatic crustaceans belonging to Superorder Peracarida, which also contains the common orders Amphipoda, Cumacea, and Isopoda (Martin and Davis, 2001). Most tanaidaceans are marine, having been reported from around the world, ranging in depth from coastal areas (1–2 m) to the deep-sea bottom at about 9000 m (cf. Kudinova-Pasternak, 1972); a few freshwater and brackish species are also known (Kakui et al., 2010). At present, more than 1000 extant and 13 fossil species are classified into 35 families (Anderson, 2010; Larsen, 2011).

For the suborder-level classification in Order Tanaidacea, two different systems have been proposed: Lang’s (1956) two-suborder system and Sieg’s (1980b) four-suborder system. Lang’s (1956) two suborders, Monokonophora and Dikonophora, were based on male external genitalia, with monokonophorans having only a single genital cone and dikonophorans having two. As diagnostic characters for the two suborders, Lang (1956) also suggested the number of antennular flagella, the presence or absence of the mandibular palp, and the number of oostegites, to which Lauterbach (1970) proposed additional 18 characters. Sieg (1980b) abolished Lang’s (1956) classification, presenting a new system comprising Anthracocaridomorpha, Apsedumorpha, Neotanaidomorpha, and Tanaidomorpha. Sieg’s (1980b) action was based on the facts that 1) several fossil species express very different characters from extant species, e.g., the pleon consisting of six pleonites and one telson; 2) there are several extant and fossil species contradicting Lang’s (1956) male genitalia-rule, i.e., the dikonophorans Hexapleomera robusta (Moore, 1894) and Pancoloides litoralis (Vanhöffen, 1914) have a single genital cone, and a monokonophoran-like fossil species, Jurapseudes friedricianus (Malzahn, 1965), has two genital cones; and 3) Family Neotanaidae, in Dikonophora, differs in various ways from other dikonophorans. In consequence, Sieg (1980b) divided Dikonophora into two suborders, Neotanaidomorpha for Neotanaidae sensu Lang (1956) and Tanaidomorpha for the other dikonophorans. Anthracocaridomorpha contains only fossil species, whereas Apsedumorpha includes both fossil and extant species. In addition, Sieg (1980b) established superfamilies within Suborders Tanaidomorpha and Apsedumorpha. Tanaidomorpha contains two superfamilies, Paratanaoidea and Tanaoidea; the former includes Paratanidae sensu Lang (1956) and the latter comprises a single family, Tanaidae. Apsedumorpha is composed of the fossil superfamily Ophthalmapseudoidea.
and the extant Apseudoidea; the latter is identical to Monokonophora sensu Lang (1956). Schram et al. (1986) revised the fossil taxa and modified Sieg's (1980b) system to the four-suborder, five-superfamily system currently accepted by most researchers (Larsen, 2005). The above two high-level classification systems are summarized in Table 1.

Previous researchers have proposed three hypotheses for relationships among extant higher-level taxa; these hypotheses differ in the position of Neotanaidae. In his two-suborder system, Lang (1956) regarded Neotanaidae as ancestral to (included the common ancestor of) Paratanidae (Fig. 1A) because 1) neotanaids show primitive states in some appendages, and 2) these two families are similar in having four paired oostegites and reduced mouthparts in males. Lauterbach (1970) and Gardiner (1975) accepted Lang's (1956) two-suborder system, but placed Neotanaidae in a different position and assumed that Tanaidae and Paratanidae shared a common ancestor (Fig. 1B), because members of the latter two families have thoracic glands, which are absent in Neotanaidae. Sieg (1980a) at first concurred with Lauterbach (1970) and Gardiner (1975), but later suggested a close relationship between Neotanaidomorpha and Apseudoomorpha (Fig. 1C), based on the reduced article 1 of the antenna (cf. Sieg, 1984: p. 90; figs. 9, 10, 29).

Among the above three hypotheses with regard to relationships, the currently accepted three-suborder, three-superfamily system is still debatable: Tanaidomorpha is paraphyletic in Lang's (1956) hypothesis (Fig. 1A). In a recent molecular phylogenetic analysis of Tanaidacea (Drumm, 2010), based on a "total evidence analysis (ingroup taxa having at least two out of the 28Sr RNA, COI, and H3 partial sequences)"; Tanaidomorpha was monophyletic with strong support and Apseudoomorpha was monophyletic with weak support. Drumm's (2010) taxon sampling, however, failed to include neotanaids, and thus the higher-level relationships and a suitable system for classifying extant tanaidaceans remained open to question. The aim of our study was to shed light on the phylogenetic position of Family Neotanaidae using 18S RNA sequences, and to clarify the phylogenetic relationships among higher taxa. On the basis of the relationships we observed, we discuss the extant tanaidacean system.

**Materials and Methods**

**Materials**

Specimen collection localities are shown in Table 2. We included two isopods (Asellus hilgendorffi Bovallius, 1886 and Colubotelson thomsoni Nicholls, 1944) as outgroup taxa (e.g., Siewing, 1963; Pires, 1987; Richter and Scholtz, 2001). 18S sequences for the following four species were obtained from GenBank (NCBI): C. thomsoni; Kalliapseudes sp. 2 (registered by Spears et al. [2005] under the name Kalliapseudes sp.); Paradoxapseudes bermudeus (Bácsescu, 1980) (registered by Wilson [2009] under the name Apseudes bermudeus); and Paratanais malignus Larsen, 2001 (Genbank accession numbers AF255703.1, AY781430.1, GO175865.1, and AY781429.1, respectively).

**Primer design**

This study used one molecular marker, the nuclear small subunit ribosomal RNA (18S rRNA) gene. Primers used for the PCR and cycle sequencing are listed in Table 3. The two outermost primers, 18S-a1F and 18S-a9R, were designed by reference to the sequence of Kalliapseudes sp. (Spears et al., 2005). Primers 18S-F2 and 18S-F3 were designed by Yamaguchi and Endo (2003). Other primers were designed by using sequences obtained during this study, taking into account the location of variable regions assessed from the secondary structure of the honeybee 18S sequence (Gillespie et al., 2006), and were checked for their adequacies with Primer3Plus (Untergasser et al., 2007).

**DNA extraction, PCR, and sequencing**

Total DNA was extracted from whole specimens or parts of specimens, using the DNeasy Blood & Tissue Kit (Qiagen GmbH) and following the manufacturer’s protocol. Exoskeletons were retained and preserved in 99% ethanol, and deposited in the Zoological Institute, Faculty of Science, Hokkaido University, Japan (ZIHU); ZIHU 4000–4028. Amplifications were performed by using a DNA thermal cycler with the following reaction conditions: 95°C for 7 min; 35 cycles of 95°C for 30 seconds, 50°C for 90 seconds, 72°C for 90 seconds; and 72°C for 7 min. All nucleotide sequences were determined by direct sequencing by using BigDye Terminator Kit ver. 3.1 with a 3730 DNA Analyzer (Life Technologies, USA).

**Phylogenetic analyses**

Nucleotide sequence pre-alignments were performed with Clustal W (Thompson et al., 1994) in MEGA 4 (Tamura et al., 2007), with the default settings: gap opening cost = 15, gap extension cost = 6.66, and transition weight = 0.5. The pre-aligned data were then realigned by eye according to the secondary
Table 2. Taxa included in this study's analysis, place of origin, and 18S sequence length.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Locality</th>
<th>Sampling site or Reference</th>
<th>Latitude (N)</th>
<th>Longitude (E)</th>
<th>Collector or vessel*</th>
<th>Sequence length (bp)</th>
</tr>
</thead>
<tbody>
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<td><strong>Suborder APSEUDOMORPHA</strong></td>
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<td>Superfamily Apseuloidea</td>
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<td>Family Apseulidae</td>
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<tr>
<td>Parapaseulidae aff. quintolongus</td>
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<td>Parapaseulidae aff. quintolongus</td>
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<tr>
<td>Family Parapaseulidae aff. serratus (G.O. Sars, 1882)</td>
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<td>Family Neotanaidae</td>
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<td>Neotanais sp. 1</td>
<td>Stn. North of Kuroshima Island</td>
<td>26°20′23.40″</td>
<td>127°26′14.40″</td>
<td>646</td>
<td>ToM</td>
<td>2296</td>
</tr>
<tr>
<td>Neotanais sp. 2</td>
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<td>29°11′28.20″</td>
<td>128°6′28.80″</td>
<td>1150</td>
<td>NaM</td>
<td>2375</td>
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<tr>
<td>Family Tanaidae</td>
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<td>Tanaiscinae</td>
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</table>

*Abbreviations: AK, Atsushi Kaneko; HY, Asaka Yamaki; HY, Hiroshi Yamasaki; KK, Keiichi Kaku; SH, Shimpei Hiruta; YH, Yoshihiro Hayashi; NaM, TR/V Nagasaki-maru; SoM, R/V Soyo-maru; TaM, R/V Tansei-maru; ToM, TR/V Toyoshio-maru.**Sequence data were obtained from NCBI.
Table 3. List of PCR and cycle sequencing (CS) primers used in this study for the 18S rRNA gene.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer name</th>
<th>Reaction</th>
<th>Primer sequence (in 5'-3' direction)</th>
<th>Direction</th>
<th>Source</th>
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<td>18S rRNA</td>
<td>18S-a1F</td>
<td>PCR &amp; CS</td>
<td>GGYGAAACCGYGAAWGTYCT</td>
<td>Forward</td>
<td>This study</td>
</tr>
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<td>18S-F2</td>
<td>CS</td>
<td>CCGGTTAAAGGCTGCTT</td>
<td>Reverse</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>18S-b4F</td>
<td>CS</td>
<td>TCGGTTAAAGGCTGCTT</td>
<td>Forward</td>
<td>This study</td>
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<tr>
<td>18S-b4R</td>
<td>CS</td>
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<td>Reverse</td>
<td>This study</td>
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<td>18S-F3</td>
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<td>GYGTTAGATTCCGCTT</td>
<td>Forward</td>
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<td>18S-b6F</td>
<td>CS</td>
<td>TCGGTTAAAGGCTGCTT</td>
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<td>This study</td>
<td></td>
</tr>
<tr>
<td>18S-a9R</td>
<td>PCR &amp; CS</td>
<td>CCTTGTTCAGCAGCTT</td>
<td>Reverse</td>
<td>This study</td>
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</tbody>
</table>

Table 4. Characteristics of the 18S sequence data set in this study. TS, total sites; VS, variable sites; PIS, parsimony informative sites (all after alignment).

<table>
<thead>
<tr>
<th>Gene</th>
<th>TS</th>
<th>VS</th>
<th>PIS</th>
<th>%A</th>
<th>%C</th>
<th>%G</th>
<th>%T</th>
<th>χ²</th>
<th>P</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>1493</td>
<td>695</td>
<td>628</td>
<td>25.7</td>
<td>22.5</td>
<td>27.8</td>
<td>23.9</td>
<td>182.50 (df = 96)</td>
<td>0.0000003*</td>
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*Significant nucleotide compositional bias.

Table 5. Optimal substitution models for maximum-likelihood (ML) and Bayesian (BI) analyses of the 18S data set, selected by AIC and BIC in jModelTest, respectively. GTR, general time reversible model (Tavaré, 1986); TIM, transitional model (Posada, 2003); I, proportion of invariant sites; G, gamma distribution shape parameter.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Analysis</th>
<th>Model</th>
<th>I</th>
<th>G</th>
<th>Base frequencies</th>
<th>Rate matrix</th>
<th>χ²</th>
<th>P</th>
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<td>18S rRNA</td>
<td>ML</td>
<td>GTR + I + G</td>
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<td>0.5530</td>
<td>A = 0.2538</td>
<td>A-C = 1.0755</td>
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<td>C = 0.2102</td>
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<td>T = 0.2579</td>
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<tr>
<td>BI</td>
<td>TIM2 + I + G</td>
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<td>0.5490</td>
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<td>A-C = 1.3015</td>
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<td>A-T = 1.3015</td>
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<td>C-G = 1.0000</td>
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<td>G-T = 1.0000</td>
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</table>

The 18S sequences for the 29 studied species ranged in length from 1818 bp to 2909 bp, and were registered in the DDBJ/EMBL/GenBank databases under accession numbers AB618174–618202. The sequences contained eight variable regions, corresponding to domains V4–V9 in Gillespie et al. (2006); as with Spears et al. (2005), regions V4 and V7 were highly divergent among taxa. The aligned data set consisted of 1493 bp. Characteristics of the data set used in this study are given in Table 4. Optimal substitution models for the ML and BI analyses are given in Table 5. Although the χ² test for homogeneity in base frequency revealed significant compositional heterogeneity for the data (Table 4), there were no differences between two ME trees under the assumption of homogeneity or heterogeneity except for minor differences in bootstrap values (data not shown). Thus, compositional heterogeneity in this data set did not seem to be problematic.

A summary of optimality values for the MP, ME, and ML analyses of 18S rRNA is as follows: three MP trees were obtained (tree length = 2560, CI = 0.494, RI = 0.740); ME-score = 1.57582; –ln L = 13204.8889. The strict consensus of three MP trees, and the ME, ML, and BI trees, are shown in Figs. 2–5. In ME, ML, and BI trees, the branches in Apeudomorpha were generally

1987). Parameters for the ML analysis were selected on the basis of the Akaike information criterion (AIC; Akaike, 1974) in jModelTest (Posada, 2008). Bootstrap values for the ML trees were calculated from 1000 pseudoreplicates analyzed by TBR searches, with the starting topology given by an NJ tree.

Finally, a Bayesian (BI) analysis was performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Parameters for this analysis were selected by the Bayesian information criterion (BIC; Schwarz, 1978) test implemented in jModelTest. A Markov-Chain Monte-Carlo (MCMC) search was performed with four chains, each of which was run for 500000 generations. Trees were sampled every 100 generations. Topological convergence diagnostics for Bayesian analysis were performed with Tracer 1.5 (Rambaut and Drummond, 2009). The first 125000 generations were discarded as burn-in. A consensus of sampled trees was computed, and the posterior probability for each internal branch was obtained to assess the robustness of the inferred relationships.

RESULTS

The 18S sequences for the 29 studied species ranged in length from 1818 bp to 2909 bp, and were registered in the DDBJ/EMBL/GenBank databases under accession numbers AB618174–618202. The sequences contained eight variable regions, corresponding to domains V2–9 in Gillespie et al. (2006); as with Spears et al. (2005), regions V4 and V7 were highly divergent among taxa. The aligned data set consisted of 1493 bp. Characteristics of the data set used in this study are given in Table 4. Optimal substitution models for the ML and BI analyses are given in Table 5. Although the χ² test for homogeneity in base frequency revealed significant compositional heterogeneity for the data (Table 4), there were no differences between two ME trees under the assumption of homogeneity or heterogeneity except for minor differences in bootstrap values (data not shown). Thus, compositional heterogeneity in this data set did not seem to be problematic.

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shorter than those in Tanaidomorpha and Neotanaidomorpha (Figs. 3–5). In all trees, Clade 12 contained all taxa included in Superfamily Paratanaoidea; Clade 18 comprised Tanaoidea + Neotanaidae (Clades 19 and 24, respectively) and formed the sister clade to Paratanaoidea (Clade 12). The position of Kalliapseudidae (Clade 10) differed among analyses. In the MP and ME trees (Figs. 2 and 3), Kalliapseudidae was included in Clade 9 with Tanaoidea, Paratanaoidea and Neotanaidae, which led Apseudomorpha to be non-monophyletic. In the ML and BI trees (Figs. 4 and 5), Kalliapseudidae was included in Clade 25 and formed the sister clade to other apseudomorphs (Clade 2). Family Sphyrapodidae (Clade 7) being the early offshoot in Clade 2 was supported in all trees. Within Clade 3, all analyses showed well-supported Paradoxapseudes and parapseudid clades (Clades 5 and 6, respectively). The positions of terminal taxa and of Clades 5 and 6 within Clade 2 were unstable among analyses.

Fig. 2. Strict consensus of the three optimal maximum-parsimony trees (length = 2560) based on 18S rRNA gene sequence data. Bootstrap values > 50% are shown, determined by analysis of 1000 pseudoreplicates. Numbers in squares indicate clades with > 50% bootstrap support. An asterisk labeling a terminal taxon indicates the sequence was obtained from NCBI. (O.G.), outgroup taxon. Bold taxon names in all capital letters labeling sidebars indicate suborder names suggested by Sieg (1980b); below the suborder names are higher taxa represented in the clade. The clade in bold lines is Neotanaidae.

Fig. 3. Minimum-evolution tree based on 18S rRNA gene sequence data, constructed by the maximum composite likelihood method assuming heterogeneity (ME-score = 1.57582). Bootstrap values > 50% are shown, determined by analysis of 10000 pseudoreplicates. Numbers in squares indicate clades with > 50% bootstrap support. An asterisk labeling a terminal taxon indicates the sequence was obtained from NCBI. (O.G.), outgroup taxon. The taxon names labeling sidebars are superfamily or family names. The clade in bold lines is Neotanaidae (Neotanaidomorpha).
DISCUSSION

The results of our analyses generally indicated that Tanaidomorpha and Neotanaidomorpha are represented by relatively long branches, compared to Apsuedomorpha (Figs. 3–5). That Neotanaidomorpha was nested within Tanaidomorpha (= Paratanida + Tanaidae) might seemingly be indicative of the long-branch attraction (LBA) artifact (Felsenstein, 1978). However, LBA is unlikely in terms of the tree topology ((Neotanaidomorpha + Tanaidae) + Paratanidae), because this relation was consistent among all the trees generated by MP, ME, ML, and BI, with high support values. The relationship ((Neotanaidomorpha + Tanaidae) + Paratanidae) contradicts the three previous hypotheses (Fig. 1A–C), but is similar to schemes suggested by Lang (1956), Lauterbach (1970), and Gardiner (1975), in which Neotanaidae is related more closely to Tanaidomorpha than to Apsuedomorpha.
Our results, in which Tanaidomorpha is paraphyletic, suggest that the currently accepted classification system for extant species should be amended. The relationship between Neotanaidomorpha and Tanaidomorpha is supported by the uniramous antennule, the mandible lacking the palp, the pereonite 1 not firmly joined with the carapace, and other features (see Lang, 1956; Lauterbach, 1970). At the same time, three taxa (Neotanaidae, Tanaoidae, and Paratanoidae) within these two suborders are well distinguishable from each other by a number of morphological features, including the number of articles on the antennules and the antennae, and the presence or absence of the thoracic glands and the uropodal exopod. It is therefore reasonable to demote Suborder Neotanaidomorpha to Superfamily Neotanaoidae within Tanaidomorpha. Consequently, the classification system for extant Tanaidacea becomes a two-suborder, four-superfamily system. As the result of the demotion of Neotanaidomorpha, it is necessary to amend the diagnoses for Suborder Tanaidomorpha and its three superfamilies.


Amended diagnosis of Tanaoidae. Eyes well defined, black (absent in Protanais). Pleonites 4 and 5, when present, are narrower than pleonites 1–3. Thoracic glands present. Antennule with three to five articles. Antenna with six to eight articles. Lacinia mobilis present on left and right mandibles. Maxillule with one endite, bearing palp. Maxilla rudimentary, oval-shaped. Maxilliped with coxae; maxillipeds coxae and bases unfused medially. Cheliped lacking ischiu (present in several species of Tanais). Pereopods lacking ischiu. Dactylus-unguis of pereopods 4–6 forming claw; claw bearing rows of spiniform setae. Pleopods three pairs. Uropod uniramous. Females with only one pair of sac-like oostegites, arising from coxae of pereopod 4.

Amended diagnosis of Paratanoidae. Eyes present or absent. Pleon never with last two pleonites fused or reduced alone. Thoracic glands present. Antennule with five or fewer articles in female, often with more than five articles in male. Antenna with seven or fewer articles. Lacinia mobilis present on left mandible. Maxillule with one endite and palp bearing two terminal setae. Maxilla rudimentary, oval-shaped. Cheliped lacking ischiu. Pereopods with ischiu. Dactylus-unguis of pereopods 4–6 forming claw in several families, but lacking rows of spiniform setae. Pleopods zero or five pairs. Uropod uni- or biramous. Females with one or four pairs of flat oostegites.


Monophyly of Apseudomorpha has been open to question, and will remain as is. Based on morphology, most tanaidacean researchers (e.g., Sieg, 1984; Larsen and Wilson, 2002) regarded the taxon as monophyletic, whereas Siewing (1953: p. 416) implied paraphyly. Drumm’s (2010) recent molecular phylogenetic analysis indicated Apseudomorpha as monophyletic, although with relatively low support values. Our results were that Apseudomorpha was monophyletic in ML and BI, and paraphyletic in MP and ME. Future studies with more reliable molecular markers must clarify the phylogenetic status of this taxon.

Among 13 families of Apseudomorpha, Kalliapseudidae and Sphyrapodidae are unique in that they produce mancas having an exopod each on pereopods 4 and 5 (cf. Hansknecht et al., 2002; Gutu, 2006). In the present analysis, Kalliapseudidae branched off earlier than the other apseudomorphs in ML and BI trees (Clade 2), as has been shown in a previous molecular phylogenetic study (Drumm, 2010). With MP and ME, Kalliapseudidae appeared as sister to Tanaidomorpha + Neotanaidae (Clade 11). Sphyrapodidae was a sister taxon to the rest of Apseudomorpha (Clade 3) in all the methods used in this study. Future analyses with denser taxon sampling are necessary to reveal the evolution of biramous appendages in Apseudomorpha.

This study is the first to use 18S sequence data and to include neotanais to analyze tanaidacean phylogeny. It strongly indicates that Neotanaidae has been erroneously positioned in the previous classification. On the other hand, the 18S data failed to resolve relationships for apseudomorphs.

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