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THE SCUTOCOXIFERA TAX. NOV. AND THE INFORMATION CONTENT OF NUCLEAR SSU RDNA SEQUENCES FOR RECONSTRUCTION OF ISOPOD PHYLOGENY (PERACARIDA: ISOPODA)

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A B S T R A C T

The nuclear ssu rRNA gene of several isopods (Crustacea, Peracarida) was sequenced to study its phylogenetic information content. Several areas had to be cut out of the alignment of 31 isopod sequences and selected outgroup arthropod sequences due to the lack of alignable patterns. The final alignment had 2,533 positions and 43 sequences. The length of the isopod nuclear ssu rRNA genes varies between 2,098 and 3,402 bp. In some clades the gene length increases; in others like the cymothoids and bopyrids, long deletions occur. Some insertions are specific for major groups (e.g., amphipods, isopods). Most elongation areas evolve rapidly and are not alignable among higher taxa. Information content is visualized with spectra of supporting positions. Only a few groups are unambiguously supported with a signal distinctly higher than background noise. The results of maximum parsimony analyses are congruent with major aspects of earlier hypotheses on isopod phylogeny. Some contradictions are discussed. The latter are mainly based on a lack of reliable information. A major monophyletic group found in the molecular phylogenies and also supported by distinct morphological characters is named Scutocoxifera tax. nov., composed of the Oniscidea, Valvifera, Sphaeromatidea, Anthuridea, and Cymothoida. SEM photographs are presented to document the apomorphic state of the coxa in the Scutocoxifera.

Isopods are peracarid crustaceans that in comparison with related taxa (e.g., Tanaidacea, Cumacea, Mictacea), are morphologically and ecologically very diverse. Of the ~10,000 known species about 3,500 are terrestrial "slaters" and "pill bugs" (Oniscidea). The remaining species are aquatic, and most of these live in marine habitats. Until recently, the more aberrant aquatic species with distinct, derived characters were grouped in separate suborders without consideration of their phylogenetic relationships. For example, gnathiid fish parasites, whose mature adults live in cryptic habitats and males bear characteristically powerful defensive mandibles, are classified in the suborder Gnathiidea; elongated, worm-like isopods with large uropods and whose exopod is folded over the telson are the Anthuridea; the amphipod-like freshwater species of the southern hemisphere are placed in the suborder Phreatoicidea, and isopods with large uropodal sympods that ventrally cover the respiratory chamber are the Valvifera. Isopod parasites of crustaceans are placed in the suborder Epicaridea; smaller benthic species with a reduced number of free pleonites and specialized sexually dimorphic pleopods are the Asellota. All remaining species belong to taxa which in their groundpattern have broad, leaf-like uropods and are united in the suborder Flabellifera. This classification is found in many textbooks and faunistic monographs (e.g., Schultz, 1969; Naylor, 1972; Kussakin, 1979; Kensley and Schotte, 1989; Roman and Dalens, 1999). Even though these isopod groups can be distinguished easily, until recently the classification was not founded on reconstructions of phylogeny.

A first attempt to describe the phylogeny of isopods together with scenarios for the evolution of lifestyles based on a Hennigian analysis of morphological data was published by Wägele (1989). A major conclusion of this study was that the Flabellifera are not monophyletic, and the taxa Anthuridea, Gnathiidea, and Epicaridea are nested within the former Flabellifera. Brusca and Wilson (1991) also used morphological characters (partly the same as in Wägele, 1989) and arrived in general to similar conclusions (the more basal groups are the Phreatoicidea and Asellota, with the Epicaridea, Gnathiidea, and Anthuridea appearing within the former Flabel-

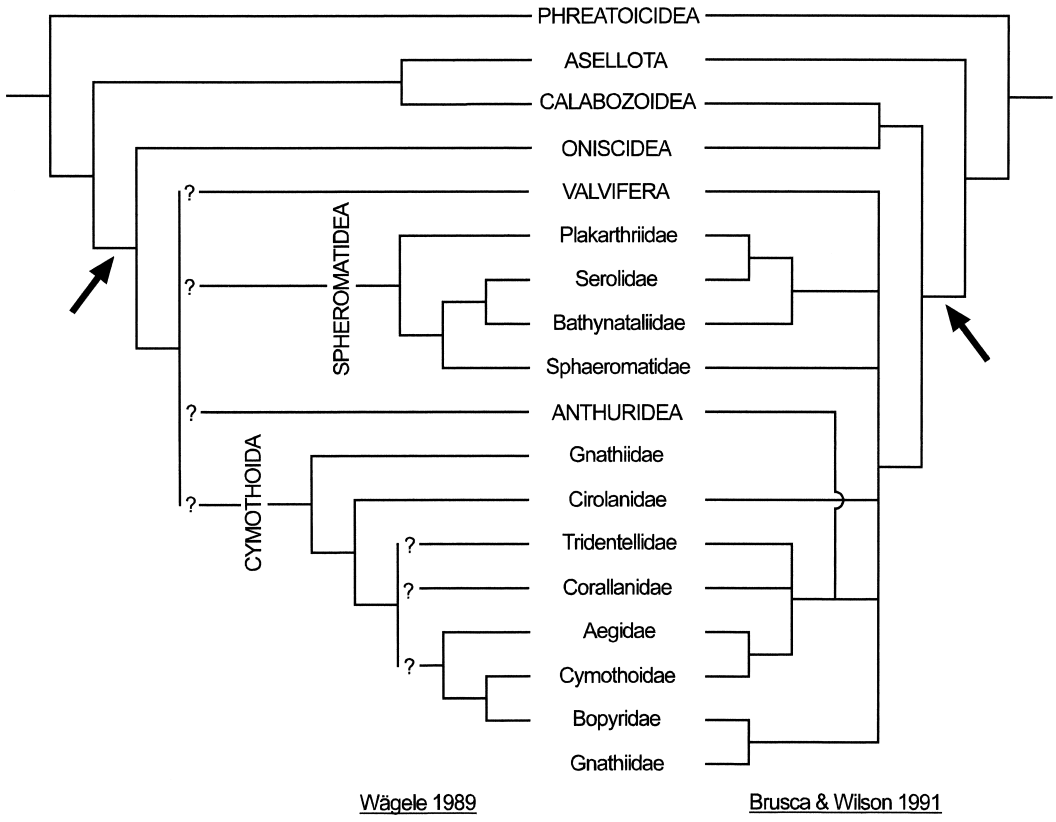

Fig. 1. Comparison of the two more recent hypotheses on isopod phylogeny reconstructed with morphological characters (modified from Wägele (1989) and Brusca and Wilson (1991)). Arrows indicate the partition separating the Scutocoxifera from the more basal isopod groups. (Some families for which no DNA sequences exist were omitted.)

lifera). Figure 1 shows a comparison of the results of these two studies, which include some differences.

One important partition of the taxa is found in both topologies: the "higher isopods" are always separated from the more basal Phreatoicidea and Asellota (arrows in Fig. 1). In the present paper, we propose to name these "higher isopods" Scutocoxifera tax. nov., and we examine the phylogenetic information content of 30 new and complete nuclear small-subunit ribosomal DNA (ssu rDNA) sequences of isopods to get further evidence apart from the known morphological characters.

Materials and Methods

Specimens.—Most specimens were collected by the authors; some were donated by colleagues. Some of the nuclear ssu rDNA sequences were already presented in a previous study (Dreyer and Wägele, 2001): Suborder Phreatoicidea: *Colubotelson thomsoni* Nicholls, 1994 (GenBank Acc. No. AF 255703); Suborder Asellota: *Janira maculosa* Leach, 1814 (Acc. No. AF 255698); Suborder Oniscidea: *Ligia oceanica* (Linnaeus, 1767) (Acc. No. AF 255698); *Oniscus asellus* Linnaeus, 1758 (Acc. No. AF 255699); Suborder Valvifera: *Glyptonotus antarcticus* Eights, 1853 (Acc. No. AF 255696); *Cleantis prismatica* (Risso, 1862) (Acc. No. AF 255697); Suborder Anthuridea: *Cyathura carinata* (Krøyer, 1847) (Acc. No. AF 332146); Suborder Sphaeromatidea: *Sphaeroma serratum* (Fabricius, 1787) (Acc. No. AF 255694); *Cassidinidea* sp. (Acc. No. AF 255693); *Cymodoce tattersalli* Torelli, 1929 (Acc. No. AF 255695); Suborder Cymothoida: Family Bopyridae: *Probopyrus pacificiensis* Roman-Contreras, 1993 (Acc. No. AF 255683); *Hemiarthrus abdominalis* (Krøyer, 1841) (Acc. No. AF 255684); Family Cymothoidae: *Riggia paranensis* (Saidat, 1948) (Acc. No. AF 255685); *Anilocra physodes* (Linnaeus, 1758) (Acc. No. AF 255686); Family Aegidae: *Aega antarctica* (Hodgson, 1910) (Acc. No. AF 255689); Family Corallanidae: *Excorallana quadricornis* (Hansen, 1890) (Acc. No. AF 255688); Family Cirolanidae: *Natatolana albinota* (Vanhöffen, 1914) (Acc. No. AF 255691); *Typhlocirolana moraguesi* Racovitza, 1905 (Acc. No. AF 255692); *Eurydice pulchra* Leach, 1815 (Acc. No. AF 255690). Note: The *Cassidinidea* sp. is a new species from the Gulf of Maracaibo; a description is in preparation.

For this study we add the following sequences (in parentheses: locality and collector, if other than the au-

thors, and GenBank accession number; system of suborders as in Wägele, 1989): Suborder Anthuridea: *Paranthura nigropunctata* (Lucas, 1846) (Spain, Galicia, Acc. No. AF 279598); Suborder Asellota: *Eurycope inermis* Hansen, 1916 (Norway, Skagerrak, *leg.* U. Englisch, Acc. No. AF 279607); *Iathrippa trilobatus* (Richardson, 1910) (Weddell Sea, *leg.* C. Held, Acc. No. AF 279606); *Jaera albifrons* Leach, 1814 (France, Bretagne, Acc. No. AF 279609); *Jaera nordmanni* (Rathke, 1837) (Spain, Galicia, Acc. No. AF 279610); *Joeropsis coralicola* Schultz and McCloskey, 1967 (Venezuela, Paria, Acc. No. AF 279608); Suborder Oniscidea: *Trachelipus rathkei* (Brandt, 1833) (Germany, Berlin, *leg.* C. Schmidt, Acc. No. AF 279605); Suborder Sphaeromatidea: *Anoplocopea lusitanica* Nolting, Reboreda, and Wägele, 1998 (Spain, Galicia, Acc. No. AF 279602); *Campecopea hirsuta* (Montagu, 1804) (Spain, Galicia, Acc. No. AF 279601); *Lekanesphaera hookeri* (Leach, 1814) (Germany, Nord-Ostsee-Kanal, Acc. No. AF 279600); *Limnoria quadripunctata* Holthuis, 1949 (France, Bretagne, Acc. No. AF 279599); Suborder Valvifera: *Antarcturus spinacoronatus* Schultz, 1978 (Weddell Sea, *leg.* C. Held, Acc. No. AF 279604); *Idotea baltica* (Pallas, 1772) (Germany, Baltic Sea, Acc. No. AF 279603).

Other sequences used for outgroup comparison: Amphipoda: *Gammarus salinus* (Germany, *leg.* U. Englisch, Acc. No. AF 356544); *Gammarus duebeni* (Germany, *leg.* U. Englisch, Acc. No. AF 356545); *Epimeria georgiana* (Weddell Sea, Acc. No. AF 356546); *Maxilliphimedia longipes* (Weddell Sea, Acc. No. AF 356547); Decapoda: *Astacus astacus* (Acc. No. U 33181); Anostraca: *Artemia salina* (Acc. No. X 01723); *Branchinecta packardi* (Acc. No. L 26512); Cladocera: *Daphnia pulex* (Acc. No. AF 014011); Ostracoda: *Stenocypris major* (Acc. No. Z 22850); Ascothoracida: *Ulophysema oeresundense* (Acc. No. L 26521); Chelicerata: *Androctonus australis* (Acc. No. X 77908); *Aphonopelma* sp. (Acc. No. X 13457).

Note: The amphipod sequences were obtained by Ulrike Englisch; a more detailed description of these sequences will be published elsewhere.

Fixation and DNA Extraction.—Most living specimens were fixed in ice-cold ethanol (80%) and kept at <4°C whenever possible. Specimens fixed during field trips in warm ethanol yielded less DNA of high quality. It was usually not possible to get suitable DNA samples from specimens fixed in Formalin. DNA was isolated from complete specimens or from tissue samples, homogenized in extraction buffer, and extracted with phenol-chloroform as described by Maniatis *et al.* (1982), or with the "blood and tissue-kit" (Qiagen), or with DTAB (see Gustincich *et al.,* 1991). The quality of the DNA was evaluated by electrophoresis.

PCR, Cloning, and Sequencing.—The 18SrRNA gene was amplified by PCR (polymerase-chain reaction) from whole genomic DNA using primers that proved to be successful in crustaceans (personal communication of Dr. T. Spears) (primer 18a1: 5′-CCTA(CT)CTGGTTGATCCTGCCAGT-3′, primer 1800: 5′-TAATGATCCTTCCGCAGGTT-3′) and with the following thermal profile: 5 min. 94°C, 36 cycles of 30 sec. 94°C, 50 sec. 52.5°C, 2.3 min. 72°C, then 10 min. 72°C and storage at <4°C. PCR products were treated with a "QIAquick" purification kit (Qiagen) and, wherever necessary, fragments were separated by electrophoresis, excised and purified using the QIAEX II–kit (Qiagen). When the quantity of the product was insufficient for sequencing the PCR product was cloned in *E. coli* using the "TOPO-TA cloning kit" (Invitrogen). The purified DNA fragments were sequenced with fluorescent-labelled primers in a Licor 4200 automatic sequencer. With exception of the universal plasmid primers (see Messing *et al.,* 1981), all other primers were designed for the isopod sequencing project (see Dreyer and Wägele, 2001). Both strands of the gene were sequenced completely, and a consensus sequence was constructed using DNASIS 2.1 (Hitachi Software).

Alignment.—Initially sequences were aligned using CLUSTAL X (Thompson *et al.,* 1997), and the result was edited by eye with GeneDoc (Nicholas and Nicholas, 1998) and corrected using secondary structure information to reduce the positional variability. Alignment of these sequences is difficult and has a strong influence on the results. Due to the enormous length variation of the isopod ssu rRNA gene, many gaps had to be inserted, and the initial alignment obtained with CULSTAL X proved to be reliable only in the most conserved areas. To reduce the variability of positions, we decided to align first groups of species that without doubt are closely related (chelicerates, branchiopods, the terrestrial isopods = Oniscidea, the Valvifera, the Asellota, the Amphipoda, Sphaeromatidae, Bopyridae, Cymothoidae) and realigned these groups with the remaining sequences conserving the gaps first introduced. Nevertheless, long insertions, especially in the V4 and V7 regions (see nomenclature in Nelles *et al.,* 1984) did not show alignable conserved patterns, except in closely related sequences (notably in oniscids and valviferans). Even though initial phylogenetic analyses of the complete alignment produced some plausible results, we decided to cut out those areas that did not show alignable patterns. We also tried to work exclusively with the highly conserved areas, but these do not contain enough phylogenetic signal, and the topologies were little resolved. Therefore, we included those parts of insertions that still showed conserved patterns (examples in Figs. 2 and 3). The complete alignment was 4,263 basepairs (bp) long. The following regions that were not alignable had to be cut out for the phylogenetic analyses (numbers in parentheses refer to the sequence of *Artemia salina*): 281–477 (insertion between 189 and 190); 600–714 (200–239); 1,285–2,494 (658–706); 2,658–2,685 (insertion between 846 and 857); 3,467–3,923 (1,365–1,385); 4,441–4,494 (1,706–1,710). The final alignment used for this study had 2,533 bp for 43 species. The alignment is available from the authors and is published on internet URL http://www.herbaria.harvard.edu/treebase/.

Phylogenetic Analyses.—The phylogenetic information content of the alignment was visualized constructing a spectrum of split-supporting positions with PHYSID (for details see Wägele and Rödding, 1998a, b). For each spectrum a tolerated degree of variability of sequence positions can be selected. For Figs. 4 and 5 we used the default value (25%) of noise per position and row (explained in Wägele and Rödding, 1998a, b). The number of supporting positions for a split is independent of any tree-constructing method and of assumptions about the substitution process, but is sensitive to the species composition of the alignment because addition of derived sequences reduces the number of conserved positions supporting a split. Working with PHYSID, the number of supporting positions is used as a measure for the amount

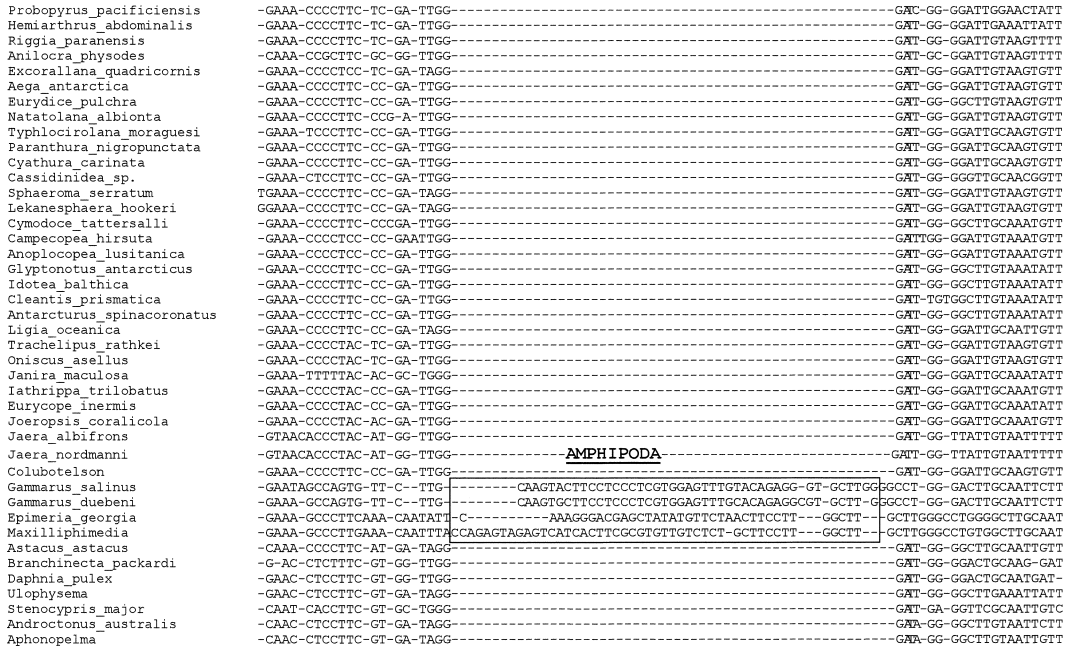

Fig. 2. Part of the alignment of ssu rDNA sequences of isopods and outgroup species (positions 2,101–2,201 of the alignment used for the phylogenetic analyses, equivalent to 1,530–1,559 of *Artemia salina*). The insertion is typical for amphipods.

of phylogenetic signal conserved in the alignments. Monophyly of a group has a higher probability if the signal is high and the group is compatible with other groups of high support. Usually, groups with the best signal (left part of the spectrum) also have a high bootstrap-support in a maximum-parsimony analysis, but not every group with good bootstrap value is also supported by many positions. Long-branch phenomena can also be detected in these spectra because highly modified sequences appear in many nonsense groupings of species due to the higher number of chance similarities (e.g., combinations of a single amphipod species with different isopods, or repeated combinations of *Jaera* spp. with a different isopod).

Visual inspection of the alignment proved to be important to discover insertions that are typical for single taxa (Figs. 2, 3). Because insertions seem to contain important information we decided to code gaps as an additional character state and also tested the effect of coding gaps as missing characters. This does not mean that absence of nucleotides is used as a character: the insertions are the positive information. Even though we had the impression that some larger insertions and deletions are characteristic for certain monophyletic groups, the high variability of the insertions does not allow one to homologise single nucleotides in many regions of the alignment.

For tree construction with distance methods (neighbor-joining: Saitou and Nei, 1987), we used Treecon 1.3b (Van de Peer and De Wachter, 1997) and PAUP 4* (Swofford, 1998), the latter also for maximum likelihood (Felsenstein, 1981) and parsimony analyses. Parameters for substitution models were also estimated with PAUP 4*. The distance trees and estimated pairwise distances were used to identify long-branch taxa.

The model for maximum-likelihood analyses was chosen with a likelihood-ratio test (Huelsenbeck and Bull, 1996; Huelsenbeck and Crandall, 1997; see also Crandall *et al.,* 2000) using the program Modeltest Version 2.0 (Posada and Crandall, 1998). The optimal model was the TrN–model (Tamura and Nei, 1993) with 3 substitution types, nucleotide frequencies estimated via maximum likelihood, gamma-distribution of rate variation among sites as well as proportion of invariable sites estimated from the data (= GTR+G+I model), the molecular clock not enforced. Other models were also used. Analyses with complex models required a reduction of the number of sequences to shorten computation times. We ran several heuristic searches; however, bootstrapping was not possible due to long computation times.

Morphology.—Morphological characters are not discussed in great detail herein (but see Wägele, 1989, 1992, 1994; Brusca and Wilson, 1991). For illustration of coxal structures, specimens were critical-point dried, sputter-coated with gold, and examined with a Zeiss DSM 950 scanning electron microscope.

RESULTS

Sequences

Isopod ssu rDNA sequences vary greatly in length due to large insertions and deletions, most of which occur in the V4 and V7 regions of the secondary structure. The sequence lengths are: *Paranthura nigropunctata* 2,385 bp; *Lekanesphaera hookeri* 2,461 bp; *Campecopea hirsuta* 2,477 bp; *Anoplocopea lusitanica* 2,515 bp; *Limnoria quadripunctata* 2,686 bp; *Idotea baltica* 2,658 bp; *Antarcturus*

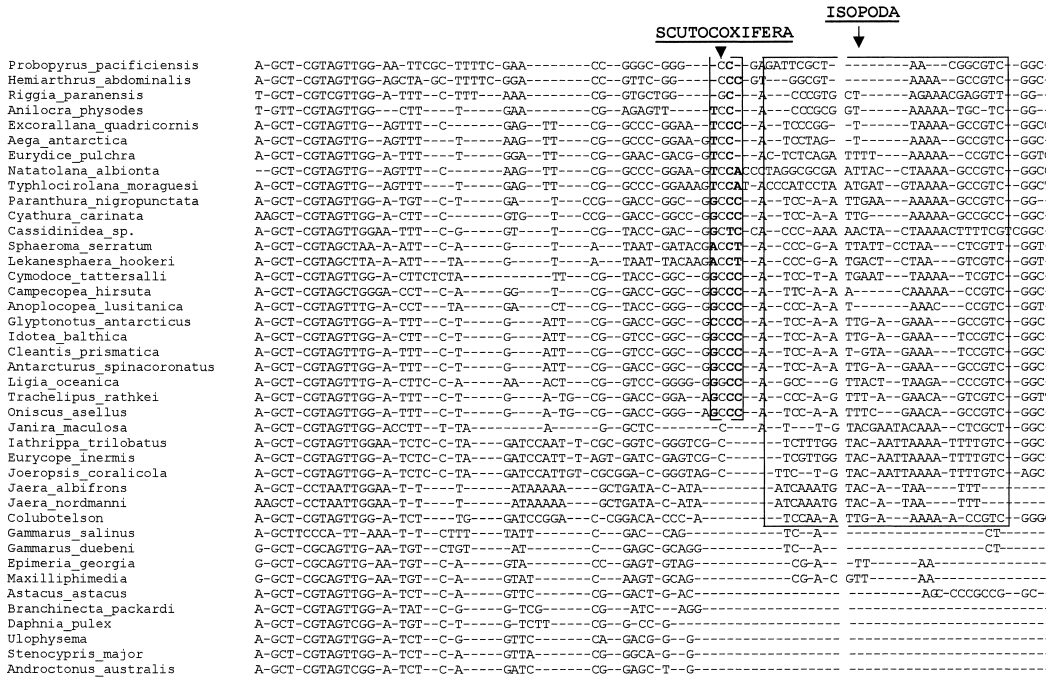

Fig. 3. Alignment as in Fig. 2, with elongations occurring in Isopoda and Scutocoxifera. The isopod insertion was drastically shortened for the phylogenetic analysis (area shown as interruption within the "Isopoda" box) because it was not alignable at the nucleotide level (positions equivalent to 658–707 of the *Artemia salina* sequence or 748–1,556 of the *Natatolana albinota* sequence were cut out). The region shown comprises alignment positions 901–1,001 (equivalent to 629–708 of the *Artemia salina* sequence).

spinacoronatus 2,367 bp; *Trachelipus rathkei* 3,402 bp; *Iathrippa trilobatus* 2,248 bp; *Eurycope inermis* 2,169 bp; *Joeropsis coralicola* 2,189 bp; *Jaera albifrons* 2,135 bp; *Jaera nordmanni* 2,137 bp. The other isopod sequences are described elsewhere (Dreyer and Wägele, 2001). All these sequences are longer than the usual size of the 18S rRNA gene of other metazoans (e.g., *Artemia salina* 1,810 bp; *Astacus astacus* 1,873 bp; *Androctonus australis* 1,812 bp), but comparable elongations are known from some insects (e.g., Sternorrhyncha: Campbell *et al.,* 1994). The fact that peracarid ssu rDNA sequences tend to have elongations was already noted by Spears (personal communication), and it has been shown for the oniscid isopod *Armadillidium vulgare* that insertions are transcribed (Choe *et al.,* 1999). In isopods, the Asellota sequenced to date have relatively short sequences (around 2,100–2,200 bp), whereas in oniscids we found the longest genes with the length varying between 2,505 bp (*Ligia oceanica*) and 3,402 bp (*Trachelipus rathkei*). In valviferans and sphaeromatids, the genes are of median length (about 2,350–2,750 bp); in cirolanids and aegids they are as long as in oniscids (2,910–3,269 bp). Assuming that the parasitic cymothoids and bopyrids evolved from cirolanid-like ancestors as reconstructed from morphological and molecular data (Dreyer and Wägele, 2001), a secondary shortening of the gene must have occurred in these parasites (sequence length 2,160–2,416 bp) in comparison to the cirolanid gene.

Areas where deletions might have occurred can be identified when the aligned sequences are compared (e.g., the region 862–1,366 of *Eurydice pulchra* is absent in bopyrids and cymothoids), but there are also insertions unique for bopyrids (e.g., positions 291–353 in the sequence of *Probopyrus pacificiensis*). With additional sequences it should be possible to identify insertions and deletions that are apomorphies for monophyletic groups with more precision. For example, visual inspection of the alignment showed patterns that suggest that some insertions are characteristic for amphipods (Fig. 2), for isopods and for the Scutocoxifera (Fig. 3, the latter with a weaker pattern). Although the alignment of single nucleotides is debatable and

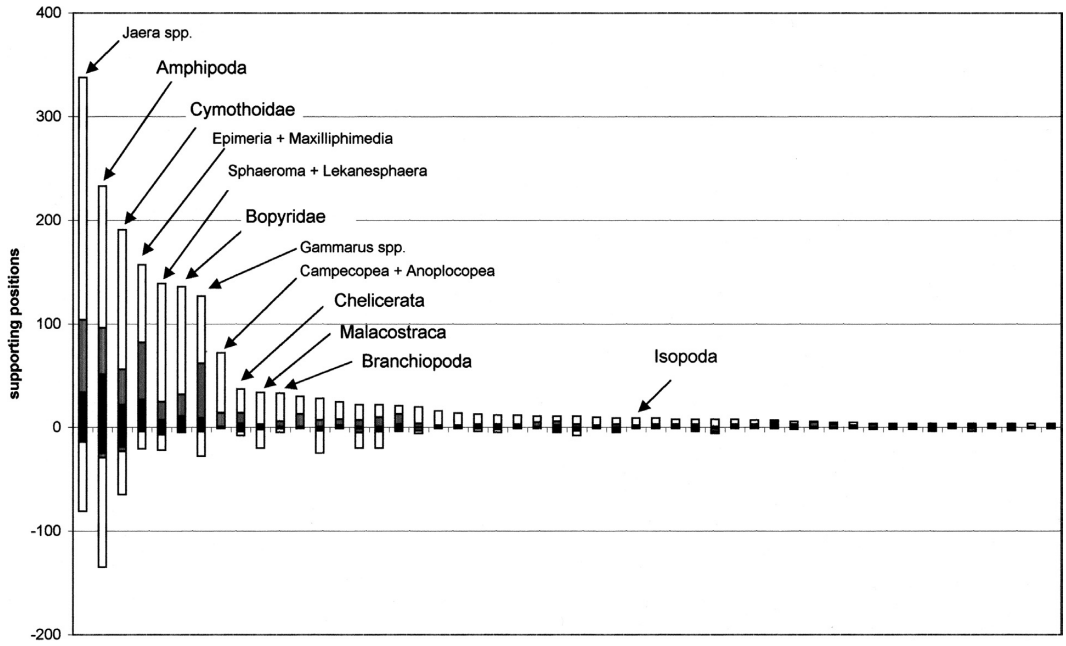

Fig. 4. Spectrum of supporting positions of the ssu rDNA alignment estimated with PHYSID (selected noise level: 25%). Columns indicate how many positions support a split (white: noisy positions; grey: character state conserved in ingroup (asymmetrical position); black: character state conserved in in- and outgroup (binary or symmetrical position)). Columns above the x-axis indicate support for the ingroup, below the line support for the outgroup is shown. Partitions without taxon names are composed of random combinations of taxa that do not appear in tree topologies (i.e., ones generally supported by chance similarities). Number of sequences: 43; number of characters: 2,533; 1,407 parsimony-informative positions and 3,762 splits, of which only the best 50 are shown.

can be modified, the presence of elongations is a fact. These are quite variable, but within groups like oniscids or valviferans these sequence areas should be useful for phylogeny reconstruction when more sequences are available.

With respect to base composition, we observed a higher G-content (between 0.27 and 0.298 in most species of anthurids, cirolanids, bopyrids, sphaeromatids, oniscids, and valviferans, and between 0.21 and 0.28 in asellotes). A slightly increased percentage of AT was noted in cymothoids (average: 0.55), and particularly in the species of *Jaera* (average 0.61); however, this had no effect in the reconstruction of dendrograms. For example, the *Jaera* sequences were not attracted by the cymothoid sequences even though some weak splits with this combination were found in the spectra of supporting positions.

Spectra of Supporting Positions

Spectra estimated with PHYSID clearly illustrate the major problems that exist with the nuclear ssu rDNA alignment: distinct phylogenetic signals in conserved positions are present only for a few taxa (Figs. 4, 5). Using the shortened alignment (2,533 positions, see above) and all 43 species, the highest signals (Fig. 4) are those favouring monophyly of the genus *Jaera,* of the Amphipoda, the Cymothoidae, a subset of amphipods (*Epimeria* + *Maxilliphimedia*), the Bopyridae, the genus *Gammarus,* the closely related sphaeromatids *Campecopea* and *Anoplocopea,* the Chelicerata, and Branchiopoda. The support for the Malacostraca and for the Isopoda is inverted, meaning that the positions are more conserved in outgroups than in ingroups. This occurs, for example, when positions evolve faster in the ingroup or when the outgroup shows only a gap, while the corresponding inserted nucleotide in the ingroup is variable. Support for the Isopoda is weak, and yet the partition is nevertheless found in all tree topologies (Figs. 6–8) and is certainly well founded on morphological grounds and by the presence of insertions (Fig. 3). Figure 4 shows only the first 50 of 3,762 splits, and only splits that occur in reconstructed trees (Figs. 6, 7) are indicated with arrows. Hence, these splits are mutually com-

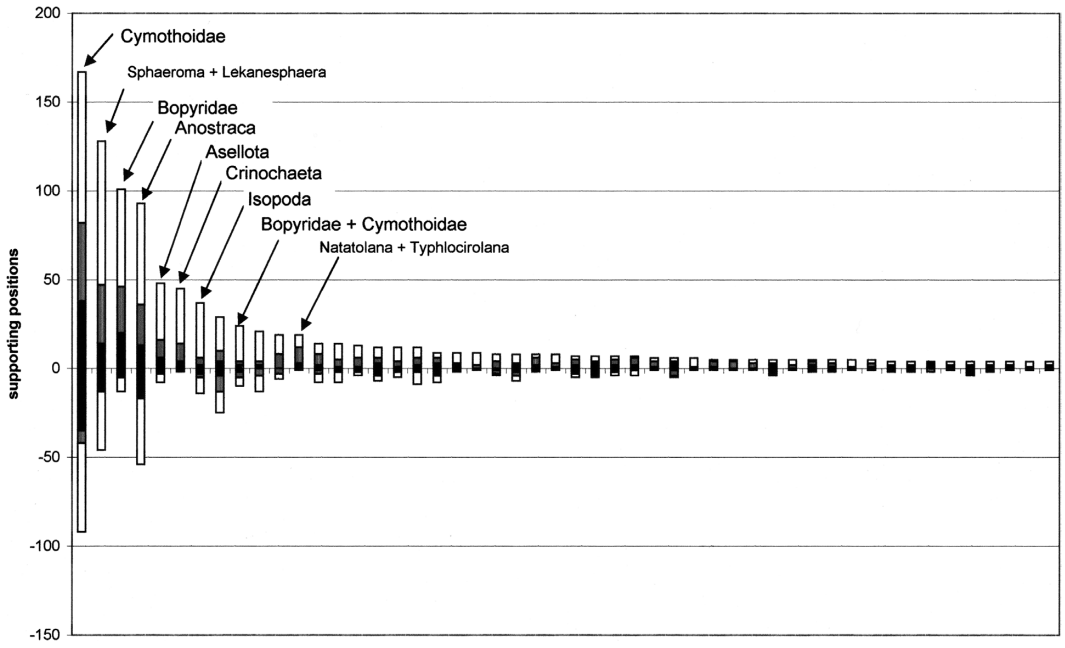

Fig. 5. Spectrum of supporting positions as in Fig. 4, however with a reduced number of species (see text). Due to the reduction of noise per position some partitions receive better support (Isopoda, Bopyridae + Cymothoidae, Asellota, Anostraca, Crinochaeta). Number of sequences: 26; number of positions: 2,533; 1,142 parsimony-informative positions and 2,379 splits, of which the 50 best are shown.

patible, they fit on a tree topology, and they are the ones with the best alignment evidence. Some weaker splits are only detected when some of the noise is deleted from the alignment. Figure 5 shows the result when only 26 species are used. Sequences of outgroup species, or sequences showing a higher AT content or attraction to distantly related groups in tree topologies or in spectra were deleted from the alignment for the spectrum of Fig. 5 (omitted species: all amphipods except *Gammarus salinus, Eurydice pulchra,* all anthurids, *Cassidinidea* sp., *Campecopea hirsuta,* and *Anoplocopea lusitanica, Ligia oceanica,* species of *Jaera, Daphnia pulex, Ulophysema oeresundense, Stenocypris major,* all chelicerates). Thus, the number of parsimony-informative positions decreased from 1,407 to 1,142 and the number of partitions decreased from 3,762 to 2,379. New well-supported splits that emerge are the Anostraca, the Asellota, and the Crinochaeta (part of the Oniscidea); the Isopoda find better support, and the group Bopyridae + Cymothoidae is recovered (see also Dreyer and Wägele, 2001). Splits corresponding to deep divergences of the isopod tree are not supported by conserved positions. Additional split-supporting positions favouring, for example, the Scutocoxifera are present in the complete alignment (which was not used for phylogeny reconstruction because of doubtful positional homology).

Neighbor-Joining

Distance methods proved to be unsuitable for this data set. Topologies differed greatly from results using parsimony and maximum likelihood. The distance trees estimated with different substitution models were poorly resolved, mainly because indels cannot be considered, and autapomorphies of terminal taxa increase distances of neighbours, an artifact that does not occur in parsimony analyses. Furthermore, we observed groupings caused by attraction of long branches (cymothoids + amphipods + *Jaera* spp.). It seems that estimated distances do not reflect the evolutionary distances for the complete gene because a large proportion of differences is caused by insertions and deletions. Therefore, we do not discuss further the topologies obtained by neighbor-joining.

Conspicuously long branches were the stem-line of cymothoids and the lines to terminal taxa of cymothoids, the stem-line of amphipods, the stem-line to *Jaera* spp., and the line to *Daphnia pulex* within bran-

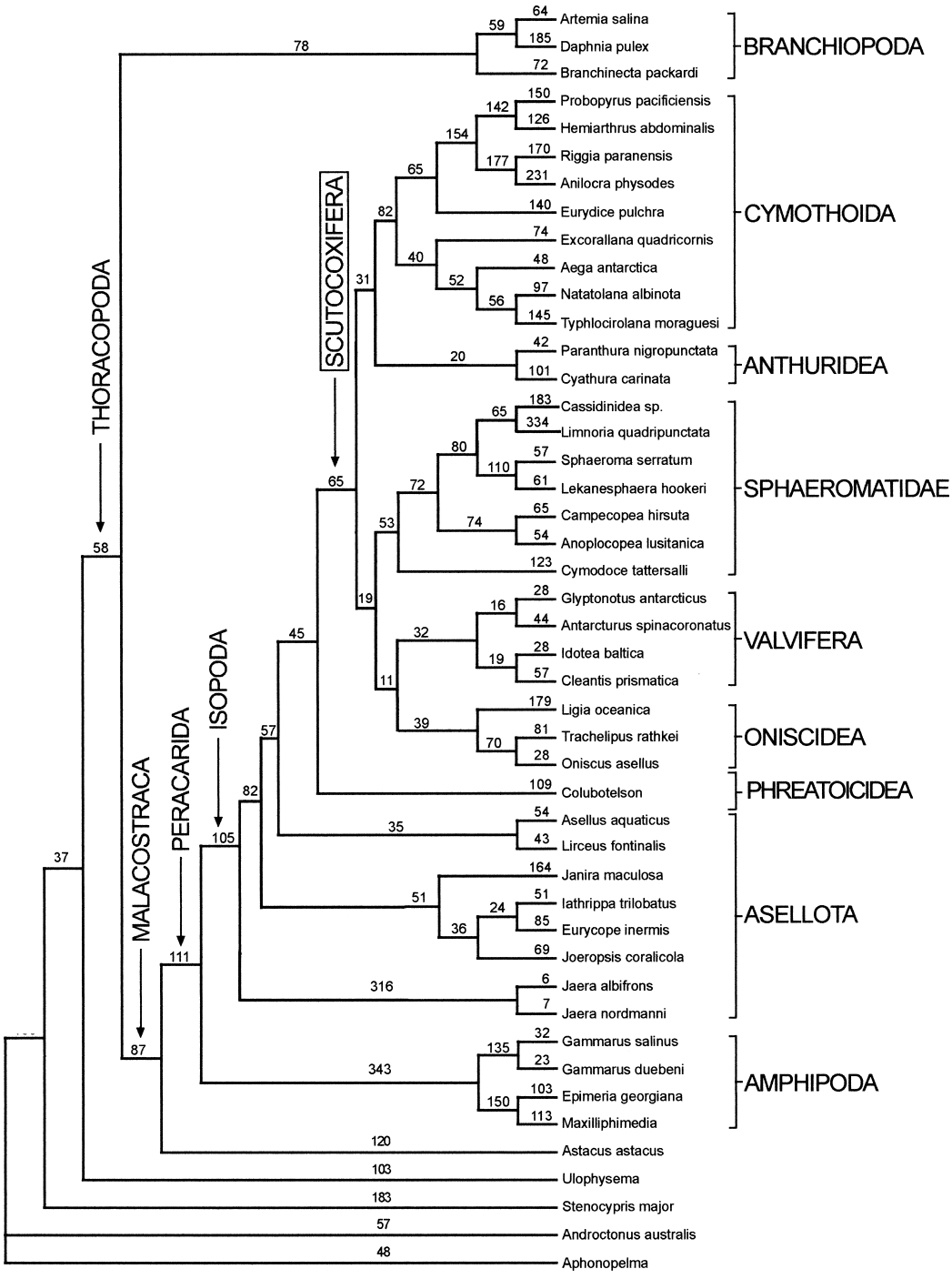

Fig. 6. The most parsimonious tree considering 46 species, gaps coded as fifth nucleotide (heuristic search and tree-bisection-reconnection branch swapping, 2,533 positions, 682 are constant, 1,403 are parsimony informative. Tree length = 7,765, consistency index 0.48, retention index 0.61). Numbers on branches are branch lengths.

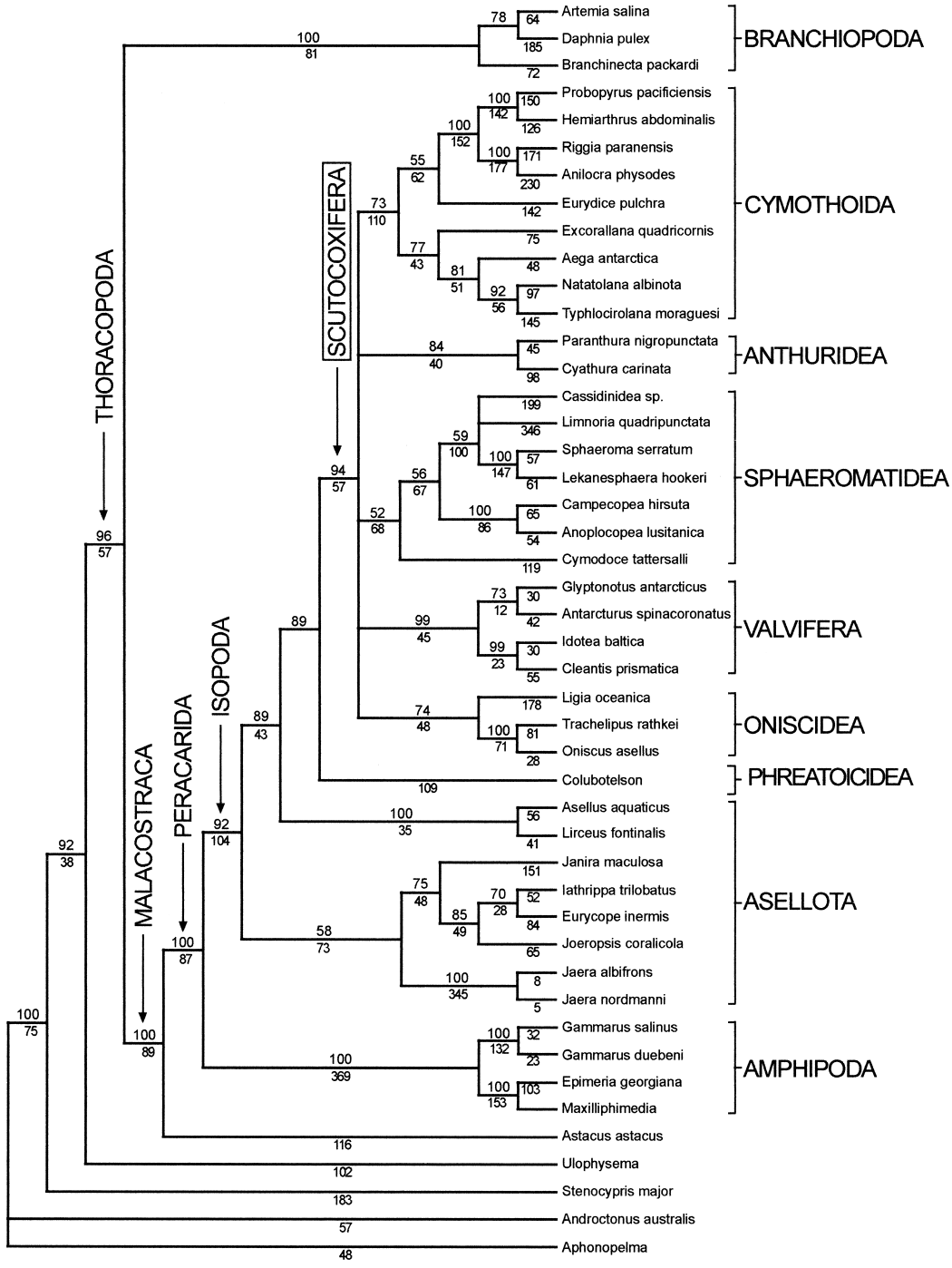

Fig. 7. Maximum parsimony 50% majority-rule bootstrap consensus tree (500 replicates) of the selected ssu rDNA alignment (same alignment as in Fig. 6, gaps coded as fifth nucleotide), for 34 isopod species and 12 outgroup sequences (numbers above the line are bootstrap support values, below the line assigned branch lengths). Some partitions strongly contradict morphology (for example, asellotes are not monophyletic) and are caused by artifacts (mainly noise in the alignment; compare also Figs. 4 and 5), even though the boostrap values may be high. (Tree length: 7,822; CI excluding uninformative characters = 0.4382; RI = 0.6023)

chiopods, and the line to *Ligia oceanica* within oniscids. In the alignment used for phylogenetic analyses, highest pairwise log-det-distances to *Androctonus australis* (outgroup sequence) occur for *Riggia paranensis* (0.200), *Anilocra physodes* (0.234), *Jaera albifrons* (0.192), *Jaera nordmanni* (0.193), *Gammarus salinus* (0.187), *Gammarus duebeni* (0.184), *Epimeria georgiana* (0.218), and *Maxilliphimedia longipes* (0.211) (assuming among-site variation of substitution rates and with an estimated proportion of invariable sites of 0.4). Long-branch attraction among these groups was observed only in distance trees.

Maximum Parsimony

A heuristic search with all sequences and the selected 2,533 positions (see Materials and Methods) yielded a single most parsimonious tree (Fig. 6). Gaps were coded as fifth nucleotide to take advantage of the information contained in insertions and deletions. With the exception of the Asellota the monophyly of all suborders with two or more sequences is confirmed. The Anthuridea appear as sister group of the Cymothoida, the Valvifera as sister group of the Oniscidea. The Scutocoxifera are monophyletic. The species of *Jaera* are found on a very long branch and are separated from the rest of the asellotes; the freshwater Asellota (*Asellus* and *Lirceus*) are separated from the marine taxa. This paraphyly of the Asellota is probably caused by erosion of signal (see Discussion).

Figure 7 shows the result of a bootstrap analysis for the same data. The following major groups are supported with bootstrap (BP) values over 90%: Thoracopoda, Malacostraca, Peracarida, Isopoda, Amphipoda, Asellidae (*Asellus* and *Lirceus*), Crinochaeta (within Oniscidea: *Oniscus* + *Trachelipus*), Valvifera, Bopyridae + Cymothoidae (*Probopyrus, Hemiarthrus,* and *Riggia, Anilocra*), and Scutocoxifera tax. nov. Monophyly of these groups does not conflict with morphological data (Wägele, 1989). Further groups recovered are the Cymothoida (including the Bopyridae), which here show only moderate support of BP = 73, the Anthuridea (BP = 84), the Oniscidea (BP = 74). The Asellota are paraphyletic as in Fig. 6. The Sphaeromatidea (in this alignment: Sphaeromatidae + Limnoriidae) find only weak support. The relation between Oniscidea, Valvifera, Sphaeromatidea, Anthuridea, and Cymothoida is not resolved. If the problematic sequences of *Limnoria quadripunctata* and the Asellidae are deleted, bootstrap values for the Sphaeromatidae and Asellota increase slightly, but the topology remains the same.

The topologies are less resolved when gaps are treated as "missing" character states (not shown). As already mentioned, specific insertions and deletions are characteristic for several isopod groups. If this information is not considered, the monophyly of the Cymothoida, Anthuridea, Sphaeromatidae, and Oniscidae is not recovered in the 50% majority-rule bootstrap consensus topologies. The most parsimonious tree contains a clade composed of the Cymothoida combined with the sequence of *Limnoria,* the sphaeromatid *Cymodoce tattersalli* in a clade with oniscids, but with monophyletic Anthuridea. In all calculations monophyly of the Amphipoda, Isopoda, Scutocoxifera, Asellota, Valvifera, and of the group Cymothoidae + Bopyridae was recovered.

Maximum Likelihood

An extensive maximum-likelihood analysis was not possible due to the intensive computational demands posed by a data set of this large size (e.g., bootstrapping would have taken a prohibitively long time). The topology in Fig. 8 was estimated with the complex GTR+G+I model (chosen with a likelihood-ratio test) and had to be calculated with a reduced data set to minimize computation times. The topology shows a few major partitions also recovered in maximum parsimony analyses (Bopyridae + Cymothoidae, Valvifera, Oniscidea, Scutocoxifera). However, the Sphaeromatidae (in Fig. 8 *Sphaeroma* + *Cymodoce*) are not monophyletic, and the monophyly of the Asellota is not resolved. These details are highly implausible and indicate that the model of sequence evolution used probably does not reflect the historical processes. A major problem here might be that models of sequence evolution do not take into account the probability that insertions and deletions occur episodically. Topologies obtained with simpler models and more sequences differed in details. For example, using the Hasegawa-Kishino-Yano model (Hasegawa *et al.,* 1985) the Cymothoida, Valvifera, Sphaeromatidae, and Asellota were recovered as monophyletic groups; however, the Oniscidea were not monophyletic, and the

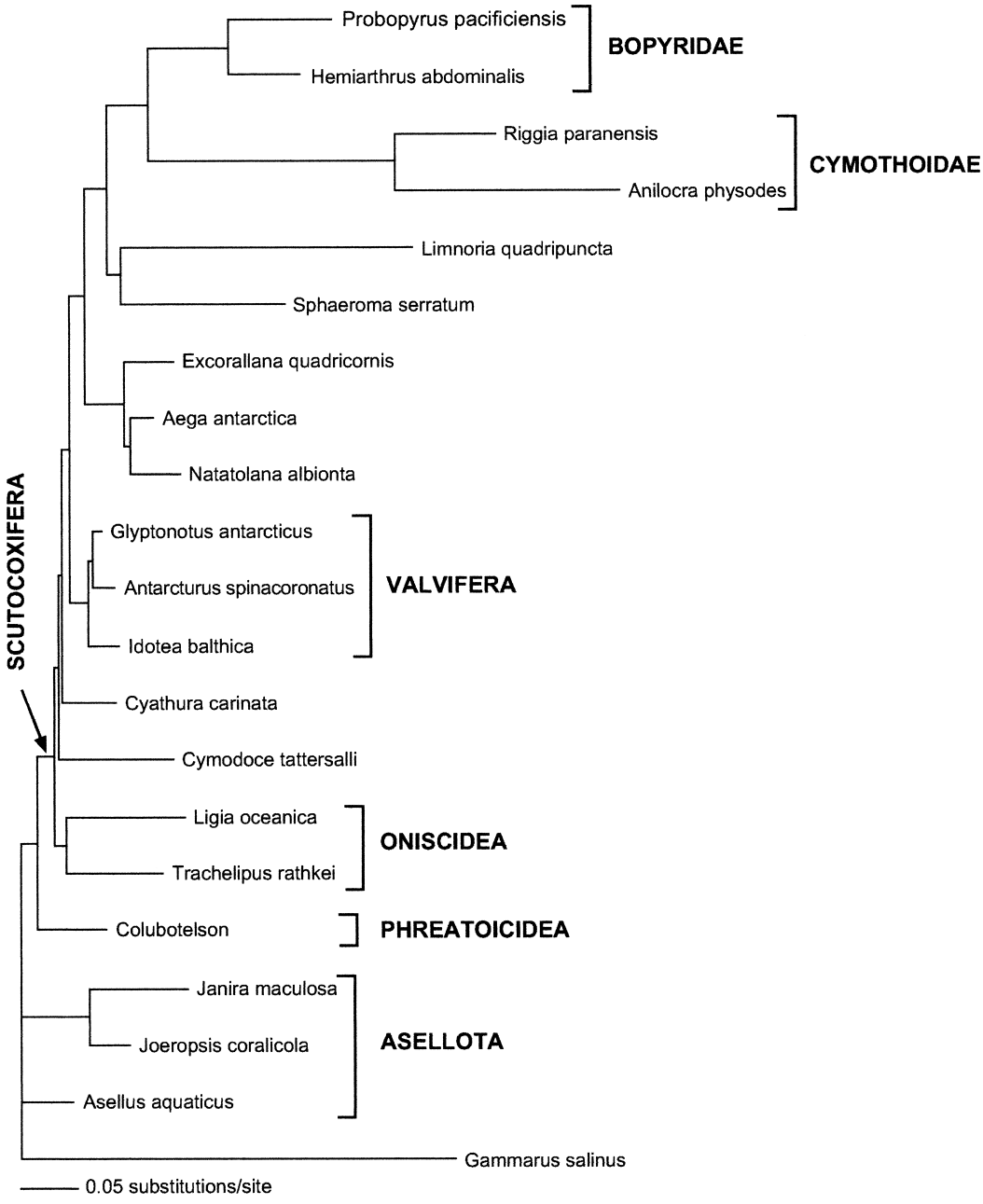

Fig. 8. Optimal maximum likelihood topology obtained with a reduced set of sequences and a complex model (GTR+G+I) chosen by a previous likelihood ratio test. The amphipod sequence of *Gammarus salinus* was selected as outgroup. Score of the best tree: 15,333.

Phreatoicidea appeared as sister taxon of *Paranthura*.

Morphology

Figures 9 and 10 show the different character states of the coxal article in phreatoicids and valviferans. In phreatoicids (Fig. 9) and also in asellotes, the coxa is roughly formed as in other peracarids (see Hessler, 1982), being a ring-like article. Most isopods have no coxal ring but have a coxal plate that has the shape of a pleurotergite (Fig. 10). The plates have a dorsal surface with a suture between the plate and the tergite, laterally a sharp lon-

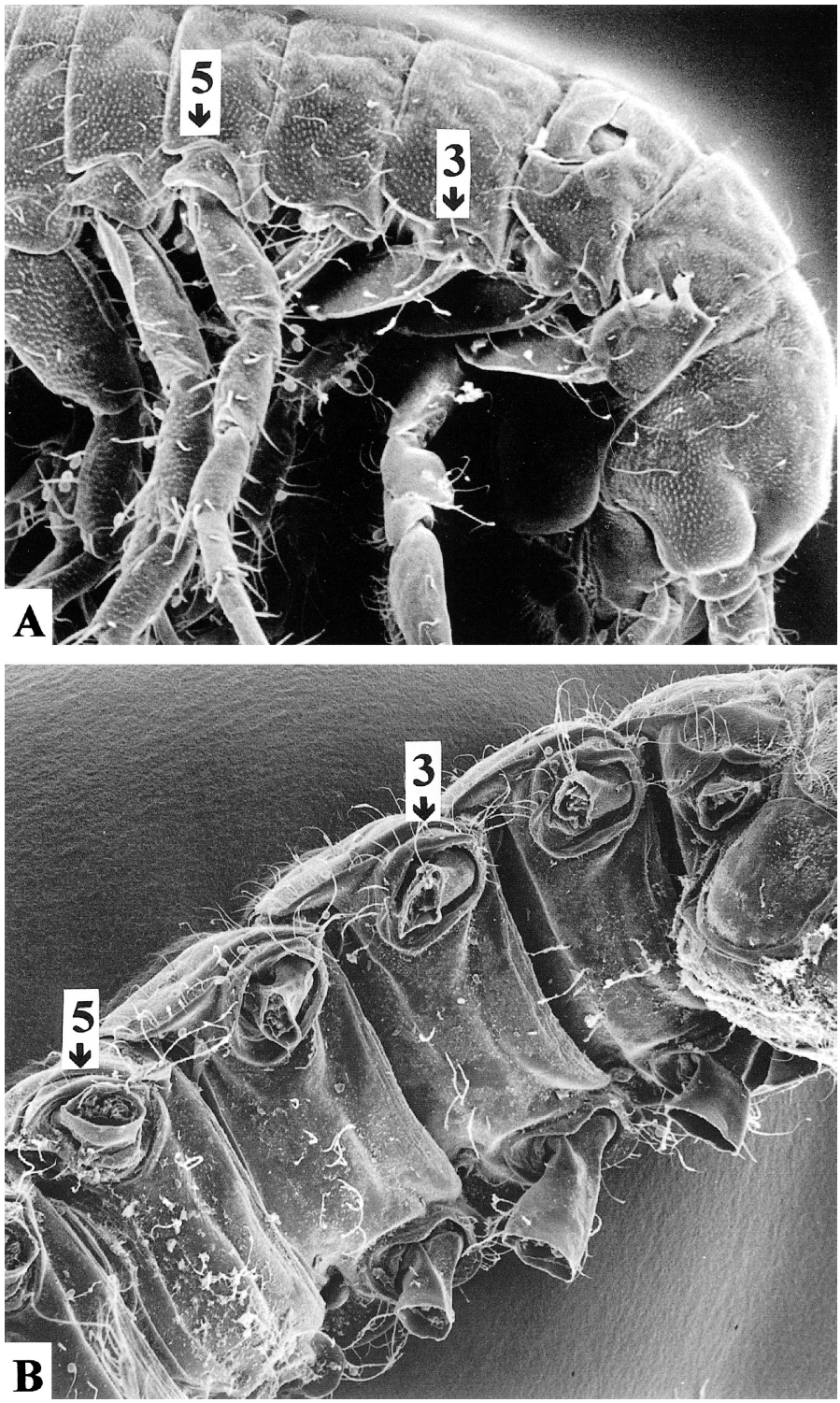

Fig. 9. SEM pictures of the South African phreatoicid isopod *Mesamphisopus capensis*. A: Lateral view of pereon (head at right). B: Ventral view (head at right), pereopods cut off. Arrows indicate the position of the coxa of pereopods 3 and 5.

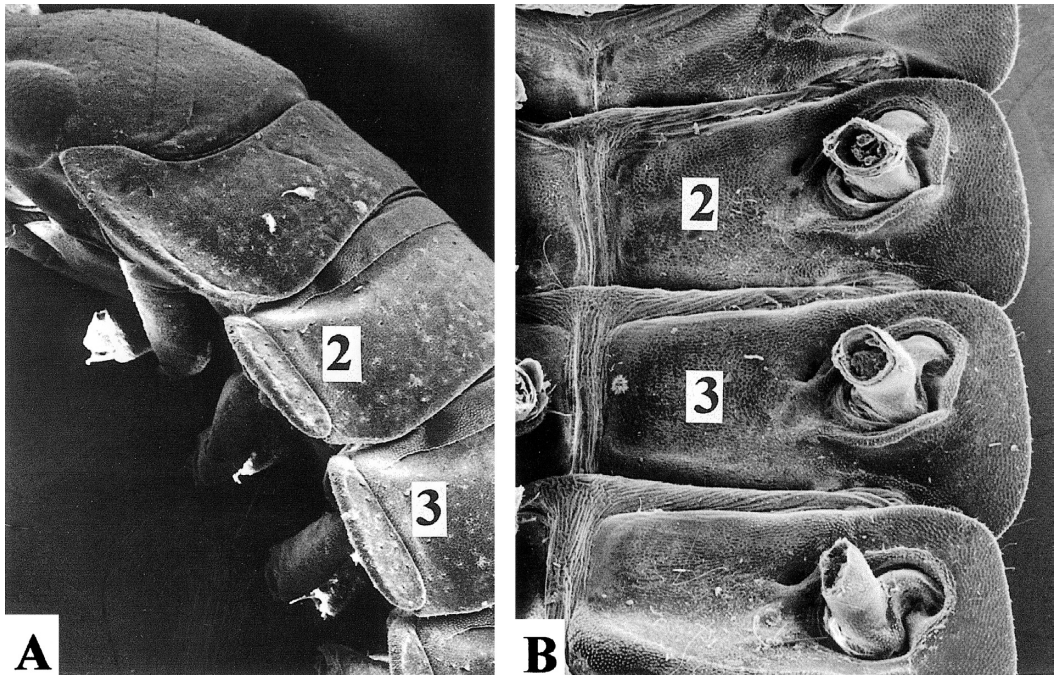

Fig. 10. SEM pictures of *Idotea baltica* (from northern Germany). A: Lateral view (head at left) with coxal plates distinct on pereonites 2 and 3. B: Ventral view of left pereonites 1 to 4, with coxal plates 2 and 3 numbered, pereopods cut off. Note that the insertion of the pereopod is at the basis-coxa articulation.

gitudinal keel separating the dorsal and the ventral surfaces, and ventrally an extended sclerotized area which can reach to the midline of the segments, as seen in *Idotea baltica* (Fig. 10). The pereopods insert on the ventral side in all species that have laterally directed plates. In the worm-like anthurids (not shown), the plates form the strongly sclerotized parts of the lateral body wall of the pereonites, and the pereopods insert laterodorsally. Such coxal plates are absent in phreatoicids and in asellotes. An example is *Mesamphisopus capensis* (Fig. 9), where each coxa (except the first one) forms a ring visible in ventral view, even though in lateral view the coxae of the posterior pereopods may look like small plates (on pereopod 5, for example: Fig. 10A). The laterally directed plates seen in asellotes are not formed by coxal plates but by the tergites and the ventral body wall, and the coxa resembles that of the phreatoicids (Fig. 9). A strongly sclerotized segmental ventral cuticle extends from one side to the other with no sutures in asellotes and phreatoicids. Functionally, in those species that have coxal plates these replace the ventral sclerotized area by large paired sclerites, often separated along the ventral midline by a soft, expandable cuticle. Such coxal plates occur in the following taxa: Oniscidea, Valvifera, Serolidae, Sphaeromatidae and related forms (Sphaeromatidea *sensu* Wägele, 1989), Anthuridea, and Cymothoida. The enigmatic isopod *Calabozoa pellucida* has no coxal ring, and it is not clear if coxal plates exist because dorsal suture lines are absent (Van Lieshout, 1983). In *Tainisopus,* the coxae are plate-like, but they are small and seemingly do not extend over the ventral surface of the segments (Wilson and Ponder, 1992). These latter two enigmatic taxa aside, we see congruence between the morphology of the coxa and the split separating the Scutocoxifera from other isopods in the molecular analyses.

Taxonomy: **Scutocoxifera** tax. nov.

Diagnosis.—Isopods with coxal articles of pereopods 1–7 transformed into plates that are in contact with the tergites along the complete length of the segments (therefore ring-like coxal articles on pereopods 1–7 absent). Stomach with a derived shape of the anterior filter channel (covered by the *clatri setarum anteriores*): channels curved laterally or in a transverse position (exception: the worm-like anthurids, see Wägele, 1989).

Remarks.—The suture lines of coxal plates are usually visible in dorsal view on pereonites 2–7, as long as the plates are not fused with the tergites. In most cases the coxal plates of the first pereonites are fused with the tergites, and in some species females show sutures. The plates often form pleurotergite-like lateral projections with a longitudinal keel forming the lateral margin of the body. The insertion of the pereopods is usually located ventrally and is homologous to the original coxa-basis articulation. Ventral expansions of the plates can cover most of the ventral body surface, leaving ventromedially a soft, expandable cuticle between the pair of plates on each segment. Oostegites are formed ventromedially of the insertion of the pereopods. The characters of the stomach were described in Wägele (1989). In other malacostracans that have a fully equipped stomach and in asellotes and phreatoicids, the filter channels have a longitudinal position; only the Scutocoxifera have channels bent laterally.

Further discussion of characters of the groundpattern of the Scutocoxifera is reserved for a future study that addresses in greater detail a comparison of isopod morphological characters.

For now, we suggest that the following taxa belong to the Scutocoxifera: Oniscidea, Valvifera, Sphaeromatidea, Anthuridea, and Cymothoida. The position of *Calabozoa* and *Tainisopus* is not clear. These species might also belong to the Scutocoxifera because coxal rings are absent, but further data are needed.

Concerning the Linnean categories available for this taxon, a rank between order and suborder would be necessary. We have to face the fact that in phylogenetic systems the number of recognized monophyla is often higher than the number of available categories. (This is one of the reasons for the recently recommended omission of categories in formal classifications: see Ax, 1988; Schander and Thollesson, 1995).

Discussion

In the present study, we concentrate on the detection of phylogenetic signal in ssu rDNA-data that help to elucidate isopod phylogeny. Molecular data cannot be regarded as infallible sources of phylogenetic information (e.g., Lecointre, 1996; Philippe and Laurent, 1998). "Molecular phylogenies" are not only sometimes incompatible with morphological evidence but, apart from being sensitive to different alignment methods (e.g., Lake, 1991; Wägele and Stanjek, 1995; Morrison and Ellis, 1997), also vary depending on the selected gene (e.g., Stock *et al.,* 1991; Otto *et al.,* 1996; Poe, 1996), the selected species (discussion in Lecointre *et al.,* 1993; Lecointre, 1996), or the chosen model of sequence evolution (e.g., Yang *et al.,* 1994; Rzhetsky and Nei, 1995; Russo *et al.,* 1996; Durbin *et al.,* 1998). Several authors (Adoutte and Philippe, 1993; Philippe *et al.,* 1994; Abouheif *et al.,* 1998) have shown that the nuclear ssu rRNA gene is not suitable to elucidate most bifurcations of Cambrian radiations of metazoans. Nevertheless, the gene is often used to resolve topologies even of Precambrian events (see, for example, early Metazoan phylogeny in Kobayashi *et al.,* 1993; Wainwright *et al.,* 1993; Cavalier-Smith *et al.,* 1996; Ecdysozoa hypothesis, discussed in Wägele *et al.,* 1999). A major problem is that topologies can also be obtained from alignments with low information content, and that groups can find high bootstrap support due to the presence of a few chance similarities or plesiomorphies (e.g., Philippe and Laurent, 1998; Wägele, 1999). Even though the nuclear ssu rRNA gene has been successfully used to reconstruct aspects of crustacean phylogeny (e.g., Abele *et al.,* 1992: origin of the Pentastomida; Spears *et al.,* 1994: phylogeny of cirripedes; Spears and Abele, 1998: Crustacea; Spears and Abele, 1999: Branchiopoda, Cephalocarida, Leptostraca), the gene is not necessarily useful for the study of all crustacean taxa or for all bifurcations in a topology. Our approach is to study the relationship between putative signals and noise in the alignment prior to phylogeny reconstruction, a methodical procedure equivalent to making distinctions between "good homologies" and "weak characters" (characters of low probability of homology) that is familiar to many morphologists (e.g., Osche, 1973; Van Valen, 1982; Wheeler, 1986), and to check the plausibility of molecular phylogenies by comparison with morphological evidence.

The best known methods to study patterns in alignments independently of any tree construction are spectral analyses (e.g., Lento *et al.,* 1995; Lockhart *et al.,* 1999). Assumptions about processes of sequence evolution can

be avoided using PHYSID (Wägele and Rödding, 1998a, b). The philosophy in this case is to start with a phenomenological analysis (i.e., "let us have a look at the raw data before we transform them") for the estimation of data quality prior to phylogeny reconstruction. The spectra (Figs. 4, 5) represent patterns of nucleotides that are present in the alignment and that can be found by anyone without the need to transform distances, to calculate the effects of multiple hits, or to estimate likelihoods. The same is true for the identification of indels by visual inspection of the alignments (Figs. 2, 3).

For the sequences used here, our conclusion is that the ssu rDNA alignment contains distinct signals in favour of few monophyletic groups (see Figs. 4, 5), some of them representing higher-level taxa (Amphipoda, Isopoda, Branchiopoda, Malacostraca), others representing lower-level taxa, such as genera or groups of genera (e.g., *Jaera,* Cymothoidae). Splits for taxa supported by few positions are drowned in background noise when the spectra are studied, but they may be detected in parsimony analysis. If only few positions have autapomorphic states for a group, high bootstrap values may be obtained when the weak signal is compatible with the overall tree topology (e.g., Wenzel and Siddall, 1999). However, the few supporting positions may be chance similarities or symplesiomorphies. The occurrence of symplesiomorphies in molecular data is discussed in Wägele and Rödding (1998b) and Wägele (1999). From our point of view additional (for example, morphological) evidence must be presented whenever the phylogenetic signal is weak.

We reserve discussion of morphological data in detail, as this information has been published elsewhere (Wägele, 1989; Brusca and Wilson, 1991). A combined analysis is planned for future studies when more sequence data are available. For unresolved questions, larger data sets are necessary (especially more genes). The general structure of the topologies obtained with our sequence data (Figs. 6–8) is compatible with hypotheses based on morphology (Fig. 1): The oldest lineages of extant isopods are the Phreatoicidea and the Asellota; the other "suborders" of the traditional classification belong to a large monophyletic taxon we propose to name Scutocoxifera. Deep relationships within the Scutocoxifera cannot be resolved owing to the lack of informative characters in the alignment as well as in morphological data sets. However, the monophyly of the taxa Oniscidea, Valvifera, Anthuridea, and Cymothoida *sensu* Wägele (1989) is confirmed. The "Epicaridea", which are parasites of other crustaceans, should not be placed in a separate suborder. Instead, we assign them to the Bopyridae, the sister group of the family Cymothoidae, and it can be shown that their morphology and way of life can be derived from that of a cymothoid-like ancestor (Dreyer and Wägele, 2001). The molecular phylogenetic information content in favour of the Sphaeromatidea is weak, and this group deserves a more detailed inspection with more data in the future.

Several details seen in the molecular phylogenies (Figs. 6–8) are probably artifacts and should be examined more closely with larger species samples and additional sequences. We noted for example that in all reconstructions the Cymothoidae do not appear close to the Aegidae, while morphologists agree that cymothoids (adults that live as permanent parasites on fishes), evolved from aegid-like ancestors (which have a similar morphology and temporarily suck blood from fishes: Menzies *et al.,* 1955; Brusca, 1981; Delaney, 1989; Wägele, 1989). Inspection of the alignment shows that cymothoid sequences are highly modified, and they show deletions and many substitutions (these also produce the long branches mentioned earlier and the high signal in Figs. 4 and 5). It is likely that apomorphies shared with the aegids have been eroded by modifications of the cymothoid sequences, and, therefore, in our alignment a group composed by *Excorallana, Aega, Natatolana,* and *Typhlocirolana* (Fig. 6) is supported by symplesiomorphies. Noise might also be responsible for some of the bifurcations within the Sphaeromatidea, the Valvifera, and the Asellota, because no distinct signals could be detected.

The Phreatoicidea appear in Figs. 6 and 7 between the asellotes and the Scutocoxifera, while in earlier studies (Fig. 1) they have been considered to be the phylogenetically oldest group of extant isopods. In the fossil record, phreatocids are the oldest known isopods (Carboniferous, see Schram, 1970). Plesiomorphic characters absent in other isopods are the presence of filter setae on maxilla 2, a subdivision of pleopod rami into two movable articles, and a laterally distinct

suture line between the cephalon and the maxillipedal segment (Wägele, 1989; Brusca and Wilson, 1991). For the time being, the molecular evidence is too weak to allow a revision of the hypotheses based on morphology (Fig. 1).

No isopod specialist would doubt that the Asellota are monophyletic. They have only a maximum of two (instead of five) free pleonites, their pleopods are not adapted for swimming but show other modifications, such as a typical apomorphic sexual dimorphism (pleopod 1 absent in females, and female pleopod 2 uniramous, male pleopod 1 uniramous, and male pleopod 2 without an appendix masculina, transformed to a characteristic gonopodium with modified, geniculate endopod), and the uropods are styliform and insert subterminally on the pleotelson (instead of laterally). This well-defined group is paraphyletic in Figs. 6 and 7, mainly because the sequences of the freshwater isopods (*Asellus* and *Lirceus*) differ markedly from those of the marine species. The latter seem to be more derived: the pairwise distances to outgroup sequences are higher for the marine species (e.g., log-det distance to *Astacus astacus* ranges between 0.122 and 0.151 for marine species, and from 0.106 to 0.118 for the asellids (freshwater species); distance to *Oniscus asellus* 0.094 to 0.21 for marine species, 0.065 to 0.075 for asellids). We therefore assume that synapomorphies of the Asellota are partly eroded in the marine species, but this problem needs a closer examination with more sequences. Interestingly, a signal in favour of the asellotes can be detected with a reduced species sample (spectrum in Fig. 5).

The clade Scutocoxifera is not strongly supported by spectral analysis of the nuclear ssu rDNA data, but it is consistently recovered in parsimony analyses. Because there are also important morphological characters favouring a hypothesis of monophyly for this group (see results and Fig. 1), we consider this taxon to be composed of descendants of a progenitor that possessed coxal plates. Though plate-like coxae also occur in amphipods, the lateral protrusion of the plate with the sharp longitudinal keel that separates the dorsal from the ventral surface of the plate is unique for these isopods. In amphipods the plates often are enlarged on pereopods 1–4, while the posterior plates are smaller, and they are directed ventrally. Our data indicate that the plate-like coxae evolved within the Isopoda because they are absent in those groups that occur at the base of the phylogenetic trees (Phreatoicidea and Asellota). Snodgrass (1952) thought that the coxal plates of isopods are not derived from appendage articles but are movable lateral tergal plates. Gruner (1954), however, showed that during embryonic development of *Porcellio scaber* the coxal article grows into a large plate that finally fuses with the lateral border of the tergite.

Unresolved relationships within the Scutocoxifera possibly indicate the occurrence of a rapid radiation, followed by a long period of divergent evolution of those groups that are now classified as suborders (named in Figs. 6, 7). To resolve the polytomy, longer sequences of slowly evolving genes are needed. We do not recommend use of the name "Flabellifera" as a synonym for the Scutocoxifera, because in traditional classifications the "Flabellifera" were composed only of those groups included in the Cymothoida and Sphaeromatidea *sensu* Wägele (1989), excluding valviferans, anthurids, gnathiids, and bopyrids.

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