JOURNAL OF NATURAL HISTORY, 2003, **37,** 20, 2461–2486

First observations on the phylogeny of the families Gammaridae, Crangonyctidae, Melitidae, Niphargidae, Megaluropidae and Oedicerotidae (Amphipoda, Crustacea), using small subunit rDNA gene sequences

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(*Accepted 21 February 2002*)

This study examines amphipod phylogeny based on small subunit (18S) rDNA sequence data. Complete sequences of 25 species representing six families were used to test the phylogenetic information content of this gene for reconstruction of amphipod phylogeny. The alignment proved to be informative for most of the studied taxa. The monophyly of the families Gammaridae, Crangonyctidae, Niphargidae and Oedicerotidae is supported. The Melitidae are not monophyletic in the reconstructed topologies, but weak molecular evidence for the monophyly of this group could be observed in spectra of supporting positions. A close relationship of Gammaridae + Melitidae or Gammaridae + Crangonyctidae is not supported, rather there are supporting positions for the incompatible sister-group relationship (Gammaridae + Niphargidae) and (Crangonyctidae + Niphargidae). The molecular evidence is in favour of the latter relationship. The evolution of cephalothoracic apodemes is discussed in the light of other phylogenetic hypotheses resulting from molecular data.

KEYWORDS: rDNA, Crustacea, Amphipoda, molecular phylogeny, parsimony, maximum likelihood, Physid.

Introduction

During the last decade considerable advances concerning the taxonomy of amphipods have been achieved (Barnard and Karaman, 1991). Nevertheless, the phylogenetic position of the major amphipod taxa is controversial. Many families, that were defined in the last century, are now abolished or fused into new families (Coleman and Barnard, 1991). A major problem is that the classification is based for the most part on diagnostic characters and not on apomorphies. Barnard and Karaman (1991: 7) described it as 'simply an artificial way to identify'. Some of the families are characterized only by plesiomorphic characters (e.g. Eusiridae). Bousfield (1977)

Journal of Natural History
ISSN 0022-2933 print/ISSN 1464-5262 online © 2003 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/00222930210144352

points to the weak concept of the Gammaridae: 'no single character used in defining the existing concept of Gammaridae (*sens. lat.*) is without exception'. These poor taxonomic definitions lead to non-monophyletic taxa that consist of groups or species that cannot clearly be identified. The classification should be based on phylogenetic analyses, not on keys of diagnostic characters.

Morphological characters have been used for years to propose a phylogenetic system of the major amphipod groups (Barnard, 1969; Bousfield, 1977, 1978, 1983; Karaman and Barnard, 1979; Barnard and Barnard, 1983; Barnard and Karaman, 1991; Kim and Kim, 1993), but because of the widespread occurrence of convergencies and reductions only a few informative characters have been found that can be used to create a phylogenetic system. The situation in amphipod phylogeny was characterized by Barnard and Karaman (1991: 5): 'Amphipoda are now well noted for their general evolutionary plan which proceeds from complex ancestral kinds to simplified derived kinds bearing many reductions or losses of complexity. At times the specialist is confronted with the feeling that most of the ''missing links'' in Amphipoda are still alive'.

Recent attempts to establish a phylogeny of amphipods based on morphological data were made by Kim and Kim (1993). They created a system of selected families based on just a few characters. The results are questionable; only the three selected corophioid families are well-founded, the remaining families are justified only by uninformative characters, such as 'cleft telson', that appear in several clearly unrelated groups. For most of the terminal taxa (e.g. Gammaridae, Oedicerotidae) the authors could not name autapomorphic characters.

The following study tries to answer some of the open questions on amphipod phylogeny using molecular data, it focuses on the relations between the families Gammaridae, Crangonyctidae, Niphargidae and Melitidae, and the question of whether these families are monophyletic. Barnard (1969) did not differentiate between Gammaridae and Melitidae, rather the genera *Maera*, *Paraceradocus*, *Elasmopus* and a few more were all considered members of the diverse family Gammaridae. Bousfield (1973) established the family Melitidae and removed the genera *Maera*, *Melita*, *Paraceradocus*, *Elasmopus*, *Jerbarnia*, *Beaudettia*, *Rotomelita*, *Paraniphargus*, *Psammogammarus* and *Eriopisa* from the Gammaridae, and placed them in the new family Melitidae which he later revised (Bousfield, 1977). The question still remains whether this family is a subgroup of the Gammaridae or an independent taxon.

In the past few years small subunit (ssu) rDNA data have been successfully used to analyse phylogenetic relationships of crustaceans (Abele *et al.*, 1990, 1992; Spears *et al.*, 1992, 1994; Held and Wägele, 1998; Spears and Abele, 1998, 1999, 2000; Dreyer and Wägele, 2001; Englisch and Koenemann, 2001). The ubiquity and homology of ssuRNA in all cell types, and its structural and functional constancy (Woese, 1987), makes it a useful evolutionary marker as long as divergence is not too long. (The phylogenetic signal before Cambrian speciations is mostly eroded by multiple substitutions: Philippe *et al.*, 1994; Philippe and Laurent, 1998.) The ssu rDNA contains regions of different degrees of variability. There are regions that are highly conserved, and there are also highly variable regions. The conserved regions allow sequences to be correctly aligned, while at the same time the variable areas may contain phylogenetic information on closely related species.

In this study we shall discuss the phylogeny derived from molecular evidence and compare it with published classifications based on morphology. A recently

discovered morphological character complex, the transverse cephalic apodemes, seems to be relevant for phylogenetic reconstruction (Coleman, 2002). Coleman (2002) shows, for example, the first apomorphy for the Gammaridae, the complete transverse apodeme bridge (plesiomorphic condition: apodemes separated). We compare these new morphological and molecular ssu rDNA data.

Material and methods

Material

Fourteen amphipod species were collected at different sites around the world (table 1) and freshly preserved in 100% EtOH. For this study the complete double-stranded ssu rDNA was sequenced for species of the families Gammaridae (eight), Melitidae (two), Crangonyctidae (one), Megaluropidae (one) and Niphargidae (one) (table 1). Additional sequences were included from Crangonyctidae (four), Niphargidae (one) and Gammaridae (two) published in Englisch and Koenemann (2001). Species from the family Oedicerotidae (four) were used as outgroup taxa within the Amphipoda, because to date no apomorphic characters that support a close relationship between this family and the above-mentioned can be found. The sequences of *Nephrops norvegicus* Linné, 1758, *Squilla empusa* Say, 1818, *Panulirus argus* (Latreille, 1804) and *Anaspides tasmaniae* Thomson, 1893 are chosen as non-amphipod outgroup taxa (table 1).

For DNA extraction only freshly preserved material was used. Three clones of the ssu rDNA for one specimen of *Gammarus duebeni* Lilleborg, 1951 and two specimens of two different populations of *Gammarus pulex* Linné, 1758 were sequenced to study intraspecific variability.

DNA extraction

DNA was obtained using the QIAmp Tissue Kit (Qiagen™). Instructions of the 'Mouse Tail Protocol' were followed exactly, with the exception of the last step: the DNA was eluted with $2 \times 100\,\mu l$ H_2O instead of $2 \times 200\,\mu l$.

PCR amplification

PCR was performed following a standard protocol: a total volume of $50\,\mu l$ consisting of $1\times$ PCR buffer, $1\times$ Q-Solution (Qiagen™), 125 pM dNTPs, 25 pM of primer small subunit F and 50 pM of primer small subunit R (table 2), 1.25 U Taq DNA polymerase (Qiagen™) and $1\,\mu l$ (50–150 μg) DNA extract. The PCR cycle was programmed as follows: 1×5 min at 94°C; 35×30 s at 94°C, 50 s at 52.5°C and 3 min 20 s at 72°C; 1×7 min at 70°C. The PCR was performed as a hot start PCR. For PCR primers (Messing *et al.*, 1981) see table 2.

The amplified PCR product was purified using the QIAquick PCR Purification Kit (Qiagen™).

DNA cloning and sequencing

The purified PCR products were ligated into the pCR®-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and cloned in heat shock component Top 10 F′ One Shot™ cells (Invitrogen).

Plasmids were purified with the S.N.A.P.™ MiniPrep Kit (Invitrogen).

Cycle sequencing was conducted with a LI-COR™ 4200 automated sequencer, using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with

Table 1. Family, sequence length, accession number and sampling site for the included taxa (the new sequences in these analyses are marked with an asterisk).

Species	Family	No. of base pairs (bp)	Accession No.	Collection site
*Arrhis phyllonyx** (Sars, 1858)	Oedicerotidae	2718	AF419235	Porsanger Anskarholmen, Norway (70°21.45′N/25°15.13′E)
*Bathymedon obtusifrons** (Hansen, 1887)	Oedicoerotidae	2613	AF419236	Porsanger Osterbotn, Norway (70°07.11′N/25°10.65′E)
*Paroedicerus propinquus** (Goes, 1866)	Oedicerotidae	2770	AF419231	Porsanger Osterbotn, Norway (70°07.11′N/25°10.65′E)
*Monoculodes carinatus** (Bate, 1856)	Oedicerotidae	2735	AF419230	Baltic Sea (57°28.01′N/11°11.70′E)
*Megaluropus longimerus** (Schellenberg, 1925)	Megaluropidae	2521	AF419234	Curacao, Netherlands Antilles (Carribean Sea)
*Paraceradocus gibber** (Andres, 1984)	Melitidae	2328	AF419232	Antarctica (61°05.40′S/55°56.40′W)
*Maera inequipes** (Costa, 1851)	Melitidae	2487	AF419229	Roses, Spain (Mediterranean Sea)
*Gammarus duebeni** (Lilleborg, 1851)	Gammaridae	2263 (Clone 1) 2259 (Clone 2)* 2260 (Clone 3)*	AF356545 AF419226 AF419227	Isle of Great Cumbrae (Scotland)
Gammarus pulex (Linné, 1758)	Gammaridae	2250 (BI)	AF202892	Schwarzbach: Bielefeld (Germany)
		2246 (SC)*	AF419225	Isle of Great Cumbrae (Scotland)
Gammarus troglophilus (Hubricht and Mackin, 1940)	Gammaridae	2307	AF202983	St. Louis Co., MO, USA (38°30.976′N/90°33.609′W)
*Gammarus locusta** (Linné, 1758)	Gammaridae	2235	AF419222	Baltic Sea
*Chaetogammarus pirloti** (Sexton and Spooner, 1940)	Gammaridae	2271	AF419228	Isle of Great Cumbrae (Scotland)
*Parapallasea lagowski** (Dybowsky, 1874)	Gammaridae	2268	AF419223	Lake Baikal
*Eulimnogammarus obtusatus** (Dahl, 1938)	Gammaridae	2361	AF419224	Baltic Sea
Niphargus fontanus (Bate, 1859)	Niphargidae	2237	AF202981	River Ruhr (Dr T. Glatzel, Oldenburg)
*Niphargus kochianus** (De Cette)	Niphargidae	2227	AF419221	River Ruhr (Dr T. Glatzel, Oldenburg)
Crangonyx forbesi (Hubricht and Mackin, 1940)	Crangonyctidae	2331	AF202980	St. Louis Co., MO, USA (38°36.952′N/90°42.056′W)
Bactrurus brachycaudus (Hubricht and Mackin, 1940)	Crangonyctidae	2322	AF202979	St. Louis Co., MO, USA (38°30.976′N/90°33.609′W)
Bactrurus mucronatus (Forbes, 1876)	Crangonyctidae	2329	AF202978	Saline Co., IL, USA (37°41.330′N/88°25.169′W)
Bactrurus pseudomucronatus (Koenemann and Holsinger, 2001)	Crangonyctidae	2319	AF202985	Oregon Co., MO, USA (36°48′54″N/91°10′51″W)
*Synurella dentata** (Hubricht, 1943)	Crangonyctidae	2315	AF419233	Preble Co., OH, USA (39°46.325′N/84°43.344′W)
Panulirus argus (Latreille, 1804)	Decapoda	1872	U19182	

Table 1. (*Continued*).

Species	Family	No. of base pairs (bp)	Accession No.	Collection site
Nephrops norvegicus (Linné, 1758)	Decapoda	1857	Y14812	
Anaspides tasmaniae (Thomson, 1893)	Anaspidacea	1827	L81948	
Squilla empusa (Say, 1818)	Stomatopoda	1817	L81946	

Table 2. Oligonucleotides used for PCR and sequencing.

Primer	Sequence (5′→3′)
PCR	
Small subunitF	CCTA(CT)CTGGTTGATCCTGCCAGT
small subunitR	TAATGATCCTTCCGCAGGTT
Cycle sequencing	
M13universal CS(-43)	CGCCAGGGTTTTCCCAGTCACGAC
M13reverse(-29)	CAGGAAACAGCTATGAC
400F	ACGGGTAACGGGGAATCAGGG
400R	CCCTGATTCCCCGTTACCCGT
700F	GTCTGGTGCCAGCAGCCGCG
700R	CGCGGCTGCTGGCACCAGAC
1000F	CGATCAGATACCGCCCTAGTTC
1000R	GAACTAGGGCGGTATCTGATCG
1155F	CTGAAACTTAAAGGAATTGACGG
1155R	CCGTCAATTCCTTTAAGTTTCAG
1250F	CCGTTCTTAGTTGGTGGAGCG
1250R	CGCTCCACCAACTAAGAACGGCC
1500R	CATCTAGGGCATCACAGACC
1600F	CGTCCCTGCCCTTTGTACACACC

F (forward) and R (reverse) indicate the orientation of the primers.

7-deaza-dGTP (Amersham™). The sequencing oligo-nucleotides were designed by Dreyer, Wollscheid and Englisch (unpublished) and are shown in table 2.

Sequence analyses

The fragments were combined to a consensus sequence using the program Dnasis (Hitatchi Software). Sequences were aligned with the software package ClustalW (Thompson *et al*., 1994) and corrected by eye in Genetic Data Environment (GDE) according to a secondary structure presented by Crease and Colbourne (1998). Additionally, a secondary structure developed by Choe *et al*. (1999) was used to determine homologous sequence positions. The alignment was scanned by eye for variable and highly variable regions where homology of sequence positions is questionable. Two more alignments were created, one without the highly variable regions, the other missing further variable positions. For length of alignments and position of removed parts see Results.

The Chi-square test of homogeneity of base frequencies across the included taxa for each of the alignments was calculated with DAMBE (Xia, 2000). Pairwise sequence differences were computed with PAUP version 4.0 (Swofford, 1998) for the gammarid taxa included in alignment 1, and are shown in table 3. Additionally,

Table 3. Pairwise sequence differences. The proportion of differing sites was calculated (PAUP version 4.0) for the complete sequences included in alignment 1 and is given as a percentage.

	G. duebeni Clone 1	*G. duebeni* Clone 2	*G. duebeni* Clone 3	*G. pulex* BI	*G. pulex* SC	*G. locusta*	*G. troglophilus*	*E. obtusatus*	*C. pirloti*	*P. lagowski*
G. duebeni Clone 1	–	0.2	0.2	2	2	2	3	4	3	2
G. duebeni Clone 2		–	0.2	2	2	2	3	4	3	2
G. duebeni Clone 3			–	2	2	2	3	4	2	3
G. pulex BI				–	0.3	2	3	4	3	2
G. pulex SC					–	2	2	4	3	2
G. locusta						–	3	3	3	3
G. troglophilus							–	6	4	3
E. obtusatus								–	4	4
C. pirloti									–	3
P. lagowski										–

a distance matrix of p-distances for alignment 1 (all positions) was calculated using PAUP 4.0.

Phylogenetic analyses

Twenty-five taxa (for *Gammarus duebeni* we used three sequences for one specimen, and with *Gammarus pulex* there were sequences of two specimens from different populations) were included in the phylogenetic analysis using the malacostracan species *Nephrops norvegicus*, *Anaspides tasmaniae*, *Squilla empusa* and *Panulirus argus* as outgroup taxa. Sequences of closer relatives (other peracarid groups) were not deposited in GenBank at the time of analyses. Three different methods of phylogeny inference, as implemented in PAUP 4.0 (Swofford, 1998), were used for each alignment:

Parsimony analyses. A heuristic search was performed using, nearest neighbour interchange (NNI) and TBR (tree bisection reconnection) for branch swapping. Steepest descent was deactivated while MulTrees was in effect. The TBR search was rerun with 1000 bootstrap replicates. A 50% majority rule consensus tree was computed.

Maximum likelihood analyses. To find the suitable model of sequence evolution a likelihood-ratio test was carried out as implemented in Modeltest 3.06 (Posada and Crandall, 1998). A heuristic search was computed in PAUP 4.0 using the determined parameters (for details see figure 4).

Distance analyses. Neighbour joining (NJ) was performed with each of the Kimura 2-parameter, logdet/paralinear and the HKY85 model for nucleotide substitution. The data set was resampled with 1000 bootstrap replicates.

PHYSID (see Wägele and Rödding, 1998) was used as a further tool to estimate the information content of the three alignments. PHYSID is a program that identifies positions supporting a particular group (Wägele and Rödding, 1998) in an alignment. Supporting sites are either putative apomorphies or plesiomorphies or chance similarities. The alignment is scanned for sites that support groups of taxa. Three classes of sites are differentiated: (1) symmetrical sites: the nucleotide for the ingroup taxa is identical and the outgroup taxa show a different nucleotide that is identical in all outgroup taxa; (2) asymmetrical sites: the nucleotide of a position is identical for all ingroup taxa while the outgroup shows different nucleotides, but not the one found in the ingroup; (3) noisy sites: like (1) or (2) but with some substitutions allowed in ingroup and outgroup taxa (cf. Wägele and Rödding, 1998 and figure 10). PHYSID allows the user to define the degree of noise permitted (Wägele and Rödding, 1998). The splits were calculated allowing 25% noisy positions in ingroup and outgroup nucleotides, both in columns and in rows.

The results of these analyses are presented in spectra that show the number of supporting positions for different putative monophyletic groups. Splits with only one or two potential apomorphies (supporting positions) are not taken into account.

Anatomical examination

The following species were examined: *Bactrurus brachycaudus* Hubricht and Mackin, 1940, *Crangonyx forbesi* (Hubricht and Mackin, 1940), *Synurella dentata* Hubricht, 1943, *Stygobromus mackini* Hubricht, 1943, *Megaluropus longimerus* Schellenberg, 1925 and *Paraceradocus gibber* Andres, 1984. Specimens were dissected using microsurgical scissors and forceps to remove the dorsal part of the cephalothorax. Heads were heated for 30 min in concentrated potassium hydroxide solution

to remove tissue. The unstained cuticle of the heads was examined in glycerol or water. To allow an undisturbed view of the transverse apodeme bridge the tendons of the mandible adductor were removed on both sides. Drawings were made up with a Wild M8 dissecting microscope and a Leica™ DMLB microscope, both fitted with a camera lucida.

Results

Sequencing and alignment

In pairwise comparison of the sequences from three clones of the ssu rDNA of a single specimen of *Gammarus duebeni*, two differences were found among the 2263 bp of the complete sequences. The sequences differ by approximately 0.1% (cf. table 3). The sequences for the two *Gammarus pulex* populations (table 1) differ in six positions of 2250, which means a difference of about 0.3% (cf. table 3). These sequence differences can be ignored in the analyses. We assume that the sequence of a single clone of a single specimen can be used as representative for a species.

All of the 16 new complete ssu rDNA sequences differ greatly in length from the approximately 1800 nucleotides (nt) regarded as typical in crustaceans and most other animals (table 1). Insertions in regions V4, V7 and V9 (Crease and Colbourne, 1998) increase the length to a range between 2277 and 2770 nt, as noted before by Englisch and Koenemann (2001). This variability of sequence length resulted in an alignment of 3274 positions (alignment 1). It was necessary to remove ambiguous parts of the first alignment to compare only positions with a high probability of homology. The 'difficult' regions were selected by eye. The second alignment (2750 bp) does not contain positions 1060–1222, 1304–1478 and 2418–2603. These correspond to sequence positions 667–783 (1060–1222 and 1304–1478) and 1371–1423 (2418–2603) of *Panulirus argus.*

To study the information content for even more conserved areas further positions of moderate variability were deleted. The third alignment (without positions 355–463, 1060–1560, 2371–2603 and 2850–2968 of the original alignment, equal to sequence positions 224–257, 667–845, 1371–1423 and 1575–1577 of *Panulirus argus*) consisted of 2312 positions (alignment 3).

The Chi-square tests for homogeneity of base frequencies across the included taxa for the three different alignments result in the following *P* values: 0.0 for alignment 1; 0.0033 for alignment 2; 0.8506 for alignment 3. There is no significant correlation between the base frequencies and the pairing of single sequences for alignment 3. The base frequencies for this alignment should not influence the topologies of the resulting trees. The results for alignment 1 and 2 indicate that for these alignments the base frequencies might influence the pairing of the sequences and probably affect the topologies.

The pairwise p-distances calculated for the oedicerotid species to all taxa included range between 0.25091 and 0.31639. Only the p-distances for *Maera inaequipes* (Costa, 1851) to the other taxa included in the analysis also show high values (0.20738–0.22821). These distances are larger or equal to the distances of the other amphipod taxa to the outgroup taxa. The highest p-distance that can be observed between the remaining amphipod taxa is 0.18402.

Phylogenetic analyses

The maximum parsimony analyses resulted in six slightly different bootstrap topologies for the three alignments and methods of analysis. The bootstrap

topologies in every case are identical to one of the shortest trees retained from heuristic searches.

Alignment 1 (all alignment positions) gives a bootstrap topology that supports the monophyly of the ingroups (amphipod taxa) against the outgroup (figure 1). The bootstrap topology that is shown in figure 1 is identical to one of the two shortest trees found in the heuristic search. (The two topologies using TBR as a branch-swapping option are 4236 steps long, six steps shorter than the six topologies resulting from the NNI search.) Within the amphipod taxa the Oedicerotidae are found to be monophyletic. Gammaridae, Crangonyctidae, Niphargidae, Melitidae and Megaluropidae cluster together as the sister-group of the Oedicerotidae. The families Gammaridae, Crangonyctidae, Niphargidae and Oedicerotidae (monophyletic groups) all have high bootstrap support (99–100%). The Melitidae are not recovered as a monophylum. The Gammaridae, Crangonyctidae and Niphargidae group together. The Crangonyctidae are

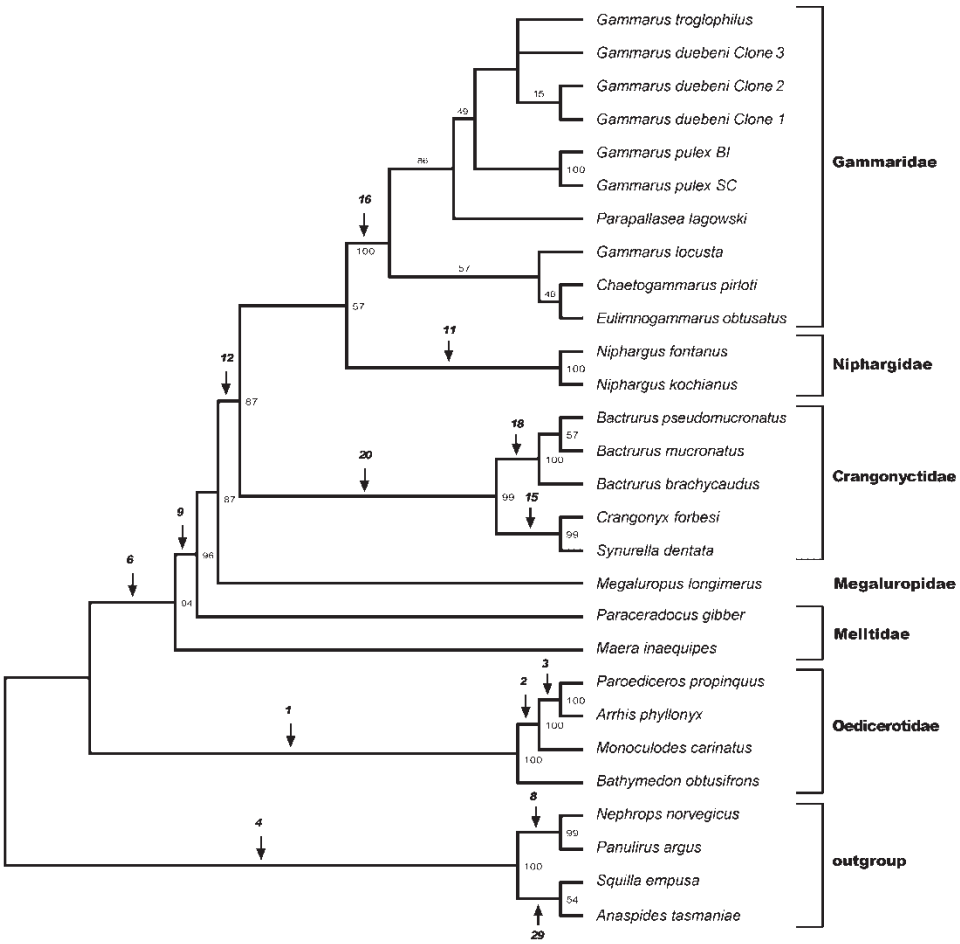


FIG. 1. 50% majority-rule consensus (1000 bootstrap replicates) for alignment 1 obtained by maximum parsimony (NNI). *N. norvegicus*, *P. argus*, *S. empusa* and *A. tasmaniae* were chosen as outgroup species and the tree was rooted with the outgroup. The numbers (italics) with arrows indicate the position of the splits in figure 7.

monophyletic. The genus *Bactrurus* is monophyletic as is the sister-group of (*Crangonyx forbesi*+*Synurella dentata*). Gammaridae and Niphargidae group together with a bootstrap value of only 58 (NNI) and 59 (TBR), respectively. This means that the relationship between the families Crangonyctidae, Niphargidae and Gammaridae is not convincingly resolved with this alignment. The basic divergences within the considered Gammaridae are not resolved [polytomy with *Gammarus locusta* Linné, 1758, *Eulimnogammarus obtusatus* (Dahl, 1938), *Chaetogammarus pirloti* (Sexton and Spooner, 1940) versus the remaining Gammaridae]. The genus *Gammarus* is not monophyletic in these topologies.

The maximum parsimony bootstrap trees for alignment 2 (2750 bp) are identical in the basic topology to the one shown in figure 1. Only the position of the gammarid taxa varies slightly (figure 2a). The heuristic searches result in trees requiring the

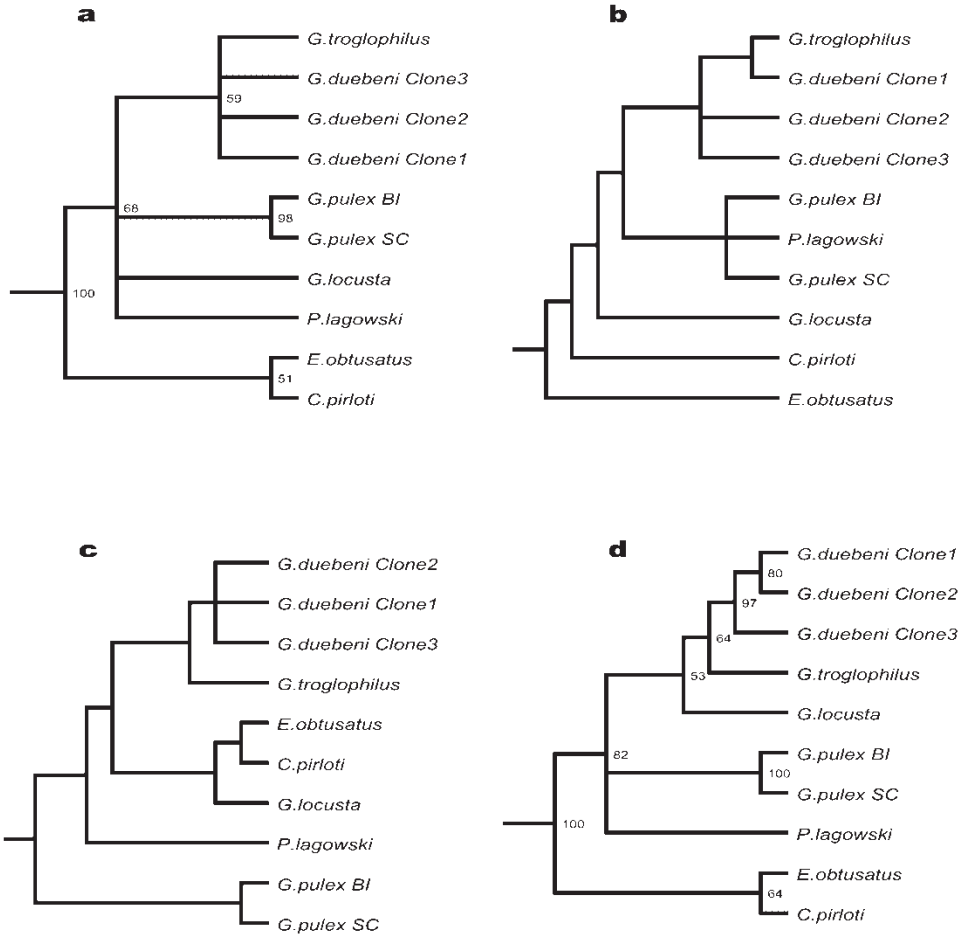


FIG. 2. Four different topologies for the included gammarid taxa, only. (a) Maximum parsimony analyses (NNI) for alignment 2 (1000 bootstrap replicates, 50% majority-rule consensus tree). (b) Maximum likelihood analyses for alignment 3 (HKY85 model, 500 bootstrap replicates, 50% majority-rule consensus). (c) Maximum likelihood analyses for alignment 2 (HKY85 model, 500 bootstrap replicates, 50% majority-rule consensus). (d) Logdet paralinear distance analyses for alignment 2 (1000 bootstrap replicates, 50% majority-rule consensus tree).

same number of changes (2747 steps/changes) for TBR (13 shortest trees) and NNI (eight shortest trees) branch-swapping options.

A comparable result (figure 3) was also found for alignment 3 (2312 bp). The heuristic searches result in trees of identical length for TBR (102 shortest trees) and NNI (72 shortest trees) branch-swapping options (1849 steps). The bootstrap topologies are identical to one of the shortest topologies.

The basic structure of the resulting trees is identical. Even though several variable positions are excluded, the information content of the more conserved positions seems to be high. The bootstrap values for the families with two or more sequences (except the non-monophyletic Melitidae) are high (98–100%). *Megaluropus longimerus* and *Paraceradocus gibber* changed their position from the topology for alignments 1 and 2. *Paraceradocus gibber* does not group with the Gammaridae, Crangonyctidae and Niphargidae as it did in the topologies for alignments 1 and 2. The Niphargidae group with the Crangonyctidae, but the bootstrap value supporting

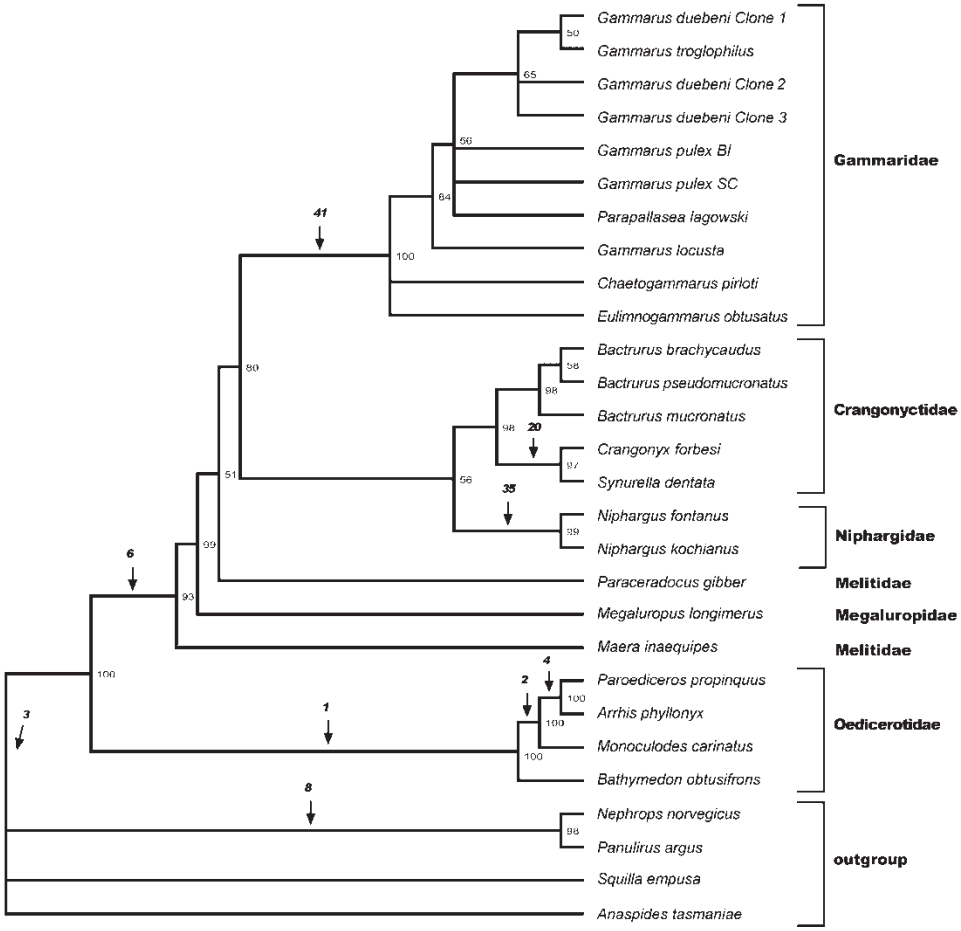


FIG. 3. 50% majority-rule consensus (1000 bootstrap replicates) tree for alignment 3 obtained by maximum parsinomy (NNI). *N. norvegicus*, *P. argus*, *S. empusa* and *A. tasmaniae* were used as outgroup and the tree was rooted with the outgroup. The numbers with arrows correspond with the splits in figure 9. Length = 1858, CI = 0.7648, HI = 0.2352, RI = 0.8275, RCI = 0.6329.

this clade is quite low (54). As mentioned for alignment 2 the topology for the grammarid taxa varies a little in the TBR and NNI trees, however the genus *Gammarus* is not monophyletic in any of the topologies.

The maximum likelihood analyses resulted in trees that show the same basic topology (figure 4) that was found in the maximum parsimony trees. The Oedicerotidae are monophyletic. The positions of *Paraceradocus gibber* and *Megaluropus longimerus* are identical for all three alignments, as in the foregoing topologies the Melitidae are not monophyletic. *Megaluropus longimerus* is the sister taxon to the group composed of Gammaridae, Crangonyctidae and Niphargidae. *Paraceradocus gibber* branches off before *Megaluropus longimerus*, and *Maera inaequipes* has the position as the sister-group of the Oedicerotidae. The Crangonyctidae are monophyletic and the Niphargidae and Gammaridae group together as in figure 1. The positions of the gammarid taxa (figure 2b, c) vary slightly in the topologies for the three alignments. The number of substitutions per site is quite high for the Oedicerotidae (0.153 (alignment 1); 0.132 (alignment 2); 0.104 (alignment 3)), which indicates that these taxa are long-branch taxa.

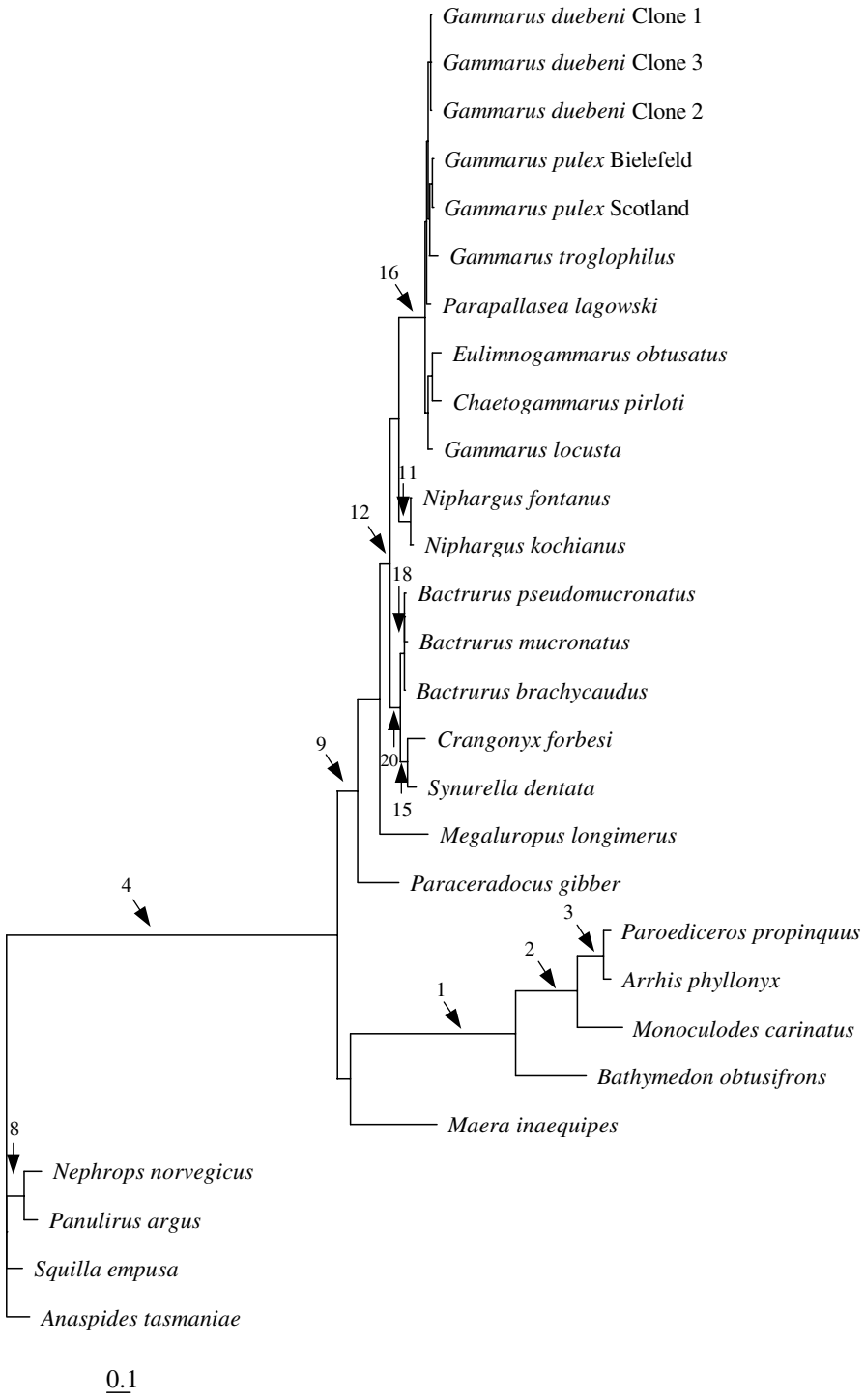
The neighbour-joining distance analyses with the logdet/paralinear, the Kimura-2-parameter or the HKY85 model produced one tree topology for each alignment, the choice of different models had no effect. The topology for alignment 1 is presented in figure 5. The topology for alignment 2 differs only in the positions of the gammarid taxa (figure 2d). The result for alignment 3 shows the same basic topology as the other distance trees but *Paraceradocus gibber* and *Megaluropus longimerus* change their position in the tree. The Niphargidae no longer group with the Crangonyctidae but with the Gammaridae (figure 6).

Spectra of supporting sites calculated with PHYSID are shown in figures 7–9, where the 49 most frequent splits for the three different alignments are presented. The different types of supporting positions are shown in different colours (symmetrical: black; asymmetrical: grey; noisy: white). For splits compatible with the clades recovered with tree-reconstructing methods, the corresponding ingroups taxa are named.

For alignment 1 (all positions) 2418 splits were found. The first split with the highest number of supporting sites (357 supporting positions) represents the Oedicerotidae [*Arrhis phyllonyx* (Sars, 1858), *Paroediceros propinquus* (Goes, 1866), *Bathymedon obtusifrons* (Hansen, 1887) and *Monoculodes carinatus* (Bate, 1856)]. The following split (345 supporting positions) supports a group of taxa within the Oedicerotidae, composed of *Arrhis phyllonyx*, *Paroediceros propinquus* and *Monoculodes carinatus*. The third split also describes a split of oedicerotid species. Three hundred and one positions support the grouping of *P. propinqus* and *A. phyllonyx*. Obviously, the number of substitutions in the Oedicerotidae is unusually high.

Thirteen of the 49 most frequent splits can be recovered in each of the

FIG. 4. Phylogram for alignment 1 obtained by maximum likelihood. The model of sequence evolution was determined by a likelihood-ratio test (GTR with gamma distributed rates: $\alpha = 0.6204$, $p_{\text{invar}} = 0.2061$, $R_{(A-C)} = 0.9022$, $R_{(A-G)} = 1.2452$, $R_{(A-T)} = 0.7955$, $R_{(C-G)} = 0.8$, $R_{(C-T)} = 2.3317$, $R_{(G-T)} = 1$, $-\ln L = 22101.95$). *N. norvegicus*, *P. argus*, *S. empusa* and *A. tasmaniae* were chosen as outgroup and the tree was rooted with the outgroup. The numbers with arrows indicate which of the splits in figure 7 correspond with the branches found in the analysis.


Gammarus duebeni Clone 1
Gammarus duebeni Clone 3
Gammarus duebeni Clone 2
Gammarus pulex Bielefeld
Gammarus pulex Scotland
Gammarus troglophilus
16
Parapallasea lagowski
Eulimnogammarus obtusatus
Chaetogammarus pirloti
Gammarus locusta
11
Niphargus fontanus
12
Niphargus kochianus
18
Bactrurus pseudomucronatus
Bactrurus mucronatus
Bactrurus brachycaudus
9
Crangonyx forbesi
20
Synurella dentata
15
Megaluropus longimerus
Paraceradocus gibber
4
3
Paroediceros propinquus
2
Arrhis phyllonyx
1
Monoculodes carinatus
Bathymedon obtusifrons
Maera inaequipes
8
Nephrops norvegicus
Panulirus argus
Squilla empusa
Anaspides tasmaniae
0.1

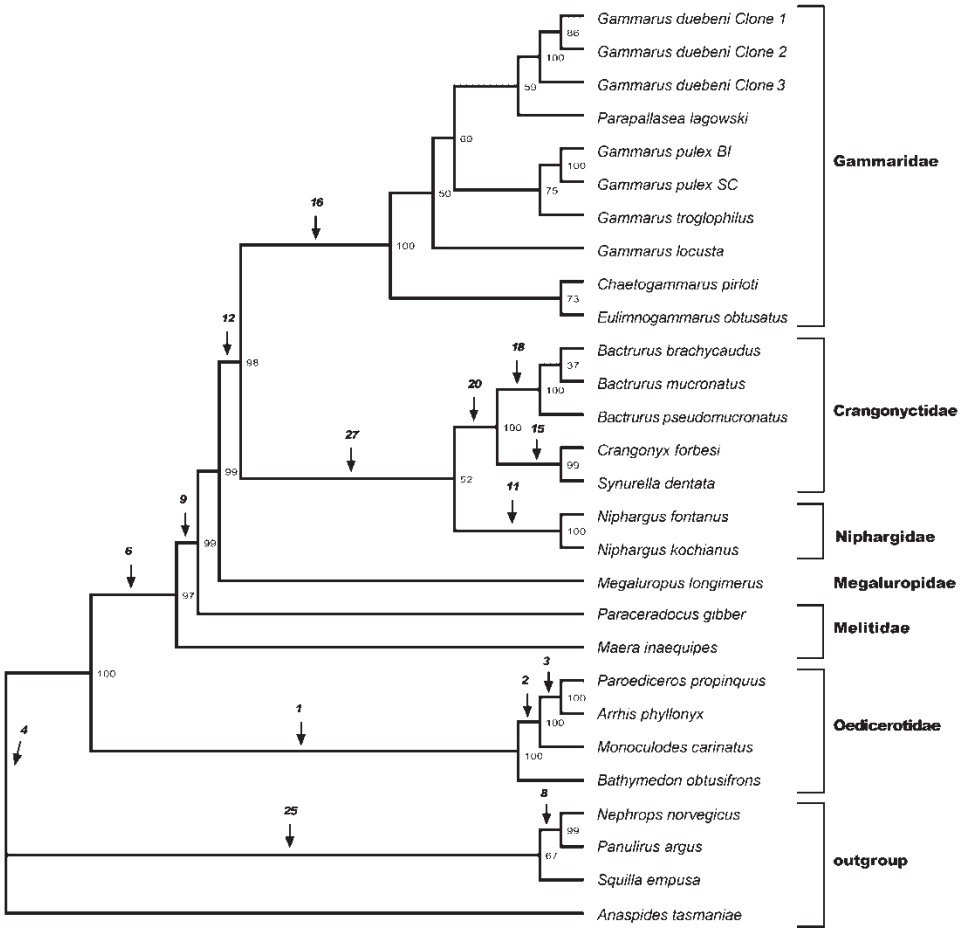


FIG. 5. 50% majority-rule consensus tree for alignment 1 obtained by a neighbour-joining distance calculation. The logdet/paralinear option was chosen as the model for the substitution process. *N. norvegicus*, *P. argus*, *S. empusa* and *A. tasmaniae* were chosen as the outgroup. The tree was rooted with the outgroup. The numbers with arrows indicate the position of the splits in figure 7.

tree topologies. Split 9 (Gammaridae + Crangonyctidae + Niphargidae + Megaluropidae + *Paraceradocus gibber*) is not compatible with the maximum likelihood analysis. Split 27 (Crangonyctidae + Niphargidae) is only accordant with the distance trees.

Similar results are found for alignment 2 (alignment without ambiguous positions). The lack of some of the variable positions of alignment 1 has the effect that the signal is lower (compare figures 7 and 8), but it seems that the number of noisy sites is still high. The Oedicerotidae (292 potential apomorphies) and the split (Amphipoda versus outgroup taxa) (276 potential apomorphies) are represented by the two most frequent splits. Splits 3 and 4 represent subgroups within the Oedicerotidae. A signal of 142 supporting sites is found for a group consisting of Gammaridae, Crangonyctidae, Niphargidae, Melitidae and Megaluropidae. There are seven more splits that are consistent with the tree topologies. Split 34 (Gammaridae + Niphargidae) is not compatible with the distance tree topologies

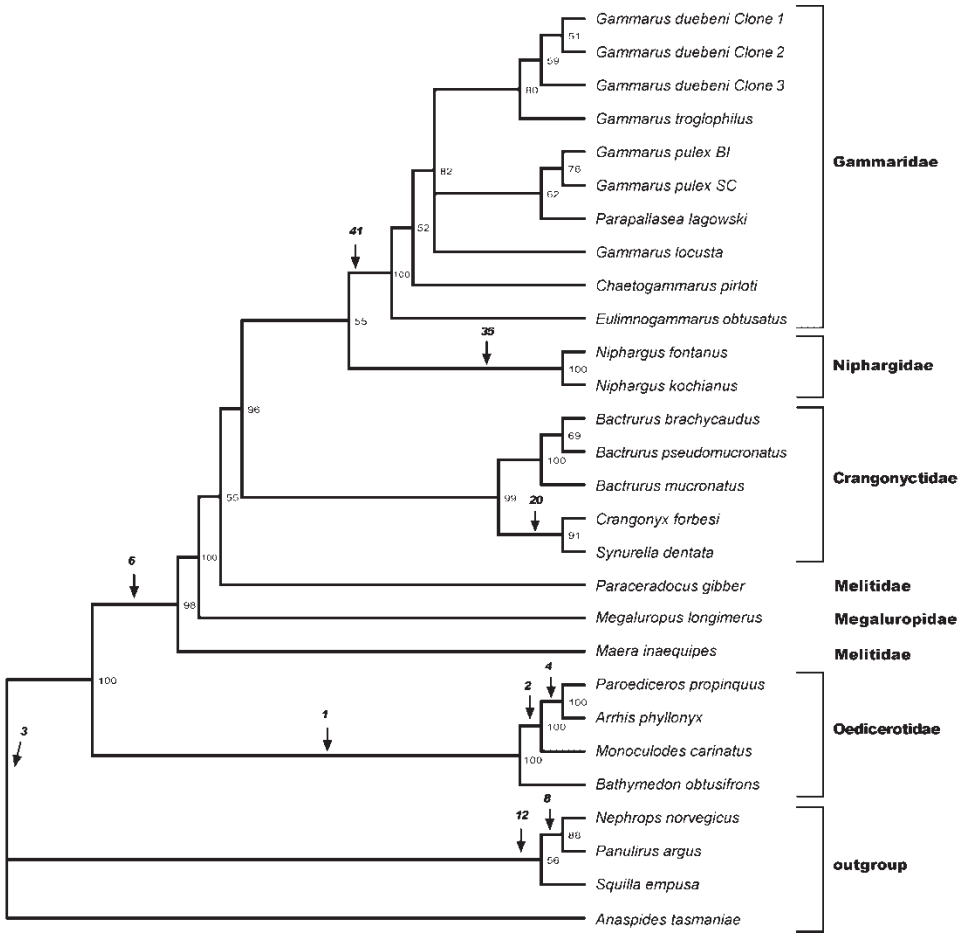


FIG. 6. 50% majority-rule consensus tree for alignment 3 obtained by distance methods. Bootstrap (1000 bootstrap replicates) values are taken from a logdet/paralinear analysis. *N. norvegicus*, *P. argus*, *S. empusa* and *A. tasmaniae* were used as outgroup and the tree was rooted with the outgroup. The numbers with arrows indicate the ranking of the splits in figure 9.

for alignment 2, only 19 positions support this group. In the distance trees split 47 (Crangonyctidae + Niphargidae) is present (figure 11). Twelve sites sustain this cluster. Compared to alignment 1 only about half of the total number of splits are present, meaning that a large number of splits caused by chance similarities (low-signal splits) are absent in alignment 2. The number of variable positions of alignment 1 (1826) was reduced to 1356 in the second alignment.

Even more variable sites were lost in alignment 3 (996 variable sites remained). The number of splits (890) decreased to two-thirds compared with alignment 2. The three most frequent of the splits again support the Oedicerotidae and the outgroup taxa. Of the 49 most frequent splits (figure 9) only nine are consistent with each of the tree topologies. The group Gammaridae, Crangonyctidae, Niphargidae and Megaluropidae (split 14) is present in the trees resulting from the maximum likelihood analyses while split 28 (Gammaridae, Crangonyctidae, Niphargidae and *Paraceradocus gibber*) can be found in distance and parsimony topologies.

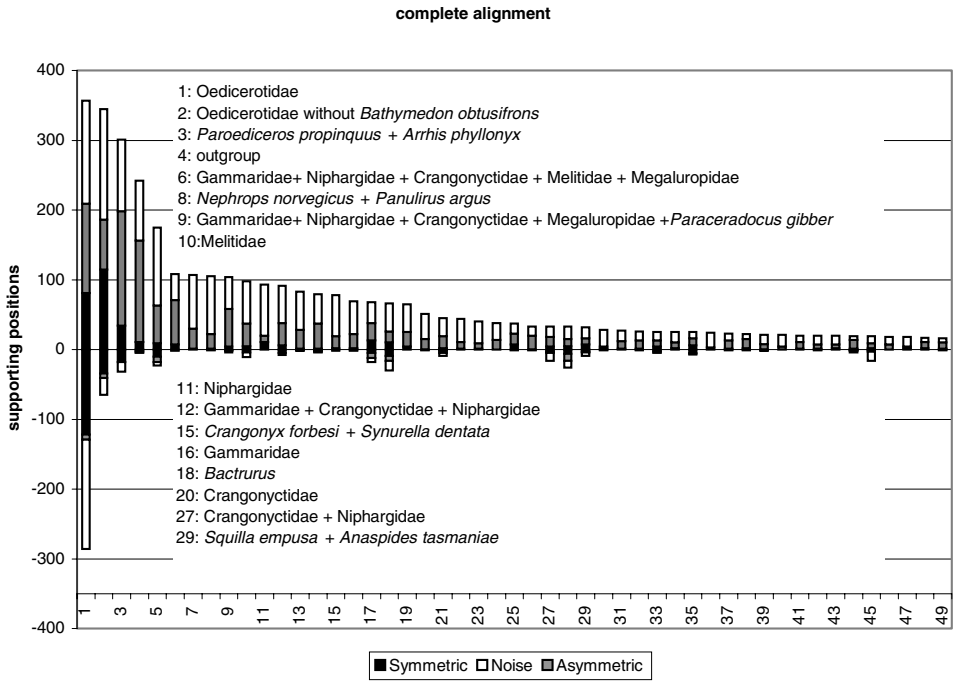


FIG. 7. Spectrum of supporting splits for alignment 1 (all positions). The first 49 splits are shown. The complete spectrum contains 2418 partitions. Those splits that are compatible with the tree topologies for alignment 1 are named. Only split 10 (Melitidae) is not recovered in the trees. The height of each column represents the number of split-supporting positions (columns below the zero line show the support for the outgroup if this is to be considered to be a monophylum). Note that the highest phylogenetic signal conserved in this alignment is present for some of the traditional families (Oedicerotidae, Niphargidae, Gammaridae) and for decapods (split 8).

The sequences of *Maera inaequipes* and *Paraceradocus gibber* represent the Melitidae in our data. These two taxa do not group together in any of the trees. Some supporting positions for this taxon are found in the PHYSID analyses (alignment 1: split 10 with 98 supporting positions; alignment 2: split 21 with 29 supporting positions; alignment 3: split 23 with 13 supporting positions). *Maera inaequipes* is part of the ingroup taxa in eight of the 49 most frequent splits for alignment 1. Seven of these splits are not consistent with any of the topologies for these analyses or known morphological data.

The remaining splits discerned by PHYSID describe nonsense groups. The sequences of *Maera inaequipes*, *Monoculodes carinatus* and *Bathymedon obtusifrons* group with almost any of the other sequences and produce these nonsense groups due to long-branch attraction. *Monoculodes carinatus* is found as ingroup member in 11 splits (of the 49 most frequent of alignment 1). Only two are compatible with the trees found in the different analyses or morphological data. A similar effect can be observed for *Bathymedon obtusifrons*: eight of nine splits containing *B. obtusifrons* as an ingroup member are not consistent with any other data. (The figures for alignment 1 are used as an example for the three different alignments because the results are similar.)

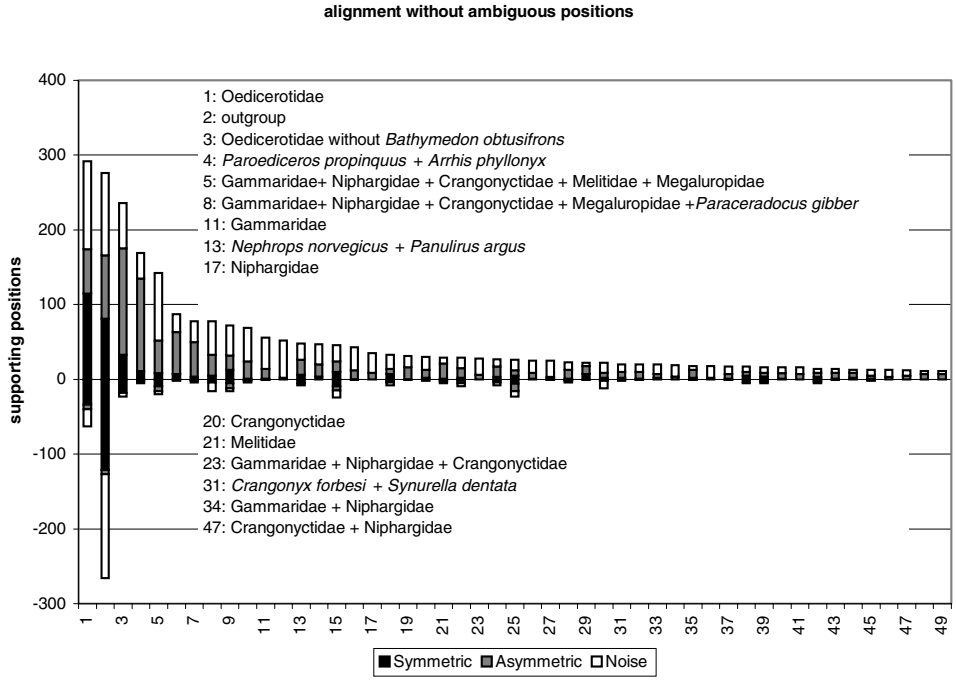


FIG. 8. Spectrum of supporting splits in alignment 2 (2750 bp). The first 49 splits are shown. The complete spectrum consists of 1364 splits. The named splits are compatible with the tree topologies. Only the Melitidae (split 21) could not be found monophyletic in the analyses.

Within the most frequent splits, partitions describing the relationship within the Gammaridae are completely missing. There is a signal found for all Gammaridae in each of the alignments. It is particularly strong for alignments 1 and 2, but information concerning the partitions within the Gammaridae is not obtained.

Anatomical examination

Previous studies of the anatomy of amphipods have yielded only few characters of taxonomic importance. Exceptions are stomach characters (e.g. Coleman, 1991, 1992) and cephalic apodemes (Coleman, 2002). The latter also show interesting variations in the taxa considered for the present study. The transverse apodemes of *Batrurus brachycaudus*, *Crangonxy forbesi*, *Synurella dentata* and *Stygobromus mackini* are club-shaped structures which do not meet medially and are only connected by tissue (figure 12a–d). A similar type of cephalothorax apodemes can be observed in *Niphargus* sp. (figure 12e). However, the shape of the distal processes is sagittate and thus different from the Crangonyctidae.

Fundamentally different from these separate transverse apodemes is the complete bridge that can be found in the Gammaridae (s. l.). *Paraceradocus gibber* and *Megaluropus longimerus* also display this character (figure 13a, b). In comparison with the other amphipods, this fusion of apodemes is an apomorphic character state of high value (high probability of homology), because the fusion must be correlated with complex functional changes.

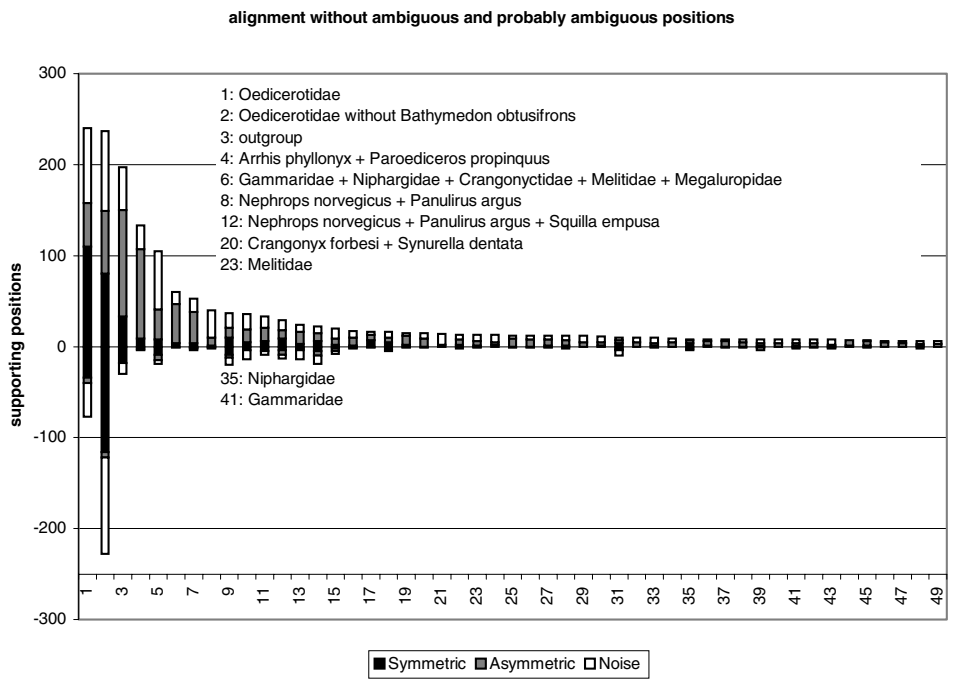


FIG. 9. Spectrum of supporting positions for alignment 3 (2312 bp). The 49 most frequent splits are shown. The complete spectrum consists of 890 splits. The named splits are those that are congruent with the topologies for this alignment. Only split 23 (Melitidae) cannot be found in any of the trees for alignment 3.

Discussion

All topologies, as well as the results obtained with PHYSID, show that ssu rDNA data strongly support the hypothesis that the amphipod taxa included here originated from a common ancestor, when compared to the chosen outgroup taxa. The Amphipoda are a group of peracarid crustaceans whose monophyly is well-founded by morphological characters (Gruner, 1993; Ax, 1999). The molecular evidence presented herein shows the amphipod taxa as monophyletic in comparison with the outgroup, but because other complete peracarid ssu sequences are missing in GenBank the monophyly is not confirmed. Representatives of all other peracarid taxa must be considered in future.

The clade Oedicerotidae (Lilleborg, 1865) is found in every topology independently of the method used for tree reconstruction. The analysis with PHYSID shows a very strong support (figure 10) for this clade. The corresponding split has the highest number of supporting positions in each of the alignments. Examples for supporting positions are shown in figure 10. The Oedicerotidae have characteristic insertions (e.g. in the region 781–816) and a large number of stem-line substitutions are conserved within this family. In view of the high number of positions showing this pattern (Oedicerotidae versus remaining species) the probability that these characters are not chance similarities but apomorphies is highest in comparison with other splits seen in our data. There are also morphological characters that can be considered to be apomorphic for the Oedicerotidae (eyes, elongate pereopod 7 and the shape of the gnathopods: Barnard, 1969). But at least two of the taxa seem to

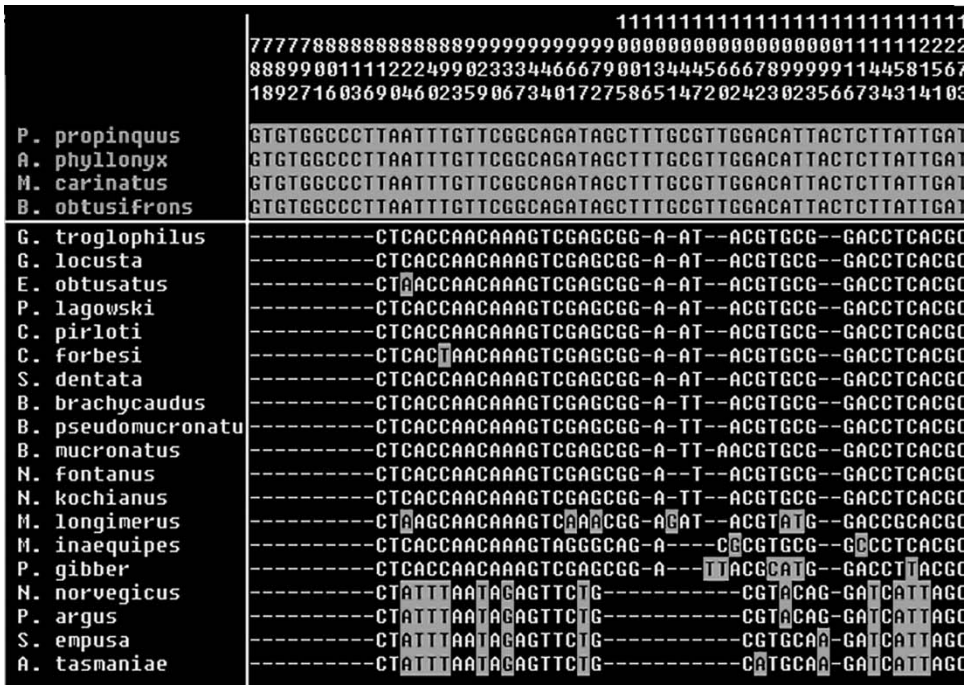


FIG. 10. Example for split-supporting positions in alignment 1. The figure shows 56 of 240 potential apomorphies for the Oedicerotidae. The first rows of numbers give the position in the alignment. The four upper sequences are the ingroup taxa (Oedicerotidae). Outgroup nucleotides with the same character state as ingroup sequences are shown on light background. Positions 1–2, 17, 18, 20, 22–24, 40, 47, 48 and 56 are symmetric positions while asymmetric positions are shown in columns 29–31, 35, 36, 45 and 55. Because of the large set of data not all taxa are shown.

originate from a long branch; the best evidence are the nonsense splits discerned with PHYSID. The fact that the two oedicerotid taxa *Bathymedon obtusifrons* and *Monoculodes carinatus* group with almost any of the other taxa in low-signal splits shows that they share many convergences with other sequences. These convergences cause splits that cannot be supported by morphological evidence. The phylogenetic signal for these taxa is noisy and information may be lost due to multiple hits in these sequences (causing 'erosion' of synapomorphies). The p-distances for the oedicerotid taxa are remarkably high compared to the other amphipod taxa, apart from *Maera inaequipes*. This also supports the notion that the Oedicerotidae or at least some of the oedicerotid taxa are 'long-branch' taxa (compare also figure 4). The maximum likelihood topology in figure 4 as well shows that the Oedicerotidae are found on a long branch. Nevertheless, the placement of these species within a single family is also plausible from a morphological point of view, as they share the oedicerotid apomorphies mentioned above.

Within the Oedicerotidae, the analyses of molecular data favour a close relationship between *Paroedicereos propinquus* and *Arrhis phyllonyx*. A high number of supporting positions as well as high bootstrap values (100) in all topologies can be observed for the group *Monoculodes carinatus*, *Paroedicereos propinquus* and *Arrhis phyllonyx*. *Bathymedon obtusifrons* appears to be the most ancient of the studied oedicerotid taxa. Whether this tree topology is realistic remains uncertain. The

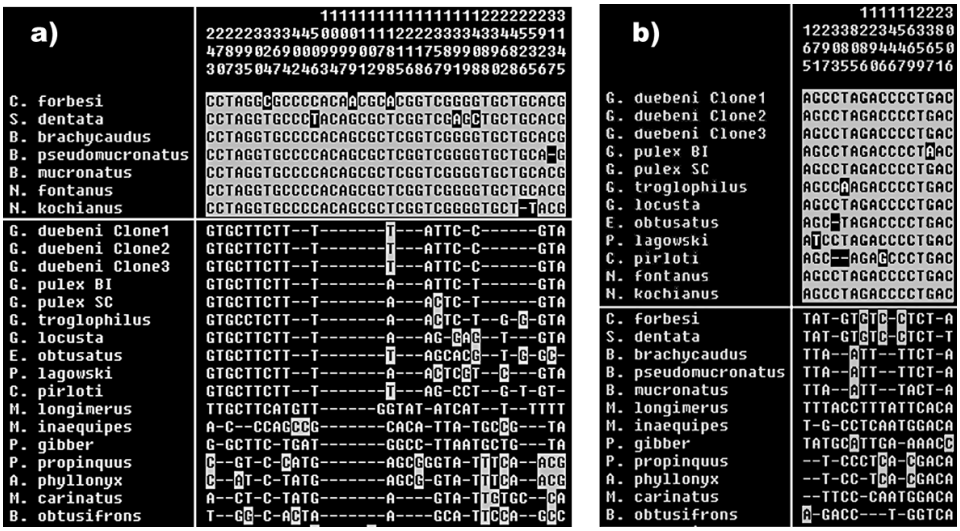


FIG. 11. Potential apomorphies for (a) Crangonyctidae and Niphargidae (ingroup) and (b) Gammaridae and Niphargidae (ingroup) in alignment 1. 25% noise was allowed in rows and columns. Outgroup nucleotides that share the same character states as the ingroup sequences are shown with a light background. Because of the large set of data not all of the outgroup taxa are shown.

Oedicerotidae consist of about 38 genera with approximately 175 species (Gruner, 1993). Some genera are restricted to certain areas (e.g. *Arrhis*: Arctic), but others are also widely distributed (e.g. *Bathymedon*: cosmopolitan). They all share the same life style, they are active burrowing benthic amphipods (Barnard, 1962, 1969; Lincoln, 1979; Gruner, 1993). Although the life style and ecology of several species, and morphological characters indicating the monophyly of the family are known, the 'oedicerotids need extensive generic revision' (Barnard, 1969). The phylogenetic analysis of ssu rDNA sequence data is a promising approach for clarifying oedicerotid systematics.

The grouping of the Gammaridae, Crangonyctidae and Niphargidae is a result that has no morphological backing. The molecular evidence, on the other hand, strongly sustains this relationship. Not only do the high bootstrap values for each of the methods used support this clade but also a number of potential apomorphies found with PHYSID (figures 7, 8), except the spectrum for alignment 3. Obviously, the phylogenetic information for this monophylum is found in the excluded variable alignment positions. Although there are few conserved supporting positions in alignment 3 for a group Gammaridae + Crangonyctidae + Niphargidae, there exist no other groupings that are incompatible with this split. All reconstructed topologies contain this monophylum, including those estimated for the shorter alignment 3.

For these three taxa three rooted topologies are possible, but only two of them occur in our analyses. The sister taxon relationship between Gammaridae and Niphargidae is supported by the trees resulting from alignments 2 and 3 whereas a group Crangonyctidae + Niphargidae is sustained by results from alignment 1. There are 38 (figure 11) potential apomorphies that support this latter relationship. In the same alignment only 16 potential apomorphies for a group Gammaridae + Niphargidae can be found (figure 11). The evidence is more strongly in favour of

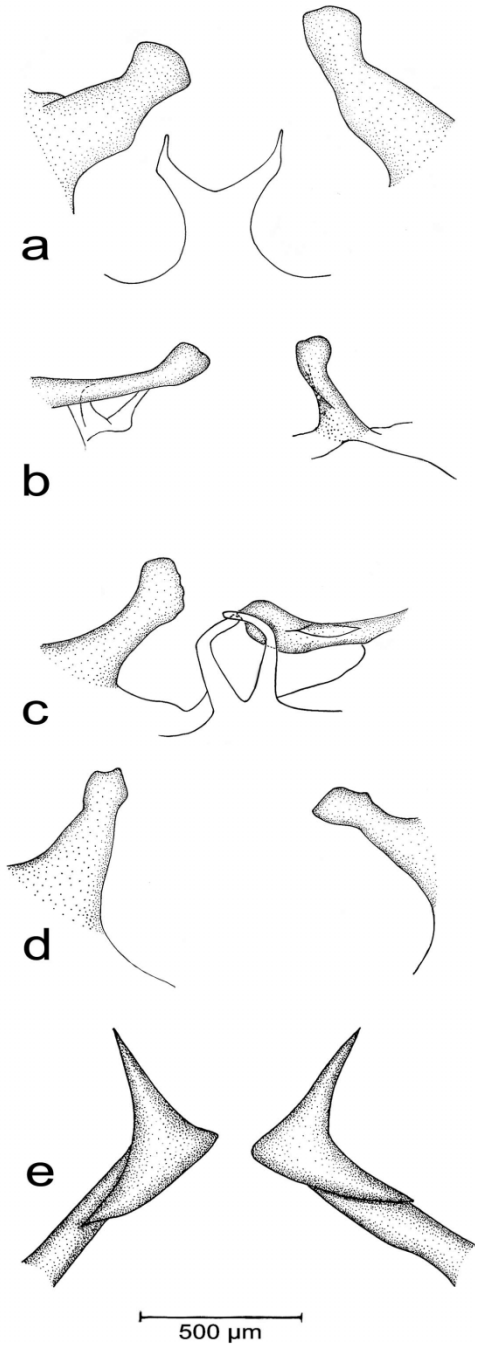


FIG. 12. Transverse apodemes of amphipod cephalothoraces (dorsal view). (a) *Bactrurus brachycaudus*; (b) *Synurella dentata*; (c) *Stygobromus mackini*; (d) *Crangonyx forbesi*; (e) *Niphargus* sp. Scale bar for (a–d): 500 μm.

the monophyly of Crangonyctidae + Niphargidae. Furthermore, the examined crangonyctid species have transverse apodemes of the same type (figure 12a–d), they are club-shaped. As in the crangonyctids, the transverse apodemes of the niphargids are separate from each other, but the shape is somewhat different (figure 12e).

The third possibility (Gammaridae + Crangonyctidae) is not observed in any of the trees. In the three alignments only one noisy position for this sister taxon relationship can be found with PHYSID. Therefore, this relationship can be ignored completely with regard to the molecular evidence.

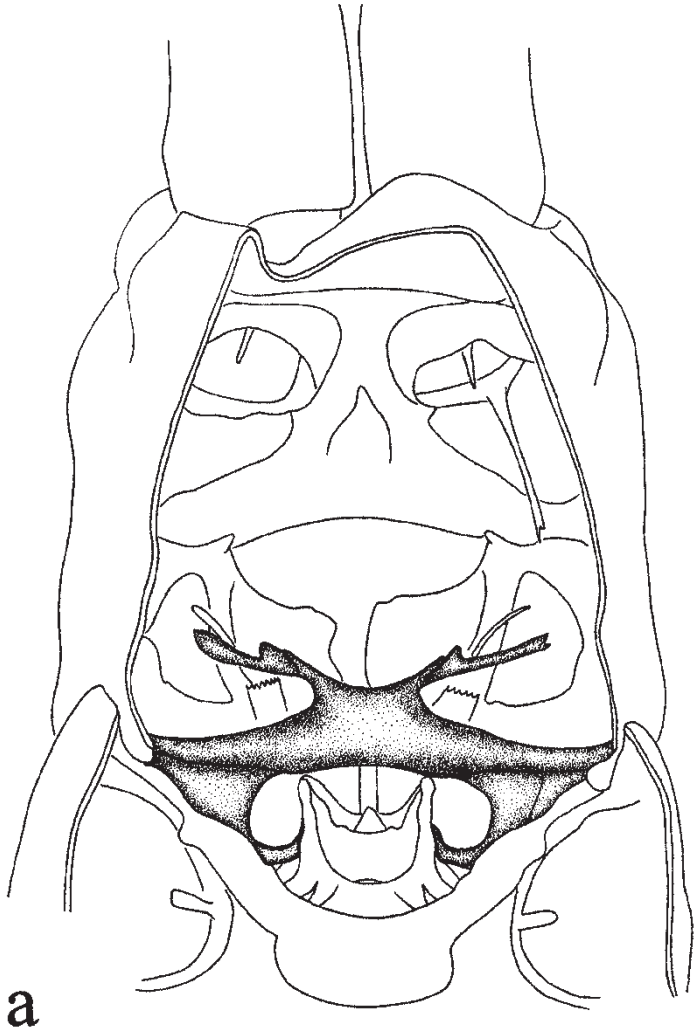
Each of the three taxa Gammaridae (*s. str.*), Crangonyctidae and Niphargidae are monophyletic. The molecular data give no evidence that is incompatible with this assumption.

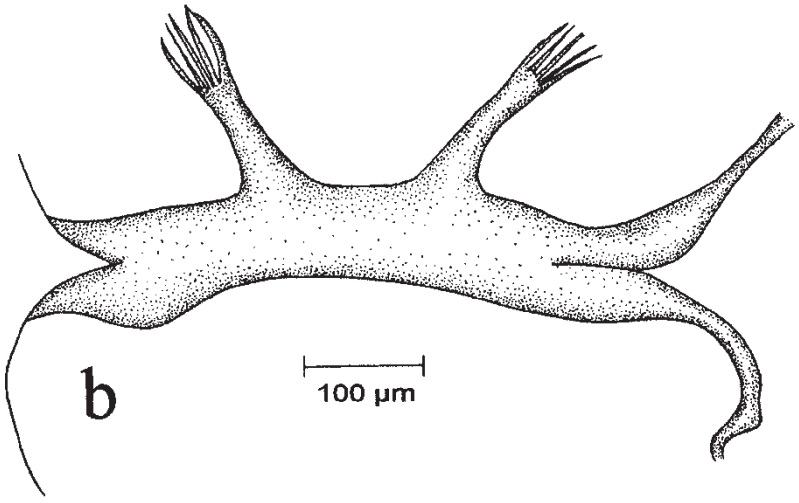
The Gammaridae (*s. str.*) are strongly supported by the phylogenetic signals in bootstrap analyses (95–100), the maximum likelihood topologies, and the PHYSID analyses for the three different alignments. The PHYSID analyses contain 20–30 potential apomorphies for this taxon. The Acanthogammaridae represented by *Parapallasea lagowski* (Dybowsky, 1874) branch within the Gammaridae (*s. str.*), a fact seen in all topologies. There is no molecular evidence for the monophyly of the Gammaridae (*s. str.*) excluding the Acanthogammaridae. If further investigations lead to the same conclusion then it will be necessary to synonymize the Acanthogammaridae with the Gammaridae (*s. str.*). The genus *Gammarus* is not monophyletic either. The baikalian amphipod *Parapallasea lagowski* (Acanthogammaridae) is branching within this genus in each topology. The amphipods from Lake Baikal are supposed to originate from freshwater ancestors, probably from the genus *Gammarus*. Therefore the concept of the genus *Gammarus* clearly needs to be revised.

The Melitidae, formerly members of the Gammaridae (*s. l.*), are found to be non-monophyletic. There is a signal for this group in the alignments (e.g. split 10 of alignment 1), but this is not compatible with the tree topologies. The sequence for *Maera inaequipes* shares more convergences with the other sequences than potential apomorphies with the sequence of *Paraceradocus gibber*. *Maera inaequipes* groups in many splits with most of the included taxa (cf. situation for *Bathymedon obtusifrons*). The p-distance for *M. inaequipes* supports the idea that this species originated from a long branch. This might be another reason why the two melitid taxa are not found to be monophyletic. It remains uncertain whether the Melitidae are monophyletic or not, because a long-branch effect may be obscuring the real phylogenetic relationships.

The family diagnosis of the Melitidae is rather unsatisfactory, because they are defined by characters that are normally found in the superfamily (Bousfield, 1977). Coleman (2002) classifies the melitid taxa again as Gammaridae, as was traditionally considered, in e.g. Barnard (1969). Coleman (2002) supported this by the complete transverse apodeme bridge that can be found in the Gammaridae (*s. str.*) and in melitid taxa such as *Paraceradocus gibber*, a hypothetical synapomorphy for the gammarid–melitid group = Gammaridae (*s. l.*). The same is true for the Megaluropidae. They show the same type of apodeme bridge that can be found

FIG. 13. Transverse apodemes of amphipod cephalothoraces (dorsal view). (a) *Paraceradocus gibber*, cephalothorax opened dorsally, showing complete transverse apodeme bridge (stippled); (b) *Megaluropus longimerus.*


a


b
100 µm

in the gammarid-melitid complex, and therefore share the same hypothetical synapomorphy as the Gammaridae (*s. l.*).

However, in our molecular analyses the Crangonyctidae and Niphargidae, and not the Melitidae and Megaluropidae, group within the Gammaridae (*s. l.*). There are several possible explanations: (1) it might be that the complete transverse apodeme bridge was separated secondarily in the common ancestor of Crangonyctidae and Niphargidae, a reversal of the plesiomorphic character state; (2) a convergent formation of the complete transverse apodeme bridge in Gammaridae (*s. str.*), Megaluropidae and melitid taxa; (3) the position of the melitid and megaluropid taxa in our molecular analyses might be incorrect due to long-branch effects.

In order to resolve this inconsistency, further melitid and megaluropid taxa should be examined using molecular methods. This study shows that older traditional concepts of the family Gammaridae probably represent aspects of the real phylogeny, as they also contain the taxa that group together in our analysis; for example Stebbing (1906) and Schellenberg (1942) included the crangonyctid, niphargid, melitid and megaluropid taxa in the Gammaridae.

Our results suggest that information for the deeper branchings within the amphipod phylogenetic tree is found in the more conserved regions of the ssu rDNA. For a few of the branchings a strong phylogenetic signal can be found. The information for divergences within the families seems to be conserved in the more variable areas of the ssu rDNA. The chances to homologize these variable regions correctly are often very weak, when the whole alignment with all taxa is studied, however within a group of closely related species the situation is more favourable. Therefore it is more informative to compare sequences within families with just a small number of outgroup taxa that preferably should be closely related. The problem can be easily observed for the Gammaridae (*s. str.*). For almost every method of phylogenetic inference (figure 2) a different topology was found. Because of the weakness of signals, the computer programs are not able to find a consistent pattern that produces a reliable topology [not one of the 49 most frequent splits in any of the alignments is within the Gammaridae (*s. str.*)].

Although the Chi-square test indicates a significant correlation of base frequency and sequence pairing for alignment 1, the insignificant differences between the resulting topologies do not imply an effect of the base frequencies, with a possible exception of the crown groups (e.g. within the Gammaridae).

The removal of positions of doubtful homology did not influence the basic topology, but the results of the PHYSID analyses show a loss of information for some of the upper parts of the topology. Only nine compatible splits are found for alignment 3 while the PHYSID analysis for alignment 1 discerns 14 compatible splits. Therefore in this case it will be better to keep all positions of the alignment than to lose information.

Acknowledgements

Ulrike Englisch was supported by a DAAD grant (Deutscher Akademischer Austauschdienst; D/99/13311) and a DFG (Deutsche Forschungsgesellschaft) grant to C. O. Coleman and J. W. Wägele (Col65/4-1). We would like to thank Dr T. Glatzel (Universität Oldenburg) for providing the *Niphargus* material and Dr E. Wollscheid-Lengeling and Dr A. Klussmann-Kolb for collecting *Maera inaequipes*. U. Englisch would like to thank Prof. Dr W. Vader and J. Berge for their

hospitality and the cruise on the 'Johan Ruud' that provided most of the oedicerotid material. The Baikal amphipod material of *Parapallasea lagowski* was kindly provided by Prof. Zerbst (Freie Universität, Berlin).

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