ORGANISMS DIVERSITY & EVOLUTION

Preliminary phylogenetic analysis of selected subterranean amphipod crustaceans, using small subunit rDNA gene sequences

Ulrike Englisch[1],*, Stefan Koenemann[2] (Authors in alphabetical order)

[1] Lehrstuhl für Spezielle Zoologie, Ruhr-Universität Bochum, Bochum, Germany
[2] Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94766, 1090 GT Amsterdam, The Netherlands

Abstract

The exclusively subterranean amphipod genus Bactrurus (Crangonyctidae) occurs in central and eastern parts of the United States. *Bactrurus* is characterized by morphologically similar species. In at least one instance, the distinction between separate species and geographic variants appears to be blurred. A sequence analysis of the small subunit (18S) rDNA gene was conducted for eight amphipod taxa. Both maximum likelihood and distance methods resulted in phylogenies for several species of *Bactrurus* that are in accordance with morphological data. These results could not be validated by a parsimony analysis. All three methods of phylogenetic inference, however, produced identical basal branching patterns. The molecular analyses do not support the recognition of what initially appeared to be a new species of *Bactrurus* from glaciated areas in Montgomery County, Illinois.

Introduction

Stygobiont amphipod crustaceans in the suborder Gammaridea occur in groundwater environments in many parts of the world. Their habitats include caves, wells, springs, and the interstitial spaces of freshwater and marine sediments. In some cases, the taxonomic classification within the suborder is unsatisfactory, be it at the species, genus or family level. Numerous discoveries of new stygobiont taxa, especially during the last two decades, call for modern taxonomic revisions.

In some recent revisions, cladistic analyses based on morphological data have been used to reorganize taxonomic groups and obtain information about their phylogenetic relationships. Examples include the families Bogidiellidae Hertzog, 1936 and Crangonyctidae Bousfield, 1973 (Holsinger, 1994; Koenemann, Vonk & Schram, 1998; Koenemann & Holsinger, 1999). Another interesting case study for phylogenetic analyses is the crangonyctid genus *Bactrurus* Hay, 1902. *Bactrurus* is endemic to parts of eastern and central United States. The distribution of the genus is characterized by both isolated, highly endemic relics and species with widespread ranges (Fig. 1). *Bactrurus mucronatus* (Forbes, 1876) is the most abundant species with the widest range (Fig. 3a). It is also the only species of *Bactrurus* that almost exclusively inhabits non-karst environments in glaciated regions of the Central Lowland Physiographic Province. All other species of the genus occur in groundwater habitats related to karst terranes. A recent collection trip led to the discovery of what appeared to be a second species within the range of *B. mucronatus* in a glaciated area (Montgomery County, Illinois). In one instance, both species were even collected from the same locality. The species that co-occurred with *B. mucronatus* was morphologically very similar to *B. brachycaudus* Hubricht & Mackin, 1940 (Fig. 3b). In contrast to *B. brachycaudus*, however, specimens from the glaciated area had sexually dimorphic telsons. Male telsons were about 20% longer than those of fe-

*Corresponding author: Ulrike Englisch, Lehrstuhl für Spezielle Zoologie, Ruhr-Universität Bochum, Universitätsstraße 150, D-44780 Bochum, Germany, Tel: ++49-234-322442; Fax: +49-234-3214114; e-mail: ulrike.englisch@ruhr-uni-bochum.de

Fig.1. Geographic distribution of *Bactrurus* in eastern and central North America. Each symbol represents approximately one locality record (the actual number of records may be slightly higher in some of the densely clustered areas). Open circles: *B. brachycaudus*; filled diamonds: *B. hubrichti*; filled circles: *B. mucronatus*; filled triangles: *B. pseudomucronatus*; dot surrounded by circle: *B. wilsoni*; filled square: *B. cellulanus*; open triangle: *B. angulus*. The large open circle in the glacial drift area (Illinois) surrounds several localities: three are the first records for *B. brachycaudus* from glacial drift habitats; one is a locality occupied by both *B. brachycaudus* and *B. mucronatus* (see text). The maximum southern extent of Pleistocene glaciation is indicated by the black dashed line. Map adapted from Koenemann & Holsinger, in press.

males. Sexually dimorphic telsons are common for *Bactrurus* and only two of the seven species of the genus, *B. brachycaudus* and *B. hubrichti* Shoemaker, 1945, have morphologically indistinguishable telsons in both sexes. Yet, despite sexually dimorphic telsons, specimens from the glacial drift area in Illinois appeared to share most diagnostic characters with *B. brachycaudus*. In a recent revision of *Bactrurus*, the specimens from Illinois were, therefore, regarded as isolated, geographic variants of *B. brachycaudus* rather than a new species (Koenemann & Holsinger, in press).

The objective of this project was to investigate the phylogenetic relationships of several similar species of *Bactrurus*, including *B. brachycaudus* and its geographic variant from glaciated parts of Illinois. Two other morphologically similar species, *B. mucronatus* and *B. pseudomucronatus* (Koenemann & Holsinger, in press), the ranges of which are adjacent to that of *B. brachycaudus*, were also included in the analyses. Because of the similarity of the species of *Bactrurus*, the limited availability of reliable diagnostic characters obstructs the application of morphological data to phylogenetic

investigations. Therefore, we performed sequence analyses of the small-subunit (SSU) rDNA gene for three species of *Bactrurus* and four other selected stygobiont, stygophile, and epigean taxa.

Material, methods and definitions

Definitions and abbreviations

Glacial drift: sediments deposited by ice sheets that repeatedly advanced and retreated in north-south movements during the Pleistocene. In the Central Lowlands Province, glacial deposits are commonly composed of layers of sand, gravel, silt or clay.
stygobiont: obligatory groundwater organism (Styx: Greek river of the underworld)
stygophile: facultative dweller of groundwater habitats
epigean: at or on the surface
B. brachycaudus GD: specimens of *B. brachycaudus* that occur in non-karst environments of glacial drift areas; found together with *B. mucronatus* in one locality (Montgomery Co., Illinois).

Table 1. Taxa used for sequence analyses. Stygobionts are obligatory groundwater organisms, whereas stygophiles can be defined as facultative (e.g. temporarily) subterranean species. The sampling sites are given in square brackets.

Hypogean Species		Epigean Species
Stygobiont Species	Stygophile Species	
Bactrurus mucronatus (Crangonyctidae) [Saline Co., Illinois, USA]	*Crangonyx forbesi* (Crangonyctidae) [St, Louis Co., Missouri, USA]	
Bactrurus pseudomucronatus (Crangonyctidae) [Oregon Co., Missouri, USA]		
Bactrurus brachycaudus (Crangonyctidae) [St. Louis Co., Missouri, USA]	*Gammarus troglophilus* (Gammaridae) [St, Louis Co., Missouri, USA]	*Gammarus pulex* (Gammaridae) Schwarzbach, Bielefeld,
Bactrurus brachycaudus GD (Crangonyctidae) [Montgomery Co., Illinois, USA]		
Niphargus fontanus (Niphargidae) [River Ruhr, Germany]		

Collection methods and selection of taxa

Specimens were collected from various subterranean habitats, including caves and springs in karst areas, and drainage systems of farm fields in glaciated areas. Moreover, a Bou-Rouch pump was employed to sample interstitial sediments of surface streams. All freshly collected material was immediately preserved in 95% ethanol.

The following species were collected during the field trips: *Bactrurus mucronatus*, *B. brachycaudus*, *B. pseudomucronatus*, *Crangonyx forbesi* (Hubricht & Mackin, 1940), and *Gammarus troglophilus* Hubricht & Mackin, 1940 (Table 1).

Sequence data from the European subterranean genus *Niphargus* represented by *N. fontanus* Bate, 1859, and the epigean freshwater species *Gammarus pulex* Linnaeus, 1758 were included in the phylogenetic analyses. *Gammarus pulex* and the stygophile freshwater species *G. troglophilus* were used as outgroup taxa. DNA extraction was performed for 6 more crangonyctid species but unfortunately failed to yield DNA.

DNA extraction

DNA was obtained using the QIAamp Tissue Kit (Qiagen). Instructions for the Mouse Tail protocol were exactly followed, with the exception of the last step: the DNA was eluted with 2 x 100 µl H$_2$O instead of 2×200 µl.

PCR amplification

Polymerase chain reaction (PCR) was performed following a standard protocol: a total volume of 50 µl consisting of $1 \times$ PCR buffer, $1 \times$ Q-Solution (Qiagen), 125 pmol dNTPs, 25 pmol of primer small subunit F and 50 pmol of primer small subunit R, 1.25 U Taq DNA polymerase (Qiagen), and 1 µl

Table 2. Oligonucleotides used for PCR and sequencing. F = forward orientation, R = reverse orientation

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

DNA extract. The PCR cycle was programmed as follows: $1 \times$ 5 min at 94° C; 35×30 sec at 94° C, 50 sec at 52.5° C and 3 min 20 sec at 72° C; 1×7 min at 70° C. The amplified PCR product was purified using the QIAquick PCR Purification Kit (Qiagen). Primer sequences are given in Tab. 2 (personal communication with T. Spears).
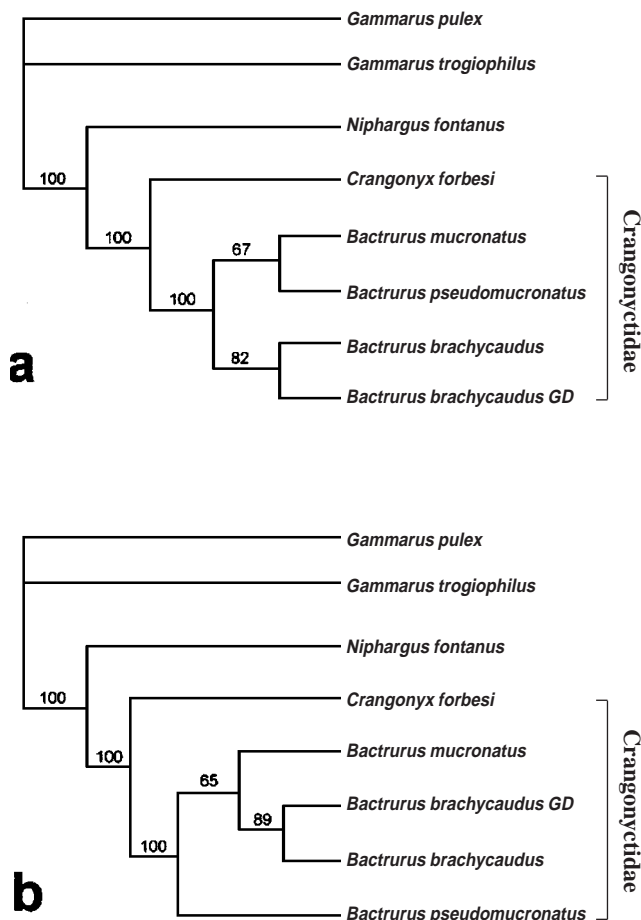
Fig. 2. Trees compatible with the 50% majority rule obtained by maximum likelihood (a) and parsimony (b) methods. Both trees were calculated using the Branch and Bound algorithm with 1000 bootstrap replicates (bootstrap values are shown for internal nodes). The trees were rooted using *Gammarus pulex* and *G. troglophilus* as outgroups.

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 competent Top 10 F′ One Shot™ cells (Invitrogen). Plasmids were purified with the S.N.A.P.™ MiniPrep Kit (Invitrogen).

Cycle sequencing was conducted in a LI-COR 4200 automated sequencer, using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) and a 6% acrylamid-bisacrylamid gel. Sequencing primers are presented in Tab. 2 [M13 universal CS (–43) and M13 reverse (–29) designed by Messing et al. (1981), internal primers designed by Dreyer, Wollscheid and Englisch].

Sequence analyses

Sequences were aligned with the software package ClustalW vers. 1.8 (Thompson et al., 1994) and corrected by eye according to a secondary structure presented by Crease & Colbourne (1998) in Genetic Data Environment (GDE). Additionally, a secondary structure developed by Choe et al. (1999) was used to homologize ambiguous positions resulting from secondary structures. A Chi-square test of the homogeneity of base frequencies and the uncorrected pairwise sequence differences were calculated in Paup 4.0 (Swofford 1998).

Phylogenetic analyses

Eight taxa were included in the phylogenetic analyses (Tab. 1). *Gammarus pulex* and *G. troglophilus* were chosen as outgroups for three methods of phylogenetic inference, each of which was computed with Paup 4.0. The following settings were chosen for the individual methods:

Parsimony Analyses: Branch-and-Bound search; both MulTrees and Sequence Addition option 'simple' were activated. Futhermore a Branch-and-Bound search with 1000 bootstrap replicates was computed and a tree compatible with the 50% majority-rule consensus was generated (Fig. 2b).

Distance Analyses: Neighbour Joining (NJ), using Kimura-2-parameter (1980) and the Tajima & Nei (1984) model for nucleotide substitution. The data set was resampled with 1000 bootstrap replicates.

Maximum Likelihood Analyses: Branch-and-Bound search, with MulTrees and the Sequence Addition "as is" (default setting) in effect. The transition/transversion ratio was 2 as required for the HKY 85 model used in this analysis. Futhermore a Branch-and-Bound search with 1000 bootstrap replicates was computed and a tree compatible with the 50% majority-rule consensus was generated (Fig. 2a).

Results

Sequencing and alignment

All of the 8 complete SSU rDNA sequences differed greatly in length from the approximately 1,800 nucleotides (nt) regarded as typical in crustaceans and most other animals (Tab. 3). Insertions in regions V4, V7 and V9 (Crease & Colbourne 1998) increased the length to a range between 2237 nt and 2331 nt.

In Tab. 4 the pairwise sequence differences are shown. Within the genus *Bactrurus* sequence differences range between 1–1.3%. Only the difference between *B. brachycaudus* and *B. brachycaudus* GD shows a value much lower than 1%. A Chi-square test of the homogeneity of base frequencies across the included taxa results in a P-value of 1. Thus, there is no significant correlation between the base distribution and the pairing of single sequences.

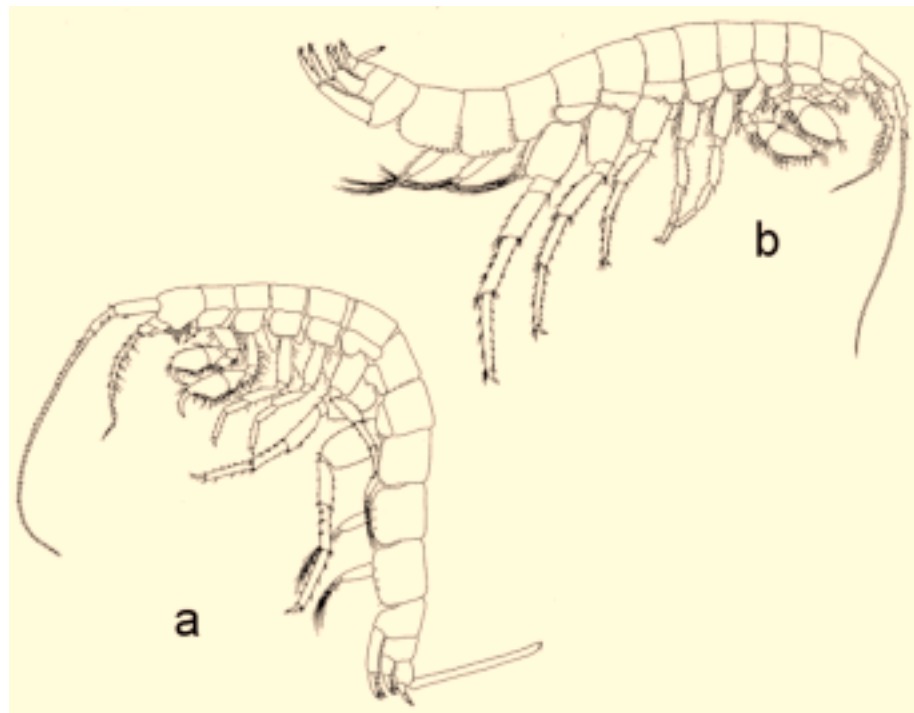The ClustalW multiple alignment was manually corrected due to secondary structure features and resulted

Fig. 3. (a) *Bactrurus mucronatus.* Male, 15.5 mm, from an old well near Champaign (Champaign County, Illinois); (b) *Bactrurus brachy-caudus.* Syntype female (19.0 mm) from Keifer Creek (St. Louis County, Missouri).

Table 3. Sequence length, GC-content and Genebank Accession numbers for the taxa included in the analyses.

Species	Sequence Length	GC Content	GB Accession No.
Bactrurus brachycaudus	2322 bp	55%	AF202979
Bactrurus mucronatus	2329 bp	55%	AF202978
Bactrurus pseudomucronatus	2319 bp	55,2%	AF202985
Bactrurus brachycaudus GD	2324 bp	55%	AF202984
Crangonyx forbesi	2331 bp	53,7%	AF202980
Niphargus fontanus	2237 bp	54,9%	AF202981
Gammarus troglophilus	2307 bp	54,7%	AF202983
Gammarus pulex	2250 bp	54,3%	AF202982

Table 4. Pairwise sequence differences (uncorrected distances) for *Bactrurus* and selected outgroup taxa, calculated in Paup 4.0. A Chi-square test was performed to test the homogeneity of base frequencies across the included taxa.

	B. mucro-natus	*B. pseudo-mucronatuis*	*B. brachy-caudus* GD	*C. forbesi*	*N. fontanus*	*G. troglophilus*	*G. pulex*
B. brachycaudus	1.2%	1.0%	0.3%	7.6%	7.3%	9.9%	9.1%
B. mucronatus	–	1.3%	1.2%	8.2%	7.8%	10.2%	9.4%
B. pseudomucronatus	–	–	1.1%	7.8%	7.6%	9.9%	9.2%
B. brachycaudus GD	–	–	–	7.8%	7.2%	10.0%	9.3%
C. forbesi	–	–	–	–	10.4%	13.3%	11.9%
N. fontanus	–	–	–	–	–	9.4%	8.7%
G. troglophilus	–	–	–	–	–	–	2.7%

in 2464 alignment positions. 2004 of these positions are constant. 203 of the remaining 460 positions are parsimony informative.

Phylogenetic analyses

For each of the three methods of phylogenetic inference one tree was found. The trees obtained for each of these methods of phylogenetic inference (maximum likelihood, distance, and parsimony) had the same basic topological structure, with *N. fontanus* as a sister-group to the crangonyctid taxa, and *C. forbesi* as a sister-group to a clade that comprised the three species (and single specimen) of *Bactrurus* (Fig. 2). Because the resulting trees of maximum likelihood and distance analyses were identical, one tree (maximum likelihood) was chosen to represent both methods (Fig. 2a). As opposed to the maximum likelihood tree, the tree produced by the parsimony analysis failed to keep the morphologically closely related species *B. mucronatus* and *B. pseudomucronatus* in a separate clade. Instead, *B. mucronatus* formed a sub-clade together with *B. brachycaudus* and *B. brachycaudus* GD (Fig. 2b). The obtained topology of the strict consensus tree is identical with the presented maximum parsimony tree.

Discussion

The consensus tree obtained by the maximum likelihood (and distance) method is in general accordance with both morphological and geographic characteristics of the species of *Bactrurus* included in the analyses (Fig. 2a):

Bactrurus brachycaudus and *B. brachycaudus* GD appear together on a sub-clade. The decision to treat the specimens of the glacial drift area as a geographic variant rather than a new species is also supported by the comparatively low sequence difference between both populations (Table 4).

Similarly, *B. mucronatus* and *B. pseudomucronatus* are rendered on a second sub-clade. Both species are morphologically distinguished from the other species of the genus by an extreme case of sexually dimorphic telsons: in males, the telson is conspicuously elongate, reaching up to 1/3 of the length of the body. Because of this distinct similarity, *B. pseudomucronatus* has been frequently misidentified as *B. mucronatus* (hence, the epithet *pseudomucronatus*, meaning "false *mucronatus*"). Yet, both species are distinguished by several less obvious diagnostic characters. Moreover, their distribution is disjunct (Fig. 1). *Bactrurus mucronatus* occurs almost exclusively in glacial drift areas, whereas *B. pseudomucronatus* inhabits karst regions of the Ozark Plateaus. The results of the analyses support the as-

sumption that some time before the Pleistocene an ancestral species with elongate male telsons split into two populations, one of which subsequently invaded and colonized the coarse grained sediments of aquifers in the glaciated parts of the Central Lowlands.

The parsimony analysis rendered a different phylogeny for *Bactrurus* (Fig. 2b). The consensus tree shows *B. pseudomucronatus* as a sister-group to *B. mucronatus* and the *brachycaudus* specimens. This branching pattern seems to contradict the morphological data. For example, it is highly unlikely that a distinct synapomorphy (extremely elongate, sexually dimorphic telson) is reduced completely back into the primitive state (normal telson in *B. brachycaudus*). This inconsistency of the tree in Figure 2b may reflect the tendency of parsimony to underestimate the amount of changes on long branches (Sullivan et al., 1997; Maley & Marshall, 1998).

Each method of phylogenetic inference resulted in identical topologies of basal branches. The selection of crangonyctid taxa appears as a monophyletic group. Interestingly, the pairwise sequence differences render the North American species *Crangonyx forbesi* closer to the European *Gammarus pulex* than to *Gammarus troglophilus* (North America), the range of which even partly overlaps with that of *C. forbesi*.

This study demonstrates that the use of the small subunit gene produces reliable results for groundwater amphipods at the species level and above. In a future project, it would be interesting to further investigate relationships of additional stygobiont and/or stygophile taxa. A subsequent approach should, for example, include several selected stygobiont taxa of all major global distribution areas, combined with co-ocurring epigean amphipods of marine or freshwater habitats.

Acknowledgements

References

Bate, C. S. (1859): On the genus *Niphargus* (Schiödte). Proc. Dub. Uni. Zool. Bot. Ass. 1: 237–244.

Bousfield, E. L. 1973. Shallow-water gammaridean Amphipoda of New England. Cornell Univ. Press; Ithaca, New York.

Choe, C. P., Hancock, J. M., Hwang, U. W. & Kim, W. (1999): Analysis of the primary and secondary structure of the unusually long SSU rRNA of the soil bug, *Armadillidium vulgare*. J. Mol. Evol. 49: 798–805

Crease, T. J. & Colbourne, J. K. (1998): The unusually long small-subunit ribosomal RNA of the crustacean, *Daphnia pulex*: Sequence and predicted secondary structure. J. Mol. Evol. 46: 307–313.

Forbes, S. A. (1876): List of Illinois Crustacea, with descriptions of new species. Bull. Illinois Mus. Nat. His. 1: 3- 25.

Hay, W. P. (1902): Observations on the crustacean fauna of Nickajack Cave, Tennessee and vicinity. Proc. U. S. Nat. Mus. 25: 417–439.

Hertzog, L. (1936): Crustacés de biotopes hypogées de la vallée du Rhin d'Alsace. Bull. Soc. Zool. France 61: 356–372.

Holsinger, J. R. (1994): Pattern and process in the biogeography of subterranean amphipods. Hydrobiol. 287: 131–145.

Hubricht, L. & Mackin, J. G. (1940): Description of nine new species of fresh-water amphipod crustaceans with notes and new localities for other species. Am. Midl. Nat. 23: 187–218.

Kimura, M. (1980): A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Koenemann, S. & Holsinger, J. R. (1999): Phylogenetic analysis of the amphipod crustacean family Bogidiellidae, s. lat., and revision of taxa above the species level. Crustaceana 72 (8): 781–816.

Koenemann, S. & Holsinger, J. R. (in press). Systematics of the North American subterranean amphipod genus *Bactrurus* (Crangonyctidae). Beaufortia/Bulletin of the Zoological Museum Amsterdam (May 2000).

Koenemann, S., Vonk, R. & Schram, F. (1998): Cladistic analysis of 37 Mediterranean Bogidiellidae (Amphipoda), including *Bogidiella arista*, new species, from Turkey J. Crust. Biol. 18: 383–404.

Linnaeus, C. (1758): *Systema Naturae*. Editio Decima, Tomus I. Holmiae (Stockholm): Laurentii Salvii.

Maley, L. E. & Marshall, C. (1998): The coming of age of molecular systematics. Science 279: 505–506.

Messing, J., Crea, R. & Seeburg, H. (1981): A system for shotgun DNA sequencing. Nucl. Acids Res. 9: 309–321

Shoemaker, C. R. (1945): Notes on the amphipod genus *Bactrurus* Hay, with description of a new species. J. Wash. Acad. Sci. 35: 24–27.

Sullivan, J., Markert, A. M. & Kilpatrick, C. W. (1997): Phylogeography and molecular systematics of the *Peromyscus aztecus* species group (Rodentia: Muridae) inferred using parsimony and likelihood. Syst. Biol. 46:426–440.

Swofford, D. L. (1998): PAUP*. Phylogenetic Analysis using Parsimony (*and other methods). Version 4 Sinauer Associates, Sunderland, Massachusetts.

Tajima, F. & Nei, M. (1984): Estimation of evolutionary distance between nucleotide sequences. Mol. Biol. Evol. 18: 387–404.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research. 22: 4673–4680.