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Phylogeny and Biogeography of Serolid Isopods (Crustacea, Isopoda, Serolidae) and the Use of Ribosomal Expansion Segments in Molecular Systematics

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In this study, a molecular phylogenetic hypothesis for 16 species of serolid isopods (Crustacea, Isopoda, Serolidae) from Antarctic waters, the deep sea, South America, and Australia is presented. The genes used are a 500-bp fragment of the mitochondrial LSU rRNA gene and a 700-bp fragment located in the variable region V4 of the nuclear SSU rRNA gene. The species composition and monophyly of morphologically defined genera of which several members were available are confirmed by the molecular data (*Ceratoserolis, Spinoserolis,* and *Cuspidoserolis*). The molecular data also support the redefinition of *Frontoserolis s.l.* and *Serolella* and the erection of the new genera *Septemserolis* and *Paraserolis,* as proposed by Wägele. The relationship among several genera is resolved differently in the molecular hypothesis than in the two existing morphological hypotheses, however. The molecular phylogeny may have important consequences for understanding the biogeography of the Serolidae, indicating that all Antarctic species in this study form a monophyletic group which has probably derived from species with closest extant relatives in South America. All 3 species included in this study living today in deep waters (>2000 m) of the Southern Ocean are most closely related to species living on the Antarctic shelf, so that parallel colonization of the deep sea by way of polar submergence can be reconstructed. In this study, a V4 expansion segment is reported which exceeds the longest crustacean sequences known until now by more than 270 bp. Although the V4 expansion segment has proven useful for phylogenetic purposes in this study, there is circumstantial evidence that its mechanism of evolution may depend not only on inheritance of single-site substitutions, making its routine use in phylogenetic studies potentially dangerous.

Key Words: **molecular systematics; nuclear small subunit ribosomal RNA gene; expansion segment V4; mitochondrial large ribosomal subunit gene; Serolidae; deep sea; biogeography.**

INTRODUCTION

The Serolidae are a family of benthic marine isopods. There are currently 86 recognized species which are distributed predominantly on the continental shelves of the southern hemisphere (Antarctica, South America, Australia), although some species have also invaded the deep sea or extended their range into the northern hemisphere (Poore and Brandt, 1997; Wägele, 1994). After a period of time during which the growing number of described species had been collected in a single genus *Serolis* Leach, 1818, several genera were erected to structure the rich morphological variation found in this family. Recently Brandt (1988, 1991) and Wägele (1994) revised the family at the genus level to identify monophyletic groups but came to different conclusions in their reconstructed phylogenies (Fig. 1). Examination of large numbers of specimens over relatively broad geographical ranges, furthermore, reveals a striking amount of intraspecific morphological variation which casts doubt on the taxonomic usefulness of several morphological features and leaves some taxonomic issues controversially discussed (Holdich and Harrison, 1980; Poore and Brandt, 1997; Wägele, 1986).

In addition to presenting different views on the phylogeny, the two hypotheses come to largely incompatible conclusions with regard to the historical biogeography of the group. Neither hypothesis allows a simple explanation of horizontal and vertical distribution patterns within the family.

In this study a first molecular phylogenetic hypothesis for the family Serolidae is presented. The aim of this study is to address open questions concerning its phylogeny and to attempt an explanation of the biogeographical patterns found among extant members of the family. It is not intended to give a full review of the taxonomy of the family at this time. This is particularly so because specimens in museum collections cannot routinely be used for molecular work due to fixation of material in formalin. The number of species at this stage representing the Serolidae is therefore limited to 16 species from 12 genera from Antarctica, South

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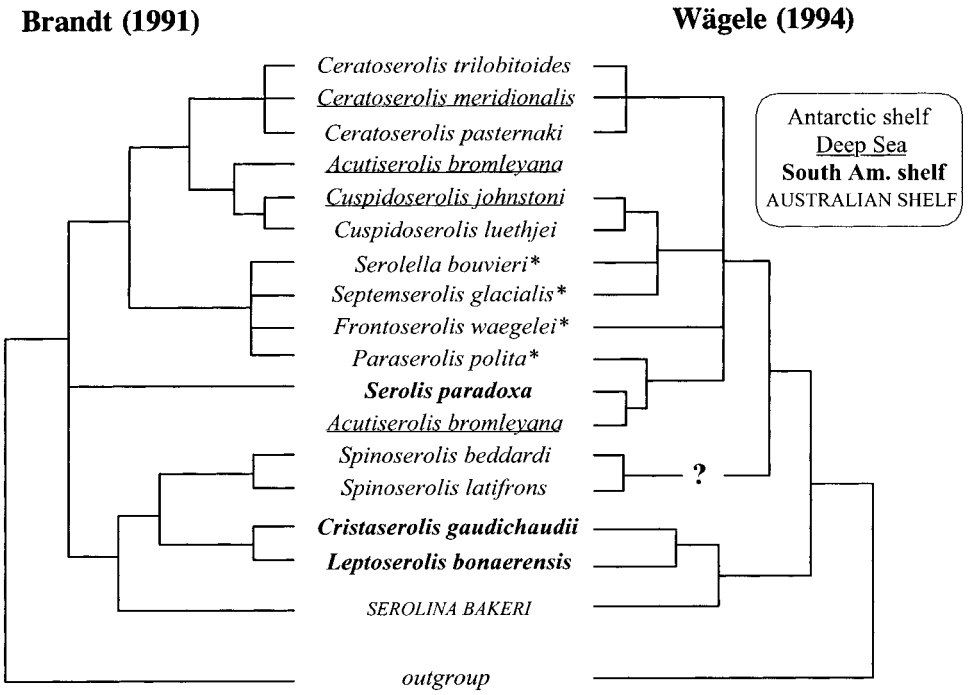

FIG. 1. Phylogenetic hypotheses of Serolidae at the genus level, modified after studies by Brandt (1991) and Wägele (1994), which are based on different sets of morphological characters. The four species marked with an asterisk (*) were placed within the genus *Frontoserolis* prior to Wägele (1994) and hence are unresolved in the left tree (see Discussion). Only a subset of species included in the studies by Brandt and Wägele is shown here to facilitate comparison with the results of this study. The deep-sea species *A. bromleyana* is included twice due to different positions in the trees.

America, and Australia; 3 isopod species from other families have been included in this study as outgroup species (Table 1).

Of the two genes applied in this study the mitochondrial LSU gene (16S rDNA) has been used over a wide range of taxa and timescales in many studies (Schubart et al., 2000). The V4 nuclear SSU ribosomal expansion segment has been described for a limited number of Crustaceans (Crease and Colbourne, 1998) and is rarely used for phylogenetic purposes in Crustaceans. The complete 18S rDNA normally varies between approx 1800 and 2000 bp in Crustacea; however, in most Peracarida and one cladoceran sequence, elongations up to 3400 bp have been found, most of which are located in four variable regions (Spears, unpubl.; Crease and Colbourne, 1998). In this study a V4 segment is reported that exceeds the longest crustacean sequences known until now by more than 270 nucleotides. Its utility for molecular taxonomical studies will be briefly discussed.

MATERIAL AND METHODS

Origin of Material and Outgroup Choice

Antarctic isopod material was collected during two cruises with the research vessel "Polarstern" to the Eastern Weddell Sea (ANT XIII/3) and to the Antarctic Peninsula (ANT XIV/2). Material was sorted out by hand from trawled gear (Agassiz' trawl, bottom trawl, mini dredge; occasionally also from epibenthic sledge, benthopelagic net, and rectangular midwater trawl) and preserved in prechilled 80% ethanol. Samples were obtained from water depths between 87 and 3720 m. Subantarctic, Australian, and South American species were collected by scuba diving or by collecting in the tidal zone (*Spinoserolis latifrons, Serolina bakeri, Cristaserolis gaudichaudii, Leptoserolis bonaerensis, Serolis paradoxa,* and *Plakarthrium punctatissimum*). Until further usage, samples were stored in ethanol at low temperatures (−30 to 4°C). Species names, sampling locality, EMBL accession number, and collection numbers in the Museum für Naturkunde (Berlin, Germany) are given in Table 1. *Plakarthrium punctatissimum* (Isopoda, Plakarthriidae), one of the closest living relatives of the Serolidae (Wägele, 1989), *Glyptonotus antarcticus* (Isopoda, Valvifera, Chaetiliidae), and *Antarcturus spinacoronatus* (Isopoda, Valvifera, Arcturidae) were chosen as members of the outgroup. Only a subset of these were eventually available for either gene, as discussed below. The *Acutiserolis* material from the deep sea was identified as *bromleyana* with difficulty, since it differs from the description in a number of respects. The author agrees with Poore and Brandt (1997) that the genus is in need of revision.

TABLE 1

Taxa, Collection Localities, and Availability of Sequences and Specimens of Isopods Used in This Study

Taxon	Loc.	Sequence acc. no. (mt LSU)	Sequence acc. no. (nSSU)	ZMB collection number
Ceratoserolis Brandt, 1988	*		*	*
Ceratoserolis trilobitoides (Eights, 1833)	EWS	AJ269799	AJ269824	27 287
Ceratoserolis meridionalis (Vanhöffen, 1914)	EWS	AJ269800	AJ269825	27 288
Ceratoserolis pasternaki (Kussakin, 1967)	EWS	AJ269801	AJ269826	27 289
Cuspidoserolis Brandt, 1988	*		*	*
Cuspidoserolis luethjei (Wägele, 1986)	EWS	AJ269802	AJ269819	27 290
Cuspidoserolis johnstoni (Hale, 1952)	EWS	AJ269803	AJ269817	27 291
Acutiserolis Brandt, 1988	*		*	*
Acutiserolis bromleyana (Willemoes-Suhm, 1874)	DP	AJ269805	AJ269818	27 292
Serolella Pfeffer, 1891	*		*	*
Serolella bouvieri (Richardson, 1906)	EWS	AJ269804	AJ269820	27 293
Septemserolis Wägele, 1994	*		*	*
Septemserolis glacialis (Tattersall, 1921)	EWS	AJ269806	AJ269821	27 294
Frontoserolis Brandt, 1991	*		*	*
Frontoserolis waegelei (Brandt, 1988)	SSI	AJ269807	AJ269822	27 295
Paraserolis Wägele, 1994	*		*	*
Paraserolis polita (Pfeffer, 1887)	SSI	AJ269808	AJ269823	27 296
Serolis Leach, 1818	*		*	*
Serolis paradoxa (Fabricius, 1775)	MS	AJ269811	AJ269827	27 297
Leptoserolis Brandt, 1988	*		*	*
Leptoserolis bonaerensis (Bastida & Torti, 1967)	ARG	AJ269812	n/a	27 305
Cristaserolis Brandt, 1988	*		*	*
Cristaserolis gaudichaudii (Audouin & Edwards, 1840)	MS	AJ269813	AJ269828	27 298
Spinoserolis Brandt, 1988	*		*	*
Spinoserolis beddardi (Calman, 1920)	SSI	AJ269810	n/a	27 300
Spinoserolis latifrons (White, 1847)	Kerg	AJ269809	n/a	27 299
Serolina Poore, 1987	*		*	*
Serolina bakeri (Chilton, 1917)	TM	AJ269814	n/a	27 301
Plakarthriidae Richardson, 1904	*		*	*
Plakarthrium punctatissimum (Pfeffer, 1887)	SSI	AJ269815	n/a	27 302§
Chaetiliidae Dana, 1853	*		*	*
Glyptonotus antarcticus (Eights, 1853)	EWS	AJ269816	n/a	27 303
Arcturidae White, 1850	*		*	*
Antarcturus spinacoronatus (Schultz, 1978)	EWS	n/a	n/a	27 304

Note. EWS, eastern Weddell Sea around Kapp Norvegia; SSI, South Shetland Islands; DP, Drake Passage; MS, Magellan Strait; Kerg, Kerguelen Islands (shallow subtidal); ARG, Argentina (shallow subtidal); ARG, Argentina (shallow subtidal); TM, Tasmania. Specimens have been deposited at the Museum für Naturkunde in Berlin (ZMB, Germany).

§ Whole specimen has been used for DNA extraction; a specimen from the same collection locality has been deposited instead. Sequence accession numbers: AJ2697799–AJ2697828.

Wägele (1994) lists *Serolis glacialis* as *species incertae sedis*. However, this species is morphologically so similar to *Septemserolis nobilis* (Brandt, 1988) that it is justified to include it in this genus until its status is clarified.

Molecular Work

Total DNA was extracted from muscle tissue samples or individual legs of single specimens using one of the two methods mentioned below. Both recipes have been modified to work with serolid isopods.

The DTAB/CTAB extraction protocol modified after Gustincich *et al.* (1991) worked well when small amounts of tissue were incubated in large buffer volumes (2-ml Eppendorf tubes) for several hours in a shaking water bath. Alternatively, a spin column extraction with commercially available columns was carried out following the recommendations of the manufacturer (Qiagen). Whereas the incubation time did not seem to influence the outcome significantly, it was critical to keep the amount of tissue as small as possible and to run multiple parallel extractions. Both recipes sometimes yielded no detectable DNA on an agarose gel, although they successfully amplified during PCR. However, increasing the amount of tissue per extraction frequently resulted in unsuccessful PCR runs, indicating that unidentified inhibitors of the PCR copurified with the DNA during the extraction process.

PCRs were carried out in 50-µl volumes (2 units Qiagen *Taq* polymerase, 5 µl 10× PCR buffer, 10 µl Q-buffer, 5 µl dNTPs, 0.5–1.5 µl DNA template, filled to 50 µl with sterile H_2O). All amplification reactions were carried out in a Perkin–Elmer thermal cycler Model

2400 or a Techne Progene cycler, with cycle sequencing reactions in the Techne cycler exclusively.

Mitochondrial Large Subunit Ribosomal RNA Gene (16S rDNA)

Individual DNA was amplified using the widely applicable primers 16Sar 5′-CGCCTGTTTATCAAAAACAT-3′ and 16Sbr 5′-CCGGTCTGAACTCAGATCACGT-3′ (Palumbi *et al.*, 1991), resulting in a product of approximately 490 bp in length (Table 2). The amplification protocol was 5 min at 94°C for initial denaturing, 35 cycles of 45 s at 94°C, 45 s at 52°C, and 1 min 20 s at 72°C, followed by 7 min for final extension.

Nuclear Small Subunit Ribosomal RNA Gene (18S rDNA)

Primers 18A1 5′-CCTA(TC)CTGGTTGATCCTGCCAGT-3′ and 1155R 5′-CCGTCAATTCCTTTAAGTTTCAG-3′ were used for amplification (Dreyer, unpubl.). On the basis of the 18S sequences from serolid isopods obtained by sequencing the above PCR products, an additional pair of internal primers, Sbeb27P 5′-AGTTGGATTTCTCTTTCGGACC-3′ and Sbeb755N 5′-TTTATCATTACCTCGGGTTCAG-3′, was designed and used successfully in the course of this study. The amplification protocol was 5 min at 94°C for initial denaturing, 36 cycles of 94°C, 48°C, and 72°C, followed by 7 min at 72°C for final extension.

DNA Sequencing

PCR products were purified using commercially available spin columns (Qiagen). Purity and amount of recovered PCR products were estimated on an ethidium bromide-stained 0.8% agarose gel; 0.5–4 µl of purified PCR product were used for dideoxy cycle sequencing using the manufacturer's protocols (Amersham). The reaction mix was reduced to 13 µl in volume and overlaid with two drops of light mineral oil. The cycle sequencing amplification protocol was 94°C for 2 min for initial denaturing and 30 cycles of 94°C for 25 s, 48°C for 25 s, and 70°C for 35 s. For sequencing the 16S amplification product, fluorescently labeled primers with the same sequence as for amplification were used and for the 18S amplification product, another set of fluorescently labeled primers was designed from isopod and amphipod sequences (Dreyer and Englisch, unpubl.): 700Fseq 5′-GTCTGGTGCCAGCAGCCGCG-3′, 600Fseq 5′-CGTATATTAAAGTTG(CT)TGC-3′, 400Fseq 5′-ACGGGTAACGGGGAATCAGGG-3′, and 1000Rseq 5′-GAACTAGGGCGGTATCTGATCG-3′. 18S rDNA PCR products which had been amplified with primers Sbeb27P and Sbeb27N were sequenced with fluorescently labeled primers with the same sequence as for amplification. In some cases, amplification of the nuclear gene resulted in more than one amplification product. In this case the band with the expected increased length (see Table 3) was excised from the gel, purified, and cloned using standard protocols; 0.5–1.5 µl of the cycle sequencing reaction was loaded onto an automated sequencer (Li-Cor) and sequenced. Gels were proofread using the image analysis software of the automated sequencer.

TABLE 2

Length of Gene Regions (in bp) per Species Prior to Removal of Positions

OTU	16S	18S
Ceratoserolis trilobitoides	484	671
Ceratoserolis meridionalis	480	670
Ceratoserolis pastemaki	486	671
Cuspidoserolis luethjei	488	699
Cuspidoserolis johnstoni	486	700
Acutiserolis bromleyana	489	699
Serolella bouvieri	489	698
Septemserolis glacialis	488	700
Frontoserolis waegelei	489	699
Paraserolis polita	492	701
Spinoserolis latifrons	488	765*
Spinoserolis beddardi	488	765*
Serolis paradoxa	487	673
Leptoserolis bonaerensis	488	—
Cristaserolis gaudichaudii	491	690
Serolina bakeri	494	735*
Glyptonotus antarcticus	482	660*
Plakarthrium punctatissimum	492	—
Antarcturus spinacoronatus	—	476*
min-max sequence length	480–494	476–765 (670–701)

* Sequence excluded from analysis due to alignment difficulties. Numbers in parentheses based on included sequences only.

TABLE 3

Length of Used V4 Region of the Nuclear SSU rRNA Gene in Selected Crustaceans (Helix E23_1, E23_2, and E23_5)

Species	Length of fragment (bp)
Astacus astacus	113
Artemia salina	104
Daphnia pulex	257
Antarcturus spinacoronatus	338
Glyptonotus antarcticus	527
Ceratoserolis trilobitoides	535

Note. Origin of species 1–3 see text, species 4–6 this study.

Sequence Alignment and Phylogenetic Analysis

Contigs of the gene regions were assembled using the DNAsis software package (Hitachi Co., Ltd.) and individual contig sequences were aligned with ClustalX (Thompson *et al.*, 1997) using the default parameters. The alignments were further improved manually by identifying secondary structure elements of the homologous molecules in *Drosophila melanogaster* (mitochondrial ribosomal LSU, Accession No. X53506; Gutell *et al.*, 1993) and *Astacus astacus* (nuclear ribosomal SSU, Accession No. U33181; Van de Peer *et al.*, 1999) using

the editing program Genedoc v2.5 (Nicholas and Nicholas, 1997). Unexcluded sequences and positions in the datamatrix (see below) were treated as single unordered characters; gaps were treated as missing information. Phylogenetic trees were inferred using maximum-parsimony (MP) and maximum-likelihood (ML).

In MP reconstructions, transitions were weighted equally (scheme TV1) relative to transversions or downweighted by factors of two or three (schemes TV2 and TV3). Bremer support was calculated by running the program Autodecay 4.0 (Eriksson, 1998) on the shortest tree or the strict consensus of the shortest trees of the separate and combined datasets. Confidence estimates of nodes in MP analysis were obtained by bootstrapping the data matrices with random addition of taxa.

To ensure the choice of a proper model of sequence evolution for ML analysis, a likelihood-ratio test (LRT) was carried out as implemented in modeltest 2.0 (Posada and Crandall, 1998). ML trees were then constructed using the model determined by the LRT. Sequence evolution parameters were either taken directly from modeltest 2.0 or estimated from within Puzzle 4.02 (Strimmer and von Haeseler, 1996), which were generally in close agreement with the parameter estimates from modeltest.

To clarify the monophyly of the genus *Cuspidoserolis,* the datamatrix was searched for positions which support either of the two topologies for group I produced by ML and MP methods (see Fig. 5). In short, molecular synapomorphies can be expected to uniquely identify the monophylum whose last common ancestor developed this evolutionary novelty (symmetrical split supporting position, SSSP) or subsequent events can have degraded this signal to some degree in the outgroup, ingroup, or both (asymmetrical and noisy split supporting positions, ASSP, ASSP + N). For a more detailed explanation of this method, see Schulenburg *et al.* (1999) and Wägele (1998). The ingroup of the MP scenario included the two *Cuspidoserolis* species in this study; the ingroup of the ML scenario comprised *C. luethjei, A. bromleyana,* and *S. bouvieri* (see Fig. 3) or, alternatively, *C. luethjei* and *A. bromleyana* (see Fig. 5). The noise parameters were varied between 25 and 45% in the outgroup. Due to the small number of ingroup species, only SSSPs and ASSPs were considered (i.e., positions which did not show heterogeneity within the ingroup).

An incongruence length difference test (ILD test) after Farris *et al.* (1994), as implemented in the partition homogeneity test in PAUP* (Swofford, 1998), was carried out to justify the combination of the datasets from the two genes. For this step, all unvaried positions were excluded from the analysis (Cunningham, 1997).

RESULTS

The sequences have been deposited at the EMBL database; the specimens, unless destroyed during DNA extraction, have been deposited at the Museum für Naturkunde in Berlin, Germany (Table 1).

Mitochondrial Large Ribosomal Subunit RNA Gene

The secondary structure of the mitochondrial large subunit ribosomal gene (16S) was in close agreement with the model proposed for *Drosophila melanogaster* (Gutell *et al.,* 1993). Only stems and loops corresponding to bases 911 to 964 in *Drosophila* could not be identified within the isopod sequences, which were highly variable in this region. This region, measuring approximately 50 bases in the isopod species in this study, was therefore excluded from the analysis of the 16S dataset, as were the first 16 bases after primer 16Sar, whose alignment strongly depended upon gap cost parameters and which could not be unambiguously aligned upon secondary structure considerations. Of the remaining 453 aligned nucleotide sites, 249 are variable and 180 are potentially parsimony informative. The fragment is AT rich with an average base frequency of pi(A) = 33.1%, pi(C) = 16.3%, pi(G) = 16.1%, and pi(T) = 34.5% and no significant evidence of base frequency heterogeneity among taxa ($\chi^2 = 66.24$; $P > 0.05$). Length variation in ingroup and outgroup is moderate, with 433 bp minimal and 441 bp maximal length.

The number of observed substitutions increases almost linearly with evolutionary distance in a pairwise sequence comparison using the GTR model and the same parameters as for phylogenetic tree estimation (Fig. 2). Only sequence comparisons involving the outgroup species show less transitions than expected from a strictly linear increase, indicating beginning saturation. This most likely accounts for stronger bootstrap support when transitions are downweighted during analysis (scheme TV2); however, the effect is weak and omission of outgroup sequences produces an identical tree for the remaining sequences (data not shown). It can therefore be concluded that the phylogenetic analysis is not misled by an excessive amount of multiple substitutions which may otherwise degrade the phylogenetic signal in the data.

The hierarchical likelihood-ratio test suggests a general time reversible model with gamma-distributed rates and a number of invariable sites (Fig. 3). The monophyly of serolids is well supported in the ML tree. The Australian species branches off first, followed by the three South American species. The branching order within the South American species suggests that they are paraphyletic with respect to the Antarctic species. All Antarctic shelf and deep-sea species cluster together with a pronounced division into two groups of taxa. One comprises the genus *Ceratoserolis;* the other comprises seven Antarctic species and will be called group I for simplicity in the following.

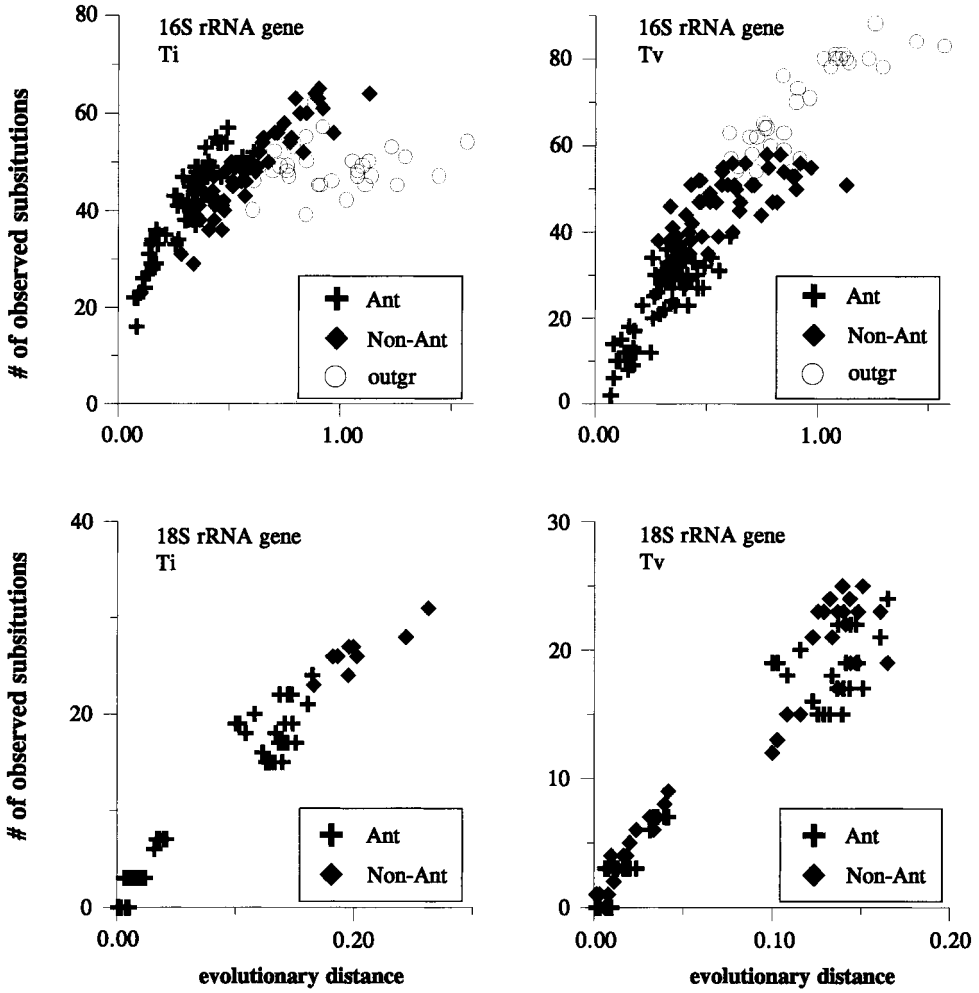

FIG. 2. Saturation plots of the mitochondrial LSU gene (16S) and the nuclear SSU gene (18S) of the serolid isopods in this study. The evolutionary distances were measured using the same models of sequence evolution as for estimating the trees (GTR + I + G and HKY + G). Ti, transition; Tv, transversion; outgr, outgroup species involved; Ant, Antarctic species; NonAnt, South American and Australian species involved.

There are two equally most-parsimonious trees when transitions and transversions are weighted equally (length 745 steps, CI = 0.529, RI = 0.490, RC = 0.259). They differ from the ML tree in having a lower resolution toward the base of the tree, but otherwise they are compatible with the ML tree. The affiliation of the Antarctic genus *Spinoserolis* to either group is not well resolved; it groups with *Ceratoserolis* in the two shortest trees but this node collapses in trees only 1 step longer. The two shortest trees differ in the position of *Acutiserolis*, which is a sister group either to *Cuspidoserolis* or to *Serolella*. The only incompatibility between the ML and the MP trees is that the genus *Cuspidoserolis* is a well-supported monophylum in the MP tree. Different weighting schemes for transition and transversion substitutions (TV2: transversions have a weight of two relative to transitions; TV3: transversions have a weight of three) result in minor changes of the topologies of the shortest trees but the topology of the consensus tree (Fig. 3) is not affected by downweighting transitions. The inconsistency between ML and MP trees is discussed below.

Nuclear Small Subunit Ribosomal Gene

Secondary structure alignment for the nuclear small subunit ribosomal gene (18S) is more problematic. The amplified region comprises the nucleotides homologous to the helices E23_1, E23_2, and E23_5 in the secondary structure model for *Astacus astacus* proposed by Van de Peer *et al.* (1999). This region is part of the highly variable region V4, which is subject to extensive variation and length differences among taxa (Crease and Colbourne, 1998; Hancock and Vogler, 1998). For the region treated in this study, length differences in excess of 100 nucleotides have been reported for Crustacea (see Crease and Colbourne (1998) and references

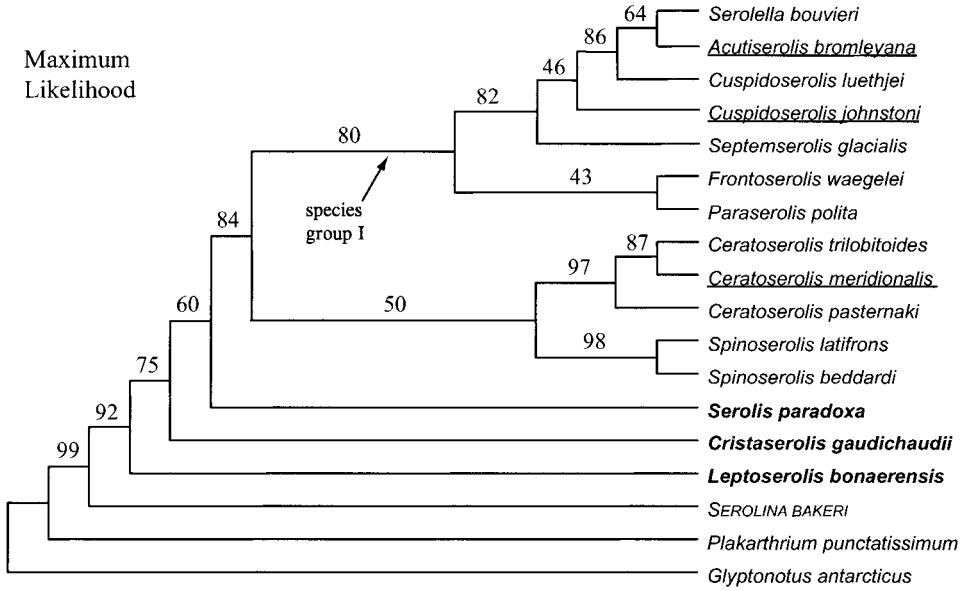

FIG. 3. Phylogenetic tree based on the 3′-terminus of the mitochondrial large subunit rRNA gene (16S) of 16 species of serolid isopods. The model of sequence evolution was chosen by a hierarchical likelihood-ratio test (GTR with gamma distributed rates approximated by four rate categories, $\alpha = 0.756$, $p_{invar} = 0.306$, $R_{(AC)} = 8.00$, $R_{(AG)} = 12.05$, $R_{(AT)} = 3.79$, $R_{(CG)} = 1.75$, $R_{(CT)} = 47.65$). Maximum-parsimony analysis produced two very similar shortest trees, a strict consensus of which is shown in Fig. 6. Note that in the MP analysis the genus *Cuspidoserolis* is monophyletic (bootstrap 83 and 62, Bremer support 3 and 4 in schemes TV1 and TV2).

therein). The serolid isopods exceed the longest crustacean sequence for the fragment used in this study (from the 5′-end of helix E23_1 to the 3′-end of helix E23_2) known until now (*Daphnia pulex,* Branchiopoda) by more than 270 nucleotides (Table 3).

Even though the helices E23_5 and the basal part of E23_1 can be tentatively identified by the presence of compensatory substitutions in stems, secondary structure is of little help in the task of achieving an unambiguously aligned dataset. There is no independently derived model of secondary structure for a crustacean nuclear small subunit ribosomal RNA harboring an extreme expansion as found in the serolids. Since RNA secondary structure *in vivo* is often modified by proteins, it often coincides only in part with predictions made on the basis of energy minimization criteria alone (see, e.g., figures in LSU rRNA structures database; Gutell *et al.,* 1993). The large number of suboptimal foldings that need to be taken into account for a large expansion segment spanning hundreds of bases as in this case make energy minimization of little help when trying to decide between two or more equally likely alternative alignments.

The following 18S sequences had to be excluded because the typical expansion was either largely absent or so strongly modified that it was impossible to align with the rest of the ingroup sequences over a wide range of alignment parameters: *Glyptonotus, Antarcturus, Serolina,* and *Spinoserolis spp* (Table 4). The remaining serolid 18S sequences were rooted with *Cristaserolis gaudichaudii* on the basis of its basal position relative to the remaining ingroup species in the mitochondrial 16S tree (Fig. 3). Approximately 49 bases in variable region V4, which correspond to the distal part of the helix E23_1 in the remaining ingroup sequences, had to be excluded from the subsequent analyses.

Of the remaining 681 positions in the 18S dataset, 285 are variable and 196 are parsimony informative. The sequence length varies between 659 and 665 included nucleotides with an average GC content of 55%. None of the sequences in this dataset deviated significantly from the mean ($P > 0.1$).

The LRT suggests the use of the HKY model (Hasegawa *et al.,* 1985) with gamma-distributed rates for the 18S dataset. Figure 2 shows a linear increase of observed transitions and transversions with evolutionary distance between taxa, thus indicating the absence of marked saturation effects in the aligned nuclear SSU sequences. The ML tree of the nuclear 18S dataset (Fig. 4) is compatible with the tree derived from the mitochondrial 16S gene, although their resolution differs. Particularly well-defined splits include the two South American vs Antarctic species and the split within the Antarctic species between the genus *Ceratoserolis* and the group I. Within group I there is also support for a group consisting of *Cuspidoserolis* spp., *Acutiserolis,* and *Serolella.* An MP analysis yields a very similar tree, the only difference between them being that *Septemserolis* branches off first in group I, although support for this node is low (Bremer support 0; bootstrap support 61 and 55% in weighting schemes TV1 and TV2, respectively).

TABLE 4

Genetic Distances in Pairwise Sequence Comparisons for Mitochondrial LSU rRNA Gene (GTR Model; Lower Triangle) and Nuclear SSU rRNA Gene (HKY Model; Upper Triangle)

		1	2	3	4	5	6	7	8
1	*Glyptonotus antarcticus*	*	*3.30413675*	*14.7152062*	*14.3595142*	*13.8306503*	*10.6471748*	*11.9074402*	*12.2802076*
2	*Plakarthrium/Antarcturus*	0.90078294	*	*6.14623404*	*6.98337555*	*5.69588372*	*5.35654736*	*5.61915493*	*5.7375885*
3	*Ceratoserolis trilobitoides*	0.75980914	1.10906732	*	0.00153428	0.00645003	0.17212565	0.17168441	0.18234748
4	*Ceratoserolis meridionalis*	0.72739416	1.5617342	0.13472591	*	0.00808763	0.17244168	0.17199968	0.182668
5	*Ceratoserolis pasternaki*	0.76908267	1.4356879	0.16626225	0.14760877	*	0.18998972	0.18921052	0.20049295
6	*Cuspidoserolis luethjel*	0.88152319	1.22432709	0.55514109	0.34533557	0.39720196	*	0.00307788	0.00966094
7	*Cuspidoserolis johnstoni*	0.72296554	1.09742415	0.45905897	0.41679555	0.31001285	0.08449046	*	0.00971693
8	*Serolella bouvieri*	0.69500041	1.25176597	0.41233584	0.48262221	0.3805766	0.13778922	0.13252535	*
9	*Acutiserolis bromleyana*	0.70263695	1.07299316	0.51990461	0.41197366	0.37874562	0.0958487	0.10932075	0.08288705
10	*Septemserolis glacialis*	0.64116013	1.053617	0.37824339	0.38709545	0.29524353	0.16813637	0.14821562	0.11735931
11	*Frontoserolis waegelei*	0.61191791	0.8428942	0.34523389	0.37572241	0.26931065	0.24903575	0.17921206	0.15290785
12	*Paraserolis polita*	0.78822678	1.07715642	0.60525805	0.39911902	0.38090894	0.34488508	0.25910783	0.27777246
13	*Spinoserolis latifrons*	0.75863814	1.12550855	0.32463613	0.33357707	0.34456381	0.48933521	0.3405754	0.33644694
14	*Spinoserolis beddardi*	0.60400558	1.02502811	0.29843563	0.32273975	0.308945	0.45665255	0.40378579	0.32004032
15	*Serolis paradoxa*	0.8453325	0.90889305	0.46356979	0.40592912	0.33668345	0.61509538	0.55199188	0.43332431
16	*Cristaserolis gaudichaudii*	0.84553593	1.28733444	0.54062855	0.64626664	0.51356733	1.12604702	0.81497353	0.59307468
17	*Serolina bakeri*	0.9190675	1.13853145	0.70040274	0.71307182	0.67194992	0.88266587	0.89475292	0.76841342

Note. Distances were calculated using gamma distributed rates with values for shape parameters alpha and proportion of invariable sites as used for constructing trees for the respective datasets (see text). Nuclear 18S sequences of species 1, 2, 13, 14, and 17 were excluded from the analysis because of alignment difficulties (upper triangle, italics).

Phylogenetic Analysis of Combined Nuclear and Mitochondrial Datasets

For this purpose the 16S dataset had to be reduced to the same taxa for which 18S data are available. The incongruence length difference test does not provide evidence for incompatible phylogenetic signal in the data partitions between the two genes for the pruned dataset ($P = 0.639$ ILD; branch-and-bound, 1000 replicates). Combining the datasets for the mitochondrial 16S and the nuclear 18S genes yields 1124 aligned nucleotide positions of which 448 are variable and 213 parsimony informative (i.e., shared by two or more taxa). For the combined data of the remaining 12 species, the modeltest identifies the general time reversible model with gamma-distributed rates and consideration of invariable sites as best suited for tree construction under the ML criterion.

The ML tree of the combined data is well resolved (Fig. 5). It shows essentially the same groups as were identified in the separate datasets, although the puzzling support values are, on average, higher in the tree based on the combined data.

An analysis of the combined datamatrix under maximum-parsimony criterion yields a single, fully resolved shortest tree which is shown in the right half of Fig. 5 (574 steps, CI = 0.708, RI = 0.648, RC = 0.456). Downweighting transitions by a factor of two or three (schemes TV2 and TV3) leaves this topology unchanged, indicating a robust phylogenetic signal in the data.

Similar to the situation in the ML tree, bootstrapping the MP tree indicates that, even though the topology shown in Fig. 5 is preferred, the available data cannot fully exclude the possibility that a monophyletic (*Frontoserolis, Paraserolis*) may be the sister group to the rest of group I and a monophyletic (*Serolella, Acutiserolis*) may be the sister to the genus *Cuspidoserolis*.

Monophyly of Cuspidoserolis: Maximum-Likelihood versus Maximum-Parsimony

As Brandt (1988) convincingly argued, monophyly of the newly recognized genus *Cuspidoserolis* is morphologically the strongly preferred option. This view is corroborated by maximum-parsimony methods, which consistently group the two *Cuspidoserolis* species in this study into one well-supported monophyletic taxon (16S and 16S and 18S combined), yet maximum-likelihood methods tend to recognize the paraphyly of the genus, even though this evidence is rather weak in the combined dataset (Fig. 5, left).

The shortest tree containing a paraphyletic *Cuspidoserolis* is 6 steps longer than the shortest tree without constraints which recognize the monophyly of the genus (580 vs 574 steps for all species in Fig. 5 or 213 vs 219 steps for species in group I alone). In the combined datamatrix there are eight positions supporting the monophyly of *Cuspidoserolis* but only a single position supporting the topology favored by the ML analysis. This situation is stable over a wide range of parameters permitting up to 45% noise in the outgroup.

TABLE 4 —*Continued*

9	10	11	12	13	14	15	16	17
10.8995655	10.8873739	10.8591765	11.3813267	3467.93	314.666	12.6948814	8.31737614	550.212
5.32922459	5.88648319	5.35651016	5.68616009	6935.87	38.175312	5.22110271	5.289985357	36.885387
0.17163825	0.16159771	0.157440066	0.20336965	*899.998*	*108.373*	0.20374218	0.39383179	*107.953*
0.17197739	0.16187334	0.15771325	0.19276258	*906.673*	*107.786*	0.19891524	0.39491528	*110.183*
0.18916198	0.17826034	0.17439346	0.224949859	*898.037*	*108.248*	0.23467578	0.43762794	*107.179*
0.00308263	0.02057778	0.0131965	0.0352237	*292.564*	*54.5140038*	0.13265078	0.28596315	*110.051*
0.00309239	0.02050214	0.01310495	0.03984421	*311.125*	*56.6472244*	0.13662355	0.28293267	*118.596*
0.00973227	0.02442037	0.01672151	0.0444871	*254.49*	*49.7058525*	0.13680431	0.31246653	*127.484*
*	0.02049657	0.1311747	0.03981629	*313.576*	*57.0868607*	0.13665777	0.28277612	*127.041*
0.15185043	*	0.02050598	0.04692317	*299.001*	*51.7074509*	0.14405501	0.30100667	*101.424*
0.17588584	0.17050368	*	0.03504891	*299.297*	*55.6314125*	0.15642296	0.30790198	*116.453*
0.2643927	0.28859192	0.21150476	*	*284.233*	*53.5266953*	0.19015117	0.30593759	*122.827*
0.3497498	0.38949189	0.32047501	0.43677524	*	0.00504084	*441.907*	*132.38*	*6935.87*
0.33737868	0.35381511	0.25610024	0.44639888	0.07132339	*	*63.5655098*	*110.763*	*6935.87*
0.47434729	0.37708876	0.28162026	0.41996697	0.62233877	0.5127669	*	0.22291665	*108.483*
0.77567106	0.63169569	0.42826229	0.57179363	0.56758422	0.47109738	0.56714034	*	*103.457*
0.84383118	0.89950794	0.61409116	0.91712201	0.7943787	0.64971763	0.9664005	0.82898688	*

On the basis of this evidence and in view of the fact that a tree containing a monophyletic genus *Cuspidoserolis* is statistically not significantly more unlikely than the ML tree under maximum-likelihood criterion (Kishino–Hasegawa test, $P > 0.2$), it may be concluded that the MP topology with *Cuspidoserolis* monophyletic (Fig. 5, right half) more likely reflects the true phylogenetic history of the species in group I.

For the remainder of the species, both genes result in a congruent topology, as shown in Fig. 5, according to which the nodes that are incongruently resolved, depending on the method of analysis, are among the most recent bifurcations in the tree. The rather weak influence of saturation effects, at least for the younger nodes (Fig. 2), makes it unlikely that homoplasies due to multiple hits account for the difficulties of the ML method in resolving relationships in group I. More likely, the small genetic distance between *Acutiserolis, Cuspidoserolis,* and *Serolella* (Table 4), indicating a short time interval between speciation events, is responsible for the difficulties of the ML reconstruction. The relatively young age of the nodes under question may account for the relatively better performance of maximum-parsimony here.

DISCUSSION

Phylogenetic Analysis of Nuclear and Mitochondrial rRNA Sequences and Usefulness of the LRT Test

The phylogenetic analysis of the nuclear small subunit rRNA and the mitochondrial large subunit rRNA gene fragments of serolid isopods is consistent with the idea that, despite their independent modes of inheritance, both genes share a common phylogenetic history. This finding increases the likelihood that the molecular data actually reflect the phylogenetic history of the species under study. Both data partitions yield congruent results, although the occurrence of unalignable V4 sequences led to the exclusion of three ingroup species from the nSSU and combined datasets.

Likelihood-ratio tests have become a useful tool in various fields, one being the choice of sequence evolution models in molecular phylogenetics (Huelsenbeck and Crandall, 1997; Huelsenbeck and Rannala, 1997). The hierarchical procedure from simpler to more complex models of sequence evolution applied with the likelihood-ratio test does not per se guarantee that the model that is finally chosen provides an optimal description of the underlying evolutionary processes (Swofford *et al.*, 1996). In principle, even in the complete absence of an adequate model, the LRT would identify a preferred model by exclusion of models with relatively worse fit of the applied test statistic. Nevertheless, the LRT makes this critical step in phylogenetic analysis (model selection) objectively justifiable and helps to avoid the circularity of tree construction that would occur if the choice of the model was based on resolution or (desired) topology of the resulting tree.

Taxonomic Implications of the Molecular Data

The molecular data yield a robust phylogeny which can be supported by morphological arguments. Some of the nodes agree with one of the morphological hypotheses or represent one possible solution for previously unresolved relationships (Figs. 1 and 6). Although a detailed analysis of morphological characters is not intended at this stage, some of the most evident implications from the molecular data shall be discussed here. *Frontoserolis* as defined by Brandt (1991) originally contained 12 species, 4 of which (*Frontoserolis*

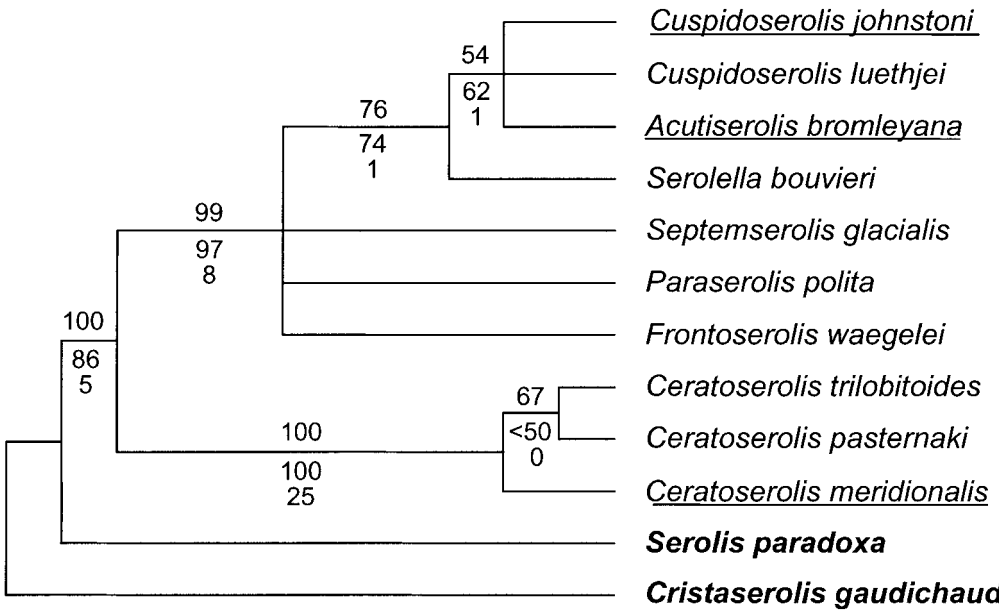

FIG. 4. Maximum-likelihood tree based on the V4 region of the nuclear small subunit rRNA gene (18S) of 12 species of serolid isopods (Puzzle 4.02; puzzling support values above branches, 1000 replicates). The choice of the model of sequence evolution is based on a hierarchical likelihood-ratio test (HKY85 Model, gamma-distributed rates approximated by eight rate categories, $\alpha = 0.18$, ti/tv = 1.024). Below the branches are the values for bootstrap support for the nodes in MP analysis (upper figure; scheme TV1, 1000 replicates, random addition of sequences, heuristic search) and for Bremer support (lower figure). This tree is rooted with *Cristaserolis* based on its basal position in the 16S tree (Fig. 3) with respect to the species included here.

waegelei, *Serolella bouvieri*, *Paraserolis polita*, and *Septemserolis glacialis*) are included in this study (see also Brandt, 1993). Examining a large collection of new material, Wägele (1994) discovered sexual dimorphism of pereiopod 7 in *Cuspidoserolis* and in a subset of the species in *Frontoserolis s. l.* and reorganized the phylogeny accordingly. *Frontoserolis s. str.* was reduced to 3 species and the other species were transferred to other genera to make the phylogeny consistent with the occurrence of the newly scored characters (Fig. 1). This study provides independent evidence from two genes that recognition of *Cuspidoserolis* Brandt, 1988 and *Acutiserolis* Brandt, 1988 indeed require splitting of *Frontoserolis sensu lato*, which would otherwise become paraphyletic (Figs. 3–6).

With the exception of *Acutiserolis*, all nodes in group I (see Fig. 5) are fully resolved and receive good support. The molecular data favor a sister group relationship between *Acutiserolis* and *Cuspidoserolis*, although the bootstrap and puzzling support values are

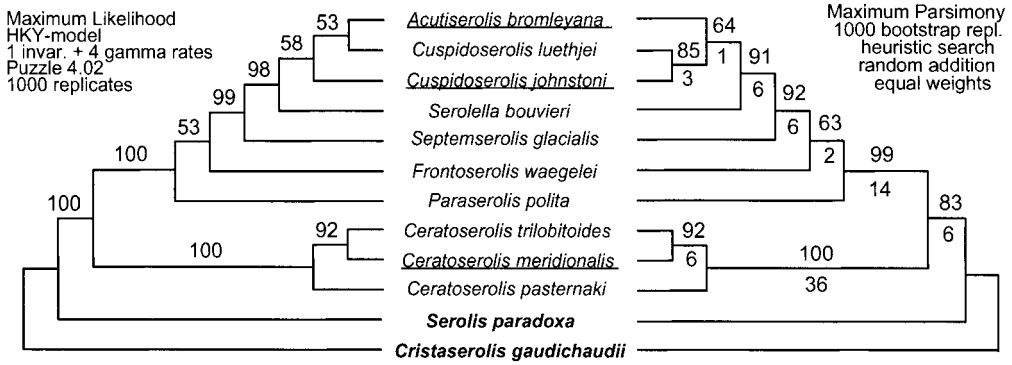

FIG. 5. Phylogenetic tree of serolid isopods based on combined mitochondrial and nuclear rRNA gene datasets (16S and 18S). For the maximum-likelihood analysis, the HKY85 model with invariable sites and gamma-distributed rates approximated by four rate categories was chosen by the LRT; sequence evolution parameters were estimated within Puzzle 4.02 ($\alpha = 0.19$, ti/tv = 1.61, $p_{invar} = 0$) with the number of replications set to 1000 quartet puzzling steps. For the MP tree, bootstrap values of 1000 replicates with random addition of taxa are shown above the branches, with equal weighting and Bremer support shown below. Alignment gaps were treated as missing information in MP trees; positions containing gaps were ignored in ML analysis.

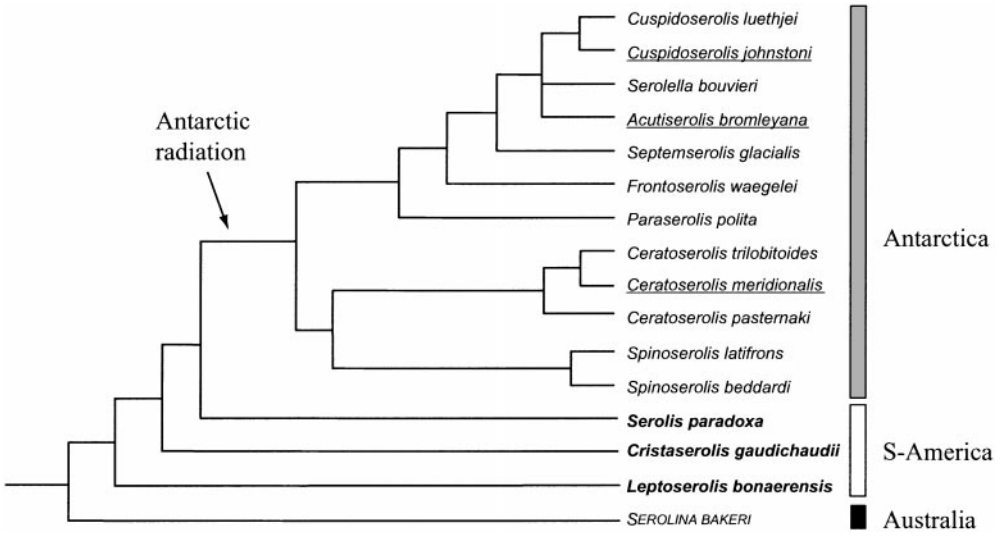

FIG. 6. Patterns of horizontal and vertical distribution of 16 species of serolid isopods. The tree represents the current best estimate of the phylogeny of this group and is based on the MP tree of the combined 16S and 18S datasets. *Serolina bakeri, Leptoserolis bonaerensis,* and *Spinoserolis spp.* have been included on the basis of their position in the 16S rRNA tree, the branches resolving the positions of *Serolella* and *Acutiserolis* have been collapsed due to ambiguous phylogenetic signal in the data (see text for details). This topology is identical to the strict consensus of the two shortest 16S trees. Underscored species names indicate occurrence in the deep sea; all other species live in shallower waters.

relatively low (Figs. 5 and 6). This is in congruence with morphology. The mediocaudal lobe or spine of the cephalothorax is a potential synapomorphy of this group (Brandt, 1991), but it is only weakly expressed in *Acutiserolis* and analogous structures occur outside this group (*Serolina, Heteroserolis;* see Wägele, 1994).

The molecular data provide solid support for a monophylum consisting of *Cuspidoserolis, Serolella, Septemserolis,* and *Acutiserolis.* For the first three genera, a sexually dimorphic pereiopod 7 is known (Wägele, 1994). This, however, is also visible in the *Acutiserolis* material from the Drake Passage (this study) and in the drawings of Poore and Brandt (1997). A sexually dimorphic pereiopod 7 therefore forms a potential morphological synapomorphy and supports the molecular phylogeny.

One of the most striking discrepancies between the two morphological hypotheses (Fig. 1) and between the molecular and the morphological data (Figs. 1, 5, and 6) is the position of *Acutiserolis bromleyana* and *Serolis paradoxa*. Wägele (1994) placed these species close together, based mainly on the bilobed shape of the endopod of the 4th pleopod (Plp4). The shortest tree that contains this group is 612 steps long, as opposed to 574 steps for the unconstrained tree. The molecular data strongly suggest that this is a polyphyletic grouping and support an alternative position instead: *Acutiserolis* groups together with *Cuspidoserolis* (see Brandt, 1991), whereas *Serolis paradoxa* occupies a more basal position (Figs. 4 and 5). This hypothesis, which is strongly supported by both mitochondrial and nuclear genes independently, casts doubts on the phylogenetic usefulness of the shape of Plp4. These doubts are further corroborated by the fact that neither *Acutiserolis* material from the Coral Sea (Poore and Brandt, 1997) nor from the Drake Passage (this study) had a bilobed Plp4. Beddard (1884), however, mentions this feature in his text (p. 60) and provides a drawing (Pl. V, Fig. 11) in which an apical division of the endopod of Plp4 is clearly visible. It must be concluded either that not all species that are currently assigned to *Acutiserolis* express that feature or that there is an extensive amount of intraspecific morphological variation. The shape of the 4th pleopod as currently scored should therefore not be treated as a reliable synapomorphy for phylogenetic purposes. As a consequence, it seems logical to reestablish *Acutiserolis* Brandt, 1988 as a valid genus (see Poore and Brandt, 1997) and not as a subgenus of *Serolis* Leach, 1818, as proposed by Wägele (1994). Whether this also applies to *Acanthoserolis* Brandt, 1988, which was also reduced to a subgenus of *Serolis s. str.*, remains to be seen when further material becomes available for molecular analysis.

Patterns of Horizontal and Vertical Distribution

The molecular phylogeny presented in this study allows a meaningful interpretation of species distribution of extant serolid isopods. Even though the results of this study should not be overgeneralized in view of the fact that a large number of taxa, especially from outside the Southern Ocean, have not been included, some pattern emerges from the molecular phylogeny.

Previously, there was no evidence for a clean biogeographic split in distribution patterns (Fig. 1). Brandt

(1991) and Wägele (1994) agree that the distribution pattern of the extant serolid fauna is partly the result of continental drift vicariance (e.g., *Serolina* in Australia), which became further modified by colonization, dispersal, and palaeoclimatic events. Both reconstructions lead to phylograms in which at least the taxa with South American and Antarctic distribution are distributed over the tree, even when only the species covered in this study are considered. The lack of a clear biogeographic pattern leads the authors to conclude that the main radiation of serolids must have taken place long before the separation of South America and Antarctica (Crame, 1997). The existence of a large monophyletic group of Southern Ocean serolid species in the molecular phylogeny is an intriguing finding.

The paraphyly of the three South American species with respect to the Antarctic species group and the well-supported monophyly of the Antarctic species in this study are presumably not independent of the geological events in this area. Two scenarios are possible: either the Serolidae colonized Antarctic waters only once, presumably from the South American shelf, and radiated after the Drake Passage provided some isolation from the South American continent or the extant Antarctic serolids are the survivors of the serolid fauna which inhabited Gondwana before the supercontinent disintegrated. In the latter case, however, we need to make additional assumptions to explain why there are no Antarctic species today that are most closely related to South American species. In the absence of an explanation for differential survival of species, the hypothesis of a radiation of the Serolidae in Antarctic waters after the opening of the Drake Passage is preferred. It remains to be seen whether this applies to all species of the Serolidae that today live in Antarctic waters when more material becomes available for molecular analysis. This is especially true of the area on both sides of the Drake Passage. The isolation is historically younger there than the separation of Antarctica and Australia and biogeographically less effective due to the presence of the Scotia Arc (Winkler, 1994).

According to the molecular phylogeny, all three deep-sea species in this study originate from within the Antarctic radiation of shallow-water serolids. The three cases of polar submergence appear not to be phylogenetically linked, however.

Acutiserolis is a deep-sea taxon that, according to morphological data, evolved independently from the blind deep-sea genera *Glabroserolis*, *Caecoserolis*, and *Atlantoserolis* (Wägele, 1994). The new molecular data suggest an origin from Antarctic shelf-dwelling serolids (Figs. 5 and 6). A number of other shelf genera also have representatives in the deep sea (*Cuspidoserolis johnstoni* and *Ceratoserolis meridionalis*) which have at least partially reduced functional eyes and prolonged coxal plates compared to their sibling species on the Antarctic shelf (*Cuspidoserolis luethjei* and *Ceratoserolis trilobitoides;* see Fig. 6). Although the depth range of the pairs of sibling species overlaps across their whole distribution range, they seem to occupy different water depths in the same area (pers. observ.). However, neither of these deep-sea species is as specialized to life in the deep sea as the presumably older blind deep-sea genera mentioned above. The last common ancestor of the deep-sea species *Cuspidoserolis johnstoni* and *Actuserolis* is unlikely to have lived in the deep sea, since the sibling species *C. luethjei* occurs exclusively on the Antarctic shelf today and possesses no adaptations to life in deep-sea environments. The serolids of the Antarctic shelf must therefore have colonized the deep sea three times independently during the relatively recent evolution of serolids. This finding agrees well with previous work based on morphological data according to which the Serolidae are a recent addition to the deep-sea fauna (Brandt, 1991, 1992; Wilson, 1998). Among other taxa, a close relationship between deep-sea and Antarctic fauna has also been found with examples of polar emergence as well as polar submergence (Barthel and Tendal, 1994; Brandt, 1991; Brey *et al.*, 1996).

All other deep-sea serolids (*Caecoserolis*, *Atlantoserolis*, and *Glabroserolis*) form a monophyletic group, according to Wägele (1994), which is presumably more closely related to South American shallow-water genera (*Cristaserolis*, *Brazilserolis*, and *Leptoserolis*) than to any of the deep-water species covered in this study. This group might therefore contain the predecessors of a single colonization event of the deep sea that originated from the South American shelf and is probably unrelated to the multiple cases of invasions into the deep sea that occurred from the Antarctic shelf.

If true, this pattern is in accordance with the idea that temperate or tropical shelf ecosystems are more strongly isolated from the deep sea than polar shelf systems. The cooling of the Southern Ocean since the mid-Eocene and the suppression of the Antarctic shelf down to 500–600 m water depth have created environmental conditions that are similar to those of the deep sea, thus facilitating a transition between these two ecosystems (Clarke and Crame, 1992).

Phylogenetic Utility of Ribosomal Expansion Segments

While at higher taxonomic levels the expansion segments are in many cases too variable and are usually discarded from the analysis (Giribet *et al.*, 1996), they turn out to be informative at the family level in serolid isopods. Their use, however, is associated with problems.

The extraordinarily high distance values for the excluded nuclear 18S sequences in Table 4 clearly result from inclusion of large stretches of unalignable nucleotides in the outgroup and three ingroup species. A more detailed account of the properties and evolution

of this part of the V4 expansion segment is in preparation (Held and Dreyer, in prep.), but some points which are directly relevant to phylogenetic inference shall be briefly presented here. For some difficult to align sequences in this study (*Glyptonotus*) it cannot be excluded on the basis of the presented data that they are simply too distant from the ingroup and hence largely saturated. The difference in length of more than 200 nucleotides between most ingroup sequences and *Antarcturus* in the outgroup, however, suggests that, in addition to inheritance of single-site substitutions, large scale insertion/deletion events also play an important role in sequence evolution of this gene fragment (Table 2).

This raises doubt concerning the homology of entire regions of nucleotides, even among sequences of similar length. For branchiopod crustaceans, insertion of noncontiguous blocks of nucleotides in different parts of the V4 and V7 regions could be shown (Crease and Taylor, 1998). Acquisition of entire new helices within these gene regions has been found in branchiopod crustacea and in tiger beetles (Vogler and Pearson, 1996). A similar mechanism might be responsible for the occurrence of the three distinctly divergent ingroup sequences (Table 4). The novel types of V4 expansion segments in *Spinoserolis* spp. and *Serolina* ("long-branch" species) must have evolved within the serolids themselves, as the outgroup comparison shows. They have to be regarded as autapomorphies of the branches leading to *Spinoserolis* and *Serolina* because the similarities between outgroup and short-branch serolids are much too complex to be explained by convergence (data not shown). It has to be concluded that the substitution process in these three species differs from that of the rest of the species for unknown reasons. Without a better understanding of why and in which way these sequences have evolved differently, these sequences cannot be used to reconstruct a reliable and historically correct phylogeny. The congruence of the results of 16S and 18S of the included species indicates that in the remaining sequences there was a signal that most likely reflects the phylogenetic history of the species. The danger of interpreting a less dramatically aberrant sequence type as phylogenetic signal, however, speaks against the routine use of the nuclear V4 expansion segment in routine studies of molecular phylogeny, especially if only a single gene is used.

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