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MITOCHONDRIAL GENES AND ISOPOD PHYLOGENY (PERACARIDA: ISOPODA)

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ABSTRACT

Molecular data are used to test whether (1) Phreatoicidea are the earliest derived living isopods, and (2) the long-tailed isopod morphology is the derived condition within the Isopoda. Small and large subunits of the mitochondrial ribosomal genes (12S- and 16S rDNA), and cytochrome oxidase *c* subunit I (COI) are used as a case study for exploring the boundaries of applicability of these genes at this taxonomic level. I evaluate three data sets, compare three differently weighted alignments, test data partitions for congruence and phylogenetic structure, and evaluate the topologies of individual and combined data partitions. The 12S- and 16S rDNA partitions are not incongruent. However, the incongruence between ribosomal and COI partitions is significant. The study provides new data for addressing generic, familial, and subordinal relationships of this large, morphologically and ecologically diverse taxon. For the three data sets investigated here, the addition of taxa increases bootstrap values at nodes, more nodes have bootstrap support greater than 50%, and clade topologies are comparable when taxa are added. These mitochondrial genes corroborate isopod clades previously recognized on morphological grounds, and in other instances, suggest relationships not previously proposed, i.e., valviferans had a sphaeromatid ancestor, and oniscids and sphaeromatids may be more closely related than previously thought.

Phylogenies based on molecular sequences, allozymes, behavior, paleontology, and other types of data are being used to test the robustness of morphological hypotheses. Although molecular data have been used to estimate various invertebrate phylogenies for more than a decade, these techniques have been applied to only a few crustacean taxa. In other taxa mitochondrial genes are routinely used to infer invertebrate relationships from populations to the level of order, and in arthropod phylogeny at the level of phylum and subphylum (e.g., Ballard *et al.,* 1992; García-Machado *et al.,* 1999). Mitochondrial phylogenetic studies are beginning to proliferate in crustacean studies as well (summarized in Wetzer, 2001). Likewise the use of multiple data sets for phylogenetic hypothesis testing is becoming more common. Multiple data sets allow more precise identification of conflict, and subsequent hypothesis testing of relative conflict among data sets.

The order Isopoda (class Malacostraca, superorder Peracarida) includes over 10,000 described marine, freshwater, and terrestrial species. Most isopod suborders were described in the early part of the nineteenth century, yet for the past 150 years classification of these suborders and their families has been unsettled. Beginning with Hansen (1905) two taxa have dominated the literature as contenders for the title of "most primitive living isopods": the Flabellifera and the Asellota. Schultz (1969, 1979) deviated markedly from this pattern, and his phylogeny depicted the Gnathiidea as the most primitive living isopod group. Schram (1974) was the only worker to have espoused the Phreatoicidea as the earliest derived isopod suborder until Wägele (1989) and Brusca and Wilson (1991) came to the same conclusion in their morphological cladistic analyses. The latter study included all 10 nominate isopod suborders. Based on the frequent suggestion that the suborder Flabellifera is not a monophyletic group, the 15 nominate flabelliferan families were included separately in the Brusca and Wilson (1991) analysis.

A key malacostracan synapomorphy, the "tailfan," and the resulting characteristic swimming and "caridoid" escape behavior in this group are relevant to discerning the most primitive isopod. The tailfan is formed by the biramous lamellar rami of the last pair of appendages, which flare out on either side of the telson. This tailfan arrangement is referred to as the "long-tail" morphology. This arrangement is characteristic of euphausids, long-

tailed (lower) decapods, and with some modifications anaspidaceans and stomatopods. Among the peracarids this long-tailed tailfan arrangement occurs in mysids, thermosbaenaceans, spelaeogriphaceans, and some isopods. In general, peracarid orders exhibit a clear trend toward the reduction of the caridoid tailfan morphology. Cumaceans, tanaids, amphipods, and many isopod taxa lack a tailfan, and in these groups the uropodal rami are styliform. In isopods, styliform uropods always consist of uniarticulate rami. This arrangement, referred to here as "short-tail," is found in the suborders Phreatoicidea, Asellota, Microcerberiidea, Calabozoidea, and Oniscidea. Broad, flattened uropods (i.e., long-tailed tailfan) occur in Flabellifera, Valvifera, Anthuridea, Gnathiidea, and Epicaridea.

The presence of both styliform uropods (short-tail) and broad, flattened uropods (long-tail) in isopods implies that either the "caridoid" tailfan was lost at least once during the history of the group or was regained at least once and represents an independent origin of the tailfan. In the latter case, the isopod tailfan is not homologous with tailfans of other malacostracans. The Brusca and Wilson (1991) analysis suggests that isopods are a monophyletic group and that phreatoicideans are the earliest derived group of living isopods, followed by the asellotan-microcerberid lineage, and then the oniscids. The long-tailed isopods form a larger clade, which is mostly unresolved. In their analysis, isopods with broad, flat uropods and elongate telsonic regions (well-developed tailfans) arose subsequent to the appearance of the phreatoicid/ asellote/ microcerberid/ oniscid lines. The apparent "caridoid" tailfan of these long-tailed isopods is thus not a primitive isopod feature but is secondarily derived within the Isopoda and not homologous with the condition seen in true "caridoid" crustaceans.

In 1882 Sars erected the "Flabellifera" for those isopods with tailfans composed of lateral uropods and an elongate pleotelson. With the subsequent description of many new taxa the original definition has become ambiguous, resulting in a paraphyletic Flabellifera (Kussakin, 1979; Bruce, 1981; and Wägele, 1989). Brusca and Wilson (1991) concluded that isopods with tailfans composed of lateral uropods and elongate pleotelsons, the long-tailed clade, is a "clearly monophyletic and easily-recognized group, with correlated anatomical and ecological attributes." They suggest that classificatory recognition of the long-tailed clade is warranted and desirable. They, however, refrain from making classificatory changes until an expanded data set and a better-resolved phylogeny are available. Additionally, they suggest that the evolution of the long-tailed morphology may have corresponded with the emergence of isopods from infaunal environments and subsequent radiation as active epifaunal swimmers, and paralleling this trend was the shift from a primary scavenging/herbivorous lifestyle to active predatory habits, and eventually parasitism.

In characterizing gene regions appropriate to address family- to order-level isopod phylogeny, I surveyed mitochondrial ribosomal 12S-, 16S rDNA, and protein-coding cytochrome oxidase *c* subunit I (COI) gene regions in a variety of taxa, and have sequenced roughly 400–700 base pair stretches of each of these genes (Wetzer, 2001). Specifically, I used molecular data to test whether (1) Phreatoicidea are the earliest derived living isopods, and (2) the long-tailed isopod morphology is the derived condition within the Isopoda. I used these genes as a case study for exploring the boundaries of applicability of these genes at this taxonomic level. I evaluated three data sets, compared three differently weighted alignments, tested data partitions for congruence and phylogenetic structure, and evaluated the topologies of individual and combined data partitions. The study also provides new data for addressing generic, familial, and subordinal relationships of this large, morphologically and ecologically diverse taxon.

Materials and Methods

Sampling of Taxa

The currently recognized isopod suborders are summarized in Table 1. Taxa from which species were sampled are denoted with asterisks following the taxon name. Taxa used in this study, their taxonomy, GenBank accession numbers, and genes sequenced (12S-, 16S rDNA, COI), are tabulated in Table 2. The sequences used in these analyses are based on highly corroborated sequences resulting from multiple amplification and sequencing events. In most instances two or more specimens were extracted, amplified, and sequenced. Sequences included in these data sets were selected based on sequence quality, and a sequence being representative of the taxonomic group (also see Wetzer, 2001). Locality data were summarized by Wetzer (2001). Generic names for species in this data set are unambiguous except for the genus *Cirolana* for which there are two species included in these

Table 1. Isopod taxonomy with suborders presently recognized. Because the monophyly of the Flabellifera is controversial (Kussakin, 1979; Bruce, 1981; Wägele, 1989), the nominate flabelliferan families are enumerated (modified from Brusca and Wilson, 1991). Species from taxa denoted with "*" are included in this study.

Order ISOPODA
Suborder Phreatoicidea*
Suborder Asellota*
Suborder Microcerberidea
Suborder Oniscidea
Infraorder Tylomorpha
Infraorder Ligiamorpha*
Suborder Calabozoidea
Suborder Valvifera*
Suborder Epicaridea
Suborder Gnathiidea
Suborder Anthuridea*
Suborder Flabellifera
Family Aegidae
Family Anuropidae
Family Bathynataliidae
Family Cirolanidae*
Family Corallanidae
Family Cymothoidae*
Family Keuphyliidae
Family Limnoriidae
Family Phoratopodidae
Family Plakarthriidae
Family Serolidae*
Family Sphaeromatidae*
Family Tridentellidae

analyses. Generic designations are used as an abbreviation for the species names, and the two *Cirolana* species are differentiated as *C. harfordi* and *C. rudicauda*. Most specimens were collected by the author and additional specimens were donated by colleagues (see Acknowledgements). DNA preservation, primers, amplification parameters, and sequencing conditions are described in Wetzer (2001). The dendrobranchiate shrimp *Penaeus* (Order: Decapoda) and its allies are an unquestioned outgroup to the Isopoda. The 12S-, 16S rDNA, and COI sequences were taken from GenBank (12S rDNA sequence for *P.* (= *Farfantepenaeus*) *notialis,* GenBank Acc. No. X84350, 16S rDNA sequence for *P.* (= *Litopenaeus*) *vannamei,* GenBank Acc. No. AJ132780, and COI sequence for *P.* (= *L.*) *vannamei,* GenBank Acc. No. X82503). *"Penaeus"* (*Farfantepenaeus* + *Litopenaeus*) was used as the outgroup in all analyses.

Data Sets

Three data sets were constructed (Table 3). Data set I is based on 11 taxa. Data sets II and III are each composed of 18 taxa. The 12S-, 16S rDNA, and COI gene sequences were generated from the same specimen whenever possible (Table 2). When this was not possible, additional specimens from the same collection lot were sequenced, and sequences were combined to comprise the partitions in data set I. Similarly, 18 taxa (individuals) were sequenced for the 16S rDNA and COI data set (data set II). The designation 12S rDNA(11), 16S rDNA(11), and COI(11) distinguishes the smaller data sets based on 11 taxa from the two larger data sets of 18 taxa each, i.e., 16S rDNA(18) and COI(18). Agosti *et al.* (1996) have suggested that the combination of nucleic acid and the translated amino acid coded character states into the same data matrix overcomes some of the problems caused by the rapid change of silent nucleotide positions, the overall slow rate of change of non-silent nucleotide positions, and slowly changing amino acids. Data set III contains the same 18 taxa as data set II; however, here the COI nucleotides are combined with the translated amino acid sequences.

Sequence Alignment Strategy

The 12S- and 16S rDNA isopod sequences were aligned with the multiple sequence alignment program CLUSTAL W 1.74 (Gibson *et al.,* 1996). The COI nucleotides were translated to amino acids based on the *Drosophila* mitochondrial code in MacClade 3.06 (Maddison and Maddison, 1997). The 12S- and 16S rDNA sequences were aligned in three separate iterations: first using the default settings (slow/accurate gap open penalty = 15, gap extension penalty = 6.66, *k*-tuple size = 2), then with gap open penalty = 12, and finally gap open penalty = 10. The three alignments of each gene were imported into a GCG (Genetics Computer Group, Madison, Wisconsin) file (i.e., file 1 contained three alignments for the 12S rDNA sequence based on the three different weighting schemes for the 10 isopod taxa; file 2 contained 16S rDNA sequence for the same 10 taxa; file 3 contained three alignments for 16S rDNA gene sequences and 17 isopod taxa). Conserved regions were aligned by eye in GCG. Files were exported to a program written by N. D. Pentcheff (unpublished) [reweight-1.2], which identifies nucleotide positions where all three alignments are identical, where two alignments are identical (one alignment differs), and positions where all three alignments differ. These positions are identified in PAUP* (Swofford, 1999) in the "charset" (character set) block, and are easily included and excluded in subsequent analyses. *Penaeus* was aligned to the isopod ingroup using the Profile Alignment feature of CLUSTAL W after the differences in alignments for the isopods had been determined. Thus, the isopod alignments (ingroup) were unaffected by the addition of the *Penaeus* sequences (outgroup). Lastly, the three differently weighted alignments were concatenated as proposed by Wheeler *et al.* (1995). Alignments are available from the author.

Phylogenetic Analyses and Tree Statistics

PAUP* (MAC version 4.062) was used for all parsimony, maximum likelihood, bootstrap, and permutation tail probability tests. PAUP* 4.0d65 for UNIX was used to calculate homogeneity partition tests. Two classes of indices are used to measure the fit of characters to a tree. One of these classes of tree statistics includes tree length, consistency, and retention indices, all of which are absolute measures of the degree of explanation of a data set. The second class of tree statistics is based on the calculation of statistical confidence limits for phylogenetic trees and uses randomization null models. Bootstrapping (Felsenstein, 1985) and permutation tail probability tests (PTP) (Faith and Cranston, 1991; Faith, 1991, 1992) belong to this class. The bootstrap procedure in phylogenetics resamples characters from the original data matrix with replacement to create new matrices of the same size as the original matrix. Although this procedure has received much discussion (e.g., Carpenter, 1992; Kluge and Wolf, 1993; Trueman, 1993; Bremer, 1994), it is an ef-

Table 2. Isopod taxa included in present study indicating genes sequenced and GenBank accession numbers. Taxonomic hierarchy represents suborder, family, genus, and species. Each species listed represents one individual; duplicated species names indicate sampling from multiple individuals.

Taxon	Genes 12S rDNA	16S rDNA	COI
Phreatoicidea			
Phreatoicidae			
Colubotelson thompsoni Nicholls, 1944	AF259525	AF259531	AF255775
Crenoicus buntiae Wilson and Ho, 1996	AF259524	AF259532	AF255776
Paramphisopus palustris Chappuis, 1939	AF259523	AF259533	AF255777
Asellota			
Asellidae			
Caecidotea sp.		AF259534	AF255778
Caecidotea sp.	AF259529		
Oniscidea			
Armadillidiidae			
Armadillidium vulgare (Latreille, 1804)	AF259522	AF259535	AF255779
Ligiidae			
Ligia occidentalis Dana, 1853		AF259536	AF255780
Valvifera			
Idoteidae			
Glyptoidotea lichtensteini (Krauss, 1843)			AF255781
Glyptoidotea lichtensteini	AF259527	AF259537	
Idotea resecata Stimpson, 1857	AF259526	AF259538	AF255782
Paridotea ungulata (Pallas, 1772)		AF259539	AF255783
Anthuridea			
Anthuridae			
Apanthura sp.		AF259545	AF225789
Flabellifera			
Sphaeromatidae			
Sphaeramene polytylotos Barnard, 1914	AF259528	AF259540	AF255784
Sphaeroma quadridentata Say, 1818			AF255785
Sphaeroma quadridentata		AF259541	
Serolidae			
Serolina bakeri (Chilton, 1917)			AF255786
Serolina bakeri		AF260864	
Cirolanidae			
Cirolana harfordi (Lockington, 1877)	AF259521		
Cirolana harfordi		AF259543	
Cirolana harfordi			AF255787
Cirolana rugicauda Heller, 1861	AF260558	AF259544	AF255788
Cymothoidae			
Lironeca vulgaris Chappuis, 1935		AF259546	AF255790
Olencira praegustator (Latrobe, 1802)		AF259547	AF260844

fective measure of support for groups within a phylogeny (Sanderson, 1989), but not between trees (Hillis and Bull, 1993). The PTP test seeks to determine whether there is significant phylogenetic signal present in the data matrix (beyond that produced by chance). This test determines whether there is a significant phylogenetic signal present in a data matrix by testing the null hypothesis that the most parsimonious tree for the data matrix is no shorter than would be expected for random data of the same character state composition, i.e., the data have no cladistic structure.

Table 3. Data partition abbreviations are 12S-, 16S rDNA, COI12 (first and second codon positions), COI3 (third codon positions), and prot (amino acids). Data set I has 11 taxa. Data sets II and III each have 18 taxa. The total number of characters and number of parsimony informative characters in each partition are noted.

Data partition	Total characters	Parsimony informative characters
Data Set I: *12S rDNA/16S rDNA/COI*		
12S rDNA : 16S rDNA : COI12 : COI3	1,189	508
12S rDNA : 16S rDNA	609	253
COI12 : COI3	508	255
12S rDNA	164	68
16S rDNA	445	185
12S rDNA : COI12 : COI3	744	323
16S rDNA : COI12 : COI3	1,025	440
Data Set II: *16S rDNA/COI*		
16S rDNA : COI12 : COI3	1,118	599
16S rDNA : COI12	924	411
COI12 : COI3	583	288
16S rDNA	535	311
Data Set III: *COI nucleotide / amino acid*		
COI12 : COI3 : prot	769	343
COI12 : COI3	583	288
COI12 : prot	575	155
prot	186	55

Six-Parameter Parsimony

Intuitively we expect greater phylogenetic accuracy when evolutionary models more accurately depict actual histories. Cunningham (1997) and Stanger-Hall and Cunningham (1998) determined that the six-parameter parsimony method showed a consistent, positive relationship between congruence of data partitions and accuracy. They found that the log-likelihood six-parameter parsimony model (6P) increased phylogenetic accuracy with known phylogenies and outperformed equally weighted parsimony, transversion parsimony, successive weighting, and invariant six-parameter parsimony. The 6P step matrices were determined for the 12S-, 16S rDNA, and COI data sets using the most parsimonious tree calculated in a heuristic search (equally weighted, unordered parsimony). Individual step matrices were calculated for first, second, and third codon positions in the COI sequence. Because step matrices for first and second codon positions were nearly identical, first and second codon positions were combined in one step matrix, yielding two partitions: one partition containing first and second codon positions (COI12) and a second partition for third codon positions (COI3).

Optimality Criteria

All parsimony analyses were heuristic searches with gaps treated as missing data, multistate characters interpreted as uncertain, starting tree(s) obtained via stepwise addition, and a simple addition sequence. The tree-bisection-reconnection (TBR) algorithm (Swofford, 1991) was used for branch swapping. Most parsimonious character reconstructions were performed with the accelerated transformation (ACCTRAN) algorithm which maximizes reversals and minimizes parallelisms (Maddison and Maddison, 1992). Maximum likelihood analyses used the general time-reversal model (GTR) (Lanave *et al.,* 1984), which takes into account unequal base frequencies and multiple substitutions and assumes all substitution probabilities are independent.

Data Partition Homogeneity Tests

The partition homogeneity test, also known as the incongruence length difference test (ILD) (Farris *et al.,* 1995a, b; also see Mason-Gamer and Kellogg, 1996; DeSalle and Brower, 1997), measures the character incongruence between data partitions under a simple reconstruction model by generating partitions of sizes equal to the original partitions and randomly resampling these newly created partitions without replacement. First, the shortest tree is obtained for each data set and the tree lengths are added to give a sum of tree lengths. The data sets are then combined and randomly repartitioned into two subsets equal in size to the original data sets. Tree lengths for the randomized partitions are determined. Random repartitioning is repeated many times (1,000 in this analysis) to generate a random distribution of the sum of tree lengths. Finally, the sum of tree lengths from the original unpermuted data set is compared to the random distribution. If the probability of randomly obtaining a smaller sum of tree lengths than that of the separate data sets is low, the data are interpreted as incongruent. Invariant characters were removed before applying the ILD, in order to make the ratio of variable to nonvariable characters between data sets comparable (Cunningham, 1997). Equally weighted and 6P step matrices were applied iteratively. The COI amino acids in the "prot" partition were not weighted.

Results

Phylogenetic Signal and Phylogeny Estimation

If a data set has no structure that is significantly different from random, then proceeding with phylogeny estimation is fruitless. The PTP test indicated that each data partition in Table 3 had significant phylogenetic structure. The value of each PTP test equaled 0.001. The null hypothesis was rejected at the $\alpha = 0.05$ level (i.e., that fewer than 50 out of 1,000 trees have a length as short or shorter than the one generated by the data set, PTP

Table 4. Summary of ILD test of data partition congruence. Data partition abbreviations are as in Table 3. Total number of characters in the data partition are followed by the number of invariant characters in the two data sets. Invariant characters were excluded from the datasets before the ILD test was applied. Parsimony informative characters and *P*-values were calculated with and without 6P stepmatrices. One thousand matrices were permuted in PAUP*. Data partitions are considered incongruent for values of $P < 0.05$. An increase (I) or decrease (D) in data partition congruence with the application of 6P stepmatrices is indicated. Incongruent data partitions are denoted with "**," and comparisons in which one partition is statistically incongruent is marked with an "(*)."

			Without 6P		With 6P		
Data partition	Total characters	Invariant characters excluded	Parsimony informative characters	$P =$	Parsimony informative characters	$P =$	Increase / decrease in congruence
11 taxa							
12S rDNA : 16S rDNA	609	232	253	0.54	265	0.19	D
12S rDNA : COI12	551	331	148	0.001	152	0.001	**
12S rDNA : COI3	357	66	243	0.014	249	0.11	I(*)
16S rDNA : COI12	832	437	265	0.17	272	0.10	D
16S rDNA : COI3	638	172	360	0.11	374	0.041	D(*)
COI12 : COI3	580	271	255	0.96	261	0.99	I
18 taxa							
16S rDNA : COI12	924	395	411	0.005	417	0.067	I(*)
16S rDNA : COI3	729	159	499	0.001	504	0.001	**
COI12 : COI3	583	236	288	0.14	289	0.96	I
COI12 : prot	575	331	155	0.94	156	1.0	I
COI3 : prot	380	95	243	0.73	243	0.99	I

≤ 0.05). Each analysis was based on 1,000 matrices, and the tests were done iteratively with and without the 6P step matrices in effect. The PTP test results with the step matrices enforced were identical to those without step matrices.

Effects of Removing Variable Alignment Regions

Phylogenetic reconstruction is predicated on the inference of sequence alignments. Arriving at homology statements, which the process of aligning sequences implies, ranges from simple for closely related protein genes, to extremely difficult or ambiguous for distantly related sequences and those coming from non-protein-coding regions of the genome. Empirical studies (e.g., Morrison and Ellis, 1997) have shown that differences in sequence alignment strategies sometimes have a greater effect on phylogenetic estimates than do differences in tree-building methods. In this study four alignments were developed for each analysis (see Methods: Sequence Alignment Strategies). Three schemes eliminating regions of variable sequence alignments were applied across the three alignments to each gene (i.e., analyses in which all genes were analyzed separately), and in analyses where genes (data partitions) were combined. Parsimony and maximum likelihood analysis were performed as follows: (1) including only nucleotide positions where all three alignments were identical, (2) where all or two alignments were identical, and (3) with no regard to alignment differences. The fourth alignment scheme was based on the concatenated sequences from the three different weighting schemes. I found these alignment differences had only minimal effect on tree topologies and bootstrap support. In a few instances, exclusion of variable regions reduced topological resolution. Based on these negligible results, variable alignment regions were not excluded in subsequent analyses and concatenated sequence alignments were not further investigated. All analyses are based on the CLUSTAL W alignment, gap open penalty = 15 with all character data included.

Homogeneity Tests (ILD)

Although numerous reasons for favoring a combined analysis have been cited (Eernisse and Kluge, 1993; Chippindale and Wiens, 1994), the importance of examining incongruence among data partitions has been stressed (Cunningham, 1997). Data partition congruence is summarized in Table 4. Applying the 6P reconstruction model (step matrix weighting) to the data partitions changed the observed congruence level in all instances, except for two partitions (12S rDNA : COI12 [11 taxa] and 16S rDNA :

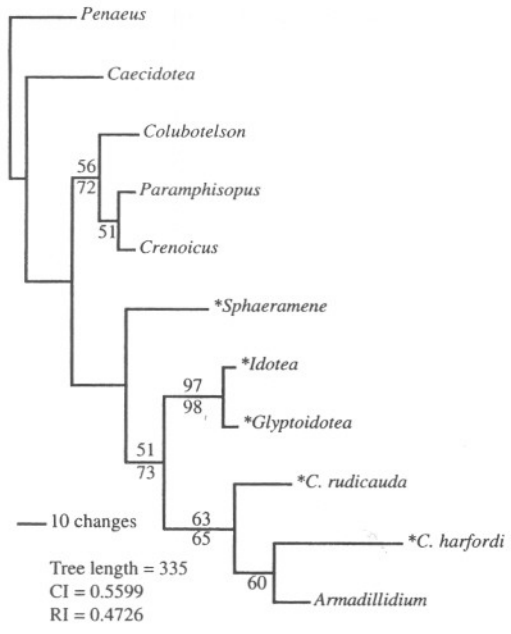

Fig. 1. Most parsimonious tree for 11 taxa based on 6P parsimony analysis of 12S rDNA data partition. Tree length, consistency index excluding uninformative characters (CI), and retention index (RI) are shown below. Numbers on branches are boostrap support based on 1,000 pseudoreplicates with >50% frequency. Numbers above branches are based on analysis with 6P step matix; numbers below branches without 6P step matrix. Long-tailed isopods are marked with "*"; all others are short-tailed.

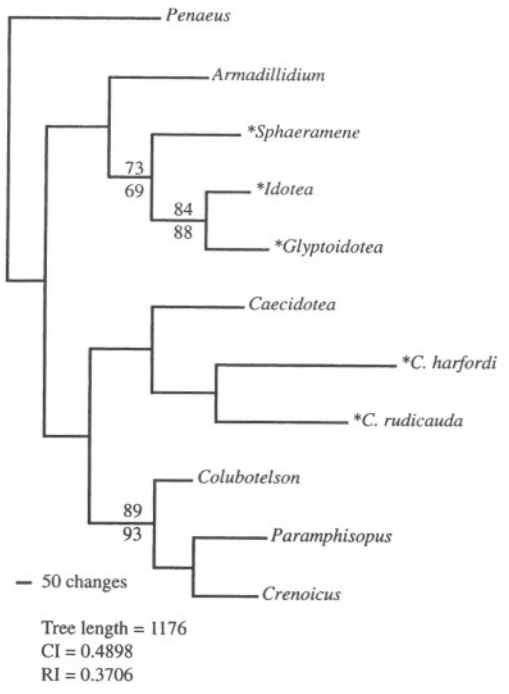

Fig. 2. Most parsimonious tree for 11 taxa based on 6P parsimony analysis of 16S rDNA data partition. Abbreviations as in Fig. 1.

COI3 [18 taxa], $P = 0.001$). The 12S rDNA : COI12 and the 16S rDNA : COI3 data partitions are statistically incongruent ($P < 0.05$). The COI12, COI3, and amino acids sequences are not incongruent with each other. Similarly, the 12S- and 16S rDNA partitions are not incongruent. However, incongruence between ribosomal and cytochrome oxidase partitions is significant.

Phylogenetic Analysis

Maximum likelihood analyses were carried out for 12S rDNA(11), 16S rDNA(11), and COI(11) partitions separately and for these same partitions in a combined analysis. Separate and combined partitions produced the same topologies as parsimony with 6P step matrices, demonstrating that the maximum likelihood and parsimony with 6P step matrices methods are comparable for these data. Parsimony was used in all subsequent analyses. Each partition was analyzed independently and then the combinable components were analyzed. The results are discussed in "Separate Analyses" and "Combined Analyses" below.

Separate Analyses

Figures 1–5 are the results of the parsimony analyses with 6P step matrices for each data partition considered separately. Parsimony analyses without 6P step matrices were also performed. These results did not greatly differ from the analysis with 6P step matrices and are discussed where applicable. The trees are not shown. All analyses produced a single most parsimonious tree, except 16S rDNA(18) analysis which produced two trees, which differ only slightly. Only one of these two trees is shown in Fig. 4. Tree lengths, consistency indices excluding uninformative characters, and retention indices are shown on the figures. Bootstrap values greater than 50% frequency based on 1,000 pseudoreplicates are shown on the branches (values above branches are from parsimony analyses with 6P step matrices, values below branches are from parsimony analyses without 6P step matrices).

The 12S rDNA(11), 16S rDNA(11), and 16S rDNA(18) analyses (Figs. 1, 2, and 4)

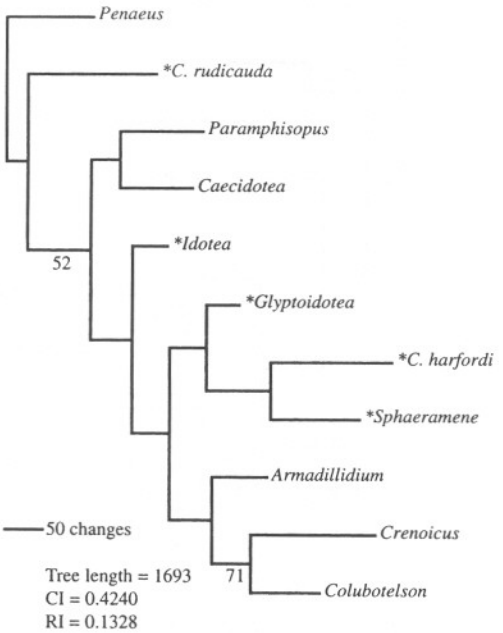

Fig. 3. Most parsimonious tree for 11 taxa based on 6P parsimony analysis of COI12 and COI3 data partitions. Abbreviations as in Fig. 1.

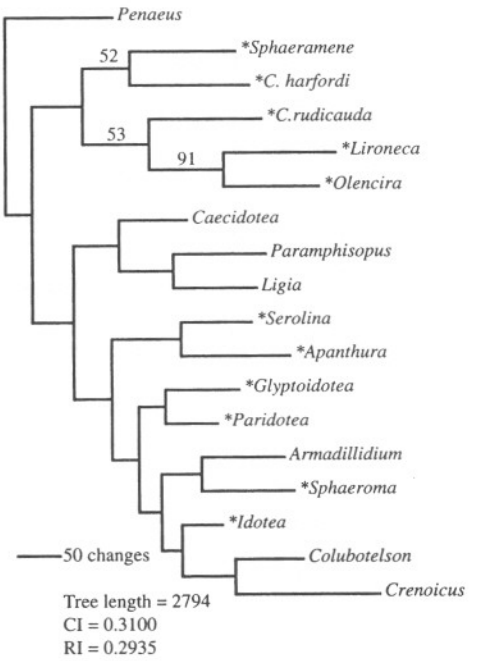

Fig. 5. Most parsimonious tree for 18 taxa based on 6P parsimony analysis of COI12 and COI3 data partitions. Abbreviations as in Fig. 1.

share several consistent features. All three analyses (with and without 6P step matrices) supported a phreatoicid clade (*Paramphisopus* (*Crenoicus* + *Colubotelson*)). A sphaeromatid-valviferan grouping with *Sphaeramene* ancestral to *Idotea* + *Glytoidotea* is indicated by the topology in Fig. 2 (16S rDNA:11). Similar topologies exist in Fig. 1 (12S rDNA:11) and Fig. 4 (16S rDNA:18); however, bootstrap support is <50%. In the two 16S rDNA(18) trees (only one tree shown) (Fig. 4), the cymothoids (*Lironeca* + *Olencira*), sphaeromatids (*Sphaeroma* + *Sphaeramene*), and valviferans (*Idotea* (*Paridotea* + *Glyptoidotea*)) are well-supported clades. The 16S rDNA(18) trees also show strong support for a cymothoid (*Lironeca* + *Olencira*) + *C. harfordi* and *C. rudicauda* + *Apanthura* relationships. A sister-group relationship of these clades to one another is supported by 72% bootstrap support. The sister-group relationship of *C. harfordi* and *C. rudicauda* occurs only in the 16S rDNA(11) tree (Fig. 2), and although one would expect strong support for two members of the same genus, bootstrap support for this relationship is less than 50%.

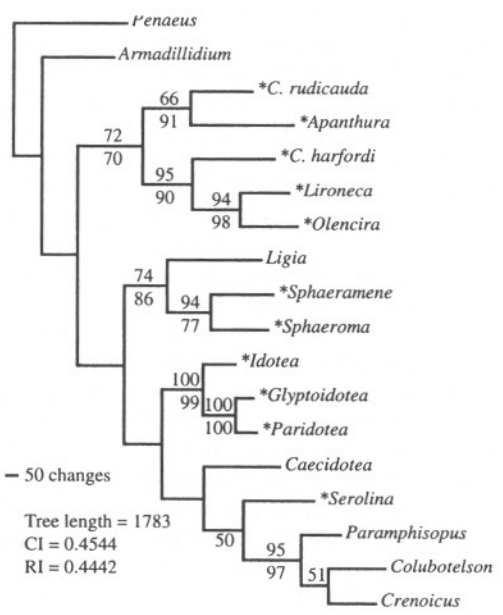

Fig. 4. One of two most parsimonious trees for 18 taxa based on 6P parsimony analysis of 16S rDNA data partition. Abbreviations as in Fig. 1.

The COI(11) tree is poorly supported (Fig. 3). In this topology only the *Crenoicus* + *Colubotelson* relationship (without 6P step matrices) has 71% bootstrap support. The remaining isopods, excluding *C. rudicauda,* are

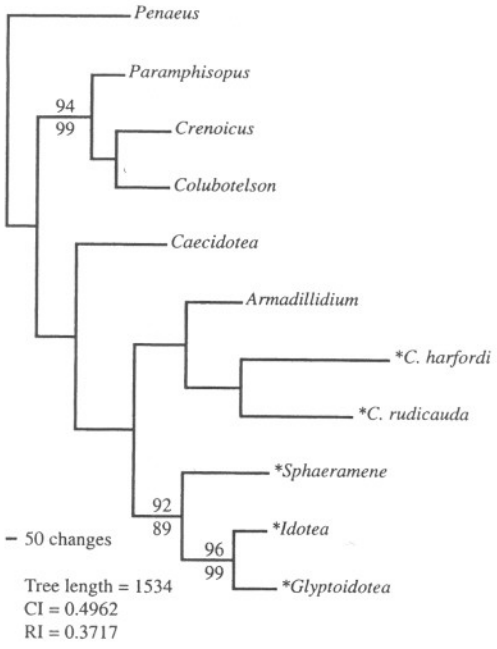

Fig. 6. Most parsimonious tree for 11 taxa based on 6P parsimony analysis of 12S- and 16S rDNA data partitions. Abbreviations as in Fig. 1.

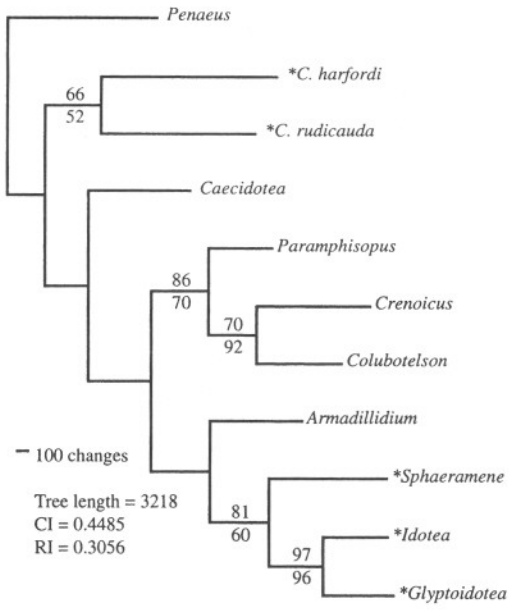

Fig. 7. Most parsimonious tree for 11 taxa based on 6P parsimony analysis of 12S-, 16S rDNA, COI12, and COI3 data partitions. Abbreviations as in Fig. 1.

united by 52% bootstrap support. The COI(18) tree has 91% bootstrap support for the cymothoid clade (*Lironeca* + *Olencira*) (Fig. 5). Greater than 50% bootstrap support is provided for a *Lironeca/Olencira* plus *C. rudicauda* relationship. In this tree *C. harfordi* is the sister taxon of *Sphaeramene*. The most parsimonious tree produced by an unweighted analysis of the amino acids (not shown) is unresolved except for a valviferan clade (*Glyptoidotea* + *Idotea* + *Paridotea*), and cymothoid clade (*Lironeca* + *Olencira*). These have bootstrap support equal to 85% and 90%, respectively.

Combined Analyses

The ILD tests of data partition congruence support the combining of the (1) 12S rDNA(11) and 16S rDNA(11) data partitions, and (2) the COI12(18), COI3(18), and amino acid data partitions (Table 4). The parsimony results of the 12S rDNA(11) : 16S rDNA(11) analyses is shown in Fig. 6. The results of the COI12(18) + COI3(18) + amino acids are not shown. COI12(11) : COI3(11) and COI12(18) : COI3(18) were considered above, see "Separate Analysis." The 12S rDNA : 16S rDNA analysis (Fig. 6) supports (1) a sphaeromatid-valviferan clade (*Sphaeramene* (*Idotea* + *Glyptoidotea*)) and (2) a phreatoicid clade (*Paramphisopus* (*Crenoicus* + *Colubotelson*)). In this tree, phreatoicids are ancestral, asellotans (*Caecidotea*) are derived from phreatoicids; oniscids (*Armadillidium*) are the sister group to the long-tailed cirolanids; and the sphaeromatid/valviferan clade is derived.

The COI(18) and amino acid data partitions result in three equally parsimonious trees (not shown). In analyses with and without 6P step matrices, the only clade supported with >50% bootstrap support (99%) are the cymothoids (*Lironeca* + *Olencira*). Eliminating third codon positions from the analyses produces similar results (not shown).

Although there are limitations of data partition combinability based on the ILD tests, data partitions were combined, and results of the combined analysis of the 12S rDNA(11) : 16S rDNA(11) : COI12(11) : COI3(11) data partitions are shown in Fig. 7. The most parsimonious tree based on 6P step matrices is identical to the general time reversal (GTR) site specific rates maximum likelihood analysis. In this topology the two *Cirolana* species are sister taxa. The long-tailed isopods are polyphyletic: *Cirolana* (basal) and (*Sphaeramene* (*Idotea* + *Glyp-*

toidotea)) clade derived. These results are consistent with the *a priori* data partition congruence test (ILD) which suggested that these data partitions are incongruent (Table 4). The tree reflects the contribution of the congruent data partitions (12S rDNA : 16S rDNA) and the conflicting signal between the ribosomal genes (12S rDNA) : 16S rDNA) and the protein coding (COI) partitions.

Discussion

Genes Are Evolving Differently

In the conditional data combination procedure used here (Bull *et al.,* 1993; Rodrigo *et al.,* 1993; Chippindale and Wiens, 1994; de Queiroz *et al.,* 1995; Huelsenbeck *et al.,* 1996), the data are divided into partitions, each independent partition is tested for homogeneity, combinable partitions are pooled, and a tree is constructed. Partitioning data (1) improves clarity in examining evolutionary forces acting on individual partitions, (2) facilitates critical data exploration, and (3) permits critical examination of the methods used for presenting data (Ballard *et al.,* 1998).

Results from these data indicate that evolutionary forces are acting in a similar fashion in the 12S- and 16S rDNA partitions, but that the COI genes may be evolving differently (Table 4). Three reasons why gene or gene regions may appear to evolve differently have been suggested (Ballard *et al.,* 1998). First, the phylogenetic signal in the partition may be swamped by homoplasy. Second, methods for investigating whether partitions should be combined may be inadequate. Finally, distinct and conflicting processes may be operating. The first point was addressed by testing for phylogenetic structure, and was rejected because each partition was demonstrated to have phylogenetic structure. Testing for homogeneity showed that the ribosomal partitions (12S rDNA(11) : 16S rDNA(11)) are combinable under the criteria of the ILD test (Table 4). Likewise, the COI partitions were found to be congruent and combinable with each other: (Analysis 1) COI12(11) : COI3(11) and (Analysis 2) COI12(18) : COI3(18) : prot.

Silent substitutions in protein-coding genes are much more frequent than replacement substitutions; thus, the third codon positions tend to become randomized quickly and convey very little information about distant phylogenetic relationships such as those being tested here. Because the fossil record of isopods is poor, their age is speculative but clearly ancient. Crustaceans appear in the Cambrian (550 mya), with terrestrial arthropods appearing in the Silurian (425 mya). The oldest isopod fossils are known from the Paleozoic Carboniferous Period 355 mya.

Additionally, base composition of the third codon position can vary systematically between some species, indicating that it can be subject to at least a moderately strong selective force that is different in different lineages (Swofford *et al.,* 1996). Applying 6P step matrices improved congruence of 12S rDNA : COI3 partitions and decreased congruence of 16S rDNA : COI12 and 16S rDNA : COI3 partitions, a difference attributed to the performance of the 6P step matrices and the method's ability to estimate the cost of the transformation from one character state to another and the possible effect of nucleotide base composition bias. Wetzer (2001) reports a roughly 7% A+T bias for all three COI codon positions. This bias was nearly eliminated when third positions were removed, yet Ts were favored over As. Ribosomal genes (12S-, 16S rDNA) had nearly equal A+T composition. Overall 12S- and 16S rDNA had about 62% and 58% A+T bias, respectively. Except that these genes produce functionally different products (components for ribosome building and participation in the electron transport chain), differences in the phylogenetic patterns revealed by isopod ribosomal and cytochrome oxidase genes remain unexplained. It is, however, not a unique result. In a study of leptodactylid frogs, the COI topology is likewise distinct from the topologies created by ribosomal, morphology, allozymes, and call partitions (see Cannatella *et al.,* 1998). Nonrandom patterns of mutations in repeated mitochondrial DNA sequences have been reported in cyprinid fish, and Brougthton *et al.* (1998) suggested that nonrandom homoplasy in molecular data may be a widespread phenomenon which currently is not recognized. They attribute the unusual character distribution to be the result of a yet unrecognized deterministic mechanism of DNA mutation, and point out that the existence of such deterministic mutation processes could skew the distribution of homoplastic characters to suggest spurious phylogenetic hypotheses.

Differing Alignments Have Little Effect

Assumptions about gap cost, nucleotide substitution cost, and alignment order are fundamental to alignment algorithms. Changes in these parameters can produce radically different alignment outcomes. Several methods to overcome subjective criteria used to distinguish regions of excessive variation (i.e., regions of ambiguous alignment) from regions of acceptable variation have been suggested. Gatesy *et al.* (1993) favor removing nucleotide positions that do not align consistently over a variety of alignment parameters, whereas Wheeler *et al.* (1995) put forth a method they call "elision." In this method they propose "agglomerating several multiple alignments into a single grand alignment," a technique not widely used, and unfortunately misnamed, as elision means removal rather than the concatenation their technique performs.

Morrison and Ellis (1997), in an extensive review of multiple-alignment procedures, attributed a greater portion of topological variation to differences in alignments than to differences in phylogenetic inference methods. They refute the assumption that similar alignments produce similar trees. In contrast, I found that eliminating the effects of three different gap open penalties (15, 12, and 10) by excluding positions where two alignments, one alignment, or all alignments differed had a negligible affect on the topology. Furthermore, applying the concatenation technique of Wheeler *et al.* (1995) also had negligible effect.

Character Weighting Protein Coding Regions Has No Effect

In a technique similar to concatenating multiple sequence alignments (described above), Agosti *et al.* (1996) proposed combining nucleic acid and translated amino acid coded character states into a single matrix for phylogenetic analysis. The authors suggest three possible outcomes of such combinations: (1) nucleotide and amino acid character sets may be entirely congruent with respect to the information they convey about the relationships, (2) one character set may contain no information about the relationships, or (3) the two character sets are entirely incongruent with respect to phylogenetic hypotheses concerning the taxa being examined. In this study nucleotide and amino acid character sets were found to be congruent (Table 4). Separate nucleotide and amino acid and combined nucleotide + amino acid analyses produced similar topologies. As expected, the amino acid partition contributed less phylogenetic information compared to the larger nucleotide data set (55, 343 parsimony informative characters, respectively).

More Taxa Yield Better Estimates Than More Characters

Is it better to add taxa or add characters to improve phylogenetic accuracy? Graybeal (1998) concluded that for a given data set, phylogenetic accuracy improved as the number of taxa increased, i.e., accuracy of the phylogenetic estimate improves with the addition of taxa even if the total number of characters examined remains the same. Graybeal's findings corroborate Kim's (1996) finding that inconsistent internal branches can be made consistent by adding one taxon to each of the two long branches in approximately the basal third of those branches (the exact position depending on the relative branch lengths).

Increasing the percentage of supported nodes within a tree is positively correlated with the number of characters and negatively correlated with the number of taxa. If the purpose is to get a strongly supported tree, it is better to analyze more characters than to investigate more taxa (Bremer *et al.,* 1999). This finding is comforting only if one has confidence that the phylogenetic signal in a data set is accurately reflecting phylogenetic relationships. This is the case for the 12S- and 16S rDNA partitions in this study, but not for the COI partitions. For the three data sets investigated here (Table 3), the addition of taxa increases bootstrap values at nodes, more nodes have bootstrap support greater than 50%, and clade topologies are comparable with the addition of taxa, thus increasing resolution (e.g., Figs. 2, 4).

Morphologically Implausible Results

The COI(18) tree depicts a plausible cymothoid clade (*Lironeca* + *Olencira*) (Fig. 5). However, the greater than 50% bootstrap support for a (*Lironeca* + *Olencira*) plus *C. rudicauda* relationship, as well as the *C. harfordi* + *Sphaeramene* relationship are questionable. The remaining tree topology is implausible as well. Similarly, morphologists would surely

consider the topology of the COI(18) and amino acid data partitions (tree not shown, but similar to Fig. 5) nonsensical, because closely related species of, e.g., phreatoicids and idoteids are distributed across the tree. These results are discussed above (see "Genes Are Evolving Differently") and remain presently unexplained.

Summary and Recommendations

Taxonomic sampling schemes can be reduced to two basic strategies: select taxa within a monophyletic group of interest that will represent the overall diversity of the group. For example, select representatives from two divergent clades in the taxon of interest, taxa purposefully chosen to best represent a taxon's diversity. Select taxa within the monophyletic group of interest that are expected (based on current taxonomy or previous phylogenetic studies) to subdivide long branches in the initial tree (Hillis, 1998).

Increasing taxon sampling of isopods is expected to improve phylogenetic resolution and accuracy. The two *Cirolana* sequences specifically, and Flabellifera sequences in general, appear to have greater substitution rates for all three genes and exhibit greater sequence variation than all other isopods examined (Wetzer, 2001). The topology of the tree in Fig. 7, with the *Cirolana* at the base and the remaining Flabellifera derived, is likely an artifact of an increased molecular evolutionary rate in the *Cirolana*, i.e., long branch attraction to the outgroup (*Penaeus*).

Eliminating COI3 partitions decreases resolution and bootstrap support. This effect is attributed in part to the reduced number of characters in the remaining data set, as well as the possible loss of phylogenetic signal. It is not possible to determine if the signal present in third positions accurately reflects phylogenetic history.

Topological incongruence may result from either random or systematic error. In the former case increasing the sample size (i.e., taxon sampling) will eliminate the observed incongruence. In the latter case, the error results from incorrect assumptions in the estimation method. The importance of *a priori* testing of data partitions for congruence is demonstrated by these data and is strongly recommended for all combined analyses.

The phylogeny estimated from the combined 12S rDNA : 16S rDNA 6P parsimony analysis (Fig. 6) places Phreatoicidea as the earliest derived living isopods, and the long-tailed isopod taxa as the derived condition within the Isopoda. In all figures, long-tailed isopods are distinguished from short-tailed isopods with an asterisk. In the 12S rDNA analysis (Fig. 1), asellotans (*Caecidotea*) are ancestral to the phreatoicids, a hypothesis favored by Schmalfuss (1989). The 16S rDNA data sets place the oniscids (*Armadillidium*) at the base, and all three trees show the phreatoicids to be the most derived (Figs. 2, 4). This variation in placement of ancestral and derived isopods suggests that additional higher order characters from slower evolving genes will be needed to more strongly support the deeper nodes of the phylogeny. Bootstrap support is low for the deeper nodes in all analyses, and the hypotheses based on these mitochondrial genes should be judged cautiously and at present inconclusive.

The Flabellifera appear to be the fastest evolving isopods, and improved sampling of additional flabelliferan taxa is recommended for future studies. Specifically, members of the families Aegidae, Corallanidae, and Limnoriidae should to be sampled. Additional genera of Cirolanidae, especially purported ancient groups such as *Bathynomus,* should be included. Like the Cirolanidae, the Sphaeromatidae are extremely speciose and morphologically diverse, and additional taxa should be sampled. Finally, additional Asellota and Oniscidea should be included.

Prospects for a Fully Resolved Isopoda

This phylogenetic analysis of mitochondrial sequences from species representing isopod suborders provides resolution of the youngest (internal) clades; however, gathering ever larger mitochondrial sequence samples from more organisms in the hope that the historical signal will eventually prevail may be futile in this group. Slower evolving nuclear sequences will likely be necessary to separate the basal groups (findings not unlike Flook *et al.,* 1999, for the insect order Orthoptera). Based on other crustacean molecular analyses (summarized in Wetzer, 2001), possible additional gene regions which may be fruitful for higher level isopod phylogeny include the small nuclear ribosomal subunit 18S rDNA and/or the nuclear protein coding EF-1α gene. Another prospect for additional higher order characters may come from char-

acters embedded in sequences, e.g., molecular data such as gene rearrangements.

Mitochondrial genes corroborate isopod clades previously recognized on morphological grounds, e.g., cymothoid fish parasites derived from a cirolanid ancestor. In other instances, these genes propose relationships not previously suggested, e.g., that valviferans may have had a sphaeromatid ancestor, and the possibility of a oniscid–sphaeromatid link. Boundaries of applicability of these genes at this taxonomic level have been clarified, and new data for addressing generic, familial, and subordinal relationships provided for a group comprising one quarter of all crustaceans.

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