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Molecular phylogeny of the Calanoida (Crustacea: Copepoda)

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ABSTRACT

The order Calanoida includes some of the most successful planktonic groups in both marine and freshwater environments. Due to the morphological complexity of the taxonomic characters in this group, subdivision and phylogenies have been complex and problematic. This study establishes a multi-gene molecular phylogeny of the calanoid copepods based upon small (18S) and large (28S) subunits of nuclear ribosomal RNA genes and mitochondrial encoded cytochrome *b* and cytochrome *c* oxidase subunit-I genes, including 29 families from 7 superfamilies of the order. This analysis is more comprehensive than earlier studies in terms of number of families, range of molecular markers, and breadth of taxonomic levels resolved. Patterns of divergence of ribosomal RNA genes are shown to be significantly heterogeneous among superfamilies, providing a likely explanation for disparate results of previous studies. The multigene phylogeny recovers a monophyletic Calanoida, as well as the superfamilies Augaptiloidea, Centropagoidea, Bathypontioidea, Eucalanoidea, Spinocalanoidea and Clausocalanoidea. The phylogeny largely agrees with previously-published morphological phylogenies, including e.g., enlargement of the Bathypontioidea to include the Fosshageniidae.

1. Introduction

The order Calanoida comprises approximately 2000 species of marine and freshwater copepods inhabiting pelagic, benthic and benthopelagic environments (Huys and Boxshall, 1991). Calanoid copepods, thought to have benthic or benthopelagic origins, are the most successful colonizers of the pelagic realm (Bradford-Grieve, 2002). Ecologically, they play essential roles in the trophic webs of diverse marine ecosystems, including highly productive upwelling systems and oligotrophic subtropical ocean gyres (Mauchline, 1998). The order has been taxonomically well-defined for many years (e.g., Giesbrecht, 1893; Sars, 1901), but subdivision has been problematical due to the wide range of characters (see review by Bradford-Grieve et al., 2010). The currently accepted taxonomic organization of the Calanoida is one established by Andronov (1974) and modified by Fosshagen and Iliffe (1985) and Park (1986). Ten superfamilies are recognized: Pseudocyclopoidea Giesbrecht, 1893; Epacteriscoidea Fosshagen, 1973; Augaptiloidea Sars, 1905; Centropagoidea Giesbrecht, 1893; Megacalanoidea Sewell, 1947; Bathypontioidea Brodsky, 1950; Eucalanoidea Giesbrecht, 1893; Ryocalanoidea Andronov, 1974; Clausocalanoidea Giesbrecht, 1893; and Spinocalanoidea Vervoort, 1951. Andronov (1974) also characterized an hypothesized calanoid ancestor using the set of morphological characters upon which his phylogeny was based (Fig. 1). More recently, Bradford-Grieve et al. (2010) published a phylogenetic analysis based upon morphological characters that tested the presumed monophyly of calanoid superfamilies and characterized relationships among species, genera and families.

Our understanding of the evolutionary history of the calanoid copepods has been markedly limited by the lack of a fossil record and the complex effects of environmental variation over the geological history of the oceans. The only published attempt to reconstruct the history of the order is by Bradford-Grieve (2002), who examined environmental, morphological and physiological characteristics; she hypothesized that representatives of two superfamilies, the Augaptiloidea and Centropagoidea, entered the pelagic realm during the Devonian; these groups occupied the oxygenated water column and radiated into deep waters during the late Carboniferous. Bradford-Grieve (2002) further hypothesized that other calanoid superfamilies radiated later, during the Permian; these groups may have escaped the anoxic conditions of the deep ocean during the Jurassic–Cretaceous–Tertiary periods and the Cretaceous–Tertiary extinction event by inhabiting shallow oxygenated waters or disoxic environments of deep benthopelagic habitats. The diatom radiation during the Jurassic–Tertiary period may have driven speciation by allowing expanded herbivory within many calanoid lineages (Bradford-Grieve, 2002).

Here we use a phylogenetic analysis of molecular markers to test current hypotheses of relationships within the order

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Calanoida. To date, several studies have used nuclear and/or mitochondrial genes to clarify relationships of calanoids at various taxonomic levels. Nuclear, ribosomal RNA (rRNA) genes were used to examine relationships from species to superfamily levels (Braga et al., 1999; Bucklin et al., 2003; Figueroa, 2011; Marszalek et al., 2009; Thum, 2004). The results of these studies differed, especially for relationships among calanoid superfamilies, and also did not agree with the recent morphological phylogenetic analysis by Bradford-Grieve et al. (2010). Published studies have also used DNA sequence variation of mitochondrial genes to resolve relationships among calanoid families, genera, species, sub-species, and populations (Bucklin and Frost, 2009; Caudill and Bucklin, 2004; Figueroa, 2011; Goetze, 2003, 2005; Goetze and Bradford-Grieve, 2005; Machida et al., 2006; Makino and Tanabe, 2009).

In this study, we analyzed DNA sequences of four genes: the 5′ regions of the nuclear large (28S) and small (18S) subunit rRNA; and mitochondrial genes encoding cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (Cyt *b*). Thirty species representing 29 of 44 families and 7 of 10 superfamilies of the order Calanoida were chosen for our phylogenetic analyses. Our selection of genes was based on the numerous studies using nuclear ribosomal genes to resolve phylogenetic relationships within and among diverse taxa (e.g., Mallatt and Giribet, 2006; Mallatt et al., 2004; Toon et al., 2009). The addition of faster-evolving mitochondrial genes was done to resolve relationships between recently diverged copepod taxa, as shown by previous studies (e.g., Bucklin and Frost, 2009; Figueroa, 2011; Machida et al., 2006) and deep nodes of other groups (Cameron et al., 2006; Gatesy et al., 1999). Our multi-gene molecular phylogenetic analysis of calanoid copepods is more comprehensive than earlier studies in terms of numbers of families, range of molecular markers, and breadth of taxonomic levels resolved. In addition, our molecular analyses are explicitly compared with a recently-published morphological phylogenetic analysis by Bradford-Grieve et al. (2010) and also with earlier studies based on diverse characters, including myelination of nerve axons (see e.g., Lenz et al., 2000).

2. Materials and methods

2.1. Samples election and DNA extraction

The material analyzed for this study included vouchered specimens and DNA stored in the Census of Marine Zooplankton (CMarZ) archives located at the Department of Marine Sciences, University of Connecticut. Individual specimens for the analysis were identified to species by CMarZ collaborators according to current species concepts. Species were selected to represent as many calanoid copepod families as possible. When available, species that are basal representatives of each family were chosen. Collection information for each specimen, metadata and other characteristics of extracted individuals can be found in GenBank records (Acc. Nos. HM997023–HM997083, HQ150023–HQ150081).

Zooplankton samples were preserved in 95% ethanol, which was changed after 24 h. DNA extractions were carried out using the DNeasy Blood & Tissue Kit (QIAGEN) from whole individuals, with an elution volume of 50–300 μl in AE buffer. DNA of two species, *Hyperbionyx athesphatos* (Bradford-Grieve, 2010) and *Bathycalanus princeps*, was extracted from two legs of each individual, due to the rarity of both species. In these cases, the kit used was QIAamp DNA Mini Kit (QIAGEN) and 25 μl was used for the final elution. All DNA samples were stored at −20 °C.

DNA was analyzed from species corresponding to 29 of 44 families of the order Calanoida, including seven superfamilies (Table 1). The Scolecitrichidae (genera *Scolecithrix* and *Scolecitrichopsis*) was represented by more than one genus due to unresolved taxonomic

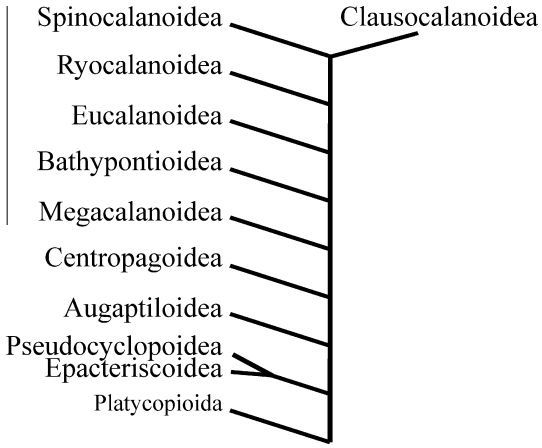

Fig. 1. Phylogeny of the Calanoida, with Platycopioida included as an outgroup. After Andronov (1974) and Park (1986).

complexity. Two genera were analyzed within the Eucalanoidea, *Rhincalanus* and *Subeucalanus*, representing two currently-accepted families, Rhincalanidae and Eucalanidae (Boxshall and Halsey, 2004; Razouls et al., 2005–2010), although the status of these families is in debate (Goetze, 2003). Representatives of the Ryocalanoidea (Ryocalanidae), Pseudocyclopoidea (Boholinidae and Pseudocyclopidae) and the Epacteriscoidea (Epacteriscidae and Ridgewayiidae) were not included in this analysis because of the unavailability of specimens. Additional 4-gene phylogenetic analyses were done, including 18S rRNA sequences for *Exumella mediterranea* (Ridgewayiidae, GenBank Acc. No. AY629259) and *Pseudocyclops* sp. (Pseudocyclopidae, GenBank Acc. No. AY626994); no data were available for the other three genes for these taxa. Eleven families for which we have other superfamily representatives were not included (Table 1).

2.2. Molecular analysis

Genes encoding the nuclear large (28S) and small (18S) subunits ribosomal RNA (rRNA) and the mitochondrial proteins cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (Cyt *b*) were used for phylogenetic analyses. The PCR and sequencing primers used for each gene, the length of the amplified regions, and the annealing temperatures used for each set of primers are specified in Table 2. PCR amplifications were performed in a total volume of 25 μl, including 5 μl of 5× Green GoTaq® Flexi Buffer, 2.5 μl of 25 mM $MgCl_2$, 1 μl of dNTPs (final concentration 0.2 mM each), 1 μl of each primer (10 mM), 0.75 units of GoTaq® Flexi DNA Polymerase (Promega) and 3 μl of DNA sample. PCR products were checked by electrophoresis on a 1% agarose/TBE gel; positive results were purified using UltraClean® PCR Clean-Up Kit (Mo Bio).

Both strands of the purified PCR product were sequenced using the same set of primers as in the original amplification and Big Dye Terminator Ver. 3.1 (Applied Biosystems Inc., ABI), and run on an ABI 3130 Genetic Analyzer capillary DNA sequencer. Sequences were edited and both strands were compared by eye using MEGA Ver. 4.1 (Tamura et al., 2007). The lengths of the sequences obtained are specified in Table 2.

2.3. DNA sequence variation

Alignments of the four genes were carried out separately in MAFFT Ver. 6.7 (Katoh and Toh, 2008), under the L-INS-i option, that uses a local pair-wise alignment with the affine gap cost (Katoh and Toh, 2008). Maximum Likelihood (ML) based pair-wise genetic distances for all sequences of the four genes were

Table 1
Taxon and gene sampling of Calanoid superfamilies analyzed for this study. Families and superfamilies assignations follow Boxshall and Halsey (2004) and Razouls et al. (2005–2010).

Superfamily	Family	Species	18S	28S	COI	Cyt *b*
Augaptiloidea	Arietellidae	*Paraugaptilus buchani*	x	x	x	x
	Augaptilidae	*Haloptilus longicornis*	x	x	x	x
	Discoidae	–				
	Heterorhabdidae	*Paraheterorhabdus compactus*	x	x	x	x
	Hyperbionychidae	*Hyperbionyx athesphatos*	x	x		x
	Lucicutiidae	*Lucicutia flavicornis*	x	x	x	x
	Metridinidae	*Metridia effusa*	x	x	x	x
	Nullosetigeridae	*Nullosetigera auctiseta*	x	x	x	x
Centropagoidea	Acartiidae	–				
	Candaciidae	*Candacia simplex*	x	x	x	x
	Centropagidae	*Centropages violaceus*	x	x	x	x
	Diaptomidae	–				
	Fosshageniidae	*Temoropia mayumbaensis*	x	x	x	x
	Parapontellidae	–				
	Pontellidae	*Pontellina plumata*	x	x	x	x
	Pseudodiaptomidae	–				
	Sulcanidae	*Sulcanus conflictus*	x	x	x	x
	Temoridae	*Temora discaudata*	x	x	x	x
	Tortanidae	*Tortanus gracilis*	x	x	x	x
Bathypontioidea	Bathypontiidae	*Temorites brevis*	x	x	x	x
Megacalanoidea	Calanidae	*Calanus helgolandicus*	x	x	x	x
	Megacalanidae	*Bathycalanus princeps*	x	x	x	
	Paracalanidae	*Paracalanus parvus*	x		x	x
Eucalanoidea	Eucalanidae	*Subeucalanus pileatus*	x	x	x	x
	Rhincalanidae	*Rhincalanus cornutus*	x	x	x	x
Spinocalanoidea	Spinocalanidae	*Spinocalanus abyssalis*	x	x	x	x
	Arctokonstantinidae	*Foxtonia barbatula*	x	x	x	x
Clausocalanoidea	Aetideidae	*Aetideus armatus*	x	x	x	x
	Clausocalanidae	*Clausocalanus arcuicornis*	x	x	x	x
	Diaixidae	*Diaixis hibernica*	x	x	x	x
	Kyphocalanidae	–				
	Euchaetidae	*Euchaeta media*	x	x	x	x
	Mesaiokeratidae	–				
	Parkiidae	–				
	Phaennidae	*Phaenna spinifera*	x	x	x	x
	Pseudocyclopiidae	–				
	Rostroclanaidae	–				
	Scolecitrichidae	*Scolecithrix bradyi*	x	x	x	x
		Scolecitrichopsis sp.	x	x	x	x
	Stephidae	–				
	Tharybidae	*Tharybis groendlandicus*	x	x	x	x

Table 2
PCR and sequencing primer names and sequences; lengths of the PCR product and the portion analyzed for this study; annealing temperature (AT). Abbreviations are: forward primer (F); reverse primer (R); base-pairs (bp).

Gene	Primer name, sequence	Product (Analyzed)	AT
18S	18SE (F), CTGGTTGATCCTGCCAGT (Hillis and Dixon, 1991) 18SL (R), CACCTACGGAAACCTTGTTACGACTT (Hamby and Zimmer, 1988)	~1650 bp (934 bp)	52 °C
28S	28S-F1a, GCGGAGGAAAAGAAACTAAC (Ortman, 2008) 28S-R1a, GCATAGTTTCACCATCTTTCGGG (Ortman, 2008)	~850 bp (716 bp)	50 °C
COI	LCO1490 (F), GGTCAACAAATCATAAAGATATTGG (Folmer et al., 1994) HCO2198 (R), TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994) Cop-COI-2189R, GGGTGACCAAAAAATCARAA (Bucklin et al., 2010)	701–710 bp (548 bp)	45 °C
Cyt *b*	UCYTB151F, TGTGGRGCNACYGTWATYACTAA (Merritt et al., 1998) UCYTB270R, AANAGGAARTAYCAYTCNGGYTG (Merritt et al., 1998)	~360 bp (328 bp)	50 °C

calculated separately in RAxML Ver. 7.2.6. (Stamatakis, 2006b). Vector analysis (Sirovich et al., 2009, 2010) was used for the graphical representation of the genetic distances. Statistical analysis was done using SPSS Ver. 15.0. Differences within and between superfamilies were tested using *t*-tests with equal or unequal variances, as indicated by the two-sample *F*-test for variances. The Mann–Whitney U-test was used was for data with non-normal distributions. Pair wise differences between COI and Cyt *b* were analyzed using paired *t*-tests in order to examine disagreements between the two mitochondrial genes. Comparisons were made both for the entire dataset and after removal of the highly divergent sequences of *Phaenna spinifera* (Phaennidae) and *Scolecithrix bradyi* (Scolecitrichidae).

2.4. Phylogenetic analysis

Two species representing different orders of Copepoda were chosen as outgroup taxa: a cyclopoid, *Paracyclopina nana* (GenBank Acc. Nos. NC012455, FJ214952) and a siphonostomatoid, *Hyalopon-*

tious typicus (for COI, Acc. No. FJ602509; all the other gene sequences are new data obtained for this study).

Partitioned phylogenetic analyses for 18S and 28S rRNA and the four genes together were carried out using Maximum Likelihood (ML) and Bayesian Inference (BI). The Maximum Likelihood analyses were computed using RAxML Ver. 7.0.3 (Stamatakis et al., 2007), under the GTRGAMMA option (i.e., GTR model of nucleotide substitution with the Γ model of rate heterogeneity) and a complete random starting tree (option -d) for the 10,000 bootstrap replicates (Pattengale et al., 2009). The Best-Known Likelihood tree search (200 inferences) was performed under GTRMIX, in which the tree inference is carried out under GTRCAT (GTR approximation with optimization of individual per-site substitution rates and classification of those individual rates into the number of rate categories specified; Stamatakis, 2006a) and a completely random starting tree. The final tree topology was evaluated under GTRGAMMA to yield stable likelihood values.

The Bayesian Inference was carried out using the MPI version of MrBayes (Altekar et al., 2004; Ronquist and Huelsenbeck, 2003). MrModeltest Ver. 2.3 (Nylander, 2004) was used to identify and select the appropriate models of sequence evolution. Each dataset was run for 3000,000 generations with a sample frequency of 1000 generations. The first 500 trees were discarded as burn-in, so 2500 trees were accepted from each run. Clade support is shown on the nodes of the trees as the Bayesian Posterior Probability (BPP) when BPP > 0.90.

Analyses were done with the unmodified sequences for the two nuclear ribosomal genes separately and for concatenated

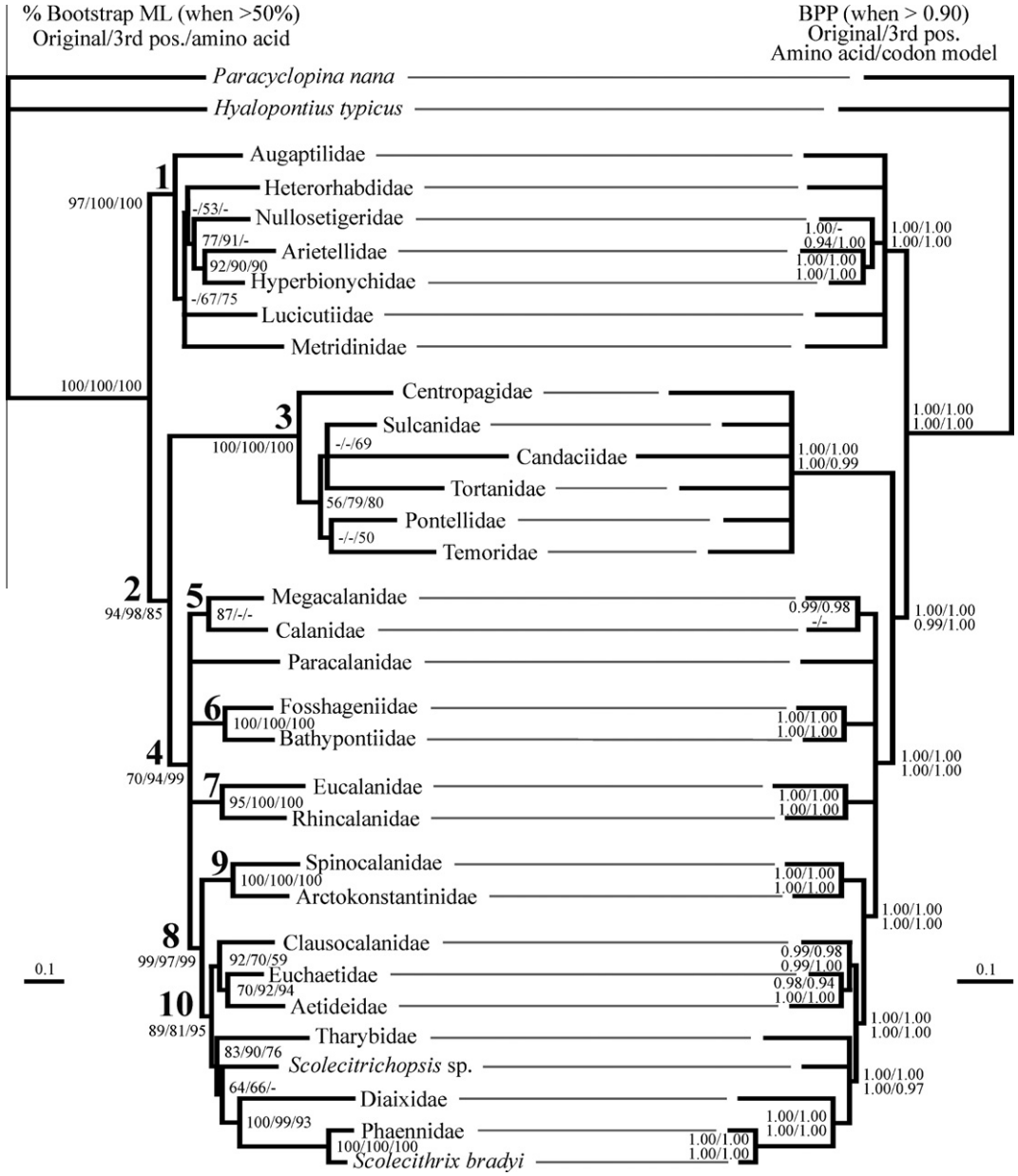

Fig. 2. Phylogram of families of the copepod Order Calanoida and outgroups. Left: RAxML Maximum Likelihood tree, with nodes indicating percentage bootstrap recovery under three analyses: unmodified sequences (original); exclusion of all third codon positions in the protein-codifying genes (3rd pos.); and translation of protein-codifying genes to amino acid sequences (amino acid). The tree topology and the branch lengths shown correspond to the original sequence analysis. Right: Bayesian Inference tree, with numbers in nodes indicating the Bayesian Posterior Probability under the same three analyses (original, 3rd pos., amino acid), plus a fourth analyzing in-frame triplets of nucleotides under a model of codon change (codon model). Branch lengths shown correspond to the unmodified original sequence analysis. For details, see Section 2. For superfamily groupings, see Table 1.

sequences of the four genes. In order to reduce the variability in the mitochondrial genes due to saturation, rapid sequence evolution, and codon bias, two additional analyses were performed:

1. All 3rd codon position bases of COI and Cyt *b* were removed from analysis, based on the assumption that they are likely to contain more homoplasy and synonymous substitutions that can confound phylogenetic reconstructions.
2. The two mitochondrial genes were translated into their amino acid sequences to reduce the effects of synonymous substitutions and rapid sequence evolution biases. The best-fit amino acid substitution model for COI and Cyt *b* was calculated using a Perl script (http://icwww.epfl.ch/~stamatak/index-Dateien/software/ProteinModelSelection.pl). The best models obtained were RTREVF for COI and BLOSUM62F for Cyt *b* using empirical base frequencies ("F" appendix). These models were implemented both in RAxML and MrBayes.

Since recent studies have questioned the performance of amino acid models compared to nucleotide and codon models (Regier et al., 2010; Seo and Kishino, 2009), an extra run was performed in the Bayesian framework with nucmodel = codon under GTR for COI and Cyt *b*. In this case, in-frame triplets of nucleotides were analyzed directly under a model of codon change. This option was not available in RAxML.

Leaf stabilities (Thorley and Wilkinson, 1999) were calculated with the tree analysis program Phyutility Ver. 2.2 (Smith and Dunn, 2008). The most unstable taxa, *Paracalanus parvus* (Paracalanidae) and *Rhincalanus cornutus* (Rhincalanidae), were then removed for additional analyses to examine their influence on the topology of the trees.

3. Results

3.1. Phylogenetic analysis

Phylogenetic analysis using all four gene sequences yielded a monophyletic clade including all 29 analyzed calanoid families (Fig. 2). Several monophyletic clades within this group were well-supported:

Clade 1 comprises the Augaptilidae, Lucicutiidae, Metridinidae, Heterorhabdidae, Nullosetigeridae, Arietellidae and Hyperbionychidae. It is sister to the remaining families in our analyses for which there were data. Within Clade 1, the Augaptilidae is sister to the remaining families, although only the relationships among the families Nullosetigeridae, Hyperbionychidae and Arietellidae were resolved and supported in most of the analyses. Relationships among families within Clade 1 were unchanged in analyses using the complete dataset and modifications to mtDNA sequences by removing 3rd codon positions and translating to amino acids.

Clade 2 includes all the remaining families in our analysis belonging to the Order Calanoida.

Clade 3 includes the families Centropagidae, Temoridae, Pontellidae, Tortanidae, Candaciidae and Sulcanidae. It is sister to the remaining taxa of Clade 4. Among the families forming this clade, the Centropagidae is sister to the remaining families, whose relationships are unresolved.

Clade 4 is a monophyletic and strongly supported clade (>89% bootstrap, 1.00 BPP) that comprises the rest of the families, which are divided into four lineages: Clades 5–7 and the Paracalanidae.

Clade 5 contains the Megacalanidae and Calanidae. This clade was not consistently supported by bootstrap value in the various analyses. Furthermore, the different analytical methods yielded different clustering patterns, including Paracalanidae (e.g., the Megacalanidae clustered with the Paracalanidae in the amino acid option analysis). The Leaf Stability analysis revealed that the Paracalanidae was one of the more unstable taxa; exclusion of this family from the phylogenetic analysis increased the support and consistency among the different methods for Clade 5. Another consequence of the removal of the unstable Paracalanidae and Rhincalanidae was an increase in bootstrap support (from <50% to 59%) of a clade containing Clades 5 and 6.

Clade 6 contains the Fosshageniidae and Bathypontiidae with 100% bootstrap support (1.00 BPP).

Clade 7 includes the families Eucalanidae and Rhincalanidae. This clade was consistently retrieved from all analyses, although the relationships with other clades were unresolved. The Leaf Stability analysis indicated the species belonging to this clade were unstable; leaving out either of these species resulted in increased support for the surrounding clades.

Clade 8 comprises two clades: one containing the Spinocalanidae and Arctokonstantinidae (Clade 9) and another (Clade 10) containing the remaining families in this analysis (Fig. 2).

Clade 9 was resolved as sister to clade 10 with analyses including the mitochondrial genes, but was not evident when only the nuclear ribosomal genes are analyzed.

Clade 10 is a monophyletic clade containing the Clausocalanidae, Euchaetidae, Aetideidae, Tharybidae, *Scolecitrichopsis* sp., Diaixidae, Phaennidae and *Scolecithrix bradyi*. Two lineages were

Table 3
Range of Maximum Likelihood genetic distances (GTR model, gamma distribution) within and between superfamilies. In parenthesis, average ± SD.

Taxon comparison	18S	28S	COI	Cyt *b*
Within Augaptiloidea	0.014–0.075 (0.037 ± 0.019)	0.073–0.165 (0.108 ± 0.025)	0.665–1.027 (0.846 ± 0.121)	0.783–1.315 (0.997 ± 0.149)
Within Centropagoidea	0.038–0.127 (0.072 ± 0.022)	0.165–0.637 (0.357 ± 0.138)	0.449–1.369 (0.751 ± 0.242)	0.427–0.945 (0.672 ± 0.174)
Within Bathypontioidea[a]	0.027	0.092	0.597	0.538
Within Megacalanoidea	0.025–0.086 (0.059 ± 0.031)	0.090	0.659–0.741 (0.688 ± 0.046)	0.582
Within Eucalanoidea	0.029	0.123	1.149	0.952
Within Spinocalanoidea	0.018	0.123	0.831	0.715
Within Clausocalanoidea	0.002–0.036 (0.018 ± 0.010)	0.043–0.178 (0.098 ± 0.041)	0.002–7.446 (3.255 ± 2.730)	0.529–2.530 (1.202 ± 0.0.631)
Within Clausocalanoidea (excluding Phaennidae and *Scolecithrix bradyi*)			0.572–1.757 (1.054 ± 0.415)	
Between superfamilies	0.021–0.238 (0.105 ± 0.050)	0.112–0.843 (0.339 ± 0.187)	0.002–7.950 (1.615 ± 1.660)	0.061–2.797 (1.063 ± 0.385)
Between superfamilies (excluding Phaennidae and *Scolecithrix bradyi*)			0.002–2.958 (1.026 ± 0.404)	

[a] Bathypontiidae + Fosshageniidae.

resolved; one including the Clausocalanidae, Aetideidae and Euchaetidae, with all other families comprising a separate clade.

When 18S rRNA sequences from GenBank for two basal families (Epacteriscidae and Pseudocyclopidae) were added to the analysis, the clade they formed appeared as sister and basal to all the other calanoid families. The missing data for the other genes for these taxa diminished the bootstrap support for many nodes throughout the phylogenetic tree (results not shown here).

3.2. DNA sequence variation

Genetic diversity of the nuclear 18S and 28S rRNA genes varied significantly among the superfamilies (Table 3; Fig. 3). There were significantly higher levels of diversity within the Centropagoidea than within the Augaptiloidea or Clausocalanoidea ($p < 0.001$ for both comparisons). There was no difference between Augaptiloidea and Clausocalanoidea for 28S rRNA ($p > 0.05$), although 18S rRNA diversity was least within the Clausocalanoidea ($p < 0.001$). Diversity within other superfamilies could not be evaluated, since only two species were analyzed for each. Distances within superfamilies were lower than between superfamilies for both 18S and 28S rRNA by a Mann–Whitney U-test ($p < 0.001$ for both genes).

Pairwise genetic distances for Cyt *b* (Table 3; Fig. 4) were lower within the Centropagoidea than the Augaptiloidea ($p < 0.05$) or Clausocalanoidea ($p < 0.001$); no significant differences were found between the last two superfamilies ($p > 0.1$). In all cases, Cyt *b* distances between species of different superfamilies were significantly larger than those between species of the same superfamily (U-test, $p < 0.01$). For COI, the Clausocalanoidea showed larger genetic distances among species than other superfamilies ($p < 0.01$ for each comparison; Table 3; Fig. 4), primarily due to the highly divergent sequences of *Phaenna spinifera* (Phaennidae) and *Scolecithrix bradyi* (Scolecitrichidae). When both species were removed, no significant differences were found and the maximum distances decreased to 2.958. Within superfamily distances were larger for COI than Cyt *b* ($p = 0.001$ by paired *t*-test, correlation $r = 0.211$ n.s.); no significant differences were found between the two mitochondrial genes after removing *P. spinifera* (Phaennidae) and *S. bradyi* (Scolecitrichidae; $p > 0.5$; correlation $r = 0.815$, $p < 0.001$).

4. Discussion

Molecular phylogenetic analysis based upon two nuclear (28S and 18S rRNA) and two mitochondrial (COI and Cyt *b*) genes clearly and consistently showed the calanoid copepods to be monophyletic (Fig. 2). The analysis resolved 6 of the 7 included superfamilies proposed by Andronov (1974) and Park (1986): Augaptiloidea (Clade 1), Centropagoidea (Clade 3), Bathypontioidea (Clade 6),

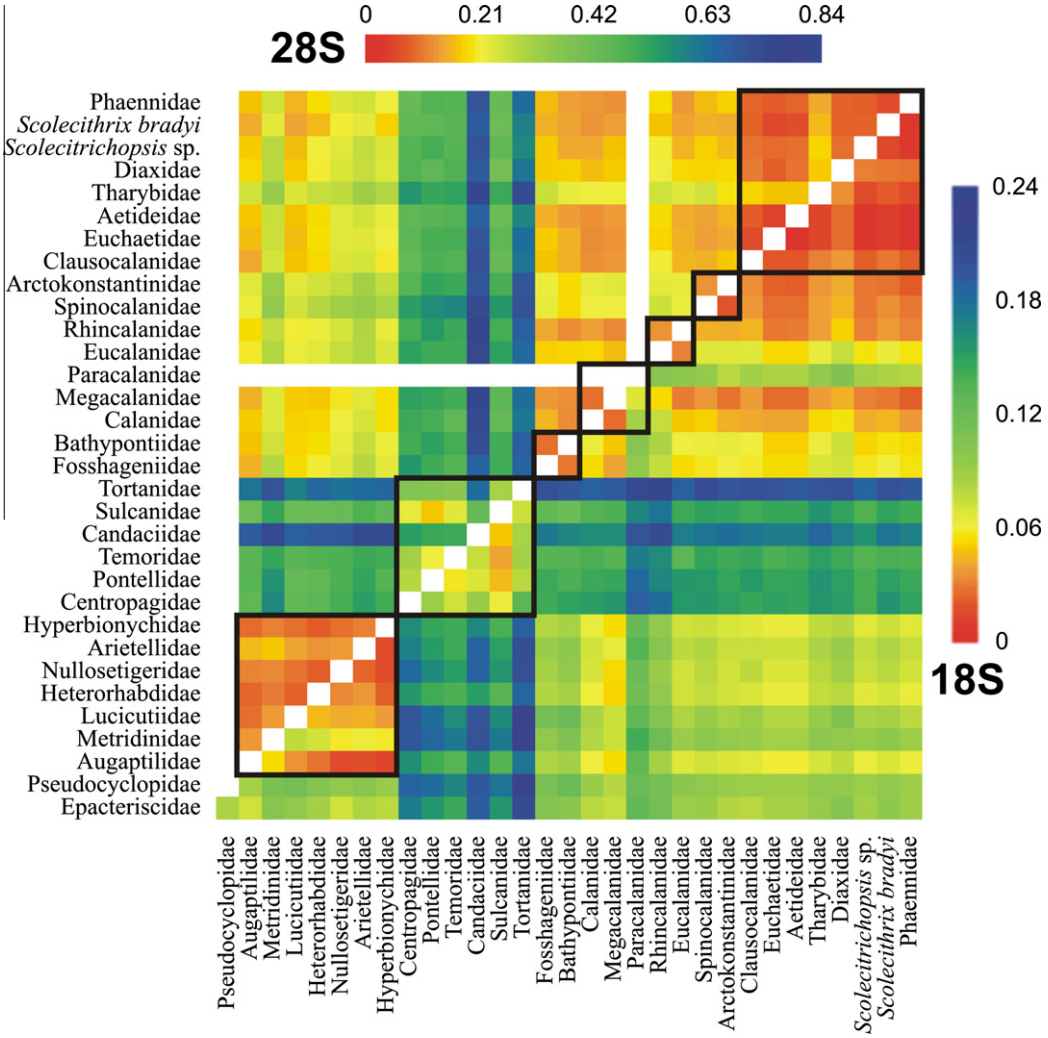

Fig. 3. Vector analysis of pair-wise Maximum Likelihood genetic distances between species for the nuclear 18S and 28S ribosomal RNA genes. Distances between taxa of the same superfamilies are outlined by black squares.

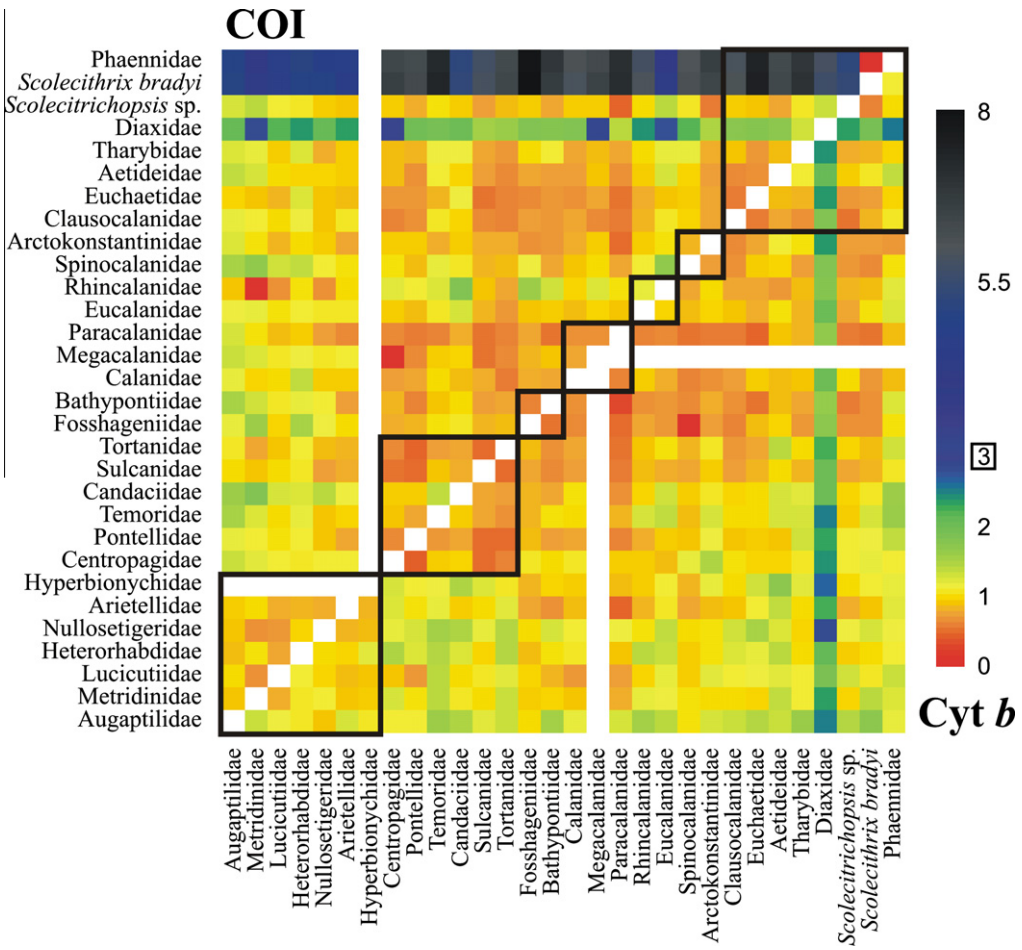

Fig. 4. Vector analysis of pair-wise Maximum Likelihood genetic distances between species for the mitochondrial COI and Cyt *b* genes. Distances between taxa of the same superfamilies are outlined by black squares. The maximum COI pair-wise distance for analyses omitting the divergent Phaennidae and *S. bradyi* equaled 2.958 (indicated by box around 3 in the scale bar).

Eucalanoidea (Clade 7), Spinocalanoidea (Clade 9), and Clausocalanoidea (Clade 10). All these superfamilies were resolved as monophyletic. Only one superfamily, Megacalanoidea, was not well-supported in this analysis (Fig. 2). Three other superfamilies (Epacteriscoidea, Pseudocyclopoidea and Ryocalanoidea) proposed by Andronov (1974) and Park (1986) were not analyzed.

These results supported conclusions based on morphological characters by Huys and Boxshall (1991) and Ho (1990) regarding the systematic integrity of the superfamilies. Our results were also congruent with a recent morphological phylogenetic analysis by Bradford-Grieve et al. (2010; Fig. 15), as well as earlier phylogenetic studies by Andronov (1974) and Park (1986). Some conclusions of the morphological phylogeny were strengthened in the current molecular analysis: a proposal by Bradford-Grieve et al. (2010) to include the Fosshageniidae within the Bathypontioidea had 100% bootstrap support here. Similarly, the Hyperbionychidae was placed terminally in the Augaptiloidea in both molecular and morphological analyses. Comparisons between our molecular phylogenetic results and recent morphological analyses are examined in detail in the following sections.

4.1. Genetic distances

Analysis of DNA sequence variation showed 18S rRNA to be more conserved than 28S rRNA; both nuclear ribosomal RNA genes were much more conserved than the mitochondrial genes COI and Cyt *b* (Table 3, Figs. 3 and 4). These findings are typical of the broad range of taxa analyzed previously (Mallatt et al., 2004; Schwentner et al., 2009; Williams et al., 2010). Our studies revealed marked variation in rates of evolution of these genes among four calanoid superfamilies analyzed for numerous species. In particular, larger distances were found among species within the superfamily Centropagoidea for the nuclear ribosomal RNA genes. This rate acceleration might be related to the characteristic habitats of these families of copepods, which typically occur in neritic, estuarine, and freshwater habitats of limited extent (Huys and Boxshall, 1991); an exception is the Candaciidae, which may be secondarily adapted to the open ocean. The Centropagoidea may thus be exposed to more frequent allopatric speciation events, resulting in smaller effective population sizes, and therefore higher rates of DNA sequence divergence. As noted by Jørgensen et al. (2010), the fixation of substitutions is likely to be more frequent in populations with smaller sizes. Distinctive variability within the Centropagoidea has been noted for morphological (e.g., female genitalia and sexual dimorphism; Barthelemy et al., 1998; Ohtsuka and Huys, 2001), and behavioral characters (e.g., reproductive strategies; Hairston and Bohonak, 1998; Kiørboe, 2006; Lindley, 1992). The high morphological, niche, and physiological diversity within the Centropagoidea may also be reflected in the genetic diversity of ribosomal RNA genes within this superfamily. Alternatively, it is possible that the extant lineages of Centropagoidea may have arisen since the superfamily diverged and survived the Cretaceous–Tertiary extinction event and more recent sea level changes in shallow waters (Bradford-Grieve, 2002). A recent divergence of

surviving lineages of the other superfamilies would explain their observed patterns of low levels of divergences of the nuclear ribosomal RNA genes versus the similarity of distances for the faster-evolving mitochondrial genes among all the superfamilies.

4.2. Effects of genes, taxon sampling, and missing data

Although the reliability of phylogenies based on nuclear ribosomal RNA genes is still in debate (von Reumont et al., 2009; Wägele and Mayer, 2007), these genes have been widely used to resolve phylogenetic relationships among many taxa as single-gene analyses (Jørgensen et al., 2010; Mallatt and Giribet, 2006; Mallatt et al., 2004; Marszalek et al., 2009) or in combination with other genes and/or morphological characters (Braga et al., 1999; Bucklin and Frost, 2009; Giribet et al., 2001; Koenemann et al., 2010; Richter et al., 2007; Toon et al., 2009; Wyngaard et al., 2010). Mitochondrial genes may in some cases lose the deep phylogenetic relationship signal due to saturation and non-phylogenetic signals, but they have been used successfully for phylogenetic studies at various levels (Bucklin et al., 2011; Machida et al., 2006). There is no *a priori* reason to exclude these genes (Cameron et al., 2004; Koenemann et al., 2010) and the noise they contain does not always overwhelm the embedded phylogenetic signal (Wenzel and Siddall, 1999). Furthermore, the addition of more genes may improve the support of phylogenies, even though phylogenies derived from single genes do not match those derived from combined genes (Cameron et al., 2004; Gatesy et al., 1999).

The tree obtained from the analysis of the concatenated 28S and 18S rRNA indicated that these two genes determined the general topology of the tree and resolved the main lineages consistently (not shown). The only node missing in this analysis was the Spinocalanoidea/Clausocalanoidea split. The missing 28S rRNA sequence for the Paracalanidae was probably the source of the instability detected for this taxon and the weak support for the superfamily Megacalanoidea. In contrast, the instability detected for families of Eucalanoidea in the multi-gene phylogeny may have resulted from variable evolution of the two nuclear ribosomal RNA genes. The single-gene phylogenies yielded different relationships for the Eucalanoidea, which appeared to be closely related to Clade 8 (Spinocalanoidea + Clausocalanoidea) based on the 18S rRNA gene tree, but was nested with Clades 5 and 6 in the 28S rRNA tree (not shown). The separate 18S and 28S rRNA gene trees also did not agree in the relationships among Augaptiloidea (Clade 1), Centropagoidea (Clade 3), and Megacalanoidea. The large distances for the species of Centropagoidea for these genes may have generated noise that overwhelmed the phylogenetic signal in the single-gene analyses. This may also explain why our results contradicted those of Braga et al. (1999), which used 28S rRNA as unique marker. Based on analysis of 18S rRNA, Figueroa (2011) suggested that the Ridgewayiidae is a sister group to the Pseudocyclopidae and that this clade is a sister group to the Augaptiloidea. These results are not inconsistent with the topology of the morphology-based tree of Bradford-Grieve et al. (2010). Figueroa (2011) also uniquely placed the Centropagoidea as a sister group to all other calanoid taxa; this conclusion has never previously been suggested (e.g. Andronov, 1974; Bradford-Grieve et al., 2010; Park, 1986). It seems likely that the use of single genes in the above analyses contributed to the disparate results. Our results indicate that – despite the discrepancy between the topologies of the 28S and 18S rRNA single-gene trees – combined analysis of sequence variation of the two genes markedly improved support for the primary nodes of the calanoid phylogeny. This observation caused concern regarding the use of only 18S rRNA sequences for Pseudocyclopidae and Ridgewayiidae; these results are not discussed further.

It is possible that the fast evolutionary rate of the mitochondrial genes might distort the phylogenetic signal. However, inclusion of mitochondrial genes in our analysis increased the support of deeper nodes (e.g., Clades 2 and 4), demonstrating possible hidden support of fast-evolving genes for deep nodes (Gatesy et al., 1999). Other nodes resolved by the mitochondrial genes, but not discriminated by the nuclear ribosomal RNA genes, were those resolving the most recently-diverged superfamilies, Spinocalanoidea (Clade 9) and Clausocalanoidea (Clade 10), and the relationships among families of Clausocalanoidea.

Data gaps resulted in differences in taxon sampling among the four genes and limited our ability to infer the roles of each gene in phylogenetic resolution across the Calanoida. When a sequence for a mitochondrial gene was missing, the superfamily integrity was not altered. For example, the missing COI sequence for the Hyperbionychidae did not compromise the resolution of the superfamily, apparently because the ribosomal RNA genes were phylogenetically informative enough to resolve relationships among these deeper lineages. We could not determine the effect of the missing Cyt *b* sequence for the Megacalanidae, since the missing Paracalanidae 28S rRNA sequence may have had a stronger effect on the tree topology, as well as the structure and cohesion of the superfamily.

4.3. Comparisons between molecular and morphological phylogenetic analyses

Our finding of the monophyly of the calanoid copepods based on the ML and Bayesian analyses of the four genes (Fig. 2) is consistent with the conclusions of Huys and Boxshall (1991) and Ho (1990) based on morphological data. Of the 10 superfamilies proposed by these authors, this analysis recovered all 6 of the 7 superfamilies for which material was available for. The lack of resolution of the Megacalanoidea may have been due to the missing 28S rRNA sequences for the Paracalanidae (see above). A major difference between this analysis and that of Bradford-Grieve et al. (2010) is the relationship between the Centropagoidea and the remaining superfamilies. The morphological analysis indicated a sister relationship between the Centropagoidea and the Augaptiloidea, although there was less than 50% jackknife support. The alternative, strongly supported here, removes the necessity of postulating two independent origins of a derived, underlying 10-segmented condition of the antennal exopod by grouping all families with this hypothesized character within Clade 2. The inclusion of families from the two most basal superfamilies of the order, Pseudocyclopoidea and Epacteriscoidea (missing here), might change the topology of the relationships of the Clades 1–3. However, the inclusion of 18S rRNA sequences for these superfamilies did not change the main tree topology and integrity of the remaining superfamilies. In fact, our analysis supported the basal state and sister condition of *Pseudocyclops* sp. (Pseudocyclopidae, Pseudocyclopoidea) and *Exumella mediterranea* (Ridgewayiidae, Epacteriscoidea), which formed a sister group to all the other calanoid copepods, consistent with Andronov (1974) and Park (1986).

The relationships among the families of the Augaptiloidea (Clade 1) partially matched the analysis by Soh (1998) based on morphology, feeding habits, and habitat. The analyses tended to separate the Augaptilidae, which are typically pelagic carnivores, from the rest of the families and indicated its basal condition within the superfamily. This supported the hypothesis of Bradford-Grieve (2002) of the earlier colonization of the pelagic realm by the Augaptilidae. Our analyses indicated the close relationship between the families Hyperbionychidae and Arietellidae, which then join the Nullosetogeridae (Fig. 2). The Hyperbionychidae and Arietellidae include benthopelagic taxa (Soh, 1998); although the Nullosetigeridae comprises only pelagic taxa, this family is thought to be derived from a hyperbenthic ancestor (Soh et al., 1999) based upon the presence of asymmetrical antennules, a characteristic linked to hyperbenthic habitats (Ohtsuka and Mitsuzumi, 1990).

Both genetic and morphological analyses yielded the terminal placement of Hyperbionychidae within the Augaptiloidea; the family share an apparently plesiomorphic character state (a seta on the outer distal border of the basis of leg 3), which may therefore result from either a character state reversal or a derived state.

The Centropagoidea (Clade 3) was shown to be the next superfamily to diverge from the main lineage. The relationships among the families consistently supported the segregation and basal condition of the Centropagidae in relation to the other Centropagoidea families.

The genetic data grouped the remaining superfamilies of Clade 4: the Megacalanoidea, Bathypontioidea, Eucalanoidea, Clausocalanoidea, and Spinocalanoidea. This grouping is consistent with Clade 3 of Bradford-Grieve et al. (2010), and is thus consistent with her hypothesis that these superfamilies may have radiated into the pelagic realm during the Permian and after the end-Permian extinction event (e.g. Bradford-Grieve, 2002). The molecular results are also consistent with hypotheses of the evolutionary innovation of myelination (i.e., formation of the myelin sheath around nerve axons) within the Calanoida. Myelination results in shorter reaction times and two- to five-times faster escape responses (Lenz et al., 2000), and is considered to be a remarkable evolutionary innovation (Bradford-Grieve et al., 2010; Davis et al., 1999; Lenz et al., 2000).

In the gene-based phylogeny, a monophyletic Megacalanoidea (Clade 5 and Paracalanidae) was retrieved, although with low bootstrap support (<50%). In the morphology-based phylogeny, the Megacalanidae and a clade containing the Paracalanidae and Calanidae were closely related (Bradford-Grieve et al., 2010; Fig. 15); a monophyletic Megacalanoidea was not retrieved. This result in the molecular analysis may have resulted from the missing Paracalanidae 28S rRNA sequence, since removal of this taxon from the analysis strengthened support for the Calanidae/Megacalanidae node (to 99% bootstrap value; 1.00 BPP). Also, the close relationship between the Megacalanoidea and Bathypontioidea + Fosshageniidae (Clade 6) was then bootstrap supported.

The clade that comprises Fosshageniidae and Bathypontioidea (6) was consistently and strongly supported in all the analyses and helped to resolve the phylogenetic position of the Fosshageniidae. In the original description of this group, Suarez-Morales and Iliffe (1996) assigned this family to a new superfamily close to the Centropagoidea (our Clade 3). But Boxshall and Halsey (2004) placed it in the Centropagoidea after determining that the earlier study (Suarez-Morales and Iliffe, 1996) was not valid, since it did not assess the relative plesiomorphic or apomorphic status of the superfamily character states tabulated by Andronov (1974). Ferrari and Ueda (2005) disagreed with this conclusion and supported the establishment of the superfamily Fosshagenioidea. Bradford-Grieve et al. (2010) suggested that the Fosshageniidae belong to the Bathypontioidea, united by several characters (i.e., the form of leg 1 endopod, supplementary geniculation between male antennular segments 14 and 15, and absence of seta on segments 21–23). Based on the support of the Bathypontiidae/Fosshageniidae node found in our analyses (100% Bootstrap, 1.00 BPP in all cases), we conclude that the Fosshageniidae should be assigned to the Bathypontioidea.

Within the Eucalanoidea, the observed sister relationship between the Eucalanidae and Rhincalanidae (Clade 7) was consistent with Goetze's (2003) phylogeny of the family, which was based upon mitochondrial 16S rRNA and the nuclear intervening transcribed spacer region, ITS-2. However, we were not able to resolve the relationship between the Eucalanoidea and the other superfamilies, perhaps due to the absence from our analyses of any representative of the closely-related Ryocalanoidea (see Andronov, 1974; Park, 1986).

The split between Spinocalanoidea (Clade 9) and Clausocalanoidea (Clade 10) appeared to be the most recent one. Our results may indicate the ancestral position of the Spinocalanoidea to the Clausocalanoidea. Since this relationship was only evident when the mitochondrial DNA sequences were included in the analysis, we conclude that this may be a recent event. The sister relationship between *Foxtonia barbatula* and *Spinocalanus abyssalis* corroborated their close taxonomic and genetic relationship (Markhaseva and Kosobokova, 2001), although the problem of whether *Arctokonstantinus* should be considered a member of the Spinocalanidae (Boxshall and Halsey, 2004) or be in a separate family, the Arctokonstantinidae, was not resolved. Within the superfamily Clausocalanoidea, our analysis supported two lineages: one comprising the Clausocalanidae, Euchaetidae and Aetideidae; and the other including the Scolecitrichidae, Phaennidae, Tharybidae and Diaixidae (the so-called "Bradfordian" families; Ferrari and Markhaseva, 1996). This finding agreed with Bradford-Grieve et al. (2010) and supported the monophyletic character of this group of families, although this is not consistent with the current morphological classification. We found a sister relationship between the Euchaetidae and Aetideidae in both phylogenies, although this was not well-supported in the morphology-based tree by Bradford-Grieve et al. (2010). These results supported the suggestion of Boxshall and Halsey (2004) that the Euchaetidae may be a derived lineage within the Aetideidae. Although sparse taxon sampling prevented us from drawing conclusions about the topology of the Clausocalanoidea, we noted a level of complexity among the "Bradfordian" families that is not consistent with the current family structure. In particular, the position of *Scolecitrichopsis* was not consistent with the analysis of Markhaseva and Ferrari (2005), who placed this genus as a sister group to *Scolecithrix*, based on morphology of the antenna, maxilla, and maxilliped. Resolution and consensus agreement regarding the family structure and phylogeny of the Clausocalanoidea must await more extensive and integrated molecular and morphological analysis.

5. Conclusions

The molecular phylogeny of the Calanoida presented here is consistent with the earlier morphological analysis by Andronov (1974) and Park (1986); this study is more comprehensive than earlier ones, in terms of the number of families, range of molecular markers, and depth of taxa resolved. All studied superfamilies except the Megacalanoidea were retrieved and were shown to be monophyletic. More specifically, this paper indicates a sister relationship between the Centropagoidea and Clade 4 (Megacalanoidea, Bathypontioidea, Eucalanoidea, Clausocalanoidea and Spinocalanoidea), and the enlargement of the Bathypontioidea to include the Fosshageniidae. The topologies of the single-gene phylogenies using nuclear ribosomal genes produced inconsistent results, apparently due to markedly high divergences between species of the Centropagoidea. In contrast, multiple-gene analyses yielded consistent and well-supported phylogenetic results for superfamilies and families of the copepod order Calanoida.

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