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First multigene phylogeny of Cumacea (crustacea: Peracarida)

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Abstract

Cumaceans are small peracarid crustaceans that can be remarkably diverse and important benthic organisms. Despite their ubiquitous presence in soft sediments, no well-resolved phylogeny currently exists, which impedes ecological and evolutionary studies of the group. We present a phylogeny based on Bayesian inference of six markers (18S, 28S, 12S, 16S, CytB and COI), which recovers monophyly of the order, a deep split between telson and pleotelson bearing groups, and monophyly of four of the seven included families, including monophyletic Pseudocumatidae, Lampropidae, Bodotriidae and Nannastacidae. The only species representing the family Gynodiastylidae in our dataset was positioned among members of Diastylidae in the phylogenetic analyses. However, this result is based on a single partial COI sequence; thus, we consider it doubtful, and the family Diastylidae are otherwise recovered as a monophyletic family. The family Leuconidae is split into two well-supported clades, a clade containing Antarctic members of the genus *Leucon* and a separate clade containing non-Antarctic members of the genera *Leucon* and *Eudorella*. The phylogeny is a great stride forwards, as it supports most families as monophyletic, making generic level phylogenies a plausible endeavour in the future.

KEYWORDS

Cumacea, Molecular, Phylogeny, Taxonomy

1 | INTRODUCTION

Cumaceans are small crustaceans (1–30 mm) with a characteristic, recognizable shape including an enlarged cephalothorax, slender abdomen and bifurcated uropods (Figure 1). The characteristic shape leads to the common names of comma shrimp and hooded shrimp. Approximately 1900 species are described worldwide (WoRMS, 2021), and since both density (maximum of 88,591/m^2, Moore et al., 2007) and diversity can be very high (Corbera & Galil, 2001), they can play important roles in the marine food web as food sources for other invertebrates, fish, birds and even whales (Jones, 1963, Moore et al., 2007, Blanchard et al., 2019). Cumaceans are ubiquitous in soft sediments and distributed in all oceans from the intertidal to trenches and have been found at hydrothermal vent sites (Corbera et al., 2008). There are some species known from fresh and brackish environments such as terrestrial waters on the Kamchatka Peninsula (Derzhavin, 1926), intertidal freshwater springs

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and estuarine rivers (Duncan, 1984), Danube and Volga rivers (Sowinsky, 1893), and the Black and Caspian Seas (Sars, 1893), but the majority are marine.

The morphology of cumaceans is consistent at the level of order, with three or more thoracic segments fused to the head, all under a carapace, with the remaining thoracic segments free, six narrow abdominal segments, and a free telson or fused pleotelson. The characters that are used for taxonomic differentiation are the shape of the carapace, antennal morphology, mandible, maxillae and maxilliped shape, patterns of exopod presence and development on the third maxilliped to the fourth pereopod, and the presence of a free telson or fused pleotelson. Sexually dimorphic characters are frequently used for discrimination of families, genera and species, with commonly used adult male characteristics including pleopod number, shape, penial lobe presence or absence, antennule and antenna morphology, and exopod numbers (Figure 1).

Cumaceans have direct development, and the development of swimming appendages depends on life stage and sex; thus, they are quite limited in their dispersal and movement capabilities. The lack of a planktonic larval stage, which cumaceans share with other peracarid orders, entails that each species is highly adapted to quite specific physical and biological conditions associated with the substrate. Environmental characteristics that affect cumacean species distributions include grain size, organic content, redox potential, depth and temperature (Brandt et al., 1999, Brandt & Schnack, 1999, Corbera & Cardell, 1995, Corbera et al., 2008, Coyle et al., 2007, Uhlir et al., 2021, Watling & Gerken, 2005). Diversity tends to increase with depth, and in some areas, density also increases with depth (Brandt & Schnack, 1999). When high local species diversity is considered, it seems obvious that cumaceans have great potential for being highly sensitive indicator organisms for environmental changes in soft sediment communities (Vassilenko, 2002). Shallow water species may have multiple generations in a single year (Bishop & Shalla, 1994), while deep-sea species have generation times of up to 3 years or more (Bishop, 1982). Reproduction is typically a terminal event in the life history, although in some species, females may reproduce up to three times. In shallow water species, it is common during the reproductive season for the adult males to vertically migrate. The majority of cumaceans are microparticle feeders, scraping sediment particles or consuming diatoms (Cartes & Sorbe, 1996), but members of the Nannastacidae may be carnivorous, based on piercing mandible morphology and the presence of polychaete jaws in the gut (Cartes & Sorbe, 1996).

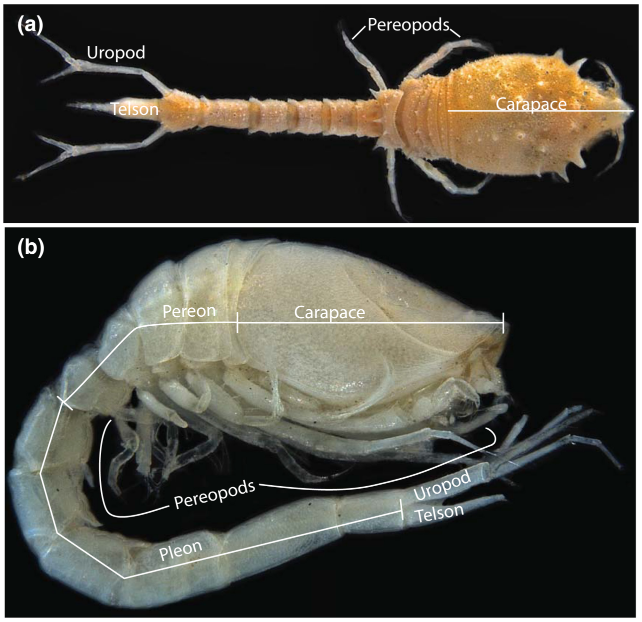

FIGURE 1 (a) *Diastylis cornuta*, dorsal view. (b) *Hemilamprops uniplicatus*, lateral view. Central anatomical body parts are indicated

Monophyly of the Cumacea is not in question, as the group is clearly circumscribed morphologically and easily recognizable. There are currently 8 families recognized within the Cumacea (Figure 2), five with a free telson (Ceratocumatidae, Diastylidae, Gynodiastylidae, Lampropidae and Pseudocumatidae) and three with a fused pleotelson (Bodotriidae, Nannastacidae and Leuconidae). The families are defined by combinations of characters, which worked well initially in the North Atlantic in the early stages of cumacean research, when the majority of species were described from this region. However, currently, there is so much overlap in family definitions that there are *incertae sedis* genera, for example *Kerguelenica* (Akiyama & Gerken, 2012) and *Atlantocuma* (Akiyama, 2012).

Even though the order Cumacea is well defined morphologically and monophyly of the order is generally accepted, the relationships between the families and the monophyly of families, subfamilies and genera have largely not been tested. Haye et al. (2004) performed a molecular phylogenetic analysis using the single mitochondrial gene COI, and they concluded that the telson fused into the pleotelson once. Rehm et al. (2020) used partial 16S from a few species per family to test relationships between the Bodotriidae, Diastylidae and Leuconidae, with the families Diastylidae and Leuconidae showing up as monophyletic, and the Bodotriidae not appearing monophyletic in their study. Bodotriidae, however, came out monophyletic in a study by Uhlir et al. (2021) also using 16S sequence, but including a larger taxon sampling covering seven of the eight existing cumacean families. In this study, Lampropidae was found paraphyletic since the single species representing Ceratocumatidae,

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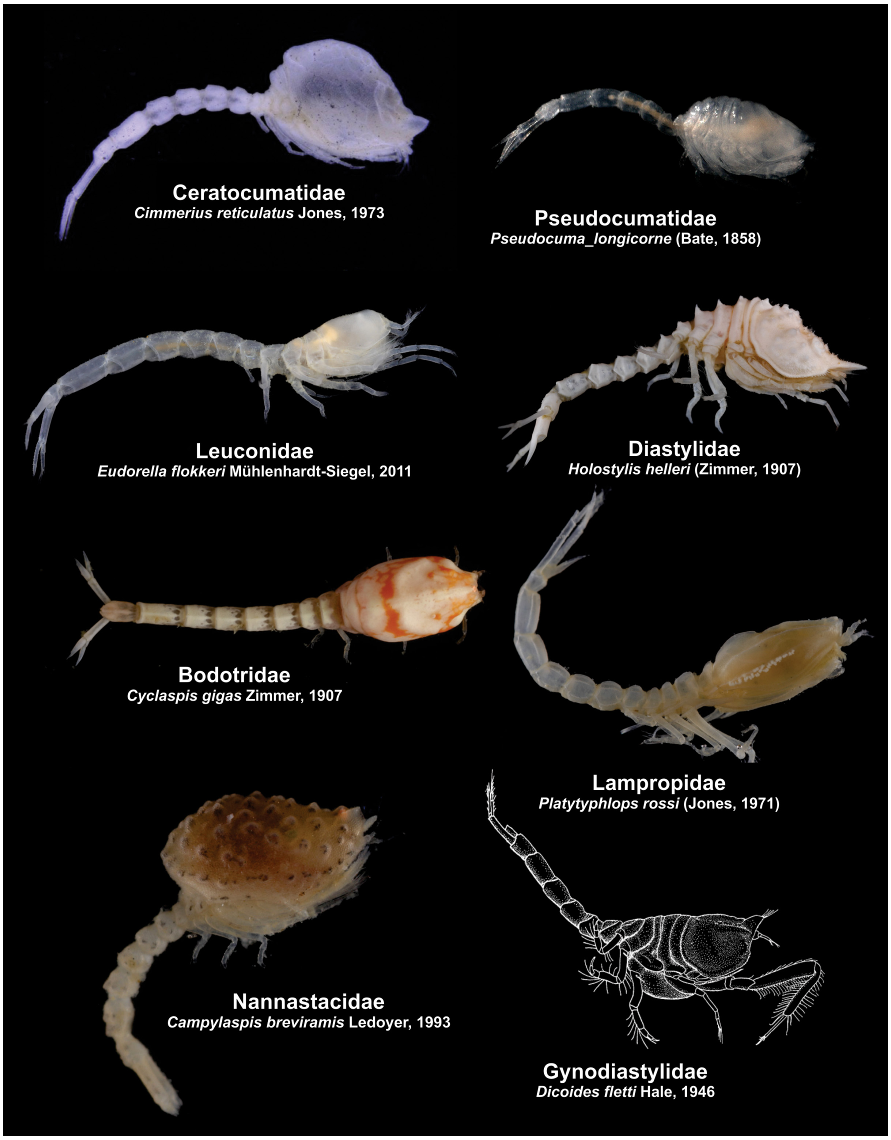

FIGURE 2 Species of the eight families of Cumacea

Cimmerius reticulatus, were positioned as sister species to *Platysympus typicus*—within Lampropidae. The few other phylogenetic analyses are based solely on morphology, such as Haye's 2007 phylogeny of the family Bodotriidae. The fossil record of cumaceans is sparse, with one fossil representative that clearly belongs in a modern family (Bodotriidae) from the Cretaceous Cenomanian (Luque & Gerken, 2019), while the few other fossils that have been assigned to the order from the Carboniferous (Schram et al., 2003), Permian (Malzahn, 1972) and Jurassic (Bachmayer, 1960) cannot be clearly associated with any of the modern families.

Morphology has been inadequate for resolving the relationships between families, largely due to families being defined by combinations of characters, and as morphological diversity has been added, family definitions have become less and less cleanly circumscribed. In terms of change due to evolutionary processes interpretation of character development can be ambiguous without a prior phylogenetic analysis, leaving the coding of characters as plesiomorphic vs. apomorphic almost impossible. In other words, without a proper analysis to test hypotheses of character evolution, directionality of change can be very difficult, and in some instances complicated further when absence or presence of a character varies within a species (Corbera & Galil, 2001).

The first molecular phylogenetic analysis of cumaceans was based on the single mitochondrial gene COI (Haye et al., 2004) and resulted in a poorly resolved phylogeny. In the current understanding, COI is considered not especially appropriate for analysis at the level of families. Since then, two other molecular phylogenetic studies have been published, but for several reasons, these have been based on another single mitochondrial gene, 16S (Rehm et al., 2020 and Uhrlir et al., 2021). Accumulation of a sufficient diversity of appropriately preserved specimens for molecular work has been difficult. Cumaceans are quite small and difficult to individually preserve; therefore,

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they are frequently preserved in bulk with sediments in formalin and sorted later, destroying molecular markers. Some families are rarely encountered, whether because they are strictly found in the deep sea, such as the Ceratocumatidae, or simply difficult to find, such as the Gynodiastylidae. In the current study, we were, for example, unable to obtain specimens of the Ceratocumatidae or the Gynodiastylidae, despite significant collection efforts in Australia and New Zealand, centres of gynodiastylid diversity. Gynodiastylidae is, therefore, in the present study represented by a single partial COI sequence from GenBank, from a specimen collected on the coast of India. Also, there have generally been challenges in successfully amplifying and sequencing certain cumacean species, although recent molecular advances and additional genetic markers to some degree have limited the issue. The lack of a family level phylogeny has been impeding research in the Cumacea in many areas. Without a solid phylogeny, diversification within the order cannot be evaluated, and hypotheses about character evolution cannot be tested. Ecological work requires a phylogenetic context to interpret patterns of diversity, dispersal and endemism. Therefore, in the present study, we conduct a thorough molecular analysis based on a carefully selected assemblage of mitochondrial and nuclear genes and broad taxon sampling. By doing this, we hope to provide a reliable phylogenic framework for future ecological and evolutionary studies of Cumacea.

2 | METHODS

2.1 | DNA extraction and amplification

In total, 92 cumacean specimens from 55 species (24 genera) covering seven of the eight accepted cumacean families are included in the molecular analyses. Total genomic DNA was extracted from the abdomens of the cumacean specimens using the Qiagen DNeasy Blood & Tissue Kit following the Qiagen DNeasy Protocol for Animal Tissues 07/2006.

DNA fragments from two nuclear ribosomal genes (28S and 18S), two mitochondrial ribosomal genes (16S and 12S) and two protein-coding mitochondrial genes (COI and CytB) were amplified and sequenced using primers listed in Table 1. Coverage of the six genes was as follows: 12S mt rDNA: 365 bp; 16S mt rDNA: 527 bp; 18S rDNA: 2555 bp; 28S rDNA: 993 bp; COI mtDNA: 634 bp; and CytB mtDNA: 392 bp.

All PCR reactions were carried out using a Bio-Rad C1000 Thermal Cycler in 25 µl volumes containing 1 µl of DNA extract, 2.5 µl 10× PCR buffer, 1.2 µl of dNTP mixture (2.5 µM each), 1 µl of each 10 µM primer and 0.75 U of Takara polymerase. Conditions for all amplifications were as follows: initial denaturation at 94°C for 5 min, then 35 cycles of 30s denaturation at 94°C, 1 min primer annealing at 52°C and 1 min extension at 72°C, with a final 7 min 72°C extension. All PCR products were visualized on 1% agarose gels and stored at 4°C prior to purification and sequencing. PCR products were cleaned by the addition of 0.1 µl (1 U) exonuclease I, 1 µl (1 U) of shrimp alkaline phosphatase and 0.9 µl of ddH$_2$O to 8 µl of PCR product. This was carried out by incubation at 37°C for 30 min and deactivation of the enzymes at 85°C for 15 min. Sequence reactions were performed using the BigDye v.3.1 Cycle Sequencing kit (Applied Biosystems, Inc.) with the same primers used for initial PCR amplification. Both strands of all PCR products were sequenced using an ABI 3730 capillary sequencer.

2.2 | Sequence alignment

All sample PCR products were sequenced in both directions in order to improve accuracy and aligned using default parameters in Genious Prime 2020 (https://www.geneious.com). Following minor improvements by eye, alignments were modified for each gene prior to further analyses. In addition to the species sequenced in the present study, 33 cumaceans and 12 out-group species were downloaded from GenBank. This allowed us to compile the most complete cumacean dataset to date, both in terms of species and DNA sequences, including data from species of seven of eight recognized cumacean families. (specimen data and sequence accession numbers for all taxa included in the study can be found in Table 2). In effect, the Bayesian inference analyses were based on two concatenated datasets, with (5760 bp) and without (5506 bp) GenBank sequences comprising six markers (18S, 28S, 12S, 16S, CytB and COI). These markers represent both nuclear and mitochondrial genes with a wide range of evolutionary rates, making them suitable for a phylogenetic resolution at all taxonomical levels in a crustacean order such as Cumacea (Toon et al., 2009; Schubart et al., 2000).

2.3 | Phylogenetic analyses

To avoid cryptic species affecting the results of our phylogenetic analyses, most species are represented by several individuals. We performed two separate analyses on two datasets. Dataset-1 included 125 taxa from both GenBank and our own material leaving many species represented by only COI mtDNA in the alignment (result of analyses in Figure 3). Expecting low support for

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TABLE 1 Primers used to amplify and sequence DNA in this study

Primer	Sequence (5′–3′)	Source	Position
28S rRNA			
1274	GACCCGTCTTGAAACACGGA	Whiting et al. 1997	810
1275	TCGGAAGGAACCAGCTACTA	Whiting et al. 1997	1150
FF	GGTGAGTTGTTACACACTCCTTAGTCGGAT	Jarman et al. 2000	1470
COI mtDNA			
LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994	1490
HCO	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994	2198
18S rRNA			
329	TAATGATCCTTCCGCAGGTT	Spears et al. 1992	1
HI	CAACTAAGAACGGCCATGCAC	Spears et al. 1992	510
F1131	AAACTYAAAGRAATTGACGG	Troedsson et al. 2008	600
A-	CAGCMGCCGCGGTAATWC	Spears et al. 1992	1220
B-	CGGGTAACGGGGAAT	Spears et al. 1992	1440
328	CCTGGTTGATCCTGCCAG	Spears et al. 1992	1800
12S mtDNA			
12Sf	GAAACCAGGATTAGATACCC	Mokady et al. 1999	330
12Sr	TTTCCCGCGAGCGACGGGCG	Mokady et al. 1999	670
CytB mtDNA			
151F	TGTGGRGCNACYGTWATYACTAA	Merritt et al. 1998	458
270R	AANAGGAARTAYCAYTCNGGYTG	Merritt et al. 1998	820
16S mtDNA			
16S ar	CGCCTGTTTATCAAAAACAT	Palumbi et al. 1991	670
16S br	CCGGTCTGAACTCAGATCACGT	Palumbi et al. 1991	1230

Note: Suggested pairing of 18S primers: 329–328, 329–F1131, 329–a, HI–B, A–328.
Suggested pairing of 28S primers: 1274–1275, 1274–FF.

such a mixed dataset, in dataset-2 (92 taxa), we removed all taxa represented by single genes from the alignment, for comparison of support values and tree topology (result of analyses in Figure 4). Independent models of sequence evolution for six genes were selected using the Akaike information criteria in MrModeltest 2.4 (Nylander, 2004). In both datasets, model testing suggested the GTR + G + I model for the entire alignment and the following models for each separate sequence: GTR + G for 18S, GTR + G + I for 16S, GTR + G for 28S, GTR + G for 12S, GTR + G + I for COI and GTR + G + I for CytB. Phylogenetic analyses were performed in MrBayes v3.2.7 (Ronquist and Huelsenbeck, 2003) on full concatenated alignments of both datasets. Sequences in each dataset were treated with separate models (partitioned) or a single model of evolution was applied to the entire dataset (non-partitioned), using Bayesian methods coupled with Markov chain Monte Carlo (MCMC) inference. For all analyses, two independent runs were performed, each consisting of four chains (1 cold and 3 hot) and proceeding for 50 million or five million generations, sampling every 2000 generations. The number of generations for each pair of runs was determined by monitoring the 'average standard deviation of spilt frequencies' (SDSF) approaching 0.01. Results were visualized in Tracer v. 1.3 (Drummond & Rambaut, 2007). For each parameter, proper mixing of the MCMC was assessed by calculating the effective sampling size (ESS). The average standard deviation of spilt frequencies (SDSF) after 50 million searches was 0.016 (partitioned dataset-1), and after five million searches 0.13 (non-partitioned dataset-1), 0.017 (partitioned dataset-2) and 0.011 (non-partitioned dataset-2). PSRF was close to 1 on all parameters. Convergence of parameter values from each run was evaluated by examining results in Tracer 1.6 (Rambaut et al., 2014). Plots from Tracer were used to determine that the initial 25% of sampled trees from each search be discarded as 'burnin'. In effect, a total of 2×18,751 trees in dataset-1 and 2×1876 trees in dataset-2 were used to summarize model parameters in MrBayes using the 'sump' command, and 'sumt' to construct a 50% majority rule consensus trees and calculate Bayesian posterior probabilities for each node (Figures 3 and 4). In addition, in order to validate the Bayesian results, we chose to apply a maximum likelihood method using RAxML

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TABLE 2 Details of specimens and GenBank accession number used in the present study

			GenBank accession numbers					
	Family/species	**Collection location**	**12S (365 bp)**	**16S (527 bp)**	**18S (2556 bp)**	**28S (994 bp)**	**COI (634 bp)**	**CytB (396 bp)**
	Bodotridae							
	Atlantocuma sp. GenBank	Antarctica		*HQ450558*				
	Bodotria cf. 221–222	New Zealand			MK635529	MK644855	MK757550	OL841397
	Cyclaspis caprella GenBank	SW Australia				*AF169712*	*DQ889092*	
*	*Cyclaspis longicaudata* 4–6	Hjeltefjorden, Norway		MK613872		MK644839	MK757518	OL841394
*	*Cyclaspis longicaudata* 7–8	Shelf, Norway		MK613873	MK635573			OL841395
	Cumopsis fagei GenBank	Siec Island, France		*AJ388111*				
	Cumopsis goodsiri GenBank	UK					*AF137518*	
	Eocuma longicorne GenBank	Kenya					*AF520445*	
*	*Iphinoe serrata* 146	Shelf, Norway	MK635039	MK613886	MK635530	MK644840	MK757512	OL841444
	Iphinoe trispinosa GenBank	UK/France			*KJ182988*		*AF137519*	
	Iphinoe truncata 1 GenBank	South Africa					*DQ351369*	
	Iphinoe truncata 4 GenBank	South Africa					*DQ351368*	
	Heterocuma sp. GenBank	Kenya					*AF520443*	
	Pseudocumatidae							
	Pseudocuma similis GenBank	UK					*AF137514*	
*	*Petalosarsia declivis* 156–158	Svalbard		MK613871	MK635534	MK644869	MK757538	OL841419
	Nannastacidae							
*	*Campylaspis costata* 120–122	Skagerak, Norway	MK635026	MK613876	MK635563	MK644859	MK757508	OL841454
*	*Campylaspis costata* 220,909–1	Fensfjorden, Norway	MK635027					OL841456
*	*Campylaspis affinis* 031109–1	Svalbard	MK635025		MK635564			OL841455
*	*Campylaspis globosa* 1–3	Skagerak, Norway		MK613874	MK635503	MK644858		OL841445
*	*Campylaspis horrida* 123	Shelf, Norway	MK635028	MK613877	MK635561	MK644856	MK757522	
*	*Campylaspis intermedia* 031109–8	Skagerak, Norway	MK635030		MK635566		MK757511	OL841446
*	*Campylaspis macrophthalma* 124–126	Shelf, Norway	MK635032	MK613879	MK635565	MK644860		OL841457
*	*Campylaspis sulcata* 133, 147–14	Shelf, Norway			MK635570	MK644864		OL841448
*	*Campylaspis sulcata* 134–139	Hjeltefjorden, Norway		MK613875	MK635571	MK644865	MK757523	OL841449
*	*Campylaspsis sulcus* (not bumpy) 200,912–3	Chile	MK635031		MK635567	MK644868		OL841442
*	*Campylaspis rubicunda* 031109–7	Svalbard			MK635568	MK644862		OL841450

(Continues)

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TABLE 2 (Continued)

	Family/species	Collection location	GenBank accession numbers					
			12S (365 bp)	**16S (527 bp)**	**18S (2556 bp)**	**28S (994 bp)**	**COI (634 bp)**	**CytB (396 bp)**
	Campylaspis rubicunda 127–129, 236	Skagerak, Norway						OL841453
*	*Campylaspis rubicunda* 130–132	Hjeltefjorden, Norway			MK635569	MK644861		
	Campylaspis rubicunda 234	Fanafjorden, Norway					MK757507	OL841452
	Campylaspis rubicunda 235	Hauglandsosen, Norway					MK757510	OL841451
*	*Campylaspis undata* 140–145	Hjeltefjorden, Norway	MK635029	MK613878	MK635562	MK644857	MK757509	
*	*Campylaspis verrucosa* ma1	Shelf, Norway				MK644863		OL841447
*	*Campylaspis* sp. 205, 219	Sagami Bay, Japan			MK635572			
*	*Cumella* sp. 200,912–8	Alaska	MK635046	MK613880	MK635505	MK644866	MK757519	OL841458
Diastylidae								
	Colurostylis longicaudata GenBank	New Zealand					*AF520446*	
	Diastylis bispinosa GenBank	Gulf of Maine, USA					*AF137511*	
*	*Diastylis cornuta* 160,409–8	Fanafjorden, Norway	MK635012	MK613898				OL841422
*	*Diastylis cornuta* 9–13	Shelf, W. Norway	MK635011	MK613897	MK635528	MK644870	MK757567	OL841421
	Diastylis crenellata GenBank	Oregon, USA					*AF352298*	
*	*Diastylis echinata* 14–15	Hjeltefjorden, Norway	MK635047		MK635514	MK644872	MK757557	OL841417
*	*Diastylis echinata* 16–19	Skagerak, Norway	MK635048		MK635513	MK644873	MK757558	OL841418
*	*Diastylis edwardsii* 031109–3	Svalbard			MK635508			OL841411
*	*Diastylis edwardsii* 031109–4	Svalbard	MK635020		MK635509			
*	*Diastylis goodsiri* 031109–12	Svalbard	MK635021	MK613904	MK635517			OL841420
*	*Diastylis laevis* 20	Skagerak, Norway			MK635531		MK757530	
	Diastylis laevis 21–22	Skagerak, Norway		MK613901		MK644871	MK757527	OL841425
*	*Diastylis lucifera* 23–25	Skagerak, Norway	MK635050		MK635533	MK644882	MK757561	OL841426
*	*Diastylis lucifera* 26–28	Skagerak, Norway		MK613911	MK635532	MK644883	MK757562	
*	*Diastylis rathkei* 031109–19	Svalbard	MK635022	MK613905			MK757524	OL841414
*	*Diastylis rathkei* 230,909–1	Island	MK635023		MK635516			OL841415
	Diastylis rathkei GenBank	Denmark		*HQ450555*			*AF069764*	
	Diastylis sculpta GenBank	Maine, USA		*DSU81512*	*AY781431*		*AF137510*	
*	*Diastylis spinulosa* 031109–16	Svalbard	MK635024	MK613906	MK635515			OL841416
*	*Diastylis stygia* 29–31	Jan Mayen		MK613902	MK635512	MK644879	MK757525	OL841409
*	*Diastylis stygia* 32–33	Eggakanten, Norway		MK613903	MK635510	MK644884	MK757526	OL841410

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			GenBank accession numbers					
	Family/species	**Collection location**	**12S (365 bp)**	**16S (527 bp)**	**18S (2556 bp)**	**28S (994 bp)**	**COI (634 bp)**	**CytB (396 bp)**
*	*Diastylis stygia* 34	Eggakanten, Norway			MK635511	MK644876		
*	*Diastylis tumida* 35–37	Hjeltefjorden, Norway		MK613899		MK644880	MK757528	OL841423
*	*Diastylis tumida* 152–153	Shelf, Norway	MK635013	MK613900		MK644878	MK757529	OL841424
*	*Diastyloides biplicatus* D4-D6	Hjeltefjorden, Norway		MK613910	MK635522			OL841405
*	*Diastyloides biplicatus* D10-D15	Lopphavet, Norway	MK635017		MK635520		MK757531	OL841404
*	*Diastyloides biplicatus* D16-D20	Førdefjorden, Norway	MK635016		MK635521			OL841403
*	*Diastyloides serratus* 38–40	Sognesjøen, Norway	MK635014	MK613907	MK635518		MK757533	OL841406
*	*Diastyloides serratus* 159–174	Førdefjorden, Norway		MK613909	MK635519	MK644841	MK757534	OL841407
*	*Diastyloides serratus* 1004–1006	Skagerak, Norway	MK635015	MK613908	MK635527	MK644867	MK757535	OL841408
	Diastylopsis thilenuisi GenBank	New Zealand					*AF520442*	
	Diastylopsis sp GenBank	??		*HQ450556*				
*	*Dimorphostylis coronata* cf. 206, 213	Sagami Bay, Japan	MK635018	MK613920	MK635526	MK644885	MK757536	OL841412
*	*Dimorphostylis elegans* cf. 207	Sagami Bay, Japan	MK635019		MK635525	MK644886	MK757537	OL841413
*	*Leptostylis longimana* 41–43	Sognesjøen, Norway		MK613921	MK635506	MK644887	MK757521	OL841435
*	*Leptostylis longimana* III 49–50	Skagerak, Norway		MK613922	MK635507	MK644888	MK757520	OL841436
*	*Leptostylis macrura* 203	Møre, Norway		MK613912		MK644881	MK757559	OL841429
*	*Leptostylis villosa* 230,909 3–4	Island	MK635043	MK613896	MK635523		MK757532	OL841388
*	*Leptostylis villosa* 57–60	Skagerak, Norway			MK635524	MK644875	MK757560	OL841428
*	*Leptostylis villosa* II 56	Skagerak, Norway				MK644874		OL841427
	Oxyurostylis lecroyal GenBank	Mississippi, USA					*AF137513*	
	Oxyurostylis smithi GenBank	Mississippi, USA					*AF137512*	
Leuconidae								
*	*Eudorella emarginata* 76–78	Fensfjorden, Norway				MK644846	MK757516	OL841398
*	*Eudorella emarginata* III 79–82	Skagerak, Norway	MK635035	MK613870	MK635552	MK644851	MK757513	OL841400
*	*Eudorella emarginata* 86–87, 98	Skagerak, Norway			MK635554	MK644852	MK757515	OL841401
*	*Eudorella emarginata* 031109–18	Svalbard					MK757514	OL841399
*	*Eudorella hirsuta* 88–93	Skagerak, Norway	MK635033	MK613887	MK635553	MK644853	MK757543	OL841402
*	*Eudorella hirsuta* 94–96, 102	Fensfjorden, Norway	MK635034	MK613888	MK635504	MK644854	MK757544	
	Eudorella pusilla GenBank	Maine, USA		*U81513*			*AF137516*	
*	*Eudorella truncatula* 97, 99	Skagerak		MK613881	MK635555	MK644844	MK757553	OL841439

(Continues)

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TABLE 2 (Continued)

Family/species	Collection location	GenBank accession numbers 12S (365 bp)	16S (527 bp)	18S (2556 bp)	28S (994 bp)	COI (634 bp)	CytB (396 bp)
* *Eudorella truncatula* 100–101	Fensfjorden		MK613884	MK635558	MK644877	MK757555	OL841440
* *Eudorella truncatula* 190, 195	Svalbard			MK635556	MK644836	MK757552	
* *Eudorella truncatula* II 200	Skagerak		MK613882	MK635557	MK644835	MK757554	OL841438
* *Eudorella truncatula* 1007–1008	Hjeltefjorden		MK613883			MK757556	OL841441
* *Eudorella truncatula* ma5	Shelf, Norway		MK613885	MK635559	MK644848	MK757551	
* *Leucon acutirostris* 103–108	Skagerak, Norway	MK635036	MK613889	MK635548	MK644842	MK757547	OL841385
Leucon antarcticus GenBank	Antarctica		*HQ450533*				
Leucon antarcticus GenBank	Antarctica		*HQ450534*				
Leucon antarcticus GenBank	Antarctica		*HQ450536*				
Leucon assimilis GenBank	Antarctica		*HQ450553*				
* *Leucon Crymoleucon tener* 73–75	Skagerak, Norway				MK644847	MK757517	OL841396
Leucon intermedius GenBank	Antarctica		*HQ450549*				
* *Leucon nasica* 109–111	Fanafjorden, Norway	MK635041	MK613895	MK635551	MK644845	MK757548	OL841389
* *Leucon nasica* 112–114	Skagerak, Norway	MK635042	MK613893	MK635549	MK644850	MK757549	OL841387
* *Leucon nasicoides* 115–116	Svalbard	MK635037	MK613890	MK635545	MK644843	MK757545	OL841393
* *Leucon nathorsti* 031109–9	Svalbard	MK635044	MK613894	MK635550			OL841390
* *Leucon nathorsti* 186, 209–211	Svalbard	MK635038		MK635546	MK644837		OL841386
* *Leucon palidus* 1001–1003	Skagerak, Norway	MK635040	MK613892		MK644838	MK757546	OL841392
* *Leucon pallidus* 117–119	Fensfjorden, Norway		MK613891	MK635560			OL841391
Leucon rossi GenBank	Antarctica		*HQ450542*				
* *Leucon* sp (big) 200,912–1	Chile			MK635547	MK644849		
Gynodiastylidae							
Gynodiastylis sp. GenBank	New Zealand					*AF520447*	
Lampropidae							
* *Hemilamprops assimilis* 187–188, ma6	Island		MK613924	MK635543	MK644833	MK757542	OL841437
Hemilamprops californicus GenBank	California, USA					*AF061781*	
* *Hemilamprops cristatus* 63–64	Skagerak, Norway	MK635052	MK613913	MK635535	MK644826		OL841430
* *Hemilamprops cristatus* 65	Skagerak, Norway		MK613914	MK635536			OL841431
* *Hemilamprops rosea* 66–67, ma8	Skagerak, Norway		MK613923	MK635542	MK644832	MK757541	
* *Hemilamprops uniplicatus* 68–70	Hjeltefjorden, Norway	MK635010	MK613915	MK635539	MK644828	MK757563	OL841432

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TABLE 2 (Continued)

Family/species	Collection location	GenBank accession numbers					
		12S (365 bp)	16S (527 bp)	18S (2556 bp)	28S (994 bp)	COI (634 bp)	CytB (396 bp)
* *Hemilamprops uniplicatus* 71–72	Shelf, W. Norway	MK635008	MK613916	MK635537	MK644827	MK757564	OL841433
* *Hemilamprops uniplicatus* 193–194	Svalbard	MK635009		MK635538	MK644834	MK757565	
* *Lamprops augustinensis* 200,912–9	Alaska	MK635045	MK613925	MK635541	MK644830	MK757568	OL841443
* *Mesolamprops denticulatus* 61–62	Shelf, W. Norway	MK635051	MK613917	MK635544	MK644831	MK757566	OL841434
* *Platysympus tricarinatus* 031109–15	Svalbard	MK635049	MK613918	MK635540		MK757540	OL841383
* *Platysympus tricarinatus* ma14	Shelf, Norway		MK613919		MK644829	MK757539	OL841384
Outgroups							
* *Nebalia* sp GenBank		*AF107606*		*EU370433*	*EU370447*	*FJ170126*	
* *Lophogaster typicus* 190,913–1	Hjeltefjorden, Norway			18S	28S	COI	CytB
* *Tanais dulongi* GenBank				*AY781428*		*HM016204*	
* *Asellus aquaticus* GenBank		*GU130252*	*GU130252*	*AF255701*	*DQ144749*	*GU130252*	*GU130252*

Note: Accession numbers in *italics* indicate sequences taken from GenBank. Remaining sequences obtained for this study. All specimens used in dataset 1. * indicates specimens used in dataset 2.

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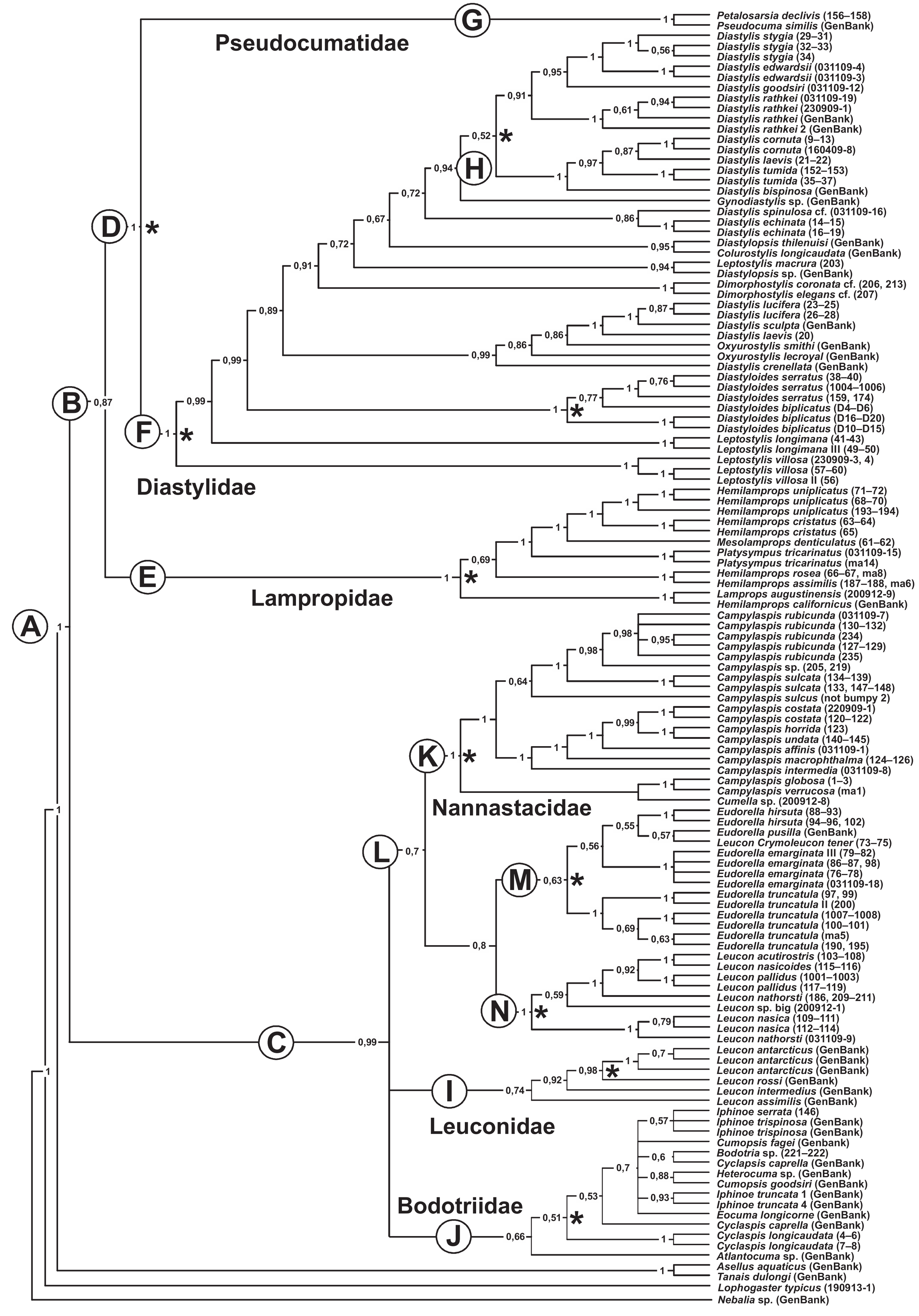

FIGURE 3 Legend on next page

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FIGURE 3 Phylogenetic relationships of *Cumacea*. Cladogram, consensus tree inferred from a Bayesian analysis (50 million generations, 37,502 trees) on a concatenated six-gene dataset (18S, 28S, 12S, 16S, CytB and COI) with letters indicating larger monophyletic taxonomic groupings. Single gene taxa taken from GeneBank are included (Dataset-1, 125 taxa). Genes were partitioned and treated as separate models. The SDSF for split frequencies was 0.016, and PSRF was close to 1 on all parameters. Nodal support is indicated in the form of Bayesian posterior probabilities (PP). Nodes with PP values less than 50 have been collapsed. '*' indicates nodes that were also supported in a Bayesian analysis having one evolutionary model applied to the entire dataset (non-partitioned) and a maximum likelihood analyses using RAxML raw trees for dataset-1 is provided in Appendices S1–S3. Major nodes in Figures 2 and 3 are labelled for reference in discussion. The monophyletic taxa: (A) Cumacea, (B) The telson bearing clades (Diastylidae, Gynodiastylidae, Pseudocumatidae and Lampropidae, (C) The clades with fused pleotelson (Nannastacidae, Leuconidae and Bodotriidae), (D) The families Diastylidae, Gynodiastylidae and Pseudocumatidae, (E) Lampropidae, (F) Diastylidae, (G) Pseudocumatidae, (H) The only gynodiastylid sequence (COI), (I) A monophyletic group of Antarctic leuconid species (Leuconidae I), (J) A clade consisting of seven different genera within a monophyletic Bodotriidae, (K) Nannastacidae, (L) An assembly of *Eudorella* and *Leucon* species closer related to Nannastacidae than the remaining Leuconidae, (M) Leuconid species within the genus *Eudorella* (Leuconidae II)—see discussion and results for more information, (N) An assembly of *Leucon* species

(Stamatakis, 2014) on our full dataset. A 50% majority tree was constructed from one thousand rapid bootstrap replicates (−f) that were calculated employing the GTRGAMMA substitution using 6 distinct data/gene partitions (applied same sequence models as those used in the Bayesian analyses) with joint branch length optimization. The parsimony random seed (−p) and bootstrap random seed (−x) were set to 1. Raw trees for all analyses, both phylograms and cladograms, with support values are provided as Appendices S1–S7.

3 | RESULTS

In our study, we focused primarily on the Bayesian analyses where each gene was treated with an independent model of evolution or with one model for all genes. These analyses were performed on a total dataset including COI sequences from GenBank, and a dataset where species represented by only COI or 16S sequences were excluded. The overall topology in all Bayesian analyses were to a large degree congruent, if not identical. With the exception of Leuconidae, the analyses retained strong support for monophyletic families. We expanded our analyses by conducting a maximum likelihood analysis on the full dataset; this was to investigate to what extent our Bayesian-based topology was retained using ML, reflecting the robustness of our dataset. As expected, the ML not only gave lower support values in deeper nodes, but also although lesser resolution in internal nodes, all families, except for Leuconidae, were retained as monophyletic. Major nodes supported by all analyses are marked with '*' in the Bayesian full dataset, partitioned gene consensus tree (Figure 3). We will continue presenting our results, both agreements and deviations, from all analyses with reference to the Bayesian, partitioned full dataset topology (Figure 3), as all additional analyses are variants of this full dataset with less data and/or less complex models.

Cumaceans are monophyletic (Figures 3 and 4). Representatives from one out-group, *Nebalia* sp., and three putative sister taxa, *Lophogaster typicus*, *Asellus aquaticu*, and *Tanais dulongi* were included in our analyses, which invariably suggested that the assembly of cumacean species define the monophyly of the taxonomic group Cumacea (Figure 3A, PP =1).

Cumaceans are profoundly divided into two major monophyletic branches (Figure 3B, PP = 0.87 & 3C, PP = 0.99), a free telson bearing clade and a fused pleotelson clade. All species that possess a free telson at the 6th abdominal segment form a monophyletic taxon (Figure 3B, PP = 0.87). The clade of telson bearing cumaceans (Figure 3B) is divided into a strongly supported dichotomy (Figure 3D,E). The branch comprising a monophyletic Lampropidae (Figure 3E, PP = 1) constitutes a monophyletic sister taxon to a clade representing the remaining telson bearing cumaceans. The sister group to the Lampropidae forms a bifurcated branch (Figure 3D, PP = 1) of which one branch leads to two species, *Petalosarsia declivis* and *Pseudocuma similis*, both belonging to the monophyletic family Pseudocumatidae (Figure 3G, PP = 1). The other branch leads to a well-supported clade (Figure 3F, PP = 1) containing all members of the family Diastylidae, and the only species in the study that represents the family Gynodiastylidae, *Gynodiastylis* sp (Figure 3H). The position of this single *Gynodiastylis* species should be taken with reservation, since only a single GenBank sequence of the COI gene is included in the analyses. The speciose genera *Diastylis*, *Hemilamprops* and *Leptostylis* are polyphyletic taxa in the analyses.

The cumacean species with a fused pleotelson form a monophyletic clade (Figure 3C, PP = 0.99), which includes the families Leuconidae, Bodotriidae and Nannastacidae. The phylogenetic analysis reveals that the pleotelson cumaceans form an unresolved trichotomy: a clade of Antarctic *Leucon* species, Leuconidae I

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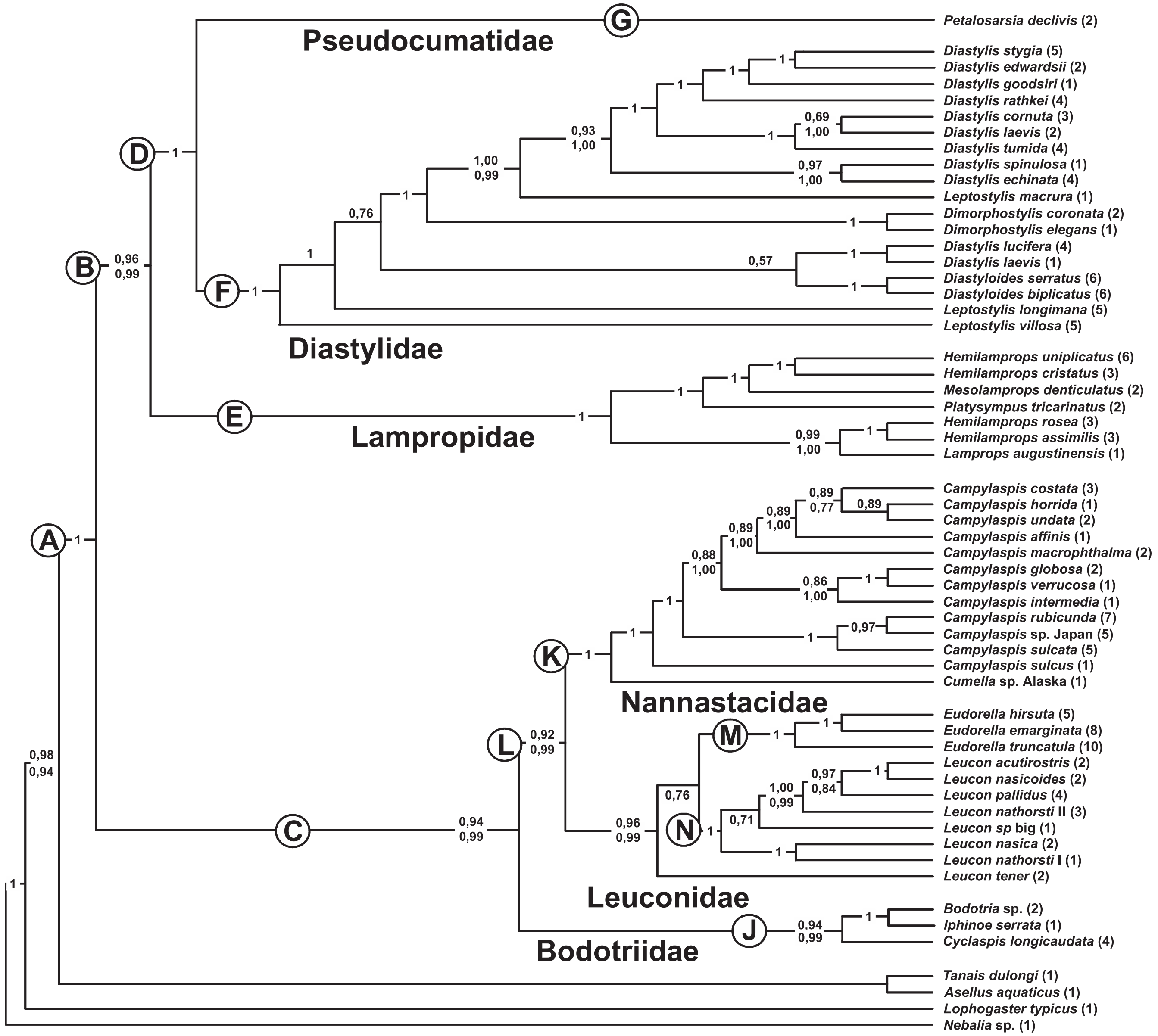

FIGURE 4 Cladogram, consensus tree from a Bayesian analysis (5 million generations, 3752 trees each) on a concatenated six-gene dataset (18S, 28S, 12S, 16S, CytB and COI). Species represented by only one gene were not included (Dataset-2, 92 taxa). The presented tree is from Analysis-1, which was a partitioned dataset, treating each gene with separate models, posterior probabilities are shown above branches (see Appendices S4 and S5 for raw trees). Analysis-2 was on an unpartitioned dataset, treating entire alignment with a single GTR + G + I model of evolution, posterior probabilities are shown below branches (see Appendices S6 and S7 for raw trees). Numbers on branches are posterior probabilities from both analyses. Outer branches are collapsed where identical species formed a monophyletic clade with full support, represented by one branch in tree. Numbers in brackets following species names indicate number of individuals used in analyses

(Figure 3I, PP = 0.74); a clade consisting of species that represent seven different genera within the monophyletic Bodotriidae representatives (Figure 3J, PP = 0.66); a dichotomous clade, which contains the monophyletic Nannastacidae (Figure 3K, PP = 1), and the second group of leuconid species, Leuconidae II (Figure 3L, PP = 0.7). Leuconidae II consists of species within the genus *Eudorella* (Figure 3M, PP = 0.63), which apart from the position of *Leucon (Crymoleucon) tener* is monophyletic, and the second group of *Leucon* species (Figure 4N, PP = 1).

By omitting species that rely solely on sequences from a single gene, the phylogeny becomes significantly more robust, illustrated by increased posterior probabilities (Figure 4). As for structure, there is virtually no difference in the topology between the phylogenies in all of our analyses, be it full data or pruned data, one model or mixed models, Bayesian or maximum likelihood.

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4 | DISCUSSION

Monophyly of cumaceans has never been in doubt morphologically, although never tested against molecular data, and is unambiguously supported by our analyses (Figures 3 and 4). Within the Peracarida, unique cumacean traits include the modification of the first three thoracic appendages as maxillipeds (rotated towards the midline and used for food handling rather than locomotion) and the fusion of the first three thoracic segments into the carapace, which is expanded and wide relative to a slender pleon, leading to the common name of comma shrimp. The sister taxon of the Cumacea is not yet known, as there have been various proposed relationships among the Peracarida, none of them with satisfactory resolution nor using modern molecular techniques and sufficient data. Using morphological data, proposed sister groups for the Cumacea have included the Tanaidacea (Schram, 1986, Watling, 1999, Richter & Scholtz, 2001, Poore, 2005), Mictacea (Wills, 1998) and Spelaeogriphacea (Siewing, 1963). Molecular analysis using a single gene proposed a sister group of the Isopoda (Spears et al., 2005). The Spears et al. paper was seminal in being the first molecular attempt at a peracarid molecular phylogeny, but suffers from the limited data that was possible at the time.

As mentioned in the results section, the phylogenetic analyses become significantly more robust by excluding species represented by only a single gene compared with the analyses where only multigenic represented species are included. We have not analysed in detail the cause of this difference, but we believe that it is likely that the species represented by a single gene during the phylogenetic analyses, to a greater extent than the multigene represented species, change phylogenetic position and thereby weaken the overall robustness of phylogeny. The phylogenetic position of these 'single gene' species in the full data analyses (e.g. G*ynodiastylis* sp and *Atlantocuma* sp.) must therefore be taken with caution. The multigenetic analyses where we excluded single gene taxa strongly support the morphology-based classification systematics with all of the well-represented families appearing as monophyletic clades. Within the Cumacea, there are five families with a free telson (Ceratocumatidae, Diastylidae, Gynodiastylidae, Lampropidae and Pseudocumatidae) and three families with a fused pleotelson (Bodotriidae, Leuconidae and Nannastacidae). There was historically some doubt as to whether telson fusion was a singular event, or occurred multiple times, suggested by characters such as the presence of a process on the pleopod endopod in adult males (Bodotriidae, Lampropidae and Pseudocumatidae) vs. absence of a process (Diastylidae, Leuconidae) (Haye et al., 2004). However, the work of Haye et al. (2004) supported a single fusion of the telson into a pleotelson, which is also unambiguously supported by our analyses (Figure 3C).

Throughout the Cumacea, reduction is a common morphological theme. Reduction is used generally to describe minimization in size or number of articles in appendages or structures, or loss of an appendage or structure entirely. For example, a reduced pleopod in the adult male is typically small, may lack articles in the rami and has few, short setae, relative to a fully developed pleopod, which is typically nearly the length of the body segment, half the width of the body segment, armed with many very long plumose setae that are used for locomotion. In phylogenetics and evolutionary theory, the loss of characters and also reductions are often hypothesized to have occurred as several independent evolutionary events and therefore fail to define monophyletic clades based on apomorphic properties.

Within the free telson clade (Figures 3B and 4B), the Lampropidae are the basal group, which is in accord with morphological characteristics that are considered primitive (Haye et al., 2004, Lomakina, 1958, Zimmer, 1941), including a large, broad telson with three or more terminal setae, three large pleopods in the adult male, a larger and more developed antenna 2 in the female and four hepatic diverticula. The Pseudocumatidae exhibit high levels of reduction, with the telson being reduced to an unarmed flap that does not contain the anus, a very reduced antenna two in the female, and zero—two pairs of reduced pleopods in the adult male. The Diastylidae likewise possesses several reduced characters compared with the likely plesiomorphic condition in Lampropidae, the sister group to Diastylidae and Pseudocumatidae. The reduction trend appears to have been strongest in Pseudocumatidae, both in terms of the degree and the number of character reductions, with a telson that is frequently shorter, with two terminal setae, and with a distinct pre and post anal division, an unreduced but small antenna two in the female, and usually two pairs of pleopods in the adult male (with rare genera with two reduced pairs, or one or zero pairs of pleopods). The families in the free telson clade that lack sufficient molecular data exhibit a range of morphological characters that suggest various possible placements. The species of the Ceratocumatidae are distinctly united by a morphological autapomorphy, a pair of small setose lobes on the propodus of the first pereopod. However, the Ceratocumatidae have a small, unarmed flap for a telson (derived), which its members share with members of Pseudocumatidae. Ceratocumatidae also possess five pairs of fully developed pleopods in the adult

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males, which they share with males of Bodotriidae. However, this character is most likely a plesiomorphy within Cumacea, and the character has therefore no relevance in a strict cladistic context. The Gynodiastylidae were initially considered to be part of the Diastylidae (Lomakina, 1958, Hale, 1946, Zimmer, 1941), although Stebbing (1912, 1913) suggested that they might be a separate family. Day (1980) resurrected Stebbing's family, and the family is defined by a high level of reduction, with a small, weakly armed telson that does contain the anus, no pleopods in the adult male and the loss of the exopod on maxilliped three in the female. The morphology suggests that the Gynodiastylidae could have a sister relationship with the Diastylidae based on the telson containing the anus and occasionally being armed with two small terminal setae. There is some support for a close relationship between these taxa by *Gynodiastylis* sp being nested within the Diastylidae (Figure 3H), albeit only represented by COI. Alternatively, there could be a sister relationship with the Pseudocumatidae, suggested by the reduced telson and reduction in pleopods.

Within the pleotelson clade, the Bodotriidae form a basal branch (Figure 4J) to a closely related clade consisting of Leuconidae and Nannastacidae, which agrees with the morphology very well. The Bodotriidae commonly have five pairs of pleopods in the adult male (or 4,3,2,0) vs. two (or 0) in the Leuconidae and none in the Nannastacidae. Species within the Bodotriidae are commonly encountered with the pleotelson fusion being less complete than in the Leuconidae or Nannastacidae, meaning that the telson is fused to the final pleonal segment and unable to move, but it extends posteriorly well between the pleopods, and there is a constriction delineating the fusion boundary of the pleotelson. This indicate that Leuconidae and Nannastacidae share a derived state of the pleotelson character, which then can act as an apomorphy for two families, while Bodotriidae possess a plesiomorphic state of the pleotelson. This can actually lead to confusion in identification of specimens, given the small and reduced telsons found in several of the telson bearing families. The Leuconidae and Nannastacidae are either nearly flat across the terminus of the pleon, or may be produced into a slight triangular or rectangular shape, but never have a large posterior protrusion with a constriction marking the fusion.

Most genera are supported, especially the morphologically well-circumscribed genera, and some genera that are known to be problematic, that is *Diastylis and Leptostylis*, are shown to be non-monophyletic. Our results disagree with those of Rehm et al. (2020), and our tree topology is very strongly supported. It is clear from the difference in support values between our 'full data' and 'single gene excluded' analyses, those incomplete datasets, or more so mixed datasets, containing taxa with one gene only, strongly affect the phylogenetic reconstruction. It then becomes clear that one cannot assess family level relationships using a single, highly variable sequence alone, such as 16S, which is more suitable for assessing species delimitations and cryptic speciation (Rehm et al., 2007).

In the full dataset analyses, the Leuconidae is split between a group of Antarctic species (Leuconidae I, Figure 3I) and non-Antarctic species (Leuconidae II, Figure 3M,N). It is possible and would be extremely interesting, if this split is reflecting a case of Antarctic isolation. However, the Antarctic species in our study are represented by 16S sequences only, and as already discussed, when species with only a single gene are excluded, nodes in our analyses gain higher support and collapsed and/or ambiguous relationships are resolved. In this case, the Leuconidae become monophyletic. By removing the Antarctic species, we are of course severely limited in presenting support for our 'limited data' hypothesis. So, until Antarctic species can be represented with complete data, it will remain unclear whether the division of the Leuconidae between Antarctic and non-Antarctic species is real or an artefact.

Within the Diastylidae (Figures 2F and 3F), the genera *Diastyloides* and *Dimorphostylis* are recovered, but none of the other genera are recovered as monophyletic. This is not surprising, as the larger diastylid genera are globally distributed and not well-circumscribed morphologically. In the case of *Leptostylis, Diastylis* and *Makrokylindrus*, it has been known for a long time that the generic definitions are not adequate and there are 'defining' morphological characters (telson length, proportions, setation; adult male antenna one and antenna two morphology) that are clearly continuous (see Day, 1980 for a discussion). None of our analyses recovers *Diastylis* or *Leptostylis* as monophyletic and the phylogeny indicates that the family Diastylidae is in need of a thorough taxonomic revision.

The family Gynodiastylidae is only represented by a partial COI sequence; thus, the placement of the family within the Diastylidae clade (Figure 3F) is uncertain, given that COI is not a suitable sequence for assessing deeper nodes, although it is useful for assessing population relationships within cumacean species (Teske et al., 2006).

The only cumacean family entirely missing in the present study, Ceratocumatidae, possesses a telson, in effect placing it in the telson bearing clade. However, the phylogenetic position of the family within the telson bearing clade is still not known, and clarification must await

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future morphological and/or molecular analyses upon collection of appropriately preserved specimens.

The phylogeny represents a great stride forward in cumacean systematics, in that families are largely recovered as monophyletic, and the strong support for the telson/ pleotelson split resolves basic questions about the evolutionary history of the group. The relationships within the telson/pleotelson clades are also strongly supported (Figures 3 and 4), providing a starting point for assessing directionality of change in morphological character transformations. The results are also very promising because it is now plausible to work on cumacean phylogenies without the concern that the families are polyphyletic, making generic level phylogenies a rewarding exercise.

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