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AN INTEGRATED PHYLOGENOMIC APPROACH FOR POTENTIAL HOST-ASSOCIATED EVOLUTION OF MONSTRILLOID COPEPODS

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Abstract Copepods, small crustaceans, exist in almost every aquatic environment on Earth, from freshwater to hypersaline and deep-sea bottom to high mountains. They play an important role in linking primary producers to higher consumers and thus contribute to the material and energy circulation in the ecosystems. The subclass Copepoda is recognised as ten orders, and each group shows highly optimised morphology and lifestyle to diverse habitat conditions. The Monstrilloida Sars, 1901 is one of the most obscure copepod groups with an unusually atrophied morphology and unique semi-parasitic lifestyle of endoparasitic juvenile and free-living, planktonic adult phases. The lack of common diagnostic features from their morphological peculiarity and little information about the endoparasitic juveniles have caused many uncertainties and ambiguities in their taxonomy and phylogeny. To elucidate phylogenetic relationships and evolutionary significance of these organisms, we first generated two genomes and three transcriptomes from four monstrilloid species (*Monstrilla grandis*, *Caromiobenella ohtsukai*, *Monstrillopsis longilobata* and *Maemonstrilla* sp.) and analysed the 25 nuclear protein-coding genes from 40 arthropod species. The molecular phylogenomic results supported the monophyly of Monstrilloida within Podoplea. Our analysis revealed that the Monstrilloida was more closely related to the Harpacticoida (Oligoarthra) than the Siphonostomatoida. These phylogenomic relationships for the Copepoda were confirmed by statistical tree topology tests, and previously known phylogenies were rejected. Our arthropod phylogeny identified a long branch leading to the Monstrilloida. Given the new phylogeny, we tested hypotheses about monstrilloid evolution by integrating the known morphological and ecological traits of four monstrilloid genera.

Keywords: Copepod Evolution; Monstrilloida; Crustacea; Phylogenomics; Divergence Time

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Introduction

Historical overview of the order Monstrilloida

Krøyer reported the first monstrilloid *Thaumatoessa typica* in 1842 by providing illustrations without descriptive text. Seven years later, the author gave an extended description and diagnosis for the species accompanied by additional figures based on the same specimen but simultaneously changed the species name to *Thaumaleus typicus* without any emendatory note (Krøyer 1849). Grygier (1994a) later re-examined the holotype of *T. typicus* and found that the specimen is probably a congener belonging to *Monstrilla* Dana, 1849, which had been more widely recognised at that time. Therefore, the generic name *Thaumatoessa* Krøyer is a senior objective synonym of *Thaumaleus* Krøyer and a senior subjective synonym of *Monstrilla* Dana. Grygier (1995b) subsequently proposed the conservation of the latter two junior names and the suppression of *Thaumatoessa* in consideration of universal awareness to the public: *Thaumaleus* had been frequently used rather than *Thaumatoessa* in about 50 publications of the last 100 years of taxonomic works, and *Monstrilla* had also been widely recognised and used for over 140 years with more than 50 nominal species assigned into this genus. On the other hand, *Thaumatoessa* has only been used as a valid name in Hesse (1868).

Another nomenclatural issue between two different names *Thaumaleus* and *Monstrilla*, both regarded as congeneric, then arose. *Monstrilla* with the type species *M. viridis* was published on 4 September 1849, while the precise publication date for *Thaumaleus* and its type species *T. typicus* in 1849 was not given (Grygier 1995a,b). Thus, the date for the latter publication was automatically taken to be the last day of the year (i.e., 31 December 1849) according to Article 21.3 (the Determination of Date) of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature, ICZN) (Grygier 1994a, 1995b). As a result, *Monstrilla* has priority over *Thaumaleus* according to Article 23 (the Principle of Priority) of the ICZN code and has eventually been the type genus of the family Monstrillidae Dana, 1849 and of the order Monstrilloida Sars, 1901.

The generic names *Thaumaleus* and *Monstrilla* continued to be used for some time before the nomenclatural revision of Grygier (1995b). During this time, *Thaumaleus* was considered a synonym of another genus *Cymbasoma* Thompson, 1888 rather than *Monstrilla* (Giesbrecht 1892a,b, Davis 1949, Isaac 1975). The incorrect synonymy resulted in *Thaumaleus* containing another form of monstrilloids. This conflicts with the diagnoses of *Thaumaleus* and *T. typicus*. Sars (1921) was clearly aware of the issue and proposed that most *Thaumaleus* species, except for *T. typicus* Krøyer, should be considered as the species of *Cymbasoma* (also see Rose 1933, Grygier 1994a). Sars suggested an additional genus *Monstrillopsis* for *Monstrilla dubia* Scott, 1904 (= *Monstrillopsis dubia*). This differs from the diagnoses of either *Monstrilla* Dana (= *Thaumaleus* Krøyer) or *Cymbasoma* Thompson. There were several other generic names, such as *Haemocera* Malaquin, 1896, *Thaumatohessia* Giard, 1900, and *Strilloma* Isaac, 1974, but these are now considered invalid or uncertain (Suárez-Morales & Gasca 2004, Grygier & Ohtsuka 2008). In the 2000s, there four additional genera were named, *Maemonstrilla* Grygier and Ohtsuka (2008), *Australomonstrillopsis* Suárez-Morales and McKinnon (2014), *Caromiobenella* Jeon, Lee and Soh (2018), and *Spinomonstrilla* Suárez-Morales, 2019 (Grygier & Ohtsuka 2008, Suárez-Morales & McKinnon 2014, Jeon et al. 2018a, Suárez-Morales 2019). Recent advances in the taxonomic methodology have provided detailed and definitive morphological characteristics for the monstrilloid taxonomy, but doubts about the validity of some genera remain (Jeon et al. 2018a,b).

General morphology of adult monstrilloids

The male body consists of nine somites including a cephalothorax fully incorporated with the first pedigerous somite (= first thoracic somite), free pedigerous somites 1–3 (= second to fourth thoracic somites), first urosomal somite, genital somite, postgenital somite, penultimate somite and anal

somite (Figure 1). The females have a genital compound somite (sensu Grygier & Ohtsuka 2008) which is formed by a fusion of the genital and succeeding postgenital somites, and thus, they present one less body somite than their corresponding males. The species of *Cymbasoma* typically have one more less urosomal somite in each sex, and thus, eight somites in males and seven in females (Thompson 1888, Suárez-Morales & McKinnon 2016). The large cephalothorax lacks common mouth appendages from mandibles to maxillipeds including antennae. Several pairs of scars that remained after discarding feeding tubes used for their endoparasitic juvenile stages are present on the ventral surface of the cephalothorax instead (Huys & Boxshall 1991, Suárez-Morales 2011) (Figure 1). The scars vary in number, size, shape and location depending on the genus and species; three pairs of the scars are common in *Monstrilla*, *Maemonstrilla* and *Caromiobenella*, and one or two pairs in *Monstrillopsis* and *Cymbasoma*. The maximum number of five scar pairs was reportedly known from *Spinomonstrilla spinosa* (Suárez-Morales 2019). A cone-shaped oral papilla is located in the ventral medial axis of the cephalothorax, but its fore-and-aft position varies by the genera or species; it is often located in the midlength of the cephalothorax in *Monstrilla*, whereas

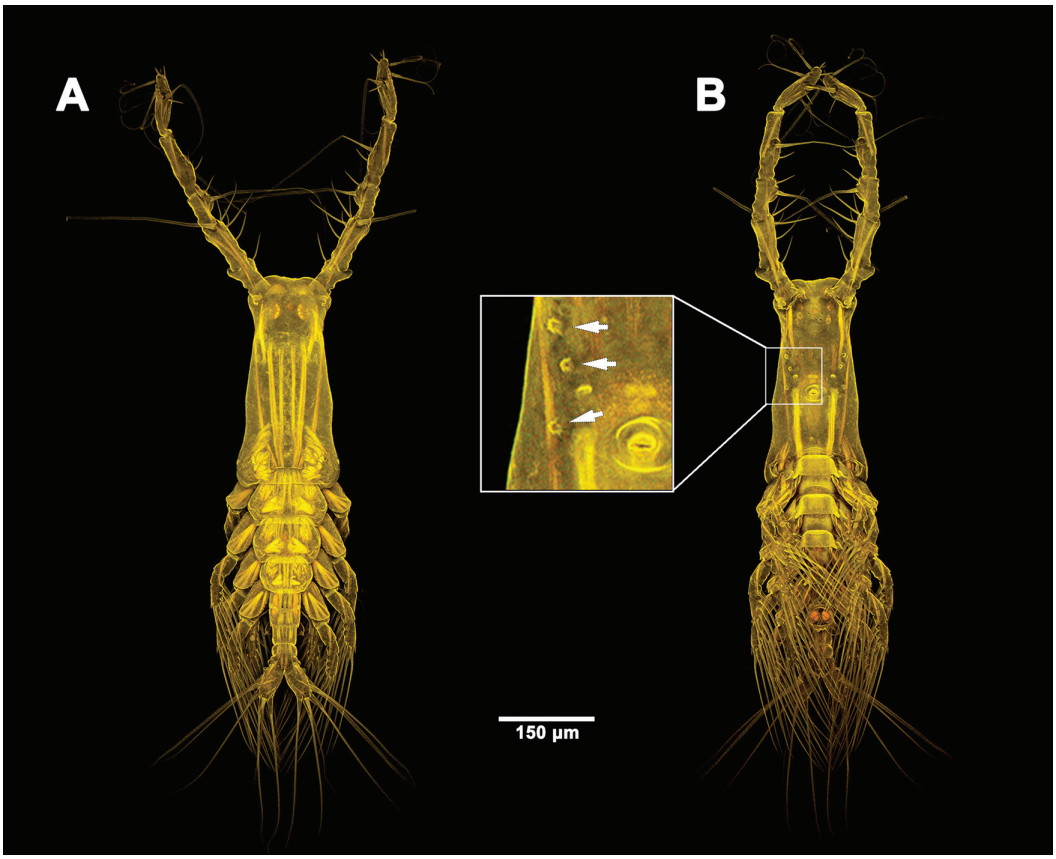

Figure 1 Confocal laser scanning microscope image of adult male *Monstrilla grandis*. (A) habitus, dorsal view. (B) habitus, ventral view. The inset illustrates three scars (arrows) on the right ventral side of cephalothorax. Monstrilloid specimen preserved in 4% borax buffered formalin was stained by dissolved Congo Red for 24 hours at room temperature. The stained specimen was washed in distilled water until no solute staining dye was present and then observed on a confocal laser scanning microscope Leica TCS5 (Leica, Germany) equipped with an optical microscope Leica DM5000 (Leica) using three visible lasers (Argon, DPSS, and HeNe). Scale bar in a micrometer. Microphotographs were provided by Dr. Seunghan Lee (Marine Act Co., Korea).

that of the other genera is more anteriorly. The developmental degree of the oral papilla also shows some differences by the genera; females of *Maemonstrilla* typically have a very large, prominent one (Grygier & Ohtsuka 2008, Suárez-Morales & McKinnon 2014), but males of *Caromiobenella* have a low, inconspicuous one (Jeon et al. 2018a).

All monstrilloids have a pair of anteriorly straight antennules each with a fundamental 5-segmented structure. Although, in females, the segmentation is often indistinct due to fusions between the segments, a quite conservative setation pattern appearing in both sexes provides a ground for assuming the putative segmentation plan for the female antennules. The last antennular segment (= fifth segment) of the males represents at least three different types of modifications, and this characteristic is regarded as one of the most evident features distinguishing the monstrilloid genera (Huys & Boxshall 1991, Suárez-Morales 2011). The first type (= type I) is a segment showing no specific modification, and this is present in the species of *Monstrilla* and *Cymbasoma*. The second type (= type II) is a segment with a hyaline bump on the inner proximal half margin and a gradually tapered distal half with a slight inner curvature. This type is currently exclusive for the males of *Monstrillopsis*. The third type (= type III) is a segment with five transverse serrate ridges on the inner distal margin and is only known from the males of *Caromiobenella*. In addition to these major types, two other modifications are also known. The male of *Cymbasoma longispinosum* presents similar ridges of the third type antennular modification, but these are much reduced (= type IV sensu Huys & Boxshall 1991). Suárez-Morales and McKinnon (2014) found another modification in which the last segment bears two inner rounded expansions along the inner margin (putatively, type V). The antennules exhibit four kinds of armature elements: aesthetascs, spines, simple setae and branched setae. The branched setae are present only in the last antennular segment, but all of these elements are replaced with simple ones in the species of *Caromiobenella* (Jeon et al. 2018a).

Monstrilloids have four well-developed pairs of swimming legs that have a similar structure (Figure 2). The protopod of each leg consists of a large coxa and a relatively small basis. The border

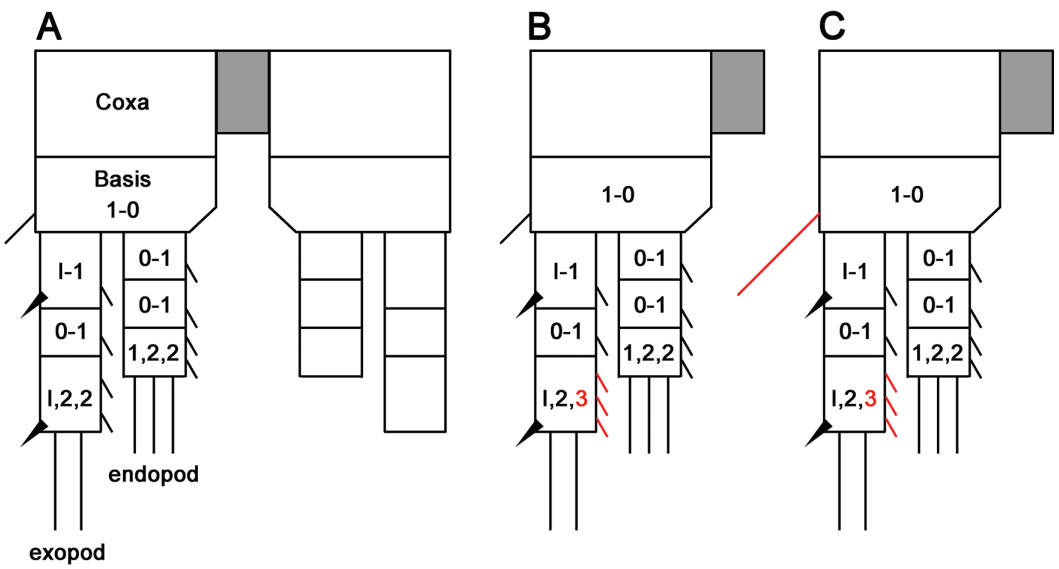

Figure 2 Schematic diagrams of swimming legs 1–4 with setation patterns. (A) pair of leg 1, each conjoined by a intercoxal sclerite (grey box). (B) legs 2 and 4. (C) leg 3. Arrowheads and lines indicate spinous and setal elements, respectively. Red-coloured numbers and elements represent the difference from the leg 1. Lines for setae, except for the basal setae, do not represent their actual length or ratio. Nomenclatural terms for describing the setation were adopted from Sewell (1949): Roman and Arabic numerals indicate the number of spines and setae, respectively.

between the coxa and basis is clearly defined from the posterior side by a clear diagonal articulation, while the anterior border is indistinctly defined by a fine seam. The basis bears two rami on its distal margin, each is typically developed into a tri-articulated endopod or exopod. Only *Cymbasoma rafaelmartinezi* is known to have a 2-segmented exopod for the swimming leg 4 (Suárez-Morales & McKinnon 2016). In addition, a similar setation pattern for the swimming legs is shared through the species. The basis of the legs 1, 2 and 4 presents a short, thin outer seta (Figure 2A and B), but this seta in leg 3 is much longer and thicker (Figure 2C). Another difference appears through the third exopodal segments of the legs. The third exopodal segments of legs 2–4 have the setation pattern of "I, 2, 3". This pattern describes one spine (in Roman numeral) on the outer distal corner, and two apical and three inner setae (in Arabic numerals) following the nomenclature of Sewell (1949). In this aspect, the third exopodal segment of leg 1 shows one less setal element on the inner margin, thus "I, 2, 2" pattern (Figure 2A). Further reduction of the setal elements occurs in the females of the *Maemonstrilla hyottoko* species group, but not for the *Maemonstrilla turgida* species group, due to the absence of inner setae from both first exopodal and endopodal segments (Grygier & Ohtsuka 2008, Suárez-Morales & McKinnon 2014).

A pair of fifth legs is another relevant morphological character for the monstrilloid taxonomy. The fifth legs are mainly present in females. The most ancestral form is biramous with unarticulate inner and outer lobes each armed with two and three setae, respectively. The most simplified structure of an elongate rod-shaped leg with two apical setae was found in the *M. hyottoko* species group (Grygier & Ohtsuka 2008). In addition, there are various types of fifth legs in the female that differ in structure and setation pattern (see section "Congruence between morphological and molecular phylogenies for Monstrilloida"). On the contrary, most males do not have a fifth pair of legs, except for some *Monstrilla* species, such as *Monstrilla grandis*, *Monstrilla longicornis* and *Monstrilla conjunctiva*. The fifth pair of legs of these latter species each are represented by a small knob-like protuberance with a long, prominent apical seta.

Males have a well-developed genital apparatus ventrally in their genital somite. The apparatus is represented by a robust, protrusive basal shaft and two lappets diverging from the distal posterior end of the shaft. The shaft can be short and broad, or elongate and cylindrical. The lappets are also various in shape including a digitiform, lamella-form or subtriangular form. The combinations of such structural variety (see Suárez-Morales 2011) result in one of the most utilisable features for identifying and distinguishing males. The genital organ of females is developed into a pair of two long spines (= ovigerous spines) and is directed posteriorly, but the females of *Maemonstrilla* have anteriorly directed spines. In *Cymbasoma*, there are two structural types of the ovigerous spines known: (1) each spine separately arises from the base as in most female monstrilloids or (2) arises as a single thread, running for a short distance, and then separates into two (especially in *C. longispinosum* species group) (Grygier 1994b, Suárez-Morales & McKinnon 2014, Üstün et al. 2014, Suárez-Morales et al. 2020).

The caudal rami present a different number of setae by the genera or species. Five or six caudal setae on each ramus are frequently found in *Monstrilla*, *Caromiobenella*, *Maemonstrilla*, *Australomonstrillopsis* and *Spinomonstrilla*. According to Suárez-Morales et al. (2006), both sexes of *Monstrillopsis* are distinct with four caudal setae. Further reduction of the number of the caudal setae is seen in *Cymbasoma*, with females always having three setae, while some males have four.

Biology and ecology

Monstrilloids have a protelean life cycle that consists of an endoparasitic juvenile phase and a free-living, planktonic adult phase (Malaquin 1901, Suárez-Morales et al. 2014). After hatching, the early lecithotrophic nauplii are initially planktonic, but soon infect various marine invertebrate hosts including polychaetes, gastropods, bivalves and sponges (Malaquin 1901, Caullery & Mesnil 1914, Pelseneer 1914, Gallien 1934, Huys & Boxshall 1991, Suárez-Morales et al. 2014). After

infection, development continues in the host, fuelled by absorbing the host's body fluids using pairs of tailored feeding tubes (Malaquin 1901, Raibaut 1985, Suárez-Morales et al. 2014). These tubes may be altered antennae, adjacent mouth appendages or both. The immature adults, presumably at their copepodid V stage, emerge from the host and undergo a final moult to the mature form which leads to a planktonic life. During emergence, maturing adults discard the feeding tubes leaving specific scars on the ventral surface of the cephalothorax. The adults are non-feeding with a lack of mouthparts and are regarded as solely reproductive (Suárez-Morales 2011).

Monstrilloid parasites can inflict negative effects on the host causing physical damage, structural alteration, castration and inducing strong inflammatory response (Malaquin 1901, Suárez-Morales et al. 2010, 2014). However, it is not clear whether those effects eventually lead the hosts to mortality. In some cases, the host is able to recover quickly from the damage. The damage itself does not appear to be very detrimental to the host (Malaquin 1901, Suárez-Morales et al. 2010). Conversely, in another instance, the damage may cause mortality (Suárez-Morales et al. 2014). In regarding to this, Suárez-Morales (2018) mentioned that the relative size of the host and the intensity of infection are also important factors determining the fate of the hosts.

Adult monstrilloids are, in general, scarce in the marine water columns but are known to occur aggregated in reef-related environments (Sale et al. 1976, Sekiguchi 1982, Suárez-Morales 2001, Grygier & Ohtsuka 2008). Suárez-Morales (2001) also found that the adult monstrilloids appear in a higher density in the water column at twilight suggesting that they remain near the bottom during the day and swim up towards the surface at night.

Diversity and geographical distribution

The review of Suárez-Morales (2011) recognised about 120 species of monstrilloids in four genera (*Monstrilla*, *Cymbasoma*, *Monstrillopsis* and *Maemonstrilla*) and noted that 73% of those were known from the North Atlantic (45% from the Northeast Atlantic including the European waters; 17% from the Northwest Atlantic; 11% from the Mediterranean Sea), whereas less than 10% were from the Asian (8%) and the Australian (~3%) waters. Since then, over 50 species have been described along with the proposal of three new genera in the past decade. In particular, Suárez-Morales and McKinnon (2014, 2016) reported 36 species in four genera (more than 20% of the species known so far), including 33 then-undescribed species and an additional genus, only from the Australian waters. As such, these studies revealed a much higher regional monstrilloid diversity in a less studied area and simultaneously suggested a promising number of new species in other unexplored regions as well (Suárez-Morales 2018).

The order Monstrilloida currently contains over 170 nominal species in a single family Monstrillidae: 51 species of *Monstrilla*, 77 of *Cymbasoma*, 21 of *Monstrillopsis*, 11 of *Maemonstrilla*, ten of *Caromiobenella*, one of *Australomonstrillopsis* and one of *Spinomonstrilla* (Suárez-Morales 2000, Razouls et al. 2005–2022, Walter & Boxshall 2022, WoRMS Editorial Board 2022). With respect to regions, Australian waters have the highest species richness (36 species) followed by the Northwest Atlantic including the Caribbean Sea and the Gulf of Mexico (34), the Northeast Atlantic including the European seas (32), the Mediterranean-Black Sea region (26), the Indonesia-Malaysia-Philippines region including southern China and Japan (i.e., Okinawa) (25), the waters around northern Japan and Korea (19), and the Brazil-Argentine region (15) (Suárez-Morales & Grygier 2021). Africa and the East Pacific regions have very low monstrilloid diversity with only three species known from African waters and 13 from the East Pacific region (Suárez-Morales 2018).

Among 172 nominal species, only 38 of them (22% of total species) are known from both sexes, whereas 134 species (78%) are described based on material for one sex; 49 of from males and 85 from females. Incomplete, sex-biased species determinations have prompted nomenclatural problems: both sexes of the same species were recorded under different names (see Grygier 1994a, Suárez-Morales et al. 2008, Rosa et al. 2021), or, conversely, each sex of different species was

identified as one species (see Suárez-Morales et al. 2006). As a result, there are still many uncertain species with taxonomic and nomenclatural problems; thus, the true diversity of the monstrilloids is likely to differ from what we know at present.

Phylogeny

Early phylogenetic investigations into the Copepoda including Monstrilloida were carried out mainly based on their morphological characteristics (Ho 1990, 1994, Huys & Boxshall 1991, Ho et al. 2003, Dahms 2004). These studies generally indicated that the monophyletic Copepoda is divided into two infraclasses, the Progymnoplea (order Platycopioida) and the Neocopepoda. The latter encompasses two superorders the Gymnoplea (order Calanoida) and the Podoplea. In the podoplean phylogeny, Monstrilloida had frequently shown a close relationship with another parasitic group, the Siphonostomatoida (Huys & Boxshall 1991, Ho 1994, Ho et al. 2003). As Huys et al. (2007) pointed out, the previous Siphonostomatoida-Monstrilloida clade (Huys & Boxshall 1991) was formed based on convergent features. Thus, the phylogenetic relationships between the two orders may not indicate a recent common ancestry. In addition to the issue of morphological convergence, inevitable errors arising from the process of the identification of structural homology, determination of character states (e.g., plesiomorphic or apomorphic) and application of different weighting criteria for morphological data have also been potential pitfalls leading to faulty phylogenetic inference (Ho 1994, Eyun 2017).

Most molecular research supports two main Neocopepoda clades (i.e., the superorders Gymnoplea and Podoplea) (Oakley et al. 2013, Eyun 2017, Schwentner et al. 2018, Lozano-Fernandez et al. 2019, Song et al. 2021) and is congruent with results from the previous morphology-based studies (Huys & Boxshall 1991, Ho 1994, Ho et al. 2003). However, some molecular-based studies have produced somewhat different copepod phylogenies (Braga et al. 1999, Schwentner et al. 2017) with respect to the possible subgroups and subsequent separations such as for the Poecilostomatoida (Tung et al. 2014) and the Harpacticoida (Schizas et al. 2015). Therefore, the relationships within the podoplean lineage remain highly controversial. Under these circumstances, the Monstrilloida has frequently been overlooked and its molecular phylogenetic position therefore remains unknown. Huys et al. (2007) suggested that the Monstrilloida appears to be nested within the Siphonostomatoida. Their molecular analyses based on 18S ribosomal RNA sequences (18S rRNA, a total aligned length of 1941 base pairs) revealed a specific molecular affinity to caligiform-family siphonostomatoids and implied the consequent demise of the order Monstrilloida. However, a similar molecular study using the same 18S rRNA data with the inclusion of the Harpacticoida taxa (a total aligned length of 1878 base pairs) indicated an unresolved polytomy among the three orders: the Harpacticoida, Siphonostomatoida and Monstrilloida (Ki et al. 2009). Thus, there is no agreed phylogenetic position for monstrilloids with either morphological or molecular data.

The main purpose of our study was to clarify the unknown phylogenetic position of the Monstrilloida among copepod orders. For this, 25 nuclear protein-coding genes were obtained from the draft genomes and transcriptomes of four monstrilloid species (*Monstrilla grandis* Giesbrecht 1891, *Caromiobenella ohtsukai* Jeon, Lee & Soh 2019, *Monstrillopsis longilobata* Lee, Kim & Chang 2016, and *Maemonstrilla* sp.) (four genera among seven monstrilloid genera). The orthologous sequences from the multiple species of Arthropoda (Hexapoda, Branchiopoda, Cephalocarida, Remipedia, Decapoda, and Thecostraca) including the four major copepod orders (Calanoida, Cyclopoida, Harpacticoida and Siphonostomatoida) were also obtained to reconstruct the phylogenomic tree. The inclusion of 21 copepod species from five groups greatly enhances our ability to make inferences regarding copepod evolution. Our results reaffirmed the previously known monophyly of Copepoda within Arthropoda and revealed the detailed phylogenetic position of the Monstrilloida within the Copepoda. The phylogenomic results detected a particularly long branch leading to the Monstrilloida. The present phylogenies and the sequence divergences also suggested

high gene evolutionary rates for this copepod group. Given all present molecular results, we discussed the hypothesis of the evolution of the Monstrilloida by integrating their morphological and ecological characteristics.

Methods

DNA/RNA sample preparation for next-generation sequencing

Genomic DNAs were extracted from *Monstrillopsis longilobata* (83 adults) and *Maemonstrilla* sp. (101) using MagAttract HMW DNA kit (Qiagen, Germany) by following the manufacturer's instructions, and the DNA libraries were constructed using TruSeq DNA Nano kit (insert size of 550 bp; Illumina, USA). Total RNA was extracted from *M. longilobata* (ten adults), *Monstrilla grandis* (15) and *Caromiobenella ohtsukai* (26) using mirVana kit (Thermofisher, USA). Total RNA libraries were constructed using TruSeq Stranded Total RNA LT sample prep kit (insert size of 300 bp; Illumina, USA). The libraries were sequenced using either 101 or 151 bp read lengths on NovaSeq6000 and HiSeq2500 (Illumina, USA) according to the manufacturer's recommended protocol (Supplementary Table 1).

Genomic/transcriptomic data and de novo assembly

Publicly available genome and transcriptome assemblies for 11 species were downloaded from the National Center for Biotechnology Information (NCBI) including two copepod genomes (*Eurytemora affinis* (Eyun et al. 2017) and *Tigriopus californicus* (Barreto et al. 2018)) (Supplementary Table 2). Additionally, next-generation sequencing data for 24 arthropods (Supplementary Table 3) were also obtained from the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra). The assembly pipeline in this work is discussed in our previous papers (Jung et al. 2020a,b). The quality control and trimming of the SRA data and the newly obtained sequencing reads were performed using `fastp` (ver. 0.20.1) (Chen et al. 2018). After read filtering process with the stringent criteria (with PHRED score Q28 or more and minimum length requirement of 75 bp for transcriptomic reads and of 125 bp for genomic reads), *de novo* assemblies were generated using Trinity (ver. 2.10.0) (Grabherr et al. 2011) and SPAdes (ver. 3.15.2) (Bushmanova et al. 2019): the transcriptomic assemblies for three monstrilloids, *Monstrilla grandis*, *Caromiobenella ohtsukai* and *Monstrillopsis longilobata*, were conducted using Trinity with the default option, and genome assemblies for *M. longilobata* and *Maemonstrilla* sp. were performed using SPAdes with the option "`--isolate`". Quality of the assemblies was estimated using BUSCO (ver. 5.3.2) (Manni et al. 2021) with the Arthropoda lineage dataset (`arthropoda _ odb10` as of September 10, 2020) containing a total of 1013 marker genes.

Gene mining, identification of orthologous genes and data preparation

Twenty-five nuclear protein-coding genes were initially obtained from the GenBank protein sequence datasets for 11 arthropods in the previous studies (Regier et al. 2008, Eyun 2017). With the acquired gene sequences as the queries, the orthologous sequences were searched using `tblastn` (Basic Local Alignment Search Tool; BLAST, ver. 2.10.0+) (Camacho et al. 2009) against the newly assembled genomes and transcriptomes for 30 arthropod species. The coding regions were predicted using `GeneWise` (ver. 2.2) (Birney et al. 2004) to determine the open reading frames and the intron/exon boundaries. The translated amino acid sequences were inspected using SMART (Letunic et al. 2021). All 25 protein-coding gene sequences from 40 arthropod species (Supplementary Table 4) were collected and verified again by `blastp` against the NCBI non-redundant (NR) protein database.

Three datasets were arranged by different taxon sampling. The arthropod dataset (AD) contained 40 arthropod species including two non-pancrustacean taxa, the blacklegged tick *Ixodes*

scapularis (Chelicerata) and centipede *Strigamia maritima* (Myriapoda). The second and third sub-datasets consisted of only the sequences of copepods with the malacostracan and thecostracan taxa as outgroups. The difference between the datasets was an inclusion or exclusion of Harpacticoida sequences; Copepoda dataset with Harpacticoida (CD) and Copepoda dataset without Harpacticoida (CDwoH). Thus, the number of taxa is 27 in CD and 24 in CDwoH. The purpose of the sub-datasets is to reconstruct the copepod phylogenies as similar to the previous molecular results in the aspect of the number of orders. We examined the robustness of our phylogeny under various taxon-sampling conditions and further compared it more directly with previous molecular phylogenies (Ho 1990, 1994, Huys & Boxshall 1991, Ho et al. 2003, Huys et al. 2007, Ki et al. 2009), regardless of taxon bias. The amino acid sequences used in this study are available in Additional File 1.

Phylogenetic analysis

Multiple sequence alignments for the 25 nuclear protein-coding genes in three different datasets (AD, CD, and CDwoH) were individually generated using MAFFT (ver. 7.475) (Katoh & Standley 2013) with the L-INS-i algorithm. The poorly aligned or many gap regions of the alignments were removed using `trimAl` (ver. 1.4.rev15) (Capella-Gutiérrez et al. 2009) with the option "`--automated1`" for further phylogenetic tree reconstruction. Best-fit phylogenetic model searches for each gene set were performed using IQ-TREE2 (ver. 2.1.2) (Minh et al. 2020) using Bayesian Information Criterion (BIC). The target models were trained only to the nuclear protein substitution models using the option "`--msub nuclear`" because all present genes have nuclear origins. The aligned protein sequences in each dataset were then concatenated into a single alignment using a custom-written Perl script (Eyun 2017) (Additional File 2). The maximum-likelihood (ML) and Bayesian inference (BI) analyses were performed by applying the best substitution models to the gene partitions in each dataset (Supplementary Tables 5–7). The ML tree was reconstructed using RAxML-NG (ver. 1.0.1) (Kozlov et al. 2019). The best tree with the highest log-likelihood score was selected among the initial 30 random and 30 parsimony trees. Bootstrap support values ($BP_{RAxML\text{-}NG}$, in percentage) were calculated with 3000 bootstrap replicates and provided with the transfer bootstrap expectation values (Lemoine et al. 2018). BI analysis was run for 3×10^6 generations, sampling every 100 generations using MrBayes (ver. 3.2.7a) (Ronquist et al. 2012). The model parameters across the partitions were unlinked to reflect probable different evolutionary rates by genes. The first 25% of the generations were discarded for the final tree reconstruction and the nodal support values ($PP_{MrBayes}$) were provided with the Bayesian posterior probability. An additional ML phylogenetic tree based on the AD was inferred by employing the protein mixture models (C20-profile mixture model) (Le et al. 2008). The best-fit model selection and subsequent best partitioning scheme determination (Supplementary Table 8) were performed in IQ-TREE2. The phylogenetic tree was reconstructed using the ultrafast bootstrapping algorithm implemented in IQ-TREE2, and bootstrap support values ($BP_{IQ\text{-}TREE2}$, in percentage) were calculated with 3000 bootstrap replicates. Graphical presentation of the phylogenies was performed using FigTree (ver. 1.4.4) (http://tree.bio.ed.ac.uk/software/figtree).

Alternative tree topology test

Statistical tree evaluations and confidence tests for the present phylogenies and those from studies by Ho (1990), Huys and Boxshall (1991) and Huys et al. (2007) were performed. An additional tree topology from Khodami et al. (2017) was also tested for support for one of our phylogenetic hypotheses, despite recent controversy (Mikhailov & Ivanenko 2021). The tree topologies were manually edited (Additional File 3) and categorised into two groups by the number of copepod taxa involved; three topologies consisting of five copepod orders (i.e., Calanoida, Cyclopoida, Harpacticoida, Siphonostomatoida and Monstrilloid) taken from other studies (Ho 1990, Huys & Boxshall 1991, Khodami et al. 2017) and one topology (Huys et al. 2007) consisting of only four copepod orders

lacking Harpacticoida. Each of these groups was compared to the two phylogenies based on CD and CDwoH of each given our 25 gene alignment. For the statistical assessment of the log-likelihood of each tree, the Kishino-Hasegawa (KH) (Kishino & Hasegawa 1989), Shimodaira-Hasegawa (SH) (Shimodaira & Hasegawa 1999) and Approximately Unbiased (AU) (Shimodaira 2002) tests were performed with 10,000 re-samplings using the RELL method in IQ-TREE2.

Sequence divergence calculation and divergence time estimation

Within-group sequence divergences for copepod orders were calculated based on the present protein and 18S rRNA sequences to detect possible high genetic mutation rates. The calculations were performed under various model parameters. For 18S rRNA sequences (see Supplemental analysis), the *p*-distance method and Kimura 2-parameter (Kimura 1980) model were used with 3000 bootstrap replicates. Similarly, the Dayhoff (Dayhoff et al. 1978) and Jones-Taylor-Thornton (JTT) (Jones et al. 1992) models including the *p*-distance method were applied for analysing protein sequence divergence. Additional attempts calculating overall mean divergence and within-group divergences at various taxonomic ranks were performed to explore the possibly different substitution rates among the arthropod groups involved in this study. This was done based only on the protein sequences under the JTT model. Divergence of each group was compared to overall mean divergence, and estimated rates were applied to the following divergence time estimation.

The divergence time of lineages was estimated using LSD2 (ver. 1.10) (To et al. 2016) with the least-squares method. The phylogenetic tree generated through ML analysis was provided as the user-defined topology. According to Wolfe et al. (2016), the minimum and maximum ages for four calibration points were applied; 497–636.1 Ma (million-year ago, in order of the minimum and maximum age boundaries) for the Branchiopoda-Hexapoda split, 405–521 Ma for the Anostraca-Cladocera split, 358.5–521 Ma for the Amphipoda-Decapoda split, and 313.7–411 Ma for the Diptera-Coleoptera split. The minimum constraint for the crown Pancrustacea was set to 525 Ma based on the fossil record of *Wujicaris muelleri* (Zhang et al. 2010). Two non-pancrustacean taxa were used as outgroups and removed from the final divergence time tree.

Results and Discussion

Genome and transcriptome assembly quality assessment

Three transcriptomes (*Monstrilla grandis*, *Caromiobenella ohtsukai* and *Monstrillopsis longilobata*) and two genomes (*Maemonstrilla* sp. and *M. longilobata*) were obtained using Illumina paired-end sequencing technology. The detailed information and statistical measurements for the sequencing and assembly results are summarised in Supplementary Table 1. The percentage of complete BUSCO gene coverage of the transcriptome assemblies ranged from 57.1% for *C. ohtsukai* to 84.4% for *M. grandis*, and those in the genome assemblies showed 45.6% for *M. longilobata* to 58.4% for *Maemonstrilla* sp. (Supplementary Figure 1). Although these assemblies generated did not contain the entire gene contents of the monstrilloids, we found many marker genes (Supplementary Table 4). We continued downstream phylogenetic analyses for the sake of providing any evidence for revealing the phylogenetic relationships of the Monstrilloida within the Copepoda.

Copepoda phylogeny within Arthropoda

Twenty-five nuclear protein-coding genes were obtained from 40 arthropod species including 21 copepods in five orders. Three concatenated sequence data (AD, CD and CDwoH) were generated, and each dataset consisted of 16,976, 17,436 and 17,478 amino acids. The ML and BI trees reconstructed based on AD showed four major splits for the pancrustacean group (Figure 3);

Hexapoda-Branchiopoda clade (HB clade) having 100/1.00/52 nodal support values (in order of $BP_{RAxML\text{-}NG}/PP_{MrBayes}/BP_{IQ\text{-}TREE2}$ analyses, see Materials for the abbreviations), Xenocarida (Remipedia-Cephalocarida clade) (Regier et al. 2010) with 65.5/1.00/100, Communostraca (Malacostraca-Thecostraca clade) (Regier et al. 2010) and Copepoda with 76.7/1.00/54. Our phylogenetic analyses at the Arthropoda level resulted in different tree topologies (RAxML-NG, MrBayes vs. IQ-TREE2 analyses). The HB clade took the most basal phylogenetic position among the given pancrustaceans in the RAxML-NG and MrBayes trees (Figure 3A), while the Xenocarida appeared as the most basal group in the IQ-TREE2 tree (the HB clade appeared as the second most basal group by following the Xenocarida; Figure 3B). The validity of the Allotriocarida clade (Oakley et al. 2013) has gained attention in recent studies (von Reumont et al. 2012, Oakley et al. 2013, Eyun 2017, Schwentner et al. 2017, 2018, Lozano-Fernandez et al. 2019). Our phylogenies failed to recover this clade. Therefore, questions on the validity of the Xenocarida or Allotriocarida remain unanswered. Nonetheless, our phylogenies still provided stable and consistent relationships within the class-level phylogeny with higher nodal support and without any unexpected inclusion of alien species into another taxon group. As the resolution of detailed relationships within the Arthropoda is beyond the scope of this study, thus the discussion should be focused on the phylogeny of the Copepoda.

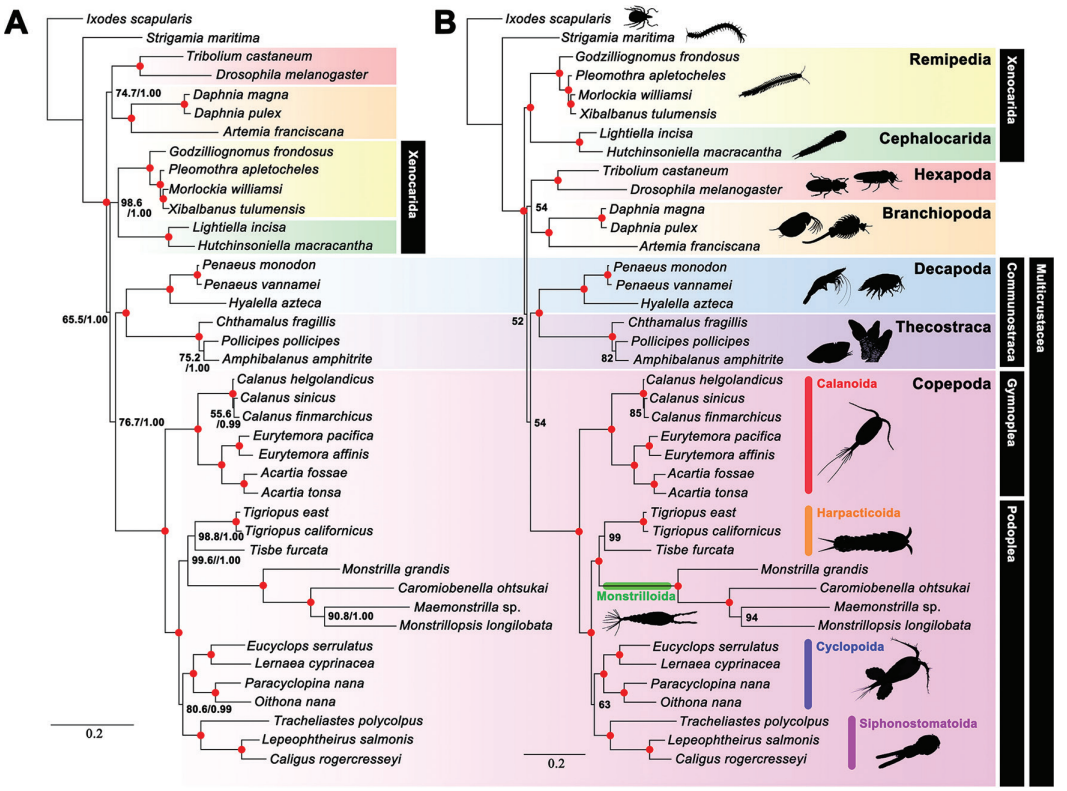

Figure 3 Phylogenies of 21 copepod and 19 other arthropod species inferred from 25 nuclear protein-coding genes. (A) Tree inferred using RAxML-NG and MrBayes. The numbers near the branching points indicate the maximum-likelihood bootstrap support values (BS, in percentage) and Bayesian posterior probabilities (PP, in probability) in order of BS/PP. (B) Tree inferred using IQ-TREE2 with the protein mixture model, C20. The numbers near the branching points indicate BS values. The red dots at the nodes indicate that BS and PP are 100% and 1.00, respectively. Non-pancrustacean species (*Ixodes scapularis*) is used as the outgroup. The clade names (Xenocarida, Communostraca, and Multicrustacea) are adopted from Regier et al. (2010).

Table 1 Statistical Comparisons among the Best Maximum-Likelihood Trees and Alternative Phylogenetic Hypotheses within the Copepoda Orders

Tree topology[a]	logL	ΔL[b]	*p*-KH[c]	*p*-SH[d]	*p*-AU[e]	References
(CAL,((HAR,MON),(CYC,SIP)))	−258924.0581	Best	1	1	1	This study
(CAL,(HAR,(MON,(CYC,SIP))))	−259028.8959	104.84	0	<0.001	<0.001	Ho (1990)
(CAL,(CYC,(HAR,(SIP,MON))))	−259059.0093	134.95	0	0	<0.001	Huys and Boxshall (1991)
(CAL,((HAR,CYC),(MON,SIP)))	−259039.0141	114.96	<0.001	<0.001	<0.001	Khodami et al. (2017)
(CAL,(MON,(CYC,SIP)))	−243919.5916	Best	1	1	1	This study
(CAL,(CYC,(nc-SIP,(MON,c-SIP))))	−244220.1269	300.54	0	0	<0.001	Huys et al. (2007)

[a] CAL = Calanoida, CYC = Cyclopoida, HAR = Harpacticoida, SIP = Siphonostomatoida, nc-SIP = non-caligiform siphonostomatoid (*Tracheliastes polycolpus*), c-SIP = caligiform siphonostomatoids (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*), MON = Monstrilloida.

[b] Δ*L*: log *L* difference from the maximal log *L* in the set.

[c] *p*-KH: *p*-value of the one-sided Kishino-Hasegawa test (Kishino & Hasegawa 1989).

[d] *p*-SH: *p*-value of the Shimodaira-Hasegawa test (Shimodaira & Hasegawa 1999).

[e] *p*-AU: *p*-value of the approximately unbiased (AU) test (Shimodaira 2002).

Within the Copepoda, two superorder divisions (Gymnoplea and Podoplea) were recovered ($BS_{RAxML\text{-}NG}=100\%$, $PP_{MrBayes}=1.00$, $BS_{IQ\text{-}TREE2}=100\%$) in congruence with previous morphological and molecular studies (Huys & Boxshall 1991, Ho 1994, Ho et al. 2003, Huys et al. 2006, 2007, Oakley et al. 2013, Tung et al. 2014, Schizas et al. 2015, Eyun 2017, Schwentner et al. 2018, Lozano-Fernandez et al. 2019). However, the interrelationships within the Podoplea showed unusual topologies involving groupings of Cyclopoida-Siphonostomatoida and Harpacticoida-Monstrilloida with strong nodal support ($BS_{RAxML\text{-}NG}=100\%$, $PP_{MrBayes}=1.00$, $BS_{IQ\text{-}TREE2}=100\%$). The multicrustacean sub-phylogenies based on CD and CDwoH also showed the same subsets of the present arthropod phylogeny (Supplementary Figures 2 and 3). Notably, none of these results provide evidence for the inclusion of the Monstrilloida within the Siphonostomatoida or a close affinity between them as in other studies (Huys & Boxshall 1991, Ho 1994, Ho et al. 2003, Huys et al. 2007). The present results were inferred based on a limited number of the five copepod orders with the inclusion of three species from the Siphonostomatoida. Given the limited availability of the genomic and transcriptomic data from copepods, we also conducted a larger phylogenetic analysis using the 18S rRNA sequences from the previous molecular phylogenetic studies (Huys et al. 2006, 2007, 2009, Khodami et al. 2017, Vakati et al. 2019) to test for other possible relationships (see Supplemental analysis). We used 129 species in our analysis from nine copepod orders and 98 species from five orders (Supplementary Figures 4 and 5). Our 18S rRNA-based analyses yielded unstable phylogenetic relationships among the copepods with a general lack of support ($BS_{RAxML\text{-}NG}>43\%$, $PP_{MrBayes}>0.13$) (Supplementary Figure 4). All the 18S rRNA-based results including those from the previous studies have shown a different tree topology to each other. Furthermore, the statistical assessments on the alternative tree topology tests including the present phylogeny and the others from the previous studies (Ho 1990, Huys & Boxshall 1991, Huys et al. 2007, Khodami et al. 2017) showed that our copepod phylogenetic hypothesis as the most likely one, while rejecting the other hypotheses at the 0.001 significance level (Table 1). The choice of suitable sequence data with sufficient phylogenetic signal and securing taxon-sampling from wider taxa are probably most important for successful phylogenetic inference (Rosenberg & Kumar 2001, 2003, Blanco-Bercial et al. 2011, Eyun 2017, Mikhailov & Ivanenko 2021). In this respect, the analyses based on partial or short single gene sequences dealing with a wide range of taxa might have a limited power to reveal true relationships.

Our phylogenomic results suggest that the Monstrilloida is a monophyletic group with a closer affinity to the Harpacticoida than the Siphonostomatoida. This contrasts with morphology-based copepod phylogenies (Ho 1990, 1994, Huys & Boxshall 1991, Ho et al. 2003). In general, previous

morphology-based phylogenies have formed an extended parasitic (or parasitic taxon-rich) clade involving not only the Siphonostomatoida and the Monstrilloida but also the Poecilostomatoida and the Thaumatopsylloida. However, the latter two orders were found to be the subgroups of Cyclopoida (Boxshall & Halsey 2004, Huys et al. 2006, 2012, Khodami et al. 2019). Thus, the parasitic copepod clustering in preceding morphology-based phylogenies does not reflect their true evolutionary relationships. Convergent evolution rules the appearance of similar phenotypic or analogous structures among phylogenetically distant species due to adaptive responses to similar environments or ecological niches (Huang et al. 2015, Speed & Arbuckle 2016, Castiglione et al. 2019). Thus, the previous morphology-based phylogenies which include convergent features (Ho 1994, Huys et al. 2007, Eyun 2017) show other relationships. Our phylogenetic results based on genetic data provide a different perhaps more objective view and suggest that the Monstrilloida has a different stem lineage from the Siphonostomatoida and a closer affinity to the Harpacticoida.

The relationship between the Cyclopoida and the Siphonostomatoida needs more research attention. It may be worth noting that the aesthetasc arising from the ancestral antennular segment XXI is typically stable within the Siphonostomatoida, even in the highly transformed genus *Spongiocnizon* (Huys & Boxshall 1991). This aesthetasc is also present in the Misophrioida and the Cyclopoida (including Poecilostomatoida) but not in the Harpacticoida and the Monstrilloida (Huys & Boxshall 1991). The loss of the key features in the Monstrilloida may be another piece of evidence to reject the close phylogenetic relationship with the Siphonostomatoida, supporting formations of two clades, the Cyclopoida-Siphonostomatoida and the Harpacticoida-Monstrilloida, in congruence with our molecular results.

Congruence between morphological and molecular phylogenies for Monstrilloida

This study provides the phylogenomic evidence for the relationships among four monstrilloid genera (*Monstrilla*, *Monstrillopsis*, *Maemonstrilla* and *Caromiobenella*) among seven valid genera (additionally, *Cymbasoma*, *Australomonstrillopsis* and *Spinomonstrilla*) and suggests that the last common ancestor (based on the phylogenetic tree) is *Monstrilla* and *Monstrillopsis* has emerged recently in evolution. Then, as a follow-up question, can the present molecular relationships explain the morphological evolutionary pattern or is there a mutual agreement between the morphological and molecular phylogenies? It is known that the copepod evolution is primarily proceeded by structural oligomerisation (i.e., a loss or fusion in body parts) and reappearance of ancestral (complex) character state from once derived (simplified) state in descendants is an extremely rare case (Huys & Boxshall 1991). These findings imply that a species or group retaining more intricate plesiomorphic features is morphologically closer to their last common ancestor.

The genus *Monstrilla*, particularly *Monstrilla grandis* involved in this study, exhibits the most complex morphological character combination including the largest number of body somites (eight in the female and nine in the male) and caudal setae (six in both sexes), a pair of a bilobed female fifth leg with a maximum of five setae (three on the outer and two on the inner lobes; i.e., 3-2 setal pattern; Figures 4 and 5A) and the presence of a pair of fifth legs (each a small knob-like protuberance with a long seta) even in males. In general, the species of *Monstrilla* have five or six caudal setae according to the species or sexes and the males usually do not have fifth legs (Suárez-Morales 2011). Most females also share the same bilobed fifth pair of legs, but only have a single seta on the inner lobe (3-1; Figure 5B). In this respect, *M. grandis* is one of the most plesiomorphic character-rich species either among the known congeners or also within the entire family Monstrillidae. On the other hand, *Monstrillopsis longilobata* has reduced structures of these characters: a presence of four caudal setae in both sexes, a fifth pair of legs in females with the inner lobe reduced and unarmed (3-0; Figure 5C), and an absence of the fifth pair of legs in males (Suárez-Morales et al.

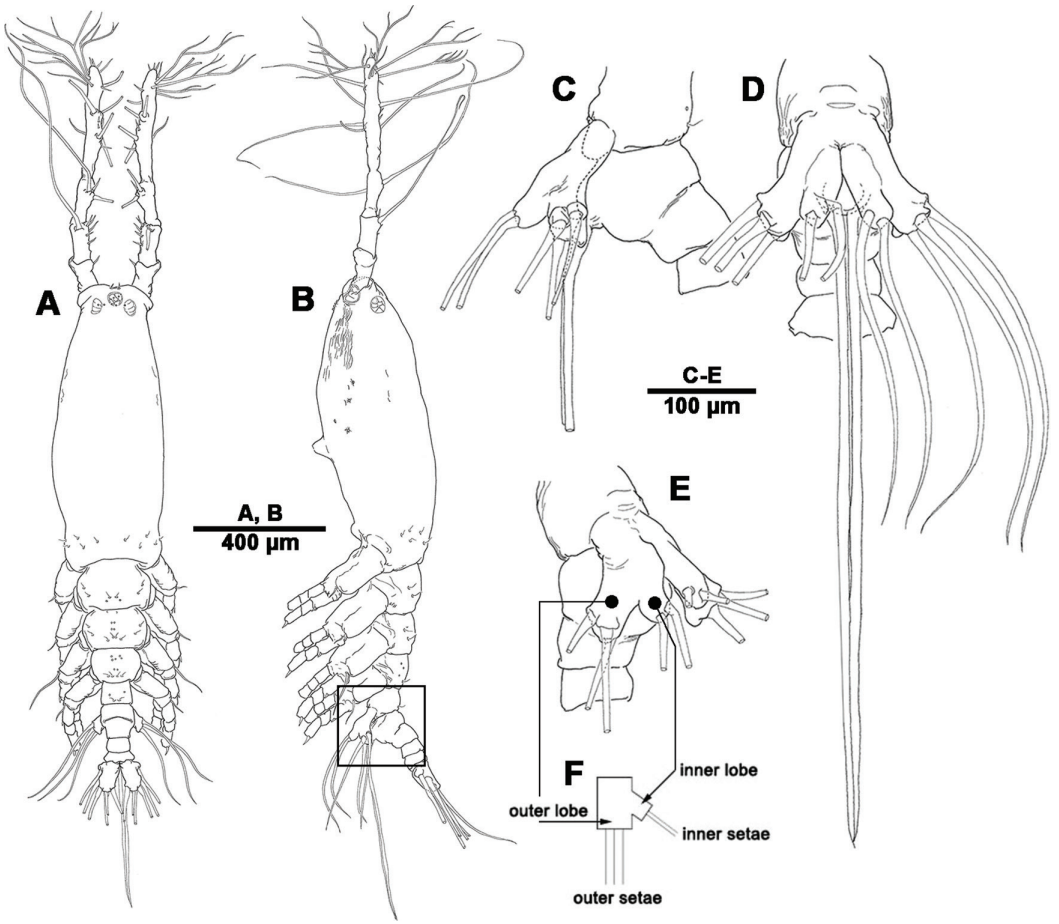

Figure 4 *Monstrilla grandis*, adult female. (A) habitus, dorsal view. (B) habitus, lateral view. Urosomal part bearing the fifth leg highlighted in the box. (C) Detail of urosomal part in B, lateral view. (D) Urosome showing a pair of fifth legs, ventral view. (E) First urosomal somite bearing a pair of fifth legs, lateroventral view. Ovigerous spines not presented. (F) Diagrammatic representation of fifth leg (cf. Figure 5).

2006, Suárez-Morales 2011). Thus, the genus *Monstrillopsis* may be a later appeared group than *Monstrilla*.

The genus *Maemonstrilla*, currently consists of two distinct subgroups, the *Maemonstrilla turgida* and *Maemonstrilla hyottoko* species groups, has only been known from the females (Grygier & Ohtsuka 2008, Suárez-Morales & McKinnon 2014). They show morphological similarities to females *Monstrillopsis* in that they have four urosomites, well-developed eyes and an anteriorly located oral papilla (Grygier & Ohtsuka 2008). The *M. turgida* species group have similarly bilobed fifth legs of *Monstrillopsis*, but simultaneously show an increase of setal elements with an additional seta on the inner lobe (3-1; Figure 5B). By contrast, the *M. hyottoko* species group which includes the present *Maemonstrilla* sp. has a derived state of the fifth leg that is reduced to an elongate rod-shape with two apical setae (Figure 5D). In addition, this group can be distinguished from the *M. turgida* group by the absence of an inner seta on each first exopodal and endopodal segment of swimming legs 1–4 (Grygier & Ohtsuka 2008). Considering the oligomerisation rule, these simplified structures could suggest that *M. hyottoko* species group might be considered to be the most recently diverged group within the Monstrilloida. However, both species

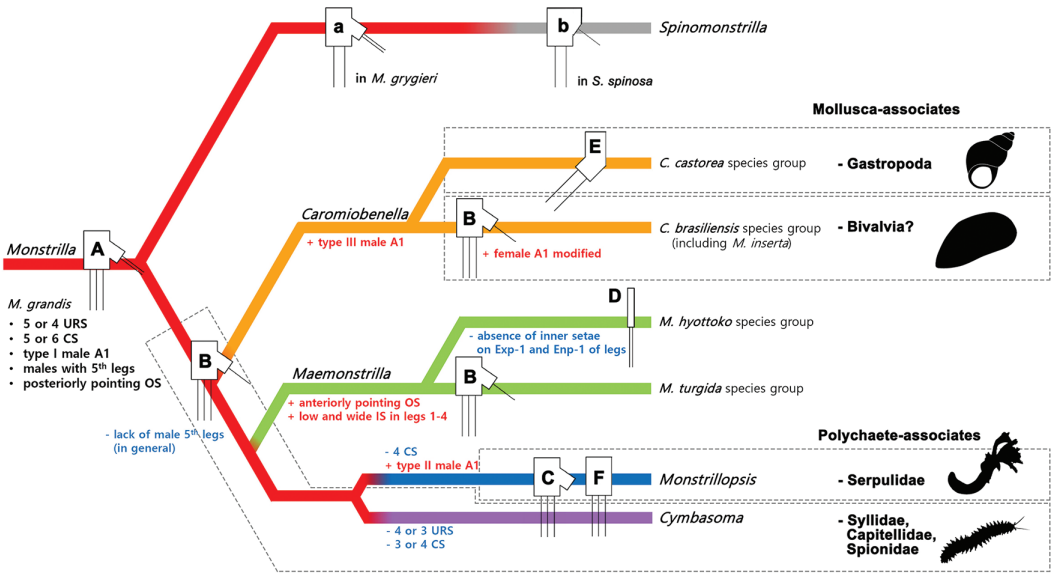

Figure 5 Schematic diagram of morphological divergence of the female fifth leg. The acquisition of new features (in red with '+' marks) and morphological oligomerisation (in blue with '-' marks) are shown along the branches. The most plesiomorphic characters from *Monstrilla grandis* were provided (in black). The host groups utilised by the monstrilloid genera were presented in dashed boxes mapping on the appropriate branches. (A) bilobed leg with 3 outer and 2 inner setae in *M. grandis*; (B) bilobed leg with 3 outer and 1 inner seta, in the majority of *Monstrilla*, *Maemonstrilla turgida* species-group and *Caromiobenella brasiliensis* species-group; (C) bilobed leg with 3 outer setae, inner lobe unarmed, in the majority of *Cymbasoma* and *Monstrillopsis*; (D) a long, rod-shaped leg with 2 apical setae, in the *Maemonstrilla hyottoko* species-group; (E), unilobed leg bent outward at half, in *Caromiobenella* species; (F) unilobed leg with 3 setae, inner lobe almost rudimentary or absent, in some *Cymbasoma* (e.g., *C. bowmani*) (Suárez-Morales & Gasca 1998) and *Monstrillopsis* (e.g., *M. planifrons*) (Delaforge et al. 2017); (a) bilobed leg with 2 outer and 2 inner setae in *Monstrilla grygieri* (Suárez-Morales 2000); (b), unilobed leg with 2 apical and 1 inner seta in *Spinomonstrilla spinosa* (Park 1967, Suárez-Morales 2019). Females of *Australomonstrillopsis* have not been reported. Branch colours of red, orange, green, blue, purple, and grey indicate the lineage of the genus *Monstrilla*, *Caromiobenella*, *Maemonstrilla*, *Monstrillopsis*, *Cymbasoma*, and *Spinomonstrilla*, respectively. Abbreviations used: URS, urosomal somites; CS, caudal setae; A1, antennules; OS, ovigerous spines; IS, intercoxal sclerites; Exp-1, first exopodal segment of leg; Enp-1, first endopodal segment of leg.

groups retain the ancestral plesiomorphic character in the caudal rami with up to six caudal setae (Grygier & Ohtsuka 2008, Suárez-Morales & McKinnon 2014). Thus, we conclude that *Maemonstrilla* is likely to be a more plesiomorphic group than *Monstrillopsis*. If this holds true, the most simplified structures inherited by the *M. hyottoko* species group are perhaps the apomorphic characters that appeared after the divergence of two *Maemonstrilla* species groups. *M. longilobata* females have mixed *Monstrillopsis* and *Maemonstrilla* morphological characteristics (Jeon et al. 2018b). This morphologically intermediate and transitional form contributes to the particularly close relationship between *Maemonstrilla* and *Monstrillopsis*.

The genus *Caromiobenella* had long been regarded as a subgroup within *Monstrilla* (Sars 1921, Huys & Boxshall 1991, Grygier & Ohtsuka 1995, Suárez-Morales et al. 2008, Jeon et al. 2018a). They are morphologically similar by sharing several ancestral plesiomorphic conditions in the aspect of the number of urosomal somites and caudal setae. Thus, it could also be considered one of the earlier clades of the Monstrilloida but might have appeared later than *M. grandis* in terms of morphological complexity. However, a distinct apomorphic character combination, each showing

the structural polarity of the simpleness (e.g., a single lamella-form female fifth leg with two apical setae (Figure 5E) and the replacement of the antennular branched setae into unbranched simple ones) and complexity (e.g., the most complicate male antennular modification with its last segments bearing an additional five serrate ridges on the inner distal margins; type III antennular modification) (Jeon et al. 2018a) had raised questions about their phylogenetic position. Recently, Rosa et al. (2021) reassigned *Monstrilla brasiliensis* into the genus *Caromiobenella* based on the morphological and molecular evidence (thus now *C. brasiliensis*). This taxonomic revision consequently expands the morphological diversity of the female *Caromiobenella* to include the presence of two prominent sensilla-like structures between the antennular bases, a peculiar round protuberance on the putative third antennular segment and a bilobed fifth leg with 3-1 setal pattern (Figure 5B). Based on the morphology of the fifth pair of legs in females in *C. brasiliensis* which is expressed in a more plesiomorphic state than those of *Monstrillopsis*, *Caromiobenella* is more primitive than *Monstrillopsis*. Given our conclusions that *Caromiobenella* and *Maemonstrilla,* respectively, have a closer affinity with *Monstrilla* (primitive character-rich taxon) and *Monstrillopsis* (derived character-rich taxon), the ancestor of *Caromiobenella* might have diverged earlier than that of *Maemonstrilla*. In conclusion, the morphological evidence depicts the relationships of (*Monstrilla*, (*Caromiobenella*, (*Maemonstrilla*, *Monstrillopsis*))), which is the same topology as our phylogenomic inferences.

Another monstrilloid genus, *Cymbasoma* is characterised by the most reduced body structures such as the least number of body somites (one less than the others in each sex) and of caudal setae (exclusively three in females, whereas males have three or four setae according to species but the maximum number of caudal seta never exceeds those of *Monstrillopsis*) (Suárez-Morales & McKinnon 2016). Moreover, the similar morphological repertoires of the fifth pair of legs in females are shared with *Monstrillopsis* (Figure 5C and F) supports their closest relationship. We did not evaluate molecular data for *Cymbasoma*, and so their molecular phylogenetic position within the Monstrilloida remains unknown. However, morphological evidence showing a general agreement with the present molecular results indicates that *Cymbasoma* is the most recent genus in the Monstrilloida with the phylogenetic relationships of (*Monstrilla*, (*Caromiobenella*, (*Maemonstrilla*, (*Monstrillopsis*, *Cymbasoma*)))).

Divergence time estimation in copepod lineages and insight into monstrilloid evolution

The divergence of Copepoda lineage (Figure 6) is estimated to have occurred 494.2 Ma (476.4–500.2 Ma; 95% confidence intervals) and further divergence into two Neocopepoda groups (Gymnoplea and Podoplea) around 433.1 Ma (372.3–454.3 Ma). This suggests that the most recent common ancestor of Copepoda might have originated between the late Cambrian and the middle Silurian. However, the minimum age for the most recent common ancestor may be earlier when considering the existence of the most primitive copepod taxon, Platycopioida (not present in this study). The podoplean lineage was diverged into several ancestral groups each leading to the present Cyclopoida, Siphonostomatoida, Harpacticoida, and Monstrilloida during the Devonian (390.3–407.1 Ma).

The potential of copepod fossilisation is considerably low because of their tiny body size and unsclerotised cuticle, and thus, few body fossil records are available from limited copepod orders (i.e., Harpacticoida, Cyclopoida, and Siphonostomatoida) (Huys et al. 2016). The first fossil records were harpacticoids (Canthocamptidae: *Cletocamptus* sp.) and unidentified cyclopoids found from the Miocene Barstow Formation (13.4–19.3 Ma) in Southern California, USA (Palmer 1960). A fossil of the siphonostomatoid (Dichelesthiidae: *Kabatarina patersoni*) was collected from the gills of a fossilised fish in the Santana Formation (110–120 Ma), Brazil (Cressey & Patterson 1973, Cressey & Boxshall 1989). The fossil of a freshwater copepod, presumably assignable to the extant harpacticoid family Canthocamptidae, was found in carboniferous bitumen (Selden et al. 2010). This latter

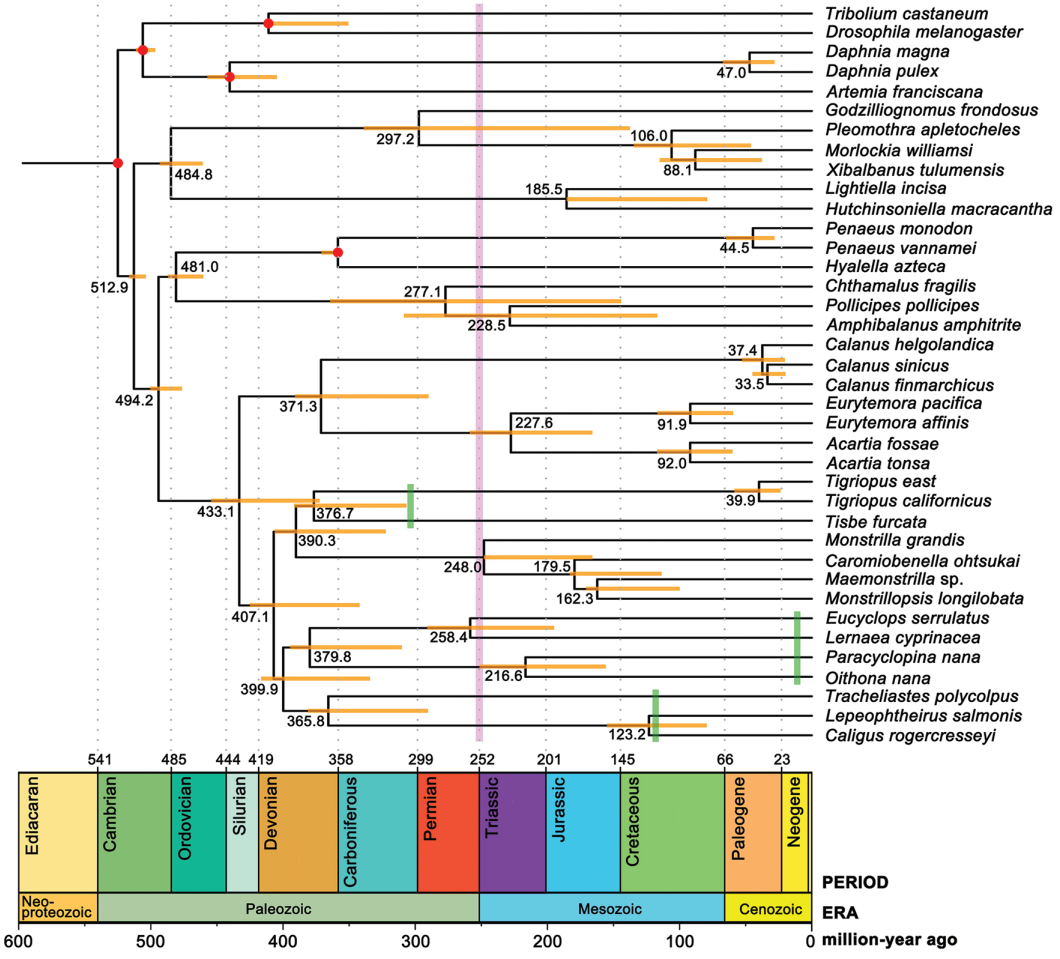

Figure 6 Estimated divergence times for arthropod species inferred using LSD2 (ver. 1.10) (To et al. 2016) with five non-copepod calibration points indicated by red dots. Orange horizontal bars near nodes indicate 95% confidence intervals and green vertical bars indicate the fossil records of Copepoda. Purple vertical bar indicates the Permian-Triassic extinction. The geologic time scale is prepared according to the International Chronostratigraphic Chart (http://www.stratigraphy.org).

find significantly extends the history of Harpacticoida back to the late Carboniferous (ca. 303 Ma). Although our divergence times were estimated without copepod fossil calibrations, the known fossil record so far fit well with our divergence time estimations. In addition, previously suggested divergence times for the copepod orders (Selden et al. 2010, Eyun 2017) are also in general agreement with our results. Boxshall and Jaume (2000) estimated that the cyclopoids probably invaded freshwaters before the breakup of Pangaea (ca. 200 Ma) (Dietz & Holden 1970, Yoshida & Hamano 2015) based on their patterns of current distribution through all continents. The suggested divergence time of 258.4 Ma for a freshwater cyclopoid *Eucyclops serrulatus* in this study also supports this hypothesis.

According to our divergence time estimates, the within-order diversifications for the Calanoida, Cyclopoida, Siphonostomatoida, and the Harpacticoida occurred in the Devonian (365.8–379.8 Ma), but it did not happen for the Monstrilloida until 248.0 Ma (165.9–248.0 Ma). The divergence time suggested for the Monstrilloida indicates that their diversification proceeded after the

Permian-Triassic mass extinction (252 Ma), which is recognised as the greatest natural disaster in magnitude and impacts (Chen & Benton 2012, Burgess et al. 2014, Stanley 2016) (Figure 6). Unfortunately, our current knowledge is insufficient to understand the relevance of the extinction event for monstrilloid evolution. We can only suggest an evolutionary hypothesis based on limited clues. Among the seven valid monstrilloid genera, at least three have been known to utilise the polychaetes as their hosts (Malaquin 1901, Caullery & Mesnil 1914, Hartman 1961, Huys & Boxshall 1991, Suárez-Morales et al. 2014). These monstrilloid genera include not only the most basal, primitive group (i.e., *Monstrilla*) but also the most recently evolved groups (*i.e.*, *Monstrillopsis* and *Cymbasoma*). Thus, extant monstrilloids may have initially stemmed from ancestors that parasitised polychaetes (Figure 5). Some marine animals, particularly polychaetes, managed to survive the mass extinction, while other marine invertebrates such as corals, brachiopods, gastropods and bivalves were severely impacted (Sepkoski 1981, Knoll et al. 2007). Monstrilloid survival relied on available parasitic hosts. Therefore, mass extinction potentially eradicated several ancestral monstrilloids that relied on parasitising other marine invertebrates rather than polychaetes due to drastic environmental changes and rapid host resource depletion. This may explain the long, seemingly trimmed branch pattern for the monstrilloid clade. The present phylogenomic results showed that the genus *Caromiobenella* evolved from the same ancestor that was associated with polychaetes. As this genus also parasitises molluscs (Pelseneer 1914, Gallien 1934), they may have undergone an independent host switch during their evolution (Figure 5). Overall bivalve and gastropod diversity prominently increased in the Mesozoic (Valentine 1969, Sepkoski 1984, Benton 1995). This pattern may also explain the later divergence of the genus *Caromiobenella* within the Monstrilloida. Coincidently, the suggested divergence time of 195.08 Ma (Lee et al. 2019) for the bivalve genus *Perna* (which is considered a potential host for *C. brasiliensis*) (Rosa et al. 2021) is congruent with the subsequent appearance of *Caromiobenella* around 179.5 Ma (113.9–182.8 Ma).

Although we confirmed monstrilloid phylogeny and parasitism pattern correlations, our comprehension is still limited by insufficient knowledge of monstrilloid hosts. Further research on endoparasitic juveniles is necessary to enhance our monstrilloid evolution understanding.

Knowledge gaps and future research priorities

In recent decades, the records of monstrilloid species have increased rapidly (Suárez-Morales 2011). On the other hand, their taxonomy based on a limited number of morphological features from adult specimens seems unable to provide sufficient criteria for their identification and classification (Suárez-Morales et al. 2017). Vague generic and specific diagnoses have caused taxonomic uncertainties along with the increase of new species. Therefore, alternative research methods in addition to traditional morphology are needed. In this context, the use of molecular tools is suggested as one of the most efficient and definitive methods for revealing their true diversity, taxonomy and phylogeny (Suárez-Morales 2011, Jeon et al. 2018b). Unfortunately, the application of molecular methods in monstrilloid research is rare.

Monstrilloida's morphological information is limited to planktonic adults, and their highly modified body structures make a direct comparative analysis with ordinary copepods for taxonomic and phylogenetic inferences almost impossible. However, their planktonic juveniles at their earliest developmental stage (i.e., nauplius stage I), which present the fundamental body plan and structures of the Copepoda (Dahms 1990, 2004, Grygier & Ohtsuka 1995, 2008), could provide additional information useful for resolving more detailed phylogenetic relationships within Monstrilloida but also among Copepoda.

As the present study shows, it would be much better to consider multilateral data together rather than a piecemeal approach to understand the monstrilloids. Particularly, research is needed to determine host diversity and the interaction between the hosts and parasites (e.g., host specificity), which are poorly known.

Table 2 Sequence Divergence within Orders Calculated Using 18S rRNA and 25 Nuclear Protein Sequences Using the *p*-Distance Method and Various Models

Copepoda Order	Sequence Divergence				
	18S rRNA		25 Nuclear Proteins		
	p-Distance	K2P[a]	*p*-Distance	Dayhoff	JTT[b]
Calanoida	0.084	0.093	0.104	0.114	0.117
Cyclopoida	0.080	0.087	0.134	0.148	0.152
Siphonostomatoida	0.070	0.076	0.143	0.161	0.165
Harpacticoida	0.074	0.080	0.104	0.115	0.117
Monstrilloida	0.095	0.105	0.223	0.271	0.275

[a] Kimura 2-parameter model.

[b] Jones-Taylor-Thornton model.

There are several studies into the host-parasite coevolution demonstrating that parasites have an accelerated gene mutation rate compared with their hosts (Hafner et al. 1994, Page et al. 1998, Paterson & Banks 2001, Nieberding et al. 2004, Huyse et al. 2005, Levin & Parker 2013, Booth et al. 2015). In this respect, the unusually higher sequence divergences within the Monstrilloida (Table 2) would deserve more attention for better understanding their evolutionary significance. Our phylogenetic results alluded that the monstrilloid genera appear to separate by the usage of the different hosts. Therefore, further molecular research targeting more specific genes involved in immune response/evasion mechanisms and neuro-signalling pathways could probably provide the primary ground for understanding the particular adaptation to various environmental conditions and testing the fast gene mutation phenomena for Monstrilloida.

The weakest point of this study is probably that the phylogenetic evaluation of Monstrilloida was considered only between the so-called major copepod taxa based on relatively small genetic information compared to other bioinformatic analyses. Accordingly, our present results show a partial resolution for the Copepoda phylogeny. Our first genome-scale phylogenetic analyses into Monstrilloida provide more detailed copepod relationships to date. It is clear that the amount of genetic information available is important, especially in resolving the deep nodes. Five copepod orders were not included in this study. Their phylogenetic relationships remain to be explored. As such, the continued accumulation and application of more genetic data for future phylogenetic research are needed for a better understanding of the phylogenetic relationships among broad taxa. These works will eventually provide further insights into the phylogeny of the Copepoda and, by extension, into the Arthropoda evolution.

Concluding remarks

The present molecular study based on the 25 orthologous nuclear protein-coding genes reaffirmed the monophyletic status of the Copepoda within the Arthropoda and further revealed the molecular phylogenetic position of the Monstrilloida within the Copepoda. The Monstrilloida appeared to have a closer affinity to the benthic copepod group, Harpacticoida, rather than the previously suggested affinity to another parasitic group, the Siphonostomatoida. Further investigations applying morphological evidence to the molecular phylogeny suggested a certain evolutionary hypothesis that the Monstrilloida have evolved in the sequence *Monstrilla*, *Caromiobenella*, *Maemonstrilla*, *Monstrillopsis* and *Cymbasoma* from their last common ancestor.

Most interestingly, the relationships between the monstrilloid genera showed two distinct patterns of their morphological evolutionary trend depending on the host type of each genus: (1) a gradual morphological reduction pattern (e.g., a decrease in the number of body somites and caudal setae, and the structural oligomerisation in the female fifth legs) mainly occur along the polychaete-associated lineages, *Monstrilla*, *Monstrillopsis* and *Cymbasoma*, while (2) an appearance of

the new feature (e.g., type III antennular modification in males of *Caromiobenella*; low and wide intercoxal sclerites and anteriorly directed ovigerous spines in females of *Maemonstrilla*) is prominent in the groups utilising non-polychaete hosts (at least in *Caromiobenella*). The adoption of the different evolutionary strategies may represent their host-associated adaptation to simultaneously thrive in challenging environments for survival. As such, this study serves as a critical starting point for explaining hypotheses on which copepod species might have evolved to adapt to diverse ecological niches. Furthermore, their intriguing characteristics in all three aspects of morphology, molecular biology and ecology will continuously give opportunities and insights to shed more light on the copepod evolution.

Data availability

All raw sequence data are deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA766505. The multiple sequence alignment used for the analyses is available for download at http://eyunlab.cau.ac.kr/monstrilloid_phylogenomics.

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