

Complicated evolution of the Caprellid (Crustacea: Malacostraca: Peracarida: Amphipoda) bodyplan, reacquisition or multiple losses of the thoracic limbs and pleons

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Abstract

The Caprellidea (Crustacea) have undergone an interesting morphological evolution from their ancestral gammarid-like form. Although most caprellid families have markedly reduced third and fourth pereopods (the walking thoracic limbs) and pleons (the posterior body parts), one family, Caprogammaridae, has developed pleon with swimming appendages (pleopods), whereas another family, Phtisicidae, possesses well-developed functional third and fourth pereopods. The unique character status of these families implies that there has been reacquisition or multiple losses of both pereopods and the pleon within the Caprellidea lineages. Although the Caprellidea are fascinating animals for the study of morphological evolution, the phylogenetic relationships among the Caprellidea are poorly understood. One obstacle to studying the evolution of the Caprellidea is the difficulty collecting samples of caprogammarid species. In this study, we obtained live samples of a Caprogammaridae species, and confirmed that its pleon and pleopods could perform similar locomotive functions and swimming movements as observed in gammarids. From the phylogenetic analyses on 18S ribosomal RNA gene sequences, we identified three distinct clades of Caprellidea. Ancestral state reconstruction based on the obtained phylogeny suggested that once lost, the third and fourth pereopods were regained in the Phtisicidae, while the pleon was regained in the Caprogammaridae, while we could not exclude the possibility of independent losses. In either case, the caprellid lineage underwent a quite complicated morphological evolution, and possibly the Caprellidea may be an exception to Dollo's law.

Key words: caprellid, molecular phylogeny, 18S rRNA, pereopod, pleon, Dollo's law

INTRODUCTION

It is generally accepted that evolution is irreversible and that once a complex morphological character is lost in the course of evolution, it never reappears. This concept of Dollo's law appeals to our statistical senses because the development of complex morphological characters requires several steps (Gould 1970). In contrast, reduction has occurred more frequently. Thus, when Dollo's law is strictly applied in phylogenetic reconstruction, one has to assume that morphological characters have undergone multiple rounds of reduction. Reduction is an important factor in producing the morphological diversity of an organism. Among metazoans, a large number of taxa show the reduction of several organs or tissues. An extreme example is a myxozoan, which has lost most aspects of its multicellular bodyplan due to its parasitic lifestyle (Jimenez-Guri et al. 2007). A crustacean of the suborder Caprellidea (Malacostraca, Peracarida, Amphipoda) also acquired a unique bodyplan by reducing its posterior body segments and thoracic limbs.

Most of the caprellid families (Caprellidae, Caprellinoididae, Cyamidae, Pariambidae and Protellidae) have strongly reduced third and fourth pereopods (walking thoracic limbs) and pleons (the body part posterior to the thorax, bearing appendages that are structurally distinct from the thoracic limbs (Schram and Koenemann 2004; Fig. 1a). The third and fourth pereopods are completely lost or are only present in a substantially rudimentary form. The pleon is also greatly reduced in size, not showing any segmental structure and bearing only 1 or 2 pairs of vestigial appendages. Some morphological and molecular phylogenetic studies strongly support the idea that the Caprellidea derived from the Gammaridea (Ito et al. 2008; Kim and Kim 1993; Myers and Lowry 2003), which is another amphipod suborder that has well-developed third and fourth pereopods and a pleon (Fig. 1b). Therefore, reduction of the thoracic limbs and posterior segments occurred within the Caprellidea.

Interestingly, there are two families that do not show the above-mentioned typical caprellid bodyplan. A family of Caprellidea, Caprogammaridae, possesses an elongated and segmented pleon with pleopods (appendages in pleon) (Fig. 2a, b), although the third and fourth

pereopods are rudimentary as in other caprellids (Kudrjaschov and Vassilenko 1966; Takeuchi and Ishimaru 1991). Although three pairs of pleopods are biramous as those of gammarids (Kudrjaschov and Vassilenko 1966; Takeuchi and Ishimaru 1991), the functional significance of the abdomen and appendages of Caprogammaridae has been largely unknown. Members of another family, Phtisicidae, show a distinct bodyplan. Although their pleons are strongly reduced, the Phtisicidae possess well-developed third and fourth pereopods, which is segmented in six parts (Fig. 2c, d). The third and fourth pereopods of the Phtisicidae function as walking limbs. Thus, the phylogeny of the caprellids is a matter of controversy, and caprellids must have experienced complicated evolutionary history in their morphology (Ito et al. 2008; Laubitz 1976; Laubitz 1993; Takeuchi 1993).

In order to elucidate the evolutionary history of these distinct bodyplans in caprellids, we previously conducted a molecular phylogenetic analysis of some caprellids and gammarids using 18S ribosomal RNA gene sequence data (Ito et al. 2008). Based on these findings, we concluded that the gammarids were likely to be paraphyletic, and thus, that the caprellid bodyplan (i.e., reduction of pereopods and posterior body segments) was derived from a gammarid-like bodyplan. Although these analyses suggested that there was a phylogenetic affinity of Phtisicidae and other caprellid families, most of the phylogenetic relationships among the caprellid families remained unclear because some families were not included in the analysis. This was considerably problematic due to the absence of sequence data from the Caprogammaridae. Caprogammaridae are difficult to collect because they are only distributed in the Northwest Pacific Oceans (Takeuchi and Ishimaru 1991) and have been reported to live at approximately 100 meters below sea-level.

Recently we successfully collected some fresh samples of a caprogammarid species, *Caprogammarus gurjanovae*. We reconstructed an 18S rRNA gene phylogenetic tree for the Caprellidea, including *C. gurjanovae* and another bizarre caprellid family, Cyamidae, which is a parasite on the surfaces of whales that was not included in our previous analysis. Molecular phylogenetic analyses covering almost all caprellid families provided an important framework for understanding the morphological transitions within Caprellidea.

MATERIALS AND METHODS

Sampling

Caprogammarus gurjanovae (Caprogammaridae; Fig. 2a) were collected at 42°48' north latitude and 144°56' east longitude at approximately 107 meters deep. *Cyamus balaenopterae* (Cyamidae) were collected from the body of Antarctic minke whale sampled by the Japanese Whale Research Program under special permit in the Antarctic (JARPA II) in the Antarctic Ocean and provided by the Institution of Cetacean Research. The collected specimens were preserved in 100% ethanol. The sequence data for *Caprella geometrica* (Caprellidae), *Perotripus* sp. (Caprellinoididae), *Pseudoprotella* sp. (Pariambidae), *Monoliropus tener* and *Protella gracilis* (Protellidae), *Phtisca marina*, and *Protomima imitatrix* (Phtisicidae) and the sequences for the two outgroup species *Jassa slatteryi* (Gammaridea, Ischyroceridae) and *Podocerus inconspicuus* (Gammaridea, Podoceridae) were obtained from GenBank (National Center for Biotechnology Information, NCBI; Table 1).

Behavioral observations

To confirm the functional significance of the abdomen, we observed the swimming behavior of *C. gurjanovae*. We released a male *C. gurjanovae* into a plastic water tank and recorded a movie of its behavior using a digital camera.

Molecular methods

Genomic DNA was extracted from the appendages or gills of specimens using the DNeasy Tissue kit from Qiagen. Polymerase chain reaction (PCR) was used to amplify the 18S rRNA gene from the extracted DNA using specific primers (Englisch et al. 2003) (Sup. Table 1). PCR was performed with Ex Taq polymerase (Takara). The PCR conditions on a DNA Engine PTC-200 Peltier Thermal Cycler (Bio-Rad) were as follows: 1 cycle for 5 min at 94°C, 35 cycles for 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and 1 cycle for 5 min at 72°C. The PCR products were treated with ExoSap-IT

(GE Healthcare, Japan) at 37°C for 15 min and 80°C for 15 min to remove the remaining primers and dNTPs. The treated PCR products were sequenced directly. The sequence analyses were performed on an ABI PRISM 310 Genetic DNA sequencer (Applied Biosystems) using the BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Primers used for PCR and sequencing was described in Ito et al. (2008).

Phylogenetic analysis

Sequence data sets were aligned using T-coffee (Notredame et al. 2000) with default settings. After correcting the alignment by eye, we excluded some stretches of nucleotide sequences using MacClade 4.03 (Maddison and Maddison 2001) because they were not confidently aligned. The alignment data is available upon request. The saturation test was then performed using DAMBE version 5.2.5 (Xia and Xie 2001) to test whether the sequences have experienced substantial substitution saturation.

Phylogenetic trees were constructed by maximum likelihood (ML) with PhyML 3.0 (Guindon and Gascuel 2003) and by Bayesian analysis with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). In both analyses, the GTR + I + G model selected by the Akaike information criterion (AIC) in Modelgenerator version 0.85 (Keane et al. 2006) was used. The estimated parameter values were as follows: nucleotide frequencies, π (A) = 0.24069, π (C) = 0.25081, π (G) = 0.28783 and π (T) = 0.22067; GTR rate parameters, R_{A-C} = 1.92, R_{A-G} = 5.54, R_{A-T} = 4.41, R_{C-G} = 1.13, R_{C-T} = 10.70 and R_{G-T} = 1.00; proportion of invariable sites = 0.57; and gamma distribution parameter α = 0.32. The maximum likelihood analysis was performed using SPR branch swapping, and 1,000 bootstrap pseudoreplicates were performed to evaluate the confidence for each node. In the Bayesian analysis, 500,000 generations were run with four simultaneous chains and every 100 generations were sampled. We confirmed that the average standard deviation of split frequencies was less than 0.01, indicating that convergence occurred.

Ancestral state reconstruction

To elucidate the evolutionary transition of third and fourth pereopods and the pleon within the Caprellidea, we reconstructed ancestral states using Mesquite version 2.6 (Maddison and Maddison 2009). Based on the phylogenetic trees that were obtained from the ML and Bayesian analyses, we constructed a multiple branching phylogram containing three caprellid clades and the Gammaridea. We coded the state of third and fourth pereopods and the pleon as discrete binary data (development or reduction; Table 1) and estimated the ancestral state on each node according to the parsimony principle.

RESULTS

Behavioral observations

C. gurjanovae showed a swimming behavior that obviously involved its abdomen and appendages. For this swimming behavior, *C. gurjanovae* stretched pereopods 5-7 and its abdomen in a posterior direction and moved from posterior to anterior by vibrating its abdominal appendages (Supplementary data: Movie S1). This behavior pattern has never been observed in other caprellids, which possess only vestigial abdomens.

Phylogenetic analyses and ancestral state reconstruction

The 18S rRNA gene from the 11 species examined ranged from 2161 to 2523 bp (Table 1). We confirmed that the GC ratio in the sequence data was approximately 52% to 54%. Therefore, a biased GC content was not likely to be problematic (Hasegawa and Hashimoto 1993). In addition, we confirmed that the sequences have experienced little substitution saturation because index of substitution saturation (Iss, 0.1024) was significantly lower ($p < 0.0001$) than the critical Iss value (Iss.c, 0.7932).

Analyses of caprellids and eleven species of gammarids supported the monophyly of the examined caprellids species (Supplementary Fig. 1). We then focused on relationships among caprellid species. In order to maximize the available nucleotide sites, we chose two close gammarid species as the outgroup. Both phylograms constructed by the ML and Bayesian analyses exhibited the same topology, and the root of the Caprellidea was placed in the same position as when the eleven gammarid species were used (Fig. 3). In these trees, the Caprellidea were divided into the following three distinct clades: Clade 1: {Caprogammaridae + (Caprellidae + Cyamidae)}, Clade 2: (Caprellinoididae + Phtisicidae) and Clade 3: (Pariambidae + Protellidae) (Fig. 3). Although the bootstrap support for the clade 1 is not particularly high (76.5%), significant supports are obtained in the Bayesian tree for all three clades (Fig. 3). Substitution rate of *Perotripus* is relatively high as reflected in its branch length, which might cause problem in resolution of phylogeny. However, we

confirmed that the monophyly of the three classes were supported and overall framework of the tree does not change after *Perotripus* is excluded from the analysis. While the tree indicated the topology of {Clade 1 + (Clade 2 + Clade 3)}, the confidence values supporting (Clade 2 + Clade 3) were low. Thus, we could not deduce the divergence order of the three clades with sufficient reliability.

Based on the deduced phylogenetic framework, we reconstructed the ancestral character states according to the maximum parsimony principle (Fig. 4). Under the assumption that character loss and reacquisition are equally likely, it was deduced that the last common ancestor of the Caprellidea (node 1) and Clade 2 (node 2) had reduced third and fourth pereopods (Fig. 4a). Similarly, the estimated states of the pleon in node 1 and node 3 were “reduced” (Fig. 4b). One might think that character loss occur more frequently compared to reacquisition. In the ancestral reconstruction under the assumption that character loss occurred twice as likely as reacquisition, it was deduced that reacquisition and multiple loss are equally likely for both pereopods and developed pleon (Fig. 4c, d). In this scenario, we note that caprellids should have lost the pereopods of the same thoracic segments in several distinct lineages.

DISCUSSION

Phylogenetic position of the Caprogammaridae, Phtisicidae and Cyamidae

In our previous analyses, although the phylogenetic affinity of the Protellidae and the Pariambidae was supported by high confidence values, most of the phylogenetic relationships among the caprellid families were still unresolved (Ito et al. 2008). In this study, we included several additional species and could find three distinct clades within the Caprellidea (Fig. 3); one clade contained the Caprogammaridae, Caprellidae and Cyamidae (Clade 1), another clade consisted of the Caprellinoididae and Phtisicidae (Clade 2), and the third clade contained the Pariambidae and Protellidae (Clade 3).

When the Caprogammaridae were first described by (Kudrjaschov and Vassilenko 1966), they were regarded as a member of the Gammaridea based on several gammarid-like morphological features, such as separation of the head from the thoracic segment bearing the first pereopod (these are partially coalesced in the Caprellidea), presence of a coxal plate (reduced in the Caprellidea), and a five-segmented pleon with pleopods. However, Takeuchi and Ishimaru (1991) noted that some caprellids also possess small coxal plates. In addition, through a detailed reanalysis of *C. gurjanovae*, Takeuchi and Ishimaru (1991) found that the head and the thoracic segment bearing the first pereopod are actually coalesced partially like other caprellids. Our phylogenetic analyses strongly support the latter idea that the Caprogammaridae should be included in the Caprellidea, not the Gammaridea (Supplementary Fig. 1).

Most of the classical studies on the Caprellidea considered the Caprogammaridae as the most basal caprellid family because their developed pleon indicate a close relationship to the Gammaridea (Laubitz 1976). However, our analyses do not support this hypothesis. Rather, the significant confidence values in our analyses indicate that the Caprogammaridae are closely related with the Cyamidae and the Caprellidae (Fig. 3). This result implies that the pleon was regained in this lineage or that pleons were lost multiple times.

The phylogenetic position of the Phtisicidae has also been problematic. Given its

morphological distinctions, Takeuchi (1993) proposed that the Phtisicidae might have derived from a different ancestor than other caprellids. Laubitz (1993) also suggested the Caprellidea may be polyphyletic. On the other hand, our previous analysis strongly supported the phylogenetic affinity of the Phtisicidae with other caprellid families, but the phylogenetic position of the Phtisicidae within the Caprellidea was unclear (Ito et al. 2008). In this study, we found that three phtisicid genera constructed a distinct clade with the Caprellinoididae (Fig. 3; Clade 2), suggesting that thoracic limbs might have been regained.

There have been a few studies concerning the evolution of the Cyamidae. The life style and body form of the Cyamidae are considerably different from other caprellid families. The Cyamidae are ectoparasitic on cetaceans (*e.g.* whales) and have a dorsoventrally flattened body, unlike general cylindrical caprellids. Their quite unique morphology makes it difficult to estimate their phylogenetic position between the cyamid and other caprellid families. Based on the morphology of the mouthparts, Laubitz (1993) suggested that the Cyamidae are phylogenetically closer to the Phtisicidae than the Caprogammaridae or the Caprellidae. However, our analyses do not support these opinions, but indicate that there is a close relationship between the Cyamidae and the Caprellidae (Fig. 3). The similarities in the mouthpart morphology must be reanalyzed from the aspect of similar functional use.

Regaining or multiple losses of third and fourth pereopods and the pleon

Even in morphologically diverse crustaceans, reduction of the pleon and third and fourth pereopods in the Caprellidea is very peculiar. In addition, the morphology of the Caprogammaridae and Phtisicidae, which apparently possess reversal phenotypes, implies that a complicated morphological evolution has occurred within the Caprellidea lineage.

Our ancestral state reconstruction based on the maximum parsimony principle suggested that third and fourth pereopods might be regained in the Phtisicidae lineage (Fig. 4a). Alternatively, third and fourth pereopods might be lost two to three times during caprellid diversification.

Reduction of the appendages is widely observed in the Crustacea, indicating that multiple losses are not highly unlikely. For example, some gammarid genera such as *Dulichia* also exhibit size-reduced third and fourth pereopods. Therefore, *Dulichia* and its allied genera have been considered to form a sister group of the Caprellidea on the basis of morphological characters (Laubitz 1979; Takeuchi 1993), but our previous molecular phylogenetic study did not support that idea and suggested that the reduction of pereopods might have occurred independently in the Caprellidea and *Dulichia* (Ito et al. 2008). On the other hand, several recent phylogenetic or developmental studies claimed that reacquisition of several complex characters might have occurred, such as the wings of stick insects, digits of lizards, feeding larval stage of marine snails (Collin et al. 2007; Collin and Miglietta 2008; Kohlsdorf and Wagner 2006; Whiting et al. 2003). Because caprellids have fully developed first, second, and fifth to seventh pereopods, it is not improbable that the gene regulation system that produces pereopods could have been re-recruited to the third and fourth pereopods in the Phtisicidae.

Ancestral state reconstruction suggested that the pleon might also be reacquired in the Caprogammaridae (Fig. 4b). Alternatively, the pleon might be lost two or three times, depending on the branching order of Clades 1-3. Reductions of the posterior body parts have occurred in some other crustaceans such as the Cirripedia (*e.g.* barnacles) (Deutsch et al. 2004). On the other hand, there has been no evidence that the pleon reoccurred in the Crustacea or the Arthropoda. The pleon and biramous pleopods of the Caprogammaridae are not rudimentary at all, and we confirmed that the pleon can be used for swimming (Supplementary data: Movie S1). Therefore, given that the pleon of the Caprogammaridae was regained, the functional muscles and nervous systems also had to be perfectly reconstructed after they were lost. This evolutionary scenario seems to be more implausible than the regaining of the pereopods mentioned above.

We do not think that the pleon with functional pleopods or thoracic limbs can be reacquired as frequently as they are lost, as assumed in our ancestral state reconstructions. In the reconstruction, under the assumption that character loss occurred twice as likely as reacquisition, it was deduced that reacquisition and multiple loss are equally likely for both pereopods and

developed pleon (Fig. 4c, d). It should also be noted that Oakey and Cunningham (2002) warned that this sort of ancestral state reconstruction might dismiss evolutionary losses based on their analysis on the evolution of a compound eye in ostracods. Therefore, based on the phylogenetic framework, we can only conclude that the caprellid lineage underwent a quite complicated morphological evolution. When Dollo's law is strictly applied, we must assume that there were three independent losses of pereopods and two losses of the pleon, or vice versa, depending on the branching order of Clades 1-3. Even in this case, it is peculiar that the pereopods were lost from the same thoracic segments. In order to better understand the true evolutionary history, it will be necessary to generate a more accurate phylogenetic framework. It will be particularly useful to resolve the relationships among the three clades that were identified in this study. In addition, information from distinct sources will also be valuable (Oakley and Cunningham, 2002). We are currently exploring the molecular development of the Caprellidea (Ito et al., submitted as an accompanying paper) and hope to decipher the developmental process and the gene network that produce the pereopods or the pleon. By comparing these genes with those of the phtisicids or caprogammarids, we will probably be able to detect not only what kind of molecular evolution caused the reduction, but also if and how the reacquisitions occurred.

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Figure legends

Figure 1. Schematic bodyplan of the Caprellidea (a) and Gammaridea (b).

The third and fourth pereopods and the pleon in the Gammaridea are hatched. The number of the pereopods is also shown. Note that the leaf-like structures in the Caprellidea (arrows) are gills, not pereopods.

Figure 2. *Caprogammarus gurjanovae* (Caprogammaridae; a), the pleon of *C. gurjanovae* (b), *Protomima imitatrix* (Phtisicidae; c) and the fourth pereopods of *P. imitatrix* (d).

The arrowhead and arrow in (b) denote the pleon and pleopods, respectively. The arrows in (c) indicate third and fourth pereopods and the arrowhead in (d) indicates the fourth pereopod.

Figure 3. Maximum likelihood tree based on 1,576 nucleotide sites of the 18S rRNA gene sequence.

Numbers under the branches in each tree represent the bootstrap values, and the numbers above the branches represent the posterior probabilities from the Bayesian analysis. Bootstrap values less than 70% and posterior probabilities less than 90% are not shown. The pleon in the Caprogammaridae and third and fourth pereopods in the Phtisicidae are hatched.

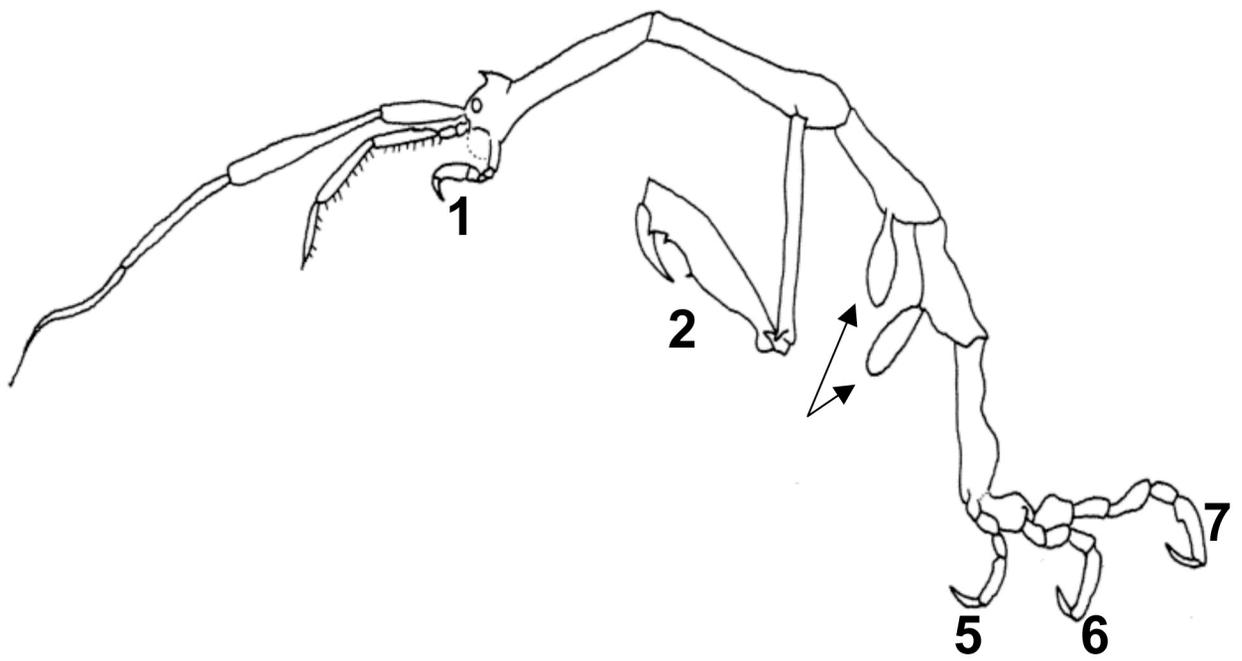
Figure 4. Ancestral state reconstruction of third and fourth pereopods (a, c) and the pleon (b, d) in the caprellid phylogeny.

In each phylogram, the black circles represent “reduced” and white circles represent “developed”. The node supporting Clade 2 and Clade 3 was collapsed because of its low confidence values (Fig. 3). The pleon in the Caprogammaridae and third and fourth pereopods in the Phtisicidae are hatched.

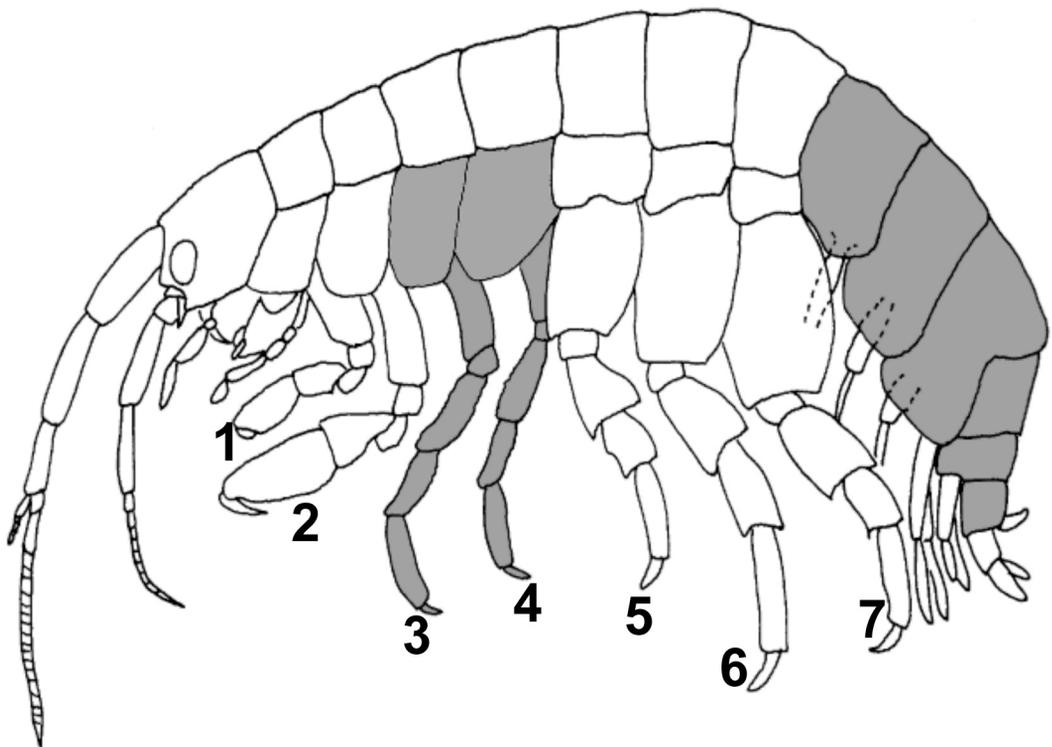
Table 1. Species included in the analyses along with the corresponding family, sequence length (bp), accession number and character state used for the ancestral state reconstruction.

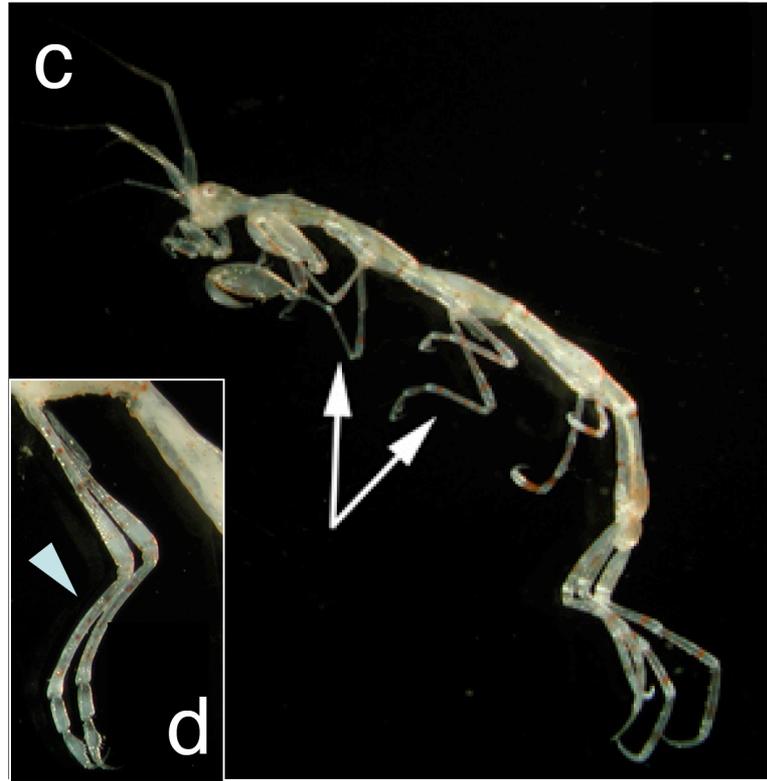
Species	Family	Sequence length (bp)	GenBank accession number	Character state	
				Pereopods 3 and 4	Pleon
Suborder Caprellidea					
<i>Caprella geometrica</i> Say, 1818	Caprellidae	2177 bp	AY781423	reduced	reduced
<i>Perotripus</i> sp.	Caprellinoididae	2256 bp	AB295401	reduced	reduced
<i>Caprogammarus gurjanovae</i> Kudrjaschov & Vassilenko, 1966	Caprogammaridae	2266 bp	AB520634	reduced	developed
<i>Cyamus balaenopterae</i> Barnard, 1931	Cyamidae	2315 bp	AB520635	reduced	reduced
<i>Pseudoprotella</i> sp.	Pariambidae	2310 bp	AB295397	reduced	reduced
<i>Protella gracilis</i> Dana, 1853	Protellidae	2523 bp	AB295396	reduced	reduced
<i>Monoliropus tener</i> Arimoto, 1968	Protellidae	2278 bp	AB295395	reduced	reduced
<i>Phtisica marina</i> Slabber, 1769	Phtisicidae	2165 bp	DQ378040	developed	reduced
<i>Protomima imitatrix</i> Mayer, 1903	Phtisicidae	2339 bp	AB295399	developed	reduced
Suborder Gammaridea (Outgroup)					
<i>Jassa slatteryi</i> Conlan, 1990	Ischyroceridae	2161 bp	AB295408	developed	developed
<i>Podocerus inconspicuus</i> (Stebbing, 1888)	Podoceridae	2325 bp	AB295409	developed	developed

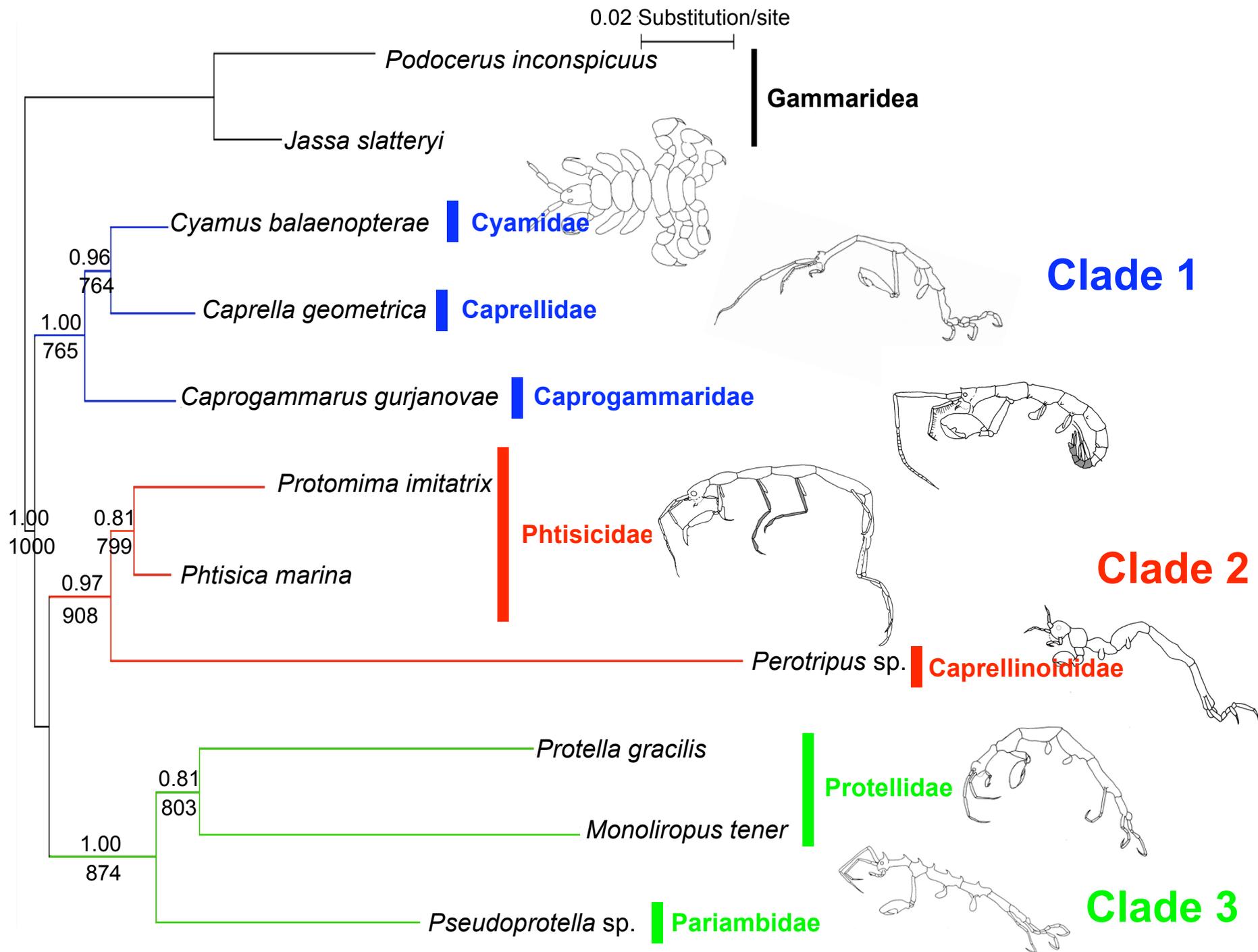
a



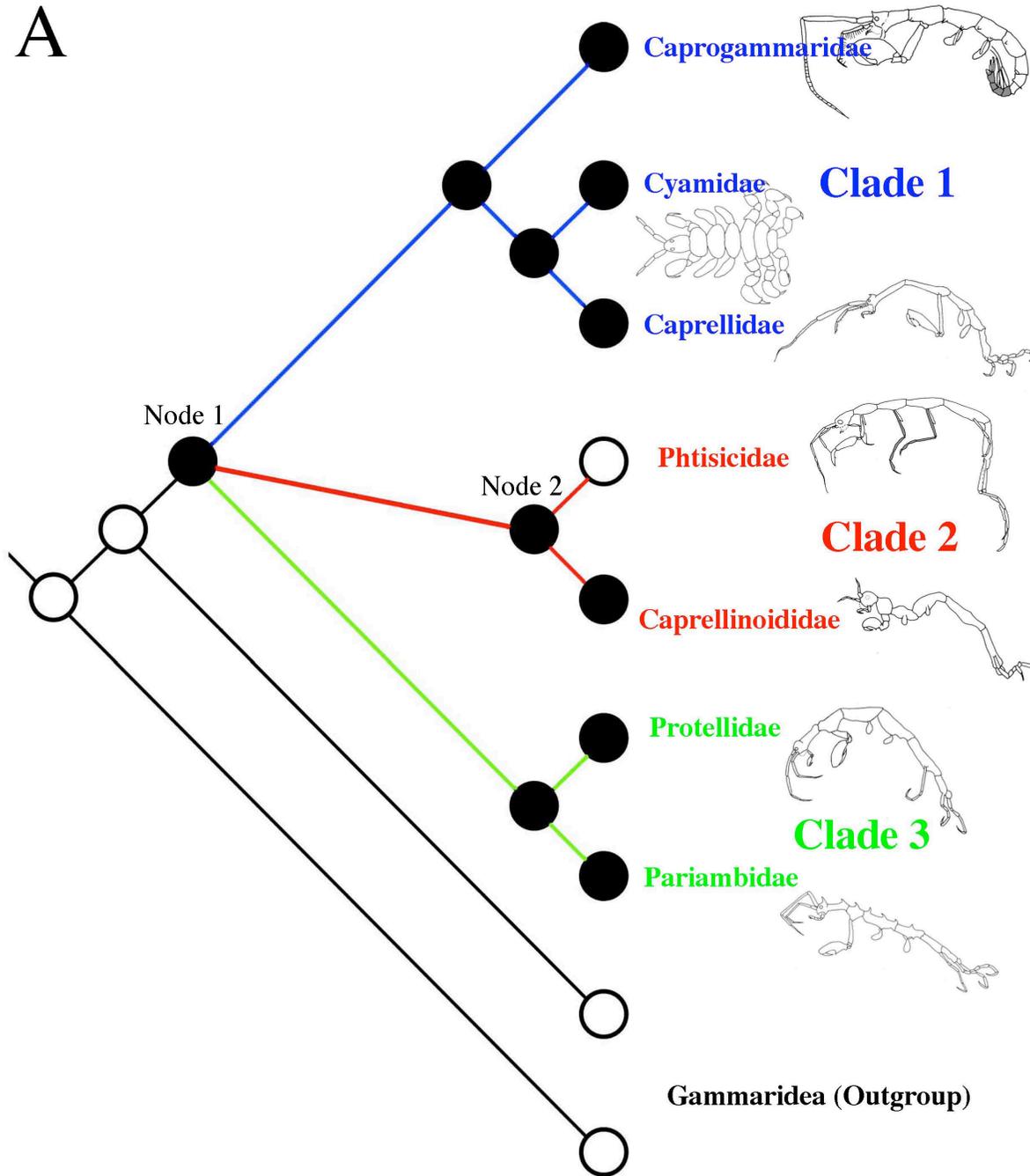
b







A



B

