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Author(s)	Takenaka, Yasuhiro; Yamaguchi, Atsushi; Shigeri, Yasushi
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HORIZONS

A light in the dark: ecology, evolution and molecular basis of copepod bioluminescence

YASUHIRO TAKENAKA^{1*}, ATSUSHI YAMAGUCHI^{2*} AND YASUSHI SHIGERI^{1*}

¹ HEALTH RESEARCH INSTITUTE, NATIONAL INSTITUTE OF ADVANCED INDUSTRIAL SCIENCE AND TECHNOLOGY (AIST), 1-8-31 MIDORIGAOKA, IKEDA, OSAKA, 563-8577, JAPAN, ² FACULTY OF FISHERIES SCIENCE, HOKKAIDO UNIVERSITY, 3-1-1 MINATO-CHO, HAKODATE 041-0821, JAPAN

*CORRESPONDING AUTHORS: pc_tert0617@yahoo.co.jp,
a-yama@fish.hokudai.ac.jp, yasushi.shigeri@aist.go.jp

SUMMARY

Within the calanoid copepods, the bioluminescent species comprise 5-59% of all bioluminescent species in the world's oceans, and 10-15% of the biomass. Most of the luminous species belong to the superfamily Augaptiloidea. The composition of bioluminescent species within the calanoid copepods shows latitudinal patterns; 5-25% of total calanoid copepods are found in high-latitude oceans, while 34-59% are in low-latitude oceans, reflecting a prey-predator relationship. Bioluminescent species of calanoid copepods are able to produce the light-emitting substrate coelenterazine. It is then transferred to higher predators through the food chain, and might be used for bioluminescence in other luminous organisms. A notable feature of copepod bioluminescence is the secreted-type, and its major function may be as an antipredatory response or a defensive behavior. Identification of more than 20 luciferase genes from calanoid copepods has revealed the highly conserved sequences those genes. This leads us the speculation that the genes for luciferase within the group of calanoid copepods have evolved independent of comparable genes outside of this group. We discuss here the ecological and biological functions of copepod bioluminescence, the significant diversity in luminous intensity, which might be evolutionarily relevant to their motility and habitat depth, and the promising directions of bioluminescent studies.

KEYWORDS: coelenterazine; crustacean; luciferase; luciferin; phylogenetic analysis; secreted reporter assay; thermostability, zooplankton

INTRODUCTION

Many luminous organisms exhibit bioluminescence from photophores, the light-emitting organs that contain their own photocytes or symbiotic luminous bacteria (Shimomura, 2006). Bioluminescence has been identified in a wide variety of organisms, such as mollusks, cnidarians, insects, and fishes (Shimomura, 2006; Haddock *et al.*, 2010; Moline *et al.*, 2013). Since Raphaël Dubois (1833 - 1877) demonstrated the first example of a luciferin-luciferase reaction (L-L reaction, Dubois, 1885), the understanding of bioluminescence at the molecular level has increased exponentially. In the late 20th century, the crystallization and structural determination of luciferin, the luminescent substrate, from several organisms was reported (Shimomura *et al.*, 1957; White *et al.*, 1961; White *et al.*, 1963; Shimomura and Johnson, 1972; Inoue *et al.*, 1975), and the efficient chemical synthesis of luciferin exploited (Moline *et al.*, 2013). With the advent of molecular biology, the gene cloning of luciferases, enzymes that catalyze the photogenic oxidation of the luciferin, was performed using fireflies (Wood *et al.*, 1984) and luminescent bacteria (Cohn *et al.*, 1985). To date, a number of different primary structures of the luciferases have been isolated from various luminous organisms, from bacteria and protista to insects and fishes (Shimomura, 2006).

In most marine environments, copepods dominate the zooplankton community, often accounting for more than 70% of the community in abundance and biomass (Mauchline, 1998). The bioluminescent abilities and characteristics of copepods have been investigated for over a hundred years (Giesbrecht 1895; Clarke *et al.* 1962; Herring 1988; Haddock *et al.*, 2010), starting with the simple observations of luminous phenomena or identifications of luminescent species. In 2002, the isolation of a

secreted-type luciferase, GLuc from *Gaussia princeps*, and its application to the microtiter plate-based DNA hybridization assay as a reporter was initially reported (Verhaegen and Christopoulos, 2002). Shortly thereafter, three other calanoid copepod luciferases (MLuc, MpLucs1 and 2) were isolated from *Metridia longa* and *Metridia pacifica* (Fig. 1C and 1D), respectively (Markova *et al.*, 2004; Takenaka *et al.*, 2008). Both species belong to the same Metridinidae family as *G. princeps*, and their luciferases showed high similarity in amino acid sequence to GLuc. Other bioluminescent copepods were collected from plankton nets off the coast of Kushiro, in the Sea of Japan, and additional copepod luciferase genes were cloned by a similar molecular cloning strategy (Takenaka *et al.*, 2012). In consequence, 21 calanoid copepod luciferases were identified, and common characteristics in their amino acid sequences were clarified (Takenaka *et al.*, 2012, 2013). Although structures and enzymatic properties of many copepod luciferases have been revealed, little is known about the potential functions of bioluminescence for copepods in the oceans. In this Horizon article, we summarize the geobiology and ecology of luminescent copepods, the possible functions of copepod bioluminescence, and the evolutionary lineage of copepod luciferases to infer the biological significance for luminescent and non-luminescent marine creatures.

DISTRIBUTION AND ECOLOGICAL ASPECTS OF CALANOID COPEPOD BIOLUMINESCENCE

Although bioluminescence has been reported in many calanoid copepod species, most of these species are found in the Augaptiloidea superfamily (Bradford-Grieve *et al.*, 2010), which contains four families: Augaptilidae, Metridinidae, Heterorhabdidae and

Lucicutiidae (Herring 1988; Takenaka *et al.*, 2012, 2013; Fig. 2). Since other families/superfamilies have little or no bioluminescence (Takenaka *et al.*, 2012), the evolution of bioluminescence may primarily have occurred in an ancestor of Augaptiloidea. This is consistent with the proposal that bioluminescent evolution occurred independently more than 40 times for marine organisms (Haddock *et al.*, 2010).

Calanoid copepods dominate both the abundance and biomass of world-wide marine zooplankton (Mauchline, 1998). Within the calanoid copepods, bioluminescent species in the four families mentioned above comprise 5-59% of all bioluminescent species in the world's oceans, and 10-15% of the biomass in the world's oceans (Fig. 3A). For various marine environments, Morin has documented that approximately 1-3% of the biomass in the surface ocean belongs to bioluminescent taxa (Morin, 1983). Compared with these values, the percentage of bioluminescent species is much higher for calanoid copepods. Interestingly, the composition of bioluminescent species within calanoid copepods showed latitudinal patterns. At high-latitude oceans ($\geq 44^\circ\text{N}$ or $\geq 44^\circ\text{S}$, arctic-subarctic or antarctic-subantarctic), bioluminescent species are relatively minor, and compose only 5-25% of the total of calanoid copepods (the mean $\pm$ SD: $14 \pm 5\%$) (Fig. 3A). While at low-latitude oceans ($< 44^\circ\text{N}$ or $< 44^\circ\text{S}$, tropical-subtropical and temperate), the composition of bioluminescent species accounted for 34-59% of the total of calanoid copepods (the mean $\pm$ SD: $45 \pm 8\%$), which is significantly higher than the amount in the high-latitude oceans ($p < 0.0001$, *U*-test). For high latitude oceans, where bioluminescence is especially important during the polar night (Johnsen *et al.*, 2014), the mass occurrence of large-sized Calanidae copepods (e.g., *Calanus* and *Neocalanus* spp., cf. Conover, 1988) may

account for the lower number of bioluminescent species. Conversely, a higher number of bioluminescent species could reflect a stable prey-predator relationship with higher complexity and biodiversity in the low-latitude oceans.

The percentage of the four families within the bioluminescent copepods also shows a different latitudinal patterns. Thus, only Metridinidae dominated at high-latitude oceans (Fig. 3A). While at low-latitude oceans, in addition to Metridinidae, Augaptilidae and Lucicutiidae were also abundant. Especially in the eastern Mediterranean Sea, Augaptilidae outnumbered Metridinidae. Since the feeding mode of Augaptilidae is carnivores (Arashkevich, 1969; Boxshall, 1985), its dominance, along with the other families at low-latitude oceans, may be related to high species diversity at low-latitude oceans (Woodd-Walker *et al.*, 2002; Rombouts *et al.*, 2009).

In summary, for pelagic calanoid copepods, the composition of bioluminescent species is much higher than for the other marine pelagic and benthic biomes. Bioluminescent copepods are also important food sources for cephalopods and myctophid and gonostomatid fishes, accounting for more than 31-89% of their food items (Fig. 3B). This suggests that bioluminescent copepods play the role of coelenterazine couriers within the marine pelagic realm. The composition of the bioluminescent species in calanoid copepods also showed latitudinal patterns; they are high in number in low-latitude oceans, which may be related to the high species diversity of these regions.

ROLE OF CALANOID COPEPOD BIOLUMINESCENCE IN TRANSFER OF LUMINESCENCE SUBSTRATE THROUGH THE TROPHIC CHAIN

The substrate for luminescence in calanoid copepods is an imidazopyrazinone

luciferin, coelenterazine. The luciferins containing the imidazopyrazinone structure are found in various other luminous marine organisms besides calanoid copepods, such as fishes, decapod shrimps, protists, molluscs, cnidarians, chaetognaths, ostracods, and radiolarians. Similar chemical structures of luciferins found in phylogenetically distant bioluminescent organisms may be derived from dietary transfer of luminescence substrates through the trophic chain (Cormier *et al.*, 1967; Thompson *et al.*, 1987, 1988a,b; Thompson and Rees, 1995; Thomson *et al.*, 1995a). It has been suggested that some luminous marine organisms are unable to produce their own luciferins and depend on a dietary supply of them for bioluminescence (Mallefet and Shimomura, 1995; Haddock *et al.*, 2001). *M. pacifica* was found to retain the biosynthetic pathway for the light-emitting substrate coelenterazine (Oba *et al.*, 2009), and can be a prey for higher consumers as the ultimate source of coelenterazine. Considering the high percentage of bioluminescent species in calanoid copepods, they might have an essential role as supplier of coelenterazine within the marine environments (Mallefet and Shimomura, 1995) (Fig. 3B). For example, the firefly squid, *Watasenia scintillans*, indigenous to northern Japan, feeds predominantly on calanoid copepods, including *M. pacifica* (Hayashi and Hirakawa, 1997). Liver tissues of *W. scintillans* contain coelenterazine and show strong chemiluminescence (Inoue *et al.*, 1975). Although the light-emitting substrate of *W. scintillans*, coelenterazine disulfate, is not identical to coelenterazine, it is probable that *W. scintillans* synthesizes its own luciferin by utilizing coelenterazine from *M. pacifica* as a starting molecule (Inoue *et al.*, 1976). Furthermore, a specialized prey-predator linkage is reported between Augaptiloidea copepods and mesopelagic myctopid and gonostomatid fishes, both having coelenterazine (Merrett and Roe, 1974; Gordon *et al.*, 1985; Lancraft *et al.*, 1988;

Hopkins and Sutton, 1998) (Fig. 3B). Except for copepods, the decapod *Systellaspis debilis* is probably able to synthesize coelenterazine (Thomson *et al.*, 1995b), although its biosynthesis has not yet been elucidated. Thus, the possibility exists that calanoid copepods may have a profound influence on marine bioluminescence with the functions of producer and courier of coelenterazine to luminous organisms utilizing imidazopyrazinone luciferins.

FUNCTIONS OF CALANOID COPEPOD BIOLUMINESCENCE

The major function of copepod bioluminescence may be an antipredatory response or a defensive behavior, although currently this is just speculation (Mauchline 1998). There is a notable relationship, for example, between bioluminescence and the jump response in the calanoid copepod *Pleuromamma xiphias* (Augaptiloidea) when tested with a quantified hydrodynamic disturbances. Weak stimuli elicited only a jump response, whereas supra-threshold stimuli resulted in both a jump and bioluminescent discharge (Hartline *et al.*, 1999). This suggests that copepods save the bioluminescence for what is perceived to be a stronger threat by a predator.

There is a large diversity in luminosity between each Augaptiloidea species (Takenaka *et al.*, 2012; Fig. 2). This may be another clue as to how calanoid copepods developed their bioluminescence. The Megacalanoidea and Clausocalanoidea, which are considered as lately diverged superfamilies after Augaptiloidea in the evolution of calanoid copepods (Bradford-Grieve *et al.*, 2010), show little or no bioluminescence (Takenaka *et al.*, 2012; Fig. 2). In these species, where they have developed myelinated nerve fibers (Davis *et al.*, 1999) and chemosensory organs, they have greater motility and faster escape responses (~2 ms)

from predators (Lenz and Hartline, 1999; Lenz *et al.*, 2000; Waggett and Buskey, 2008). Whereas the Augaptilodea organisms, which have only unmyelinated axons, show significantly slower responses (~6 ms) to initiate an escape behavior (Davis *et al.*, 1999). Thus, differences in an escape response among calanoid copepod superfamilies could be relevant to the function of their bioluminescence as a defensive mechanism to escape from surrounding predators. Bioluminescent species in the Augaptiloidea family evolved several defensive behaviors, including a strong escape jump and bioluminescence, to avoid predation, whereas species in the Megacalanoidea and Clausocalanoidea families may have developed a reliance on improved speed and strength of the escape response itself, made possible by the evolution of myelinated axons. For the ancestral species in the Megacalanoidea and Clausocalanoidea, bioluminescence as a defensive function might have been less essential than for the non-myelinated Augaptiloidea superfamily, including the Metridinidae family, resulting in a gradual loss of luminescence activity as myelination evolved.

The habitat depth of calanoid copepods also seems to be an important factor that can affect the intensity of bioluminescence. The Heterorhabdidae family also belong to the Augaptiloidea superfamily, but inhabits mesopelagic and bathypelagic zones (200 - 3000 m) (Yamaguchi and Ikeda, 2000), and shows much weaker bioluminescence than Metridinidae species (Takenaka *et al.*, 2012, 2013; Fig. 2). Compared with Metridinidae species, such as *M. pacifica* that inhabits the epipelagic layer (0 - 200 m), Heterorhabdidae would be exposed to lower predation pressure by mesopelagic and bathypelagic fishes. Furthermore, counter-illumination is not available at such great and dark depths. These ecological aspects might have led to the evolution of diversified bioluminescence in the Augaptiloidea superfamily.

Besides the defensive function of bioluminescence in copepods, it could also be used for communication as a warning signal between individuals of *Metridia longa*, was proposed by Buskey and Swift (1985); however there are no reports of a possible function for bioluminescence in sexual recognition by copepod species. A potential antioxidative function of bioluminescence proposed for other organisms (Rees *et al.*, 1998) also remains unsupported in copepods.

FUNCTIONS OF COPEPOD BIOLUMINESCENCE INFERRED FROM STRUCTURE OF COPEPOD LUCIFERASES

The biological functions of copepod bioluminescence could be interpreted by comparing structures of luciferases isolated from different copepod species and how the luciferase gene has evolved from a common ancestral origin in each group of luminous species. More than 20 genes coding for the luciferase have been isolated so far from luminous calanoid copepods. All natural copepod luciferases identified so far contain about 20 amino acids of an N-terminal signal sequence for extracellular secretion. Even recombinant copepod luciferases expressed in mammalian cultured cells were also efficiently secreted into the culture medium (Markova *et al.*, 2004; Tannous *et al.*, 2005; Takenaka *et al.*, 2008, 2012). This property of the protein can provide calanoid copepods the ability to produce the secreted-type luminescence. When they are secreted with luciferin from the luminous glands (Fig. 1B and 1D), the display of a cloud of sparkles may serve as counterillumination to hide the silhouette of the body and/or as smoke screen in a bioluminescent defense (Haddock *et al.*, 2010). Another function of bioluminescence in copepods is startling the approaching predators by a strong flash. Many of the copepod luciferases exhibit spike-like, strong light when

they are mixed with the substrate luciferin. This flash-type bioluminescence can act effectively as a repellent or distractor to predators. In contrast, stable and long-lasting luminescence is more suitable for counter-illumination and smoke-screen roles.

In *Metridia* and *Pleuromamma* species, multiple luciferase genes were cloned from the same cDNA library (Takenaka *et al.*, 2008; Borisova *et al.*, 2008; Takenaka *et al.*, 2012). The MpLuc1 and MpLuc2 from *M. pacifica* would be transcribed from two different loci or alleles, since a survey of their genomic sequences revealed the presence of different lengths of the introns at different positions in two MpLuc genes (Takenaka *et al.*, 2008), and also in MoLuc genes (Takenaka *et al.*, 2012). There are distinct differences in the enzymatic properties of MpLuc1 and MpLuc2, as seen in their luminescence kinetics. MpLuc1 sharply emits light upon mixing with the substrate coelenterazine, whereas MpLuc2 shows a much slower build-up and gradual decay of luminescence (Takenaka *et al.*, 2008). Although details about the expression and localization of MpLuc1 and MpLuc2 in the body of *M. pacifica* are still unclear, it is plausible that the copepod uses two isoforms with different enzymatic characteristics for different defensive roles, such as startling and counter-illumination.

EVOLUTION OF COPEPOD BIOLUMINESCENCE

The multiple sequence alignments of copepod luciferases isolated so far revealed the presence of two tandem consensus sequences of C-x(3)-C-L-x(2)-L-x(4)-C-x(8)-P-x-R-C (x, amino acid residue) (Takenaka *et al.*, 2013), which suggests that all known calanoid copepod luciferases originated from the same ancestral gene. Each consensus sequence was thought to act as a core of the

functional domain. It has been reported that each of two domains in GLuc exhibited weak, but notable, luciferase activity when they were expressed in *E. coli* and characterized independently (Inoue and Sahara, 2008). As is obvious from the presence of four cysteine residues in the consensus sequence, there are a lot of cysteine residues in the full amino acid sequence of copepod luciferases. The presence of tandem consensus sequences in catalytic domains mentioned above, and the abundant cysteine residues, can be the characteristic benchmarks for the identification of copepod luciferases.

Among the many luciferases, beetle luciferases are probably the most studied luciferase in the field of evolution (Viviani, 2002). Notable similarities in catalytic mechanisms (McElroy *et al.*, 1967) and amino acid sequences (Suzuki *et al.*, 1990) between a luciferase from the North American firefly and acyl-CoA synthetase have been reported. It was proven that firefly luciferase possesses both catalytic activities of the bioluminescence reaction (ATP-dependent monooxygenase) and the fatty acyl-CoA synthetic reaction (Oba *et al.*, 2003). In marine organisms, it has been suggested that aequorin and obelin, the coelenterate photoproteins, originated from ancestral calcium-binding proteins, such as calmodulin, based on the amino acid sequence similarity and their reactivity to calcium ion (Tsuji *et al.*, 1995). In copepods, the BLAST search uncovered no protein showing higher similarity to the overall structure of any copepod luciferase or consensus sequences described above. Thus, this strategy, which was successful in studies of other organisms to reveal evolutionary origins, has not yet produced this outcome for copepods. The lack of significant homologies between copepod luciferase sequences and any other luciferase sequences suggests that copepod luciferases evolved from their ancestral origins

independent of non-copepod luciferases. This strongly supports the idea that the capacity to generate light-emission has arisen through convergent evolution of bioluminescence-related genes among different lineages.

Takenaka et al. tried to estimate an amino acid sequence of an ancestral copepod luciferase by *in silico* phylogenetic analyses of all known luciferases derived from the Augaptiloidea superfamily (Takenaka et al., 2013). As a consequence of the ancestral sequence reconstruction, two candidates for the ancestral genes, aCopLuc43 and aCopLuc48, were predicted. The aCopLuc43 and aCopLuc48 were the putative common ancestors for the Metridinidae/Lucicutiidae families and for the Heterorhabdidae family, respectively. The aCopLuc43 and aCopLuc48 expressed in mammalian cells exhibited remarkable luciferase activities in the intracellular lysates and culture media. In addition, they only reacted with coelenterazine, not with *Cypridina* luciferin, just as seen in native copepod luciferases. The results suggest that ancestral species acquired the ability to produce bioluminescence before evolutionary diversification of related copepod species.

FUTURE DIRECTIONS

Although the application of copepod luciferases as a reporter in biomedical studies has developed rapidly over the last ten years (Tannous *et al.*, 2005; Remy and Michnick, 2006; Morlighem *et al.*, 2007; Wu *et al.*, 2007; Wurdinger *et al.*, 2008; Kim *et al.*, 2011), mechanisms of production, secretion, and regulation of copepod bioluminescence remain almost entirely unknown. A future, basic biological study of copepod bioluminescence could be the morphological recognition of copepod bioluminescence with techniques such as immunostaining or whole mount *in situ* hybridization; these

will provide further information about the expression and localization of bioluminescent-related proteins, such as luciferases and other unknown accessory proteins. Besides these *in vitro* studies, observation of bioluminescent behaviors in copepod culture will give us clues to understand how bioluminescence works in nature. Development of a long-term copepod culture system equipped with a high-speed camera may afford researchers in bioluminescence and zooplankton studies a tool to observe live copepod subjects directly, with or without its predators. The system also would enable us to sample copepods in good condition for molecular and morphological analyses in the lab.

To further clarify the evolution and origin of copepod luciferases, we may need more information about the primary structure of luciferase and luciferase-like proteins from a variety of marine creatures, including copepods. For example, the tiny poecilostomatoid copepod *Oncaea* is known to have bioluminescence (Herring *et al.*, 1993) (Fig. 2), but there is no information about its luciferase gene structure. Structural and enzymatic comparison of luciferase genes isolated from calanoid and poecilostomatoid copepods might provide phylogenetic and evolutionary insights for understanding marine bioluminescence. Furthermore, metagenomic approaches will also uncover the evolutionary trait of bioluminescence in marine organisms. On the other hand, we cannot rule out the possibility that non-bioluminescent calanoid copepods, such as the Megacalanoidea and Clausocalanoidea, still possess luciferase genes that previously coded the active luciferase, but lost function subsequently. Luciferase gene loss was suggested in the dinoflagellate *Gonyaulax spinifera*, which shows very weak luminescence (Baker *et al.*, 2008). These kinds of pseudogenes, or the remnants of the genes, may be the clues to how luciferase genes evolved in the

copepods during myelination.

Another approach to reveal the evolution of copepod bioluminescence is identification of the pathways of coelenterazine synthesis in calanoid copepods. The *de novo* synthesis of coelenterazine from two L-tyrosines and an L-phenylalanine has been shown by feeding isotopically labeled L-amino acids to *M. pacifica* (Oba *et al.*, 2009; Markova and Vysotski, 2015). The chemistry of coelenterazine biosynthesis and features of related enzymes will be a promising area for future bioluminescence studies. Phylogeny of the biosynthetic proteins might shed light on the relationship among marine bioluminescent and/or non-bioluminescent organisms. Also, as a biotechnological application, if genes encoding the metabolic enzymes involved in luciferin biosynthesis were identified, we could develop autoluminescent organisms equipped with luciferases and luciferin biosynthesis systems.

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Figure legends

Fig. 1. Bioluminescent calanoid copepods. (A) microscopic image of *Metridia okhotensis*; (B) fluorescent image of *M. okhotensis* illuminated by UV light. Arrowheads indicate the possible position of luminous glands; (C) bright-field image of *Metridia pacifica*; (D) fluorescent image of the urosome of *M. pacifica*. Arrowheads indicate the possible luminous glands on the caudal rami and anal segment; (E) *Pleuromamma abdominalis*; (F) *Pleuromamma xiphias*. (Photos: Y. Takenaka).

Fig. 2. Taxa of bioluminescent and non-bioluminescent copepods depicted based on molecular phylogeny of 18S rRNA nucleotide sequences. The Augaptiloidea superfamily contains a number of bioluminescent species. Most luciferases were isolated from species in the Metridinidae family, comprising *Gaussia*, *Pleuromamma* and *Metridia* genera. Some species in different phyla are also luminous, but their distribution is patchy. The fluorescent proteins were identified from several species in the Centropagoidea superfamily. *Oncaea conifera* is known as a bioluminescent species and belongs to the Poecilostomatoida superfamily (Herring *et al.*, 1993). Although the complete 18S rRNA sequence of *Oncaea* was not available in the database, it was depicted with a dashed line in the tree based on the partial 18S and ITS1 sequences.

Fig. 3. (A) Composition of bioluminescent families relative to total calanoid copepod abundance (circles) or biomass (in dry mass [DM] = squares or carbon [C] = triangles) from cold-water region (Arctic-subarctic region, left) and warm-water region (temperate-tropical region, right). Data sources are ¹Deevey and Brooks (1977),

²Ward and Shreeve (1999), ³Auel and Hagen (2002), ⁴Yamaguchi et al. (2002),
⁵Koppelman and Weikert (2007), ⁶Homma and Yamaguchi (2010), and ⁷Kosobokova
and Hopcroft (2010). (B) Prey-predator linkage between bioluminescent copepod
families and their predators (cephalopods, myctophids, gonostomatids, and cnidarians).
Cn: percentage composition in number of bioluminescent copepods in their food items.
Data sources are ⁸Lancraft *et al.* (1988), ⁹Passarella and Hopkins (1991), and ¹⁰Williams
et al. (2001).

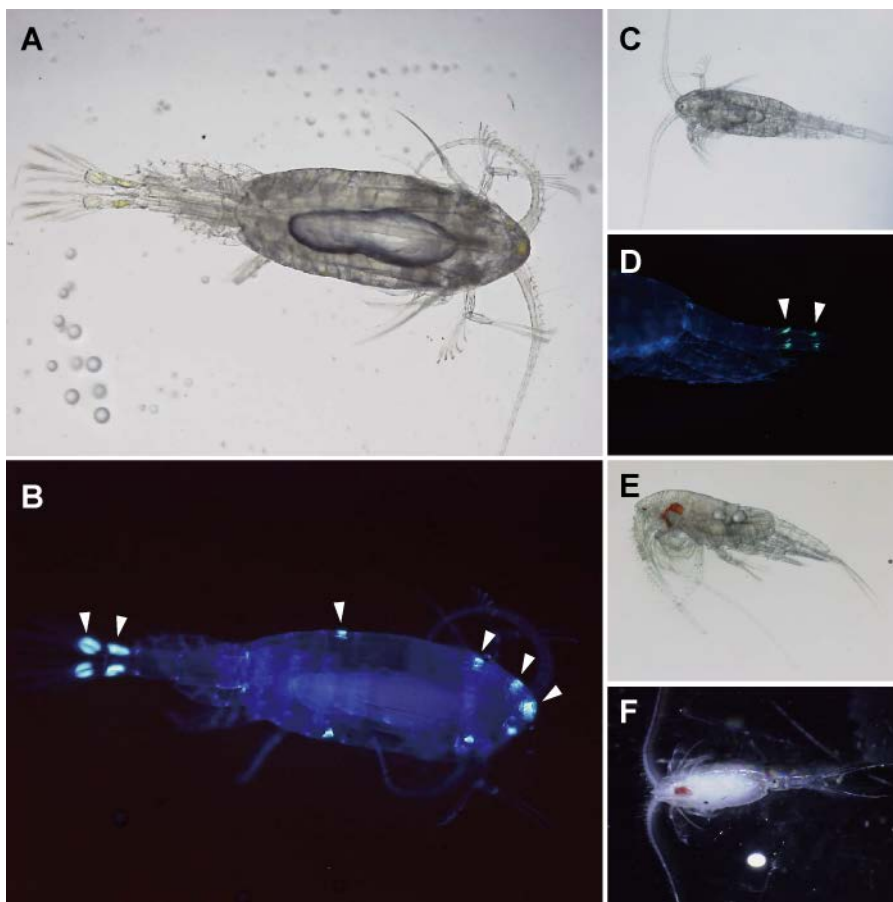


Fig. 1

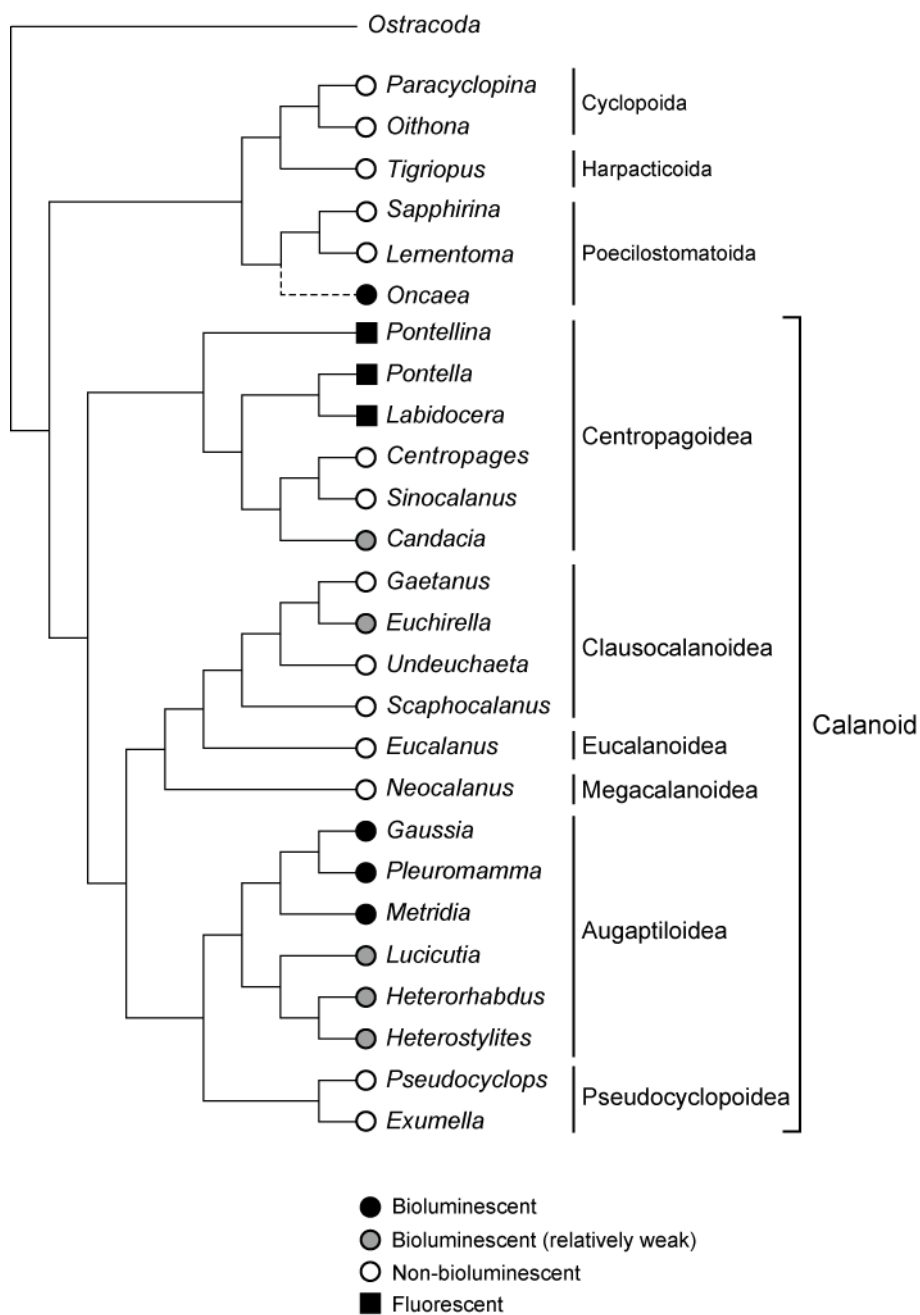
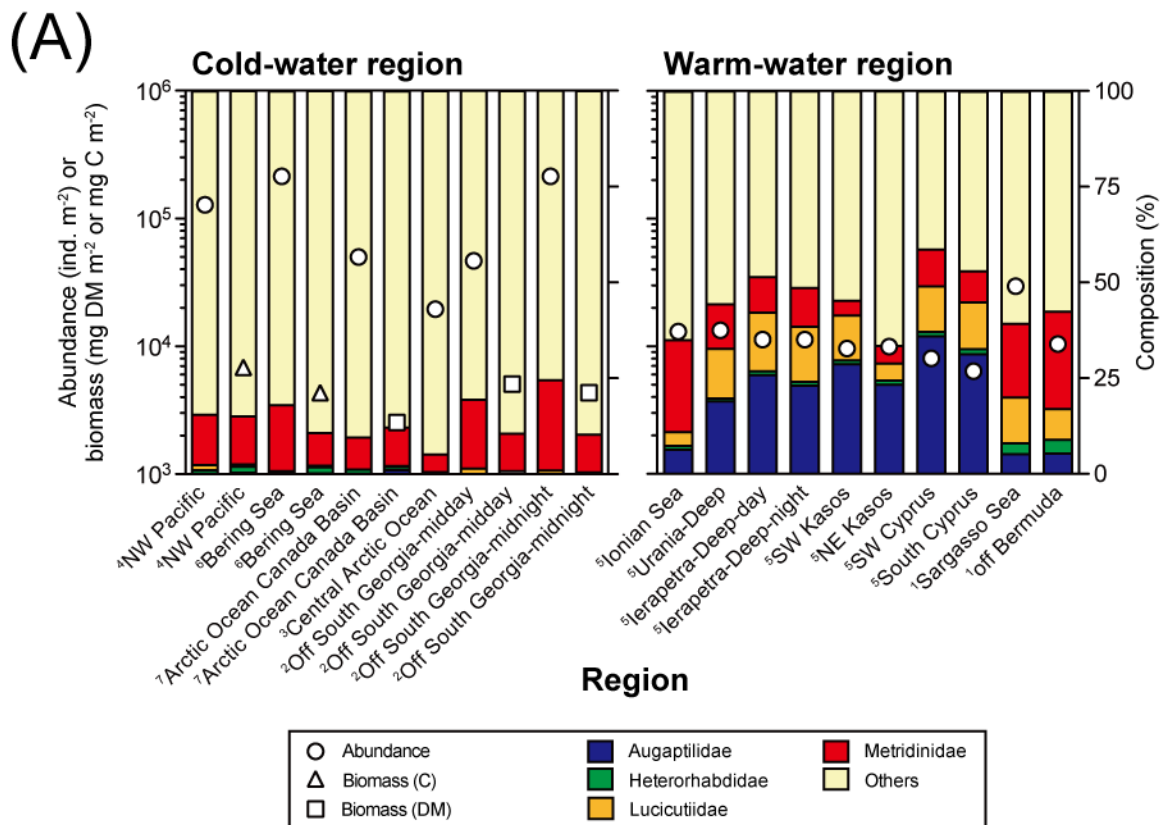


Fig. 2



(B)

Bioluminescent copepods
(Augaptilidae, Heterorhabdidae, Lucicutiidae, Metridinidae)

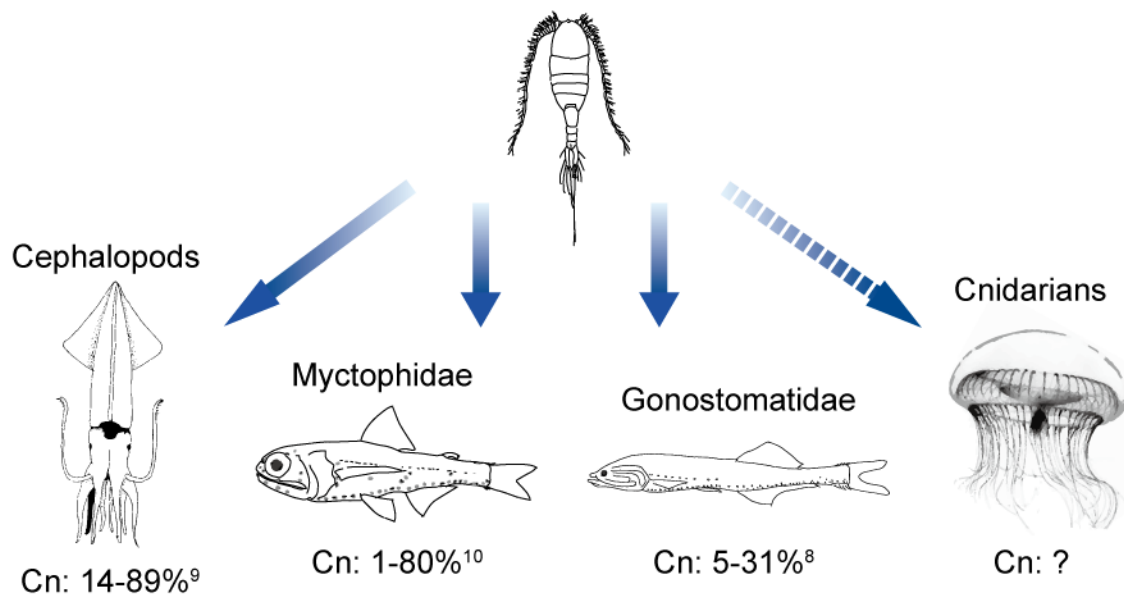


Fig. 3