Evolution of Bioluminescence in Marine Planktonic Copepods

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Abstract

Copepods are the dominant taxa in zooplankton communities of the ocean worldwide. Although bioluminescence of certain copepods has been known for more than a 100 years, there is very limited information about the structure and evolutionary history of copepod luciferase genes. Here, we report the cDNA sequences of 11 copepod luciferases isolated from the superfamily Augaptiloidea in the order Calanoida. Highly conserved amino acid residues in two similar repeat sequences were confirmed by the multiple alignment of all known copepod luciferases. Copepod luciferases were classified into two groups of Metridinidae and Heterorhabdidae/Lucicutiidae families based on phylogenetic analyses, with confirmation of the interrelationships within the Calanoida using 18S ribosomal DNA sequences. The large diversity in the specific activity of planktonic homogenates and copepod luciferases that we were able to express in mammalian cultured cells illustrates the importance of bioluminescence as a protective function against predators. We also discuss the relationship between the evolution of copepod bioluminescence and the aspects of their ecological characteristics, such as swimming activity and vertical habitat.

Key words: copepoda, luciferase, zooplankton, crustacean, phylogenetic analysis.

Introduction

Copepods are the most numerous taxa of zooplankton fauna in the ocean worldwide. Their bioluminescent abilities and characteristics have been investigated for over a 100 years (Giesbrecht 1895; Clarke et al. 1962; Haddock et al. 2010). Luminescence of calanoid copepods is produced by glandular cells in the luminous glands, which are largely located on the caudal rami and legs (fig. 1), although their number and location vary considerably among families. Most of the luminous species reported to date belong to the superfamily Augaptiloidea, which contains the families Arietellidae, Augaptilidae, Heterorhabdidae, Lucicutiidae, Metridinidae, and Nullosetigeridae (Herring 1988). Within these families, the luminescent behavior of Metridinidae and most Heterorhabdidae, Lucicutiidae, and Augaptilidae are well known (Herring 1988). Based on a previous cladistic analysis of morphological features, the Augaptiloidea is believed to have diverged very early in the evolution of calanoid copepods (Bradford-Grieve et al. 2010). Except for the Augaptiloidea superfamily, a single species in Megacalanoidea (*Megacalanus princeps*) was reported as luminous (Herring 1988) with very limited evidence, and it remains unverified. It is also unclear whether species in other superfamilies, such as Centropagoidea, Clausocalanoidea, and Eucalanoidea, are luminous. This indicates that among the ten calanoid superfamilies, bioluminescent copepods are found almost exclusively in the superfamily, Augaptiloidea. Concerning bioluminescence behavior other than calanoid copepods, that of tiny pseudoplanktonic poecilostomatoid *Oncaea* spp. is only notable (Herring et al. 1993). One reason for the limited information on copepod bioluminescence is that correct copepod identification is very difficult. In addition to the species identification challenges, zooplankton sampling, especially in deep water, requires special equipment, including large-sized plankton nets, to collect sufficient numbers of specimens. Furthermore, macro- and microscopic detection of weak and flash-type bioluminescence from tiny copepods is also difficult, especially if the specimen was damaged during the collection or manipulation by an investigator. Therefore, a device, such as a luminometer, to detect weak bioluminescence of zooplankton is needed.

One strong piece of evidence for the presence of bioluminescence in a particular organism is the molecular identification and functional analysis of luciferase, which is the enzyme directly involved in producing luminescence in an organism. In 2002, the luciferase gene was cloned from calanoid *Gaussia princeps* (family Metridinidae), which is a large (~10 mm) gray-black–pigmented copepod usually found in the mesopelagic zone (at the depths from 200 to 1,000 m) (Verhaegen and Christopoulos 2002). A novel feature of *Gaussia* luciferase (GLuc) is its relatively small size (~20 kDa) and secreted luminescence, not only in planktonic

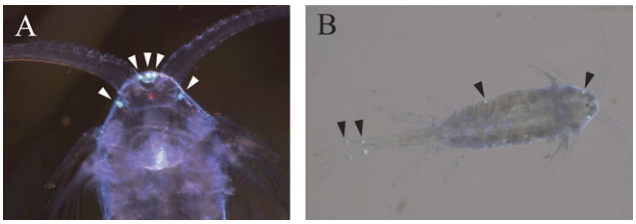

FIG. 1. (*A*) Fluorescent microscopic image of bioluminescent Copepoda, *Metridia pacifica*, illuminated with ultraviolet. Arrowheads indicate the position of putative luminous glands on head. (*B*) Bright-field and fluorescent image of *M. pacifica*. Putative luminous glands were on the legs and abdomen adjacent to the head.

expression but also when expressed in mammalian and insect cells, with robust stability and great luminescent activity. GLuc catalyzes the oxidation of a luciferin called coelenterazine to produce the light. Luciferase genes have also been found in *Metridia longa* (MLuc) (Markova et al. 2004) and *Metridia pacifica* (MpLuc1 and MpLuc2) (Takenaka et al. 2008), both of which belong to the Metridinidae family just as *G. princeps* does. These GLuc-homologues also react with the substrate coelenterazine to produce light. The bioluminescence in these species was confirmed by identification and functional analysis of their luciferases.

In this study, we isolated and characterized novel luciferase genes from five species of calanoid copepods and conducted the quantitative analyses of copepod bioluminescence using zooplankton homogenates and recombinant luciferases. By quantitative measurements, we could reveal the presence or absence of luminescence of unverified species with confirmation of verified ones. Furthermore, we compared the luminous intensity of homogenates among calanoid superfamilies and those of luciferases among genera in Augaptiloidea to infer the defensive function and molecular evolution of copepod bioluminescence.

Materials and Methods

Collection of the Copepods

Zooplankton samples were collected with vertical tows of a closing net (60 cm mouth diameter, 100 μm mesh) from 0 to 300 or 100 to 900 m at several stations located 41°54′–42°24′N, 141°10′–144°36′E off south of Hokkaido from 25 September to 3 October 2008. After collection, samples were immediately and gently transferred into chilled seawater, examined under a stereomicroscope, and live copepods, such as *Metridia okhotensis*, *Pleuromamma abdominalis*, *Lucicutia ovaliformis*, *Heterorhabdus tanneri*, and *Heterostylites major*, were sorted out. One to 20 live copepods (number varied with body size of copepods) were immersed into RNAlater (Qiagen, Japan), kept at 4 °C in the dark for 1 day, and then stored in a −30 °C freezer. For the luminescence assay of zooplankton lysate, 1–10 live copepods were frozen at −30 °C without any preservative immediately after sorting. Fluorescent images of *M. pacifica* were observed under a fluorescence stereomicroscope (VB-G25/S20/L11; Keyence, Osaka, Japan), equipped with a BFP filter pair (excitation at 387/28 nm, emission at 430 nm), and cooled CCD camera (VB-7010; Keyence).

Luminescence Assay for Zooplankton Homogenate

Frozen specimens were transferred into 150 μl of 20 mM Tris–HCl (pH 8.0) with 10 mM $MgCl_2$ and an appropriate amount of Complete Protease Inhibitor Cocktail (Roche Applied Science, Japan) and thoroughly homogenized in a 1.5-ml microtube using a plastic pestle (As one, Japan). The homogenate was centrifuged at 15,000 rpm for 5 min, and the supernatant was used for the luminescence assay and determination of protein concentration. Luminescence activity was measured by mixing 5 μl of zooplankton lysate and 10 μl of 1 ng/μl coelenterazine in 20 mM Tris–HCl (pH 8.0), with 50 mM $MgCl_2$, followed by 10 s of counting. Protein concentration was determined by the Bradford method (Bradford 1976).

Sequencing and Analyses of Zooplankton Ribosomal RNA Genes

Nuclear ribosomal RNA (rRNA) sequences were searched and obtained from GenBank. If they were not available in database, we cloned and sequenced as follows. The specimens fixed in 100% ethanol were transferred into 200 μl of Buffer ATL (DNeasy Tissue Kit; Qiagen, Japan) containing 2 μg/μl of proteinase K and thoroughly homogenized in 1.5-ml microtubes using a plastic pestle (As one). Genomic DNA was purified using a DNeasy Tissue Kit (Qiagen, Japan) according to the manufacturer's instructions. The primers to amplify the 18S rRNA genes were 21F, 5′-ATCTGGTTGATCCTGCCAGT-3′ and 1778R, 5′-AATGATCCTTCCGCAGGTTC-3′. The thermal cycling conditions for the polymerase chain reaction (PCR) amplification were 98 °C for 2 min for denaturation, followed by 30 cycles of 98 °C for 10 s for denaturation, 52 °C for 15 s for annealing, and 68 °C for 90 s for extension. PCR products were purified with AMPure (Beckman Coulter, Japan), and both strands were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit using a 3130xl Genetic Analyzer (Applied Biosystems, Japan) following the manufacturer's instructions. The sequencing primers were used according to an earlier protocol (Kurtzman and Robnett 2003). The trimmed 18S sequences were deposited in the DDBJ/EMBL/GenBank databases with accession numbers AB625956 (*M. pacifica*), AB625957 (*M. okhotensis*), AB625962 (*M. curticauda*), AB625967 (*M. asymmetrica*), AB625961 (*M. longa*), AB625958 (*P. scutullata*), AB625963 (*P. abdominalis*), AB625964 (*P. xiphias*), AB625970 (*G. princeps*), AB625965 (*L. ovaliformis*), AB625959 (*H. tanneri*), AB625968 (*H. major*), AB625960 (*Gaetanus variabilis*), AB625966 (*Scaphocalanus magnus*), AB625971 (*Euchirella amoena*), AB625972 (*Undeuchaeta major*), AB625973 (*Candacia bipinnata*), AB625974 (*Candacia columbiae*), and AB625969 (the ostracod *Vargula hilgendorfii*). All sequences were aligned using ClustalW with default parameters (http://www.genome.jp/tools/clustalw/). Cladistic analyses among calanoid species using 18S rRNA genes were determined using maximum likelihood (ML) and neighbor joining (NJ) programs of

MEGA5 (Tamura et al. 2011). The reliability of the trees was computed by 1,000 replications of bootstrap analyses.

PCR Cloning and Molecular Phylogeny of Copepod Luciferases

Total RNA was prepared from 1 to 5 specimens using RNeasy Micro kit (Qiagen, Japan). cDNA was synthesized from total RNA using the SMART RACE cDNA Synthesis Kit (Takara Bio, Japan). Internal sequences of copepod luciferase cDNAs were amplified using the White_luc UP1, 5′-GGCTGCACYAGGGGATGYCTKATMTG-3′ and White_luc UP2, 5′-GCTATTGTTGAYATYCCYGARAT-3′ as forward primers and the White_luc LP1, 5′-ACATTGG-CAAGACCYTTVAGRCA-3′ and White_luc LP2 5′-TCAA CTTGWTCAATRAAYTGYTCCAT-3′, which were designed based on the conserved region of MLuc (AAR17541, *M. longa*), GLuc (AAG54095, *G. princeps*), MpLuc1, and MpLuc2 (AB195233 and AB195234, respectively, *M. pacifica*) luciferases. Thermal cycling proceeded in a PTC-200 (Bio-Rad, Japan) at 96 °C for 1 min, followed by 35 cycles of 96 °C for 5 s, 55 °C for 10 s, and 68 °C for 30 s with Advantage 2 DNA polymerase (Takara Bio). PCR products (200–300 bp) were gel purified and cloned into pCR2.1 vector (Invitrogen, Japan). The plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen, Japan) and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The 5′- and 3′-ends of copepod luciferase cDNAs were amplified using the gene-specific primer and Universal Primer Mix following the manufacturer's instructions. After sequencing the 5′- and 3′-RACE products, the full-length cDNA was amplified using gene-specific primers. cDNA sequences of novel luciferases were deposited in the DDBJ/EMBL/GenBank databases with accession numbers as follows; MoLuc1, AB519698 and MoLuc2, AB519699 (*M. okhotensis*), PaLuc1, AB519704 and PaLuc2, AB519705 (*P. abdominalis*), LoLuc, AB519710 (*L. ovaliformis*), HtLuc1, AB519711, and HtLuc2, AB519714 (*H. tanneri*), HmLuc1, AB519715, and HmLuc2, AB519716 (*H. major*). Both MoLuc1 and MoLuc2 genes were amplified from genomic DNA isolated from *M. okhotensis* with gene-specific primers. Sequences of both PCR products were deposited in the DDBJ/EMBL/GenBank databases (AB674505 and AB674506). For phylogenetic analyses, the deduced amino acid sequences of copepod luciferases were trimmed manually to domains 1 and 2 with internal sequences because of their divergence at their N- and C-terminals. Resulting core regions consisting of 135–142 amino acid residues were aligned using the web-based ClustalW program with default parameters (http://www.genome.jp/tools/clustalw/). Phylogenetic trees were generated with the neighbor joining (NJ) and ML analyses using MEGA5 program (Tamura et al. 2011).

Vector Construct and Transfection of Copepod Luciferases in Cultured Cells

The full coding sequences of the copepod luciferases were amplified by *Pyrobest* DNA Polymerase (Takara Bio) from the template plasmids that harbor the full-length cDNAs and subcloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to express luciferase proteins tagged with 6× histidines at their C-terminus in mammalian cultured cells. All expression vectors were purified using an EndoFree Plasmid Maxi Kit (Qiagen, Japan). HEK293 cells (Health Science Research Resources Bank, Japan) were maintained in Mega-Cell MEM (Sigma-Aldrich, Japan) supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. For transfections, 5×10^5 cells per well were seeded in six-well culture plates, and FuGENE HD (Roche Applied Science) was used according to the manufacturer's instructions. The culture medium was incubated for 72 h after transfection and stored at −30 °C until immunoblotting and luciferase assay.

Assays of Copepod Luciferases

Bioluminescence in all experiments, except for analyses of the spectra and kinetics, was measured using a MiniLumat LB 9506 luminometer (Berthold, Japan). The basic procedure for the luciferase assay was as follows: 5 μl of protein solution containing each of the luciferases was transferred into the luminometer tube and then placed in the luminometer. A 10-s measurement was initiated immediately after injecting 10 μl of 1 ng/μl coelenterazine in 20 mM Tris–HCl (pH 8.0) with 50 mM $MgCl_2$ into the protein solution. Luminescent spectra of recombinant copepod luciferases were measured using an AB-1850C spectrofluorometer (Atto, Japan) (slit width, 1 mm; spectral resolution, 0.5 nm) for 1 min after mixing 20 μl of copepod luciferases expressed by cultured cells and 200 ng of coelenterazine in a 0.2-ml PCR tube. To determine thermostability of copepod luciferases, 20 μl of copepod luciferases expressed by cultured cells was incubated in a block heater at 60 or 80 °C for 30 min and then cooled on ice for 5 min. Bioluminescence was measured by injecting 10 μl of coelenterazine solution (1 ng/μl) into 5 μl of heat-treated luciferases at room temperature using a MiniLumat LB 9506 luminometer.

Immunoblotting

Hexahistidine-tagged luciferases were concentrated from 1 ml of HEK293 culture medium by applying to a centrifugal filter unit, Ultrafree-MC (Millipore, Japan), containing 100 μl of Ni Sepharose 6 Fast Flow resin (GE Healthcare, Japan). The resin was washed four times with 0.5 ml of 20 mM Tris–HCl (pH 8.0) with 10 mM $MgCl_2$ and 5 mM imidazole. The bound proteins were eluted with 0.1 ml of 20 mM Tris–HCl (pH 8.0) with 10 mM $MgCl_2$ and 500 mM imidazole. Samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (13%) electrophoresis, transferred to a PVDF membrane, and incubated in 1% Western blocking reagent (Roche Applied Science) at room temperature for 1 h. The membrane was then incubated overnight with anti-His_6(2) antibody (Roche Applied Science) diluted (1:2,500) in 0.5% Western blocking reagent. The blot was developed with HRP-conjugated sheep anti-mouse IgG antibody (GE Healthcare) using SuperSignal West Femto substrate (Pierce, Japan). The chemiluminescent signal was imaged and quantified by LAS 4000mini (GE healthcare).

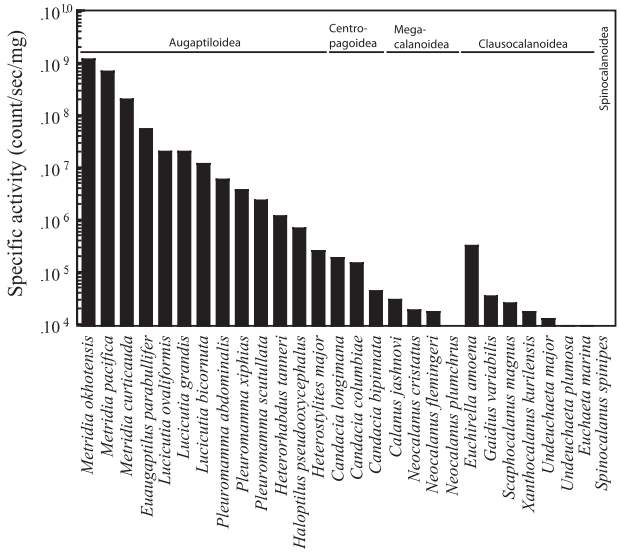

FIG. 2. Bioluminescent activity of zooplankton homogenates. Luminous activity of homogenized copepods was measured by mixing coelenterazine solution and the supernatant of the lysate. Total protein concentration was determined to compare specific luciferase activities among copepod species.

Results

Bioluminescent Activity of Zooplankton Homogenate

Many calanoid copepods have been reported to have luminous capability. However, many investigations were simply descriptive observations of luminous species, and little quantitative analysis of their bioluminescence has been reported. Therefore, we examined the measurement of luminous activity quantitatively in homogenized samples from 23 calanoid species. Figure 2 shows the specific bioluminescence activity of zooplankton homogenates after mixing them with the luminous substrate coelenterazine. Within four superfamilies investigated in this study, significant activity was observed predominantly in species of Augaptiloidea but also in *E. amoena* of the Clausocalanoidea family. The presence of bioluminescence in homogenates of genera *Metridia*, *Lucicutia*, *Pleuromamma*, *Euaugaptilus*, *Heterorhabdus*, and *Heterostylites* was consistent with previous reports (Clarke et al. 1962; Herring 1988). Luminous activities of homogenates from three other superfamilies, Centropagoidea, Megacalanoidea, and Clausocalanoidea, were extremely low or indistinguishable from the blank sample that contained only coelenterazine. We also tested reactivity of the planktonic homogenates to the chemically synthesized *Vargula* (*Cypridina*) luciferin, but there was no sample that showed significant luminescence upon injection of *Vargula* luciferin (data not shown).

Phylogenetic Analysis of Copepod rRNAs

Table 1 lists luminous and nonluminous calanoid copepods that were investigated using the bioluminescent assay of planktonic homogenates as shown in figure 2. Cladistic analysis for the calanoid copepods has been examined largely by morphology-based analysis (Bradford-Grieve et al. 2010), and molecular phylogeny using ribosomal or mitochondrial genes also has been reported (Bucklin et al. 1995, 2003; Braga et al. 1999; Blanco-Bercial et al. 2011; Figueroa 2011). In this study, we have confirmed the phylogenetic relationships among luminous and nonluminous calanoid copepods using approximately 1738 nt of 18S ribosomal DNA sequences using ML (fig. 3) and NJ analyses (supplementary fig. S1, Supplementary Material online). The 18S alignment contained 35 species of 22 genera of copepods plus four malacostracan (*Gonodactylus viridis*, *Odontodactylus havanensis*, *Squilla rugosa*, and *Euphausia superba*) and two ostracod (*V. hilgendorfii* and *Conchoecia* sp.) species as outgroup taxa. The 18S ML analysis reconstructed a monophyletic clade containing the Augaptiloidea and Pseudocyclopoidea with low bootstrap support (67%, not shown in fig. 3), which was positioned as a sister taxon to the other calanoid superfamilies. After division of the Augaptiloidea/Pseudocyclopoidea, there are two clades in Calanoida; one contains the monophyletic Centropagoidea and the other was the Megacalanoidea/Eucalanoidea/Clausocalanoidea. The NJ analysis of 18S also reproduced a tree with similar topology as seen in the ML tree, but with the notable difference that the Centropagoidea family is sister to the remaining calanoid superfamilies (supplementary fig. S1, Supplementary Material online). For the cluster of the Augaptiloidea, both methods of 18S reconstructed a monophyletic clade with high bootstrap values (99% in ML and 100% in NJ) containing the Metridinidae family (genus *Metridia*, *Pleuromamma*, and *Gaussia*) that contains many highly luminous species. Each monophyletic clade is sister to the other Metridinidae families (fig. 3 and supplementary fig. S1, Supplementary Material online). The topology among 13 species in the families Metridinidae, Heterorhabdidae, and Lucicutiidae is fundamentally the same between ML and NJ trees.

Molecular Cloning of Novel Calanoid Luciferases

The reactivity of homogenates with coelenterazine indicates the expression of luciferase in these luminous species tested (fig. 2), as previously reported molecular identification of luciferase genes from single *Gaussia* and two *Metridia* species has indicated (Verhaegen and Christopoulos 2002; Markova et al. 2004; Takenaka et al. 2008). We amplified full-length luciferase genes from five copepod cDNAs with degenerate primers designed based on the nucleotide sequences of the *G. princeps*, *M. longa*, and *M. pacifica* luciferases. At least two forms of luciferase genes, as identified in *M. pacifica* (MpLuc1 and MpLuc2) (Takenaka et al. 2008), were also identified from *M. okhotensis*, *P. abdominalis*, *H. tanneri*, and *H. major* cDNAs. The gene names and biochemical features of these proteins are summarized in table 2. They are relatively small proteins, ranging from 19.0 to 23.5 kDa, and they are efficiently secreted into the culture medium when they are expressed by cultured mammalian cells. The N-terminal 17–22 amino acid sequences of all novel luciferases seem to have the features of a consensus sequence that signals secretion based on the

Table 1. Luminescent and Nonluminescent Calanoid Copepods Examined for the Luminous Assay of Planktonic Homogenates.

Superfamily, Family, Species	Bioluminescence	Feeding Type	Myelinated Sheath	Diel Vertical Migration	Pelagic Zone	Density (ind. m^{-3})
Augaptiloidea						
Metridinidae						
Metridia pacifica	+(Luc)	S	A	++	E	87.2–265
Metridia longa	+(Luc)	S	A	++	E	ND
Metridia okhotensis	+(Luc)	S	A	++	M	2.78–5.11
Pleuromamma abdominalis	+(Luc)	S	A	++	E	ND
Pleuromamma scutullata	+	S	A	++	M	3.35–4.49
Pleuromamma xiphias	+	S	A	++	M	ND
Gaussia princeps	+(Luc)	S	A	++	M	ND
Lucicutiidae						
Lucicutia ovaliformis	+(Luc)	S	A	+	M	1.28–1.42
Lucicutia grandis	+	S	A	ND	B	0.00421–0.0172
Lucicutia bicornuta	+	S	A	ND	B	ND
Heterorhabdidae						
Heterorhabdus tanneri	+(Luc)	C	A	+	M	1.50–1.80
Heterostylites major	+(Luc)	C	A	+	B	0.303–0.479
Augaptilidae						
Euaugaptilus parabullifer	+	C	A	ND	B	0.00842
Centropagoidea						
Candaciidae						
Candacia longimana	−	C	A	+	E	ND
Candacia columbiae	−	C	A	+	E	0.0813–0.338
Candacia bipinnata	−	C	A	+	E	0.338
Megacalanoidea						
Calanidae						
Neocalanus cristatus	−	S	P	−	E	15.1–21.5
Neocalanus flemingeri	−	S	P	−	E	11.6–71.5
Neocalanus plumchrus	−	S	P	−	E	9.30–150
Calanus jashnovi	−	S	P	−	E	ND
Clausocalanoidea						
Scolecitrichidae						
Scaphocalanus magnus	−	D	P	−	B	0.222–0.225
Aetideidae						
Gaetanus simplex	−	S	P	−	M	4.21–5.05
Gaetanus variabilis	−	S	P	−	B	0.834–2.86
Euchirella amoena	+?	S	P	+	M	ND
Euchaetidae						
Euchaeta marina	−	C	P	+	E	ND

Note.—Symbols of bioluminescence were as follows: +, positive in the assay shown in figure 1 or already confirmed in previous reports; (Luc), luciferase gene was identified in this study or elsewhere; and −, negative in the assay shown in figure 1. Feeding type (C, carnivore; D, detritivore; and S, suspension feeder) (Ohtsuka et al. 1997), myelinated sheath (P, present; A, absent) (Lenz et al. 2000), DVM (++, strong; +, present; −, absent; and ND, no data) (Yamaguchi et al. 2004), pelagic zone (E, epipelagic, 0–200 m; M, mesopelagic, 200–1000 m; and B, bathypelagic, 1000–3000 m) (Roe 1984; Yamaguchi et al. 2002), and density (ND, no data) (Yamaguchi et al. 2002; Homma and Yamaguchi 2010).

prediction by PSORT II (http://psort.nibb.ac.jp/). MoLuc1, MoLuc2, PaLuc1, and PaLuc2 retained more than half of their initial activity even after 30-min incubation at 60 °C but were largely inactivated at 80 °C. Thermostability of HtLuc1, HtLuc2, HmLuc1, and HmLuc2 could not be determined correctly because of their low initial activity. The identity of the amino acid sequence of these luciferases was notably high at the internal and C-terminal regions rather than N-terminals (fig. 4*A*). Inouye and Sahara (2008) reported the identification of two catalytic domains in *Gaussia* luciferase, and we confirmed the presence of two short repeat sequences in the primary structures of these novel copepod luciferases. Alignment of two repeat sequences consisting of 62–64 amino acid residues (referred as domains 1 and 2, respectively) from 12 luciferases revealed consensus sequence of C-x(3)-C-L-x(2)-L-x(4)-C-x(8)-P-x-R-C (X, amino acid residue) in both domains (fig. 4*B*). Substitution of all cysteine residues with alanine in domains 1 and 2 of MpLuc1 resulted in complete loss of activity when it was expressed in Escherichia *coli* (data not shown), suggesting an essential role of cysteine residues in luciferase activity.

Phylogeny of Copepod Luciferases

In phylogenetic analyses of aligned amino acid sequences of copepod luciferases, 48 substitution models for proteins used in ML estimates were tested by MEGA5 to find the most appropriate model. The best model showing the lowest Bayesian information criterion score was Whelan and Goldman plus gamma (gamma distribution), therefore, an ML bootstrap consensus tree was generated with the model (fig. 5*A*). NJ analysis of 13 luciferases was also conducted based on the equal input model; the NJ bootstrap consensus tree (supplementary fig. S2, Supplementary Material online) is almost identical to the ML tree except for different topology of GLuc.

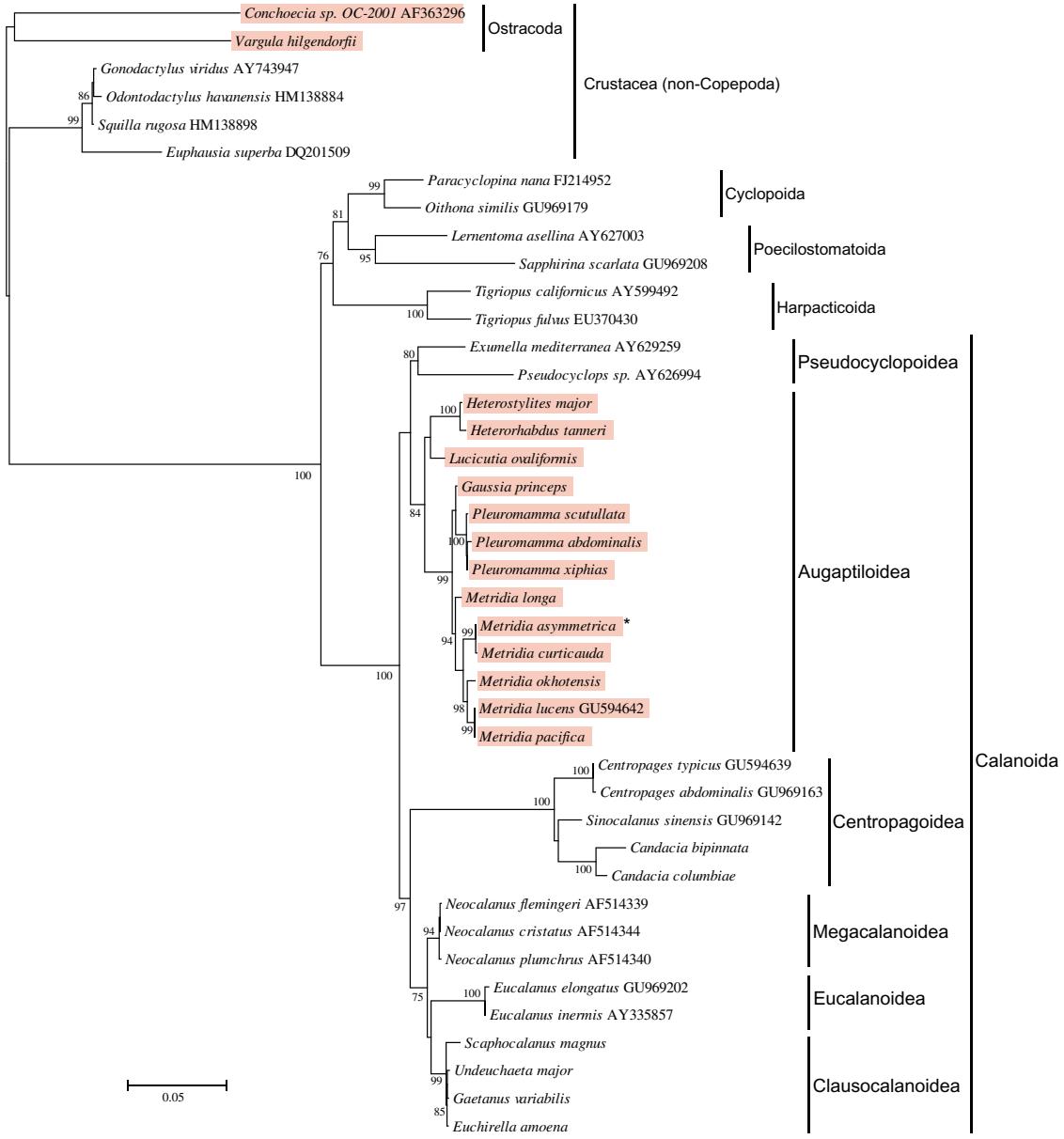

Fig. 3. ML analysis of the 18S rRNA nucleotide sequences from calanoid copepods and marine bioluminescent organisms. Species with bioluminescence assay in this study or reported elsewhere are shown in red boxes. Accession numbers are indicated with names of species whose 18S rRNA sequences are available from public databases. Bootstrap values are shown on only highly or moderately supported nodes (>74%) as a percentage of 1,000 replicates. Scale bar indicates genetic distance. *Metridia asymmetrica* (*) was not included in the luminescence assay of zooplankton lysate (fig. 2) but is capable of bioluminescence (Takenaka Y, unpublished data).

Luciferases from the family Metridinidae constitute a monophyletic clade (clade 1) that is sister to those from the family that contains Heterorhabdidae (clades 6 and 7) and Lucicutiidae (clade 5) (fig. 5A). The Metridinidae clade further diverges into two monophyletic clades (clades 2 and 3). Clade 2 contains three type-I luciferases in addition to MLuc, whereas clade 3 consists of their type-II counterparts and GLuc. In contrast to Metridinidae luciferases, paralogous type-I and type-II luciferases of Heterorhabdidae (HtLuc and HmLuc) formed monophyletic clades within species (fig. 5A, clades 6 and 7). Lucicutiidae luciferase (LoLuc) that has a relatively unique amino acid sequence, comparable with those of other Augaptiloidea luciferases, but is associated with neither Metridinidae nor Heterorhabdidae luciferases (clade 5).

Specific Activity of Recombinant Luciferases

Luminous activity of zooplankton homogenates varies greatly among the copepods (fig. 2). For instance, luminous activity of an *M. pacifica* homogenate was ~500-fold higher than that of a *H. tanneri* homogenate, even though both species belong to the same superfamily. One possible explanation is very different levels of luciferase expression among bioluminescent copepod species. The other is a substantial divergence of specific activity of copepod luciferases. To compare the specific activity of copepod

Table 2. Summary of Novel Copepod Luciferases and Their Properties.

Luciferase	Organism	Deduced Molecular Weight (kDa)	Luminescence Emission Maximum (nm)	Secreted Protein (%)	Thermostability (%)
MoLuc1	*Metridia okhotensis*	23.5	493	98.2	88.9/5.4
MoLuc2	*M. okhotensis*	20.4	493	99.2	74.6/3.6
PaLuc1	*Pleuromamma abdominalis*	23.1	493	98.8	98.5/6.7
PaLuc2	*P. abdominalis*	20.2	490	99.3	68.4/5.1
LoLuc	*Lucicutia ovaliformis*	24.2	493	84.2	42.8/0.1
HtLuc1	*Heterorhabdus tanneri*	19.0	ND	95.8	ND
HtLuc2	*H. tanneri*	21.1	ND	91.7	ND
HmLuc1	*Heterostylites major*	22.6	ND	82.6	ND
HmLuc2	*H. major*	22.8	ND	87.6	ND

NOTE.—Percentages of secreted protein were calculated by measuring luciferase activities in cellular lysate and culture medium of HEK293 cells transfected with luciferase expression vector. Thermostability denotes remaining luciferase activities after incubating copepods luciferases at 60 (left) or 80 (right) °C for 30 min relative to those of proteins at 4 °C. ND, not determined.

luciferases isolated in this study, 11 luciferases were expressed in HEK293 cells as recombinant proteins tagged with hexahistidine at the C-terminus, concentrated from culture medium by a nickel-chelating column, and then tested for their protein expression and activity. Specific activity was estimated by dividing the luminous activity of the culture media by signal intensity of polyhistidine-tagged copepod luciferases detected by an anti-polyhistidine antibody (fig. 6*A*). Results showed that there is a large difference in specific activity among copepod luciferases (fig. 6*B*) but only a slight difference in their expression levels (fig. 6*A*). In several experiments, we consistently observed that MpLuc1 showed the highest level of specific activity among tested luciferases, which was approximately 10^6-fold higher than that of HtLuc2. The specific activity of luciferases isolated from copepods in the family Metridinidae (MpLuc1, MpLuc2, MoLuc1, MoLuc2, PaLuc1, and PaLuc2) was notably higher than that of luciferases from the Heterorhabdidae family (HtLuc1, HtLuc2, HmLuc1, and HmLuc2). LoLuc, isolated from *L. ovaliformis*, which belongs to the Lucicutiidae family, showed intermediate levels of activity. These results are consistent with the luminous activity obtained by homogenate assays of calanoid copepods (fig. 2). Thus, species differences in copepod bioluminescence was reflected in the substantial divergence of specific activity of the expressed luciferases.

Discussion

Calanoid Luciferases

We have isolated nine novel copepod luciferases using the homology-based PCR cloning strategy. A BLAST search using the amino acid sequences of copepod luciferases as the query revealed no highly similar proteins in the database, except for other calanoid luciferases (GLuc, MLuc, and MpLuc). This may be due to the limited number of deposited sequences of copepod proteins. So far, all of copepod luciferase genes, including GLuc and MLuc reported elsewhere, have been isolated from species in the Augaptiloidea superfamily in Calanoida. We have tried to isolate luciferase or luciferase-like genes from other superfamilies of Calanoida, such as Pseudocyclopoidea or Centropagoidea using degenerate PCR primers. The result was no amplification or no luciferase-like sequences in the amplified DNA product (data not shown). However, we assume that luciferases or luciferase-like genes may exist, not only in Augaptiloidea but also in other superfamilies, because the luminous assay of zooplankton lysate from these superfamilies all had a measurable, though very weak, level of activity compared with that of negative control samples (fig. 2). Further screening of the calanoid luciferase or luciferase-like genes by different cloning strategies may reveal the structure and evolution of a direct ancestral copepod luciferase gene.

Consensus amino acid residues and domain structure were revealed by an alignment of 13 copepod luciferases obtained in this study (fig. 4*A*). Highly conserved amino acid residues, C-x(3)-C-L-x(2)-L-x(4)-C-x(8)-P-x-R-C, which are present in both domains, would be one of the criteria for the copepod luciferase, although the reason for this cysteine-rich area in the conserved sequence remains obscure. Because the similarity in the structure of the two domains was found in all of the copepod luciferases isolated in this study (fig. 4*B*), we assume this similarity in the structure of the two domains is very characteristic of the luciferases isolated from Augaptiloidea species.

Cladistic Analysis of rRNAs and Luciferases

The current ML analyses using 18S rRNA sequences essentially support previous morphology- (Bradford-Grieve et al. 2010) and sequence-based phylogeny (Blanco-Bercial et al. 2011). The Augaptiloidea family contained numbers of bioluminescence-positive species (fig. 3, red shadow) and was recovered as monophyletic with a moderate bootstrap value (84%) and closely related with the Pseudocyclopoidea (fig. 3). In NJ analysis of 18S, the notable difference is in the family Centropagoidea, which forms a monophyletic assemblage and is a sister to other calanoid superfamilies (supplementary fig. S1, Supplementary Material online). The topology is supported with a high bootstrap value (100%) and is consistent with the results from several sequence-based analyses of Calanoida (Braga et al. 1999; Figueroa 2011). Inconsistent results between our ML and NJ trees might indicate that the use of a single gene (18S) in the phylogenetic analysis is likely to produce

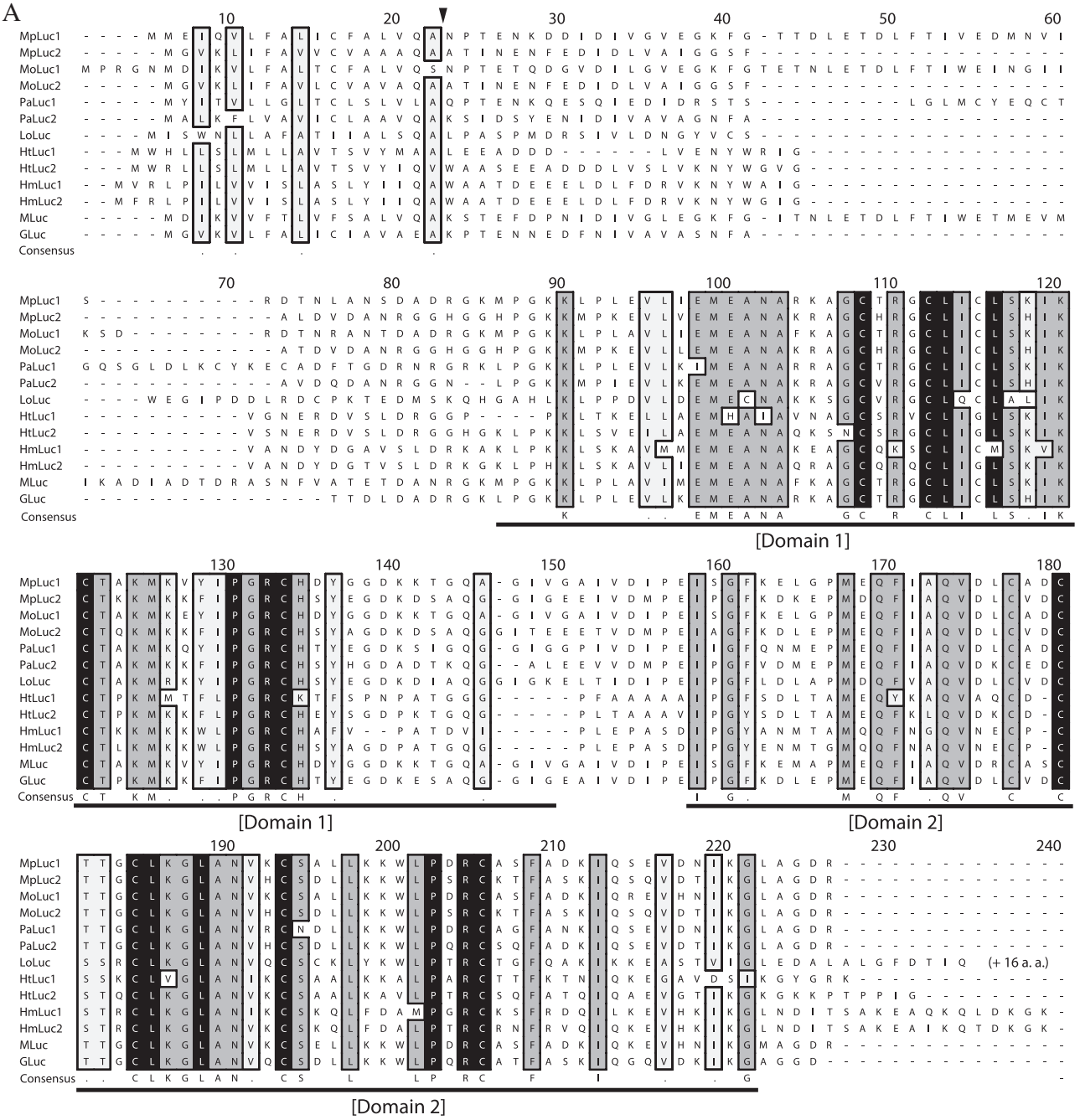

FIG. 4. (*A*) Multiple amino acid sequence alignment of 13 copepod luciferases. Positions of two putatively similar domains are underlined. Outlined letters represent at least 90% identical (dark shade) or chemically similar (light shade) amino acid residues. White letters in the black boxes are highly conserved residues in both domains. Arrowhead indicates proposed cleavage sites of N-terminal secretion signals of copepod luciferases. (*B*) Multiple amino acid sequence alignment of tandem repeat domains of luciferases. Residues are darkly shaded and outlined if they are conserved at a level of more than 90% among all aligned sequences. Similar residues are lightly shaded and outlined. The distance between the two domains in each species is indicated as number of amino acids in parentheses at the end of the sequence. Those amino acids that are conserved in both domains (consensus), domain 1 only (consensus_domain1) or domain 2 only (consensus_domain2), are indicated in the last three rows.

unstable results. Nevertheless, ML analysis of 18S genes produced a tree with more similar topology to a multigene molecular phylogeny of the Calanoida (Blanco-Bercial et al. 2011), in comparison with NJ analysis. We also sequenced the D1–D2 regions of 28S rRNA and analyzed phylogenetic relationships among calanoid species by ML and NJ analyses. The results were similar to the 18S analysis, but many clades were very poorly supported, which is probably due to relatively high interspecific divergence of D1–D2 sequences within each superfamily (data not shown).

The ML phylogenetic analysis based upon 18S also clearly showed the intrafamilial relationships of the Augaptiloidea. One of the Augaptiloidean families, Metridinidae, in which many species are capable of strong bioluminescence, consists

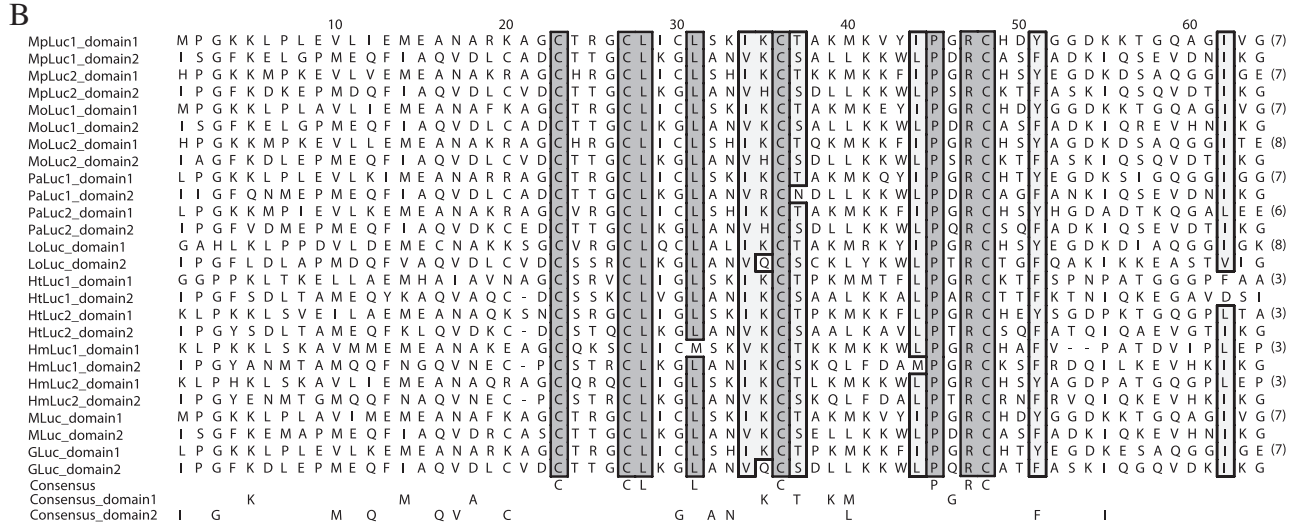

FIG. 4. (Continued)

of three genera *Metridia*, *Pleuromamma*, and *Gaussia*. The genus *Metridia* contains sibling species in the North Atlantic (*M. lucens*) and North Pacific (*M. pacifica*). In the same way, their large body relatives (e.g., *M. longa* in the North Atlantic and *M. okhotensis* in the North Pacific) also have similar ecological characteristics in each ocean (Mauchline 1998). In the monophyletic clade of the Metridinidae family, morphologically similar species of *M. pacifica* and *M. lucens* are paired with a high bootstrap value (99% in ML and 100% in NJ trees), which is consistent with a previous report (Bucklin et al. 1995). Furthermore, phylogenetic relationships among additional luminous species of the genus *Metridia* (*M. okhotensis*, *M. curticauda*, and *M. asymmetrica*) also was revealed in our analyses. *Metridia okhotensis*, which is a mesopelagic inhabitant and dominant in the same region of the subarctic Pacific as *M. pacifica* (Yamaguchi et al. 2004), is a sister to the clade containing *M. pacifica* and *M. lucens* (fig. 3), which indicates the close relationships between *M. okhotensis* and these two species. *Metridia curticauda* and *M. asymmetrica* are potentially luminous (fig. 2 and Takenaka Y, unpublished data) and are strongly associated with a high bootstrap value (100%). On the other hand, *M. longa* is unexpectedly set apart from its North Atlantic counterpart, *M. okhotensis*. *Gaussia princeps* is also a calanoid of the family Metridinidae, and it has distinct characteristics if compared with other genera in Metridinidae, such as the large body size, tropic and temperate distribution, and detailed morphology (Soh et al. 1998). Both ML and NJ analyses showed that *Gaussia* and *Pleuromamma* form a monophyletic clade that is sister to *Metridia* (fig. 3 and supplementary fig. S1, Supplementary Material online). Although bootstrap support for the *Gaussia*–*Pleuromamma* clade is weak (64% in ML, not shown in fig. 3), our results are similar to the morphology-based phylogeny of *Gaussia* (Soh 1998). Further investigation with additional sequences from other *Gaussia* species will clarify relationships among genera in the family Metridinidae.

The phylogeny of 13 calanoid luciferases suggests a different evolution of the Metridinidae and Heterorhabdidae families' luciferases (fig. 5A). In the clade containing Metridinidae luciferases (clade 1), two proteins in a single species are likely to have evolved independently. The similarity of primary structures among MpLuc1, MoLuc1, and PaLuc1 (type-I) luciferases were greater than those of their counterparts (MpLuc2, MoLuc2, and PaLuc2) (type-II) luciferases (fig. 4A). The mRNA coding for MoLuc1 or MoLuc2 would be transcribed from two different loci or alleles since studies of genomic sequences revealed the presence of different lengths and positions of the introns in MoLuc genes (fig. 5B), as seen in MpLuc genes (Takenaka et al. 2008). Gene duplication events of luciferase probably happened in the ancestral species of the *Metridia* and *Pleuromamma* families, thereafter, paralogous luciferases might have diverged independently. Conversely, it would be expected that specialization of Heterorhabdidae species preceded the duplication and diversification of luciferase genes since pairs of luciferases (HtLuc1 and HtLuc2; HmLuc1 and HmLuc2) are monophyletic (clades 6 and 7) within species. Nucleotide identity between coding sequences of HmLuc1 and HmLuc2 is 91.8%, whereas it is 62.6% between HmLuc1 and HtLuc1, which were isolated from species of the same Heterorhabdidae family. Extensive genome survey of two lineages of Metridinidae and Heterorhabdidae families may shed light on the multigene structure of luciferase genes and how copepod luciferases have evolved. From the present study, we could not interpret any evolutionary novelty derived from gene duplication of luciferases in calanoid copepods. Because phenotypic observation on live copepod bioluminescence is difficult without special video equipment and relevant technical skills, we are trying to evaluate any possible evolutionary novelty of the gene duplication by revealing different biochemical characteristics between type-I and type-II luciferases identified in this study.

Forming a monophyletic clade containing both MLuc (*M. longa*) and MoLuc1 (*M. okhotensis*) (fig. 5A) is consistent with the fact that both species are closely related.

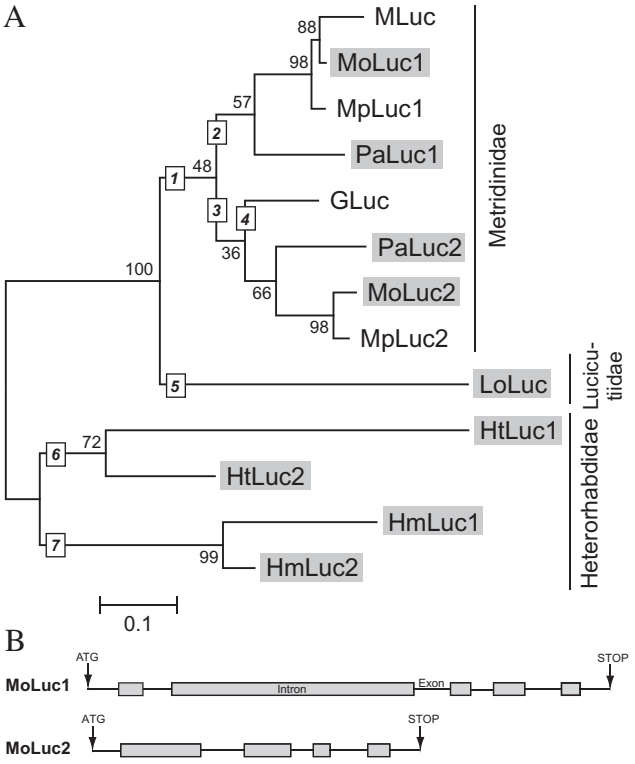

FIG. 5. (*A*) ML phylogeny of copepod luciferases based on an amino acid sequence alignment of domains 1 and 2 with a linker sequence. The tree is midpoint rooted, and numbers on the nodes indicate % bootstrap values from 1,000 replicates. Luciferases isolated and analyzed in this study are shown in shaded boxes. Scale bar indicates evolutionary distance and is in the units of the number of amino acid substitutions per site. (*B*) Gene structures of MoLuc1 and MoLuc2. Introns and exons are shown as open boxes and lines, respectively.

However, *M. longa* is basal to other *Metridia* species in the 18S rRNA tree, and *M. okhotensis* is nested within that tree (fig. 3). The discordance is probably due to the different number of analyzed species and different evolutionary mechanisms between luciferase and the 18S rRNA gene. The inclusion of additional luciferases from *Metridia* species, such as *M. curticauda* and *M. asymmetrica*, might change the topology of the clades 1–4 in figure 5*A*. The relationships among luciferases from *Metridia* and *Pleuromamma* are basically the same between type-I and type-II groups. GLuc, *Gaussia* luciferase, is weakly associated with Metridinidae type-II luciferases (clade 3). A low bootstrap support value for clade 3 (<50%) indicates the branching position of GLuc could not be determined with high confidence. In fact, GLuc clustered with Metridinidae type-I luciferases as the sister to a monophyletic clade containing all type-I luciferases and MLuc in NJ analysis (supplementary fig. S2, Supplementary Material online). The result was inconsistent depending on the method of the phylogeny analysis, substitution model, and parameters used in the analysis. This is probably explained by the neutral characteristic of the GLuc sequence, which possesses an equal ratio of amino acid residues specifically found on type-I or type-II luciferases at different sites (data not shown).

Factors Affecting the Intensity of Copepod Bioluminescence

Zooplankton lysates (fig. 2) and luciferases (fig. 6*B*) from *Metridia* spp. and *Pleuromamma* spp. (both in the Metridinidae family) (MpLuc, MoLuc, and PaLuc) showed significantly higher specific activity than those from *Heterorhabdus* spp. and *Heterostylites* spp. (family Heterorhabdidae) (HtLuc and HmLuc), although both families belong to the same Augaptiloidea superfamily. As the factors that may affect the intensity of bioluminescence, diel vertical migration (DVM), feeding patterns, and selection pressure from predators are considered.

DVM is a copepod behavior to stay in darker deeper layers during daytime and migrate upward to food-rich surface layers at night for feeding. A most important function of copepod DVM is considered to be avoidance from predation by visual-feeding fishes (Gliwicz 1986; Neill 1992). Species characterized with strong bioluminescence (Metridinidae) are all medium-sized suspension feeders known to perform strong nocturnal ascent DVM (Haury 1988; Hays 1995). The dominance of Metridinidae in oceans worldwide can be explained by the fast swimming speed and strong DVM intensity (Mauchline 1998). Because of its numerical dominance, Metridinidae is selectively captured by various mesopelagic lantern fishes (Merrett and Roe 1974; Hopkins and Sutton 1998). The bioluminescent behavior of copepods is considered to have a function of avoidance from vision-dependent predators (Herring 1988; Widder 1992). To distract or blind a predator of mesopelagic lantern fish predator, Metridinidae with reinforced luciferases would have a selective advantage for their stronger bioluminescent system (MpLuc, MoLuc, and PaLuc). We suggest that MpLuc from *M. pacifica* (fig. 1) shows the highest bioluminescence (fig. 6*B*) because the habitat depth of *M. pacifica* is the epipelagic layer (0–200 m) (table 1) where a large number of predators exist.

The species that are characterized with weak bioluminescence (figs. 2 and 6*B*), such as *H. tanneri* and *H. major* (Heterorhabdidae) (HtLuc and HmLuc), inhabit the deeper darker depths and at low density (table 1) and show a low intensity of DVM due to their slower swimming behavior (Yamaguchi and Ikeda 2000). They are also known to be carnivores, evolutionarily having switched from suspension feeders to predators with a specialized feeding mechanism that inject venom or anesthetic from the mandibular ventralmost tooth into the prey animal (Nishida and Ohtsuka 1996; Ohtsuka et al. 1997). This specialized feeding behavior of *Heterorhabdus* may facilitate easy capturing of their prey and reduce the need for motion during predation. In contrast to the Metridinidae, their low motility might be insusceptible to the motion-dependent predators. Taken together with these characteristics, the Heterorhabdidae may be exposed to low predation pressure by mesopelagic fishes, resulting in less selective evolutionary advantage for any remarkable bioluminescence.

Lucicutia ovaliformis (family Lucicutiidae) (LoLuc) is a small-sized suspension feeders that dominates the mesopelagic layer (200–1,000 m) (Yamaguchi et al. 2002). All of

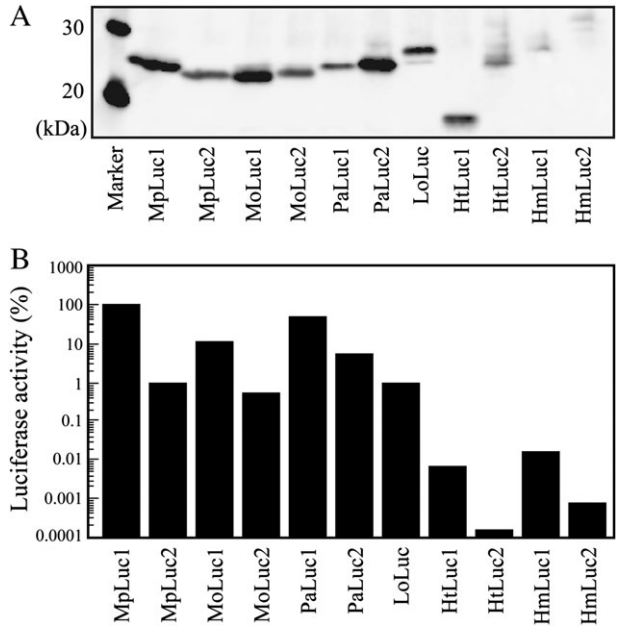

Fig. 6. (*A*) Immunoblot of polyhistidine-tagged copepod luciferases expressed by HEK293 cells. Proteins were concentrated from culture media on a Ni-chelating column. The same volume of eluents was loaded on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, blotted, and detected by anti-polyhistidine tag antibody. (*B*) Specific activity of secreted copepod luciferases expressed by HEK293 cells. The initial light intensity was counted for 10 s and then normalized to the protein expression level obtained by immunoblotting shown in figure 5*A*. Luciferase activities are expressed as percentages of normalized luminescence activity (light intensity/protein expression level), using the value of MpLuc1 defined as 100%. The data represent the mean for triplicate measurements.

the Lucicutiidae species are known to be distributed in the meso- to abyssopelagic zones of the ocean (Brodskii 1967). Although the feeding type and numerical abundance of Lucicutiidae (table 1) are comparable to those of Metridinidae, the bioluminescent intensity of Lucicutiidae is lower than that of Metridinidae (fig. 2). One of factors that might explain the intensity difference between two families is their habitat depth. Because the Lucicutiidae occurs much deeper than the shallower habitat Metridinidae does, they could be difficult to perceive and may not attract predators in the meso- to abyssopelagic layer. This is especially the case if the predators are feeding selectively (Merrett and Roe 1974; Hopkins and Sutton 1998). Therefore, predation pressure on Lucicutiidae has been relatively low, resulting in a lower intensity of bioluminescence than seen in the Metridinidae.

Bioluminescence in Calanoid Superfamilies Except for Augaptiloidea

Copepod luciferases, isolated in this study and previous reports, are all derived from the Augaptiloidea superfamily in Calanoida. There are few to no reports of bioluminescence in other calanoid superfamilies. The reason for the difference in the luminous ability between the Augaptiloidea and other superfamilies could be explained by their ecology

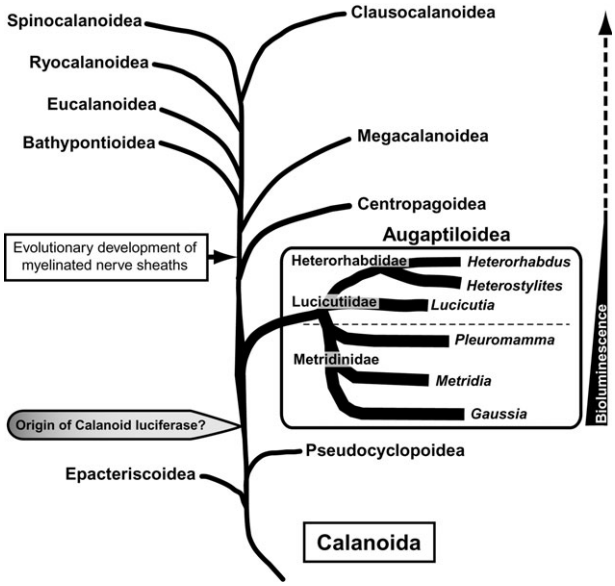

Fig. 7. Speculative scheme of evolution of calanoid bioluminescence. Phylogeny of the calanoida is based on previous reports (Park 1986; Bradford-Grieve et al. 2010).

(e.g., motility, habitat, and body size). Although precise evolutionary positions of the Pseudocyclopoidea and Epacteriscoidea are not determined yet, these superfamilies are believed to be the most primitive Calanoida (fig. 7) (Bradford-Grieve et al. 2010). Comparing with other eight superfamilies illustrated in figure 7, they are highly specialized because of their adaptation to the epibenthic habitats. They are found only at shallow to shelf depth (Ohtsuka et al. 1999) or in submarine caves (Ohtsuka et al. 2002), in contrast to the Augaptiloidea, which has a greater diversity of distribution within the pelagic zones (Yamaguchi et al. 2004). These observations imply little change, if any, of habitat in the evolutionary history of the Pseudocyclopoidea and Epacteriscoidea (Bradford-Grieve 2004). Living on-bottom– or in-bottom–dwelling habitat would be a very effective means to find nutrients and hide from predators. There is no report, so far, describing the bioluminescence of these primitive copepods, probably because of the very limited research on these minor species in Calanoida so far. However, considering their stable benthic habitat and very small body size (below 1 mm) (Ohtsuka et al. 1999), evolving bioluminescence as a defense against the predators seems to be unnecessary for them.

Based on the cladistic analysis of morphological features, the Augaptiloidea is believed to have diverged very early in the evolution of calanoid copepods (Bradford-Grieve et al. 2010). The seven other superfamilies (Centropagoidea, Megacalanoidea, Bathypontioidea, Eucalanoidea, Ryocalanoidea, Spinocalanoidea, and Clausocalanoidea) could be grouped into a monophyletic assemblage after the separation of the Augaptiloidea (fig. 7). The Megacalanoidea and Clausocalanoidea (and Eucalanoidea), of which several species were bioluminescence negative in this research (fig. 2), have developed a mechanism of "myelinated" nerve fibers (Davis et al. 1999) (table 1) and chemosensory organs. Thus,

they have gained greater motility with faster responses to their predators (Lenz and Hartline 1999; Lenz et al. 2000; Waggett and Buskey 2008). Meanwhile, for the ancestral species in these superfamilies, an advantage of bioluminescence as a defense system might have turned into a disadvantage because once luminescent substances were secreted, it could not be reduced or quenched and eventually allowed their predators to target them easily. This might have caused a loss of luminescent functions in the Megacalanoidea and Clausocalanoidea (fig. 7). A very weak luminous activity in some species of Centropagoidea superfamily (fig. 2), which is suggested to have diverged next to the Augaptiloidea (Park 1986), might be a trace of the loss of bioluminescence in Calanoida.

Concerning the bioluminescent copepods other than Calanoida, *Oncaea* belonging to Poecilostomatoida is known to have bioluminescence (Herring et al. 1993). Since many of the bioluminescent copepods are predatory and live moderately deep, it is unique that the tiny poecilostomatoid copepod *Oncaea* is one example that is relatively shallow water habitat and has pseudoplanktonic behavior (Böttger-Schnack and Schnack 2005). The most notable feature of *Oncaea* bioluminescence is their nonsecretory and repetitive fast flashes. The reason for this different characteristic of *Oncaea* bioluminescence compared with those of other bioluminescent copepods might lie in their smaller size and reduced motility. Throwing off bioluminescence as a decoy against potential predators is not an effective protection for *Oncaea*, which cannot escape rapidly from surrounding luminescent materials ejected from themselves, unlike calanoid copepods (Herring et al. 1993). Furthermore, the bioluminescence emission spectra of *Oncaea* (~470 nm) are slightly different from those of other calanoid copepods (480–500 nm), possibly due to the structural difference of their luciferase and/or luciferin.

The origin of the bioluminescence of calanoid copepods must be carefully verified with an assessment of bioluminescence, including nonluminescent primitive species, such as the Pseudocyclopoidea, and luminescent *Oncaea* species. Nonetheless, we postulate that bioluminescence in calanoid copepods could have originated from an ancient species between the families Pseudocyclopoidea and Augaptiloidea (fig. 7). The ancient Augaptiloidea may have ventured into the surface water, which was probably caused by unexpected food or oxygen demands (Bradford-Grieve 2002, 2004), and then encountered a number of difficulties for adapting to the pelagic habitat. Until myelinated nerve axons evolved, the earlier evolution of bioluminescence might have been a highly efficient strategy for them to avoid surrounding predators. Further analysis of copepod bioluminescence in the world's oceans could be a clue for understanding the ecology and evolution of marine organisms.

Supplementary Material

Supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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