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Invited Review

Selected Least Studied but not Forgotten Bioluminescent Systems†

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ABSTRACT

Bioluminescence is a form of chemiluminescence generated by luminous organisms. Luminous taxa have currently been reported from about 800 genera and probably over 10 000 species in the world. On the other hand, their bioluminescent systems, including chemical structures of luciferins/chromophores and the genes encoding luciferases/photoproteins, have been elucidated from only a few taxonomic groups, for example beetles, bacteria, dinoflagellates, ostracods and some cnidarians. Research efforts to understand unknown bioluminescence systems are being conducted around the world, and recently, for example, novel luciferin structures of luminous enchytraeid potworms and fungi were identified by the authors. In this study, we review the current status and perspectives, in the context of postgenomic era, of most likely novel but less-revealed bioluminescence systems of ten selected organisms: earthworm, parchment tubeworm, fireworm, scaleworm, limpet, millipede, brittle star, acorn worms, tunicate and shark, which indeed are the next focus of our international collaboration.

INTRODUCTION

Bioluminescence is visible light produced by a chemical reaction in living organisms. Basically, it is explained to be the oxidation process of "luciferin" (Fig. 1) with molecular oxygen by "luciferase." Here, "luciferin" is a general term for organic compounds that exist in luminous organisms and provides the energy for light emission by being oxidized, and "luciferase" is a general term for enzymes catalyzing the oxidative light-emitting reaction of luciferin in luminous organisms (by the definition from Shimomura, 2006) (1). Some bioluminescence systems do not conform to explanation by luciferin–luciferase reaction. For example, the luminous hydromedusa *Aequorea* emits light by intramolecular reaction of the protein named aequorin. This reaction does not require molecular oxygen and is triggered by the binding of calcium ions. Aequorin is consumed by the reaction; thus, this process cannot be assigned to the category of enzymatic reaction. As we show in the following sections, the bioluminescence of the parchment tubeworm *Chaetopterus*, scaleworms, millipede *Motyxia* and bivalve *Pholas* occurs in a similar manner, and the proteins of this kind are termed "photoprotein." Photoprotein is currently defined as a general term for proteins that occur in the light organ of a luminous organism and are capable of emitting light in proportion to the amount of the protein (1). Photoproteins contain a prosthetic group bound to apoprotein playing a role of luciferin, such as coelenterazine (Fig. 1A) in aequorin; thus, photoproteins can be regarded as a stable luciferin–luciferase complex (1).

Bioluminescent organisms are found in 800 genera of *ca*. 13 phyla (2–5). Each group uses an independent bioluminescence system; in other words, they evolved their bioluminescent traits independently in their lineages. The history of modern views on bioluminescence mechanism dates back to the Dubois' "classic luciferin–luciferase experiments," which uses heat-stable and heat-unstable components of luminous organisms to reconstitute light production by mixing together, for the luminous piddock *Pholas dactylus* (Fig. 2) and click beetle *Pyrophorus* (6). To date, the chemical structures of luciferin/chromophore were determined and the genes of luciferase/photoprotein were isolated for some major bioluminescent organisms (e.g. bacteria, ostracods, beetles, dinoflagellates, *Pholas* and some jellies) (1). Regarding luciferin chemistry, since the chemical structure of firefly luciferin was determined in 1961–1963, seven different types of luciferin have been identified: firefly luciferin, bacteria luciferin (long-chain aldehyde and $FMNH_2$), cypridinid luciferin, earthworm luciferin, coelenterazine-type luciferin (incl. *Watasenia* preluciferin), dinoflagellate luciferin and krill luciferins (tetrapyrroles) and *Latia* luciferin (Fig. 1C) (7). Actually, the last structural characterization of novel luciferin, dinoflagellate luciferin, dates back 25 years, but recently the author's group added two novel luciferin structures in this list: enchytraeid worm luciferin (8) and fungi luciferin (7,9). On the other hand, there are still many luminous organisms in which bioluminescence

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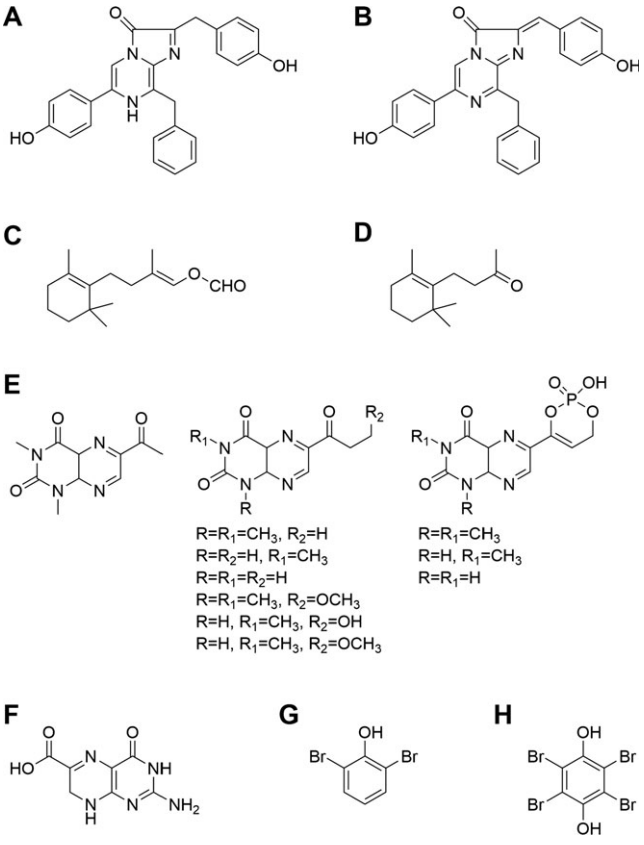

Figure 1. (A) Coelenterazine, (B) dehydrocoelenterazine, (C) *Latia* luciferin, (D) *Latia* oxyluciferin, (E) lumazines in the fireworm *Odontosyllis* cf. *undecimdonta*, (F) fluorescence compound in the cuticle of the luminous millipede *Motyxia sequoiae*, (G) 2,6-dibromophenol, (H) 2,3,5,6-tetrabromohydroquinone from acorn worms.

systems are mechanistically novel but less studied, especially in inconspicuous worms and slugs.

In this study, we review ten selected bioluminescence systems, which are "least studied" and not fully explored bioluminescence systems, especially focusing on the recent progress after Shimomura's comprehensive book for chemistry and biochemistry of bioluminescence was published in 2006 (1). These worms and slugs may not be good-looking, but potentially have novel bioluminescence systems useful for next biotechnological application tools. We do not deal the perfectly known systems and the almost unknown systems. Indeed, the organisms examined in this review are the next targets for us to solve. We also do not approach the biological function and evolution of the bioluminescence. These aspects have been reviewed in the recent papers (4,5,10).

ANNELIDA

The phylum Annelida has been traditionally classified into three extant classes, Polychaeta (bristle worms), Oligochaeta (earthworms and potworms) and Hirudinea (leeches). Luminous species have been reported from five families in oligochaetes (11) and ten families in polychaetes (4), but none from Hirudinea. In these various luminous annelids, we focus on the earthworms (Acanthodrilidae, Octochaetidae, Megascolecidae and Lumbricidae) and potworms (Enchytraeidae) from Oligochaeta, and parchment tubeworm (Chaetopteridae), fireworms (Syllidae) and scaleworms (Polynoidae) from Polychaeta.

Earthworms and potworms

Luminous earthworms discharge luminescent fluid upon mechanical stimulation. The mechanisms of earthworm luminescence have been best studied in the North American earthworm *Diplocardia longa* (Acanthodrilidae), wherein the luciferin was identified as *N*-isovaleryl-3-aminopropanal (12), the luciferase was purified as 300-kDa heterotrimeric Cu^{2+} metalloprotein and the luminescence reaction was triggered by hydrogen peroxide (13,14).

The bioluminescence of other earthworm species, including the genera *Diplotrema*, *Microscolex* (Acanthodrilidae), *Octochaetus* (Octochaetidae), *Fletcherodrilus*, *Pontodrilus* and *Spenceriella* (Megascolecidae), is also triggered by hydrogen peroxide, and their luminescence can be further enhanced by the addition of either *D. longa* luciferases or luciferin (whereas the enhancement of the luminescence in *Diplotrema* and *Fletcherodrilus* by *D. longa* luciferin was negative) (15,16). These results indicated that the bioluminescence mechanisms in these three families are basically identical.

A novel type of luciferin structure was recently determined in the Siberian luminous potworm *Fridericia heliota* (Enchytraeidae) (7,8). Cross-reaction test of luciferin–luciferase between *F. heliota* and *Microscolex phosphoreus* (Fig. 3) was negative (V. N. Petushkov, personal communication). Another Siberian enchytraeid potworm *Henlea* sp. is also luminous, but the luciferin–luciferase cross-reaction between this species and *F. heliota* was negative (17).

It has been suggested that the vast amount of riboflavin stored in coelomic fluid plays an important role in the luminescence of the European luminous earthworm *Eisenia lucens* (Lumbricidae) (18,19), but involvement of riboflavin in the bioluminescence reaction was not demonstrated.

Luciferase genes have not been isolated from any luminous earthworms and potworms.

Parchment tubeworms

Chaetopterus variopedatus (Fig. 4) is widely distributed in the world, although a recent molecular phylogenetic analysis suggested its status as a species complex (20). It is a filter feeder, living in a U-shaped tube in the mud of marine shallow water, both ends of which stick out of the seafloor. Upon physical stress, the worm emits a flash of blue light and also secretes glowing luminescent slime when disturbed more aggressively (20).

In the 1960s, Shimomura and Johnson showed that bioluminescence of *Chaetopterus* "tissue" extracts peaks at 453–455 nm and is enhanced in the presence of Fe^{2+} and hydrogen peroxide (1,21,22). Based on their analyses, Shimomura suggested the involvement of a photoprotein in *Chaetopterus* luminescence and stated that the luminescent system of purest prepared photoprotein needs five factors: oxygen, Fe^{2+}, peroxide and two unknown cofactors (1). The photoprotein was also crystallized by ammonium sulfate precipitation, but the protein structure was not determined. The molecular mass of the photoprotein was measured to be 130 kDa and 184 kDa before and after crystallization, respectively (1). Fluorescence of the purified photoprotein is characterized by an emission peak at 453–

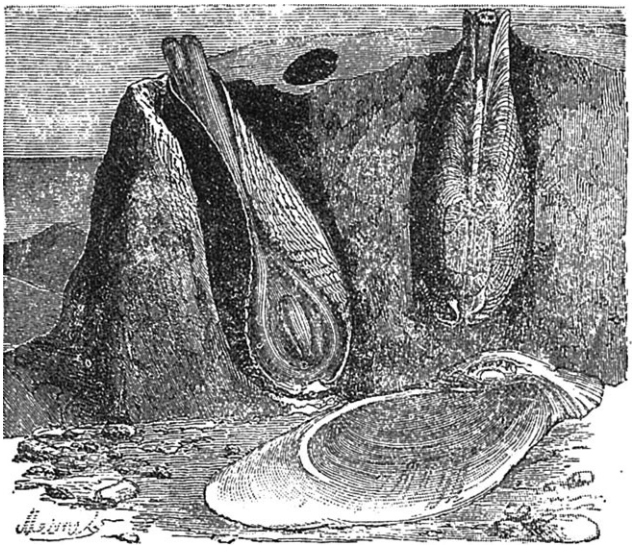

Figure 2. Origin of the modern bioluminescence chemistry and biochemistry. Engraving of the piddock *Pholas dactylus* in the historical book "La Vie et la Lumière" by Raphaël Dubois, 1914.

455 nm, which matches its bioluminescence spectrum. The bioluminescence reaction does not significantly change the fluorescence spectrum (1).

Recently, Deheyn's group focused on the luminescent "mucus" of *Chaetopterus* (20,23). Classic luciferin–luciferase analysis of the mucus was negative. Fluorescence spectrum measured immediately after cessation of luminescent reaction revealed peaks at 461 and 519 nm. After 2-h incubation at room temperature, 461-nm peak nearly disappeared, while a single peak at 525 nm persisted. According to the LC-MS analyses, a 525-nm fluorescent peak corresponds to riboflavin. They suggested that the *Chaetopterus* luminous mucus contains a photoprotein, and riboflavin (or its derivative) is the light emitter of bioluminescence (20,23).

Fireworms

Bioluminescence in Polychaeta is also recorded in some genera of the family Syllidae, and the most well-known taxa are the marine "fireworm" *Odontosyllis* spp. Males and females release a luminous secretion for mating. The secretion is also observed upon mechanical stimulation, which probably functions to deter predation (24).

A classic luciferin–luciferase reaction using the whole body of *Odontosyllis enopla* and *Odontosyllis phosphorea* has been demonstrated (25). The luciferin of the Bermudian *O. enopla* was extracted with boiling ethanol and purified by several precipitations and chromatography steps (26). The purified *O. enopla* luciferin is colorless and nonfluorescent, and it emits light in the presence of Mg^{2+}, molecular oxygen and crude luciferase. The oxyluciferin produced by this luminescence reaction has a fluorescence peak at 507 nm, which is almost identical to the bioluminescence spectrum *in vitro* and *in vivo* (26). The chemical structure of the luciferin and oxyluciferin has not been determined.

Bioluminescence of *O. phosphorea* collected in San Diego was re-examined by Deheyn and Latz (27). The classic luciferin–luciferase reaction of the mucus was not fully reconstituted, and the mucus persisted intense bioluminescence under anoxic conditions. Based on these results, they suggested that the luminescence of the secreted mucus involves a photoprotein rather than luciferin–luciferase reaction and that this mechanism is different from that of internal flash light production.

Several derivatives of 6-propionyllumazine (Fig. 1E) were identified in the extract of crude luciferin and oxyluciferin from the Japanese luminous *Odontosyllis* sp. (near *Odontosyllis undecimdonta*; H. Kakoi, personal communication) (28,29) (Fig. 5). The relationship between luciferin/oxyluciferin and these lumazine compounds is not clear.

Scaleworms

Several species of the scaleworm (Polynoidae) emit light from elytra, and the scales are arranged in two rows on a dorsal side, by mechanical stimulation. With further irritation, the elytra detach from the worm's body probably to distract and confuse predators.

The bioluminescence of the luminous polynoid *Acholoë squamosa* (formerly, *Acholoë astericola*) requires molecular oxygen, but the luciferin–luciferase reaction has never been demonstrated (25,30). Later, a temperature- and trypsin-sensitive protein was isolated as a photoprotein from the luminous scales of polynoid *Malmgrenia lunulata* (formerly, *Harmothoë lunulata*) by chromatography and named as polynoidin (originally, polynoïdin) (31). The purified *M. lunulata* polynoidin was nonfluorescent either before or after luminescence, and the molecular mass was estimated as 500 kDa by gel filtration chromatography (31) and 65 kDa by SDS-PAGE (32). The light emission could be induced upon the addition of sodium dithionite, xanthine–xanthine oxidase system and Fenton's reagent (hydrogen peroxide and Fe^{2+}) (31), suggesting the involvement of radical anion superoxide in the reaction. The emission spectrum of the *M. lunulata* polynoidin peaks at 510 nm, which is close to the *in vivo* emission peak (31). Recently, other polynoidin proteins were purified up to 80–90% homogeneity from four polynoid species, *Harmothoë imbricata* (Fig. 6), *Harmothoë areolata*, *Lepidonotus squamatus* and *Lepidonotus clava* (33). Their molecular sizes were estimated to be about 65 kDa by ultracentrifugation in sucrose medium (33). Interestingly, *Lepidonotus* species are regarded to be nonbioluminescent, but their polynoidins emitted light by xanthine–xanthine oxidase system (33).

Nicolas *et al.* showed that the detached scales of *M. lunulata* exhibit green fluorescence after luminescence, and the fluorescent entity is riboflavin by TLC analysis (31). They suggested that radical anion superoxide involved in the bioluminescence reaction is generated by the oxidation of reduced riboflavin in the presence of Ca^{2+} ion (31). On the contrary, a fluorescent substance generated by luminescence reaction of the scales was isolated as a complex of low molecular mass protein and chromophore from *A. squamosa* (34). The fluorescence emission spectrum was similar to the *in vivo* bioluminescence (λ_{max}, 520 nm), suggesting that the fluorescent substance is likely the reaction product of the bioluminescence (34).

GASTROPODA

The phylum Mollusca contains a large number of luminous species, especially in the class Cephalopoda (squids and octopus). In contrast, small numbers of luminous species are recorded from the other molluscan classes Bivalvia and Gastropoda. The bioluminescence system of the Mediterranean bivalve *Pholas*

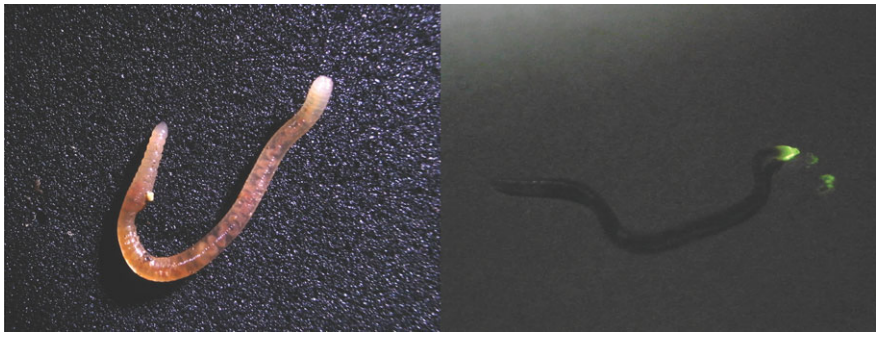

Figure 3. Earthworm *Microscolex phosphoreus*. The heads are shown on the left side of these photographs. Photograph by Yuichi Oba.

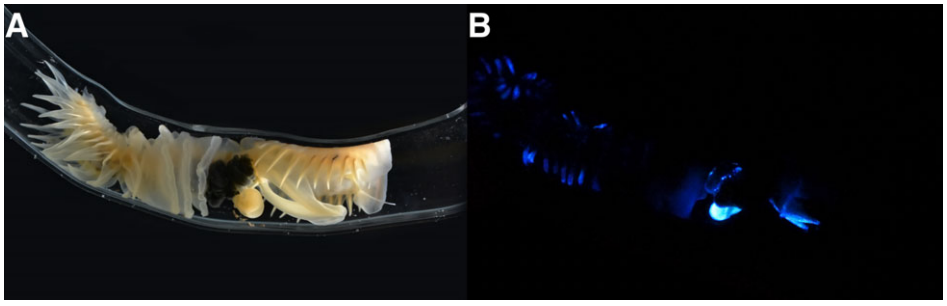

Figure 4. Parchment tubeworm *Chaetopterus variopedatus* under illumination (A) and its bioluminescence along the body in parapodia, mucous gland and tail (B). Photograph by Anderson G. Oliveira.

dactylus has been well studied: The gene of the photoprotein pholasin was isolated (35), and the chromophore was identified to be dehydrocoelenterazine (Fig. 1B) (36). Here, we focus on the bioluminescence of gastropods as a least studied group in Mollusca.

Gastropod bioluminescence is observed in some marine nudibranchs, tiny marine snails *Angiola*, *Hinea* and *Melanella*, the land snail *Quantula striata* and the freshwater limpet *Latia neritoides* (2,5). Studies on the bioluminescence mechanisms in these luminous gastropods are very limited except for *L. neritoides*.

New Zealand freshwater limpet (*Latia neritoides*)

The freshwater limpet *L. neritoides* (Fig. 7) is endemic in the North Island of New Zealand, where it is widely distributed in shallow, fast-flowing streams and some lakes (37). Notably, this species is the only luminous animal known to date that spends its entire life cycle in freshwater. It clings to the submerged rocks and logs, and discharges a yellow-green luminescent mucus when disturbed.

A classic luciferin–luciferase reaction in *L. neritoides* was demonstrated by Bowden (38). *Latia* luciferin was purified as a hydrophobic colorless liquid, and the chemical structure was determined to be (*E*)-2-methyl-4-(2,6,6-trimethyl-1-cyclohex-1-yl)-1-buten-1-ol formate (Fig. 1C) (39). In the presence of *Latia* luciferase, unknown purple protein and molecular oxygen, *Latia* luciferin is converted to oxyluciferin (Fig 1D) with a yellow-green light emission (λ_{max} = 536 nm) (40). However, the presence of the purple protein was considered to be inessential for the bioluminescence reaction (1). Shimomura argued in his book that the function of the purple protein was to serve as an activator or enhancer of the light-emitting reaction (1). *Latia* luciferase and purple protein were purified, and their molecular masses were determined to be 173 and 39 kDa, respectively (40). Ohmiya *et al.* suggested that this luciferase is a homo-hexameric glycoprotein comprising 31.6-kDa subunits (41). The

Figure 5. Fireworm *Odontosyllis* cf. *undecimdonta*. The heads are shown on the left side of these photographs. Photograph by Yuichi Oba.

light emitter of the *Latia* bioluminescence was not determined, but the results above suggest that the chromophore is bound to luciferase protein. Shimomura predicted that the light emitter is a flavin or a flavin-like compound, because *Latia* luciferase showed fluorescence in KCN solution and the emission spectrum is close to the bioluminescence spectra and also to the fluorescence of flavins (1).

DIPLOPODA

Arthropoda contains huge numbers of luminous species, especially in crustaceans and hexapods, which constitute about 25% of all genera comprising luminous species (5). On the other hand, a few luminous species have been reported only in Chilopoda (centipedes) and Diplopoda (millipedes) if crustaceans and hexapods are excluded (2). Bioluminescence systems of some crustaceans and insects, such as ostracods, copepods, deep sea shrimp *Oplophorus*, fireflies and click beetles, have been well studied (1). On the other hand, the mechanistic studies on centipedes and millipedes luminescence are very limited. In this section, we focus on luminous millipede *Motyxia sequoiae* as a least studied case.

Sierra luminous millipedes

Only 11 of 12 000 described millipede species are bioluminescent, nine of them belonging to the genus *Motyxia* (Xystodesmidae) (42) (Fig. 8). There are two more luminous species described, the tropical cosmopolitan *Paraspirobolus lucifugus* (Spirobolellidae) (43,44) and New Caledonian *Dinematocricus* sp. (Rhinocricidae) (45), but the biochemistry of their bioluminescence has not been studied.

Motyxia sequoiae (Syn. *Luminodesmus sequoiae*) is a millipede of about ~35 mm length and ~7 mm width, distributed across the southern Sierra Nevada Mountains (46). Its bioluminescence is greenish blue peaking around 495 nm and emitting spontaneously with 20–40% oscillation of intensity (47). Physical stimulation triggers light emission of higher intensity. The cuticle of *M. sequoiae* exhibits a greenish-blue fluorescence, as for some nonluminous millipedes. Interestingly, the emission maximum of the *M. sequoiae* cuticle is similar to its bioluminescence (48).

Figure 6. Scaleworm *Harmothoë imbricata*. Photograph was taken at the White Sea Biological Station of the Moscow State University, Murmansk region, Russia, by Alexander Semenov.

In the 1980s, Shimomura proposed the involvement of a photoprotein to explain *M. sequoiae* bioluminescence (49). The photoprotein was purified by chromatography, and the molecular mass was estimated to be 60 kDa by gel filtration analysis. The protein emits light in the presence of ATP, Mg^{2+} and oxygen, and the spectral maximum matches exactly to the *in vivo* luminescence (49). Later on, the same author determined the molecular mass of photoprotein to be 104 kDa by SDS-PAGE analysis and proposed the involvement of a porphyrin chromophore with maximum absorbance around 410 nm in the bioluminescence reaction (50). The chromophore separated from purified photoprotein by HCl treatment showed fluorescence peak at 595 and 650 nm under acidic condition, which strikingly matches the *in vivo* bioluminescence. Further experiments demonstrated the porphyrin does not contain Fe^{3+} (50). Shimomura suggested that the porphyrin present as a chromophore in the photoprotein is the emitter in bioluminescence of *Motyxia* millipedes.

Twenty years later, a greenish-blue fluorescent compound from the cuticle of *M. sequoiae* was determined to be 7,8-dihydropterin-6-carboxylic acid (emission max, 505 nm) (Fig. 1F) together with pterin-6-carboxylic acid (emission max, 450 nm) (48). As the fluorescence peak of the former compound is close to that of *in vivo* and *in vitro* luminescence of *M. sequoiae*, Kuse *et al.* suggested it as the light emitter. It is of note that same fluorescent compounds were also isolated from the cuticle of the nonluminous xystodesmid millipede *Parafontaria laminata armigera* (51).

ECHINODERMATA

The phylum Echinodermata consists of five extant classes: Crinoidea (sea lilies), Ophiuroidea (brittle stars), Asteroidea (starfishes), Echinoidea (sea urchins) and Holothuroidea (sea cucumbers) (2,4). Luminous species are found in all classes except Echinoidea, and there are only few studies on their bioluminescence systems except for brittle stars.

Brittle stars

Brittle stars are animals belonging to the class Ophiuroidea of the phylum Echinodermata. Currently, about 2000 ophiuroid species have been recorded worldwide (52), of which about 70 species are recognized as luminous (53,54).

The luminescence color of the brittle stars is mostly green, but blue luminescence has been reported in some species (53). These animals emit flashes of light along the arms by mechanical stimulation (Fig. 9). Some species eject luminous mucus (54).

In 1986, Shimomura proposed the involvement of a photoprotein in the bioluminescence of the luminous brittle star *Ophiopsila californica* (55). He purified the photoprotein, Ophiopsilin (53), whose fluorescence is greenish blue (λ_{max} = 482 nm) by UV irradiation and emitted the same green light when triggered by H_2O_2. The molecular mass of the photoprotein was estimated to be approximately 45 kDa by gel filtration (1,55). Shimomura also found the presence of another green fluorescent substance in the extract and suggested its involvement in the *in vivo* green luminescence as the fluorescence emission spectrum matched to *in vivo* bioluminescence (broad peak at about 510 nm) (1,55). In 2009, Mallefet reported the bioluminescence system in the luminous brittle star

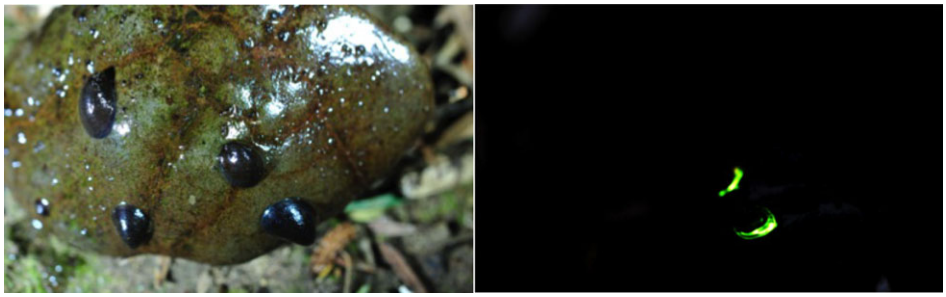

Figure 7. New Zealand freshwater limpet *Latia neritoides* and its luminescence secretion. Photographs by So Yamashita.

Amphiura filiformis to be a luciferin–luciferase reaction involving coelenterazine (Fig. 1A, λ_{max} = 475 nm) (56). The molecular mass of the luciferase is 23 kDa, and therefore, the luminescence mechanism of *A. filiformis* is probably different from that of *O. californica* (1).

Noticeably, the draft genome of the luminous brittle star *Ophionereis fasciata* was reported with low coverage (57), and it will be useful for identifying photoprotein or luciferase genes of luminous brittle stars in the future.

HEMICHORDATA

The Phylum Hemichordata consists of three extant classes, Enteropneusta, Pterobranchia and Planctosphaeroidea, of which only Enteropneusta contains luminous species (2,4,25).

Acorn worms

There are about 70 species recorded worldwide in Enteropneusta (58), of which only a few species of the genera *Balanoglossus* and *Ptychodera* in the family Ptychoderidae are known to be luminescent (59). Luminosity of the *Glossobalanus* species is dubious because one species of the genus *Glossobalanus* sp. was reported as bioluminescent by Harvey in 1922 (30), but not listed in his later books (25,60). The luminous acorn worms discharged a bluish light following mechanical (25), electrical (Herring, 1978) or chemical stimuli such as diluted H_2O_2 (61).

A luciferin–luciferase reaction was demonstrated in *Balanoglossus biminiensis*. The *in vitro* luminescence of luciferin and luciferase mixture was triggered by the addition of H_2O_2 (62). All active luciferin preparations had iodoform-like odor, and 2,6-dibromophenol (Fig. 1G) isolated from *B. biminiensis* was found to be responsible for the said odor (63). Kanakubo *et al.* determined 2,3,5,6-tetrabromohydroquinone (Fig. 1H) and riboflavin as a possible luminous compound and light emitter, respectively, from *Ptychodera flava* (Fig. 10) (64). A mixture of 2,3,5,6-tetrabromohydroquinone and riboflavin elicited chemiluminescence at λ_{max} = 521 nm, which is close to the *in vivo* bioluminescence at λ_{max}= 528 nm by the addition of diluted H_2O_2 in 70% 1,4-dioxane at pH 12. They also isolated several brominated and chlorinated quinone derivatives as other potential luminous compounds, which showed chemiluminescence (65). They did not demonstrate the classic luciferin–luciferase reaction of *P. flava* (64).

The *in vivo* bioluminescence mechanisms of acorn worms remain unknown. It is worth noting that the draft genome sequence of *P. flava* was recently reported (66).

Figure 8. (A) Bioluminescent millipede *Motyxia sequoiae* collected from Tulare County, California. (B) *Motyxia bistipita* collected from San Luis Obispo County, California. Photographs kindly provided by Paul Marek.

CHORDATA

Tunicates

The Subphylum Urochordata is the invertebrate taxon most closely related to vertebrates and consists of three classes: Ascidiacea, Thaliacea and Appendicularia. Each class contains small numbers of luminous species: the sea vase tunicate *Clavelina miniata* in Ascidiacea (67); pyrosomatid *Pyrosoma* spp. and doliolid *Doliolula equus*, *Pseudusa bostigrinus* and *Paradoliopsis harbisoni* in Thaliacea (4,68); and all *Oikopleura* (subgenus *Vexillaria*) spp. and *Stegosoma magnum* in Appendicularia (69). The old description of the bioluminescence in ascidiacean *Ciona intestinalis* is dubious (67). Bioluminescence in Salpida has not been confirmed (4). Special care is needed to identify the bioluminescence of these transparent filter feeders, because they sometimes trap luminous microorganism, such as luminous dinoflagellates or a colony of luminous bacteria, which may induce extrinsic luminescence (70,71).

The brilliant blue-green luminescence of the free-floating colonial tunicate *Pyrosoma* (Fig. 11) has been well recorded in the old literature (ref. 25, and references therein), but the bioluminescence systems are not much studied. The bioluminescence of *Pyrosoma* had been attributed to luminous bacteria, but currently, this symbiotic hypothesis is almost rejected (1). Its luciferin–luciferase reaction has not been demonstrated, and no further studies have been reported (1).

Another colonial tunicate *Clavelina miniata* is sessile in adult phase. It emits strong green light when stimulated (67). The luminescence was recovered by adding water to the lyophilized material. The addition of ATP or hot water extract of lyophilized power did not enhance the luminescence intensity of spent solution (72).

Bioluminescence system of the appendicularians has been briefly reported only in a book chapter (70). They considered that the bioluminescence is coelenterazine-related luciferin–luciferase system based on their unpublished experiments using house rudiment (nonexpanded and preliminarily secreted "house" of appendicularians) of *Oikopleura labradoriensis*: The addition of coelenterazine to the spent solution enhanced the luminescence, and the methanol extract induced the luminescence of luciferase from a sea pen (*Ptilosarcus* sp.).

The draft genome sequence of *Oikopleura dioica* was recently reported (73), but the bioluminescence aspect was not addressed in this works.

Lantern sharks

Most of the luminous taxa within vertebrates are marine bony fishes, which include about 1500 species in 43 families (74). Some luminescent teleosts, such as anglerfishes, possess luminous bacteria in photophores (symbiotic bioluminescence). Some teleosts, such as the midshipman *Porichthys* and myctophid lanternfishes, use cypridinid luciferin and coelenterazine as a luciferin, respectively, which are probably obtained through the food chain (5). Other fishes possess intrinsic bioluminescence. However, chemical studies on fish luminescence have been mostly hampered by difficulties in obtaining specimens, as most luminous fishes inhabit deep sea and carry very small photophores.

In cartilaginous fishes (the class Chondrichthyes), bioluminescence is not as common as among bony fishes. Only dozens of species in three families of luminescent shark, Dalatiidae, Etmopteridae and Somniosidae, are recorded in the world (74). These sharks possess many small light-producing organs in a complex array on their bodies (Fig. 12).

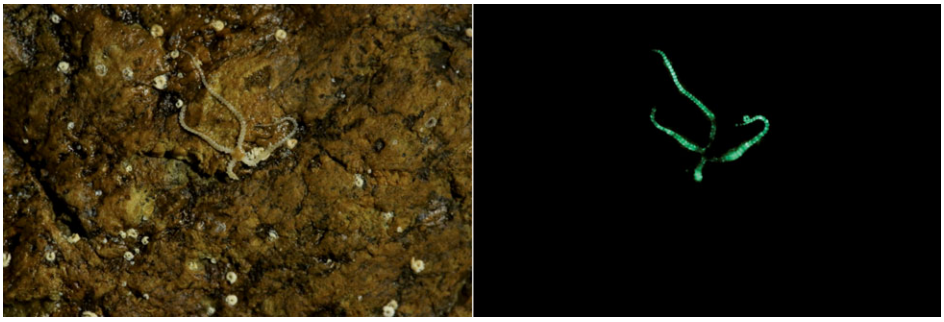

Figure 9. Luminous brittle star *Amphipholis squamata* and its luminescence. Photographs by Takehito Miyatake.

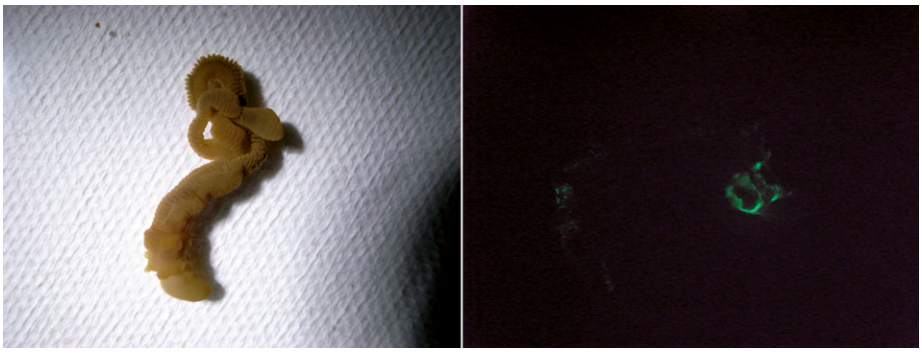

Figure 10. *Ptychodera flava* and its luminescence by H_2O_2 stimulation. Photographs by Yuichi Oba.

So far, no bacterial symbiosis has been reported in luminous sharks, and Renwart *et al.* showed by microscopic analyses the absence of bacteria inside the photocytes of the deep-sea lantern shark *Etmopterus spinax* (75). These results suggested that the light production of the photocytes in luminous sharks is intrinsic.

Renwart and Mallefet carried out biochemical studies on the shark luminescence using *E. spinax* (76). They examined cross-reactions between the cold distilled water extract of photophores with three known luciferins in marine luminous organisms: krill luciferin, coelenterazine and cypridinid luciferin (76). They also examined cross-reactions between the methanol extract of photophores with three known luciferases, which utilize krill luciferin, coelenterazine or cypridinid luciferin. To prevent the possibility that coelenterazine is stored as inactive forms, such as protein-bound form, enol sulfate form and dehydrolyzed form (Fig. 1B), they also performed pretreatments of the methanol extract to make coelenterazine active free form. However, all cross-reaction analyses were negative. Although the cross-reactivity between water extract and methanol extract of shark photophore was not confirmed, the results suggested that the bioluminescence of shark refers to unknown system, involving a new luciferin molecule, a new storage form of known luciferin or photoprotein system (76). No further studies on the biochemistry of shark luminescence have been reported so far.

CONCLUSION

In 2006, Osamu Shimomura, a Nobel Prize winner and the leading authority in the chemical aspects of bioluminescence, published a monumental book entitled "Bioluminescence: Chemical principles and Methods" (1). This book compiled most of the chemical and biochemical researches mainly published since the 1960s, when the techniques of chemical and biochemical analyses were rapidly developed. Ten years after this publication, analytical methods were further refined, especially in the field of omics sciences such as transcriptome, whole-genome analysis, metabolomics and bioinformatics. NMR and mass spectrometry technologies became also more sophisticated and currently allow the determination of chemical structures of complicated and less stable compounds. Therefore, the authors of this review expect that we are now in the time of the next breakthrough of the studies on bioluminescence systems.

The selected bioluminescent systems described in this review are indeed the focus of current research by the authors. The animals listed in the review are all relatively easy to collect in sufficient amounts for chemical, biochemical and genomic/transcriptomic analyses. On the other hand, considering recent worldwide environmental destruction that diminishes valuable gene resources, we need to solve novel bioluminescence systems rapidly with focusing on selected targets close to the goal by international collaboration. Additionally, the taxonomy and systematics of several luminous organisms were revised recently. We therefore keep in mind this fact especially when we revisit the organism studied long years before; that is, old studies may be mixed up with the results from more than two species. Finally, we hope that studies on unknown bioluminescence systems will provoke new applied techniques on their base.

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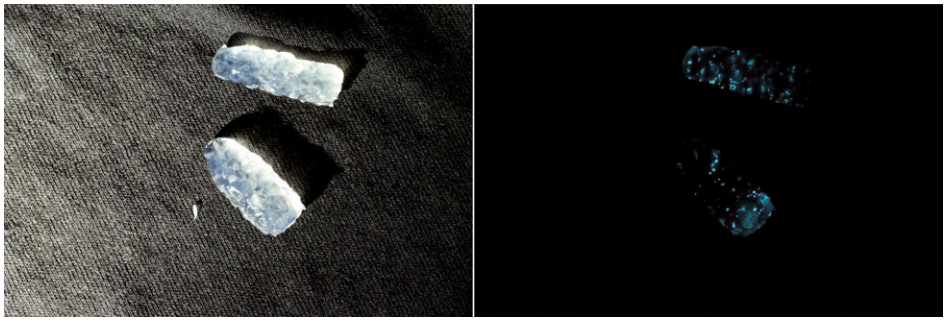

Figure 11. *Pyrosoma atlanticum* and its luminescence. Photographs by Yuichi Oba.

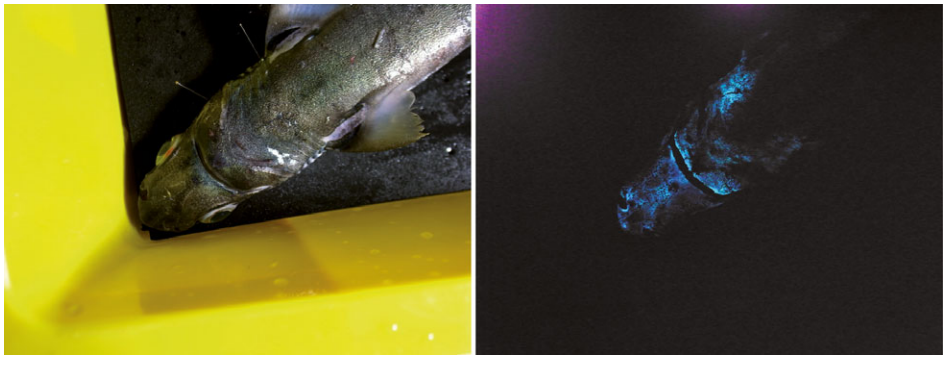

Figure 12. Ventral view of the head of the luminous shark *Etmopterus lucifer* and its luminescence. Photographs by Yuichi Oba.

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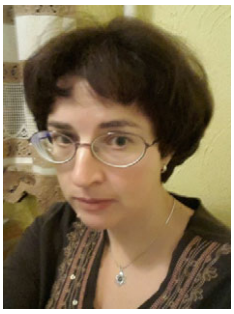

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