Bioluminescence and Fluorescence in Littoral Earthworms

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Abstract

Pontodrilus litoralis is a cosmopolitan littoral earthworm known to exhibit bioluminescence. Recently, a congeneric species *Pontodrilus longissimus* from Thailand was described. These species are sympatric but their burrowing depths on Thai beaches are different. In this study, we examined the *in vivo* and *in vitro* bioluminescence properties of *P. longissimus* and *P. litoralis*. Mechanical stimulation induced *in vivo* luminescence in *P. litoralis*, as reported previously, but not in *P. longissimus*. *In vitro* cross-reaction tests between these species revealed the absence of luciferin and luciferase activities in *P. longissimus*. *P. litoralis* had strong fluorescence in a coelomic fluid that matches to the spectral maximum of its bioluminescence, but *P. longissimus* did not. These results suggest that *P. longissimus* does not have luminescence ability due to the lack of all bioluminescent components, luciferin, luciferase, and light emitter, despite its close relationship to the luminous *P. litoralis*. The presence of both luminous and non-luminous species in a single genus is uncommon, and our present findings will shed insight on the possible functions of bioluminescence in the earthworm, such as avoiding predation by littoral earwigs.

Introduction

The earthworm genus *Pontodrilus* Perrier, 1874 displays various unique characteristics. The littoral earthworm *P. litoralis* (Grube, 1855) distributes in the tropical and sub-tropical coasts of the Atlantic, Indian and Pacific Oceans [1–3], and is known to be bioluminescent [4–7]. The luminescent system of *P. litoralis* has been shown to be a luciferin-luciferase type reaction triggered by hydrogen peroxide, with a fluorescence compound acting as a light emitter [7], although the chemical structure of the luciferin remains uncertain and the luciferase gene has not been determined. Recently, the littoral earthworm *Pontodrilus longissimus* Seesamut & Panha, 2018 was described from the coastal areas in Thailand and Peninsular Malaysia [8] based on the morphological differences in the size of the body, the number of segments and the diverticulum from other congeners.

In the present study, the bioluminescence and fluorescence of *P. litoralis* and *P. longissimus* were examined *in vivo* and *in vitro*, and the results suggested that *P. longissimus* lacks luminescence ability despite its genetically close relationship to *P. litoralis*. Based on these findings, we discuss the biological function of earthworm bioluminescence and a convenient parataxonomy method for *Pontodrilus* species.

Results

In vivo and in vitro bioluminescence

After the live specimens of both *Pontodrilus* species were stimulated by electricity or rough handling, *P. litoralis* exuded a green luminescent fluid, whereas the fluid exuded from *P. longissimus* was not luminescent (Fig. 1A). Under a handheld long-wave UV lamp (365 nm), almost the entire body of *P. litoralis* emitted strong yellow fluorescence, which was most conspicuous at the rows of setae, whereas *P. longissimus* did not emit fluorescence under the same condition (Fig. 1B).

The cross-reactivities of the crude luciferase and crude luciferin in *P. litoralis* and *P. longissimus* were examined (Fig. 2A). For certainty of the results, we used a concentration of *P. longissimus* extract that was 5-fold higher than the concentration of *P. litoralis* extract. The results showed that significant luminescence was detected only when mixing the luciferin extract from *P. litoralis* with the luciferase extract from *P. litoralis*. On the other hand, both luciferin and luciferase activities in the extracts of *P. longissimus* were negative (the levels of both activities were almost the same as in a negative control). This finding suggested that *P. longissimus* is non-luminous due to the lack of both luciferin and luciferase.

Fluorescence and luminescence spectra of luminous *P. litoralis*

Fluorescence spectra were measured using a crude coelomic fluid extract of *P. litoralis* (Fig. 2B). The peaks of the excitation spectra were 370 and 453 nm, whereas the emission peaks were 450 and 523 nm, indicating the presence of at least two fluorescence compounds in *P. litoralis*. The luminescence spectrum of *P. litoralis* had a maximum wavelength of 528 nm *in vivo* and 524 nm *in vitro* (Fig. 2C). Wampler and Jamieson showed that the spectral maximum of bioluminescence (540 nm) in the Bermudan *P. bermudensis* (which is now considered synonymous with *P. litoralis* [2]) matched the fluorescence maximum of the coelomic fluid, and suggested the fluorescence substance is a light emitter [7]. Although the spectral maximum values of our present study were different from their results, probably due to genetic differences in the specimens examined or differences in the spectrophotometers used, our results also showed a spectral match between fluorescence and bioluminescence *in vitro*. The small red-shift of the *in vivo* spectrum might have been due to a reflection effect from the reddish earthworm body.

Comparison of the coelomic fluid cells and protein bands between the two littoral earthworms

The coelomic cells of these littoral earthworm species were observed under a fluorescence microscope (Fig. 3). The results showed that the *P. litoralis* coelomic cells emitted fluorescence but the *P. longissimus* did not. The size of the coelomic cells was approximately 15 µm in diameter, and numerous small fluorescent particles were detected in the coelomic cells of *P. litoralis*. SDS-PAGE of the coelomic fluids showed different protein constitutions between the two species (Fig. 4).

Discussion

In this study, we confirmed that *P. longissimus* is non-bioluminescent, despite its close relationship to the luminous *P. litoralis*. The presence of both luminous and non-luminous species in a single genus is uncommon; in general, bioluminescence is shared among all members of the same genus, sometimes at the family level. For example, the family Lampyridae (fireflies) consists of over 67 genera and 2,000 species around the world, and all are considered to be bioluminescent, at least in the larval stage, which uses the same luciferin molecule and homologous luciferase [9]. In contrast, we can list only a few exceptions, such as *Vibrio* and *Photobacterium* (marine bacteria) [10], *Epigonus* (deep-sea fishes) [11] and *Eisenia* (terrestrial earthworms) [12]; these genera have been reported to contain both luminous and non-luminous species. *P. litoralis* and *P. longissimus* are easily collectible at the same beach [8] and

rearable in a laboratory; thus they are suitable materials for studying the ecology and evolution of bioluminescence.

In vitro luciferin-luciferase cross-reaction tests of *P. longissimus* and *P. litoralis* confirmed that the lack of luminescence ability of *P. longissimus* is due to the absence of all bioluminescent components, i.e., luciferin, luciferase and the light emitter in coelomic fluid. It has previously been suggested by cross-reaction tests that the luminous earthworms in the genera *Pontodrilus* (Megascolecidae), *Microscolex* and *Diplocardia* (Acanthodrilidae) share the same basic bioluminescence mechanisms [5, 7, 13, 14], in spite of their far-distant relationship to each other [15, 16]. It is expected that the ancestral state of *Pontodrilus* is non-bioluminescent, because the nearest extant relatives of *Pontodrilus* belong to the genus *Plutellus* Perrier, 1873, and all members of this group are non-bioluminescent [6, 17]. These findings suggested that *P. litoralis* secondarily acquired the bioluminescent properties as a parallel evolution, similar to the case of bioluminescence in lampyrid and elaterid beetles [18]. We detected a clear difference in protein composition of the secreted fluid between *P. litoralis* and *P. longissimus*. The luciferase and other bioluminescent components of luminous earthworms were not determined, and further comparative analyses between the proteins and substances of these secreted fluids will be useful to understand the mechanism of bioluminescence and its parallel evolution.

In Thailand, *P. longissimus* was found sympatrically with *P. litoralis* at the beaches along the coast, but the microhabitats of the two congeners are different; *P. litoralis* was collected on the beach surface (under trash or leaf litter on sandy beaches), whereas *P. longissimus* was found at a greater depth than *P. litoralis*, i.e., a depth of more than 10 cm, where trash and leaves are scarce [8] (Fig. 5A–5D). It has been hypothesized that the biological function of bioluminescence in Annelida, including *P. litoralis*, is to stun or divert attention as an anti-predator defense [19–25], but experiments and observations of the prey are limited. Sivinski & Forrest [25] reported the luminescence of *Microscolex phosphoreus* deterred from predation of the mole cricket *Scapteriscus acletus* (but the specimen was finally consumed) under laboratory conditions. A British television program [26] presented by David Attenborough showed that the French luminous earthworm *Avelona ligra* glowed when attacked by the carabid beetle, but the beetle consumed the luminescent worm without any hesitation. We consider that the absence of bioluminescence in *P. longissimus* may correlate with the habitats with low-predation pressure, whereas *P. litoralis* acquired a bioluminescence property during evolution that enables it to enter the surface environment of the beach, which is rich in nutrition and food sources [3, 27] as well as in potential predators.

Indeed, no possible predator was found to live alongside *P. longissimus*. In contrast, various carnivorous invertebrates, such as earwigs, robe beetles, carabid beetles, crabs, and hermit crabs, were found to live along with *P. litoralis* on the beaches in Thailand and Japan (Seesamut pers. obs.). In this context, we performed a feeding experiment using the maritime earwig sympatrically distributed in the *P. litoralis* habitat. The maritime earwig *Anisolabis maritima* (Dermaptera, Anisolabididae) was a cosmopolitan, also distributed in Japan. It has developed compound eyes (Fig. 5E) and is considered a carnivorous animal that forages its prey at night [28, 29]. We found *A. maritima* (body length ≤ 30 mm) predominantly

at the beach where *P. littoralis* was collected (Fig. 5F). Some robe beetles (Coleoptera, Staphylinidae) were also found at the same habitat, but they seemed to be too small (< 10 mm) for predation of *P. littoralis*, and under our laboratory observations, the robe beetle did not attack the worm. Thus, we think *A. maritima* is major potential predator of *P. littoralis* at the beach in Japan. Living *P. littoralis* and *A. maritima* were collected from the same beach on the same day, and we observed the predation behavior in the laboratory in a dark cage with beach sand spread on the bottom. Our observation of the predation of *P. littoralis* by the earwigs (Supplementary Video 1) may have provided an important insight into the function of bioluminescence in *P. littoralis*. The earwigs immediately started to aggressively attack the worm with their mandibles and abdominal cerci, a pair of scissors-like pincers; the worm secreted luminescent mucus from its wounds (Supplementary Video 1), and it appeared that the retention of the gluey luminescent mucus on the mouths and forelegs of the earwigs was unpleasant to them, since they struggled to remove the glue by frequent grooming (Fig. 5E, Supplementary Video 2). Indeed, after aggressive attacks, the earwigs finally abandoned their consumption of the worm, and thus the worm survived. To the best of our knowledge, this is the first observation of earthworm bioluminescence by predation under almost natural conditions. Based on these observations, we hypothesized that the luminous glue of *P. littoralis* may function to deter and/or divert from the predation, and that the luminescence might even enhance the avoidance learning of the predator. Nevertheless, in terms of the function of luminescence, we consider that the global distribution of *P. littoralis* is a consequence of its adaptation to the beach surface, which provided the opportunities for dispersal by current, whereas *P. longissimus* is endemic to the coast of the Thai-Malay peninsula [8, 30] due to its deeper inhabitation in sand.

Based on microscopic observations, we confirmed that both species secrete coelomic cells by stimulation, but neither bioluminescence nor fluorescence was observed in *P. longissimus*. The presence and absence of fluorescence in the same genus of earthworm was also reported in the terrestrial genus *Eisenia*; *E. andrei* showed fluorescence in coelomic fluid, while *E. fetida* did not. Although both species are non-bioluminescent and the fluorescence emission maximum in *E. andrei* was in the UV region, 370 nm, they suggested the difference in fluorescent characteristics was useful to delimitate these closely related species as a “fluorescence fingerprint” by using a fluorescent probe [31]. In the case of *Pontodrilus*, on the other hand, the fluorescence emission maximum of *P. littoralis* was in the visible region and the fluorescence intensity was strong enough to be observed by the naked eye under portable UV light, without using any additional fluorescent probe. Therefore, the fluorescence fingerprint method was also applicable to *Pontodrilus*. Moreover, we found that the protein band patterns by SDS-PAGE were clearly different between species, thus this may have been useful as a protein fingerprint for the taxonomic delimitation of these closely related species [32, 33]. The littoral zones have rich species diversity of both macro- and microorganisms [34, 35]. They comprise a front of human pressure in marine ecology and one of the most important zones for conservation [36, 37]. Therefore, understanding of littoral fauna is unavoidable. Earthworms have principally strong effects on soil ecosystems [38–40]. *Pontodrilus* is a major “ecosystem engineer” [40] that inhabits the littoral habitat. Thus, species identification of *P. littoralis* and *P. longissimus* is significant to assess the littoral environment. They are actually distinguishable by

the internal morphology of the spermathecal diverticulum, but special skills and equipment are necessary for the morphological analyses. In this study, we showed the differences in the bioluminescence, fluorescence, and protein-fingerprinting characteristics between *P. littoralis* and *P. longissimus*, and demonstrated that the analysis of these differences provides an easy *in situ* methodology to identify these earthworms for marine ecological studies and conservation of littoral zones in Southeast Asia.

Methods

Specimens and species identification

The littoral earthworm *P. littoralis* was collected at a sandy region of one of the following beaches [3, 8]: Wonnapa beach, Amphoe Mueang Chon Buri, Chonburi, Thailand (13°15'55.6"N 100°55'29.3"E), Kowa beach, Chita, Aichi prefecture, Japan (34°46'23.3"N 136°54'52.7"E) and Kira Waikiki Beach, Nishio, Aichi prefecture, Japan (34°46'55.2"N 137°05'48.3"E). *P. longissimus* was collected from Tambon Muang Klang, Amphoe Kaper, Ranong, Thailand (9°37'26.7"N 98°28'08.6"E). The earthworms were maintained in native sand in plastic containers sprayed with artificial seawater to keep the sand moist. Species identification was performed based on morphological characteristics by Seesamut et al. (2018) [8]. *In vivo* bioluminescence was photographed in darkness with a Nikon D5500 digital camera (Nikon, Tokyo). *Pontodrilus* were stimulated by electricity or rough handling to induce bioluminescence, and *in vivo* fluorescence was photographed under a handheld UV lamp (365 nm) without mechanical stimulation.

Extraction of the luminescent substance

To prepare the crude *Pontodrilus* luciferase and luciferin, the live earthworms were rinsed with distilled water and transferred for 24 h to Petri dishes in wet tissue paper moistened with artificial seawater to avoid the contamination of their stomach content when extracting coelomic fluid. All experiments were carried out on ice except for the measurements of light intensity and spectra. Coelomic fluid was extracted as follows: twenty live worms of each species (2.72 g wet weight of *P. littoralis* and 7.4 g wet weight of *P. longissimus*) were put on a mortar and stimulated with a pestle to induce exudation of coelomic fluid, then 10 ml of 50 mM Tris-HCl at pH 7.2 was added. After removing the specimens, the solution was centrifuged at 15,000 × g for 15 min at 4 °C in a TOMY MX-100 high speed refrigerated microcentrifuge, and the supernatants were collected as the crude extracts. The crude luciferin and luciferase fractions were prepared based on the method by Bellisario [41]. In brief, the crude extract was filtered using a 10K centrifugal filter device (Merck, Germany), and the first flow through was used as a crude luciferin extract and the retentates on the membranes were collected as crude luciferase extract.

Cross-reaction experiment and spectral measurement

The total protein concentrations of crude luciferase extracts measured using a protein assay kit (Bio-Rad, USA) were 19.56 µg/ml in *P. littoralis* and 102.78 µg/ml in *P. longissimus*. The luminescent activity was monitored using a luminometer (Centro LB960, Berthold). Ten µl of crude luciferase was mixed with 40 µl of crude luciferin and 10 µl of 0.3% hydrogen peroxide was injected to initiate the luminescence reaction.

The luminescence was recorded in relative light units (RLUs) for 120 s accumulation after injection of hydrogen peroxide.

Spectral measurement

Luminescence and fluorescence spectra were recorded with a spectrofluorometer (JASCO, FP-777W). For the luminescence spectra measurements, the excitation light source was shut off. Smooth data were applied using the binomial method and the spectral response was not corrected. An *in vivo* luminescence spectrum was obtained using a single living specimen put into a quartz cuvette immediately after stimulation by rough handling. To obtain an *in vitro* luminescence spectrum, 100 µl of crude luciferase and 300 µl of luciferin were mixed with 400 ml of 50 mM Tris-HCl at pH 7.2 and 40 µl of 0.3% hydrogen peroxide, and immediately measured. Fluorescence spectra of coelomic fluid in *P. littoralis* were obtained using crude extract suspended in 500 µl of 50 mM Tris-HCl at pH 7.2. The bandwidths used for the emission and excitation were 5 nm.

Coelomic cells photography and SDS-PAGE

Coelomic cells of *Pontodrilus* were isolated by stimulating earthworms on microscope slides, observed under a fluorescence microscope (Nikon Eclipse E600, Japan) with a 60x objective lens (Nikon CFI Plan Fluor Series, Japan), and photographed using an attached digital camera (Nikon D5500, Japan). The fluorescence excitation was 380 nm.

The protein of crude coelomic extract of both species was run by 15% SDS-PAGE gel using a 1D Gel Electrophoresis Mini Gel, AE-6530mPAGE (ATTO), followed by silver staining (Silver Stain MS Kit, FUJIFILM Wako Pure Chemical Corporation).

Video recording

Video recording of the live specimens was performed using a Nikon D500 and Micro NIKKOR 60 mm lens (Nikon) with the following settings: ISO 64000, F2.8, expose 1/60 s, under red light (LED Lenser T²QC).

Declarations

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Author Contributions

Conceptualization, T.S. and Y.O.; Fieldwork, T.S., I.K. and S.P.; methodology, T.S., D.Y., J.P. and I.K.; investigation, T.S., D.Y., J.P. and Y.O.; writing original draft preparation, T.S.; writing, review and editing, T.S.; Y.O.; supervision, S.P. and Y.O. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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Figures

Figure 1

(A) Bioluminescence of littoral earthworms after mechanical stimulation. Left smaller specimen, *Pontodrilus litoralis*; right larger specimen, *Pontodrilus longissimus*. (B) Fluorescence observed under a handheld long-wave UV lamp (365 nm); *P. litoralis* (left), *P. longissimus* (right). Inset, magnified image of *P. litoralis*. The arrow indicates a row of setae.

Figure 2

(A) Cross-reaction between crude luciferase and luciferin of the littoral earthworm genus *Pontodrilus*. Plit represents *P. litoralis*, Plon represents *P. longissimus*, and Buff represents buffer (50 mM Tris-HCl, pH 7.2). Data are shown as mean $\pm$ standard deviation ($n=3$). (B) Fluorescence spectra of crude coelomic fluid extract from *P. litoralis*. The solid line was obtained with an emission at 523 nm, the dotted line with an excitation at 453 nm and the dashed line with an excitation at 370 nm. (C) The solid line shows the in

vivo luminescence spectrum of living *P. litoralis*. The dotted line shows the in vitro luminescence spectrum of crude coelomic fluid extract from *P. litoralis*, which contained 500 μ l 50 mM Tris-HCl, pH 7.2 and 50 μ l 0.3% hydrogen peroxide. Data are the means of five measurements.

Figure 3

(A) Bright field image of *P. litoralis* coelomic cells (arrowheads). (B) Bright field image of *P. longissimus* coelomic cells. (C) *P. litoralis* coelomic cells under UV irradiation at 380 nm. (D) *P. longissimus* coelomic cells under UV irradiation at 380 nm. The photography conditions in (C) were the same as in (D), but no fluorescence images were observed in latter image.

Figure 4

SDS-PAGE of the crude coelomic extract of *P. litoralis* and *P. longissimus* (detection by silver staining). Molecular weight marker sizes in kDa are provided on the left.

Figure 5

Photographs showing (A) the microhabitat of *Pontodrilus litoralis* from Aichi Prefecture, Japan and (B) the microhabitat of *P. longissimus* from Ranong, Thailand, which was locality where both *Pontodrilus* species were sympatrically collected (Seesamut et al., 2018). (C) *P. longissimus* was found at 10–30 cm depth in muddy sand; the earthworm is shown by an arrow. (D) Bright field image of *Pontodrilus* species. Small size (left), *P. litoralis*; large size (right), *P. longissimus*. (E) An earwig *Anisolabis maritima* (a

potential *Pontodrilus* predator) grooming its forelegs after attacking *P. litoralis*. (F) *Anisolabis maritima* (arrowhead) was found at the same microhabitat as *P. litoralis* in Aichi Prefecture, Japan.

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