

Kleptoprotein bioluminescence: *Parapriacanthus* fish obtain luciferase from ostracod prey

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Through their diet, animals can obtain substances essential for imparting special characteristics, such as toxins in monarch butterflies and luminescent substances in jellyfishes. These substances are typically small molecules because they are less likely to be digested and may be hard for the consumer to biosynthesize. Here, we report that *Parapriacanthus ransonneti*, a bioluminescent fish, obtains not only its luciferin but also its luciferase enzyme from bioluminescent ostracod prey. The enzyme purified from the fish's light organs was identical to the luciferase of *Cypridina noctiluca*, a bioluminescent ostracod that they feed upon. Experiments where fish were fed with a related ostracod, *Vargula hilgendorfii*, demonstrated the specific uptake of the luciferase to the fish's light organs. This "kleptoprotein" system allows an organism to use novel functional proteins that are not encoded in its genome and provides an evolutionary alternative to DNA-based molecular evolution.

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INTRODUCTION

The evolutionary acquisition of novelties is not always achieved through genetic mutations. At times, novelty can be achieved by "stealing" components that have evolved elsewhere. Poison dart frogs and monarch butterflies obtain toxic molecules from invertebrates and plants in their diets (1, 2), and some jellies and fishes obtain bioluminescent substances from their prey (3–6). In each of these cases, small organic molecules are acquired. Other animals go further and steal intact organelles and cells from their prey. Some sea slugs incorporate and maintain chloroplasts (kleptoplastids) from algal prey to generate photosynthetic products in their own tissues (7–9). Many gastropods, as well as flatworms and comb jellies, steal and house "cnidocytes" from cnidarian prey to use as their own stinging cells (kleptocnidae) (10). Proteins, however, have been thought to be too fragile and too easily digested to be stolen. In this study, we describe the first example, to our knowledge, of protein theft to acquire an evolutionary novelty, bioluminescence.

Bioluminescence, light emission by living organisms, is commonly invoked as an example of the evolution of novelty. Across the tree of life, more than 800 genera contain bioluminescent species, of which ~200 genera are ray-finned fishes (Actinopterygii) (3, 5, 11, 12). A recent molecular phylogenetic analysis indicated that bioluminescence in ray-finned fishes has evolved independently as many as 27 times (12). Bioluminescence in fishes is generated either by symbiotic bacteria or intrinsically, and both systems have evolved multiple times (3, 12).

In general, a bioluminescent reaction requires a substrate (generically called the luciferin), an enzyme (luciferase), and the presence of molecular oxygen with or without cofactors (3, 4). The molecules coelenterazine and vargulin have been identified as two luciferins used by intrinsically luminescent fishes (3–5, 13). Coelenterazine is found in a variety of luminous marine organisms, including deep-sea

fishes in the orders Myctophiformes and Stomiiformes (3–5, 13). It is expected that these animals acquire the coelenterazine from their diets (3–6), for example, directly through the consumption of coelenterazine-producing copepods (14) or indirectly through the copepods' consumers. Vargulin, also called *Cypridina* luciferin (see Supplementary Text) (3, 15), was originally discovered as the bioluminescent substrate of the crustacean *Vargula hilgendorfii* (cypridinid; Ostracoda), and it was later found in some coastal luminous fishes of three lineages, Pempheridae, Apogonidae, and Batrachoididae (3–5, 12, 16–19). These fishes probably obtain vargulin from their ostracod diet (3–6, 19). For example, the midshipman fish *Porichthys notatus* (Batrachoididae) is typically capable of luminescence along the coast of California but is nonluminous in Puget Sound where suitable bioluminescent ostracods do not occur (19). The *Porichthys* in Puget Sound lacks the capability of light production because of the absence of vargulin but is able to emit light if vargulin is supplied by direct injection or if fed luminous ostracods (19). In contrast to the increasing knowledge of luciferin in fishes as outlined above, although bioluminescent fish are believed to have endogenous luciferase, no luciferase genes or proteins have been identified from any intrinsically bioluminescent fishes.

The golden sweeper *Parapriacanthus ransonneti* (Pempheridae; Fig. 1A) is a shallow-water fish distributed along the West Pacific and Indian Ocean coastlines (20). A nocturnal fish known for schooling under rocky and coral shelters during daytime (20), *Parapriacanthus* has two types of ventral light organs (Fig. 1B and fig. S1): a Y-shaped thoracic light organ, which extends from the first pair of pyloric caeca and is located from the isthmus to the base of the pelvic fins beneath the thoracic translucent muscle, and a linear anal light organ, which emerges from the rectum and anus (16). In 1958, Haneda and Johnson used hot- and cold-water extracts from the light organs in *Parapriacanthus* to demonstrate a light-producing luciferin-luciferase (L-L) reaction; they also found an interphylum cross-reaction of *Parapriacanthus* light organ extracts with whole body extracts from the luminous ostracod *V. hilgendorfii* (16, 21, 22). Additionally, these authors showed that *Parapriacanthus* luciferin was concentrated in pyloric caeca, and that the crystallized compound from these organs was chemically equivalent to vargulin. Coupled with the fact that "*Cypridina*" ostracods were found in the fish's stomach,

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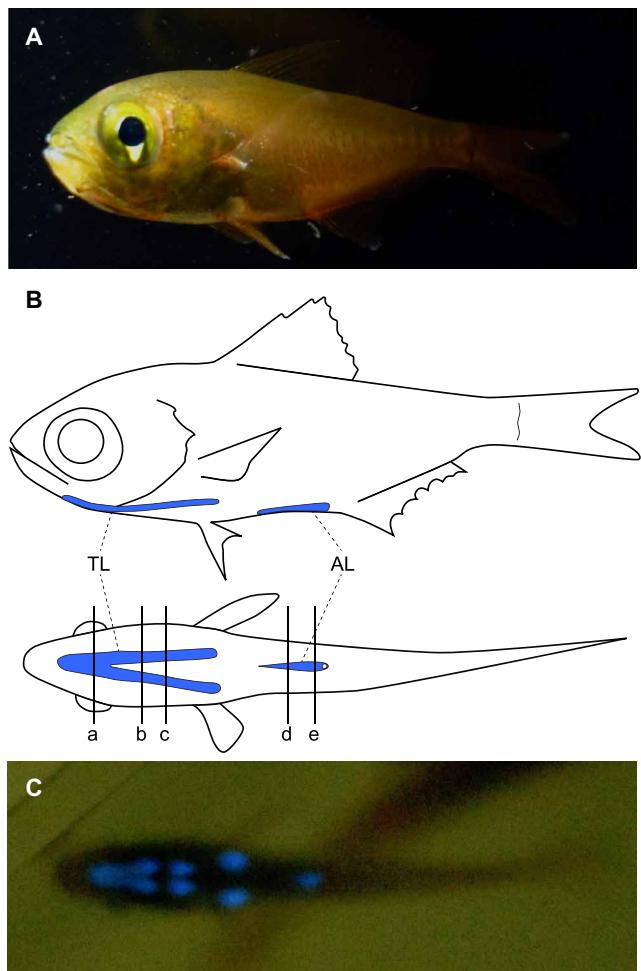


Fig. 1. *P. ransonneti* and its bioluminescence. (A) Lateral view of the fish under white light. Body length, 8 cm. (B) Lateral and ventral views of the thoracic and anal luminous organs (TL and AL; blue lines). The cross sections for immunohistochemistry (Fig. 3) were made at lines a to e. (C) A ventral view of in vivo bioluminescence. Photo credit: Manabu Bessho-Uehara, MBARI.

these findings suggested that *Parapriacanthus* uses the luciferin vargulin obtained from its ostracod prey for bioluminescence (21, 22). Here, we report the identification of luciferase from *Parapriacanthus*, and the unexpected discovery that this enzyme is not produced by the fish but instead is sequestered from its ostracod diet.

RESULTS

Living *Parapriacanthus* emitted dim blue light from thoracic and anal light organs simultaneously when presented with weak overhead light (Fig. 1C, fig. S2, and Supplementary Text). This behavior is similar to that of *P. notatus*, which uses its ventral bioluminescence to cancel out its own silhouette in a strategy termed “counterillumination” (3, 11, 23).

We confirmed that the luminescence reaction of *Parapriacanthus* could be recreated by mixing crude luciferase extracts from light organs with luciferin extracts from the pyloric caeca, producing a blue light emission ($\lambda_{\text{max}} = 456$ nm; Fig. 2A). Luciferase extracts cross-reacted with vargulin, while luciferin extracts reacted with crude luciferase from *V. hilgendorfii*, as reported previously (16, 17). In vitro lumi-

nescence spectra were identical to the in vivo luminescence spectra of both *P. ransonneti* and *V. hilgendorfii* (Fig. 2A and fig. S3).

Luciferase activity was predominantly detected in extracts from the thoracic light organ and anal light organ (Fig. 2B). Protein purification was performed using thoracic light organs from 200 specimens by anion exchange chromatography and size exclusion chromatography techniques to give specific activity about 115 times greater than that of the crude extract (Table 1, fig. S4, and Supplementary Text). Mass spectrometry (MS) analyses demonstrated that the peptide fragment pattern of the purified *Parapriacanthus* luciferase matched exactly to the luciferase of luminous ostracod, *Cypridina noctiluca*, with coverage of 41% of the full length (Fig. 2D). Western blot analysis with anti-cypridinid luciferase polyclonal antibody detected an immunoreactive band corresponding to the size of cypridinid luciferase (ca. 62 kDa) in extracts from both thoracic and anal light organs but not in extracts from the pyloric caeca, intestine, or muscle (Fig. 2C), coinciding with the distribution of luciferase enzymatic activity (Fig. 2B). Immunohistochemistry showed positive reactions in the lateral cell membrane and in the cytoplasm of the light organ cells (Fig. 3 and fig. S5). These results suggest that *Parapriacanthus* uses luciferase protein virtually identical to that of *C. noctiluca*.

To test whether the ostracod luciferase gene is encoded in the fish genome, we examined the presence/absence of the ostracod luciferase-like gene and transcripts. RNA sequencing (RNA-seq) analyses showed no transcripts corresponding to the ostracod luciferase in thoracic and anal light organs or other tissues of *Parapriacanthus*. The most similar transcript to the ostracod luciferase in our RNA-seq data had homology to the zonadhesin-like protein of the nonluminous fish *Seriola lalandi* (*e* value, 5.66×10^{-11}); in vitro translation assays using mRNA from the light organs by either wheat germ or rabbit reticulocyte extracts failed to produce any protein with luciferase activity (fig. S6). Polymerase chain reaction (PCR) analyses using gene-specific primers failed to amplify the ostracod luciferase from the *Parapriacanthus* genomic DNA (fig. S7). These results suggest that horizontal gene transfer of the luciferase gene from *Cypridina* to *Parapriacanthus* is unlikely.

To test the hypothesis that *Parapriacanthus* acquires exogenous protein, we performed long-term feeding experiments. The luciferase activity of *Parapriacanthus* specimens decreased after being kept for several months in aquaria while being fed nonluminous fish meat. Subsequent feeding with *V. hilgendorfii*, not *C. noctiluca*, for several weeks resulted in the recovery of luciferase activity in the light organs (Fig. 4A). MS analysis of the immuno-pull-down fraction using the anti-cypridinid luciferase antibody (fig. S8) showed the presence of peptide fragments unique to *V. hilgendorfii* luciferase in the light organs of the fish specimens after feeding with *V. hilgendorfii* (Fig. 4, B and C). This incorporation study using identifiably *Vargula*-derived, not *Cypridina*-derived, luciferase demonstrated the presence of a foreign protein uptake system in *Parapriacanthus*.

Enzyme stability of ostracod luciferases was tested. The purified native *V. hilgendorfii* luciferase and recombinant *C. noctiluca* luciferase were stubbornly resistant to the treatment of proteases, heat, or urea, but the activity was lost in the presence of the reductant dithiothreitol (DTT) (fig. S9).

DISCUSSION

We observed the bioluminescence of *Parapriacanthus* from a living specimen and confirmed a cross L-L luminescence reaction between

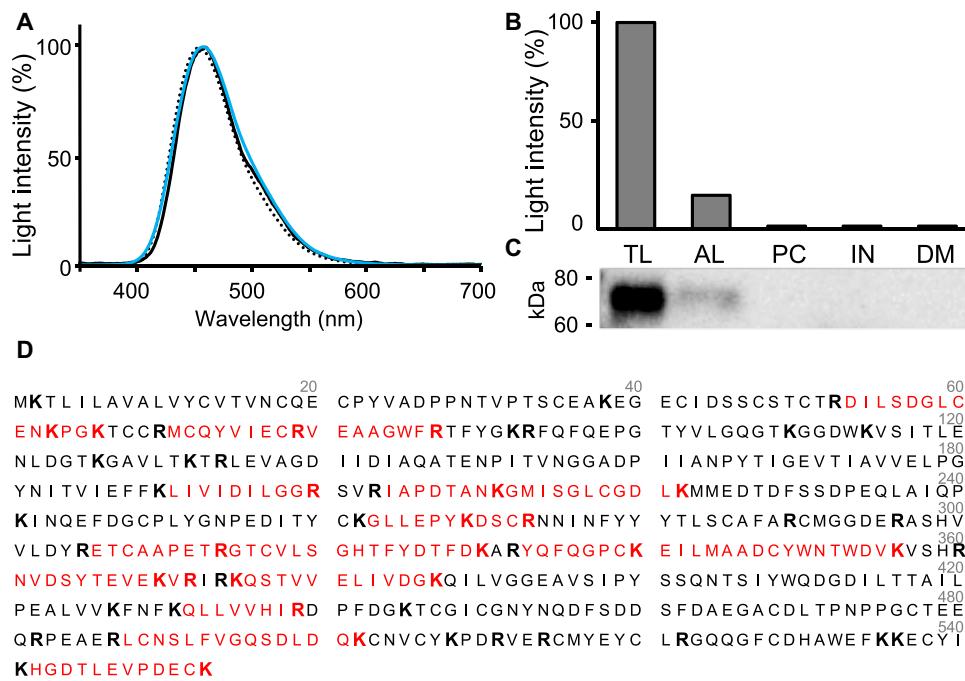


Fig. 2. Properties and identification of the *P. ransonneti* luciferase. (A) Bioluminescence spectra of *V. hilgendorfii* (dotted line) from the dissected thoracic light organ of *Parapriacanthus* (solid black line) and of in vitro L-L reaction using *Parapriacanthus* luciferase and vargulin (solid blue line). (B) Distribution of luciferase activity. PC, pyloric caeca; IN, intestine; DM, dorsal muscle. (C) Western blot using anti-cypridinid luciferase antibody. The relative molecular weight of the band detected in TL and AL corresponds to that of *V. hilgendorfii* luciferase. (D) Peptide fragments of the purified *Parapriacanthus* luciferase detected by quadrupole orthogonal acceleration-time-of-flight tandem mass spectrometry (qTOF-MS/MS) mapped onto the amino acid sequence of *C. noctiluca* luciferase (red). Theoretical cleavage positions by trypsin and lysyl endopeptidase, lysine (K) and arginine (R), are shown in bold.

Table 1. Purification of *Parapriacanthus* luciferase. Protein was calculated by the extract volume and concentration as determined by absorbance at 280 nm. rlu, relative light unit; HPLC, high-performance liquid chromatography.

Purification method	Activity (rlu)	Protein (mg)	Specific activity (rlu/mg)	Purity (fold)
Crude extract	19714076	261.20	75475	1
HiTrap Q	13280465	2.35	5651262	75
Sephadex G-75	12062759	1.82	6646148	88
HPLC Mono Q 1 (fractions 21-27)	1718422	0.17	10231190	136
HPLC Mono Q 2 (fractions 35-42)	209020	0.02	8709167	115

the fish and the ostracod. The purified luciferase from the light organ of *Parapriacanthus* was identified as *C. noctiluca* luciferase by MS analysis (Fig. 2). Transcriptome, genomic PCR, and in vitro translation analyses suggested that the luciferase gene is unlikely to be encoded in the fish genome. Feeding experiments determined that the luciferase is of dietary origin. Together, we demonstrated that the *Parapriacanthus* luciferase in the light organ is supplied from dietary ostracods. This is the first example, to our knowledge, not

only of the sequestration and reuse of an enzyme from a dietary source, but also of this mechanism leading to an evolutionary novelty, bioluminescence. This “stolen protein” can be compared to kleptoplastids (7–9) and kleptocnidae (10). By analogy with these phenomena, we propose the term “kleptoprotein” for the phenomenon that we found in this study.

Kleptoprotein in bioluminescent fish was unexpected because ingested proteins are usually decomposed in digestive systems, including pyloric caeca in fishes, into amino acids or oligopeptides and absorbed through the gut wall as nutrients. This means that the original structure and enzymatic activity of ingested proteins are typically not maintained during their passage through digestive tracts. However, the phenomenon of protein uptake without full digestion has been reported in some vertebrate immune systems. For example, M cells in mammalian intestinal epithelia play an important role for the immune system by taking macromolecules or even microbes into the cell as antigens via pinocytosis (24). Similar antigen-sampling functions were reported in the intestinal epithelium of cyprinid fishes, and the presence of M cell-like cells was reported in the intestine of a salmonid fish (25–27). While this mechanism for protein uptake is not selective and the protein is not used for its original function, these immune systems may have been co-opted to serve a kleptoprotein role in *Parapriacanthus* bioluminescence during evolution. We expect that the reason why ostracod luciferase was exploited for kleptoprotein bioluminescence of *Parapriacanthus* is due partly to its high proteolytic resistance and highly stable nature (fig. S9). Kleptoprotein bioluminescence using the ostracod luciferase might have also evolved in parallel in some

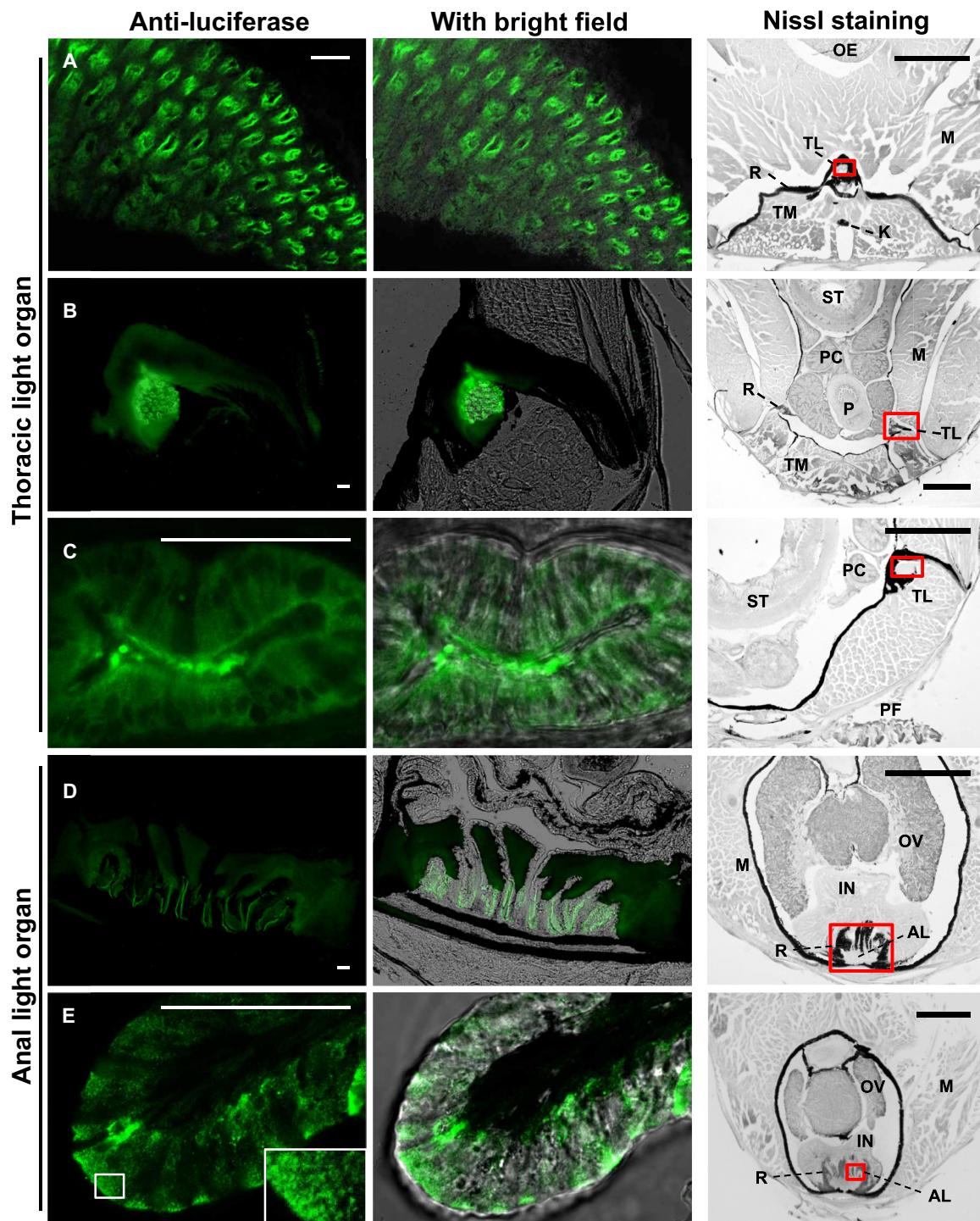


Fig. 3. Localization of luciferase in the thoracic and anal light organs. The levels of sections in (A) to (E) correspond to the positions a to e shown in Fig. 1B. Left: Fluorescent signals of anti-cypridinid luciferase antibody labeled by Alexa Fluor 488 (A, C, and E) or fluorescein (B and D). Scale bars, 100 μ m. Middle: Merged images of fluorescent and phase contrast microscopic images. Right: Nissl-stained sections. Scale bars, 1 mm. The left and middle panels correspond to the red boxes in the right panel. Light organs are surrounded by reflectors (thick black area in the bright field). (A to C) The thoracic light organ is composed of tubular structures with the luciferase signal. The number of tubes decreases from anterior to posterior, but the diameter of each becomes larger (A to C). Luciferase is detected on the epidermal cell surface and in the cytosol. (D) The anal light organ has villi-like structures and is separated by a reflector from the intestine. The luciferase signal is primarily on the epidermal cell surface. (E) Granular substructures with the luciferase signals are spread in the cytosol of the epidermal cells in the villi. K, keel; M, muscle; TM, translucent muscle; OE, oesophagus; OV, ovary; P, pylorus; PF, pelvic fin; R, reflector; ST, stomach.

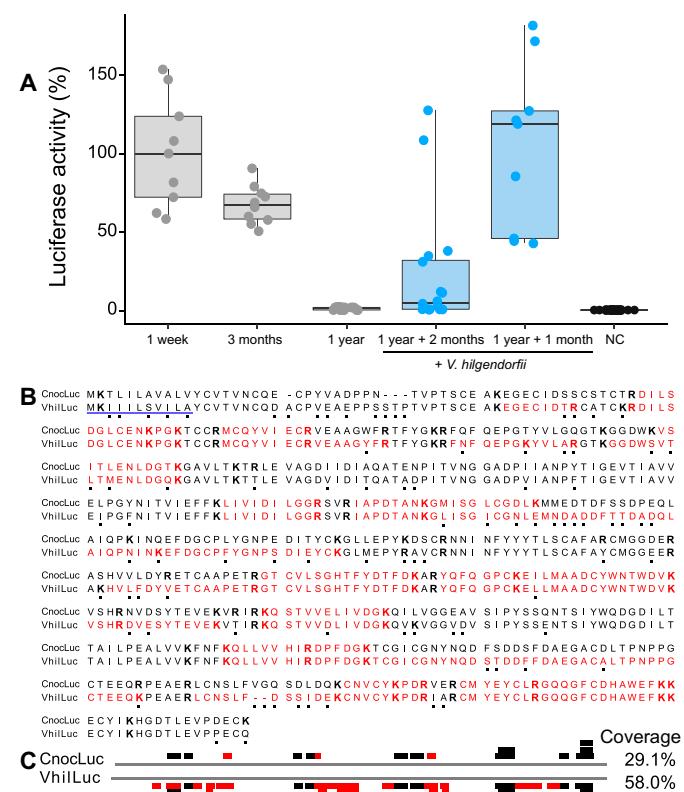


Fig. 4. *Vargula* feeding experiment. (A) The values are given in percent relative to the mean of the original luciferase activities of extracts from freshly caught fish (1 week). Activity of specimens fed with nonluminous fish for 1 week, 3 months, and 1 year (gray) declined and subsequently increased when fed with *V. hilgendorfii* for 2 weeks (1 year + 2 weeks) and 1 month (1 year + 1 month) (blue). NC, no enzyme control. (B) Peptides detected by qTOF-MS/MS are highlighted in red on the amino acids of *C. noctiluca* luciferase (CnocLuc) and *V. hilgendorfii* luciferase (VhilLuc). It can be seen that in varying regions, peptide fragments matched VhilLuc, indicating a dietary source. The amino acid identity between CnocLuc and VhilLuc is 84%. The disagreements of amino acid residues between two luciferases were indicated by dots under the alignment. Theoretical cleavage positions by protease treatment are shown in bold. Putative signal peptide at the N terminus (33) is indicated by the blue underline. (C) Schematic view of the detected peptides. Full-length luciferase sequences are indicated by gray lines. The peptides that are common to both luciferases are shown by bold black lines. Peptides specific to either luciferase are shown as bold red lines.

other teleost lineages, e.g., the apogonid fishes *Jaydia ellioti* and *Rhabdamia cypselura*, which use vargulin for their luminescence and whose light organs are connected to the digestive tract (16, 17), as in *Parapriacanthus*.

In this study, we have demonstrated that the shallow-water luminous fish *P. ransonneti* uses an ostracod luciferase acquired from its prey *C. noctiluca* for its bioluminescence. The use of kleptoproteins is a novel category of bioluminescence, adding a third process to the two other known bioluminescence types, which generate light using symbiotic bacteria or endogenous luciferase, respectively. Our results suggest the possibility that kleptoproteins might be found in biological processes other than bioluminescence, and thus serve as another means of evolutionary innovation alongside canonical genome-based molecular evolution.

MATERIALS AND METHODS

Ethics statement

All the animal work was performed according to the guidelines of the Regulations of Animal Experiments of Chubu University (approval number: 2910075) and Institutional Animal Care and Use Committee guidelines.

In situ bioluminescence of *P. ransonneti*

The bioluminescence of the living *P. ransonneti* was observed. Less than 2 weeks after being caught in the wild, the *P. ransonneti* specimens were gently transferred into a transparent tank. The specimen was kept in the dark for an hour at 23°C before bioluminescence observation. To evoke the light emission, a dim white light-emitting diode covered with paper to diffuse the light was shone above the tank.

L-L reaction

Luciferase activities were measured as follows unless otherwise specified. The light intensity was measured by using a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany) for 20 s at 0.5-s intervals after the injection of 90 μ l of luciferin solution. Ten microliters of fish or ostracod luciferase solution was applied for a 96-well plate. The luciferin solution was freshly prepared on the day of use as follows. Approximately 10 to 20 individuals of freeze-dried *V. hilgendorfii* were homogenized in 200 μ l of 0.5 M HCl in 95% ethanol, followed by centrifugation at 4°C for 10 min at 15,000g. The supernatant was filtered using a 0.45- μ m membrane filter (MilliporeSigma). The filtrate was diluted 1000-fold with 20 mM tris-HCl (pH 8.0) and used as luciferin solution.

To determine the tissue distribution of luciferase activity, crude luciferase extracts were prepared as follows. The living fish specimen was anesthetized on ice for the dissection. The dissected thoracic and anal light organs, pyloric caeca, intestine, and dorsal muscle were washed with 300 μ l of deionized water and homogenized with 200 μ l per tissue of extraction buffer [20 mM tris-HCl, 50 mM NaCl, and 1:200 protease inhibitor cocktail III (pH 8) (MilliporeSigma)], followed by centrifugation at 4°C for 10 min at 15,000g. The pellet was suspended with the same buffer to extract additional protein, and the homogenate was centrifuged again. The supernatant was combined and was filtered using a 0.20- μ m membrane filter (DISMIC-AS25; Advantec Toyo Kaisa, Tokyo).

The L-L reaction for the firefly luminescence system was performed as previously described (28, 29). Briefly, 10 μ l of a solution (5 μ g/ml) of purified firefly *Aquatica lateralis* Luc1-type luciferase (FLuc) was added to 90 μ l of firefly luciferin solution [20 μ M D-luciferin, 100 μ M adenosine triphosphate (ATP), and 10 mM MgSO₄ in 20 mM tris-HCl (pH 8.2)]. The luminescence activity was measured by using a Centro LB 960 luminometer for 20 s in 0.5-s intervals after injection of the luciferin solution.

Measurement of bioluminescence spectra

A single specimen of *V. hilgendorfii* was placed in a quartz cuvette containing seawater. In this process, the specimen was physically stimulated and discharged a luminous cloud, and the luminescence spectrum was immediately measured using an FP-777 W fluorescence spectrophotometer (JASCO, Tokyo) with the excitation light source turned off. The data of five biological replicates were normalized and averaged. Thoracic and anal light organs were dissected from a single living *P. ransonneti* specimen. These tissues emitted continuous

light, and the luminescence spectrum was measured using the same method as described above.

For cross-reaction tests, crude luciferase extracts were prepared by homogenization of the thoracic/anal light organ of a single specimen of *P. ransonneti* or five whole-body specimens of *V. hilgendorfii* in 200 μ l of 20 mM tris-HCl and 50 mM NaCl (pH 8), followed by centrifugation at 4°C for 10 min at 15,000g, and the supernatant was filtered using a 0.45- μ m membrane filter (Advantec Toyo Kaisha). Fish luciferin was extracted by homogenizing the pyloric caeca of a single specimen of *P. ransonneti* in 200 μ l of 0.5 M HCl in 95% ethanol, followed by centrifugation at 4°C for 10 min at 15,000g, and the supernatant was filtered using a 0.45- μ m membrane filter (MilliporeSigma). The luciferin from *V. hilgendorfii* was prepared as described above (see the “L-L reaction” section). The L-L reaction was initiated by the addition of 10 μ l of luciferin extract into 200 μ l of luciferase extract. The luminescence spectrum of each reciprocal cross-reaction between the crude protein extracts and luciferins was immediately measured using an FP-777 W fluorescence spectrophotometer with the excitation light source turned off. The measured data of three experimental replications were normalized and averaged.

Protein extraction and purification from *P. ransonneti*

For protein purifications, crude luciferase extract was prepared from the anesthetized fish specimen. The dissected thoracic light organ was washed with 300 μ l of deionized water and homogenized with 200 μ l of extraction buffer [20 mM tris-HCl, 50 mM NaCl, and 1:200 protease inhibitor cocktail III (pH 8) (MilliporeSigma)] per specimen, followed by centrifugation at 4°C for 10 min at 15,000g. The pellet was suspended with the same buffer to extract additional protein, and the homogenate was centrifuged again. The supernatant was combined and was filtered using a 0.20- μ m membrane filter (Advantec Toyo Kaisha).

The filtered extract from thoracic light organs from 200 specimens was adsorbed on a 1-ml HiTrap Q HP (GE Healthcare, Fairfield, CT) anion exchange column equilibrated with 20 mM tris-HCl (pH 8.0) at 4°C. The proteins were eluted in a stepwise fashion with buffers containing 0.25, 0.40, and 2.0 M NaCl. Luciferase activity and absorption at 225 and 280 nm were measured for each fraction (500 μ l). A 10- μ l aliquot of each fraction was used to measure the luciferase activity. The fractions containing luciferase activity (fraction numbers 6 to 8) were combined and further separated by gel filtration using Sephadex G-75 (ϕ , 15 mm by 155 mm; the bed volume is 27.5 ml). The separation buffer [20 mM tris-HCl and 0.15 M NaCl (pH 8.0)] was pumped at 1.0 ml/min using a Perista Pump (ATTO, Tokyo) peristaltic pump. Vargulin was added to a 10- μ l aliquot of each fraction (1.5 ml) to measure luciferase activity. The active fractions (fraction numbers 7 to 12) were concentrated by anion exchange chromatography using a HiTrap Q HP column as described above. The buffer in the concentrated active fraction was exchanged for 20 mM MES-NaOH at pH 6.0 using PD-10 (GE Healthcare). The resulting solution was further subjected to high-performance liquid chromatography (HPLC) on a SMART System using a Mono Q anion exchange column (Pharmacia), and the absorbance at 280 nm indicating the protein concentration was monitored using a μ Peak Monitor (Pharmacia). The adsorbed protein was eluted by changing the NaCl concentration in the MES-NaOH buffer under the following gradient conditions: 0.0 to 10.0 min (0 to 300 mM NaCl), 10.0 to 15.0 min (300 mM), and 15.0 to

57.0 min (300 to 500 mM) at a flow rate of 100 μ l/min. For each fraction, 100 μ l of eluent was collected. This experiment resulted in three active peaks, and we then separated the second peak by HPLC. The second peak was combined and separated again with the Mono Q column under the following gradient condition: 0.0 to 5.0 min (100 to 200 mM NaCl), 5.0 to 50.0 min (200 to 400 mM), and 50.0 to 60.0 min (500 mM) at a flow rate 100 μ l/min. For each fraction, 100 μ l of eluent was collected.

SDS-polyacrylamide gel electrophoresis

Protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) using 10, 12.5, 15, or 4 to 12% gradient polyacrylamide gel (ATTO) under reducing conditions. A one-fourth volume of 5 \times SDS-sample buffer [250 mM tris-HCl, 10% SDS, 100 mM DTT, 50% glycerol, and a trace of bromophenol blue at (pH 6.8)] was added to the protein sample and heated at 70°C for 10 min. For the MS analysis, 1 μ l of 1% acrylamide monomer was added to the samples after heating to form a propionamide group on the reduced free sulfide of Cys residues. After electrophoresis, protein bands were visualized by Coomassie brilliant blue staining or a Silver Stain MS kit (Wako Pure Chemical, Osaka, Japan). For mass spectrometric sequencing, the excised band from the gel was cut and destained, followed by drying.

Protein identification by MS

The purified protein was digested, and the resulting peptides were analyzed using MS for identification by searching against protein databases. The protein in gel or solution was digested with Trypsin Gold (10 ng/ μ l; Promega, Madison, WI, USA), lysyl endopeptidase (2.5 ng/ μ l; Wako), and 0.01% MAX surfactant (Promega) in 50 mM ammonium bicarbonate for 1 hour at 50°C. The digestion reaction was terminated by acidification (lowering the pH below 3) with trifluoroacetic acid. The peptides were further purified with GL-Tip SDB (GL Sciences, Tokyo). The peptides were analyzed by LC-MS/MS (tandem MS) by a TripleTOF 5600+ mass spectrometer (SCIEX, Concord, Canada), essentially as described previously (30). MS/MS spectra were interpreted, and peak lists were generated using Mascot Server version 2.4.0 (Matrix Science, Boston, MA, USA). Searches were performed by using the SEQUEST algorithm against the in-house build *P. ransonneti* protein database (see the “Open reading frame prediction and building a proteome database for MS sequence analysis” section) and the public National Center for Biotechnology Information nonredundant protein database (NCBInr). Search parameters were the following: enzyme selected as used with two maximum missing cleavage sites, a mass tolerance of 45 parts per million for peptide tolerance, 0.1 Da for MS/MS tolerance, fixed modification of propionamide (C), and variable modification of oxidation (M). The maximum expectation value for accepting individual peptide ion scores [$-10^{\ast}\log(p)$] was set to ≤ 0.05 , where p is the probability that an observed match is a random event. Protein identification and modification information returned from Mascot were manually inspected and filtered to obtain confirmed protein identification and modification lists of collision-induced dissociation MS/MS.

Cloning of the luciferase gene from *C. noctiluca*

Total RNA was extracted from a single specimen of *C. noctiluca* using TRIzol (Invitrogen) according to the manufacturer’s instruction. To determine the 5' and 3' ends of the cDNAs, the rapid amplification of cDNA ends (RACE) technique was carried out using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) with

gene-specific primers designed on the basis of the internal fragment sequences described previously (31). The complete coding sequence (CDS) of the *C. noctiluca* luciferase (*CnocLuc*) was amplified using a high-fidelity PrimeSTAR Max DNA Polymerase (Takara, Shiga, Japan) with gene-specific primer sets CnLuc-f_5UTR (5'-GATCATCCCG-GTGTGATCCAC-3') and CnLuc-r_3UTR (5'-CTTCTTGGTTCAAT-GAATGC-3'), designed on the basis of the sequences of the noncoding region of the 5' and 3'ends. PCR conditions to amplify *CnocLuc* CDS were the following: an initial 2.0 min at 96°C, followed by 30 cycles of 10 s at 98°C, 10 s at 52°C, and 40 s at 72°C, with a final extension of 5.0 min at 72°C. The nucleotide sequence was determined using a BigDye Terminator kit (Thermo Fisher Scientific, Waltham, MA, USA) and an ABI PRISM 3130 genetic analyzer (Thermo Fisher Scientific). The GenBank accession number of the transcript is LC427371.

Heterologous expression of *CnocLuc* and purification

The full CDS of *CnocLuc* excluding the predicted signal sequence at the N terminus end was amplified by PCR using PrimeSTAR Max DNA Polymerase with specific primers CnLas-InPaA-f (5'-TCGG-TACCTCTGAGCCGCGGATACTGCGTCAGTAACT-GTC-3') and CnL-InPaA-r (5'-GAGTTTTGTTCTAGATGTTG-CATTCTGACTTCTAG-3'). PCR conditions to amplify *CnocLuc* CDS were the following: an initial 2.0 min at 96°C, followed by 30 cycles of 10 s at 98°C, 10 s at 52°C, and 40 s at 72°C, with a final extension of 5.0 min at 72°C. The amplicon of *CnocLuc* was inserted into the pPICZαA expression vector using the Gibson assembly system (New England Biolabs, Ipswich, MA, USA) to give the plasmid pPICZαA-CnLas. The plasmids were sequenced to confirm that no undesired mutations were generated during the cloning processes. The expression plasmids were linearized using Pme I restriction enzyme and transformed into the wild-type *Pichia pastoris* X-33 strain (Invitrogen) to give pPICZαA-CnLas/X-33. The electroporation was performed in a 2-mm-gap cuvette at 1.5 kV, 25 μF, and 400 ohm for 6.5 ms using a Gene Pulser (Bio-Rad). Immediately after cells were pulsed, 1 ml of ice-cold 1 M sorbitol was added to the cuvette. Transformants were screened for a phenotype on minimal dextrose medium (13.4 g of yeast nitrogen base, 20 g of dextrose, and 0.4 mg of biotin per liter of medium) agar plates and colony PCR. The transformants were grown for 21 hours in 10 ml of buffered complex glycerol medium (BMGY) broth [0.1 M potassium phosphate, 2% peptone, 1% yeast extract, 1% (v/v) glycerol, and 1.34% yeast nitrogen base (pH 6.0) (Invitrogen)] containing Zeocin (100 μg/ml; Invitrogen) at 37°C with an agitation speed of 250 rpm. Yeast cells were collected by centrifugation at 4°C for 5 min at 2000g. Pellet cells were suspended with 400 ml of buffered complex methanol medium (BMMY) broth [0.1 M potassium phosphate, 2% peptone, 1% yeast extract, 0.5% methanol, and 1.34% yeast nitrogen base (pH 6.0)] and cultured for 3 days at 16°C in a shaking incubator (250 rpm). Every 24 hours, methanol was added to the medium to a final concentration of 0.5% methanol. The culture medium was centrifuged at room temperature for 10 min at 630g. The supernatant (400 ml) was neutralized with 4.84 g of 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris base) and filtered using a 0.45-μm membrane filter. The expressed protein secreted in the medium was adsorbed on a 20-ml Ni-chelating column at 4°C. *CnocLuc* was purified by changing the concentration of imidazole in 20 mM phosphate-buffered saline (PBS) (pH 7.5). The purified *CnocLuc* was desalting using a PD-10 (GE Healthcare) column and concentrated by anion exchange chromatography using a 1-ml HiTrap Q HP anion exchange column

as described above. Briefly, the luciferase-binding column was washed with 50 mM NaCl and then eluted with 250 mM NaCl buffer. The luciferase activity was assayed after fractionation as described above.

Purification of luciferase from *V. hilgendorfii*

The native *V. hilgendorfii* luciferase (VhilLuc) was purified from wild-caught *V. hilgendorfii* based on the report of McElroy and Chase (32). Ten grams of freeze-dried *V. hilgendorfii* was homogenized with an ice-cooled motor. The luciferase was extracted twice with 100 ml of extraction buffer [20 mM PBS, 0.1 M NaCl, and 0.05% Tween 20 (pH 6.0)], followed by centrifugation at 4°C for 10 min at 15,000g. Cold acetone was slowly added to the supernatant to a final concentration of 30% and mixed using a magnetic stirrer at 4°C for 5 hours. The resulting precipitate was discarded after centrifugation (4°C, 10 min, 20,000g). Additional cold acetone was slowly added to the supernatant to raise the concentration to 60% and mixed at 4°C for 10 hours. The resulting soluble proteins were removed by centrifugation (4°C, 10 min, 20,000g). Further fractionation was carried out with (NH₄)₂SO₄. The acetone precipitation was suspended with 90 ml of 0.1 M PBS (pH 7.5), and 24.3 g of solid (NH₄)₂SO₄ was slowly added to 40% saturation. After incubation at 4°C for 3 hours, the precipitation was removed by centrifugation and discarded. An additional 14.7 g of solid (NH₄)₂SO₄ was slowly added to raise the concentration to 60%. The mixture was incubated at 4°C for 18 hours, and the precipitate was collected by centrifugation at 4°C for 10 min at 20,000g. The precipitate was dissolved in 8 ml of deionized water and dialyzed against 20 mM PBS (pH 6.0). Further purification was carried out with a HiTrap Q HP column as described above. Briefly, the luciferase-binding column was washed with 50 mM NaCl and then eluted with 250 mM NaCl buffer. The luciferase activity was assayed after fractionation as described above.

Preparation of anti-cypridinid luciferase antibody

Rabbit polyclonal anti-cypridinid luciferase antibody was raised against the purified recombinant *CnocLuc* (see the “Heterologous expression of *CnocLuc* and purification” section) by Eurofins Genomics K.K. (Tokyo). This antibody was affinity-purified with the recombinant *CnocLuc* by Eurofins Genomics K.K. To improve the specificity of the immunological reaction, the antibody was further purified by using a 1-ml HiTrap NHS-activated HP column (GE Healthcare) coupled with the purified native VhilLuc (see the “Purification of luciferase from *V. hilgendorfii*” section). The purified antibody was mixed with glycerol to a final concentration of 40% (v/v) and stored at -25°C.

Western blotting

Western blotting analysis was performed as described previously (28). Proteins in the crude extracts were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (MilliporeSigma). The membrane was incubated in blocking buffer {5% skimmed milk in TBS-T [20 mM tris-HCl (pH 8.0), 137 mM NaCl, and 2.7 mM KCl]} and probed with the primary antibody, anti-cypridinid luciferase antibody (0.7 μg/ml), followed by horseradish peroxidase-linked anti-rabbit immunoglobulin G (IgG) mouse secondary antibody (dilution: 1:4000). The immunological reaction was visualized by the addition of ImmunoStar LD (Wako) and detected by a cooled charge-coupled device camera (LAS-1000, Fujifilm, Tokyo).

Feeding experiment of *V. hilgendorfii*

P. ransonneti fishes kept for 1 year in the Shima Marineland aquarium were transferred into an aquarium tank at the university laboratory and used for feeding experiments. In the Shima Marineland aquarium, the fish were fed sliced nonluminous fishes such as mackerel, horse mackerel, and sardines but not luminous ostracods. In the tank in the university laboratory, *P. ransonneti* was fed with thawed frozen krill and/or *V. hilgendorfii* between 18:00 and 24:00. Sunlight and room light were cut using black vinyl boards or filtered using red transparent plastic boards.

The crude luciferase solution was extracted from fish specimens kept under five conditions: in an aquarium for less than 1 week after capture, in an aquarium for ~3 months or for more than 1 year without feeding of ostracods, or in an aquarium without ostracod feeding for 1 year then feeding *V. hilgendorfii* for 2 weeks or for 1 month. The dissected thoracic and anal light organs were homogenized in the extraction buffer [20 mM tris-HCl, 50 mM NaCl, and 1:200 protease inhibitor cocktail III (pH 8.0) (Novagen)], followed by centrifugation at 4°C for 10 min at 10,000g. The supernatant was used for the L-L reaction and immunoprecipitation and pull-down assay.

To determine the amino acid sequences of luciferase, the protein was affinity-purified using Protein G Mag Sepharose (GE Healthcare) with anti-cypridinid luciferase antibody from the *P. ransonneti* specimen after feeding of *V. hilgendorfii* for 2 weeks or 1 month. The 200-μl crude extract from light organs was incubated with 200 μl of gel suspension containing 2.5 μl of affinity gel for 12 hours at 4°C. The gel was washed three times with 1 ml of 20 mM tris-HCl and 50 mM NaCl (pH 8.0) and then incubated at 70°C for 10 min with 15 μl of 1× SDS sample buffer to elute adsorbed protein. The immunoprecipitation and pull-down samples were analyzed by SDS-PAGE and MS.

Histology

For immunohistochemical analysis of the luciferase, the fish specimens were anesthetized with ethyl 3-aminobenzoate methanesulfonate salt and fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M PBS at pH 7.4. The sample was decalcified with 0.5 M EDTA (pH 8.0) for a week and then transferred to PBS (pH 7.4). The concentration of sucrose in the PBS was gradually increased up to 20% for a week. Preparations were then embedded in low-melting point agarose (MilliporeSigma) and frozen in *n*-hexane at -80°C. The prepared block was cut with a cryostat (section thickness, 20 μm). Sections were stained with cresyl violet (Nissl staining) or processed for immunohistochemistry.

The sections on slides were washed with PBS and incubated in 1% normal goat serum in 0.1 M PBS containing 0.03% Tween 20 (PBST). The anti-cypridinid luciferase antibody (0.5 μg/ml) in PBST was reacted with the sections at 4°C overnight (8 to 12 hours), subsequent to washes in PBST (once) and PBS (twice). For fluorescence microscopic observation, the washed sections were reacted with biotinylated secondary monoclonal anti-rabbit IgG antibody (200 times diluted) produced in mouse (VECTASTAIN Elite ABC kits; Vector Laboratories) in PBST, subsequent to washes in PBST (once) and PBS (twice). The washed sections were reacted with 1% fluorescence probe solutions (aminomethylcoumarin-, fluorescein-, or Texas Red-conjugated streptavidin; Vector Laboratories, Peterborough, UK) in PBST and mounted with 1,4-diazabicyclo[2.2.2]octane in 10% polyvinyl alcohol. For confocal laser microscopic observation, washed sections after primary antibody reaction were reacted with anti-rabbit IgG antibody produced in mouse conjugated with Alexa Fluor 488

and mounted with Dako fluorescence mounting medium (Dako, Glostrup, Copenhagen, Denmark).

Treatment of luciferase with urea

Effects of urea treatment on the purified native VhilLuc, purified recombinant CnocLuc, and recombinant firefly luciferase FLuc [Luc1-type luciferase cloned from Japanese firefly *A. lateralis* (28)] were examined. Fifty nanograms of the luciferases in 20 mM tris-HCl containing 0, 2.0, 4.0, or 6.0 M urea were incubated at 30°C for 5 min. After the incubation, the ostracod luciferase activities were assayed using vargulin; ten microliters of treated luciferase solution was added to 90 μl of vargulin in 20 mM tris-HCl (pH 8.2), as described above. Ten microliters of the treated firefly luciferase was added to 90 μl of firefly luciferin solution [20 μM d-luciferin, 100 μM ATP, and 10 mM MgSO₄ in 20 mM tri-HCl (pH 8.2)]. The luminescence activity was measured by using a Centro LB 960 luminometer for 20 s in 0.5-s intervals after the luciferin solution injection. Three experimental replicates were performed.

Stability assay of luciferase at different temperatures

Heat stability of the VhilLuc, CnocLuc, and FLuc was examined. Fifty nanograms of the luciferases in 20 mM tris-HCl were incubated on ice or at 30° or 50°C for 30 min. After the incubation, the ostracod luciferase activities were assayed using vargulin; ten microliters of treated luciferase (50 ng) solution was added to 90 μl of vargulin in 20 mM tris-HCl (pH 8.2), as described above. Ten microliters of the treated firefly luciferase was added to 90 μl of firefly luciferin solution [20 μM d-luciferin, 100 μM ATP, and 10 mM MgSO₄ in 20 mM tris-HCl (pH 8.2)]. Luminescence activity was measured by using a Centro LB 960 luminometer for 20 s in 0.5-s intervals after the luciferin solution injection. Three experimental replicates were performed.

Treatment of luciferase with DTT

Effects of the reducing reagent DTT on the VhilLuc, CnocLuc, and FLuc were examined. Fifty nanograms of the luciferases in 20 mM tris-HCl containing 0 or 50 mM DTT were incubated at 30°C for 5 min. After the incubation, the ostracod luciferase activities were assayed using vargulin; ten microliters of treated luciferase solution was added to 90 μl of vargulin in 20 mM tris-HCl (pH 8.2), as described above. Ten microliters of the treated firefly luciferase was added to 90 μl of firefly luciferin solution. The luminescence activity was measured by using a Centro LB 960 luminometer for 20 s in 0.5-s intervals after the luciferin solution injection. Three experimental replicates were performed.

Digestive tolerance assay of luciferase

The digestive tolerance of luciferases was evaluated. Five hundred nanograms of CnocLuc, VhilLuc, and FLuc was digested with Proteinase K (QIAGEN, Hilden, Germany) or Trypsin Gold (Promega). One hundred microliters of the digestive reaction mixture was composed of 500 ng of luciferase in 20 mM tris-HCl (pH 8.2) with a protease: 600 μAU (activity unit) of Proteinase K or 200 ng of Trypsin Gold. The reaction mixture was incubated for 1 hour at 30°C. After the digestion reaction, luciferase activities were assayed using vargulin; two microliters of luciferase was added to 98 μl of vargulin in 20 mM tris-HCl (pH 8.2), as described above. Ten microliters of the digested firefly luciferase was added to 90 μl of firefly luciferin solution. The luminescence activity was measured using a Centro LB 960 luminometer for 20 s in 0.5-s intervals after the luciferin solution injection.

RNA extraction, library preparation, and sequencing

RNA-seq libraries were prepared from five different tissues (thoracic light organ, anal light organ, pyloric caeca, intestine, and dorsal muscle) of *P. ransonneti* and the whole bodies of five individuals of *V. hilgendorfii* (table S1). Total RNA was extracted from the dissected tissues using TRIzol (Invitrogen) with deoxyribonuclease (QIAGEN) in solution and then cleaned using the RNeasy Mini Kit (QIAGEN). The cDNA libraries were generated from the total RNA (500 ng from each sample) using a TruSeq RNA Sample Preparation Kit v2 (Illumina) according to the manufacturer's protocol (low-throughput protocol), except that all reactions were carried out at half scale. The fragmentation of mRNA was performed for 4 min. The enrichment PCR was done for 6 cycles. A subset of six libraries (thoracic light organ, anal light organ, pyloric caeca, intestine, dorsal muscle, and *V. hilgendorfii*; table S1) was multiplexed and sequenced in a single lane of HiSeq 1500 101 × 101-base pair (bp) paired-end reads. The sequence quality was inspected by FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

De novo transcriptome assembly

To build a comprehensive set of reference transcript sequences, reads derived from the five libraries (thoracic light organ, anal light organ, pyloric caeca, intestine, and dorsal muscle; table S2.6.1) were pooled and used for de novo assembly. Illumina reads were cleaned with Cutadapt (v1.0): Low-quality ends (<QV30) and adapter sequences were trimmed, while reads shorter than 50 bp were discarded. The cleaned reads were assembled de novo with Trinity (version R2013-02-25) in the paired-end mode (parameters: -min_kmer_cov = 1).

Open reading frame prediction and building a proteome database for MS sequence analysis

Open reading frames (ORFs) were extracted from the Trinity contigs (see above) by using a custom pipeline OkORF (<https://github.com/shujishigenobu/OkORF>). We generated two sets of ORFs, set A and B, with different filtering criteria. Set A is composed of 71,654 predicted ORFs filtering out low-quality models, while set B is composed of 294,515 predicted ORFs capturing all possible ORFs without quality filtration. To build a comprehensive proteome database for MS analysis, we chose set B as reference sequences, allowing false-positive ORF prediction, to maximize the sensitivity in peptide identification by MS. Partial ORFs (i.e., start codon or stop codon missing) were allowed. We added pig trypsin and human keratin to the reference proteome database as common contaminants. Anal and thoracic light organs from 6 specimens were dissected and were used for total RNA extraction using TRIzol (Invitrogen). Messenger RNA were purified from total RNA using Oligo-dT30 super mRNA purification kit (Takara). Cell-free in vitro protein expression was performed according to the manufacturer's instruction. For Wheat Germ Extract (Promega), 20 µg/ml for anal light organ, 10 µg/ml for thoracic light organ, and 10 µg/ml for firefly luciferase control of mRNA at a final concentration in the 20 µl of reaction mix were translated at 25°C for 2 h. For Rabbit Reticulocyte Lysate System (Promega), 4 µg/ml of mRNA at a final concentration in the 20 µl of reaction mix were translated at 30°C for 90 min.

Genomic DNA extraction, amplification, and sequencing

Genomic DNA was prepared from 20.0 mg of dorsal muscle of the frozen *P. ransonneti* specimen and 7.5 mg of lyophilized *V. hilgendorfii* specimens using a QIAamp DNA mini kit (QIAGEN) according

to the manufacturer's instruction. PCR for amplifying *luciferase* gene from *P. ransonneti* and *V. hilgendorfii* (33) using a primer set PrLsf-5 (5'-GACAAAGCAAGATATCAATTCCAG-3') and PrLsr-5 (5'-GTAGCATTCTTCTTGAACCTCCC-3') using an initial 5.0 min at 94°C, followed by 30 cycles of 30 s at 94°C, 60 s at 55°C, and 4.0 min at 72°C with a final extension of 5.0 min at 72°C. This primer set, PrLsf and PrLsr, was designed at the identical sequences among *C. noctiluca* and *V. hilgendorfii* luciferases. PCR for amplifying 18S ribosomal RNA gene (34) of *V. hilgendorfii* using a primer set, Vhil18S-367f (5'-CTGGTTGATCCTGCCAGT-3') and Vhil18S-1635r (5'-TAATGATCCTCCGCAGGTTCACCT-3'), and *Hoxc6a* gene of *P. ransonneti* (35) using a primer set, hoxc6a_F215 (5'-ATGGAT-CAAACGTGTTCTCA-3') and hoxc6a_R1129 (5'-GATCTAC-CCGTGGATGCAGCG-3') for the first reaction and hoxc6a_F386 (5'-GCGATYTCGATGCGTCTGCG-3') and hoxc6a_R1129 for the second reaction, was performed under the following condition: an initial 5.0 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1.0 min at 56°C, and 1.5 min at 72°C, with a final extension of 5.0 min at 72°C. PCR for amplifying *COI* gene (36) from *P. ransonneti* and *V. hilgendorfii* with primer set LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') used an initial 1.0 min at 94°C, followed by 30 cycles of 40 s at 94°C, 40 s at 45°C, and 1.0 min at 72°C, with a final extension of 5.0 min at 72°C. GoTaq polymerase (Promega) was used for all the PCR. The *COI* nucleotide sequences from three individuals of *P. ransonneti* were determined using a BigDye Terminator kit (Thermo Fisher Scientific) and an ABI PRISM 3130 genetic analyzer (Thermo Fisher Scientific). The GenBank accession numbers are LC427372 and LC427373 (specimens from Shima) and LC427374 (a specimen from Chiba).

Phylogenetic analysis

The *COI* nucleotide sequences of *Pempheris* spp. and *Glaucosoma* spp. were obtained from the Barcode of Life Data (BOLD) system (37). The sequences showing more than 99% sequence identity were collapsed using CD-HIT-EST version 4.7 (38). The sequences of *COI* genes from our analysis were aligned with those of *P. ransonneti* from various localities, *Pempheris* spp. and *Glaucosoma* spp. (as an out-group) from the BOLD database using MAFFT alignment (version 7.309). The phylogenetic relationship was inferred by the neighbor-joining method using the aligned 623 bp with default parameters in Geneious (version 9.0.1). Bootstrap analysis was performed with 1000 replicates.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/2/eaax4942/DC1>

Supplementary Text

Fig. S1. Light organ and luciferin storage organ in *P. ransonneti*.

Fig. S2. Bioluminescence of *P. ransonneti*.

Fig. S3. Luminescence spectra.

Fig. S4. Purification of *P. ransonneti* luciferase.

Fig. S5. Immunohistochemistry of thoracic and anal light organs.

Fig. S6. In vitro luciferase expression.

Fig. S7. Genomic PCR.

Fig. S8. Immuno-pull-down assay.

Fig. S9. Proteolytic resistance and stability of luciferases.

Fig. S10. Phylogenetic relationship of *P. ransonneti* specimens used in this study.

Table S1. Materials for RNA-seq

External database S1. DRA file of RNA-seq (DRA submission DRA008133) includes raw reads generated from thoracic light organ, anal light organ, pyloric caeca, intestine, and dorsal muscle of *P. ransonneti* and whole body of *V. hilgendorfii*.

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[View/request a protocol for this paper from Bio-protocol.](http://advances.sciencemag.org/)

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