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Discoveries

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Functional Characterization of Luciferase in a Brittle Star Indicates Parallel Evolution Influenced by Genomic Availability of Haloalkane Dehalogenase

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Abstract

Determining why convergent traits use distinct versus shared genetic components is crucial for understanding how evolutionary processes generate and sustain biodiversity. However, the factors dictating the genetic underpinnings of convergent traits remain incompletely understood. Here, we use heterologous protein expression, biochemical assays, and phylogenetic analyses to confirm the origin of a luciferase gene from haloalkane dehalogenases in the brittle star *Amphiura filiformis*. Through database searches and gene tree analyses, we also show a complex pattern of the presence and absence of haloalkane dehalogenases across organismal genomes. These results first confirm parallel evolution across a vast phylogenetic distance, because octocorals like *Renilla* also use luciferase derived from haloalkane dehalogenases. This parallel evolution is surprising, even though previously hypothesized, because many organisms that also use coelenterazine as the bioluminescence substrate evolved completely distinct luciferases. The inability to detect haloalkane dehalogenases in the genomes of several bioluminescent groups suggests that the distribution of this gene family influences its recruitment as a luciferase. Together, our findings highlight how biochemical function and genomic availability help determine whether distinct or shared genetic components are used during the convergent evolution of traits like bioluminescence.

Keywords: parallel evolution, convergent evolution, bioluminescence, luciferase, haloalkane dehalogenase

Introduction

Similar traits evolve convergently using shared or distinct genetic pathways, depending on the interplay between function, mutation, and phylogenetic history (Christin et al. 2010; Stern 2013). Similar traits may originate repeatedly via parallel evolution in distinct lineages by recruiting homologous genes; especially when genetic pathways are shared among lineages (Shubin et al. 2009; Rosenblum et al. 2014), or when functional evolution is constrained by limited genetic solutions (Lau et al. 2024). Conversely, similar traits may originate repeatedly by using distinct and nonhomologous genes if there are many possible genetic pathways that produce the same function (Tomarev and Piatigorsky 1996; Foster et al. 2022) or if shared genetic pathways are not maintained (Oakley 2024). Here, we explore the factors shaping the repeated evolution of coelenterazine-based bioluminescence.

Bioluminescence, the production of light by a living organism, is an excellent system for studying patterns of convergence. Bioluminescence repeatedly evolved at least 94 times across distantly related taxa (Lau and Oakley 2020) and is produced when enzymes, generally called luciferases, oxidize any of several substrates generally called luciferins (Shimomura and Yampolsky 2019). Across convergent origins of bioluminescence, many luciferases are nonhomologous and taxon-specific, whereas the same luciferin may be used in many bioluminescence systems, even across vast phylogenetic distances (Delroisse et al. 2021). The most widespread luciferin in marine bioluminescence systems is called coelenterazine, which is

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produced by a few taxa, such as the shrimp *Systellaspis debilis* (Thomson et al. 1995), the copepod *Metridia pacifica* (Oba et al. 2009), and the ctenophores *Mnemiopsis leidyi* and *Bolinopsis infundibulum* (Bessho-Uehara et al. 2020). Other luminous organisms, such as the jellyfish *Aequorea* (Haddock et al. 2001), the shrimp *Gnathophausia ingens* (Frank et al. 1984), and the brittle star *Amphiura filiformis* (Mallefet et al. 2020), obtain coelenterazine through their diets. Despite using the same luciferin, most organisms that use coelenterazine evolved luciferases by recruiting nonhomologous genes (Markova and Vysotski 2015), revealing a diversity of genetic solutions for coelenterazine-based light production. This previous work suggests that coelenterazine-based bioluminescence typically evolves convergently, rather than in parallel.

Surprisingly, sea pansies and brittle stars may have repeatedly recruited members of the haloalkane dehalogenase gene family as coelenterazine-based luciferases (Delroisse et al. 2017; Chaloupkova et al. 2019). The sea pansies *Renilla* sp. use a luciferase that was first cloned in 1991 (Lorenz et al. 1991) and has been structurally (Loening et al. 2007) and biochemically (Schenkmayerova et al. 2023) well-characterized. The brittle star *A. filiformis* may use a luciferase homologous to haloalkane dehalogenases, based on the immunohistochemical detection of *Renilla* luciferase-like proteins in the light-emitting spines of their arms (Delroisse et al. 2017). However, while several candidate genes were identified from the genome of *A. filiformis* (Delroisse et al. 2017; Parey et al. 2024), the luciferase gene has not yet been identified and biochemically characterized. Determining whether these distantly related taxa share a common biochemical mechanism—and if so, understanding the processes that shape the repeated recruitment of this gene family during the evolution of coelenterazine-based bioluminescence—requires identifying the luciferase gene of *A. filiformis* and investigating the distribution of this gene family across luminous organisms.

We recombinantly expressed and functionally tested eight haloalkane dehalogenase/luciferase (hereafter HLD/LUC) proteins corresponding to six gene models from the genome of *A. filiformis* (Parey et al. 2024). Of the HLD/LUC genes tested, we identified one gene (Gen313061), which we named *Amphiura* luciferase, or "*afLuc*", encoding a protein with robust luciferase activity. We also identified a gene, which we named *A. filiformis* dehalogenase, or "*dafA*", encoding a protein with dehalogenase activity and low luciferase activity. Similar to *Renilla* luciferase (RLuc), AfLuc lacks dehalogenase activity with a common substrate, 1,2-dibromoethane, while DafA exhibits activity with 1,2-dibromoethane and other halogenated compounds. AfLuc produces luminescence with an emission spectrum similar to RLuc's, with maximum light emission at a wavelength of 482 nm, and exhibits a similar affinity for coelenterazine. Haloalkane dehalogenase genes in metazoans may have originated via a horizontal gene transfer from bacteria to a cnidarian–bilaterian ancestor (Delroisse et al. 2017) and subsequent gene losses may have influenced the availability of this gene family for recruitment during the evolution of bioluminescence. Altogether, our results provide functional evidence for the evolution of luciferases in brittle stars in parallel with sea pansies, a finding that deviates from the typical pattern of convergent genetic recruitment in coelenterazine-based systems, highlighting how genetic processes such as horizontal gene transfer and gene loss impact the predictability of convergent evolution and subsequent biodiversity.

Results

The Genome of *A. filiformis* Encodes a Gene With High Luciferase Activity

We identified one gene, *afLuc*, which encodes a protein with luciferase activity but no dehalogenase activity, and one gene, *dafA*, which encodes a protein with dehalogenase activity and low luciferase activity in *A. filiformis* (supplementary fig. S1, Supplementary Material online). The gene *afLuc* corresponds to two previously identified gene models (Gen313061 and AFI20122.1) and the gene *dafA* corresponds to two previously identified gene models (AF37308.1 and AFI06958.1). Based on a luciferase assay, which measures light production upon the addition of coelenterazine (Fig. 1a, top), AfLuc exhibited statistically significant luciferase activity (Dunnett's test, $P < 2e^{-16}$), compared to a negative control of bovine serum albumin (BSA). DafA exhibits low luciferase activity that was significantly higher than the negative control (Dunnett's test, $P < 2e^{-16}$), but produced light four and five orders of magnitude lower than that of AfLuc and *Renilla* luciferase (RLuc), respectively (Fig. 1b).

Based on a halide oxidation (HOX) assay (Fig. 1a, bottom), a fluorescence-based assay that quantifies dehalogenase activity, only DafA exhibited statistically significant dehalogenase activity (Dunnett's test, $P = 0.00402$) with the substrate 1,2-dibromoethane (Fig. 1c, supplementary figs. S2 and S3, Supplementary Material online). Further functional tests revealed that DafA exhibits maximum activity toward 1,2-dibromoethane at 30 °C (supplementary fig. S4, Supplementary Material online), and at this temperature, it also catalyzed the dehalogenation of 1-iodohexane, 1,3-dibromopropane, 1-bromo-3-chloropropane, and 3-chloro-2-methylpropene (supplementary fig. S5, Supplementary Material online). The highest specific activity was measured toward 1,2-dibromoethane (73.79 ± 0.44 nmol s^{-1} mg^{-1}), while the lowest activity was measured toward 1-iodohexane (6.47 ± 3.50 nmol s^{-1} mg^{-1}). The activity of DafA is comparable to those of characterized haloalkane dehalogenases in bacteria (supplementary table S1, Supplementary Material online).

RLuc and AfLuc Exhibit Similar Emission Spectra and Catalytic Properties

We conducted a conventional biochemical characterization (Fig. 2a and c, supplementary fig. S6, Supplementary Material online) followed by a global numerical analysis (Johnson 2019) incorporating new standards for the collection and fitting of steady-state kinetic data (supplementary fig. S7, Supplementary Material online and supplementary table S2, Supplementary Material online). Unlike the traditional analysis of initial velocity, the updated numerical approach enables direct estimation of the turnover number k_{cat} without requiring complex luminometer calibration or quantum yield determination (Schenkmayerova et al. 2021).

The kinetic analysis indicates that AfLuc has kinetic parameters comparable to those of RLuc, with substrate affinity $K_m = 1.21 \pm 0.03$ μM and $k_{cat} = 4.12 \pm 0.05$ s^{-1} for AfLuc, and $K_m = 0.91 \pm 0.05$ μM and $k_{cat} = 4.2 \pm 0.2$ s^{-1} for RLuc. The kinetic parameters of RLuc are consistent with previously reported values of $K_m = 1.5 \pm 0.1$ μM and k_{cat} 4.7 ± 0.1 s^{-1} (Schenkmayerova et al. 2021), as determined using the updated protocol for collecting and fitting steady-state kinetic data (see supplementary Methods, Supplementary Material online).

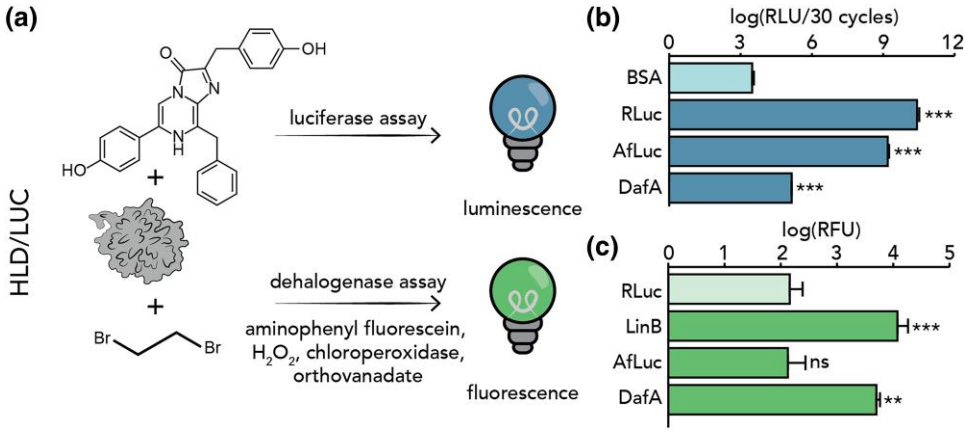

Fig. 1. *Amphiura* luciferase (AfLuc) and DafA exhibit significant luciferase activity, but only DafA exhibits significant dehalogenase activity. a) We tested HLD/LUC proteins for luciferase activity with coelenterazine substrate and dehalogenase activity with 1-2-dibromoethane substrate (supplementary figs. S1 to S3, Supplementary Material online). b) Results of luciferase activity using 25 nM purified protein in 1X TBS (pH = 7.4) and 10 μM coelenterazine reveal that AfLuc and DafA exhibit significant luciferase activity when compared to the negative control of BSA. Luciferase activity was measured at room temperature and quantified in relative light units (RLU). The positive control is *Renilla* luciferase (RLuc), and the negative control is bovine serum albumin (BSA). c) Dehalogenase assay using crude protein extract reveals that DafA, but not AfLuc or other HLD/LUC proteins tested (supplementary figs. S2 and S3, Supplementary Material online), exhibits dehalogenase activity with 1-2-dibromoethane (DBE) as a substrate (0.3 mM DBE, 1 mM orthovanadate in 20 mM phosphate buffer, pH = 8.0). Dehalogenase activity was measured at 30 °C and quantified in relative fluorescence units (RFU). We compared dehalogenase activity at 60 min for AfLuc and DafA, using LinB as a positive control and RLuc as a negative control. Data in this figure are expressed as average ± standard deviation represented by the error bars (N = 3). * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$, and ns denotes non-significance ($P \geq 0.05$).

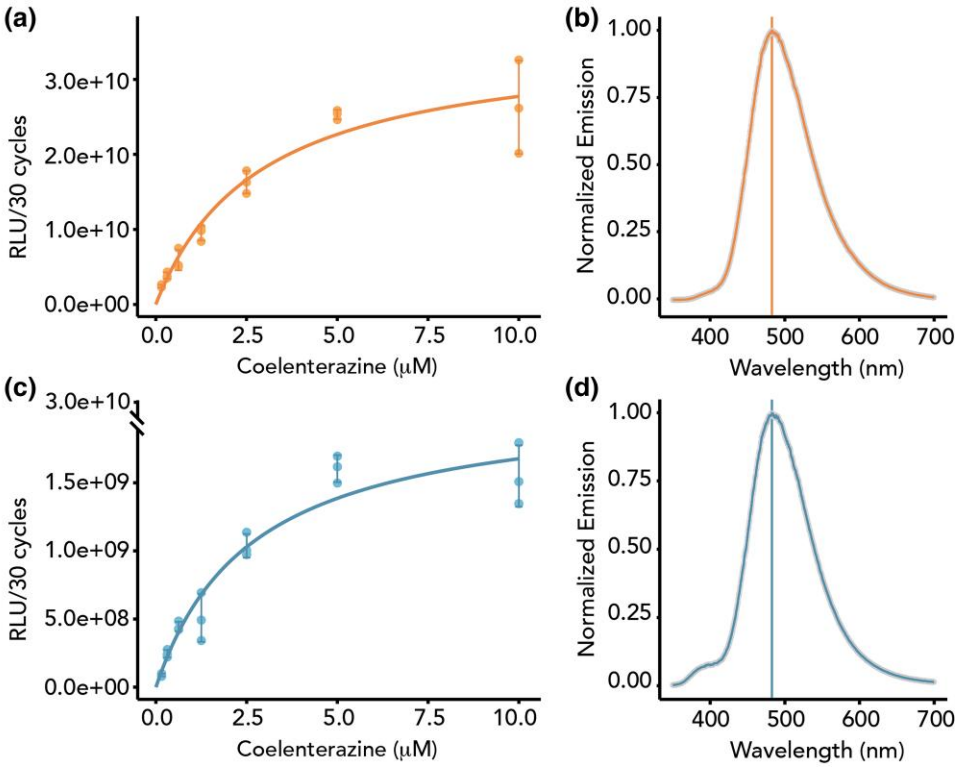

Fig. 2. Biochemical properties of RLuc (a, b) and AfLuc (c, d). a, c) Steady-state kinetic data recorded upon mixing 25 nM of protein with varying concentrations of coelenterazine. We averaged these data (N = 3) and fit them to a Michaelis–Menten model. Data are expressed as average ± standard deviation represented by the error bars (N = 3). b, d) RLuc and AfLuc emit bioluminescence with similar maximum wavelengths and have similar emission spectra, but AfLuc's emission spectrum has a small shoulder at around 400 nm.

Interestingly, the global kinetic analysis further indicated that AfLuc does not undergo the irreversible inactivation observed in RLuc and other tested variants. The absence of inactivation is also clearly visible from the luminescence decay data (supplementary fig. S8, Supplementary Material online). In this analysis, AfLuc is the only variant displaying a consistent single-exponential decay of luminescence activity over time, whereas the other variants demonstrate a significant slowdown in kinetics, characterized by a biexponential decay model (supplementary table S3, Supplementary Material online).

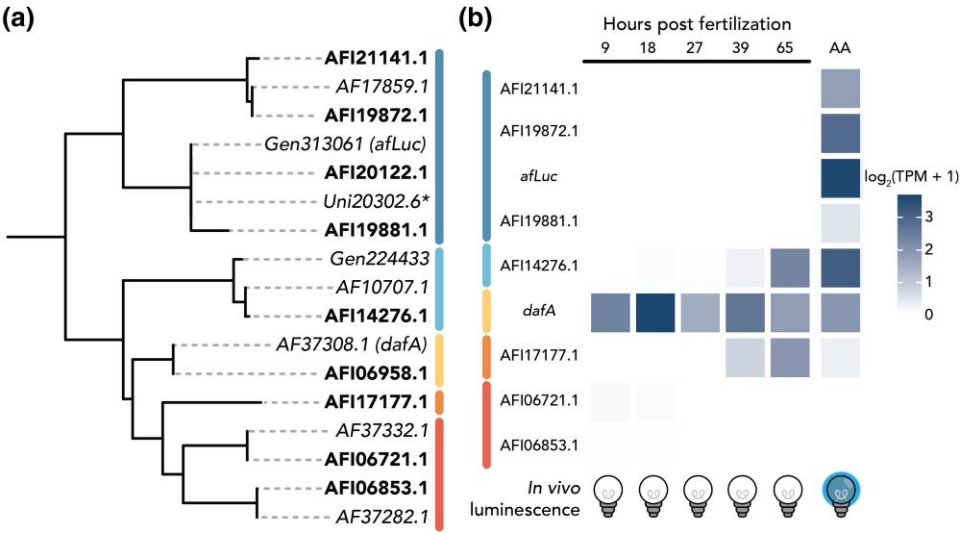

Fig. 3. Closely related HLD/LUC genes exhibit similar expression patterns during development. a) Maximum likelihood, midpoint rooted phylogeny of HLD/LUC protein sequences from preliminary gene models and the final gene models (bolded) as published in Parey et al. (2024). Protein sequences we tested are italicized. *Uni20302.6 is a transcript sequence derived from a transcriptome of the adult arm and encodes a truncated protein sequence, which is otherwise identical to the protein sequence encoded by Gen313061. b) Heatmap showing gene expression of HLD/LUC genes during development and in the adult arm (AA). Gene expression dataset is from a publication (Parey et al. 2024), which quantified gene expression levels in $\log_2$(Transcripts Per Million (TPM) + 1). During the development of *Amphiura filiformis*, luminescence ability emerges after larvae metamorphose into juveniles (Coubris et al. 2024). The gene *afLuc* is most highly expressed in the arms of adult specimens, where bioluminescence is produced. The gene *dafA* is highly expressed during earlier developmental time points and in the adult arms.

Both AfLuc and RLuc exhibit similar emission spectra (RLuc $\lambda_{max} = 482.20$ nm and FWHM = 91.92, AfLuc $\lambda_{max} = 482.93$ nm and FWHM = 91.77). However, one difference is that AfLuc's emission spectrum shows a minor shoulder around 400 nm (Fig. 2b and d).

afLuc Is Mainly Expressed in Adult Tissues While *dafA* Is Expressed Throughout Development

The gene *afLuc* appears to be the most highly expressed HLD/LUC gene in the adult arm tissue, but has little to no expression during other developmental time points (Fig. 3). Only AFI06958.1/*dafA*, and to a much lesser extent the gene models AFI17177.1 and AFI14276.1, are expressed during development. We observed that closely related HLD/LUC genes exhibit similar gene expression patterns across development. For instance, AFI21141.1, AFI19872.1, AFI20122.1/*afLuc*, and AFI19881.1 are expressed primarily in the adult arms, while AFI06721.1 and AFI06853.1 are lowly expressed only during the early developmental stages.

Amphiura filiformis and *Renilla* Catalyze Light Production by Using Homologous Genes

Dehalogenase genes in *Renilla* and *A. filiformis* independently evolved luciferase activity with coelenterazine. They may have evolved from a haloalkane dehalogenase gene family that originally was horizontally transferred from bacteria to metazoans (Fig. 4). We identified dehalogenase-like proteins—containing conserved alpha–beta hydrolase domains (Chovancová et al. 2007)—in bacteria and eukaryotes, namely Fungi, Porifera, Cnidaria, Annelida, Hemichordata, Echinodermata, and Chordata (Tunicata, Cephalochordata, and *Homo sapiens* Vertebrata). Our phylogenetic analysis identified three distinct clades of alpha–beta hydrolases originating from dehalogenases, each clade containing at least one representative bacterial dehalogenase from subfamilies HLD-I, HLD-II, and HLD-III (Chovancová et al. 2007).

All eukaryotic sequences are found within a clade containing sequences from bacterial dehalogenases in subfamily HLD-II. Within this clade, fungal and metazoan sequences are polyphyletic, which suggests multiple horizontal transfers of a subfamily II dehalogenase gene from bacteria to eukaryotes. AfLuc is found within a clade of sequences from nonluminous metazoans, while RLuc is found in a clade of sequences from bacteria and nonluminous octocorals. Altogether, these results support the parallel evolution of luciferases in *Renilla* and *A. filiformis* from a subfamily HLD-II dehalogenases, which may have originated in metazoans from an ancient bacterial horizontal gene transfer (Fig. 4).

Discussion

Convergently evolved traits may recruit homologous or non-homologous genes, depending on the range of possible genetic solutions and the availability of raw genetic material. In this study, we provide functional evidence supporting the parallel evolution of luciferases in sea pansies and brittle stars. While the genome of *A. filiformis* encodes multiple genes homologous to the luciferase from the sea pansy *Renilla* and other members of the haloalkane dehalogenase gene family, we identify only one gene (*afLuc*) encoding a functional luciferase. We present several lines of evidence supporting that *afLuc* is a functional luciferase gene in the bioluminescence system of *A. filiformis*. First, the light produced by AfLuc is strongly detectable and four orders of magnitude higher than that of DafA, a dual-function enzyme we find to have dehalogenase activity and low luciferase activity. Interestingly, the same pattern of dual activity was achieved by site-directed mutagenesis of a single active-site residue of RLuc (Chaloupkova et al. 2019) and by ancestral sequence reconstruction of dehalogenase and luciferase sequences

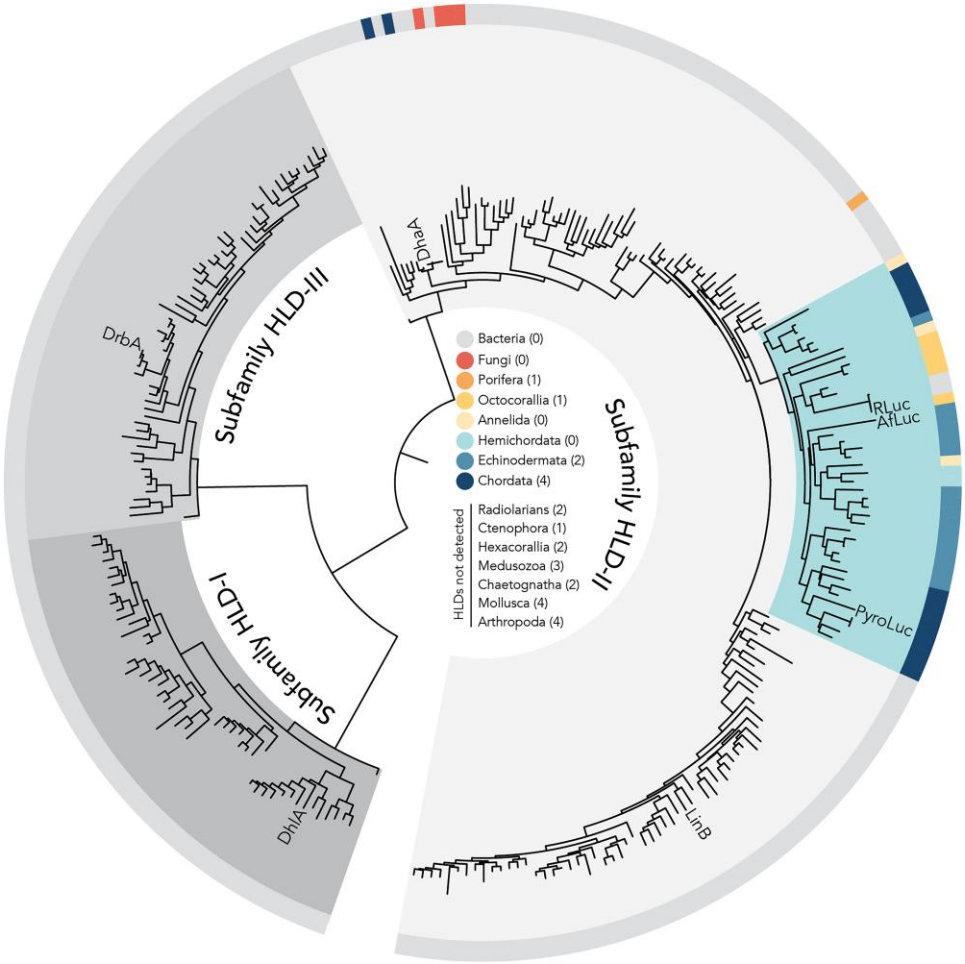

Fig. 4. Maximum likelihood phylogeny of alpha–beta hydrolase domains from haloalkane dehalogenase-like sequences. We used characterized haloalkane dehalogenase sequences from bacteria (DhlA, DrbA, DhaA, and LinB) and luciferases (RLuc, AfLuc, and PyroLuc) as query sequences to identify haloalkane dehalogenase-like proteins in the UniRef90 database. Clades of dehalogenase subfamilies are colored in shades of gray. All metazoan sequences are found within the subfamily HLD-II and most sequences are found in one clade (blue), supporting a horizontal gene transfer from bacteria to a cnidarian–bilaterian ancestor. The outer arc is colored based on taxonomy (gray = bacteria, colors = eukaryotes), as denoted in the legend found in the center of the tree. Numbers next to each taxon name indicate the number of times coelenterazine-based bioluminescence repeatedly evolved (supplementary table S4, Supplementary Material online).

(Schenkmayerova et al. 2021). Second, similar to RLuc, AfLuc does not exhibit dehalogenase activity with the substrate 1,2-dibromoethane, supporting a shift from dehalogenase to luciferase function. Third, *afLuc* is highly expressed in *A. filiformis*'s arms, which produce bioluminescence. While *afLuc* has little to no expression during early developmental stages, it shows low expression during early stages of arm regeneration and strong expression during the late stages of regeneration (Parey et al. 2024). Consistent with this expression pattern, luciferase activity is detected in juvenile *A. filiformis* only after their arms start to develop (Coubris et al. 2024). Taken together, these lines of evidence strongly support AfLuc's organismal role in the bioluminescence system of *A. filiformis*. Previous work detected *Renilla* luciferase-like proteins in the arms of *A. filiformis* (Delroisse et al. 2017). Since multiple HLD/LUC genes, including *afLuc* and dehalogenase genes encoding proteins with no luciferase activity, are expressed in the adult arms, the protein detected using the anti-*Renilla* luciferase antibody remains an open question. Out of all the HLD/LUC genes in the genome, *afLuc* is the most highly expressed in the adult arm and is a likely candidate for encoding the protein responsible for the antibody signal identified by Delroisse et al. (2017). Additional in situ hybridization experiments may identify the HLD/LUC gene(s) expressed in the light-producing cells, providing evidence supporting the in vivo usage of the HLD/LUC gene(s) at a cellular level. At the time of our experiments, we identified and tested all six HLD/LUC genes from a draft genome of *A. filiformis*. Recently, a more complete and chromosome-level assembly of *A. filiformis* was published (Parey et al. 2024) and identified nine HLD/LUC genes, three of which did not correspond to the gene models we tested here. Future tests for luciferase and dehalogenase activities encoded by these three genes (AFI21141.1, AFI19881.1, and AFI17177.1), in combination with in situ hybridization experiments, will provide insight into whether in vivo bioluminescence in *A. filiformis* may be produced by one or more HLD/LUC genes, a pattern seen with fatty acyl-CoA synthetase genes during the separate origin of bioluminescence in fireflies (Bessho-Uehara et al. 2017).

Phylogenetic analysis of dehalogenase sequences supports the parallel evolution of luciferases in *A. filiformis* and *Renilla*. The phylogenetic distribution of dehalogenase genes is widespread in bacteria (Janssen et al. 2005) but sparse in fungi and metazoans. Our phylogeny, which contains

representative sequences from the three bacterial haloalkane dehalogenase subfamilies (Chovancová et al. 2007) and similar sequences in eukaryotes, suggests that the conserved alpha–beta hydrolase domains from haloalkane dehalogenase genes may have been horizontally transferred, multiple times, from bacteria to eukaryotes. Additionally, the sequence of the alpha–beta hydrolase domain in RLuc is identical to the hydrolase domain found in the bacterial cluster UniRef90_A0A941CXK5, with a representative aminoglycoside phosphotransferase sequence from the bacteria *Allobacillus saliphilus*, which suggests a secondary transfer of the hydrolase domain from *Renilla* to bacteria. In bacteria, dehalogenases are often associated with molecules implicated in the transfer of genetic material (e.g. integrase and invertase genes and insertion elements), which implicates horizontal transfer as a mechanism for genetic recruitment in the evolution of xenobiotic degradation (Janssen et al. 2005). The presence of conserved alpha–beta hydrolase domains from bacterial dehalogenases in multi-domain proteins in prokaryotes and eukaryotes suggests that horizontal transfer may be an important genetic mechanism in the evolution of various functions, even besides light production and xenobiotic degradation. Overall, our phylogenetic results support an origin of haloalkane dehalogenase genes in metazoan genomes via a horizontal gene transfer from bacteria to an early cnidarian–bilaterian ancestor—as previously hypothesized by Delroisse et al. (2017)—and reveal that this gene family may have been subsequently lost in many metazoan lineages, including those with taxa that produce coelenterazine-based bioluminescence (supplementary table S4, Supplementary Material online). Lineage-specific gene loss is a common pattern in metazoan evolution (Albalat and Cañestro 2016; Fernández and Gabaldón 2019) and has been hypothesized to play a role in shaping evolutionary diversification (Guijarro-Clarke et al. 2020). The factors influencing the retention of haloalkane dehalogenase genes for potential co-option remains an open question for further investigation. Other possible explanations for the limited distribution of this gene family in Metazoa involve an initial horizontal gene transfer from bacteria to a metazoan, followed by multiple metazoan-to-metazoan horizontal gene transfers. Nevertheless, the limited availability of this gene family, coupled with the numerous alternative genetic solutions that can converge to produce coelenterazine-based bioluminescence (Lau and Oakley 2020), may help explain why haloalkane dehalogenases have not been more frequently recruited in the multiple evolutionary origins of this trait.

In addition to octocoral cnidarians and the brittle star *A. filiformis*, the bioluminescence system of the chordate *Pyrosoma atlanticum* may also use a luciferase homologous to haloalkane dehalogenases (Tessler et al. 2020). However, its usage in the pyrosome bioluminescence system remains suspect for several reasons. Primarily, the study that identified the pyrosome luciferase did not demonstrate the presence of coelenterazine in vivo (Tessler et al. 2020). In addition, we recombinantly expressed the putative luciferase gene from *P. atlanticum*, *pyroLuc*, and detected only low luciferase activity (supplementary fig. S9, Supplementary Material online). Specifically, the amount of light produced by PyroLuc is six orders of magnitude lower than RLuc, five orders of magnitude lower than AfLuc, and one order of magnitude lower than DafA. Similar to DafA, we were unable to detect enough luminescence to measure spectral emission for PyroLuc. Lastly, a later publication identified bioluminescent bacteria in the light-producing organs of *P. atlanticum* (Berger et al. 2021). For these reasons, the biochemical mechanism of bioluminescence in pyrosomes remains controversial and will benefit from future biochemical studies. Aside from pyrosomes, RLuc-like proteins are not found in other chordates with coelenterazine-based bioluminescence systems. The lanternfish *Diaphus watasei*, which does not express RLuc-like genes in its light-producing organs, may use a nonhomologous luciferase (Yano et al. 2023). Similarly, we were unable to identify RLuc-like sequences in the genomes of *Oikopleura* spp. and the stomiiform fish *Borostomias antarcticus*. We identified one transcript similar to RLuc in the transcriptome of *Etmopterus spinax*, GHAY01109793.1 Unigene82405_All (*e*-value $3e^{-28}$). However, since this sequence matches those from the a-proteobacteria *Bradyrhizobium* (*e*-value 0.0) in the core_nt NCBI database, this transcript may be a bacterial contaminant. These findings, coupled with the inability to detect haloalkane dehalogenase genes in most chordate genomes, suggest that haloalkane dehalogenases may not have been recruited as luciferases in most chordate bioluminescence systems.

While most convergently evolved bioluminescence systems use nonhomologous luciferases, there are several instances of parallel evolution (Delroisse et al. 2021) which provide intriguing insights into the factors that may shape the repeatability of molecular evolution. For example, fireflies and click beetles, members of the same order (Coleoptera) in the phylum Arthropoda evolved luciferases in parallel at least three times by recruiting members of the fatty acyl-CoA synthetase gene family (He et al. 2024). These luciferases use ATP as a cofactor to adenylate D-luciferin, a luciferin substrate unique to fireflies and click beetles, which is then oxidized to produce light (McElroy et al. 1969). This pattern of evolution suggests that for D-luciferin-based bioluminescence systems, luciferase evolution may be more repeatable, perhaps due to constraints imposed by the functional requirement of activating D-luciferin via adenylation and the widespread availability of the acyl-CoA synthetase gene family for genetic recruitment (Karan et al. 2001). Unlike D-luciferin, coelenterazine does not need to be activated via biochemical modification, is used as a luciferin substrate across at least nine phyla, and reacts with a diversity of nonhomologous luciferases to produce light, indicating the functional evolution of coelenterazine-based bioluminescence is not genetically constrained and often unrepeated. Deviating from this typical pattern of distinct genetic evolution, octocoral cnidarians and echinoderms each evolved luciferases by recruiting homologous haloalkane dehalogenases, a gene family with a sparse distribution across the tree of life as a result of horizontal transfer and subsequent gene loss. These findings underscore the role of historical contingency in shaping patterns of genetic recruitment during functional evolution. Divergent genetic histories, contingent on past mutational events, in combination with the number of potential genetic solutions, may explain when and why similar phenotypes evolve by recruiting similar versus distinct genes.

Materials and Methods

For a comprehensive list of materials and more detailed methods used in this study, please refer to the methods in the supplementary material.

Obtaining Gene Sequence and Expression Data

We obtained HLD/LUC sequences from *A. filiformis* from various sources. We obtained the sequences Gen224433 and

Gen313061 (named *afLuc*) from a previous transcriptomic dataset (Delroisse et al. 2017), and the sequence Uni20302.6 from an initial de novo transcriptome of the species (Delroisse et al. 2014). The sequences AF10707.1, AF17859.1, AF37282.1, AF37308.1 (named *dafA*), and AF37332.1 originated from a set of preliminary gene models predicted from a genome of *A. filiformis*—new versions of these gene models are now published in Parey et al. (2024). Based on percent sequence identity, we synonymized all sequences from the transcriptomic dataset, preliminary gene models, and final gene models from Parey et al. (2024) (supplementary fig. S10, Supplementary Material online, supplementary table S5, Supplementary Material online). Additionally, we obtained the gene expression dataset used in this study from Parey et al. (2024), which combined expression data from several publications (Delroisse et al. 2014, 2015; Dylus et al. 2016).

Primer Design, *A. filiformis* Sampling, Genomic DNA Extraction and Amplification of Dehalogenase Sequences

We performed genomic DNA-based validation PCRs to confirm portions of the HLD/LUC gene sequences (supplementary fig. S11, Supplementary Material online). For each gene, we designed primer pairs using the Primer3 software (v4.1.0, http://bioinfo.ut.ee/primer3) (supplementary table S6, Supplementary Material online). We collected *A. filiformis* individuals from a depth of 30 to 40 m in the Gullmars fjord near the Kristineberg Marine Research Station (University of Gothenburg, Fiskebäckskil, Sweden) and extracted genomic DNA from arm tissues using Qiagen DNeasy® Blood & Tissue kit. We performed PCR amplifications using Red'y'Star Mix (Eurogentecs) or Q5® High-Fidelity DNA Polymerase (New England BioLabs) and purified PCR products before sending samples for Sanger sequencing (Eurofins Genomics, Germany). We aligned these sequences with the reference HLD/LUC genes to verify their identities. For more detailed protocols for DNA extractions and PCR, please refer to the methods in the supplementary materials.

Expressing Recombinant Proteins and Testing Crude Cellular Extracts for Luciferase Activity

We codon-optimized and synthesized DNA sequences corresponding to the luciferase sequence of *Renilla reniformis* (UniProt Accession P27652) and dehalogenase sequences from *A. filiformis*, namely Gen224433, Gen313061 (named *afLuc*), and Uni20302.6 as reported in Delroisse et al. (2017), and AF10707.1, AF17859.1, AF37282.1, AF37308.1 (named *dafA*), AF37332.1 (sequences predicted from a draft genome of *A. filiformis*), and the pyrosome luciferase (*pyroLuc*), identified by Tessler et al. (2020) (supplementary fig. S12, Supplementary Material online). We cloned these sequences into the bacterial expression vector pET21b, transformed competent *Escherichia coli* cells for propagating plasmids, then extracted and used Sanger sequencing to confirm successful cloning. Then, we transformed competent BL21 cells via electroporation with these plasmids for protein expression. We grew transformed BL21 cells in Terrific Broth containing ampicillin, at 37 °C and shaking at 250 rpm, until cultures reached the mid-log phase. We then added isopropyl-β-D-thiogalactopyranoside (IPTG) to induce protein expression, moved the cultures to a shaker at room temperature, and continued protein expression for 16 to 18 h. We centrifuged the cultures to harvest bacterial cells, removed the supernatant, and froze the cell pellets. We lysed bacterial cells in a lysis buffer and sonicated the cells on ice. Then, we centrifuged the lysed cells and collected the supernatant, which contained our recombinant proteins. We tested the clarified supernatant from lysed bacterial cells for luciferase activity by adding coelenterazine and measuring luminescence using a microplate reader.

Recombinant Expression and Purification of AfLuc, RLuc, DafA, and PyroLuc Using Immobilized Metal Chelate Affinity Chromatography

We expressed recombinant proteins using the protocol as described above. After harvesting and freezing cell pellets, we extracted recombinant proteins by resuspending bacterial cells in lysis buffer containing imidazole and sonicating the cells on ice. We centrifuged these lysates to pellet cellular debris, collected the clarified supernatants, added it to Ni-NTA agarose beads, and then incubated the samples at 4 °C overnight while mixing on a rotary mixer. Next, we loaded the Ni-NTA slurry into gravity-flow chromatography columns, discarded the flow through, washed the column twice with wash buffer, and eluted proteins bound to agarose using an elution buffer. We performed spin ultrafiltration (10 kDa molecular weight cutoff) to concentrate and buffer exchange the eluates into a storage buffer. After running SDS-PAGE to assess protein purity and quantifying proteins via a Bradford assay, we flash-froze single-use aliquots of recombinant protein and stored them at −80 °C until use.

Estimating Michaelis–Menten Kinetic Profiles and Luminescence Decay Parameters

We characterized the enzyme kinetics of AfLuc, DafA, and PyroLuc, using *Renilla* luciferase (RLuc) as a positive control. Since coelenterazine may produce low amounts of chemiluminescence with proteins such as BSA (Vassel et al. 2012), we used BSA as a negative control. We added varying concentrations of coelenterazine to recombinant protein (final concentrations 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM, 0.15625 μM) and measured light production. Specifically, we used a plate reader (Tecan Spark) to measure the background luminescence for five cycles prior to injecting coelenterazine and measuring luminescence for 30 cycles, with a 1 s integration time for each cycle. We repeated each sample measurement in triplicate, and for each measurement, we subtracted the background luminescence. For detailed methods for kinetic data analysis and statistics, please refer to the supplementary Methods, Supplementary Material online section.

We used nonlinear model fitting and comparison to estimate parameters describing the decay of light production between different proteins measured in the plate reader, as above. Identified from the literature, we fit four different models of exponential decay separately to each dataset in R using nlsLM, which uses the more robust LM method to find suitable parameter estimates. For each protein, we then compared models using the corrected Akaike Information Criteria; the model with the lowest value was considered the best fit. However, we note that for one sample (AfLuc) some models were less than 2 AICc values apart, indicating model equivalency. We also visually examined the predicted fit of every model to the data in each dataset.

Luminescence Emission Spectra Measurements

We measured emission spectra using a custom spectroradiometer setup at UCSB, as detailed in a previous study (Hensley

et al. 2021). In brief, we added coelenterazine to recombinant proteins diluted with 1X Tris Buffered Saline (TBS), and measured the emission spectra using a spectroradiometer (Acton SpectraPro 300i) with a charge-coupled device camera detector (Andor iDus). We corrected these spectral data using correction factors calculated from the spectrum of a black body-like light source (Ocean Optics LS-1) and subtracted background emission spectra data of 1X TBS from the experimental data. We repeated each sample measurement in triplicate, then normalized and averaged these data.

Testing Whole Cell Extracts for Dehalogenase Activity

We prepared whole cell extracts by transferring transformed *E. coli* BL21 cells into sterile 96-well plates, then incubated the plates for 3 h at 37 °C while shaking at 200 rpm. We added IPTG to induce protein expression to each well and incubated the plates at 20 °C for 18 h while at 200 rpm. We centrifuged the 96-well plates to pellet cell cultures, washed the pellets with reaction buffer twice, and centrifuged again to harvest cell pellets. We resuspended cell pellets in the reaction buffer and lysed them by freezing them at −70 °C.

To screen whole cell extracts for dehalogenase activity, we used a HOX assay (Aslan-Üzel et al. 2020). In a new 96-well plate, we added the assay master mix, which consists of 25 µM aminophenyl fluorescein, 26 mM H_2O_2, 1.1 U *Curvularia inaequalis* histidine-tagged vanadium chloroperoxidase, 1 mM orthovanadate, 20 mM phosphate buffer, pH = 8.0. We then added resuspended cells to each well for a final OD600 ~0.02 and added the 1,2-dibromoethane (DBE) substrate. We measured fluorescence with an excitation at 488 nm and emission detention at 525 nm, at 30 °C (Synergy™ H4 Hybrid Microplate Reader), with results normalized to OD600 = 1. We measured all data in triplicate and calculated means and standard deviations.

Measurements of Specific Dehalogenase Activity at Varying Temperatures

We measured the temperature profile and dehalogenase activity with various substrates for DafA using the capillary-based droplet microfluidic platform MicroPEX (Buryska et al. 2019; Vasina et al. 2022), which enables us to measure specific enzyme activity within droplets for multiple enzyme variants in one run. Briefly, we generated a custom sequence of droplets (Mitos Dropix), then incubated these droplets with the halogenated substrate in a reaction solution and a complementary fluorescent indicator, 8-hydroxypyrene-1,3,6-trisulfonic acid. Then, fluorescence was measured using an optical setup with an excitation laser (450 nm), a dichroic mirror with a cutoff at 490 nm filtering the excitation light, and a Si-detector. We processed raw data using LabView and used MatLab to calculate specific activities.

Phylogenetic Analysis of Dehalogenase Sequences

Using AfLuc (accession PP777633), RLuc (accession P27652), PyroLuc (accession PP777641), DhaA (accession P59336), LinB (accession D4Z2G1), DhlA (accession P22643), and DrbA (accession G3XCP3) as query sequences, we used DIAMOND blastp (v 0.9.12.113) (Buchfink et al. 2015) to identify the top 50 proteins with the lowest *e*-value scores from the UniRef90 database (downloaded March 2024). We used HMMER (v 3.4) to identify alpha/beta hydrolase domains (PF00561) present in all haloalkane dehalogenases and aligned these domain sequences using MAFFT (v 7.453) (Katoh and Standley 2013). We used IQ-TREE (v 2.0.3) (Minh et al. 2020) to infer a maximum likelihood phylogeny using the best-fit substitution model (LG + R7) as determined by ModelFinder (Kalyaanamoorthy et al. 2017) according to Bayesian Information Criterion and performed ultrafast bootstrap approximation with 1,000 replicates. We visualized trees using iToL and annotated the phylogeny based on the taxon ID of the representative sequence for each UniRef90 accession.

Searching for RLuc-Like Sequences in Chordates With Coelenterazine-Based Bioluminescence

Besides pyrosomes, other chordates that may use coelenterazine to produce bioluminescence include the larvacean *Oikopleura labradoriensis*, the lanternshark *Etmopterus*, the lanternfishes Myctophidae, and stomiiform fish *Vinciguerria attenuata* (supplementary table S4, Supplementary Material online). Using RLuc (accession P27652) as a query sequence, we used TBLASTN (*e*-value threshold 0.05) to search for RLuc-like sequences in the genome of *Oikopleura longicauda* (GCA_004367895.1), *Oikopleura dioica* (GCA_907165135.1), *Oikopleura vanhoeffeni* (GCA_004367855.1), *Oikopleura albicans* (GCA_004367875.1), and *E. spinax* (GHAY00000000.1). Due to the lack of assembled transcriptomes and genomes for *Vinciguerria* (Stomiiformes), we searched for RLuc-like sequences in the genome of another stomiiform fish, *B. antarcticus* (GCA_949987555.1).

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Data Availability

Protein sequences are available in GenBank: PP777633 (AfLuc), PP777634 (DafA), PP777635 (AF10707.1), PP777636 (AF17859.1), PP777637 (AF37282.1), PP777638 (AF37332.1), PP777639 (Gen224433), PP777640 (Uni20302.6), and PP777641 (PyroLuc). Data files and code used to run analyses are available in Dryad: https://doi.org/10.5061/dryad.rv15dv4gm.

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