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Selection, drift, and constraint in cypridinid luciferases and the diversification of bioluminescent signals in sea fireflies

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Abstract

Understanding the genetic causes of evolutionary diversification is challenging because differences across species are complex, often involving many genes. However, cases where single or few genetic loci affect a trait that varies dramatically across a radiation of species provide tractable opportunities to understand the genetics of diversification. Here, we begin to explore how diversification of bioluminescent signals across species of cypridinid ostracods ("sea fireflies") was influenced by evolution of a single gene, cypridinid-luciferase. In addition to emission spectra ("colour") of bioluminescence from 21 cypridinid species, we report 13 new c-luciferase genes from de novo transcriptomes, including *in vitro* assays to confirm function of four of those genes. Our comparative analyses suggest some amino acid sites in c-luciferase evolved under episodic diversifying selection and may be associated with changes in both enzyme kinetics and colour, two enzymatic

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functions that directly impact the phenotype of bioluminescent signals. The analyses also suggest multiple other amino acid positions in c-luciferase evolved neutrally or under purifying selection, and may have impacted the variation of colour of bioluminescent signals across genera. Previous mutagenesis studies at candidate sites show epistatic interactions, which could constrain the evolution of c-luciferase function. This work provides important steps toward understanding the genetic basis of diversification of behavioural signals across multiple species, suggesting different evolutionary processes act at different times during a radiation of species. These results set the stage for additional mutagenesis studies that could explicitly link selection, drift, and constraint to the evolution of phenotypic diversification.

1 | INTRODUCTION

Why and how some groups of species diversify more than others are enduring questions in biology, with broad implications for the origin and maintenance of biodiversity. Particularly challenging is to understand the genetic underpinnings of diversification, because numerous genes typically underlie quantitative phenotypic differences that vary across many species (Flint & Mackay, 2009; Hansen, 2006; Mackay, 2001). In addition, wide disparities across species also exist in particular traits that often accompany rapid speciation, such as morphology or courtship behaviours (Brand et al., 2020; Ellis & Oakley, 2016; Lall et al., 1980; Mendelson & Shaw, 2005; Nagel et al., 2017). During rapid divergence, particular traits are more likely to diversify quickly if they possess a simple genetic architecture (Ellison et al., 2011) or if diversity is driven by few loci of large effect (e.g., effector genes *sensu* Stern & Orgogozo, 2008; Templeton, 1981; Wright, 1984), but such genes for these traits are often difficult to identify. Rapidly diversifying phenotypes have therefore inspired genome-scale studies of species radiations, which show that positive and purifying selection are involved in species differences and sometimes can act on a limited number of loci to promote such variety (Brawand et al., 2014; Supple et al., 2014). When possible, another approach to the genetics of diversification is to identify cases where one or a few genes are linked to diverse phenotypes across many species. Visual pigment genes (opsins) are a prime example of a gene family that underlies an organismal phenotype (colour sensitivity) with a shared genetic basis across species (Henze & Oakley, 2015; Spaethe & Briscoe, 2004; Yokoyama, 1997). While most genomic and single-gene studies highlight how selection and/or epistasis impact phenotypic diversification (Brawand et al., 2014; Yokoyama et al., 2015), a role for neutral processes has been largely overlooked at causal loci, despite its theoretical prevalence (Mendelson et al., 2014; Shaw & Parsons, 2002) and known contribution at the phenotypic level (e.g., Campbell et al., 2010; Irwin et al., 2008; Martin & Mendelson, 2012; but see Picq et al., 2020, who explicitly consider the neutral alternative).

Bioluminescent ostracods (family Cypridinidae) and their phenotypically disparate signals provide a particularly attractive system for integrative understanding of diversification (Ellis & Oakley, 2016; Gerrish & Morin, 2016; Hensley et al., 2019; Morin, 2019; Rivers & Morin, 2008). Bioluminescent cypridinids globally use light as an antipredator display, including Caribbean species that also use light for courtship. This Caribbean clade diversified into ~70 species (Ellis, 2019; Torres & Gonzalez, 2007), each with a courtship display that is largely conserved within species, but variable between species (Cohen & Morin, 2010; Morin, 2019). Although both sexes use light for antipredator displays by expelling light-producing chemicals mixed with mucus, as far as we know, only males of Caribbean species produce courtship signals. In contrast to the light cloud produced during antipredator signals, courtship signals are composed of delicate, discrete pulses of light formed as males rapidly swim (Morin, 1986; Rivers & Morin, 2008). Multiple species commonly live in geographical sympatry, yet produce signals above different microhabitats. These courtship signals also vary in other parameters including: the display angle (relative to the ocean floor), specific time of display onset during the night (Gerrish et al., 2009), and time and distance between light pulses (Gerrish & Morin, 2016). Pulses can vary in brightness, kinetics, and colour (Harvey, 1924; Hensley et al., 2019; Rivers & Morin, 2008). As ostracods externally secrete the pulses that comprise bioluminescent signals, the phenotype of a single pulse is dictated by biochemistry instead of behaviour, and depends largely on well-understood chemical reactions between cypridinid luciferase ("c-luciferase") and the substrate, luciferin. Because the substrate is shared within this ostracod family (Harvey, 1924), biochemical differences in light production depend largely on differences in the protein. Therefore, cypridinid ostracods provide a system whereby some critical aspects of signals may be separated from the complexities of behaviour and connected directly to genetic changes in enzymes, allowing detailed insights into the relationships between genotype, phenotype and diversification.

The decay rate of light production is one target for connecting genotype, phenotype and diversification of signals in cypridinid

ostracods. Recent work shows decay rates vary considerably across species of luminous ostracods, and are correlated with the temporal duration of light in the individual pulses of courtship signals (Hensley et al., 2019). Hensley et al. (2019) measured the kinetics of light production by fitting an exponential decay function to each species' bioluminescence. Cypridinid bioluminescence is a single-order biochemical reaction whose rate depends on the concentration of substrate (Stevens, 1927). Although other components within the mucus alter the overall reaction dynamics, including varying levels of luciferin, light-production fades exponentially once substrate becomes limiting and is a reliable metric of c-luciferase function. We hypothesize that differences in sequences of c-luciferase enzymes influence differences in the light decay kinetics, which vary dramatically across species and may affect the light-duration of both antipredation or courtship displays (Hensley et al., 2019; Rivers & Morin, 2012). The duration of antipredator signals could be under natural selection, and the duration of courtship pulses could be under sexual selection, perhaps for species recognition or choosing among conspecific mates.

In addition to the kinetics of light decay, the colour of cypridinid bioluminescence could be dictated by differences in c-luciferase proteins. However, unlike kinetics, emission spectra ("colours") are not well characterized in many cypridinids. Previously published experiments hinted at variation in colour of ostracod bioluminescence (Harvey, 1924; Huvard, 1993), yet interspecific comparisons were impossible due to differences in methods and lack of replication (Table S1). Harvey (1924) first noted a difference in colour between two species when he cross-reacted crude preparations from *Vargula hilgendorfii* and an unknown Jamaican species. He noted the luciferase preparation of *V. hilgendorfii* catalysed a "bluish" light and the Jamaican luciferase a "yellowish" light, concluding that the protein (luciferase) dictated the colour. The qualitative observations of Harvey nearly 100 years ago are consistent with the more recently published emission spectra of ostracods (Huvard, 1993; Nakajima et al., 2004; Tsuji et al., 1974; Widder et al., 1983) that suggest some Caribbean species have higher peak emission spectra (λ_{max} values) than other species. If so, λ_{max} may be another phenotype that varies across species and is dictated by c-luciferase. Like the rate of light decay, differences in the colour of bioluminescence could be functionally neutral, or could be influenced by natural selection, mediated through its appearance to would-be predators, and/or sexual selection, mediated through its appearance to would-be mates or competitors (Orteu & Jiggins, 2020).

Because c-luciferases may be expressed *in vitro* to test biochemical functions (Tochigi et al., 2010)—including light production, decay kinetics, and colour—this system could fit squarely into the "functional synthesis" research paradigm (Dean & Thornton, 2007), which combines phylogenetic analyses of sequences and manipulative biochemical experiments to allow mechanistic understanding of adaptation and evolutionary constraints. However, despite the potential value of bioluminescence for genotype–phenotype relationships, including some work in other organismal groups of independent evolutionary origins (Fallon et al., 2018; e.g., Viviani et al., 2011), previous studies characterizing luciferase genes of cypridinid bioluminescence exist for only two species (Nakajima et al., 2004;

Thompson et al., 1989). These show cypridinid luciferases evolved independently of other luciferases, have a signal peptide leading to secretion outside cells, and possess two Von Willebrand Factor-D (hereafter called: VWD) domains (Oakley, 2005). In c-luciferase, a plethora of disulphide bonds between conserved cysteines (Hunt et al., 2017; Inouye & Sahara, 2008; Nakajima et al., 2004) as well as post-translational *N*-linked glycosylation are both thought to be important for proper folding and function (Mitani et al., 2017; Yasuno et al., 2018). Here, we characterize new c-luciferase genes for phylogenetic analyses in combination with functional characterizations of bioluminescence. In particular, we hypothesize that differences in biochemical kinetics, namely the rate of decay of light production ("decay"), and emission spectra of bioluminescence ("colour") can be linked to genetic variation in c-luciferase genes during the diversification of both antipredator and courtship signals. Moving toward that goal, we report patterns of molecular evolution in newly sequenced c-luciferases, new data on the colour of cypridinid bioluminescence, previous mutagenesis studies, and correlations between genotypes and phenotypes. These results suggest molecular evolution of c-luciferase contributed to signal diversity in cypridinids, setting the stage for additional functional syntheses in the future.

2 | MATERIALS AND METHODS

2.1 | Specimen collection

We collected animals using small nets while SCUBA diving, or by setting baited traps, as previously described (Cohen & Oakley, 2017). We collected multiple species from each of four Caribbean locations: Discovery Bay, Jamaica; Bocas del Toro, Panama; South Water Caye, Belize; and Roatan, Honduras. We also analysed one species from Catalina Island, California, and two from Isla Magueyes, Puerto Rico. We purchased dried *V. hilgendorfii* (Carolina Biological), which originates in Japan, to measure the emission spectrum of its bioluminescence. We report specific collection localities and size measurements in Table S2. For transcriptomes, we preserved multiple specimens together in RNALater (Invitrogen). We carried some animals alive to University of California, Santa Barbara (UCSB) to measure emission spectra and others we dried using different methods depending on what tools were available on-site. To induce bioluminescence, we crushed live or dried specimens in seawater using a small plastic pestle, usually one animal at a time.

2.2 | c-Luciferase discovery and amplification

We discovered 13 putative luciferase genes ("c-luciferases") from transcriptomes of cypridinid ostracods, one each from a single species. To find these, we queried a previously published transcriptome of *Vargula tsujii* (Oakley et al., 2013) and 32 new transcriptomes using whole bodies of luminous cypridinids with the published c-luciferases from *V. hilgendorfii* and *Cypridina noctiluca* as queries using BLAST-P. We will

use these transcriptomes for a future phylogenetic study, but we present them here as paired-end transcriptomes submitted to BioProject PRJNA589015. Metadata from sequencing, including library prep and instrumentation, as well as NCBI accessions are included in Table S2. Briefly, RNA was extracted either via RNAEasy (Qaigen) or using TRIzol (Invitrogen), and sequencing was performed on a NextSeq, or Illumina HiSeq or MiSeq machine. All paired-end raw reads were quality controlled (Phred cut-off > 20) and adapters were trimmed using TRIMGALORE version 0.4.1 (Krueger, 2012) and assembled using TRINITY version 2.2.0 (Grabherr et al., 2011; Haas et al., 2013). In many cases, we found complete assembly of putative luciferase genes from transcriptomes to depend on which assembly programs and parameters we used (see Table S2 for assembly quality information, as assessed by BUSCO; Waterhouse et al., 2018). If we did not recover a complete c-luciferase from the initial TRINITY assembly for a species (as for *Photeros morini*, *Maristella* sp. SVU, *Maristella* sp. SVD, and *Kornickeria hastingsi carriebowae*), we used TRINITY contigs as input into a subsequent CAP3 (Huang & Madan, 1999) assembly. For many species, we never recovered clear evidence of a c-luciferase (Table S2), and we exclude these transcriptomes from the BioProject. We believe the VWD domains contained in luciferases (Oakley, 2005) diversified wildly in ostracods, leading to challenges when assembling transcriptomes from multiple individuals. In some cases, the final assembly in our BioProject differs from early assemblies we used to design primers (Table S7).

For six species, we amplified putative c-luciferase sequences via PCR (polymerase chain reaction) and confirmed nucleotide sequences with Sanger sequencing. We analysed the Sanger-sequenced genes and provide a multiple sequence alignment of corresponding sequences (Figure S1). Throughout the text we refer to each c-luciferase orthologue by the name of the species from which it came, either the official taxonomic name or the field identification code with nominal genus assignment.

2.3 | c-Luciferase expression in vitro

We expressed new, putative c-luciferases from a representative species from each of four major clades of cypridinids: *Maristella*, *Photeros*, *Kornickeria* and *Vargula*. Using mammalian cells, we expressed three new c-luciferase proteins (from species *M.* sp. SVU, *V. tsujii* and *P. morini*) to confirm their gene function. In *Pichia* yeast, we expressed three new c-luciferases (from *V. tsujii*, *M.* sp. SVU and *K. hastingsi carriebowae*), and one that had been previously expressed from *C. noctiluca* (Nakajima et al., 2004) as a positive control. These proteins are secreted into culture media, to which we added luciferin substrate to test the hypothesis that they are luciferases and catalyse a light reaction. More details are given in the Supporting Information.

2.4 | Light catalysis assay to test protein function

To assess the ability of expressed genes to catalyse a light reaction, we harvested cell culture media from mammalian or yeast cells from each transfection (~10–25 ml per transfection), and concentrated it using 30,000 MWCO centrifugal filters (Amicon), spun from 30 to 240 min at 4,000 *g*. After centrifugation, the protein solution was immediately collected for the assay. Varying volumes of concentrated protein solution and luciferin assay mix were added together in a plate reader (Wallac). We measured luminescence in counts per second (CPS) for 10 s. For mammalian cultures, cypridinid luciferin was prepared to the manufacturer's specifications but at an unknown absolute concentration (Targeting Systems). For yeast, we procured vargulin from NanoLight Technology and suspended it in 10 mM Tris to a working concentration of 0.01 ng/µl before using in each light catalysis assay.

2.5 | Measuring colour with emission spectra

We used a home-built fluorimeter (spectroradiometer) at UCSB to measure emission spectra. We imaged the output orifice of the integrating sphere or a cuvette cell's transparent wall by a relay lens onto the entrance slit of a spectroradiometer (Acton SpectraPro 300i) equipped with a charge-coupled device (CCD) camera detector (Andor iDus). Because of the limited time span of bioluminescence, we collected a series of data frames upon introduction of specimens. We usually sampled at 10 time points every 2 s for each emission with 2 s integration time, although sample numbers ranged from 5 to 20, depending on the species. The spectral data acquired by the CCD camera were corrected for instrumental response artefacts by measuring the spectrum of a black body-like light source (Ocean Optics LS-1) and calculating appropriate correction factors. We also took background spectra for each experiment to subtract from the experimental emission spectra (see Supporting Information for further details). For the high-quality spectra, we employed Savitzky-Golay smoothing using the signal package (Ligges et al., 2015) in R (Team et al., 2013) and then calculated λ_{max} (the wavelength with the highest emission value) and full width half maximum (FWHM, the width of the spectrum in nanometres where emission is half the value of maximum). The bulk of our analyses relate to λ_{max}, and we report variation in FWHM in Figure S4.

2.6 | Phylogeny of c-luciferase

We generated a gene tree of translated c-luciferase candidates and closely related genes. We used a published c-luciferase from *V. hilgendorfii* as a query in a similarity search using BLAST, retaining the top 20 hits from each transcriptome searched. As mentioned above, in some cases, we did not obtain full-length luciferase transcripts in the assembly created by TRINITY, and which we attribute to polymorphism from pooling individuals. In these cases, we did a second assembly of the TRINITY contigs, including luciferase fragments, using CAP3 and selected the longest open reading frames (ORFs) as putative c-luciferases. We aligned these sequences using MAFFT (Katoh & Standley, 2013). We used IQ-TREE version 1.6.12 (Nguyen et al., 2015)

to select the best-fit model of protein evolution and to estimate the maximum likelihood phylogeny. We rooted this phylogeny using midpoint rooting to identify putative c-luciferases from new transcriptomes as orthologues to previously published c-luciferases.

2.7 | Quantifying synonymous and nonsynonymous rates of substitution

We aligned luciferase DNA by codon, first aligning amino acids in MAFFT (Katoh & Standley, 2013), then matching DNA codons using PAL2NAL (Suyama et al., 2006). We used HYPHY (Pond et al., 2020) to compare ratios of nonsynonymous to synonymous substitutions using various models. Many models and methods exist to quantify substitution rates of a gene under the assumption that rates of synonymous substitution represent rates of neutral mutation (Kimura, 1977). As such, ratios of nonsynonymous to synonymous (dN:dS) substitution rates can be informative regarding evolutionary mechanisms, including positive selection (Hughes & Nei, 1988). Different approaches address different questions, such as whether entire genes, individual branches, or individual sites show signs of selection. For methods focused on individual sites, some methods assume a single dN:dS ratio at a site for the entire history of that site ("pervasive" *sensu* Kosakovsky Pond & Frost, 2005). Other methods allow for different ratios at a site along different branches of the gene tree ("episodic" *sensu* Murrell et al., 2012). In HYPHY, we explored different patterns of nucleotide substitution at the gene, branch, and site level using different tests based on biological hypotheses, which we outline here.

Because we believe c-luciferase may have important functional characteristics tied to organismal fitness, we initially used busted[s] to test the hypothesis that positive selection occurred at some (unspecified) point in the history of the gene (Wisotsky et al., 2020). We also tested for episodic positive selection among the branches of the tree using aBSREL (Smith et al., 2015), which can be used to identify branches that show elevated signatures of dN:dS without *a priori* specification. We hypothesized that a relatively lengthy branch leading to *Photeros* may indicate episodic positive selection because the genus *Photeros* shows strong differences in our phenotypic measures: a minor shift in colour, and some species possess very rapid courtship pulses.

At the codon level, we used fixed effect likelihood (FEL) tests to look for evidence of pervasive diversifying (high dN:dS) or purifying (low dN:dS) selection on individual sites (Kosakovsky Pond & Frost, 2005). Finally, we tested for episodic diversifying selection using mixed effects model of evolution (MEME; Murrell et al., 2012) at individual sites. We believe episodic selection, where dN:dS is allowed to differ in different lineages across sites, fits the biology of signal diversification better than pervasive selection because optima of signal phenotypes are expected to change commonly during evolution, such as to track shifts in mating preferences by courtship signals (i.e., phenotypic tango of the Fisher-Lande process; Arnold & Houck, 2016), and/or as predator behaviours co-evolve to combat the efficacy of defensive displays in a Red Queen scenario (Van & Van Valen, 1973).

The accuracy of these statistical methods is impacted by deviations from their assumptions, such as constant rates of synonymous substitutions across the phylogeny or independent mutations (so-called multinucleotide substitutions, or MNS). Both raw evolutionary rates (e.g., site heterogeneity in dS) and MNS can increase rates of false positives (Venkat et al., 2018; Wisotsky et al., 2020). The sensitivity of these methods means they must be interpreted with caution. At the same time, interesting patterns of substitutions generate testable functional hypotheses, especially in systems where enzymatic function can be measured following mutagenesis and *in vitro* expression. First, simply given our number of sequences ($N = 15$), we expect initial identification of false positive sites due to sampling to be relatively low (i.e., 0%–1%; Murrell et al., 2012). Second, we used tests that either account for site heterogeneity, have models incorporating MNS, or implement a model that can account for both site-level rate heterogeneity and MNS events to qualify the potential for false positives in our data due to these two processes. At the gene level, busted[s] accounts for site-level changes in dS and busted[s]-mh can account for both changes in dS and multiple hits. At the branch and site levels, all three aBSREL, FEL and MEME tests incorporate site-level heterogeneity into their models. Finally, at single sites, we used MULTIHIT (Lucaci et al., 2020) to assess the evidence supporting a double or triple substitution over a single substitution at every site (i.e., incidences of MNS).

Given multiple statistical tests, it is necessary under many conditions to perform a correction for false discovery rate (FDR). We performed a number of tests aimed at different levels of analysis (whole gene, among branches, and between sites), each with different null hypotheses, which makes FDR inappropriate across tests (Murrell et al., 2012). Within any given test that is composed of multiple likelihood ratio tests (such as FEL and MEME), FDR is still required. However, the specific arguments for the best practices of correction vary depending on the level of conservatism and the specific hypotheses under question. Namely, FDR *q*-values may be too conservative when the *a priori* expectation on a gene of interest is previously established, as for c-luciferases. In line with this view, we adopted the methods used by Murrell et al. (2012) to control for the site-wise error instead of the family-wise error. The interpretation of these *q*-values may be thought of as the expected number of false positives (at a certain *q*-value level) for a given set of *p*-values at a specified threshold. Because MEME is already a conservative test, we performed an FDR correction for FEL and MEME sites with a threshold of $p \leq .1$, and report *q*-values (Table 1; Tables S9 and S10). Depending on the level of stringency, these values can be set to accept any percentage of false positives. As this is the first exploration into the molecular diversification of c-luciferase, we adopt a slightly permissive *q*-value threshold of 0.05 to allow for 5% false positives, which provides context for interpretation and the future hypothesis exploration.

Throughout our results, we refer to individual codon positions by their corresponding number to the codon site in the unaligned *C. noctiluca* c-luciferase (GenBank accession BBG57195), and provided in Table S8 for easy reference.

2.8 | Correlating genetic variation with c-luciferase function

We looked for amino acid changes associated with changes in three functions: λ_{max}, FWHM and light decay constant. To find mutations in luciferase that shift λ_{max}, we analysed previously published data from mutagenesis experiments on *C. noctiluca* luciferase (*Cn-luc*) and data on luciferases from nine other cypridinid species with both luciferase and emission spectrum data. Kawasaki et al. (2012) created 35 variants of *Cn-luc* and measured λ_{max} for each variant plus wild type *Cn-luc*. To measure λ_{max} they added luciferin to *Cn-luc* variants expressed heterologously, finding λ_{max} to vary between 435 and 463 nm. They did not report complete spectra or FWHM for most of the mutants, instead only reporting λ_{max}. Therefore, our subsequent analyses of FWHM use only data from natural c-luciferase sequences and our newly reported FWHM data from emission spectra. For the mutational analysis, each variant contained one to eight mutated sites compared to the wild type, and across all 35 variants, a total of 23 different amino acid sites contained mutations. For the non-*Cn-luc* luciferases, we aligned sequences to *Cn-luc* using MUSCLE (Edgar, 2004) to identify amino acids at sites homologous to those mutated in *Cn-luc* variants. We refer to sites as numbered for the homologous positions in *Cn-luc*, which differs from their aligned position. For light decay constant, we used previously published data (Hensley et al., 2019).

We analysed candidate mutations with an ANOVA to test for significant associations between variant amino acid sites and functions of c-luciferases, similar to methods used for opsins and spectral absorbances (Yokoyama, Yang, et al., 2008). To determine which sites were most highly correlated with each function, we used a model selection approach in the R package MUMIN (Bartoń, 2018). We used the dredge function to test combinations of amino acid sites regressed against each trait, which formed different models. Dredge sorts models by Akaike information criterion (AIC) score. We tallied amino acid sites present in the highest number of best-fit models, and then performed phylogenetically informed least squares regression (PGLS) using a linear model with those sites and incorporating branch lengths from the gene tree transformed with a Brownian-motion model of evolution as a correlation structure into the residuals. We compared corrected and uncorrected models (i.e., with and without the phylogeny) using AICc of the maximum likelihood model fits. In most comparisons, AICc scores favoured uncorrected, ordinary least squares (OLS) fit models (Table S3). In one case, OLS and PGLS fits were equivalent and we default to reporting results from a phylogenetically corrected model (annotated in Table 1).

3 | RESULTS

3.1 | Discovery of 13 new, putative c-luciferases

We identified 13 new putative luciferases from whole-body transcriptomes of cypridinid ostracods, one each from the species included in our study. These genes form part of a monophyletic gene family along with the only two previously published cypridinid luciferases from *V. hilgendorfii* and *C. noctiluca* (Figure 1; Figures S1 and S2). These new, putative luciferases bear multiple hallmarks of c-luciferase, including two VWD domains, a signal peptide and conserved cysteine sites (Nakajima et al., 2004; Oakley, 2005; Thompson et al., 1989). From the transcriptomes of both *Photeros annecohenae* and *P.* sp. WLU, we recovered two additional genes (in-paralogues) in this clade, whereas all other species had one direct orthologue of published c-luciferase in the clade. We excluded the two in-paralogues from further analysis because we did not confirm these transcriptome sequences with PCR and because the in-paralogue sequence from *P. annecohenae* lacks a signal peptide and is therefore unlikely to be a functional c-luciferase. For the full tree, see Figure S2.

3.2 | c-Luciferases from exemplar species are functional

Using *in vitro* expression in mammalian and/or yeast cells (Figure 2), we tested the ability of putative c-luciferases to catalyse light reactions. We selected four exemplar species representing different genera of bioluminescent Cypridinidae. For each luciferase construct, light levels increased significantly after the addition of the substrate luciferin or compared to negative controls (Figure 2; Table S4). Adding luciferin to biological media often produces light (Viviani & Ohmiya, 2006) that varies across biological replicates. We note this variation, yet also report statistically significant differences in light production after adding substrate (for yeast) or between c-luciferase secreting cells and cells or media alone (for HEK293 cells and yeast) (Figure 2). This is consistent with the putative c-luciferases across multiple genera of Cypridinidae being functional c-luciferases. During deposition of our plasmids with Addgene and at the proof stage of manuscript editing, it has come to our attention that the plasmids used in our yeast transformations, and which contain c-luciferases from species *M. sp.* "SVU" (Addgene plasmid # 160426) and *Vargula tsujii* (Addgene plasmid # 160425), have previously undetected insertions in the open reading frames of the vectors (39 bp and 140bp, respectively). The plasmid for the c-luciferase from species *K. hastingsi carriebowae* remains as originally reported. Although our functional results and conclusions herein remain unchanged, these additions may affect conclusions from future experiments, and the discrepancies should be noted. Please see the Addgene deposition comments for further details.

3.3 | Emission spectra vary across species of bioluminescent Cypridinidae

By crushing whole specimens to elicit light production in front of a spectroradiometer (see Section 2), we obtained new data on emission spectra from 20 species (Figure 3; Tables S5 and S6). The wavelength of maximum emission (λ_{max}) varies from 458.7 ± 1.80 nm in *V. hilgendorfii* to 468.0 ± 1.80 nm in *Photeros* sp. EGD. Previously published

TABLE 1 Sites inferred to be associated with phenotypic changes. Candidate sites are from ANOVAs with associated phenotypes (colour, decay), and labelled with inferred processes of molecular evolution. Results are based on estimated rates of nonsynonymous to synonymous substitution ratios from HYPHY MEME and FEL tests. FDR-corrected q-values for FEL or MEME results, respectively. Columns from MULTIHIT list the evidence ratio preferring a model of di- or trinucleotide substitution, respectively, over a model of a single substitution at that site. Data sets refer to which data subsets collected and collated in this study were used for ANOVA, with their corresponding source ("Ref")

Phenotype	Site (Cypridina)	Site (aligned)	Process	ANOVA p-value	HYPHY MEME p-value	HYPHY FEL p-value	Respective q-value	MULTIHIT Two hits	MULTIHIT Three hits	Data set	Ref
Lambda max	178	207	Neutral	<.0001	.67	.723	–	0.437	0.981	Previous mutagenesis, new colour, & transcriptome combined	Kawasaki et al. (2012)
	280	311	Neutral	.0002	.67	.25	–	1.34	1.09	Previous mutagenesis, new colour, & transcriptome combined	Kawasaki et al. (2012)
	375	406	Neutral	.018	.32	.506	–	2.218	0.704	Previous mutagenesis, new colour, & transcriptome combined	Kawasaki et al. (2012)
	404	435	Purifying	.0209	.67	.007	0.0201	0.986	1.023	Previous mutagenesis, new colour, & transcriptome combined	Kawasaki et al. (2012)
	375:404	406:435	Epistatsis	.029[b]	–	–	–	–	–	Previous mutagenesis, new colour, & transcriptome combined	Kawasaki et al. (2012)
	74	102	Diversifying	.0005	.0906	.47	0.0906	1.394	0.778	Newly collected colour & transcriptome only	This study
	114	142	Diversifying	.0033	.0015	.325	0.0062	6.659	1.819	Newly collected colour & transcriptome only	This study
	160	189	Diversifying	.0053	.0509	.347	0.0803	0.169	1.044	Newly collected colour & transcriptome only	This study
Rate of light decay	19	41	Diversifying	.1384	.0256	.793	0.0555	35.124	6.27	Published decay & new transcriptome only	Hensley et al. (2019)
	160	189	Diversifying	.0032[a]	.0509	.347	0.0803	0.169	1.044	Published decay & new transcriptome only	Hensley et al. (2019)
	232	261	Diversifying	.1123[a]	.0005	.266	0.0062	65.506	0.583	Published decay & new transcriptome only	Hensley et al. (2019)

[a]Results from a PGLS model where PGLS and OLS fits were equivalent. All other ANOVA p-values are reported from models preferring an OLS fit based on comparison of AIC scores.

[b]Results from a GLS model predicting an interaction between differences in 375-R and 404-N with those residues only. Note that we do not have a large enough data set to predict the effect of all residue changes at these two sites.
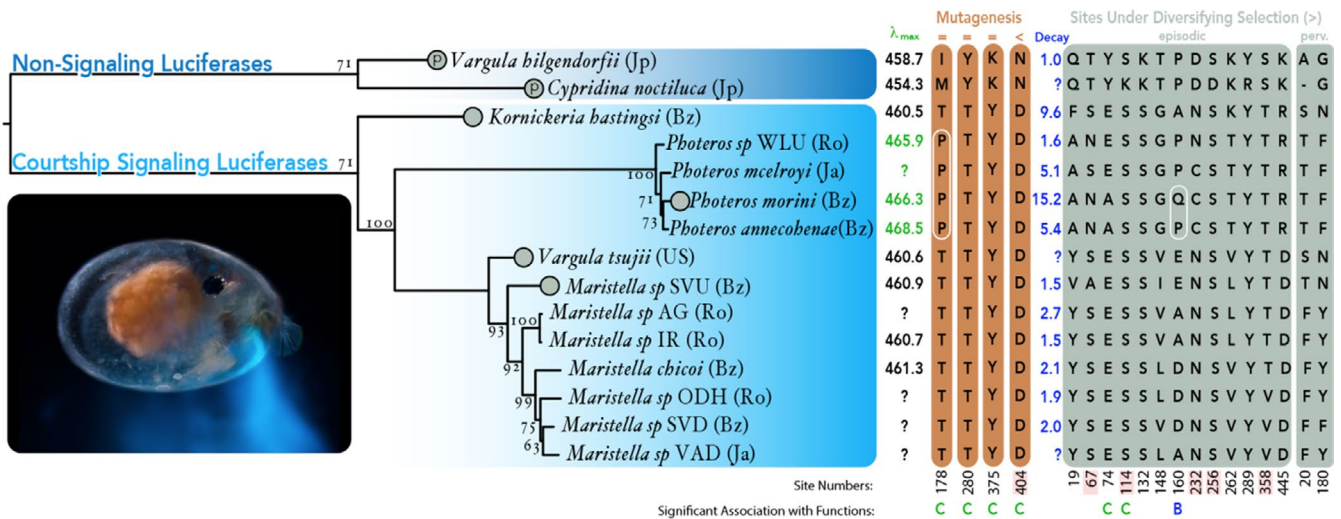
FIGURE 1 Maximum likelihood phylogeny of new and published c-luciferase genes using codon-aligned DNA and GTR models with fast bootstraps (percentages at nodes) in IQ-TREE. Circles at tips of the phylogeny illustrate six genes expressed in vitro that show light catalysis. Two circles at tips with "P" inside are previously published, while the other four are newly expressed in this study and explained herein (Figure 2). Orthologues are designated by their species name or nominal assignment to genus and field identification code. Country of origin in parentheses (Bz = Belize, Ja = Jamaica, Jp = Japan, Ro = Roatan, US = United States). On the right are two biochemical phenotypes (colour data as λ_{max} and the rate of light decay constant), and interesting amino acid sites, including four that affect λ_{max} in previously performed mutagenesis experiments (orange shading), 13 potentially under episodic diversifying selection (HYPHY MEME, $p < .1$), and two potentially under pervasive diversifying selection (HYPHY FEL, $p < .1$). Numbers of sites at the bottom correspond to the unaligned *Cypridina noctiluca* luciferase sequence. We label the dN:dS ratio above the sites with ">" indicating diversifying/positive selection, "<" indicating purifying selection and "=" indicating failure to reject the null, neutral model. White ellipses around sites are amino acid patterns discussed in the main text. At the far bottom, we label sites significantly associated with molecular phenotypes via ANOVA as: colour (C), or both colour and kinetics (B). Red shading behind site numbers indicates sites with FDR-corrected q-values ≤0.05 from either FEL or MEME, respectively. Inset: adult female *Photeros annecohenae* (~1.5 mm long) secreting bioluminescence.
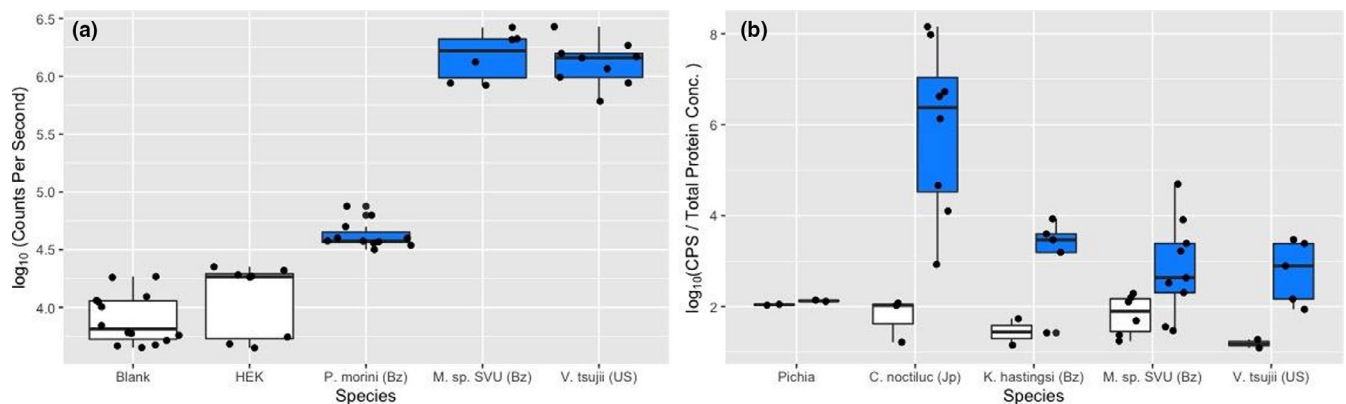Photo courtesy of Elliot P. Lowndes

FIGURE 2 Exemplar luciferases are functional. (a) Light produced after addition of luciferin to putative c-luciferase constructs from different species, measured in log counts per second. Controls include blank cell culture media and HEK cells with no construct. Constructs are labelled by species name or nominal genera assignment with field designation and country of origin in parentheses. (b) Log counts per second normalized by total protein concentration for four c-luciferase constructs expressed in yeast *Pichia*. Measures are before the addition of the substrate luciferin (grey) and after (blue). Control is *Pichia* cells alone. Constructs are: (i) the host strain of *Pichia* without a luciferase as a negative control, (ii) a sequence known from *Cypridina noctiluca* as a positive control, (iii) a novel sequence from *Kornickeria hastingsi carriebowae*, (iv) a novel sequence from an undescribed species from Belize, "SVU" (nominal genus *Maristella*), and (v) a novel sequence from the California sea firefly *Vargula tsujii*. Country of origin in parentheses (Bz = Belize, Jp = Japan, US = United States). Each datum is an average of three technical replicates

data extend this range, with *C. noctiluca* at 454.3 nm and *Photeros graminicola* at 471.1 nm. We found λ_{max} from species of *Photeros* do not overlap in value with λ_{max} of other species. The lowest λ_{max} from any *Photeros* we measured is 465.6 ± 0.78 nm, whereas the highest of any non-*Photeros* species is 461.5 ± 2.70. Assuming *Photeros* is monophyletic, we infer an evolutionary increase in λ_{max} along the

branch leading to *Photeros*. Monophyly of *Photeros* is supported by published morphological (Cohen & Morin, 2003) and molecular (Hensley et al., 2019) phylogenetic analyses, by the phylogeny of c-luciferase we present here (Figure 1), and by a diagnostic ratio of length:height of the carapace (Gerrish & Morin, 2016; Morin & Cohen, 2017; Reda et al., 2019), which we also used here to classify undescribed species into genera (Table S2). FWHM is a common parameter to describe the variation (width) of wavelengths in emission spectra. We found FWHM values to also vary between species of Cyprinidae (Figure S4), ranging from 75.05 ± 0.91 to 85.44 ± 0.32, although we noted no apparent phylogenetic pattern.

3.4 | Patterns of natural selection on and in c-luciferase

At the level of the gene, our analysis using busted[s] yields very strong statistical support ($p < .0001$) for episodic positive selection, without specifying which sites or branches are under selection. However, busted[s]-mh, which uses a model aimed to account for nonindependent, adjacent nucleotide substitutions, did not support the alternative hypothesis of episodic diversifying selection ($p = .1835$). Incorporating a conservative alpha of .025 to correct for multiple BUSTED tests (two), we see a significant effect with busted[s] and a model that does not incorporate multiple hits, but an insignificant one for busted[s]-mh and a model that does. We argue that dN:dS tests may be overly conservative when using models that incorporate MULTIHIT substitutions because comparative data often fail to distinguish simultaneous substitutions versus multiple, single substitutions (see Section 4 for further commentary).

Our aBSREL analysis indicated two branches of the c-luciferase phylogeny are under positive selection, both which are terminal branches (*M. chicoi* and *P. mcelroyi*, Bonferroni-corrected p-values <.002). aBSREL did not support our a priori hypothesis of increased selection on the branch leading to *Photeros*, the only genus with very rapid courtship pulses, and as we report here, slightly green-shifted λ_{max}.

Looking at specific sites using FEL (p-value cut-off ≤.1), 250 c-luciferase sites have significantly low rates of dN:dS, consistent with purifying (negative) selection, and two sites with significantly high dN:dS, consistent with pervasive positive selection (elevated dN:dS across the entire gene tree). After FDR correction (q-value cut-off ≤0.05), 144 of these sites remain and all under purifying selection, although the two sites under pervasive positive selection are marginally significant ($q < 0.0642$, Table S10). Of note, our alignment with newly discovered c-luciferases shows that 32 of the 34 cysteine residues are conserved, and after FDR correction, 19 demonstrate significant patterns of purifying selection (Table S10). With MEME (p-value cut-off ≤.1), we found ratios of nonsynonymous to synonymous substitution rates in c-luciferase to indicate 13 sites, consistent with significant episodic diversifying selection distributed throughout the gene (Figure 1). "Episodic" diversifying selection refers to sites with elevated dN:dS along some proportion of branches of the gene tree. After FDR correction (q-value cut-off ≤0.05), five sites remain confidently under episodic diversifying selection (Table S9).

MULTIHIT indicates that models incorporating either two, three, or three hit islands substitutions at specific sites are preferred to single mutation models or other, less complex models (likelihood ratio tests, $p < .001$). However, the impact of this varies by site (Figure S3). Across the gene alignment, 4.09% of codons (25/610) strongly prefer a multinucleotide substitution event (Evidence ratio ≥5).

3.5 | Genetic correlations with shifts in colour (λ_{max}) from previous mutagenesis experiments

By analysing previously published mutagenesis studies along with new c-luciferase sequences and new colour data, we identified amino acid sites that influence λ_{max}. The site most strongly associated with λ_{max} is 178 (ANOVA, $p < .0001$; Table 1). This effect is clear from published mutagenesis experiments: wild-type luciferase from *C. noctiluca* (hereafter *Cn-luc*) had λ_{max} of 454 nm with a methionine at 178 (M-178-M) while mutants had lower λ_{max} (M-178-R = 435 nm; M-178-K = 447 nm), although these sequences also had other mutations at sites besides
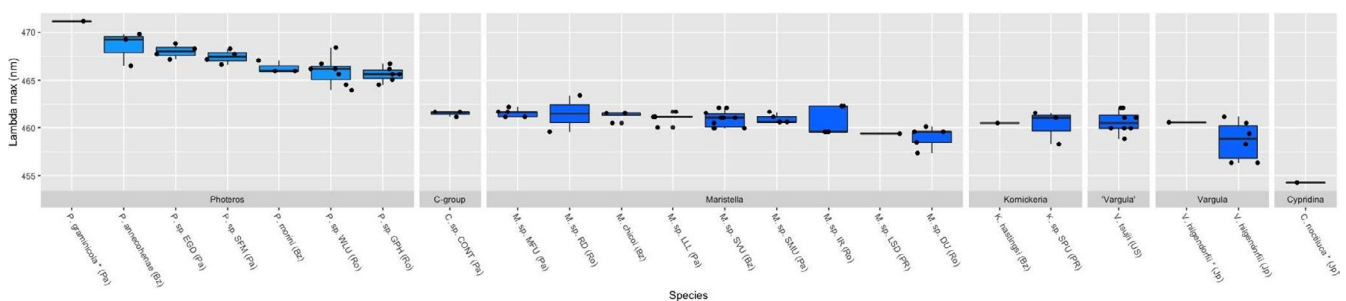

FIGURE 3 *Photeros* (left) has higher values of λ_{max} than other genera (right). Wavelength value with peak emission (λ_{max}; "Lambda max" on the y-axis) in nanometres (nm) from new emission spectra of 20 species, sorted by genus assignment, and previously published spectra from three species (marked with an asterisk *). Note that the genus *Vargula* is probably polyphyletic and has been separated based on current taxonomic understanding into "Vargula," and true *Vargula* species. Country of origin follows each species in parentheses (Bz = Belize, Jp = Japan, Ro = Roatan, Pa = Panama, PR = Puerto Rico, US = United States). See Table S2 for collection details
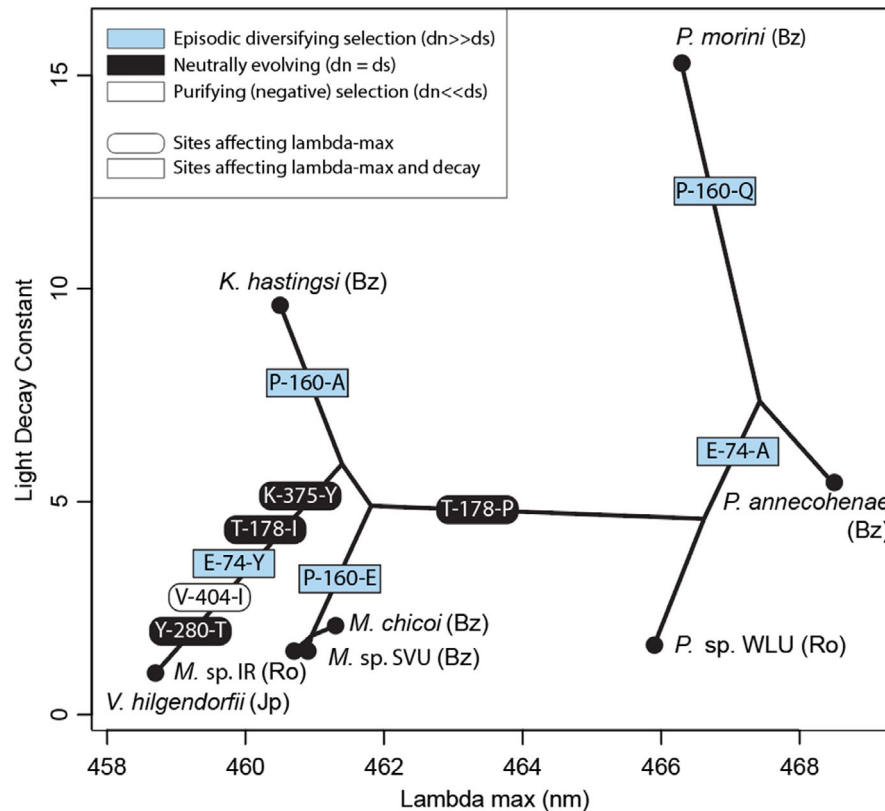
FIGURE 4 Bioluminescence phenotypes, including λ_{max} ("Lambda max" on the x-axis) of emission spectra and decay of light emission ("Light decay constant" on the y-axis), diversified during the evolution of Cypridinidae, perhaps through different mutations in c-luciferases, and influenced by different evolutionary processes. Points are different species ($N = 8$) as they occupy this phylophenospace of bioluminescence, connected by branches representing phylogenetic relationships. Shapes and colours on branches represent inferred mutations and evolutionary processes acting on c-luciferases and correlated with bioluminescence phenotypes. Rectangular sites are correlated with both decay and colour, or those with rounded edges only affect colour (solid border). The colour of the shapes represent evolutionary processes inferred at those sites, blue is positive selection ($dN \gg dS$), white is purifying selection ($dN \ll dS$), and black are sites indistinguishable from neutral evolution. Each site has a number corresponding to the homologous site in *Cypridina noctiluca* and two single-letter abbreviations for amino acids representing evolved changes at each site. Species names or identifying codes are as in Table S2. Country of origin abbreviation in parentheses (Bz = Belize, Jp = Japan, Ro = Roatan)

178 (Kawasaki et al., 2012). Site 178 also varies in c-luciferases of living species (Figure 1), and notably differs in *Photeros*, the genus with higher λ_{max}. In addition to site 178, variation at sites 280, 375, and 404 shows significant correlation with λ_{max}. (Figure 1; Table 1). Our ANOVA also showed a significant interaction between sites 375 and 404. Although there are also other explanations, such covariation could be caused by persistent epistatic interactions (Yokoyama, Yang, et al., 2008).

Next, we examined patterns of dN:dS rates of mutation in these four sites from mutagenesis experiments that most strongly influence λ_{max}. Three sites that affect colour (178, 280, 375) did not reject the null model of neutral evolution. One site (404) had significantly lower dN:dS (FEL, $q = 0.0201$), indicative of purifying selection. Three of these sites (280, 375, 404) are fixed within courtship-signalling cypridinids, fixed within noncourtship species, but different between courtship-signalling and noncourtship cypridinids. These significant correlations between genotype and phenotype provide hypotheses for future mutagenesis studies that could test how different processes of molecular evolution, including diversifying and purifying selection, may have contributed to the diversification of biochemical functions that could be important for cypridinid signals (Figure 4).

3.6 | c-Luciferase and the diversification of functions (colour and light decay)

Comparison of c-luciferases and bioluminescence phenotypes suggests multiple evolutionary forces may have acted on one gene to diversify signals. We used candidate sites identified from mutagenesis experiments along with sites identified from HYPHY analyses (FEL, MEME) in different ANOVAs to better understand the relationship between patterns of selection, enzyme function and bioluminescent phenotypes (λ_{max} and light decay).

From FEL and MEME, we found two and 13 sites with dN:dS ratios consistent with pervasive or episodic diversifying selection ($dN > dS$), respectively (Figure 1). After FDR ($q \leq 0.05$) correction, only five sites under episodic diversifying selection confidently remained. However, FDR may be considered too conservative an approach for two reasons: (a) genes with an *a priori* expected relationship with a phenotype are philosophically different than those without, and (b) the null model of neutral evolution where p-values are sampled from, and therefore corrected by with an FDR, is rarely accurate for sites in a protein. As p- and q-values

represent the upper bound on the statistical evidence for a relationship between variables, we present both uncorrected and FDR-corrected results with the goal of generating hypotheses for future exploration.

Of these 13 sites identified by MEME, two under episodic diversifying selection are significantly correlated with changes in c-luciferase function. Our ANOVA results indicate that site 114 is significantly associated with a change in λ_{max} (ANOVA, $p = .0033$; MEME, $q = 0.0062$; Figure 1; Table 1). One site in particular, site 160, is correlated significantly with both λ_{max} (ANOVA, $p = .0053$) and light decay of c-luciferases (ANOVA, $p = .0032$), and may be under diversifying (positive) selection (MEME, $q = 0.0803$; Figure 1; Table 1). Two other sites (19, 232) were initially identified by our modelling approach as significantly related to light decay but these relationships dissolved with iterative removal of sites in the ANOVA.

4 | DISCUSSION

The genetic basis of species diversification remains understudied because species differences usually involve many genetic differences across species. At the same time, scientists know very few cases where a single gene contributes to important and extensive phenotypic variation across radiations of species (but see Yokoyama, 1997), which could provide tractable systems to study the molecular evolution of diversification. The bioluminescent signals of cypridinid ostracods provide a prime example of signals that vary in multiple parameters across species, and here we show variation in a single gene, c-luciferase, is associated with variation in phenotypes. Only two previously published c-luciferase genes existed in the literature and we report multiple new c-luciferases and biochemical functions from diverse cypridinid species. Our comparative analyses suggest various evolutionary forces acted on c-luciferase. In addition to some sites in c-luciferase that control colour evolving neutrally or under purifying selection, we found episodic diversifying selection on luciferases at amino acid sites to be correlated with changes in both colour and light decay rates. Beyond proposing that these candidate sites are important for the diversification of enzyme functions, we also hypothesize how organismal and biochemical phenotypes relate to each other during evolution of this system. As with most biological phenomena, variation in phenotypes could be directly under selection, nonfunctional, and/or influenced by phylogenetic or biochemical constraints.

4.1 | Selection may shape biochemical functions such as light decay

First, differences in molecular phenotypes could be shaped by natural and/or sexual selection. Patterns of variation in nonsynonymous:synonymous substitutions in c-luciferase sequences are consistent with selection and correlated to phenotypic

variation of biochemical properties of light production. We found at least one site (160) in c-luciferase evolving under episodic diversifying selection is correlated with the rate of decay of light. This is of particular interest because it is one of a handful of sites that differs between *P. annecohenae* and *P. morini*. Regardless of strong similarity in c-luciferases between these two species, their enzymes have dramatic differences in measures of decay rate (Figure 1) that are related to duration of courtship pulses (Hensley et al., 2019). Although these substitution patterns suggest selection acting at the molecular level, the selective forces that influence rates of light decay at the organismal level are uncertain because there are very few experiments on the fitness effects of different ostracod signals (Rivers & Morin, 2013). We hypothesize that pulse duration, in part dictated by enzyme function (Hensley et al., 2019), may be important for fitness via interspecific antipredator displays and/or through mate recognition or choice. Consistent with this hypothesis, there is extensive variation among species, with pulse durations in courtship signals ranging from ~0.15 to 9 s (Cohen & Morin, 2010; Hensley et al., 2019). Because the relationship between enzyme kinetics and pulse duration varies across species (Hensley et al., 2019), selection on single sites associated with changes in c-luciferase function may be more episodic.

In addition to sexual selection, c-luciferase kinetics could also be under selection at the organismal level due to changes in environmental factors such as temperature, pH or salinity. Cypridinid luciferase is secreted externally and bioluminescent ostracods are globally distributed, so environmental factors affecting enzyme function could vary widely. Indeed, previous *in vitro* expression of *C. noctiluca* luciferase showed temperature-dependent differences in activity (Nakajima et al., 2004; Yasuno et al., 2018). Increased sampling of taxa living in different habitats, including deep-sea cypridinids, could allow testing of varying conditions and the role that adaptation may play in constraining or facilitating changes in rates of light decay.

4.2 | Drift and phylogeny may shape phenotypic evolution of colour

Another possibility is that variation in some bioluminescent functions are selectively neutral, which seems to be true for colour, including emission width (FWHM) and perhaps λ_{max}. We see no clear pattern in variation of FWHM, and no correlation of this parameter with any positively selected sites in c-luciferase. Although we do not yet have good candidate mutations for the genetic basis of FWHM, we do have such candidates for λ_{max} thanks to previous mutagenesis experiments (Kawasaki et al., 2012). One site (178) strongly affects λ_{max} and has a dN:dS ratio indistinguishable from 1, consistent with a minimal impact on fitness, suggesting it evolved neutrally. In contrast, three sites (74, 114, 160) are correlated with changes in λ_{max} and under positive selection, which could indicate selection drove some changes in colour. The patterns of amino acid differences in these selected sites also show differences between noncourtship

and courtship-signalling species. At the organismal level, λ_{max} is nonrandom with respect to phylogeny (Figures 1 and 3) and we observe small evolutionary shifts in λ_{max} between noncourtship and courtship-signalling species, and separately, along the branch leading to the genus *Photeros*. If the colour change between noncourtship and courtship-signalling species is robust to greater taxon sampling, it could be adaptive, perhaps linked to environmental differences and/ or differences in predator vision. In contrast, the colour change in *Photeros* does not seem adaptive and could be neutral. Because *Photeros* are among the few signalling species that live in seagrass (Morin, 2019), the "reflectance hypothesis" (Endler, 1992) would predict the ancestor of *Photeros* underwent an adaptive shift to greener signals to increase signal efficacy with a correlated shift to grass bed habitat. However, even *Photeros* species that live in nongrass bed habitats (e.g., *P. morini*) have green-shifted λ_{max} (Figures 1 and 3). Furthermore, an adaptive green-shift is not supported by patterns of substitution in c-luciferase because none of the sites putatively under positive selection are fixed in *Photeros*, which is also different from non-*Photeros* species. Finally, despite many amino acid changes, we did not find evidence of positive selection along the branch leading to *Photeros*. If λ_{max} and FWHM are in fact neutral for organismal fitness, other factors such as constraints could instead dictate the changes we observed in colour, especially λ_{max}.

4.3 | Phenotypic change may be genetically constrained

Biochemical constraints such as pleiotropy could influence the evolution of bioluminescent phenotypes. One site in c-luciferase (160) may be under positive selection and is correlated with both λ_{max} and light decay. This suggests a possible role of pleiotropy in phenotypic evolution if mutations in c-luciferase affect multiple phenotypes at once. However, counter to a pervasive role for pleiotropy throughout the entire history of cypridinid luminescence, we do not find a strong relationship between λ_{max} and light decay constant across all species in our study (Figure S5). Instead, all *Photeros* have similar λ_{max} while rates of light decay vary considerably between those species. Still, we cannot fully rule out biochemical constraint as a driver for the evolution of emission spectra because such constraints may have changed during evolution, for example during the shift in λ_{max} in early *Photeros*. If so, modern *Photeros* could have biochemical constraints that differ from ancestral species, now allowing rates of light decay to change independently of λ_{max}. Testing an evolutionary change in biochemical constraint would entail extensive mutagenesis and expression experiments guided by reconstructing the history of *Photeros* luciferase.

Constraints may also arise from epistatic interactions among sites to structure phenotypic differences between species. Although we lack statistical power to exhaustively cover all site-by-site interactions, we found a significant interaction in our ANOVA between sites 375 and 404 (Table 1). As enzymatic phenotypes such as colour and/or light decay evolve, previous changes at certain sites (such as 404) may influence the magnitude of functional changes due to new

mutations. In sea fireflies, we find a natural pattern of replacement from arginine (N) to aspartic acid (D) residues between noncourtship and courtship-signalling cypridinids at site 404. In the luciferase of the noncourtship signalling species *C. noctiluca*, site 404 is part of a *N*-glycosylation site (Mitani et al., 2017), and mutagenesis from N to D residues decreases relative light production (Yasuno et al., 2018). The magnitude of this decrease is also epistatic with both sites 182 and 184 (both part of the same *N*-glycosylation site; Yasuno et al., 2018). Although site 404 does not appear to be part of a putative glycosylation site in c-luciferases of courtship-signalling species, these results indicate epistatic effects on protein functions in some species. It is possible that site-specific epistatic interactions changed during the evolution of c-luciferases, as in other proteins (Ortlund et al., 2007; Yokoyama et al., 2014).

5 | CONCLUSIONS

We believe our results represent potentially important functional sites in a single gene—some verified via previous mutagenesis experiments—that impact phenotypic diversity. Although some methods to detect diversifying selection are sensitive to false positives, we remain confident that our conservative estimates of function are promising. First, we provide evidence of which sites may or may not be impacted by MNS, one source of false inference (Table 1; Figure S3). While some have most likely been impacted by at least two mutations, others remain confidently as single mutations that influence organismal phenotypes (colour or kinetics). On older genes or across wider scales of phylogenetic diversity, these models may not be adequate to estimate the substitution process because of the time for increased numbers of mutations to arise and fix. And although MNS may increase false positives at sites assuming single hits, this does not mean that sites with true MNS may not be under positive selection as well. Without more complex molecular models, we cannot account for these biological possibilities.

Second, we agree with others (Murrell et al., 2012; Yokoyama, Tada, et al., 2008) that a model of pervasive selection at any single site is not expected *a priori* in many biological scenarios, as well as in our system. Numerous forces may act to drive functional evolution of c-luciferase across species, and besides purifying selection acting to maintain critical light-production function, none of these regimes are expected to act towards the same phenotypic optima necessarily. As such, selection may be more transient on sites than strict models may be able to detect, as has been found in functionally verified opsins (Murrell et al., 2012). Further understanding of how site-specific changes alter enzyme function will clarify their roles in the evolution of signal diversity.

Although it is intriguing to seek direct parallels between the molecular evolution of c-luciferase and other bioluminescent systems, we urge caution for a number of reasons. First, bioluminescence in cypridinid ostracods has independently evolved from other, more well-known cases such as terrestrial insects (fireflies), cnidarians, or even other crustaceans (Haddock et al., 2010; Lau & Oakley, 2020).

In many cases, the molecular underpinnings of these bioluminescent reactions are unknown or only characterized from a few species, which prevents studies looking at patterns of molecular evolution and diversification, although this trend is slowly being reversed (Bessho-Uehara et al., 2020). For example, besides ostracods, the best systems for comparison are either fireflies (Viviani, 2002) or copepods (Markova et al., 2019; Takenaka et al., 2013), neither of which use the same biochemical substrate to ostracods or to one another. These mechanistic differences make comparisons across wide taxonomic scales difficult and potentially erroneous if not carefully approached. With more study, ostracods are one such system where we can begin to ask questions of molecular evolution thanks to shared biochemistry paired with high diversity.

Taken together, our results are consistent with a hypothesis that molecular evolution of c-luciferase impacted the diversity of signal phenotypes in species of sea fireflies. These results further suggest the potential for varied interactions between molecular function, evolution and phenotypes during diversification—even when considering a single gene such as c-luciferase. When extrapolated to other genes, such as the presumably many genes affecting behavioural phenotypes and in different environments, the possibilities of new combinations become enormous, providing a perspective on how life became so incredibly diverse. Such a pluralistic view of evolution, incorporating many different processes at different levels of organization (Seilacher, 1970; Tinbergen, 1963), allows for a more holistic understanding of how biodiversity originates. Cyprinid luciferases, whose molecular phenotypes impact organismal phenotypes and can be measured during *in vitro* expression, provide great opportunities for studying the genetic basis of such phenotypic diversification.

AUTHOR CONTRIBUTIONS

N.M.H. designed experiments, performed research, analysed data and wrote the paper. E.A.E. designed experiments, collected emission spectra data, and collected and analysed transcriptomes. N.Y.L. and D.A.T. collected mammalian cell culture data, and N.Y.L. also generated cDNA libraries for cloning. J.C. generated yeast fusion constructs. A.M. designed emission spectra experiments and assisted with data collection and quality control. M.T. and D.F.G. contributed resources for transcriptome generation and analysis. A.W.D. provided resources for mammalian cell cultures. Y.M. provided oversight and resources to yeast culture expression. T.J.R., G.A.G. and E.T. provided resources, and collected specimens and morphological data. T.H.O. provided resources, designed experiments, analysed and curated data, and wrote the paper. All authors contributed to editing.

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SUPPORTING INFORMATION

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