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Firefly genomes illuminate the evolution of beetle bioluminescent systems

Yuichi Oba[1] and Darrin T Schultz[2]

Fireflies are one of the best-known bioluminescent organisms, and the reaction mechanism and ecological utility of bioluminescence have been well-studied. Genome assemblies of six species of bioluminescent beetles have recently been published. These studies have focused on the evolution of novelties; luciferase, and the biosynthesis of luciferin and defensive chemicals. For example, clustering of the luciferase gene with acyl-CoA synthetase genes on a chromosome in luminous beetle genomes suggests the involvement of tandem gene duplications and neofunctionalization during the evolution of beetle bioluminescence. Several candidate genes for critical roles in beetle bioluminescence have been identified, but their functional analyses are still ongoing. The establishment of a long-term mass-rearing system and strain will be the key for the post-genome research on bioluminescent beetles. Lastly, the application of contemporary chromosome-scale genome assembly techniques to luminous beetles will help resolve outstanding evolutionary questions, such as how many times bioluminescence evolved in this clade.

Addresses
[1] Department of Environmental Biology, Chubu University, Kasugai 487-8501, Japan
[2] Monterey Bay Aquarium Research Institute, Moss Landing, CA 95039, United States

Corresponding author: Oba, Yuichi (yoba@isc.chubu.ac.jp)

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Introduction

Genomics research on bioluminescent organisms is still at an early stage. However, there is much to gain from studying the genomes of luminous organisms, such as the potential for developing novel bio-imaging tools from luciferase genes and luciferin biosynthetic enzymes, and the possibility of studying bioluminescence genes as a model of evolutionary gene co-option [1] or in the evolution of novelty. Whole genome sequences have been reported in some bioluminescent animals: the ctenophore *Mnemiopsis leidyi* [2], the cnidarian sea pansy *Renilla* spp. [3], the larvaceans *Oikopleura* spp. [4,5], and the acorn worm *Ptychodera flava* [6]. However, these works were motivated not by bioluminescence, but primarily by an interest in these species' phylogenetic positions, body plans, and development. The luciferases are known in the ctenophore *Mnemiopsis* and in the cnidarian *Renilla*, but not in *Oikopleura* or *Ptychodera*. The genome sequences of several luminous mushroom species have also been published [7]. While fungi are not metazoans, the genome sequences were used to help find the bioluminescence-related genes. The fungal luciferase gene *luz* was found to be only several kilobases away from luciferin biosynthesis and recycling genes [7]. These genes have since been used to generate self-luminescing plants [8].

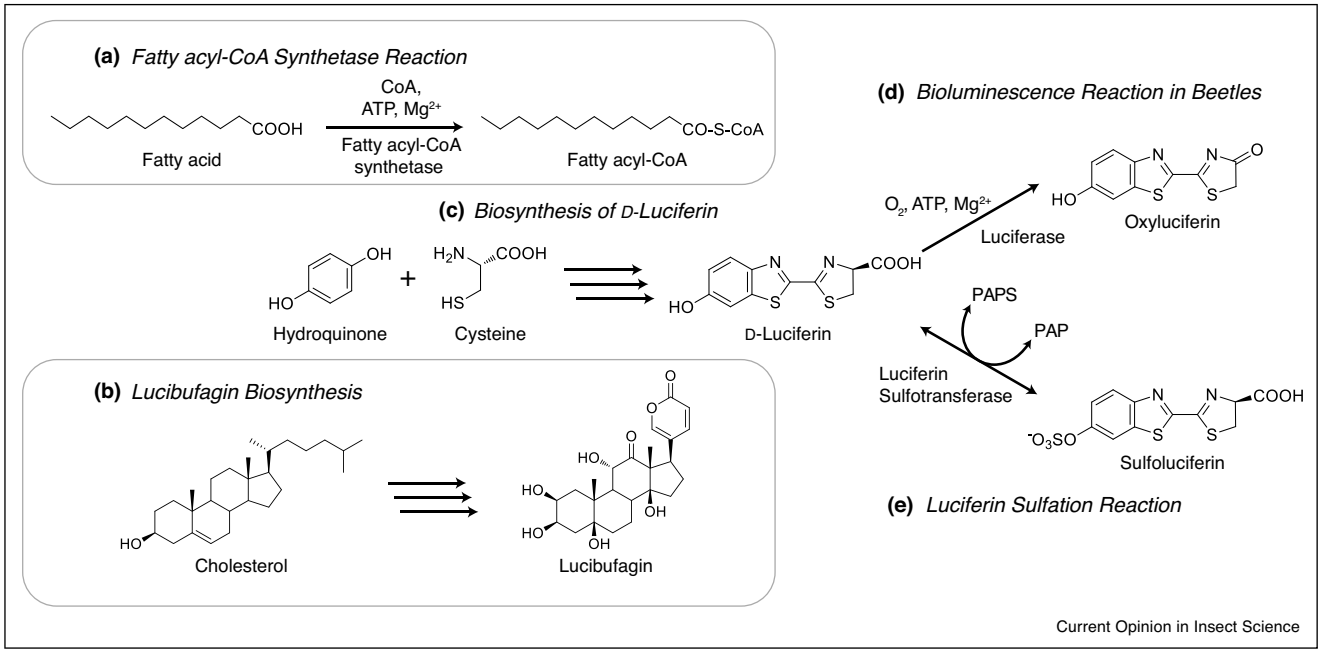
In insects, most genome sequencing has been performed on model organisms, agricultural pests, or pathogen vectors. Because bioluminescent beetles do not fit any of those categories, they have only recently become targets for genomic research.

Beetle bioluminescence

Fireflies are iconic bioluminescent organisms. They appear in the yards of residential neighborhoods, along riverbanks, and in forests. Children enjoy collecting fireflies, and firefly watching is a tradition in many cultures. The mechanism of bioluminescence in fireflies and other luminescent beetles has been studied for more than 130 years. In 1885 and 1886, a French physiologist, Raphaël Dubois, demonstrated the molecular basis of bioluminescence in a now-historical experiment. He prepared both hot water and cold water extracts from the photophores of the Caribbean luminous click beetle, *Pyrophorus*, and based on this experiment and further experiments using a luminous bivalve *Pholas dactylus*, he established the concept that bioluminescence is an enzymatic oxidation reaction of the molecular substrate. He later defined the enzyme catalyzing the bioluminescence reaction as the general term 'luciferase', and the substrate oxidized by luciferase is called by the general term 'luciferin' [9–11].

Firefly luciferase and luciferin were crystallized seventy years later [12,13]. The chemical structure of firefly luciferin was determined (Figure 1d) [14,15]; and the firefly luciferase protein was isolated and cloned [16–18]. These studies were performed using the big dipper firefly common to the eastern United States, *Photinus pyralis*. Later, it was found that all firefly species (Lampyridae) share

Figure 1



Biochemical reactions studied from luminous beetle genomes.
Schematic representation of **(a)** the fatty-acyl-CoA synthetase reaction, **(b)** the biosynthesis of the defensive steroid lucibufagin, **(c)** the biosynthesis of firefly luciferin, **(d)** the bioluminescence reaction of beetle luciferin, and **(e)** the firefly luciferin sulfation reaction.

homologous luciferases, and the same luciferin, with the luminous beetles in the families Phengodidae, Rhagophthalmidae, and Elateridae [19]. All bioluminescent beetles share a common bioluminescence mechanism: they use same luciferin compound, D-luciferin, and a highly homologous luciferase (>40% amino acid identity) with O_2, ATP and Mg^{2+} (Figure 1d). Only one genus of bioluminescent beetle has a completely unknown bioluminescence system, the genus *Sinopyrophorus*. However, as the family Sinopyrophoridae is closely related to the families listed above, it is likely *Sinopyrophorus schimmeli* use the same luciferin and luciferase as in other beetles. Luciferase genes have been cloned from over 30 species of fireflies, phengodids and rhagophthalmids, and the difference in bioluminescence color, from green to orange, is determined by the amino acid sequence of the luciferase [20].

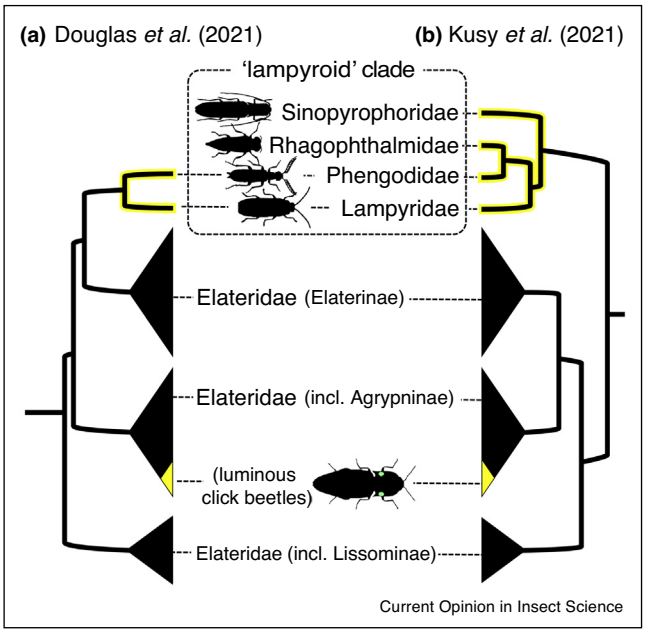
Physiological, ecological, and behavioral studies have also been performed in luminous beetles. For example, the blinking mechanism of firefly luminescence is fed by the oxygen supply from the trachea to the peroxisomes where luciferin and luciferase are situated in photocytes [21]. Chemical defense using a toad toxin-like steroid, lucibufagins, were discovered in various firefly species (Figure 1b) [22]. The courtship behavior of fireflies and the unique aggressive mimicry in firefly 'femmes fatales' have also been extensively studied [23–25].

Luminous beetle phylogenomics

Bioluminescent beetles are currently known from five families: the Lampyridae, Phengodidae, Rhagophthalmidae, Elateridae, and Sinopyrophoridae. The Lampyridae (fireflies, glowworms, ~2000 species recorded in the world), Phengodidae (~270 species), and Rhagophthalmidae (~60 species) form the 'lampyroid' clade (Figure 2). It is thought that all species in this clade are bioluminescent at least during the larval stage [26•]. In contrast, the family Elateridae (click beetles) comprises over 10 000 species [27], but relatively few elaterid species are bioluminescent. The elaterid subfamily Agrypninae contains at least 200 luminous species, and the subfamily Campyloxeninae contains a single species known to be luminous, *Campyloxenus pyrothorax* [28].

Recently, a newly described luminous click beetle species, *S. schimmeli*, was reported to be a member of the Elateridae [29]. However, a more recent phylogenomic analysis using genomic data showed that, instead, *Sinopyrophorus* is the sister clade to the lampyroid clade [26•]. Based on this finding, a new family Sinopyrophoridae was established for this single species [26•]. Other phylogenomic results suggest that the lampyroid clade is placed within the click beetles, therefore fireflies are click beetles [30•]. Phylogenomic approaches now challenge the existing morphology-based systematics of

Figure 2



Luminous beetle phylogenomics.
The phylogeny of the Lampyridae and Elateridae is not completely resolved, however all luminous fireflies and click beetles share a single common ancestor (clade shown in red). Luminescence is thought to have evolved twice in the beetles: once in the lampyroids and once in click beetles (yellow highlights).

bioluminescent beetles, and have forced a reconsideration of the evolution of bioluminescence in beetles (Figure 2).

Genomic sequencing of luminous beetles

The first luminous beetle genome was reported in 2017 [31]. The authors assembled the genome of a single female adult firefly *Pyrocoelia pectoralis*, subfamily Lampyrinae, mainly for the purpose of firefly conservation [31]. The genome assembly had a high N50 (3.04 Mb), however the assembly was not chromosome-scale. This species is terrestrial and consumes land snails, suggesting that this species could be employed as a possible biocontrol agent against land snails [31].

Two additional firefly genomes, and one luminous click beetle genome, were published in 2018 [32••]. The firefly species were the North American terrestrial firefly *P. pyralis* (subfamily Lampyrinae), the Japanese firefly *Aquatica lateralis* (subfamily Luciolinae), and the Caribbean luminous click beetle *Ignelater luminosus* (family Elateridae, subfamily Agrypninae). The genome assembly of the American firefly *P. pyralis* is chromosome-scale (N50 47.02 Mb), however the genome assemblies of *A. lateralis* (N50 0.69 Mb) and *I. luminosus* (N50 0.10 Mb) are not (Table 1).

The firefly *P. pyralis* is the most common American endemic firefly, and uses luminescence for aposematic warnings as larvae, and for sexual communication as adults. *A. lateralis* is one of the most common firefly species in Japan. The larvae, as suggested in the genus name *Aquatica*, live in bodies of water, such as rice paddies and marshes. Like the American *P. pyralis*, *A. lateralis* produces light for both aposematic and sexual displays. Both firefly species possess large double light organs at the 6th and 7th abdominal segments in adult males, or a single light organ in the 6th abdominal segment in adult females. Lastly, adult *I. luminosus* click beetles possess a pair of oval light organs on the dorsal surface of the prothorax and a single ventral light organ between the metathorax and abdomen. The bioluminescent behavior of luminous click beetles is not well documented, but click beetle bioluminescence is thought to be used in courtship behaviors [33].

The most essential finding in the analysis of the firefly genomes are that fireflies possess two luciferase genes, called Luc1-type and Luc2-type, and that Luc1-type luciferases are clustered with acyl-CoA synthetases tandemly on single chromosomes in each species [32••]. Firefly luciferases (both Luc1 and Luc2) have significant amino acid sequence similarity to fatty acyl-CoA synthetase in *Drosophila* (41%), and have acyl-CoA synthetic activity to some fatty acids (Figure 1a) [34]. Given the sequence similarity of firefly luciferases and acyl-CoA synthetases, and the proximity of these two genes in the firefly genomes, it was hypothesized that the firefly luciferase Luc1 evolved from an acyl-CoA synthetase (probably fatty acyl-CoA synthetase) after a tandem gene duplication (Figure 3a). Luc2 could have evolved by gene duplication and translocation (Figure 3a). Interestingly, the luciferase gene in the luminous click beetle genome also occurs on the same scaffolds as acyl-CoA synthetases, but the gene order of the cluster is different from that of fireflies. The size of the gene clusters in all three species is between 100–300 kb. The authors argue that click beetle luciferase may have instead evolved from an acyl-CoA synthetase after two tandem gene duplications, independently from the evolution of luciferase in fireflies. In short, the common ancestor of the lampyroid clade and luminous click beetles was non-luminescent and luciferase evolved independently in each clade. The authors also identified, in the *P. pyralis* genome, candidate genes for a specialized enzyme to produce a storage form of luciferin, luciferin sulfotransferase [35], and a lucibufagin biosynthetic enzyme [32••].

The fifth and sixth luminous beetle genomes were published in 2020 [36•]. The genomes sequenced were two fireflies from the People's Republic of China, *Lamprigera*

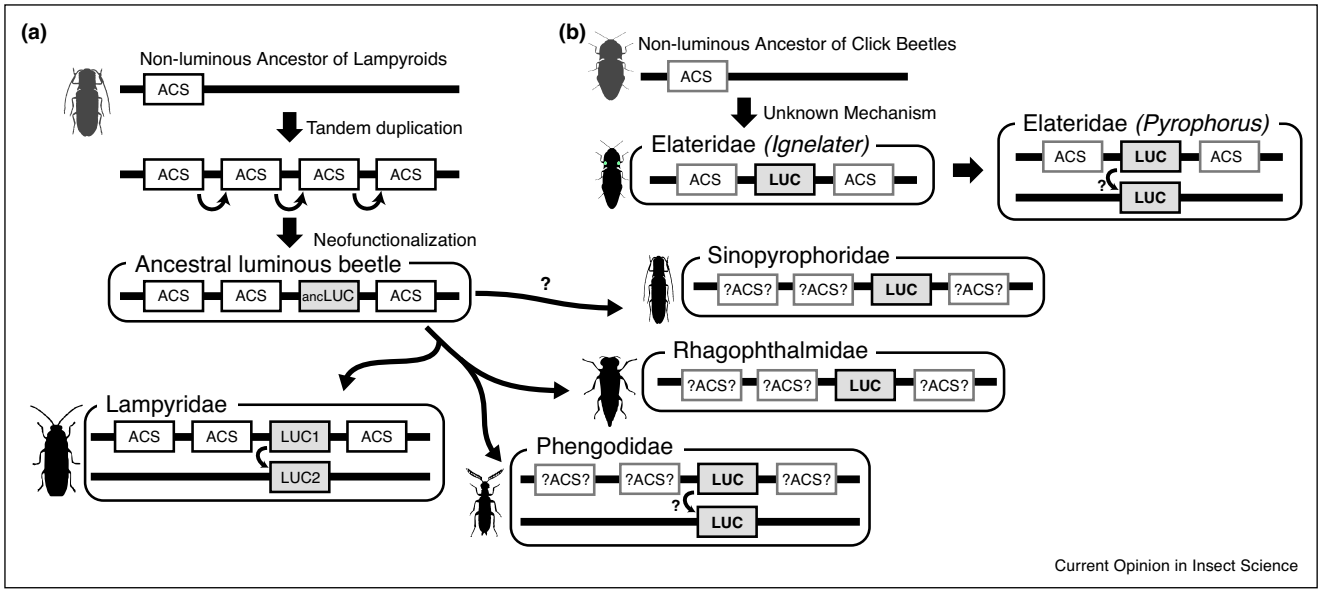
Table 1

Statistics of luminous beetle genome assemblies

Family (subfamily)	Species	Chromosome-scale assembly	Number of coding genes	Scaffold N50 (Mb)	Scaffold L50	Scaffold Count	Contig Count	Assembly Size (Mb)	Source
Lampyridae (Lampyrinae)	*Photinus pyralis*	Yes	15 773	47.02	5	2160	7909	471.51	[32••]
Lampyridae (subfamily unknown)	*Lamprigera yunnana*	No	19 443	3.51	79	2684	2684	1052.93	[36•]
Lampyridae (Lampyrinae)	*Pyrocoelia pectoralis*	No	23 092	3.04	79	474	474	760.42	[31]
Lampyridae (Luciolinae)	*Abscondita terminalis*	No	21 024	1.22	105	3187	3273	499.65	[36•]
Lampyridae (Luciolinae)	*Aquatica lateralis*	No	14 285	0.69	381	5365	32 698	908.42	[32••]
Elateirdae (Agrypninae)	*Ignelater luminosus*	No	27 557	0.10	1967	91 322	106 585	842.91	[32••]

Rows are sorted by the scaffold N50.

Figure 3



Luciferases evolved at least twice in beetle genomes.
Genome sequencing suggests that **(a)** luciferase (LUC) evolved in the lampyroids in a several step mechanism. In a nonluminous ancestor to the lampyroids, an ACS (acyl-CoA synthetase) gene underwent a tandem duplication on a single chromosome. The function of one ACS gene on that chromosome changed to a luciferase (neofunctionalization). Another duplication, and translocation, resulted in another luciferase gene on a separate chromosome. Because only the *P. pyralis* (Lampyridae) genome is chromosome-scale, it is unclear: how many luciferase genes are in the Phengodidae, Sinopyrophoridae, and Rhagophthalmidae species' genomes, whether ACS and luciferase genes reside on the same chromosomes in species of these families, and whether the luciferase duplication in the Lampyridae occurred at the common ancestor to the Lampyridae-Phengodidae-Rhagophthalmidae clade or independently. **(b)** Luciferase evolved independently from lampyroids in the click beetles. Because of fragmented genome assemblies, it is unclear whether there was a luciferase duplication in the genus *Pyrophorus*, and whether luciferase and ACS genes reside on the same chromosome.

yunnana (subfamily unknown) [37] and *Abscondita terminalis* (subfamily Luciolinae). Neither assembly is chromosome scale, however the contig N50 of the *L. yunnana* is high (3.51 Mb). Several findings from this study were related to bioluminescence. The authors found that the Luc1 gene and acyl-CoA synthetases occur on single scaffolds in both species. Based on the light organ-specific gene expression and the hypothetical biosynthetic pathway of firefly luciferin, they picked out candidate genes for luciferin biosynthesis (Figure 1c). They especially

focused on acyl-CoA thioesterase, which can convert an L-luciferin precursor to D-luciferin, the luciferin form used in the bioluminescence reaction. The authors showed the activity of recombinant firefly acyl-CoA thioesterase *in vitro*.

Fireflies as model organisms

Generally, the life cycle of firefly species takes between one to two years. However, the adult stage only lasts as long as a few days or weeks. Firefly larvae are carnivorous. Some species only eat live snails, and some species only eat live earthworms. In the Japanese *Luciola cruciata*, their aquatic larvae feed on a single species of freshwater snail *Semisulcospira libertina*. The *L. cruciata* pupate in soil, then as adults spawn on moss. Because of their complicated life styles and unbalanced diets, laboratory rearing of fireflies is considered to be difficult. For this reason, the firefly genome sequencing studies introduced above were performed using wild-caught specimens, except for *P. pectoralis* [31] and *A. lateralis* [32••].

The population of *A. lateralis* used for genome sequencing [32••] was established from several individuals collected from rice paddies in Yokohama, Japan, in 1989 and 1990. This population has been maintained for the past 31 years in laboratory aquaria at a stable 19−21°C without the addition of any wild individuals. Using this culturing system, a single laboratory could supply dozens of individuals at any life stage every few weeks. This culture appears to be the only inbred strain of firefly in the world. This strain, 'Ikeya-Y90', is named after the dedicated high school teacher from Kanagawa prefecture, Mr. Haruyoshi Ikeya, who established the culture and rearing techniques. Given these strengths, the firefly *A. lateralis*, and the laboratory strain 'Ikeya-Y90', is the most-established model firefly for post-genomic research. RNA interference (RNAi) and transient CRSPR/Cas9 have been used in lampyrid species [36•,38], suggesting that functional genetic assays could also be applied also to *A. lateralis*.

Concluding remarks

The genomes of 6 species of bioluminescent beetles have been sequenced and assembled (5 species in the Lampyridae and 1 species in the Elateridae), of which one assembly is chromosome-scale and reference quality (*P. pyralis*). These data revealed new details about the evolution of bioluminescence in fireflies and other luminous beetles, the biosynthesis of luciferin, the biosynthesis of beetle toxins, and more. However, many open questions remain that must be solved using chromosome-scale genome assemblies. For example, chromosomal macrosynteny analyses of luciferase gene-containing firefly and click beetle chromosomes will help clarify the evolutionary provenance of firefly luciferases. Namely, chromosome-scale genomes may help determine what evolutionary events lead to luciferase-acyl-CoA-synthetases colocalization on single chromosomes and duplication onto multiple chromosomes. Future luminous beetle genome sequencing projects would benefit from using contemporary proximity ligation-based genome scaffolding techniques, such as Hi-C [39,40]. One unresolved question that can also be answered with chromosome-scale genomes and karyotyping is whether luminous beetles have sex chromosomes, and the possible role of sex chromosomes in firefly sexual dimorphism. A small Y chromosome was detected in the firefly *A. lateralis* by karyotyping, and the chromosome constitution was found to be 2n = 14A + XY in males [41]. The presence of sex chromosomes is very rare in Lampyridae [42], therefore the determination of sex chromosomes in *A. lateralis* will useful for studying sexual dimorphism in fireflies.

The publication of more reference-quality luminous beetle genomes will accelerate post-genomic research in this clade. One next step in advancing our understanding of beetle luminescence includes confirming the enzymatic function of the candidate genes discovered by the genome analyses mentioned above. RNAi and genome editing will be possible in the future if the firefly strain Ikeya-Y90 is further developed as a model organism. Population resequencing of fireflies species with existing genome assemblies will be helpful to assess the most diverse populations for targeted conservation [43]. Local populations of many fireflies have declined rapidly by human activities, such as light pollution, agricultural chemicals, and water pollution especially for Asian aquatic fireflies. Given the diversity of open questions in luminous beetle biology, and the growing threat of species extinction or decline, the next several years will likely yield exciting advances in luminous beetle genomics.

Conflict of interest statement

Nothing declared.

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