# Firefly bioluminescence outshines aerial predators

Gareth S. Powell[1*], Natalie A. Saxton[1], Yelena M. Pacheco[2], Kathrin F. Stanger-Hall[2], Gavin J. Martin[3], Dominik Kusy[4], Ladislav Bocak[4], Marc A. Branham[5], Seth M. Bybee[1]

[1]Department of Biology and Monte L. Bean Museum, Brigham Young University, 4102 LSB, Provo, Utah, 84602, USA
[2]Plant Biology Department, University of Georgia, 403 Biosciences Building, Athens, GA, 30602, USA
[3]Department of Math and Sciences, Laramie County Community College, 1400 E. College Dr., Cheyenne, WY, 82007, USA
[4]Laboratory of Biodiversity & Molecular Evolution, Czech Advanced Technology Research Institute - CRH, Slechtitelu 27, Palacky University, 783 71 Olomouc, Czech Republic
[5]Department of Entomology & Nematology, University of Florida, P.O. Box 110620, Gainesville, FL 32611, USA
*corresponding author: garethpowell@byu.edu

***Abstract***

Bioluminescence is found across life and has many functions. Yet we understand very little about its timing and origins, particularly as a predator avoidance strategy. Understanding the timing between bioluminescence and predator origins has yet to be examined and can help elucidate the evolution of the ecologically important signal aposematism. Using the most prevalent bioluminescent group, fireflies, where bioluminescence primarily functions as aposematic and sexual signals, the timing for the origins of both potential predators of fireflies and bioluminescence is explored. Divergence time estimations were performed using a genomic-scale phylogenetic reconstruction Lampyridae, and multiple fossil calibration points, allowing for a robust estimate for the origin of beetle bioluminescence as both a terrestrial and aerial signal. Our results recover the origins of terrestrial beetle bioluminescence at 141 mya and aerial bioluminescence at 133 mya. These ages predate the origins of all known extant aerial predators (i.e., bats and birds) and support the much older terrestrial predators (frogs, ground beetles, lizards, snakes, and hunting spiders) as the most likely drivers of bioluminescence in beetles.

***Keywords***
Aposematism, predation, divergence time estimation, phylogeny, Lampyridae

***Introduction***

Bioluminescence has evolved independently almost 100 times across both eukaryotes and prokaryotes (e.g., insects, crustaceans, other marine invertebrates, fish, protists, fungi, and bacteria; Hastings and Nealson, 1977; Herring, 1987; Haddock et al., 2010; Wilson and Hastings, 1998; Widder, 2010; Lau and Oakley, 2021). The function of bioluminescence varies across organisms including: prey attraction (Lloyd, 1975; Redford, 1982; Faust et al., 2012; Souto et al., 2019), predator avoidance (Grober, 1988; Marek et al., 2011), counterillumination (Jones and Nishiguchi 2004; Martini and Haddock 2017), sexual communication (Lloyd, 1983; Herring, 2007), and spore dispersal (Desjardin et al., 2008; Oliveira et al., 2015). In the terrestrial environment, bioluminescence is principally used as an aposematic signal (Porter and Porter, 1979; Grober, 1988; De Cock and Matthysen, 2001, 2003; Marek et al., 2011; Stanger-Hall and Oakley, 2019).

While Lampyridae (fireflies) are perhaps the most well-known group of bioluminescent beetles, there are four additional extant families that contain bioluminescent species: Elateridae, Sinopyrophoridae, Phengodidae, and Rhagophthalmidae. All known larvae in the Lampyridae, Phengodidae, and Rhagophthalmidae are bioluminescent (Branham and Wenzel, 2001), suggesting larval bioluminescence is the ancestral state for each group or their common ancestor. These larvae are also largely terrestrial. Oba et al. (2020) timed the origin for luciferase at ~102.55 MY using seven taxa and a single gene tree (18S). Larval bioluminescence in fireflies of larvae likely originated as a predator avoidance strategy (Branham and Wenzel, 2003; Martin et al., 2017; Oba et al., 2020; Sivinski, 1981). Bioluminescence allows ground dwelling invertebrates to advertise chemical defenses to potential predators (De Cock and Matthysen, 2001, 2003; Marek et al., 2011). For example, Underwood et al. (1997) found that predators learned to avoid distasteful prey when it was associated with light, ultimately resulting in 100% predator avoidance. This demonstrates the effectiveness of bioluminescence, when accompanied by chemical defenses, as a strong aposematic signal.

Herein we consider larval and larviform or wingless (apterous or brachypterous) females as terrestrial bioluminescence. Adult bioluminescence in fireflies was co-opted for sexual communication in the form of complex aerial bioluminescent courtship displays (called aerial bioluminescence going forward), usually by male fireflies (Branham and Wenzel, 2003). However, there is evidence that adult bioluminescence also accelerates avoidance learning and functions as an aposematic signal for aerial predators (*i.e.* bats; Moosman et al., 2009; Leavell et al., 2018).

Potential predators contributing to the origins of terrestrial and aerial (above-ground) bioluminescent signals in fireflies, would have to predate or coincide with the respective origins of terrestrial or aerial bioluminescence. The origin and evolution of terrestrial bioluminescence as an aposematic signal would have been driven by terrestrial predators (e.g., carabid beetles, spiders, amphibians, reptiles, and rodents; Lloyd, 1973). Similarly, in order for aerial bioluminescence to arise as an aposematic signal, aerial predators (e.g., bats, dusk/night active birds; Dillon, 1967; Lloyd, 1973) would have to predate or coincide with the origin of aerial bioluminescence displays. Despite the modern interactions between fireflies and predator groups, none of these predators can yet be attributed to the origin of bioluminescence, terrestrial or aerial, as an aposematic signal in beetles.

Estimating the origins of bioluminescence in fireflies (and close relatives), in relation to the origins of potential predators, is central to understanding the ecology and evolutionary adaptations of bioluminescence as an aposematic visual signal. Here, we take advantage of four described fossils, including one recent elateroid fossil representing the oldest known bioluminescent beetle fossil (see *Fossil selection* below). These fossils, combined with a recent phylogenomic-scale phylogeny in Lampyridae (Martin et al., 2019), allow for the most robust estimate of the origin of firefly bioluminescence to date. We report potential terrestrial and aerial predator groups at the origins of both terrestrial bioluminescence and aerial bioluminescence in beetles. The results shed light on potential selective agents for firefly bioluminescence, ultimately giving rise to some of the most captivating displays seen in the terrestrial environment.

## ***Material and methods***

### *Dataset for divergence time estimation*

Bayesian divergence time estimation combines phylogenetic hypotheses with prior knowledge of molecular clocks and/or the fossil record allowing for investigations into the time of origin for organisms and their ecological innovations (Drummond and Rambaut, 2007; Peters et al., 2017). Fossil calibrations are known to have significant impact on divergence time estimation depending on their placement and the age distribution provided (Saladin et al., 2017; Toussaint et al., 2017). Recently described fossils (e.g., Li et al., 2021) and a phylogenetic reconstruction of bioluminescent beetles (Martin et al., 2020) provides us with the opportunity to explore the time of origin for both terrestrial and aerial beetle bioluminescence with greater confidence than ever before. We used an existing large-scale phylogeny to estimate the age for the origin of bioluminescence. Tree files were obtained from Martin et al. (2019), who focused on sub-familial placement and tribes within Lampyridae.

*Fossil selection*

We placed a total of four fossils across the topology (see Table 2). Other described fossils were not included (e.g., Heer, 1849; Wickham, 1912) when older fossil representatives were used. Fossil calibrations were placed on topologies based on morphological similarities with extant taxa discussed within the literature and their currently proposed placements. We placed one fossil at the base of Luciolinae (*Protoluciola albertalleni*; Kazantsev, 2015) and one at the node Phengodidae + Rhagophthalmidae (*Cretophengodes azari;* Li et al., 2021). We also placed a fossil at the base of the subfamily Lamprohizinae (*Lamprohiza fossilis;* Beier 1952; Fanti, 2017), and a fourth at the base of an extant genus (Photinus), using the fossil *Photinus kazantsevi* (Alekseev, 2019).

We obtained relative ages for each fossil from the age of each deposit provided by Paleobiology Database (Clapman, 2020). When using fossil specimens as calibration points, the relative ages of those deposits can be used as "hard" minimums as there is no need to sample ages younger than the fossil evidence being used; maximum ages are "soft" allowing the divergence time estimation to sample ages older than that proposed maximum, but do so by decreasing the probability of those ages as they get further from that maximum age (Ho and Phillips, 2009). Exponential fossil priors with hard minimums were placed on the following groups across both topologies; (*Lamprohiza* + *Phausis*), Luciolinae, *Photinus*, and (Rhagophthalmidae + Phengodidae). Soft maximum ages for the subfamily and family level fossils were placed at 223 mya based on the oldest known Polyphagan beetles (Chatzimanolis et al., 2012). We also used a soft maximum for the *Photinus* calibration, set to the age of the oldest known Lampyridae (99 mya) (Li et al., 2021).

*Divergence time estimation*

We used *chronoPL* implemented in the R package APE (Paradis and Schliep, 2019) to give relative node ages to the Martin et al. (2019) topology to ensure our starting node ages were within the bounds of the fossil prior distributions for subsequent analyses (Brewer et al., 2012; Huie et al., 2020). The resulting ultrametric trees from *chronoPL* were used as fixed topologies in subsequent Baysian analysis to estimate divergence times (Bouchaert et al., 2019). BEAUTi (Bouchaert et al., 2019) was employed to set analysis parameters such as fossil placement and tree models. Multiple combinations of tree and clock models were used to check the sensitivity to model choice. To estimate a rooted time-calibrated phylogeny we used BEAST v.2.6.0 (Bouckaert et al. 2019) via the CIPRES Science Gateway v.3.3 (www.phylo.org). Analyses were run with a chain length of 200,000,000, and to ensure convergence of our analyses the log file for each analysis was viewed in Tracer v.1.7.1 (Rambaut et al., 2018). ESS values >200 were

considered sufficiently converged. Lastly, TreeAnnotator v.1.10.4 (Bouckaert et al., 2014) was used to generate consensus trees after a burn-in of 10% (20,000,000) was discarded.

*Ancestral state reconstruction*

Terrestrial and aerial bioluminescence was coded for the extant taxa in our phylogeny based on whether a light organ was present/absent in the terrestrial larvae (or larviform females) and/or the aerial adults respectively. The resulting matrix was analyzed in Mesquite v.3.6 using both parsimony and maximum likelihood ancestral state reconstructions (Maddison and Maddison, 2019).

*Potential predators*

If beetle bioluminescence arose to advertise a chemical defense in terrestrial, firefly larvae then there must have been predation pressure by terrestrial predators selecting for this trait. Adult bioluminescence is used during aerial displays (often by males), and these aerial bioluminescent individuals are preyed upon by aerial predators. Terrestrial and aerial animal groups (e.g., insectivores) known to prey on fireflies were compiled from Day (2011), Lloyd (1973) and authors' observations (see results). Extinct insectivorous groups (e.g., Pterosauria) were also considered. To assess the potential role of each predatory group in the origin of beetle bioluminescent displays, predator group ages were compared with our estimates for the origin of terrestrial and aerial bioluminescence. The ages of each firefly predator group were gathered from the literature (Coddington and Levi, 1991; Evans, 2003; Teeling et al., 2005; Pyron, 2011; dos Reis et al., 2012; Prum et al., 2015; Toussaint et al., 2017)

***Results and Discussion***

Terrestrial bioluminescence is an ancestral trait in Lampyridae (and their relatives: Phengodidae, and Rhagophthalmidae) preceding the origin of adult aerial bioluminescence (Figure 1 & Supp Figure 1) that is used widely as a sexual signal in modern fireflies (Branham et al., 2001; 2003; Stanger-Hall and Lloyd 2015). Our results recover the terrestrial bioluminescence at ~141.17 (122.63–161.17) mya. This was followed by the origin of aerial bioluminescence at ~133.18 (117.86–152.47) mya (Figure 1).

*Divergence time estimation*

Accurate divergence time estimation relies on a combination of broad taxon sampling, an accurate phylogenetic reconstruction, and sufficient breadth and depth of fossil sampling (Lemmon et al., 2009; Heath et al., 2008; Saladin et al., 2017; Toussaint et al., 2017). The limitations of the fossil record, reliable placement of extinct taxa, and immense extant diversity for a group like Elateroidea (21,000 described species), have led to continued confusion and disagreement for the ages of these groups.

Previous estimates for the origin of Elateroidea ranged from 220–130 mya, and estimates for the origin of Lampyridae ranged from 130 –75 mya (Bocak et al., 2016, McKenna et al., 2015). These large ranges were likely due to limited taxon sampling, for both ingroup lampyrids and fossil calibration points used. For example, Bocak et al. (2016), hypothesized the age of Elateroidea to be ~220 mya and Lampyridae as ~130 mya based on two extant firefly taxa and a single fossil calibration point (15 gene dataset). Similarly, McKenna et al. (2015) estimated the age of Elateroidea to be ~130 mya and Lampyridae to be ~78 mya, with a dataset of four firefly taxa (eight gene dataset). In a subsequent analysis McKenna et al. (2019) reconstructed a phylogeny of Coleoptera and estimated the of Elateroidea to be ~187 mya and ~120 mya for the branch leading to Lampyridae, however, this study included only a single lampyrid species and no internal elateroid fossil calibration, instead utilizing an ancestral click beetle at the base of Elateroidea+Byrroidea (4800 gene dataset).

Our analyses based on Martin et al. (2019) with 436 loci, 88 firefly species (98 taxa total), and four fossil calibrations across the topology recovered a root age for Lampyridae of 133 mya. The divergence time estimates for Lampyridae are older than previously published estimates. McKenna et al. (2015, 2019) had limited lampyrid and broader elateroid taxon sampling with limited fossil calibrations that resulted in much younger ages for Lampyridae, and Coleoptera overall, which was already noted by others (Gunter et al., 2016; Toussaint et al., 2017; Che et al., 2021). Our age estimates are closely aligned to the estimates of Bocak et al. (2016), which is likely due to their larger elateroid sampling and ingroup fossil calibration (e.g., *Elaterophanes*). With updated clade ages, and the species-level biological information gathered for bioluminescence across our phylogeny, we can now examine the validity of previous hypotheses relating to predation and firefly bioluminescence.

*Age of bioluminescence in beetles*

Clade ages for bioluminescence were largely congruent between all parameter combinations implemented with minor variations in estimated ages reported between the different clock and tree models (Supp. Table 2). The Yule model does not account for any extinction and elateroid beetles have experienced at least two global extinction events, thus we have focused our discussion going forward on the ages resulting from Birth-Death tree models. The age estimates for each clock model combination largely overlapped for the Birth-Death models, here we discuss the relaxed lognormal clock model here, which has been shown to more precisely estimate ages and is widely used across the discipline (Drummond et al., 2006; Lepage et al., 2007). Our ancestral state reconstruction placed the origin of terrestrial bioluminescence (aposematism) ~141.17 mya, during the early Cretaceous period, followed by the origin of aerial bioluminescence (sexual communication in extant fireflies), estimated to be ~133.18 mya in the common

ancestor of fireflies. Terrestrial bioluminescence is an ancestral trait in Lampyridae and relatives (Phengodidae, and Rhagophthalmidae) preceding the origin of adult aerial bioluminescence that is used widely as a sexual signal in modern fireflies (Branham et al., 2001, 2003; Stanger-Hall and Lloyd, 2015). Assuming that the original function of bioluminescence was predator deterrence, it begs the question as to what predator, or predators, could have driven the evolution of firefly bioluminescence.

*Predators*

Clade ages for potential predators that fireflies likely encountered during their early history, that would have contributed to the origin of bioluminescence as an aposematic signal, were compiled. We identified potential groups of firefly predators that were hypothesized to feed on these groups and prevalent and broad enough in distribution to potentially function as significant firefly predators. Terrestrial predators included: Araneae (spiders) (Coddington and Levi, 1991), Carabidae (carabid beetles) (Toussaint et al., 2017), Anura (frog and toads) (Pyron, 2011), Squamata (lizards and snakes) (Evans, 2003), and Rodentia (dos Reis et al., 2012). Aerial predators included: Aves (birds) (Prum et al., 2015) and Chiroptera (bats) (Teeling et al., 2005). An estimation for the origins of terrestrial bioluminescence allows us to discuss the likeliness of these predator groups as drivers of aposematism. Several diverse terrestrial predator groups, including Anura, Araneae, Carabidae, and Squamata emerged prior to the Jurassic (>200 mya), and therefore could have contributed to the origin of aposematic bioluminescence that arose 141 mya.

In contrast, several other insectivore clades (Aves, Chiroptera, Rodentia) originated much later (<70 mya) than beetle bioluminescence and thus could not have been selective agents in either terrestrial or aerial bioluminescence. It has been suggested that modern aerial predators, specifically bats, could have driven the origin of aerial bioluminescence as a predator avoidance strategy (Leavell et al., 2018). This is contradicted by our data, with bats originating at ~65 mya (Teeling et al., 2005), considerable after (52-87 million years) the origin of aerial bioluminescence. However, it is possible that once bioluminescence is used by adult fireflies in aerial displays, potential aerial predators like bats and birds can utilize adult firefly bioluminescence as an additional cue to avoid distasteful prey (Leavell et al., 2018). The sensory systems of insectivorous bats can detect both the wavelength of firefly light emissions and the ultrasonic wingbeat clicks emitted by flying fireflies (Hope and Bhatnagar, 1979; Krivoruchko et al., 2021), and they also learn to discriminate flying insects based on their different echo signatures in their echolocation calls (von der Emde and Menne, 1989). Bats have indeed been shown to reject flying fireflies based on their chemical defenses, reinforced by their sonar profile and/or bioluminescent signal that speed up avoidance learning (Leavell et al., 2018). Whether predators like bats impose selection on the aerial bioluminescence of fireflies and possibly contribute to the maintenance of aerial light signals in beetles

remains to be tested. Other predators such as nocturnal birds and rodents (Lloyd, 1973) that could have preyed on bioluminescent beetles also originated significantly after the origin of both terrestrial and aerial bioluminescence (Fig. 2). For example, modern birds originated ~75 mya (Prum et al., 2015), and rodents at ~61 mya (dos Reis et al., 2012), placing them ~65-79 million years after the estimated origin of terrestrial bioluminescence and ~58-72 million years after aerial bioluminescence (Fig. 2). Our age estimates for bats, birds, and rodents are almost certainly older than the actual insectivorous lineages within each. Given that larval bioluminescence evolved first and operated in a terrestrial environment, aerial predators, such as bats, could not have been the original receivers or drivers of these aposematic signals. The signal would have been directed toward contemporary predators of elateroids in the early Cretaceous.

There are several extinct vertebrate insectivore groups that could have also preyed on elateroid beetles prior to or during the early Cretaceous (>100 mya) (Brusatte et al., 2015; Xing et al., 2018; Grossnickle et al., 2019). Most of these are limited in both known diversity and distribution in the fossil record and would have only added to the already existing predation pressures in the terrestrial environment. One potential exception could have been the aerial predatory group, Pterosauria. Pterosaurs were the first vertebrate group to develop true flight in the late Triassic (~227 mya), with the common ancestor of the group hypothesized to be insectivorous (Organ and Shedlock, 2009; Zou et al., 2017). Furthermore, Pterosauria had a broad enough distribution to have encountered bioluminescent beetles (Witton, 2013), and some species are assumed to have been crepuscular or even night (Schmitz and Mortani, 2011), and thus could have been early receivers of the aposematic signals. Although tantalizing, there is no current evidence to support that pterosaurs fed on elateroid beetles.

Our more extensive taxon sampling and well-placed fossils across our topology, recovered more reliable ages for bioluminescent beetles. These ages are older than previous estimations and preclude modern aerial predators as selective agents for the origin of beetle bioluminescence. However, several groups of modern terrestrial predators predate the origin of beetle bioluminescence and thus could have functioned as selective agents for the origin of terrestrial bioluminescence. Based on the present-day use/abundance of terrestrial bioluminescence as aposematic signals and the presence of terrestrial insectivores at the origin of beetle bioluminescence strongly suggests that terrestrial beetle bioluminescence arose as an aposematic signal. In contrast, aerial beetle bioluminescence, which is widely used as a sexual signal during mate search in extant beetles, likely originated as such. We do not rule out that extant insectivores such as bats, birds, and rodents that came after the origin of bioluminescence may operate as contributing factors in conjunction with older predatory groups (e.g., anurans, araneae) that predate beetle

bioluminescence. What is clear is that the origin of aerial bioluminescence predates any extant aerial predator. Thus, we find that beetle bioluminescence outshines, or predates, the origins of extant aerial predators by roughly 60 million years.

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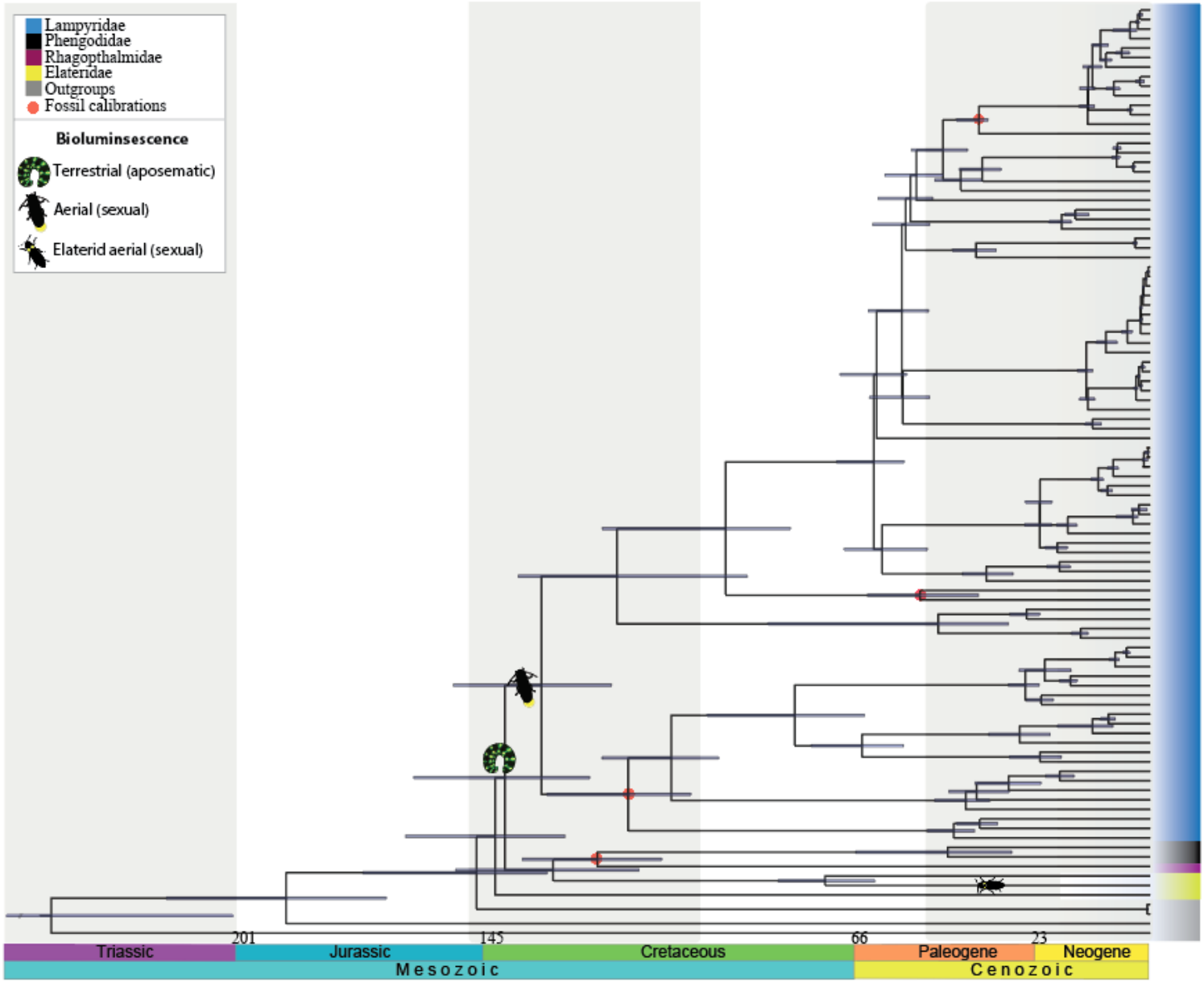



Figure 1. Divergence time calibrated phylogeny of Elateroidea using LN and BD (based on data from Martin et al., 2019) with results of ancestral state reconstruction for both terrestrial and aerial bioluminescence depicted at appropriate nodes. Grey bars to the back of the figure represent 50 million years.

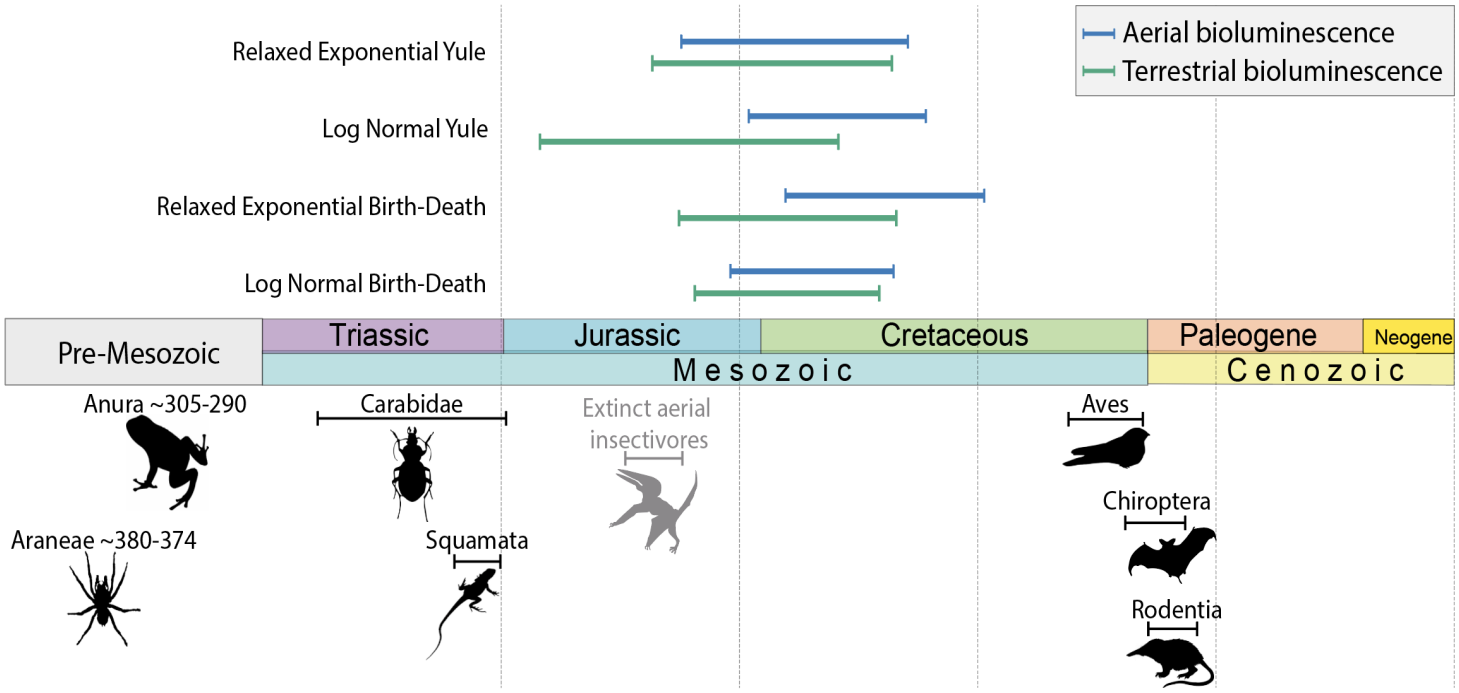



Figure 2. Comparison of divergence time estimates for nodes representing the origins of both terrestrial (blue line) and aerial (blue line) bioluminescence and with published clade origin ages for potential extant (black) and extinct (grey) predator groups. Times written out for Anura and Araneae due to age and figure constraints. Dotted vertical lines represent 50 million-year intervals.

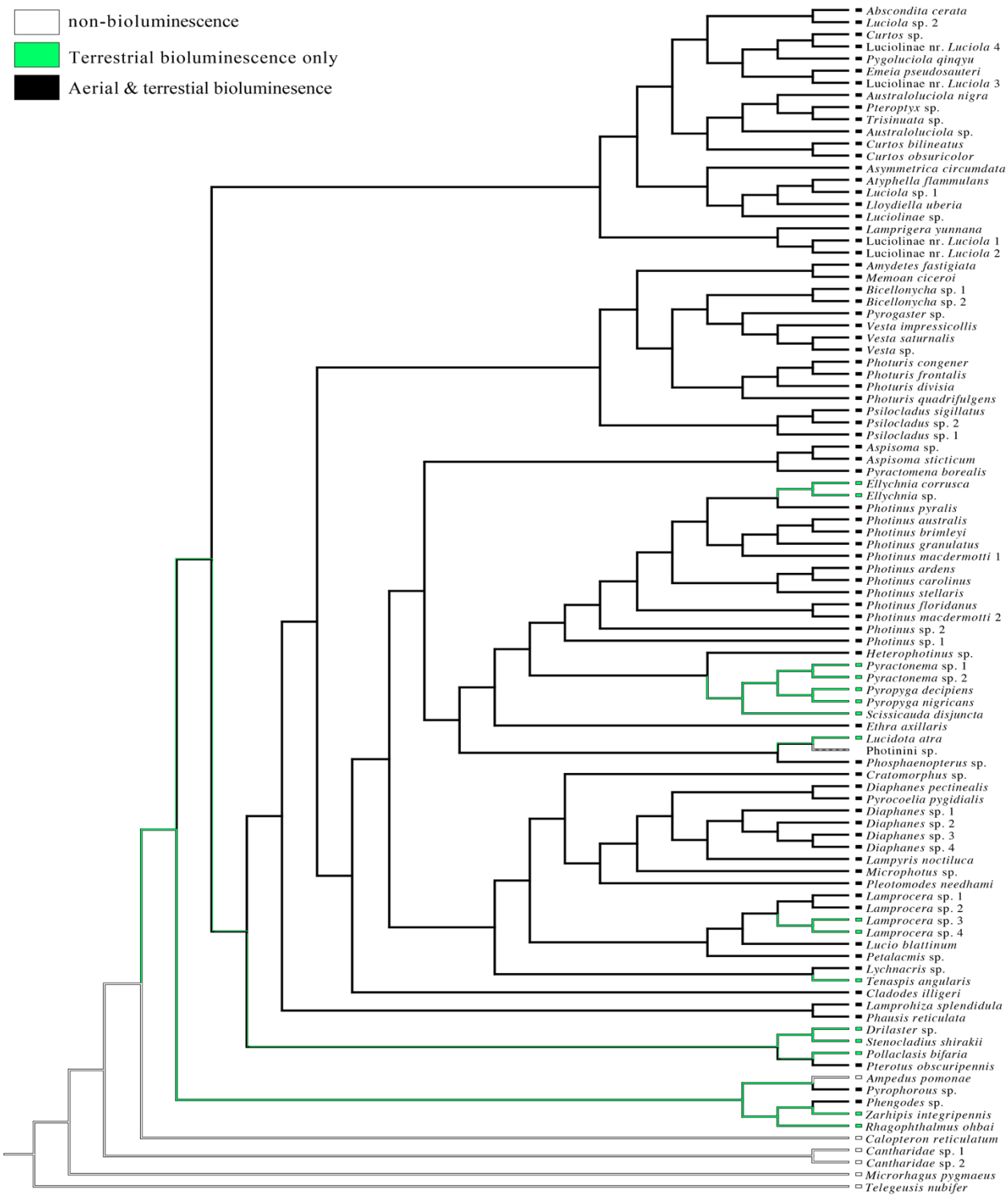
**Supplementary Material**

| Fossil | Age (mya) | Clade |
|---|---|---|
| *Protoluciola albertalleni* | 99 | Luciolinae |
| *Cretophengodes azari* | 99 | (Rhagophthalmidae + Phengodidae) |
| *Photinus kazantsevi* | 36.2 | *Photinus* |
| *Phausis fossilis* | 25 | Lamprohizinae |

Supp Table 1: Fossils used in divergence time estimation with their relative age, placement, and dataset they were applied to.

| Tree | Clock | Terrestrial | Aerial |
|---|---|---|---|
| BD | LN | 141.17 (122.63–161.17) | 133.18 (117.86–152.47) |
| BD | RE | 129.87 (117.39–164.44) | 110.30 (99.36–140.97) |
| Yule | LN | 160.77 (129.73–192.69) | 124.06 112.65–148.25 |
| Yule | RE | 139.51 (118.78–169.69) | 134.60 (115.69–163.76) |

Supp Table 2. Divergence time estimates for nodes reconstructed to be the origins of terrestrial and aerial bioluminescence under different tree and clock models with each dataset. LN- Log Normal, RE- Relaxed Exponential, BD- Birth-Death.




Supp. Figure 1. Maximum parsimony ancestral state reconstruction of bioluminescence in beetles.