Total evidence phylogeny and the evolution of adult bioluminescence in fireflies (Coleoptera: Lampyridae)

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Abstract

Fireflies are some of the most captivating organisms on the planet. They have a rich history as subjects of scientific study, especially in relation to their bioluminescent behavior. Yet, the phylogenetic relationships of fireflies are still poorly understood. Here, we present the first total evidence approach to reconstruct lampyrid phylogeny using both a molecular matrix from six loci and an extensive morphological matrix. Using this phylogeny we test the hypothesis that adult bioluminescence evolved after the origin of the firefly clade. The ancestral state of adult bioluminescence is recovered as non-bioluminescent with one to six gains and five to ten subsequent losses. The monophyly of the family, as well as the subfamilies is also tested. Otoretinae, Cyphonocerinae, Luciolinae (incl. *Pristolycus*), Amydetinae, "cheguevarinae" sensu Jeng 2008, and Photurinae are highly supported as monophyletic. With the exception of four taxa, Lampyrinae is also recovered as monophyletic with high support. Based on phylogenetic and morphological data *Lamprohiza*, *Phausis*, and *Lamprigera* are transferred to Lampyridae incertae sedis.

1. Introduction

1.1. Bioluminescence

Bioluminescence is a beautiful signal system among animals that is used both as an aposematic and a sexual signal. Within the Hexapoda, bioluminescence is only found in three orders: Collembola, Diptera, and Coleoptera (Viviani 2002, Viviani *et al.* 2002, Oba *et al.* 2011). Within Coleoptera, bioluminescence occurs members of the elateriform families Elateridae, Rhagophthalmidae, Phengodidae, and Lampyridae, as well as in the distantly related Staphylinidae (Lloyd 1978; Costa *et al.* 1986; Branham & Wenzel 2001; Sagegami-Oba *et al.* 2007, Rosa 2010). The light production of Staphylinidae has yet to be studied in depth to exclude the possibility that the bioluminescence is the result of bacteria or some other ingested luminous organism (Oba 2014). Elateridae, Rhagophthalmidae, Phengodidae, and Lampyridae produce light through the same chemical pathway involving the compound luciferin and a number of structurally similar luciferases (Day *et al.* 2009). Within Elateriformia, Bocakova *et al.* (2007) hypothesized bioluminescence to have originated at least twice, however they did not include any of the bioluminescent Elateridae in their analysis, which would represent a third origin. Fireflies (Coleoptera: Lampyridae Rafinesque, 1815) are the most diverse terrestrial group of organisms that produce bioluminescence. There is a large body of research documenting firefly life history and especially their bioluminescence (Barber 1951; Buschman 1977; Lall *et al.* 2000; Lloyd 1983; Sagegami-oba *et al.* 2007).

All known firefly larvae are bioluminescent. The behavioral context of these emissions, *i.e.*, glowing more brightly when harassed, is consistent with use as an aposematic signal (Sivinski 1981, Marek *et al.* 2011). Fireflies contain defensive steroids known as lucibufagins (Eisner *et al.* 1978) and predators can learn to associate a glow with lucibufagins (Underwood *et al.* 1997; De Cock & Matthysen 1999). Larval bioluminescence used in an aposematic context is hypothesized to represent the first function and earliest origin of bioluminescence in Lampyridae (see Branham & Wenzel 2003 for discussion). Bioluminescence is hypothesized to then have been co-opted in the adult life stage of several lineages, where it serves primarily as a sexual signal for courtship (Lloyd 1989; Branham & Wenzel 2003; Moosman *et al.* 2009; Faust 2010). Branham and Wenzel (2003) hypothesized the ancestral state of adult courtship communication in Lampyridae to be pheromones with no bioluminescence, followed by adult bioluminescence being gained four times and lost at least four times. Stanger-Hall *et al.* (2007) proposed

that the loss of bioluminescence is more likely than a gain and suggested the ancestral state of adult communication to be bioluminescence with one gain and nine subsequent losses. However, if gains and losses of bioluminescence are considered equally likely, their data suggest the same hypothesis put forward by Branham and Wenzel (2003) (see above, Stanger-Hall *et al.* 2007).

1.2. Classification

Lampyridae is a cosmopolitan family composed of seven subfamilies, 67 genera, and ~2,000 species, with the majority of diversity found in the tropical regions of the world (Branham 2010). Firefly phylogenetic hypotheses have remained controversial. Species of some genera appear to be morphologically identical and can at present only be identified with knowledge of the species-specific bioluminescent flash patterns emitted by adults during courtship (e.g., *Photuris* Dejean, 1833; Lloyd 1969). Other species often possess such unique combinations of morphological characters that their taxonomic placement is challenging (McDermott 1964; Crowson 1972; Branham and Wenzel 2001; Branham 2010). Elucidating the phylogenetic relationships within this family will contribute to the development of a stable, natural classification scheme and serve as a framework for studying bioluminescent character evolution.

There has been debate over identifying the correct sister group to Lampyridae. Branham and Wenzel (2001) recovered Rhagophthalmidae in a polytomy with Lampyridae and several other families based on a dataset of 212 morphological characters. In 2007, Stanger-Hall *et al.* recovered Cantharidae + Lycidae as sister to Lampyridae in a study based on three molecular markers (18S, 16S, and COI), however they indicate another study in prep which recovered Lycidae as the sister group, a relationship which is also supported by Hunt *et al.* 2007 (18S, 16S, and COI). Bocakova *et al.* (2007) recovered Cantharidae as sister to Lampyridae (18S, 28S, 16S, and COI). In the most recent large-scale molecular analysis including the largest beetle sampling to date, McKenna *et al.* (2015) recovered Phengodidae + Rhagophthalmidae as sister to Lampyridae (28S, 18S, EF1- α , wingless, CAD, arginine kinase, alpha-spectrin, and PEPCK).

F.A. McDermott, one of the prominent workers on Lampyridae, stated that the tribal classification of Lampyridae was “more or less arbitrary” and while the generic arrangement within Lampyridae was “logical” and of some utility in understanding fireflies, the classification should not be misconstrued as representing phylogenetic relationships (McDermott 1964). In his classification of Lampyridae, Olivier recognized nine subfamilies based mostly on head and antennal morphology (Olivier 1907, 1910). Later, Green instituted a tribal classification specific to the subfamily Lampyrinae, which McDermott then applied to the whole of Lampyridae (Green 1948, 1959; McDermott 1964). In his classification, McDermott recognized seven subfamilies with sixteen tribes/subtribes (McDermott 1966). R.A. Crowson then proposed a classification of Lampyridae with a system that set aside the tribal classifications of McDermott and Green, and recognized eight subfamilies (Crowson 1972).

Suzuki (1997) was the first to conduct a formal phylogenetic analysis of lampyrid taxa. This investigation was based on molecular data (16S only) and taxon sampling that was focused on the Japanese fauna (Fig. 1a). Branham and Wenzel (2001, 2003) reconstructed the first lampyrid phylogeny encompassing the major diversity within the Cantharoidea (to which Lampyridae had been assigned) using morphological data (Fig. 1b). They recovered a trichotomy between Lampyridae, Rhagophthalmidae, and a clade comprising several other families. Within Lampyridae only Luciolinae and Photurinae were recovered monophyletic. As a result of this topology, Branham and Wenzel (2001) removed *Drilaster* Kiesenwetter, 1879, *Harmatelia* Walker, 1858, and *Pterotus* LeConte, 1859 from Lampyridae and placed them as Elateriformia *Incertae Sedis* status. See Branham and Wenzel (2001) for a brief history of the taxonomic placement of these genera. Stanger-Hall *et al.* (2007) performed a phylogenetic analysis using mostly North American taxa based on three genetic markers (18S, 16S, and COI) and recovered both *Pterotus* and *Rhagophthalmus* Motschulsky, 1854 as members of Lampyridae (Fig. 1c), however no formal taxonomic changes were proposed. In 2008, Jeng reconstructed a phylogeny based on a morphological analysis of 410 characters that included the largest and broadest in-group sampling to date representing 78% of lampyrid genera, as well as representatives from nine other related families (Fig. 1d). However, at the current time, this thesis remains unpublished. In this analysis, *Pterotus* was recovered basal to what he considered Lampyridae, while (Rhagophthalmidae + (Phengodidae + Telegeusidae)) was recovered in a clade sister to Lampyridae. Jeng subsequently presented a classification of Lampyridae, which included nine subfamilies, eight of which were recovered as monophyletic in his analysis. The only subfamily *not* recovered as monophyletic

was Ototretinae, with respect to Ototretadrilinae. For purposes of classification and to preserve the monophyly of the group, Ototretinae was broken into two groups: the monophyletic Ototretinae, and the paraphyletic pan-ototretinae which included the non-traditional Ototretinae + Ototretadrilinae (Jeng 2008). Jeng also proposed a taxonomic change to the subfamily Megalophthalminae Olivier, 1907, recognizing that this name was unavailable. He proposed the new name Cheguevarinae for the genera *Cheguevaria* Kazantsev, 2006 and *Magnoculus* McDermott, 1964. As this work is not published, we will refer to this proposed subfamily as: "cheguevarinae" *sensu* Jeng (2008).

The purpose of the present study is to reconstruct the first lampyrid phylogeny using both molecular and morphological data for an extensive sample of taxa representing most major firefly lineages. The phylogeny is used to address three main goals: (1) perform ancestral state reconstruction and investigate the evolutionary pattern of adult bioluminescence; (2) assess how many times adult bioluminescence has been gained and lost throughout the evolution of Lampyridae; and (3) test Jeng's (2008) hypotheses of lampyrid monophyly including subfamilial relationships.

2. Materials and Methods

2.1. Taxon sampling

2.1.1. Molecular taxon sampling: A total of 82 in-group taxa (Table 1) were included. Specimens of 29 lampyrids collected from around the world were combined with 53 additional taxa (16S, 18S, & COI) from GenBank. Our taxon sampling for molecular data represented six of the seven currently defined lampyrid subfamilies and 36 of the 67 genera. We were unable to acquire molecular grade specimens from the less diverse and geographically restricted Amydetinae, and "cheguevarinae" *sensu* Jeng 2008, however they are included in the morphological partition (see below). Out-group taxa included exemplars from Elateridae, Phengodidae, Lycidae, Cantharidae, Rhagophthalmidae, Drilidae, and Omalisidae (Table 1). Because the sister relationship to Lampyridae is in question, we choose *Chrysochroa tonkinensis* Descarpentries, 1948, from Buprestidae (Buprestoidea) as the most distant out-group to root our topology.

2.1.2. Morphological taxon sampling: A morphological matrix of 410 male characters was adapted from Jeng 2008 and included in our analyses. Because Jeng's taxonomic species-level coding overlapped little with our taxon sampling, we revised the matrix by consolidating all species scorings across a genus into a single generic "composite" scoring. When this scoring resulted in polymorphic states, the characters were coded as polymorphic. The composite coding was applied to each species in the genus for all taxa in our dataset. Buprestidae, Cantharidae, and Elateridae were not included in Jeng's (2008) analysis and as such, these taxa had no associated morphological data. See Table 1 for data overlap.

2.2. DNA extraction and amplification

Specimens were stored at -80 °C in 95% ethanol. Muscle tissue was removed from one metacoxa for each specimen. The rest of the body was vouchered in 95% ethanol at -80 °C in the BYU Insect Genomics Collection. DNA was extracted using the Qiagen DNAeasy™ extraction kit. Portions of three mitochondrial (12S, 16S, COI) and three nuclear (18S, 28S, wingless) genes were amplified using polymerase chain reaction (for primers and PCR conditions see tables 2 & 3).

2.3. Alignment & Phylogenetic methods

Sequences were aligned using MAFFT v 7.017 (Katoh *et al.* 2002) under the L-INS-i strategy implemented in Geneious v 7.1.7. All gene alignments were concatenated using Geneious for downstream phylogenetic analysis (see table 5 for individual gene lengths). The morphological data were analyzed independently to ensure that our modification to the scoring was congruent with Jeng's analysis (2008). We then analyzed the molecular data and a combined dataset of both molecular and morphological data. Appropriate models of evolution for Maximum likelihood (ML) and Bayesian molecular phylogenetic reconstruction were generated in PartitionFinder v1.1.1 (Lanfear *et al.* 2012) with model selection set to "BIC" and search criteria set to "greedy" (Table 4). The morphological partition was analyzed using the MK model (Lewis 2001). All trees were visualized in FigTree v. 1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

A combined parsimony analysis was performed in TNT v.1.1 (Goloboff 2008) with xmult level 10, fuse set to 5, and drift set to 30. Sectorial searching was also used. The ratchet was set at 50 iterations with up and

down weighting set to 12. Bootstrap (BtS) values based on 1000 pseudo-replicates were also calculated in TNT. The morphological data alone were analyzed in TNT with the same settings, and the resulting topology, while being less resolved, showed no incongruence with Jeng's original analysis (Jeng 2008) thus validating our scoring scheme.

Maximum likelihood analyses were carried out using GARLI v.2.01 (Zwickl 2006). Default parameters were used with searchreps set to eight and attachments per taxon set to 186. Analyses were re-run until at least four reps produced topologies with lnL within one lnL of each other, indicating the best tree had been found. Bootstrap support was conducted in the SumTrees v.3.3.1 program of the Dendropy v. 3.8.0 package (Sukumaran & Holder 2010).

Bayesian analyses were performed utilizing MrBayes 3.2 (Ronquist *et al.* 2012). Two parallel runs with eight chains each (one cold and seven hot) were run, sampling every 1000 generations. Log files were then analyzed in Tracer v.1.4.1 (Rambaut and Drummond, 2007) to confirm that stationarity was reached. The molecular analysis ran for 97,883,000 generations and finished with an average standard of split frequencies of 0.002788. When the morphological data were added, the analysis ran for 56,844,000 generations and finished with an average standard of split frequencies of 0.033348. All sampled generations prior to stationarity were discarded (burn-in). Posterior probabilities were also calculated in MrBayes. Maximum likelihood and Bayesian analyses were performed on the Fulton Supercomputer at Brigham Young University.

2.4 Ancestral state reconstruction

The ancestral state of adult bioluminescence was reconstructed using both the Bayesian (which was nearly identical to the Parsimony phylogenetic estimate) and Maximum Likelihood topologies (both inferred from the full dataset) using the ancestral state reconstruction package in Mesquite (Maddison & Maddison 2011) under both a parsimony and likelihood (MK1 model) framework.

Bioluminescence was coded as follows: 0= bioluminescence absent (adults only); 1= bioluminescence present (male and/or female; adults only). A specimen was coded as bioluminescent if it had a photic organ as an adult. The scoring of actual flash pattern is not currently possible due to the lack of empirical flash data for the majority of firefly species. Data on the presence or absence of bioluminescence/photic organs was generated from the literature and through direct observation of specimens.

3. Results

3.1. Alignment

Our analyses resulted in a total alignment of 6,122 base pairs (Table 5). The TNT analyses of the molecular and total evidence datasets resulted in tree lengths of 12,418 and 15,233 respectively. The Garli analyses resulted in lnL of -59889.0786 for the molecular dataset and -71681.2039 for the total evidence dataset.

3.2. Phylogenetic reconstruction

Phylogenetic reconstruction of molecular data alone recovered two major clades (Fig. 2): clade 1) Otoretinae, *Pterotus*, Cyphonocerinae and Luciolinae (plus *Lamprigera* Motschulsky, 1853 and *Pristolycus* Gorham, 1883), and clade 2) Photurinae and Lampyrinae. When morphology was added to the analyses, clade 1 became a pectinate assemblage at the base of Lampyridae (Fig. 3). All reconstruction methods (*e.g.*, parsimony, likelihood, Bayesian) recovered the same topologies based on dataset analyzed. Otoretinae was recovered sister to or as part of a clade that is sister to all other lampyrids in most topologies (see supplemental figs. 1 + 2). By contrast, *Pterotus* was recovered as sister to all lampyrids in both the Bayesian and maximum parsimony analyses that incorporated morphology and molecular data. With the exception of the parsimony and likelihood total evidence analyses, Photurinae were recovered as sister to the Lampyrinae with high support (Table 6). The monophyly of the Otoretinae and Cyphonocerinae were highly supported in all analyses while the Photurinae and Luciolinae were reconstructed as monophyletic only in the total evidence topology (Figs. 2, 3, Table 6). In our total evidence phylogeny, "cheguevarinae" *sensu* Jeng (2008) was recovered as monophyletic with high support (Fig. 3). The lampyrine genus *Vesta* Laporte, 1833 was recovered sister to or included within the Photurinae in all analyses (Fig. 2 & Table 6). There were also three other lampyrine genera that proved difficult to place: *Lamprohiza* Motschulsky, 1853, *Phausis* LeConte, 1852, and *Lamprigera* (see discussion).

3.3. Ancestral state reconstruction:

The ancestral state for adult sexual communication in the Lampyridae was optimized as either non-bioluminescent in the M.L. reconstruction or ambiguous in the Bayesian reconstruction. Adult bioluminescence arose from one to six times with five to ten subsequent losses in regardless of reconstruction method. The ancestor of the Luciolinae is hypothesized to have utilized bioluminescence as adults, as well as the ancestors of the Photurinae and Lampyrinae (Fig. 4 a & b).

4. Discussion

Pristolycus is recovered within or sister to Luciolinae in all analyses. *Pristolycus* has had a complex taxonomic history, having been originally placed in Lycidae and subsequently transferred to different subfamilies within Lampyridae (Lucidotinae and Lampyrinae; for a full history see Jeng 2008). Winkler (1953) erected Pristolycini for the sole genus *Pristolycus* within the Lampyrinae. McDermott (1964) included *Pristolycus* in the Lampyrinae but offered no morphological explanation (Jeng et al. 2002). While not congruent with the analyses of Branham and Wenzel (2001, 2003) *Pristolycus* was recovered within the Luciolinae by Suzuki (1997) and Stanger-Hall et al. (2007), as well as Jeng (2008) who was the first to formally transfer the genus to the Luciolinae. Kazantsev (2010) later erected Pristolycini within the Luciolinae, with no mention of Winkler 1953 or of a transfer of *Pristolycus*. The previous description of Winkler renders Kazantsev's Pristolycini an objective synonym. Olivier (1907) described Luciolinae based on the following characters:

“Body elongate, parallel or oblong oval, convex; head visible when looking at the insect from above, concave in males, almost flat in females; eyes large, occupying almost all of the head, very prominent in males, much less in females; antennae short, filiform, gradually attenuated, of 11 segments; labrum distinct; prothorax short, transverse, shrunken and truncate or rounded in the front with posterior angles well marked, often projecting or arched; abdomen consisting of six segments in males, the last ventral segment of variable form, and seven in females; males and females winged, except *Lampyroidea*.” (pp. 48–49)

While Olivier describes the abdomen as consisting of six segments in males, examination of the Luciolinae reveals that this should in actuality be abdomen with six *visible sternites* in males, belonging to abdominal segments II to VII. For a detailed discussion of abdominal segment fusion/loss in Luciolinae, see (Ballantyne and McLean 1970; Ballantyne 1987). *Pristolycus* shares all of Olivier's characters except two: eyes small and widely separated; antennae long and serrate. Jeng et al. (2002) confirmed that *Pristolycus* shares many lucioline features, however based on taxon sampling and lack of a robust phylogenetic framework, were unwilling to propose the taxonomic change. This was remedied with his 2008 thesis (Jeng 2008). As a result, the diagnosis of Olivier (1907) for the subfamily is herein amended as follows: eyes large *or small*; antennae short, filiform *or long, serrate*, abdomen with six *visible sternites* in males.

We are confident that this change will more accurately define the subfamily, as eye and antennal morphology are often associated with adult sexual signaling (bioluminescence vs. pheromone) and have been shown to vary widely between closely related groups (e.g. *Ellychnia* and *Photinus*: Stanger-Hall et al. 2015). The morphological matrix presented herein (adapted from Jeng 2008) provides putative synapomorphies for *Pristolycus* + Luciolinae sensu stricto in characters 186 (state 1), 210 (state 1), 291 (state 0), 299 (state 1), 318 (state 0), 346 (states 1&2), and 382 (states 1&2). In short, these characters involve the elytra, sternites and genitalia (see Jeng 2008 for morphological character description). Since our analyses included 12 species from three of the nine genera assigned to Luciolinae, further testing of these putative synapomorphies is across all of Luciolinae is encouraged.

In our molecular analyses, Lampyrinae and Photurinae are consistently recovered as non-monophyletic and with the addition of the morphological dataset, only Lampyrinae is recovered as non-monophyletic. The change in monophyly of Photurinae is due to *Vesta* sp, a genus that has is not well defined. When only the molecular data is considered, *Vesta* is recovered within the photurine clade sister to *Bicellonycha* Motschulsky, 1853 with low support, whereas support for the entire Photurinae + *Vesta* clade is high

(Bootstrap 96; Posterior Probability 1). When the morphological data is added, *Vesta* sp. is recovered as sister to Photurinae, with little to no support (except in the Bayesian reconstruction; PP 0.96). McDermott (1966) classified *Vesta* in the subfamily Amydetinae. Jeng (2008) recovered *Vesta* as paraphyletic with some species of the genus in a polytomy that included Photurinae and Lampyrinae. Given that our morphological partition is adapted from Jeng (2008), it is not surprising that *Vesta* is difficult to place. As we have only one representative of the genus in our analyses, it is not possible to hypothesize the placement of *Vesta* in relation to the Photurinae. These relationships will likely become clearer with the inclusion of more taxa from *Vesta* and Amydetinae.

Three genera of Lampyrinae are difficult to place phylogenetically: *Lamprohiza*, *Phausis*, and *Lamprigera*. Jeng (2008) found these genera difficult to place phylogenetically due to significant morphological differences from Lampyrinae (unmodified mandibles, dorsal abdominal spiracles, and the symmetrical aedeagal sheath), their hypothesized subfamilial group. In our molecular analyses these three genera are distributed throughout the topology (*Lamprigera* sister to the Luciolinae, *Phausis* as sister to the Photurinae + Lampyrinae, and *Lamprohiza* within the traditional Lampyrinae; see Supp. figs 1 & 3). When morphological data is added, *Phausis* and *Lamprohiza* are recovered as sister to each other with high support (BtS 99, PP 1; See Supp. figs 2 & 4) and sister to the Photurinae + Lampyrinae. *Lamprigera* is recovered in a polytomy with the Amydetinae and "cheguevarinae" *sensu* Jeng (2008) + Photurinae + Lampyrinae. Based on all data, as well as the morphological differences stated above, it is clear these taxa do not belong within the Lampyrinae. Thus, for the purposes of this paper we define Lampyrinae *sensu stricto* as without these genera, and place these taxa in Lampyridae *incertae sedis* **new placement**.

The relationship between *Ellychnia* Blanchard, 1845 and *Photinus* Laporte, 1833 is also interesting. Several studies suggest that *Ellychnia* may in fact be a highly derived lineage of *Photinus* (Stanger-Hall *et al.* 2007, Stanger-Hall & Lloyd 2015; and Lewis & Cratsley 2008). In our molecular analyses, *Ellychnia* is recovered within the *Photinus* clade, but high support is only found at the node subtending the entire clade (BtS 100 & 100, PP 1; figs. 2 & 3). When morphology and molecules are analyzed together, *Ellychnia* is recovered as sister to *Photinus* in all analyses with moderate to high support (BtS 79 & 96; PP 1; in our parsimony analysis, *Ellychnia* is recovered in a four branch polytomy with three *Photinus* branches). While molecular evidence alone (16S, 18S, COI) places *Ellychnia* as a derived lineage of *Photinus*, there is solid support for a sister relationship between *Ellychnia* and *Photinus* when molecular and morphological data are combined.

The evolution of bioluminescence across Lampyridae has been addressed several times in the literature (Branham and Wenzel 2001, 2003, Martin *et al.* 2015, Stanger-Hall & Lloyd 2015) yet, due to the different phylogenetic hypotheses, there is still no consensus. Adult bioluminescence is clearly used for sexual communication, with only a few known exceptions (*Photuris* femme fatales; Lloyd 1965 & 1975). Branham and Wenzel (2003) was the first study to recover the ancestral state of adult signaling in Lampyridae as non-luminous and employing pheromonal signals, while adult bioluminescence likely evolved via multiple origins and multiple losses. Our analyses support these hypotheses (Fig. 4). The present analysis recovers one to six gains and five to ten losses of adult bioluminescence across the evolutionary history of the group, regardless of topology. *Pterotus* represents the first gain of adult bioluminescence. In his thesis on *Pterotus obscuripennis* LeConte, 1859, Dean (1979) suggested that *Pterotus* represented a potential step or progression in the process that lead to adults utilizing bioluminescence for mate recognition. Mate recognition in *Pterotus* is facilitated by a pheromone signal from the female to draw the male near her position. Then the male can use the female's glow for close range adjustments (Dean 1979). Bioluminescence is not widespread across the topology until the trait becomes ubiquitous in the Luciolinae, Photurinae and Lampyrinae. M.L. and Bayesian topologies are not congruent at the base of Lampyridae, however, the ancestral state reconstruction is equivalent as the likelihood of the ancestral state being "pheromone only" dips from 0.83 in the M.L. topology to 0.65 on the Bayesian topology. We see that bioluminescence was lost in the "cheguevarinae" however it is conserved in all members of the Photurinae. What is especially interesting, and what needs to be studied further, is the evolution of bioluminescence in Lampyrinae. In this subfamily alone we see up to seven losses of adult bioluminescence, with one potential loss in the relatively diverse *Ellychnia* + *Photinus* clade.

5. Conclusions

Lampyridae, including Pterotinae and Ototretinae are recovered as monophyletic with high support. All subfamilies included in our analyses except Lampyrinae are recovered as monophyletic when both molecular and morphological data are combined. The sister relationship between Lampyrinae and Photurinae is well supported but the relationships between the remaining subfamilies, particularly whether Luciolinae is sister group to the Lampyrinae + Photurinae, is unclear. Further estimates that include taxa with molecular data from all subfamilies, particularly Amydetinae, are needed. The phylogenetic placement of the genera *Phausis*, *Lamprigera*, *Lamprohiza*, *Vesta*, still needs to be resolved. When analyzing all data (both morphological and molecular), it is apparent that *Ellychnia* is related to, but not a member of, *Photinus*. The first gain of adult bioluminescence in Lampyridae appears to have arisen within *Pterotus* where it represents the first use of adult bioluminescence mainly as an aposematic signal, with little, if any, function in mate recognition. Bioluminescence then became the main mode of sexual communication in the Luciolinae, Photurinae and Lampyrinae, where it likely has contributed to the rapid diversification of these taxa. Through the first combined analysis of both morphological and molecular data in Lampyridae, we were able to advance the understanding of their evolutionary relationships by evaluating the monophyly of several subfamilies, addressing certain taxonomic issues, and adding support to the hypothesis that adults of the ancestral firefly lineage did not use bioluminescence in sexual communication, but rather resembled the modern *Pterotus*.

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Figure 1. Summary of previous phylogenetic hypotheses; 1a: Suzuki 1997; 1b: Branham & Wenzel 2001; 1c: Stanger-Hall et al. 2007; 1d: Jeng 2008. Redrawn from Jeng 2008

Figure 2. Bayesian reconstruction based on molecular dataset only, aligned in MAFFT. Numbers at nodes indicate posterior probabilities. Only those nodes with support above 0.7 are labeled.

Figure 3. Bayesian reconstruction based on molecular and morphological datasets. Molecular dataset aligned in MAFFT. Numbers at nodes indicate posterior probabilities. Only those nodes with support above 0.7 are labeled.

Figure 4a. Ancestral State Reconstruction of adult bioluminescence reconstructed onto the Bayesian total evidence phylogeny. 4b. Ancestral state reconstruction of adult bioluminescence estimated onto the Maximum Likelihood total evidence phylogeny. Black bars indicate presence of bioluminescence in the adult life stage, white bars indicate absence, reconstructed under a parsimony framework. Circles at nodes represent likelihood of presence of bioluminescence based on likelihood reconstruction framework.

528 Table Legends
529 Table 1: Taxa included in the present study.
530 Table 2: Primer sequences.
531 Table 3: Amplification profiles.
532 Table 4: Model selection and partitioning schemes.
533 Table 5: Total and individual gene alignments lengths.
534 Table 6: Summary of support for phylogenetic relationships. Support for Parsimony and Maximum
535 likelihood analyses based on_Bootstrap (BtS) values. Support for Bayesian analyses based on Posterior
536 Probabilities.
537
538

Figures

Figure 1

Fig. 1a. Suzuki 1997

Fig. 1c. Stanger-Hall et al. 2007

Fig. 1b. Branham & Wenzel 2001

Fig. 1d. Jeng 2008

Figure 2

Figure 3

Figure 4

Table 1

	12S	16S	18S	28S	COI	WNT	Morphology
Amydetes_apicalis	N/A	N/A	N/A	N/A	N/A	N/A	Yes
Amydetes_fastigiatus	N/A	N/A	N/A	N/A	N/A	N/A	Yes
Aspisoma_sp._1	KY317726	KY317758	KY317790	KY317824	KY317853	KY317885	Yes
Aspisoma_sp._2	N/A	EU009285	EU009248	N/A	EU009322	N/A	Yes
Bicellonycha_wickershamorum	KY317749	KY317783	KY317791	KY317825	KY317854	KY317886	Yes
CANTHARIDAE_1	KY317756	KY317787	KY317792	KY317826	KY317855	KY317887	N/A
CANTHARIDAE_2	KY317757	KY317788	KY317793	KY317827	KY317856	KY317888	N/A
Ceylanidrilus_sp.	N/A	DQ198682	DQ100524	N/A	DQ198605	N/A	Yes
Cheguevaria_taino	N/A	N/A	N/A	N/A	N/A	N/A	Yes
Chrysochroa_tonkinensis	KY317755	KY317789	KY317794	KY317828	KY317857	KY317889	N/A
Curtos_costipennis	N/A	AB671250	AB298848	N/A	AB671258	N/A	Yes
Curtos_okinawanus	N/A	AB671252	AB298849	N/A	AB671262	N/A	Yes
Curtos_sp.	N/A	DQ198671	DQ100513	N/A	DQ198594	N/A	Yes
Cyphonocerus_ruficollis	N/A	DQ198670	DQ100512	N/A	DQ198593	N/A	Yes
Diaphanes_formosus	N/A	EU009280	EU009243	N/A	EU009317	N/A	Yes
Drilaster_axillaris	N/A	AB436506	AB298853	N/A	AB608756	N/A	Yes
Drilaster_borneensis	N/A	DQ198680	DQ100522	N/A	DQ198603	N/A	Yes
Drilaster_sp.	N/A	DQ198675	DQ100517	N/A	DQ198598	N/A	Yes
Drilus_flavescens	N/A	DQ202597.1	AY745559.1	DQ202663.1	KM450710.1	N/A	Yes
ELATERIDAE	KY317753	KY317786	KY317795	KY317829	KY317858	KY317890	N/A
Ellychnia_californica	N/A	EU009255	EU009218	N/A	EU009292	N/A	Yes
Ellychnia_corrusca	N/A	EU009262	EU009225	N/A	EU009299	N/A	Yes
Ellychnia_sp._1	KY317729	KY317777	KY317796	N/A	KY317859	KY317891	Yes
Ellychnia_sp._2	KY317734	KY317760	KY317797	KY317830	KY317860	KY317892	Yes
Ellychnia_sp._3	KY317736	KY317762	KY317798	KY317831	KY317861	KY317893	Yes
Eropterus_nothus	N/A	N/A	DQ181082.1	DQ181156.1	DQ181230	N/A	Yes
Erythrolychnia_sp.	KY317744	KY317771	KY317799	KY317832	KY317862	KY317894	Yes
Flabellotreta_obscuricollis	N/A	DQ198677	DQ100519	N/A	DQ198600	N/A	Yes
Flabellotreta_sp.	N/A	DQ198678	DQ100520	N/A	DQ198601	N/A	Yes
Lamprigera_sp.	KY317754	N/A	KY317800	N/A	KY317863	KY317895	Yes
Lamprocera_sp.	KY317735	KY317761	KY317801	N/A	KY317864	KY317896	Yes
Lamprohiza_splendidula	N/A	EU009282	EU009245	N/A	EU009319	N/A	Yes
Lampyris_noctiluca	KY317730	KY317773	KY317802	KY317833	KY317865	KY317897	Yes
Lucidina_biplagiata	N/A	AB009922	AB298844	N/A	N/A	N/A	Yes
Lucidota_atra	KY317752	KY317782	KY317803	KY317834	KY317866	KY317898	Yes
Lucidota_luteicollis	N/A	KY317759	KY317804	N/A	KY317867	KY317899	Yes
Luciola_cruciata	N/A	AB009904	N/A	N/A	AF360953	N/A	Yes
Luciola_filiformis_yayeyamana	N/A	AB436493	AB298850	N/A	N/A	N/A	Yes
Luciola_italica	N/A	AB436494	N/A	N/A	N/A	N/A	Yes
Luciola_kuroiwae	N/A	AB009907	N/A	N/A	N/A	N/A	Yes
Luciola_lateralis	N/A	AB009906	AB298851	N/A	N/A	N/A	Yes

Luciola_ovalis	N/A	DQ371179	N/A	N/A	N/A	N/A	Yes
Luciola_parvula	N/A	AB436504.1	AB298852	N/A	AB608763	N/A	Yes
Luciola_sp.	N/A	EU009281	EU009244	N/A	EU009318	N/A	Yes
Lychnuris_formosana	N/A	EU009279	EU009242	N/A	EU009316	N/A	N/A
Magnoculus_sp_a	N/A	N/A	N/A	N/A	N/A	N/A	Yes
Magnoculus_sp_b	N/A	N/A	N/A	N/A	N/A	N/A	Yes
Micronaspis_floridana	N/A	EU009277	EU009240	N/A	EU009314	N/A	N/A
Microphotus_angustus	N/A	EU009264	EU009227	N/A	EU009301	N/A	Yes
Microphotus_octarthrus	KY317732	KY317776	KY317805	KY317835	KY317868	KY317900	Yes
Microphotus_sp.	KY317733	KY317775	KY317806	N/A	KY317869	KY317901	Yes
Omalisus_fontisbellaquei	N/A	DQ202607.1	AF451948.1	DQ202680.1	KM450050.1	N/A	Yes
Oxynopterus_sp.	N/A	HQ333710	HQ333800	N/A	HQ333982	N/A	N/A
Paraphausis_eximia	N/A	EU009260	EU009223	N/A	EU009297	N/A	Yes
Phausis_reticulata	N/A	EU009274	EU009237	N/A	EU009311	N/A	Yes
Phengodes_frontalis	N/A	N/A	KY317807	KY317836	N/A	KY317902	Yes
Phosphaenus_hemipterus	N/A	EU009283	EU009246	N/A	EU009320	N/A	Yes
Photinus_australis	N/A	EU009261	EU009224	N/A	EU009298	N/A	Yes
Photinus_brimleyi	KY317742	KY317766	KY317808	KY317837	KY317870	KY317903	Yes
Photinus_collustrans	KY317741	KY317767	KY317809	KY317838	KY317871	KY317904	Yes
Photinus_curtatus	KY317743	KY317768	KY317810	KY317839	KY317872	KY317905	Yes
Photinus_floridanus	N/A	EU009269	EU009232	N/A	EU009306	N/A	Yes
Photinus_macdermotti	KY317740	KY317769	KY317811	KY317840	KY317873	KY317906	Yes
Photinus_punctulatus	N/A	EU009275	EU009238	N/A	EU009312	N/A	Yes
Photinus_pyrallis	KY317737	KY317763	KY317812	KY317841	KY317874	KY317907	Yes
Photinus_sp._1	KY317738	KY317764	KY317813	KY317842	N/A	KY317908	Yes
Photinus_sp._2	KY317739	KY317765	KY317814	KY317843	KY317875	KY317909	Yes
Photinus_stellaris	KY317746	KY317770	KY317815	KY317844	KY317876	KY317910	Yes
Photinus_tanytoxis	N/A	EU009278	EU009241	N/A	EU009315	N/A	Yes
Photuris_aff._lucicrescens	N/A	EU009253	EU009216	N/A	EU009290	N/A	Yes
Photuris_congener	N/A	EU301845	N/A	N/A	N/A	N/A	Yes
Photuris_divisa	KY317750	KY317785	KY317816	KY317845	KY317877	KY317911	Yes
Photuris_pennsylvanica	N/A	N/A	PPU65129	N/A	AY165656	N/A	Yes
Photuris_quadrifulgens	N/A	EU009273	EU009236	N/A	EU009310	N/A	Yes
Photuris_tremulans	N/A	EU009271	EU009234	N/A	EU009308	N/A	Yes
Pleotomodes_needhami	N/A	EU009268	EU009231	N/A	EU009305	N/A	Yes
Pleotomus_pallens	N/A	EU009254	EU009217	N/A	EU009291	N/A	Yes
Pleotomus_sp.	KY317731	KY317774	KY317817	KY317846	KY317878	KY317912	Yes
Pollaclasis_bifaria	N/A	EU009258	EU009221	N/A	EU009295	N/A	Yes
Pristolycus_sagulatus	N/A	AB009925	N/A	N/A	N/A	N/A	Yes
Pterotus_obscuripennis	N/A	EU009266	EU009229	N/A	EU009303	N/A	Yes
Pyractomena_angulata	N/A	EU009270	EU009233	N/A	EU009307	N/A	Yes
Pyractomena_borealis	N/A	EU009259	EU009222	N/A	EU009296	N/A	Yes

Pyractomena_dispersa	KY317727	KY317779	KY317818	KY317847	KY317879	KY317913	Yes
Pyractomena_palustris	N/A	EU009272	EU009235	N/A	EU009309	N/A	Yes
Pyractomena_sp.	KY317728	KY317778	KY317819	KY317848	KY317880	KY317914	Yes
Pyractonema_sp.	KY317747	KY317781	KY317820	KY317849	KY317881	KY317915	Yes
Pyrocoelia_abdominalis	N/A	AB009921	N/A	N/A	AB608766	N/A	Yes
Pyrocoelia_amplissima	N/A	DQ371190	N/A	N/A	N/A	N/A	Yes
Pyrocoelia_discicollis	N/A	AB436511	N/A	N/A	AB608768	N/A	Yes
Pyrocoelia_fumosa	N/A	AB436510	AB298846	N/A	AB608769	N/A	Yes
Pyrogaster_sp.	KY317751	KY317784	KY317821	KY317850	KY317882	KY317916	Yes
Pyropyga_decipiens	N/A	EU009263	EU009226	N/A	EU009300	N/A	Yes
Pyropyga_nigricans	KY317748	KY317780	KY317822	KY317851	KY317883	KY317917	Yes
Rhagophthalmus_ohbai	N/A	AB009931.1	AB298864.1	N/A	AB608775.1	N/A	Yes
Robopus_sp.	KY317745	KY317772	KY317823	KY317852	KY317884	KY317918	Yes
Vesta_sp.	N/A	DQ198669	DQ100511	N/A	DQ198592	N/A	Yes

Table 2

	Primer	Sequence	Source
12S	12S ai	5'- AAA CTA CGA TTA GAT ACC CTA TTA T -3'	Svenson & Whiting 2009
	12S bi	5'- AAG AGC GAC GGG CGA TGT GT -3'	Svenson & Whiting 2009
16S	16S a	5'- CGC CTG TTT ATC AAA AAC AT -3'	Svenson & Whiting 2004
	16S b	5'- CTC CGG TTT GAA CTC AGA TCA -3'	Svenson & Whiting 2004
18S	18S a0.7	5'- ATT AAA GTT GTT GCG GTT -3'	Whiting 2002
	18S bi	5'- GAG TCT CGT TCG TTA TCG GA -3'	Whiting 2002
28S	F2	5'- AGA GAG AGA GTT CAA GAG TAC GTG -3'	Belshaw <i>et al.</i> 2001
	3DR	5'- TAG TTC ACC ATC TTT CGG GTC -3'	Belshaw <i>et al.</i> 2001
COI	J-2195	5'- TTG ATT TTT TGG TCA TCC AGA AGT -3'	Simon <i>et al.</i> 1994
	PAT	5'- TCC AAT GCA CTA ATC TGC GAT ATT A -3'	Simon <i>et al.</i> 1994
WNT	Wg 550f	5'- ATG CGT CAG GAR TGY AAR TGY CAY GGY ATG TC -3'	Wild & Maddison 2008
	Wg AbrZ	5'- CAC TTN ACY TCR CAR CAC CAR -3'	Wild & Maddison 2008
	Wg 578f	5'- TGC ACN GTG AAR ACY TGC TGG ATG -3'	Ward & Downie 2005
	Wg Abr	5'- CAC TTN ACY TCR CAR CAC CAR TG -3'	Abouheif & Wray 2002

Table 3

	Hot start	Denature	Anneal	Extension	Final Extend	Cycles
12S	94°C (2 min)	94°C (45 sec)	50°C (45 sec)	72°C (45 sec)	72°C (15 min)	30
16S	95°C (2 min)	94°C (45 sec)	54°C (45 sec)	72°C (45 sec)	72°C (15 min)	35
18S	95°C (2 min)	94°C (45 sec)	55°C (45 sec)	72°C (1 min)	72°C (15 min)	35
28S	94°C (2 min)	94°C (45 sec)	59°C (45 sec)	70°C (1 min 30 sec)	70°C (7 min)	40
COI	94°C (2 min)	94°C (30 sec)	51°C (1 min) TchDown 0.2°C per cycle	70°C (1 min 30 sec)		10
		94°C (30 sec)	50°C (1 min)	70°C (1 min 30 sec)	70°C (7 min)	25
WNT Template	94°C (2 min)	94°C (1 min)	54°C (1 min 30 sec)	72°C (45 sec)	72°C (10 min)	35
WNT Nested	94°C (2 min)	94°C (1 min)	54°C (1 min 15 sec)	72°C (45 sec)	72°C (10 min)	35

Table 4

	Max. Likelihood		Bayesian	
	Model	Part. Subset	Model	Part. Subset
12S	GTR + Γ + I	1	GTR + Γ + I	1
16S	GTR + Γ + I	1	GTR + Γ + I	1
18S	TrN + Γ + I	2	GTR + Γ + I	2
28S	TrN + Γ + I	2	GTR + Γ + I	2
COI pos1	GTR + Γ + I	1	GTR + Γ + I	1
COI pos2	GTR + Γ + I	1	GTR + Γ + I	1
COI pos3	TVM + Γ	3	GTR + Γ	3
WNT pos1	TrN + Γ + I	2	GTR + Γ + I	2
WNT pos2	TrN + Γ + I	2	GTR + Γ + I	2
WNT pos3	GTR + Γ + I	1	GTR + Γ + I	1

Table 5

	Length
12S	368 bp
16S	545 bp
18S	1886 bp
28S	1370 bp
COI	1497 bp
WNT	456 bp
Total	6122 bp

Table 6

	Parsimony		Max. Likelihood		Bayesian	
	Molecular	Total Evidence	Molecular	Total Evidence	Molecular	Total Evidence
Lampyridae	78	98	100	100	100	100
Ototretinae	98	99	100	100	100	100
Luciolinae	<70	85	<70	89(<70)	73	100
Photurinae and Vesta	incl. Vesta 96	100	incl. Vesta 96	100	incl. Vesta 100	100
Cyphonocerinae	<70	100	97	100	100	100
Lampyrinae sensu stricto	93	84	96	86	100	100
Photurinae + Lampyrinae	90	<70	97	<70	100	99
Pterotinae	N/A	N/A	N/A	N/A	N/A	N/A