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A Molecular Phylogeny of Lampyridae

with Insight into Visual and

Bioluminescent Evolution

Gavin J. Martin

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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December 2014

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ABSTRACT

A Molecular Phylogeny of Lampyridae
with Insight into Visual and
Bioluminescent Evolution

Gavin J. Martin
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Master of Science

Fireflies are some of the most captivating organisms on the planet. Because of this, they have a rich history of study, especially concerning their bioluminescent and visual behavior. Among insects, opsin copy number variation has been shown to be quite diverse. However, within the beetles, very little work on opsins has been conducted. Here we look at the visual system of fireflies (Coleoptera: Lampyridae), which offer an elegant system in which to study visual evolution as it relates to their behavior and broader ecology. They are the best-known case of a terrestrial organism that communicates through the use bioluminescence. The molecular basis for this communication is relatively simple: one gene-family (opsins) controls the detection of the signal, and one gene family (luciferase) controls the production of the signal. We use a transcriptomic approach to sample for and investigate opsin evolution in fireflies. We also present the first total evidence approach using both an extensive molecular matrix and a robust morphological matrix to reconstruct the lampyrid phylogeny. We then use this phylogeny to assess the hypothesis that adult use of bioluminescence occurred after the origin of Lampyridae.

We find evidence for only two expressed opsin classes in each of the nine firefly species studied, one in the ultra-violet sensitive and one in the long-wavelength sensitive areas of the visible spectrum. Despite the need for most adult fireflies to respond to a clearly sexual and colorful visual signal (bioluminescence) to maximize fitness, their visual system is relatively simple, and does not match the trend for opsin duplication found in other insect groups. All subfamilies except for Lampyrinae are recovered as monophyletic; Pterotinae and Ototretinae are recovered within the Lampyridae. The ancestral state of adult bioluminescence is suggested to be non-bioluminescent, with at least three gains and at least three losses.

Keywords: phylogeny, Coleoptera, Lampyridae, opsin, transcriptome, bioluminescence

ACKNOWLEDGEMENTS

I would like to thank all of my committee members for countless hours of tutoring as well as deep and meaningful discussions. I want to thank my committee chair, Dr. Seth Bybee for many hours spent in his office learning not only about visual systems and science in general, but also about becoming a productive part of the scientific community, and perhaps most importantly how to balance the rigors of academia with the joys and trials of family life. I thank Dr. Michael Whiting for taking me on my first international collecting trip, and for pushing my mind and helping me start to find my place in academia.. I thank Dr. Marc Branham for helping me develop a love for one of the most fascinating creatures on Earth. I also want to thank my fellow lab members, Katie Manwaring, Joey Mugleston, Anton Suvorov, and Haley Wightman for numerous fruitful discussions, for many, many reviews of manuscript drafts, and for their friendship. I also wish to express my gratitude all of the faculty, staff, and graduate students of the Department of Biology and the College of Life Sciences at Brigham Young University.

I especially want to thank Dr. Nathan Lord who has been like an older brother to me in my scientific endeavors. Working with him has taught me that what is most important in our field is love and passion for what we do.

I would most like to acknowledge and thank my wife Tara Marie. Her constant dedication and love have proven to help me get through the trying times, and to remember to keep the truly important things in perspective. I also thank my children Emma Rae and Charlotte Grace for keeping me happy, and making me feel like a rock star every day.

This research was supported financially by grants from the National Science Foundation, American Philosophical Foundation, and by a BYU Graduate Student Fellowship.

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Chapter 1: Review of the firefly visual system (Coleoptera: Lampyridae) and evolution of the opsin genes underlying color vision.

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Keywords: phylogeny, Coleoptera, Lampyridae, opsin, transcriptome, bioluminescence

ABSTRACT

Among insects, opsin copy number variation has been shown to be quite diverse. However, within the beetles, very little work on opsins has been conducted. Here we look at the visual system of fireflies (Coleoptera: Lampyridae), which offer an elegant system in which to study visual evolution as it relates to their behavior and broader ecology. They are the best-known case of a terrestrial organism that communicates through the use bioluminescence. The molecular

basis for this communication is relatively simple: one gene-family (opsins) controls the detection of the signal, and one gene family (luciferase) controls the production of the signal. We use a transcriptomic approach to sample for and investigate opsin evolution in fireflies. We also use a phylogenetic estimate of Lampyridae to examine the evolution and ancestral modality of adult courtship communication.

We find evidence for only two expressed opsin classes in each of the nine firefly species studied, one in the ultra-violet sensitive and one in the long-wavelength sensitive areas of the visible spectrum. Bioluminescent communication in adults is not optimized to be present ancestrally, and was gained two to three times with six or seven subsequent losses. Despite the need for most adult fireflies to respond to a clearly sexual and colorful visual signal (bioluminescence) to maximize fitness, their visual system is relatively simple, and does not match the trend for opsin duplication found in other insect groups.

INTRODUCTION

Vision plays a central role in the lives of most animals. From predator avoidance to prey detection, from mate to habitat selection, the ability to sense one's surroundings using visual cues has long fascinated scientists (Warrant & Nilsson 2006). The components of visual communication between animals can be extremely complicated for science to tease apart. When one considers the need for animals to discriminate what signal is being transmitted, what medium or media the signal is being transmitted through, and how the signal is being perceived, there are so many variables, that the study of vision in its totality can seem daunting. However, the presence of visual pigments allows for a more direct, preliminary examination and understanding of visual communication. Visual pigments are composed of an opsin protein covalently bound to a chromophore, the specific molecule responsible for light absorption, (Wald 1967), and are contained within the photoreceptor cells of the eye. Opsins are responsible for light detection and overall vision, but multiple opsin copies can allow for color discrimination. Color discrimination is achieved by comparing the given stimulation of one opsin with another opsin that is sensitive to a different portion of the visible light spectrum (Cuthill 2006). There are currently four known "types or groups" of opsin: C-type, R-type, Cnidops and Group 4 (Porter *et al.* 2012). Here we restrict our discussion to only the R-type opsins, which are largely responsible for arthropod vision. Changes in the amino acid sequence of the opsin protein or in the structure of the chromophore can alter the overall spectral sensitivity of these pigments. The number of expressed opsins and the range in sensitivity is known to vary across animals (Rivera & Oakley 2009; Briscoe and Chittka 2001; Land & Nilsson 2012). For example, in stomatopods (Crustacea), 6–15 different expressed opsins have been found (Porter *et al.* 2009).

Arthropods, specifically insects, are the most diverse group of animals in the world, and their visual systems reflect this diversity (Rivera & Oakley 2009). Three major opsin classes have been identified in insects: ultra-violet sensitive (UVS), blue sensitive (BS) and long-wavelength sensitive (LWS). Based on a phylogenetic analysis of 54 arthropod species, it is hypothesized that the ancestral state for insect vision is a single copy of each of the three classes, (Briscoe & Chittka, 2001). However, current research demonstrates varying numbers of copies within each of these classes. In *Panorpa cognata* Rambur (Mecoptera) there is evidence for only one opsin (LWS; Burkhart & De LaMotte 1972, K. Manwaring unpublished data), while in *Papilio glaucus* Linnaeus (Lepidoptera) six total opsin copies across the three classes have been recovered (Briscoe 2000). Some dragonflies appear to have at least five copies across the three classes (Land and Osorio, 1991), with some species having as many as three LWS opsin copies alone (Bybee unpublished data; Meinertzhagen *et al.* 1983), and some butterflies possess two copies of UVS opsins (*e.g.*, Briscoe *et al.* 2010; Bybee *et al.* 2012). Other insects (*e.g.* beetles, *Tribolium castaneum* (Herbst)); owlflies, *Ascalaphus macaronius* Scopoli; cockroaches, *Periplaneta americana* Linnaeus) appear to have lost the blue opsin class entirely (Briscoe & Chittka 2001; Jackowska *et al.* 2007; Gogala 1967; Paul *et al.* 1986; Mote & Goldsmith 1970).

Given the large diversity in opsin copy number across insects, and especially within the hyper-diverse Holometabola (Diptera, Lepidoptera, & Hymenoptera) we expect a similar diversity in the beetles (Coleoptera). Surprisingly, opsins do not appear to have diversified across beetles as in other holometabolous insect groups, but opsin copy variation for relatively few beetles has been studied. Only one copy of the LWS and one copy of the UVS was recovered in the red flour beetle *T. castaneum* (Jackowska *et al.* 2007). However, Maksimovic *et al.* 2009 recovered three copies in the larval stage of the sunburst diving beetle *Thermonectus*

marmoratus (Gray) and Crook *et al.* 2009 found ERG evidence for four sensitivities in the emerald ash borer *Agrilus planipennis* Fairmaire. It is important to note that ERG data does not necessarily correlate one-to-one with opsin number (see below). The beetles are a cosmopolitan group of extraordinarily diverse insects that occupy a vast number of ecological niches, both terrestrial and aquatic. Coleoptera exhibit a range of eye types from anophthalmy (loss), to microphthalmy (reduction), to simple ocelli, to large, compound eyes. Given the niche diversity occupied by beetles, and by extension the diversity of visual conditions and eye morphologies, we expect that their modes of communication, specifically their visual communication, would also be highly variable and adaptive. Thus, the underlying molecular systems may also exhibit equal degrees of variability (Rivera & Oakley 2009). This is best tested in those lineages in which it is clear that visual communication plays an important role in life history. Perhaps one of the best examples of a visual coleopteran is the firefly (lightning bug; family Lampyridae), easily recognizable for their bioluminescent flash patterns, which are used to communicate both inter- and intra-specifically.

Fireflies are arguably the most well-known bioluminescent organism in the animal kingdom. In the marine environment, particularly in the deep ocean, examples of bioluminescence are prolific and found among many lineages of organisms, but terrestrial examples are comparatively rare in scope and diversity. Light production in fireflies is hypothesized to have originated as an aposematic warning signal among larvae (Branham & Wenzel 2001; 2003). Many fireflies are chemically defended by distasteful steroids called lucibufagins (Eisner *et al.* 1978) that are likely advertised via bioluminescence (Crowson 1972; Sivinsky 1981; Underwood *et al.* 1997; De Cock & Matthysen 2003; Branham 2010). It is hypothesized that this aposematic, larval bioluminescence was then co-opted as a method of adult visual communication (Branham &

Wenzel 2003). In addition to bioluminescence, many fireflies also use pheromones to communicate as adults (Fig. 1:A) and several different sexual communication systems have been proposed that incorporate different degrees of bioluminescence and/or pheromones.

Lloyd (1971) suggested two main groups of sexual communication among fireflies: signal system I: fireflies that employ little to no bioluminescent sexual communication and rely on the use of chemical pheromones, (*e.g.* members of *Ellychnia* Blanchard), and signal system II: fireflies in which females glow and/or flash and males are either non-luminescent (*e.g. Microphotus* LeConte, some *Phausis* LeConte), or luminescent (*e.g. Photinus* Laporte, *Photuris Dejean*). Based on a study of Japanese fireflies, Ohba (1983; 2004) suggested a system based on six types: 1) the *Hotaria parvula* (HP) system in which the male flashes and the female issues a consistent delayed response; 2), the *Luciola lateralis* (LL) system in which the timing of the female response varies; 3) the *Luciola cruciata* (LC) system in which the male flash pattern changes upon perching near a female; 4) the *Pyrocoelia rufa* (PR) system in which both sexes emit a continuous light; 5) the *Cyphonocerus ruficollis* (CR) system in which pheromones are predominantly used, but shortly after sunset a weak glow is given off by the males; and 6) the *Lucidina biplagiata* (LB) system in which both sexes are non-luminescent and use only pheromones. Branham and Wenzel (2003) investigated the evolution of firefly signal systems through a phylogenetic analysis of worldwide taxa. In that analysis, courtship behavior was investigated by categorizing signal systems solely on the basis of the signal modality used (e.g. chemical and or photic emissions). Branham and Wenzel (2003) recognized three signal systems: 1) pheromone only, 2) pheromones with bioluminescence, and 3) bioluminescence only. In a study on the evolution of bioluminescence in North American fireflies, Stanger-Hall *et al.* (2007) used a combination of previously-described systems and recognized four groups: 1) use of

pheromones only, 2) continuous glows mixed with pheromones, 3) flashes, whether short or long, and 4) pheromones accompanied by a weak glow during daylight or dusk (See Table 1 for summary). Focusing on how these sexual communication systems may play a role in opsin evolution is a potentially rich subject.

Bioluminescent emission data, as well as peak spectral sensitivity, has been recorded for several firefly species (Fig. 1:B & C) (Lall 1981; Lall *et al.* 1980; 1988; Eguchi *et al. 1984;* Lall & Worthy 2000). In all but one firefly species, *Photinus pyralis* (Linnaeus), the peak wavelength in visual sensitivity is within 5 nm of the peak intensity of the emission. Eguchi *et al*. (1984) suggested that, while the luciferase emission peak is in concordance with peak spectral sensitivity, especially in his study of Japanese fireflies, this does not mean that the opsin is specifically tuned to the emission, rather it suggests that the emission of particular wavelengths evolved to take advantage of a pre-existing spectral sensitivity. Thus, an important question is how diversity between opsin(s) and luciferase(s) are interlinked.

Oba & Kainuma (2009) found a correlation between diel pattern and opsin expression in *Luciola cruciata* Motschulsky. *Luciola cruciata* was found to have two expressed opsin classes - one in the UVS (360nm) and one in the LWS (560nm) portions of the spectrum. In male *L. cruciata*, neither UVS nor LWS opsin expression varied significantly throughout the day. In the female, however, expression in the long-wavelength opsin peaked at 20:00 hours, while UVS expression remained relatively consistent. Additionally, Oba & Kainuma recovered different opsin expression levels between sexes, with higher expression in males than in females, regardless of time of day. The timing of peak female expression also coincided with the peak bioluminescent activity of *L. cruciata* (Oba & Kainuma 2009).

Lall and Lloyd (1989) established that the visual sensitivities of adult fireflies vary based on when the firefly is active. Some night-active fireflies utilize a single broad peak sensitivity across the green portion of the visible spectrum, whereas some crepuscular fireflies utilize a narrow peak sensitivity in the yellow in addition to a "marked attenuation in the green region" (Lall *et al.* 1988; Lall & Worthy 2000; Lall *et al.*1982). Seliger *et al.* (1982) hypothesized that the broad green peak sensitivity in night-active fireflies is to detect flashes in a green foliage environment. A broad peak sensitivity, which includes a wider portion of the color spectrum, allows for less specificity in signal detection but a greater ability to detect low intensity signals. The narrow yellow visual peak in dusk-active fireflies corresponds to the peak of that species' bioluminescent emission for an increased spectral specificity. As a result, a greater amount of ambient and non-informative light is filtered out (Lall & Lloyd 1989). A second peak sensitivity has been reported in all fireflies in the near-UVS (Lall *et al.* 1980; Lall *et al*. 1982). These visual system data were obtained via electroretinographic (ERG) methods (Fig. 1:C).

Lall *et al.* (1982) were the first to experimentally demonstrate three spectral sensitivities in a firefly eye. They found support for sensitivity in the near-UVS, violet and long-wavelength (green-yellow) ranges in the firefly *Photuris lucicrescens* Barber. They also discussed the difficulty in isolating the violet mechanism using ERG methods, commenting on the possibility of hidden signal due to overlap of the UV and the LW sensitivity curves. While they recovered three spectral sensitivities, their results do not necessarily translate directly to the presence and expression of three distinct opsin classes underlying those sensitivities, as it is common for organisms to tune visual pigments/opsins to different sensitivities (Briscoe & Chittka 2001; *e.g.* use of screening pigments to screen out particular wavelengths of light, thus narrowing the band of light reaching the opsin molecule). In 1982, Seliger *et al.* proposed that spectral tuning could

occur through the screening pigment pathway as opposed to the opsin pathway. As suggested by Cronin *et al.* 2000, this assumes that either the visual pigments and/or opsins could be identical, and that variation is due to different species applying different filtration methods of visual light via screening pigments and/or a photoreceptor cell array. Cronin *et al.*'s (2000) findings indicated that variations exist in both the screening pigments *and* visual pigments, between the twilight active fireflies *Photinus scintillans* (Say) and *Photinus pyralis* and the night active *Photuris versicolor* Barber.

This diversity of signal systems across multiple modalities within this family of highly visual organisms predisposes fireflies as a study system for investigating the evolution of signals and the mechanisms used to perceive them. In this paper, we seek to understand how firefly visual systems relate to adult bioluminescence. We provide opsin DNA sequence data generated from the first transcriptomes of the firefly eye and place these data in a phylogenetic context with other insect opsin sequences. Because the color of the bioluminescent sexual signals fireflies emit appears tuned to specific opsin spectral peaks (Fig. 1:B & C), we investigate whether the family Lampyridae will have a more complex visual system at the molecular level (*i.e.*, more copies within each opsin class, Lall *et al.* 1982) than is currently known from other coleopteran families. We also investigate whether a higher diversity of opsins will be recovered in fireflies that utilize bioluminescence as the major component of their sexual communication versus those that use only pheromones or a combination of bioluminescence and pheromones. A phylogenetic estimate of Lampyridae is presented to place adult bioluminescence in an evolutionary context in order to visualize the evolutionary history of this bioluminescence.

MATERIALS AND METHODS

Taxon Sampling and Data

Transcriptomics: Specimens were collected from North and South America and preserved in an RNAlater®. Tissue for transcriptome assembly was prepped from the following taxa: *Micronaspis floridana* Green (male), *Pyractomena dispersa* Green (male), *Photinus pyralis* Linnaeus (male), a male and female *Bicellonycha wickershamorum* Cicero, two undetermined *Photuris* spp. (males) an undetermined *Aspisoma* sp. (male), *Ellychnia* sp. (male), and *Microphotus* sp. (female). These taxa represent two subfamilies and afford us an excellent opportunity to examine the evolution of visual systems at the molecular level among lineages that are well-documented both behaviorally and ecologically (Barber 1951; Cicero 1982; Fender 1970; Green 1948; 1956; 1957; 1959; Lloyd 1966; 1968; 1969; McDermott 1967). Tissue was prepped from the head and abdominal regions separately. In addition, some specimens had tissue prepared from the entire body. When both head and full body tissues were used, separate individuals collected at the same locality and at the same time of day were used (Table 2). Total RNA was extracted from each taxon using NucleoSpin columns (Clontech) and reverse-transcribed into cDNA libraries using the Illumina TruSeq RNA v2 sample preparation that both generates and amplifies full-length cDNAs. Some of the prepped mRNA libraries were sequenced on an Illumina HiSeq 2000 utilizing 101-cycle paired-end reads by the Microarray and Genomic Analysis Core Facility at the Huntsman Cancer Institute at the University of Utah, Salt Lake City, UT, USA, while the remainder were sequenced on a GaIIX utilizing 72 paired-end reads by the DNA sequencing center at Brigham Young University, Provo, UT, USA. Sequencing resulted in an estimated ~50x coverage.

Phylogenetic Analyses: To aid in the construction of a comprehensive phylogeny, 66 additional in-group lampyrid taxa representing six of the nine subfamilies (32 genera) recognized by Jeng 2008 and three outgroup taxa (one each of the families Elateridae, Rhagophthalmidae and Lycidae) were downloaded from Genbank® (Table 3). Three genes were selected from the Genbank® data that represented the most complete dataset available for fireflies: 16S (98% of taxa), 18S (83%) and COI (83%). These three genes have been shown from previous studies to be useful at resolving insect relationships (see Miller *et al*. 2007; Lord *et al.* 2010)

Sequence Data

Transcriptome Assembly: Quality control, assembly, annotation, and transcriptome analysis using existing computational tools that have been combined into a Galaxy pipeline for the Bybee Lab (Suvorov *et al., in prep*.) was performed to facilitate downstream phylogenetic analyses. RNA-seq reads were trimmed using the Mott algorithm implemented in PoPoolation (Kofler *et al.* 2011), with a minimum read length = 40 and quality threshold = 20. The de novo assembly of the transcriptome contigs was carried out using Trinity (Grabherr *et al.* 2011) under the default parameters.

Putative light-interacting genes were isolated from each transcriptome by utilizing the Phylogenetically-Informed Annotation (PIA) tool (Spieser *et al.*, *submitted*; http://galaxy-dev.cnsi.ucsb.edu/pia/), implemented in Galaxy (Goecks *et al.* 2010; Blankenberg *et al.* 2010; Giardine *et al.* 2005). As the PIA tool is optimized to identify an array of light-interacting genes involving circadian cycles, eye development, phototransduction, pigment synthesis, *etc*., resultant matches in the transcriptomes were then vetted for opsin-specific genes. All individual reads isolated by the PIA tool were BLASTed, implemented in Geneious®, utilizing the "nr"

database option (GenBank, RefSeq, EMBL, DDBJ, and PDB databases) and the "blastn" program set to 100 maximum hits. Similar hits were then assessed for e-value and sequence type/description. All non-opsin contigs were ignored, and all putative opsin contigs, regardless of length, were mapped in SWISS-MODEL (available from http://swissmodel.expasy.org/) (Biasini *et al.* 2014; Arnold *et al.* 2006; Bordoli *et al.* 2009; Kiefer *et al.* 2009; Kopp and Schwede 2006) to verify the presence of the seven trans-membrane regions and aid in the exclusion of partial reads. Additional opsin data from other insects were downloaded from GenBank (see Table 4 for accession numbers) for assistance in constructing an opsin phylogeny.

Phylogenetic Reconstruction: For the opsin data, entire opsin genes were reduced to the coding sequence (CDS) by trimming untranslated regions (UTRs) for each sequence in Geneious. All opsin genes were then aligned in MAFFT v 7.017 (Katoh and Standley 2013) under the G-INS-i strategy, implemented in Geneious v. 7.1.2, and checked for open reading frames. Genes compiled for the lampyrid phylogeny were aligned independently in MAFFT under the L-INS-i strategy, implemented in Geneious. Other alignment strategies (G-INS-i & E-INS-i) were tested in MAFFT, however the L-INS-i strategy provided the shortest, least gap-filled alignment, as well as the log likelihood value closest to zero. Genes were concatenated using Geneious. Maximum Likelihood analyses were run on the aligned datasets independently in RAxML (200 replicates) (Stamatakis & Rougemont 2008) using the GTR + Γ model as recommended through analysis in PartitionFinder (Lanfear *et al.* 2012) on the BYU Fulton Supercomputer. Bootstrap support values were based on 1000 bootstrap pseudoreplicates.

Ancestral State Reconstruction: Adult bioluminescence was reconstructed onto the ML tree using the ancestral state reconstruction package in Mesquite (Maddison & Maddison 2011) under both a parsimony and ML framework. Bioluminescence was coded under the system

implemented by Lloyd (see above) and modified 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 additional flash pattern information is not currently possible across the breadth of taxa needed for comprehensive analyses due to the lack of empirical data for the majority of firefly species and as such, Lloyd's system was chosen over the more complex character scoring alternatives of Ohba, Branham & Wenzel or Stanger-Hall *et al.* (above) as a conservative estimate of bioluminescence. Data on the presence or absence of bioluminescence/photic organs is widely available, both in the literature and through direct observation.

Tree Figures: Trees were visualized in Figtree v. 1.4 (http://tree.bio.ed.ac.uk/software/figtree/) and tree figures were constructed in Adobe Illustrator CS5.

RESULTS

Transcriptome assembly: Results from the transcriptome assemblies are displayed in Table 2. On average, transcriptome sequences derived from the head and whole-body region were longer than those generated from the abdominal region. Expressed opsins were recovered in both the head-only and full-body transcriptomes, demonstrating the utility of full-body transcriptomes for the isolation of opsin genes. This can be useful in the case of smaller insects (*i.e. Phausis*) in which extracting only head tissue is difficult and yields small quantities of RNA.

Opsin expression in Lampyridae: BLAST searches conducted using the PIA pipeline on the assembled firefly transcriptomes resulted in the recovery of one copy of an expressed long-wavelength sensitive (LWS) opsin and one copy of an expressed ultra-violet sensitive (UVS)

opsin in each of the ten taxa sampled. No evidence of expression of a blue sensitive opsin was detected, and no additional expressed copies of LWS or UVS opsins were recovered.

Opsin gene tree: All recovered firefly opsins were extracted from the transcriptomes and aligned and analyzed with an additional sampling of 69 opsin sequences (GenBank data, see Materials and Methods, Table 4). The resulting alignment of the opsin genes (including only CDS regions) was 1,404 bp long. The opsin maximum likelihood tree with the highest log likelihood score (-59449.717677) (Fig. 2) recovered the three opsin spectral classes with strong support (bootstrap values of 91, 91 and 100 for BS, UVS, and LWS respectively). In both the UVS and LWS portions of the tree, the opsins from fireflies form monophyletic clades (bootstrap support 100 for each) sister to the other Coleoptera.

Phylogeny of Lampyridae: A concatenated alignment of the data derived from GenBank (16S, 18S, COI) was 4,677 bp long. Our tree supports 2–3 gains and 6–7 losses of adult bioluminescence across Lampyridae (Log likelihood: -48695.074634; Fig. 3). The ancestral state for adult Lampyridae is non-bioluminescent. With the exception of the Lampyrinae and Ototretinae, all subfamilies sampled were recovered as monophyletic (see Figure 3 for bootstrap values). Lampyrinae was rendered paraphyletic by Photurinae, and Ototretinae was rendered paraphyletic with *Brachylampis blaisdelli* Van Dyke as sister to the Photurinae + Lampyrinae.

DISCUSSION

We recovered no evidence of a blue opsin class among the species studied. It appears that, although many fireflies produce and respond to complex visual signal patterns, the opsins are not particularly diverse in class and/or copy number. While this is similar to *Tribolium* (Jackowska

et al. 2007), this does set fireflies apart from both *A. planipennis* (Crook *et al.* 2009) and *T. marmoratus,* (Maksimovic *et al.* 2009). We also found the number of opsin copies to be consistent between both the lineages that are capable of adult bioluminescence and those lineages that are not.

Our findings are surprising given the highly visual nature of fireflies, when compared to other visual beetle groups such as Buprestidae (Crook *et al.* 2009). The lack of opsin diversity among fireflies, however, is at least partially supported through most of the available electroretinographic data, with the exception of Lall *et al.* (1982), who recovered three spectral sensitivities in *Photuris lucicrescens* (see above). No study to date has demonstrated duplicate copies within a particular class.

Even with this significant contribution to what is currently known about the molecular basis of firefly visual systems, only a minority of firefly species have been examined in reference to opsin copy number and diversity. More taxa representing all the major lineages of the family need to be explored in order to robustly demonstrate a lack of diversity in opsins across this family (*i.e.* only two of the nine subfamilies were studied herein). If fireflies are not gaining increased specificity from duplicating opsin copies, then another mechanism could be in use, such as variation in visual and screening pigments as suggested by Seliger *et al.* (1982, see above). In order to truly decipher the variables impacting firefly spectral tuning, sensitivities, and color vision, additional research needs to be undertaken and a robust phylogenetic estimate is needed. This phylogenetic estimate would allow for a more targeted sampling of visual systems among fireflies while also placing existing data within a phylogenetic context to better understand how firefly visual systems have evolved across their entire diversity.

As a first attempt at this phylogeny, we performed ancestral state reconstruction on the lampyrid phylogeny for adult bioluminescence only. This analysis resulted in 2–3 independent gains (or instances of maintenance and expression) of bioluminescence in the adult life stage coupled with 6–7 losses (Fig 3). While a single ancestral gain of adult bioluminesce would only increase the parsimony analysis by two steps, ML reconstruction suggests a single gain as highly unlikely (proportional likelihood value of 0.185). This same evolutionary pattern has been reconstructed by numerous authors who conducted phylogenetic analyses of datasets that differ in both taxon sampling and types of characters used, e.g. morphology or molecules (Susuki 1997; Branham and Wenzel 2003; Jeng 2008).

The subfamily Ototretinae was recovered as completely non-bioluminescent. In the subfamilies Photurinae and Pterotinae, adults of all known species are bioluminescent, whereas the other subfamilies included were found to have both luminous and non-luminous members. Opsin data was not mapped on the phylogeny because our data do not show any compelling evidence for particular visual system complexities (i.e., there were no recovered duplication events) and are not yet extensive enough for studies of molecular evolution (*i.e.,* rates of evolution). In the future, we plan to sequence a more phylogenetically representative sample of fireflies, including those that purportedly have three opsin classes such as *P. lucicrescens* (Lall *et al.* 1982).

Although not the focus of our study, the phylogenetic estimate allows us to comment on the status of lampyrid classification. McDermott (1964) stated that the classification of Lampyridae is largely artificial, with generic arrangement being “logical” but not representative of phylogenetic relationships and with the tribal classification “more or less arbitrary.” Jeng (2008) confirmed an artificial tribal classification with morphological data and suggested abandoning it

altogether, (see also Archangelsky & Branham 2001 for corroborating data from larval morphology). Jeng's phylogenetic estimate corroborated McDermott's sub-familial classification with the exception of the Ototretadrilinae-Ototretinae complex. Not all phylogenetic analyses have reached this conclusion (Branham & Wenzel 2003, Stanger-Hall *et al.* 2007). In so much as our taxa overlap, our results agree in large part with Jeng (2008), as only the subfamilies Ototretinae (defined as the traditional Ototretinae and the pan ototretinae) and Lampyrinae were recovered as non-monophyletic.

CONCLUSIONS

Our results suggest a deviation in the general trend of opsin copy duplication, as recovered in many other insect groups such as Lepidoptera, Odonata, Hymenoptera, and Diptera. These data identify several areas of study that will further illuminate lampyrid visual system evolution. For example, an increased taxon sampling from some of the under-represented subfamilies (*e.g.* Luciolinae & Ototretinae) will be needed to truly investigate opsin evolution across Lampyridae. Physiological (ERG) and mechanical (spectral tuning) data for the lampyrid eye will be central to understanding the evolution of firefly visual systems. Also, a phylogeny of Lampyridae that includes a large and diverse taxon sampling built on an extensive molecular matrix is long overdue and is essential to further studies of firefly evolution.

ACKNOWLEDGEMENTS

We would like to thank the members of the Branham lab (Gainesville, FL), the Whiting and Bybee labs (Provo, UT), as well as Jim Lloyd (Gainesville, FL) and Tara Martin (Provo, UT) for specimen donation. We thank Derek Houston, Kelsey Johnson, Yelena Pacheco and Mike Swindle (Dept. of Biology, BYU, Provo, UT) for assistance with Illumina library preparation. We also thank the BYU DNA Sequencing center and the Microarray and Genomic Analysis Core Facility at the Huntsman Cancer Institute at the University of Utah for transcriptome sequencing. We thank Perry Ridge, David Morris, Ryan Hillary, Anton Suvorov and Perry Wood for assistance with transcriptome assembly and analyses. We want to thank BYU for access to the Fulton Supercomputer, as well as the Oakley lab for access to and assistance with the PIA pipeline. We also thank Joseph Mugleston, Haley Wightman, Jerry Johnson (BYU, Provo, UT) for reviewing previous versions of this manuscript. We especially thank Michael Whiting (BYU) for specimen donation and extensive help with revisions to the manuscript. This study was supported in part by a BYU Graduate Student Fellowship (GJM), as well as grants from the National Science Foundation (SMB; IOS-1265714) and the American Philosophical Society (SMB).

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CHAPTER 1 TABLES

Table 1: Summary of the classification systems of adult lampyrid bioluminescence from Lloyd 1971, Ohba 1983, Branham & Wenzel 2003, & Stanger-Hall *et al*. 2007.

Lloyd		**Ohba**	
Signal System I	No Bioluminescence	HP	Male flashes; female issues a consistent delayed response
Signal System II	Bioluminescence	LL	Male flashes; the timing of the response of the female varies
		LC	Male flash pattern changes upon perching near a female
		PR	Both sexes use a continuous light
		CR	Mainly pheromones, shortly after sunset a weak glow is given off by the males
		LB	Only pheromones

Stanger-Hall *et al.*		**Branham & Wenzel**	
Group 1	Pheromone only	1	Pheromone only
Group 2	Continuous glow + pheromone	2	Pheromone + Bioluminescence
Group 3	Short or long flashes	3	Bioluminescence only
Group 4	Pheromones + weak glow during daylight/dusk		

Table 2: Summary of transcriptome assembly statistics. Head and abdomen values are from the same individual; when full body tissue was used in addition to head + abdomen (*Photinus pyralis, Micronaspis floridana, Pyractomena dispersa*) a different individual that was collected at the same locality at the same time and date was used. Min. and max. refer to the shortest and longest contig assembled respectively.

Species	Part of Body Sequenced	N50	Min	Max	# of Contigs
Pyractomena dispersa	Head	1,071	201	7,179	31,768
Pyractomena dispersa	Tail	1,428	201	10,244	31,013
Photinus pyralis	Head	2,005	201	14,093	32,337
Photinus pyralis	Tail	947	201	6,976	19,676
Photuris "A"	Head	1,496	201	10,320	35,203
Photuris "A"	Tail	1,437	201	11,868	25,980
Micronaspis floridana	Head	1,468	201	7,515	30,157
Micronaspis floridana	Tail	1,070	201	10,189	23,848
Photinus pyralis	Full body	1,581	201	11,606	29,288
Micronaspis floridana	Full body	1,718	201	18,075	31,188
Pyractomena dispersa	Full body	1,302	201	9,150	30,632
Aspisoma sp.	Head	1,841	201	10,180	33,589
Aspisoma sp.	Tail	1,835	201	18,824	31,370
Photuris sp. 1	Head	1,817	201	12,265	31,346
Photuris sp. 1	Tail	1,321	201	7,162	29,237
Ellychnia sp.	Full body	2,478	201	23,792	56,511
Bicellonycha wickershamorum Female	Full body	1,988	201	16,382	46,760
Bicellonycha wickershamorum Male	Full body	2,324	201	24,599	54,138
Microphotus sp.	Full body	2,538	201	15,518	63,016
Photinus marginalis	Full body	2,504	201	20,728	61,254
Photuris sp. Larva	Full body	1,802	201	19,236	44,634
Photuris sp. 2	Full body	2,346	201	16,690	59,181
Phausis reticulata	Full body	2,837	201	25,672	73,905

Table 3: GenBank accession numbers for taxa used in the lampyrid phylogenetic estimation.

Family	Species	18S	16S	COI
Lampyridae	*Aspisoma* sp.	EU009248	EU009285	EU009322
Lampyridae	*Bicellonycha wickershamorum*	EU009228	EU009265	EU009302
Lampyridae	*Brachylampis blaisdelli*	EU009230	EU009267	EU009304
Lampyridae	*Ceylanidrilus* sp.	DQ100524	DQ198682	DQ198605
Lampyridae	*Curtos costipennis*	AB298848	AB671250	AB671258
Lampyridae	*Curtos okinawanus*	AB298849	AB671252	AB671262
Lampyridae	*Curtos* sp.	DQ100513	DQ198671	DQ198594
Lampyridae	*Cyphonocerus ruficollis*	DQ100512	DQ198670	DQ198593
Lampyridae	*Diaphanes formosus*	EU009243	EU009280	EU009317
Lampyridae	*Drilaster axillaris*	AB298853	AB436506	AB608756
Lampyridae	*Drilaster borneensis*	DQ100522	DQ198680	DQ198603
Lampyridae	*Drilaster* sp.	DQ100517	DQ198675	DQ198598
Lampyridae	*Ellychnia californica*	EU009218	EU009255	EU009292
Lampyridae	*Ellychnia corrusca*	EU009225	EU009262	EU009299
Lampyridae	*Flabellotreta obscuricollis*	DQ100519	DQ198677	DQ198600
Lampyridae	*Flabellotreta* sp.	DQ100520	DQ198678	DQ198601
Lampyridae	*Lamprohiza splendidula*	EU009245	EU009282	EU009319
Lampyridae	*Lampryis noctiluca*	EU009247	EU009284	EU009321
Lampyridae	*Lucidina biplagiata*	AB298844	AB009922	
Lampyridae	*Lucidota atra*	EU009219	EU009256	EU009293
Lampyridae	*Luciola cruciata*		AB009904	AF360953
Lampyridae	*Luciola filiformis yayeyamana*	AB298850	AB436493	
Lampyridae	*Luciola italica*		AB436494	
Lampyridae	*Luciola kuroiwae*		AB009907	
Lampyridae	*Luciola lateralis*	AB298851	AB009906	
Lampyridae	*Luciola ovalis*		DQ371179	
Lampyridae	*Luciola parvula*	AB298852	AB436504.1	AB608763
Lampyridae	*Luciola* sp.	EU009244	EU009281	EU009318
Lampyridae	*Lychnuris formosana*	EU009242	EU009279	EU009316
Lampyridae	*Micronaspis floridana*	EU009240	EU009277	EU009314
Lampyridae	*Microphotus angustus*	EU009227	EU009264	EU009301
Lampyridae	*Paraphausis eximia*	EU009223	EU009260	EU009297
Lampyridae	*Phausis reticulata*	EU009237	EU009274	EU009311
Lampyridae	*Phosphaenus hemipterus*	EU009246	EU009283	EU009320
Lampyridae	*Photinus australis*	EU009224	EU009261	EU009298
Lampyridae	*Photinus floridanus*	EU009232	EU009269	EU009306
Lampyridae	*Photinus punctulatus*	EU009238	EU009275	EU009312
Lampyridae	*Photinus pyralis*	EU009239	EU009276	EU009313
Lampyridae	*Photinus tanytoxis*	EU009241	EU009278	EU009315
Lampyridae	*Photuris aff. lucicrescens*	EU009216	EU009253	EU009290
Lampyridae	*Photuris congener*		EU301845	
Lampyridae	*Photuris pennsylvanica*	PPU65129		AY165656
Lampyridae	*Photuris quadrifulgens*	EU009236	EU009273	EU009310
Lampyridae	*Photuris tremulans*	EU009234	EU009271	EU009308
Lampyridae	*Pleotomodes needhami*	EU009231	EU009268	EU009305
Lampyridae	*Pleotomus pallens*	EU009217	EU009254	EU009291
Lampyridae	*Pollaclasis bifaria*	EU009221	EU009258	EU009295
Lampyridae	*Pristolycus sangulatus*		AB009925	

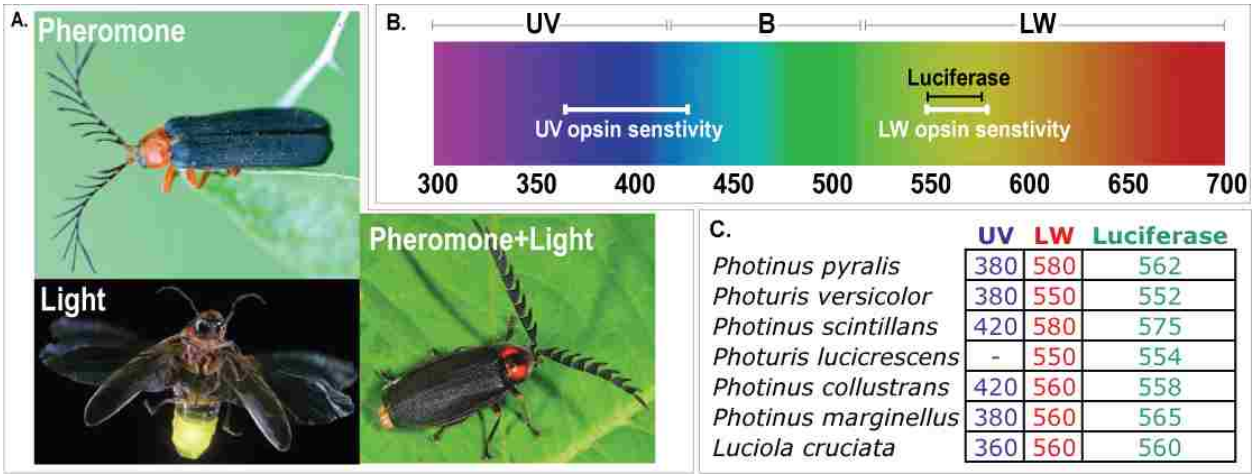
Lampyridae	*Pterotus obscuripennis*	EU009229	EU009266	EU009303
Lampyridae	*Pyractomena angulata*	EU009233	EU009270	EU009307
Lampyridae	*Pyractomena borealis*	EU009222	EU009259	EU009296
Lampyridae	*Pyractomena palustris*	EU009235	EU009272	EU009309
Lampyridae	*Pyractomena dispersa*	XXXXXX	XXXXXX	XXXXXX
Lampyridae	*Pyrocoelia abdominalis*		AB009921	AB608766
Lampyridae	*Pyrocoelia amplissima*		DQ371190	
Lampyridae	*Pyrocoelia atripennis*	AB298845	AB009915	AB608767
Lampyridae	*Pyrocoelia discicollis*		AB436511	AB608768
Lampyridae	*Pyrocoelia fumosa*	AB298846	AB436510	AB608769
Lampyridae	*Pyrocoelia rufa*		AB009913	
Lampyridae	*Pyropyga decipiens*	EU009226	EU009263	EU009300
Lampyridae	*Pyropyga nigricans*	EU009220	EU009257	EU009294
Lampyridae	*Vesta saturnalis*		DQ371195	
Lampyridae	*Vesta* sp.	DQ100511	DQ198669	DQ198592
Elateridae	*Oxynopterus* sp.	HQ333800	HQ333710	HQ333982
Lycidae	*Plateros* sp.	DQ181109	FJ390407	FJ390409
Rhagophthalmidae	*Rhagophthalmus ohbai*	AB298864.1	AB009931.1	AB608775.1

Table 4: GenBank accession numbers for taxa used in the opsin gene phylogenetic estimation.

Order	Family	Binomen	Description	Data Source	GenBank Accession Number
Coleoptera	Lampyridae	*Aspisoma* sp.	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Bicellonycha wickershamorum*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Ellychnia* sp.	Long Wavelength-sensitive opsin UV-sensitive opsin 1	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Micronaspis floridana*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Microphotus* sp.	UV-sensitive opsin	G.J. Martin, this paper	########
Coleoptera	Lampyridae	*Phausis reticulata*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Photinus marginellus*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Photinus pyralis*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Photuris* sp. larva	UV-sensitive opsin	G.J. Martin, this paper	########
Coleoptera	Lampyridae	*Photuris* sp. 1	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Photuris* sp. 2	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Pyractomena dispersa*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Pyropyga nigricans*	Long Wavelength-sensitive opsin UV-sensitive opsin	G.J. Martin, this paper G.J. Martin, this paper	######## ########
Coleoptera	Lampyridae	*Luciola cruciata*	Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank	AB300328 AB300329
Coleoptera	Tenebrionidae	*Tribolium castaneum*	Similar to UV-sensitive opsin Long Wavelength-sensitive opsin	GenBank BeetleBase, this paper	XM_965251 ########
Coleoptera	Dytiscidae	*Thermonectus marmoratus*	Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank	EU921225 EU921226 EU921227
Hymenoptera	Apidae	*Apis cerana*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	AB355817 AB355818 AB355816
Hymenoptera	Apidae	*Apis mellifera*	Blue-sensitive opsin Long Wavelength-sensitive opsin 1 Long Wavelength-sensitive opsin 2 UV-sensitive opsin	GenBank GenBank GenBank GenBank	NM_001011606 NM_001011639 NM_001077825 NM_001011605
Hymenoptera	Agaonidae	*Ceratosolen solmsi*	Blue-sensitive opsin Long Wavelength-sensitive opsin 1 Long Wavelength-sensitive opsin 2 UV-sensitive opsin	GenBank GenBank GenBank GenBank	JX402132 JX402130 JX402131 JX402133
Lepidoptera	Nymphalidae	*Heliconius erato*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank GenBank	AY918906 AY918907 AY918904 AY918905
Lepidoptera	Nymphalidae	*Heliconius sapho*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank GenBank	GU324692 GU324705 GQ451907 GQ451908
Lepidoptera	Sphingidae	*Macroglossum stellatarum*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	KF539426 KF539444 KF539456
Lepidoptera	Sphingidae	*Manduca sexta*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	AD001674 L78080 L78081
Lepidoptera	Nymphalidae	*Danaus plexippus*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	AY605544 AY605545 AY605546
Lepidoptera	Pieridae	*Pieris rapae*	Blue-sensitive opsin Long Wavelength-sensitive opsin 1 UV-sensitive opsin Violet-sensitive opsin	GenBank GenBank GenBank GenBank	AB208675 AB177984 AB208673 AB208674
Orthoptera	Gryllidae	*Dianemobius nigrofasciatus*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	AB291232 FJ232921 AB458852
Orthoptera	Gryllidae	*Gryllus bimaculatus*	Blue-sensitive opsin Green-sensitive opsin 1 Green-sensitive opsin 2 UV-sensitive opsin	GenBank GenBank GenBank GenBank	HM363622 HM363620 HM363621 HM363623
Hemiptera	Delphacidae	*Laodelphax striatella*	Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank	AB761153 AB761154 AB761155
Hemiptera	Delphacidae	*Nilaparvata lugens*	Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank	AB761147 AB761148 AB761149
Hemiptera	Delphacidae	*Sogatella furcifera*	Long Wavelength-sensitive opsin UV-sensitive opsin 1 UV-sensitive opsin 2	GenBank GenBank GenBank	AB761150 AB761151 AB761152
Hemiptera	Cicadellidae	*Nephotettix cincticeps*	Blue-sensitive opsin Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank GenBank	AB761157 AB761156 AB761158

Hemiptera	Aphididae	*Megoura viciae*	Long Wavelength-sensitive opsin UV-sensitive opsin	GenBank GenBank	AF189714 AF189715
Diptera	Drosophilidae	*Drosophila melanogaster*	Blue-sensitive opsin (UV3_UVA) Long Wavelength-sensitive opsin 1 (violet) Long Wavelength-sensitive opsin 2 (blue) UV-sensitive opsin 1 UV-sensitive opsin 2	FlyBase, this paper FlyBase, this paper FlyBase, this paper FlyBase, this paper FlyBase, this paper	######## ######## ######## ######## ########

CHAPTER 1 FIGURES



	UV	LW	Luciferase
Photinus pyralis	380	580	562
Photuris versicolor	380	550	552
Photinus scintillans	420	580	575
Photuris lucicrescens	-	550	554
Photinus collustrans	420	560	558
Photinus marginellus	380	560	565
Luciola cruciata	360	560	560

Figure 1: A: Representatives of three generalized communication systems, pheromone, light, pheromone + light. B: Visual spectrum (in nm). White bars indicate UV and LW sensitivities; black bar indicates luciferase emission spectrum. C: Summary of known sensitivities for UVS and LWS opsins (from ERG data) and luciferase color emission.

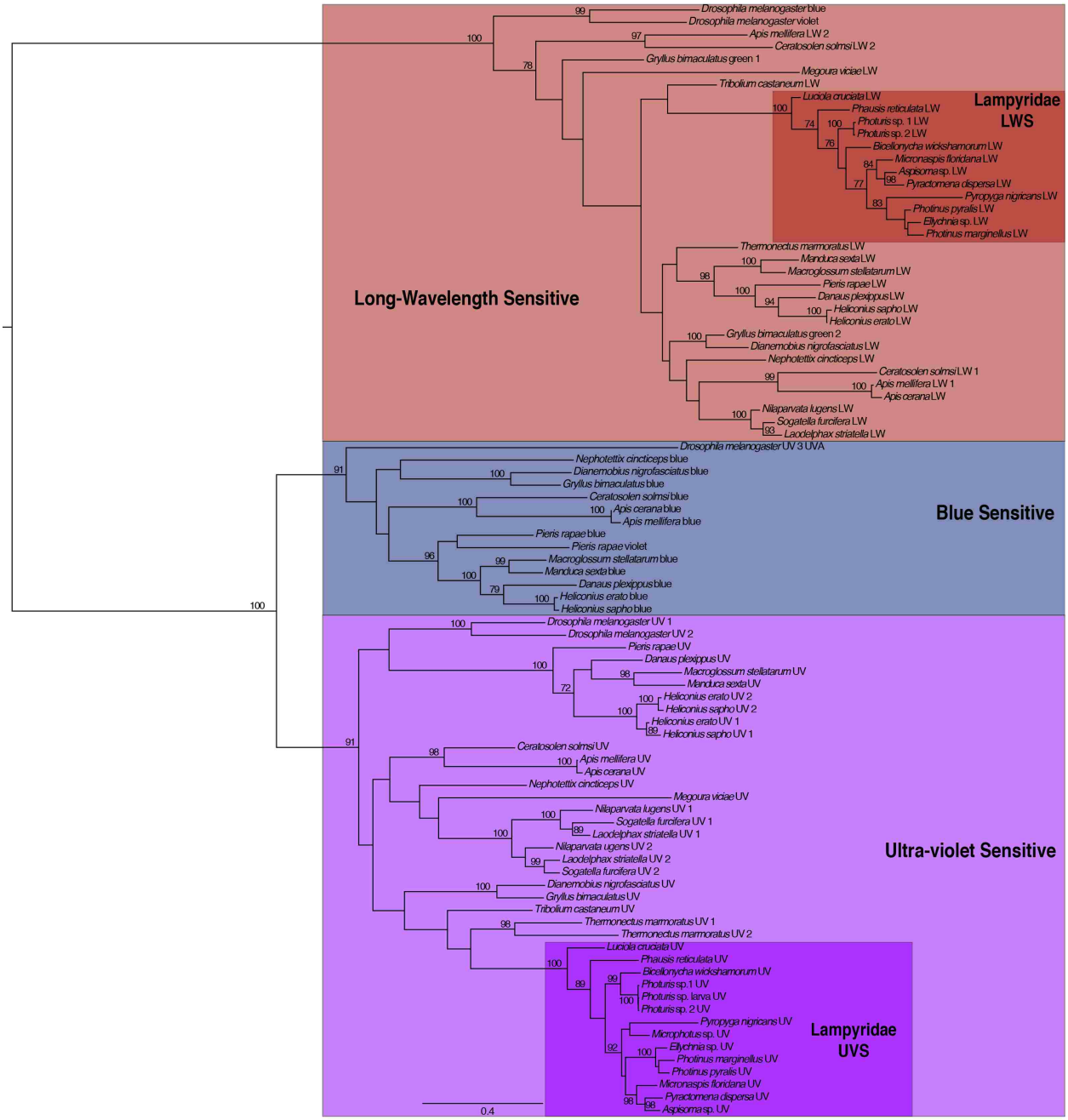


Figure 2: Opsin phylogeny; Best scoring Maximum Likelihood tree from 200 replicates (Log likelihood: -59449.717677).

Bootstrap values based on 1000 replicates over 70 indicated at nodes.

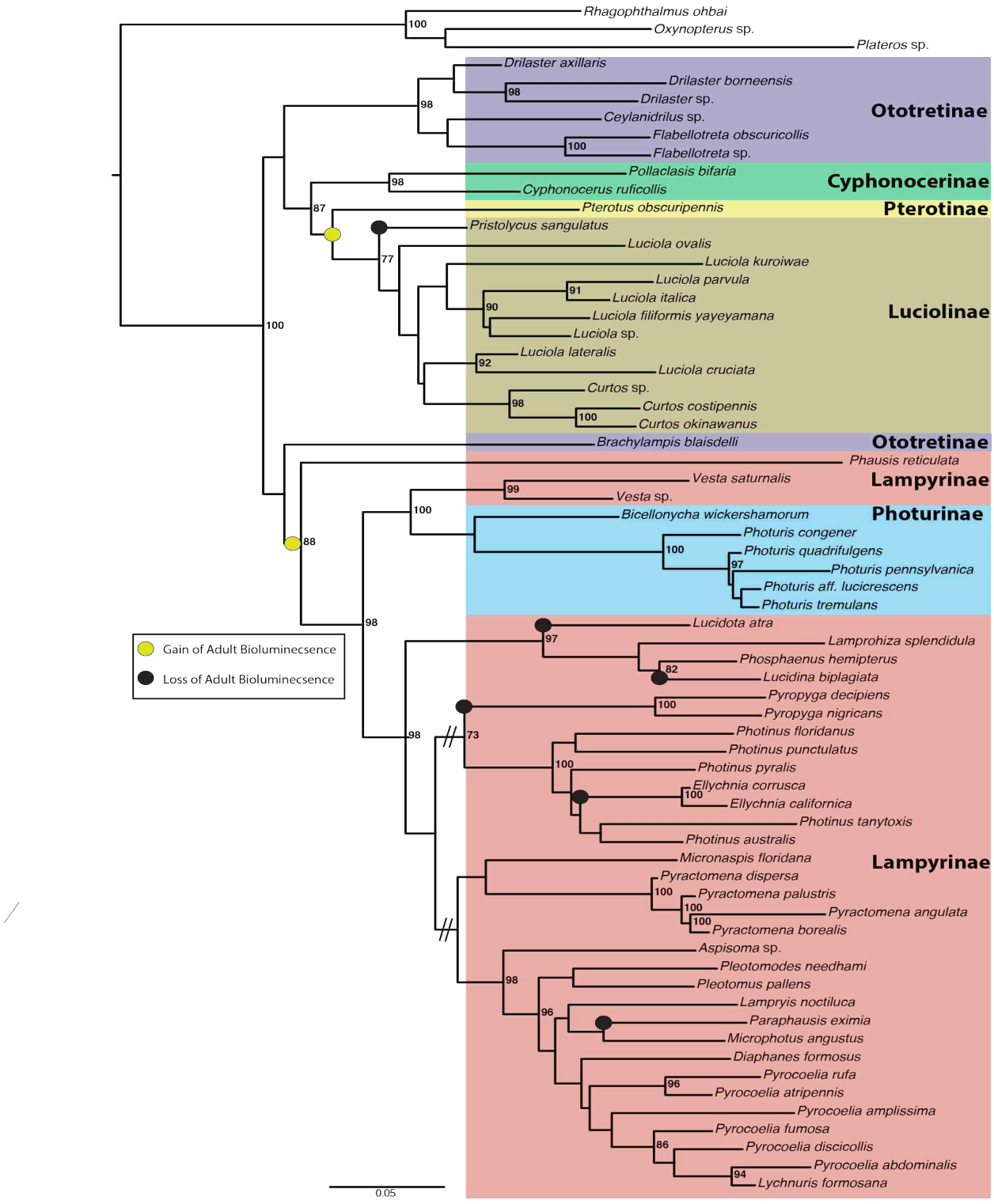


Figure 3: Best scoring Maximum Likelihood tree of 66 taxa from 200 replicates (Log likelihood: -48695.074634). Bootstrap values based on 1000 replicates over 70 indicated at nodes. Parsimony ancestral reconstruction of bioluminescence according to system proposed by Lloyd 1982. Gains of adult bioluminescence represented by yellow circle; losses by a black circle.

Chapter 2. Molecular phylogeny of Lampyridae (Insecta: Coleoptera): with implication into bioluminescent evolution

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Keywords**:** Phylogeny, Coleoptera, Lampyridae, Bioluminescence

ABSTRACT

Fireflies are some of the most captivating organisms on the planet. Due to this, they have a rich history of study, especially concerning their bioluminescent behavior. Despite this history of research, firefly relationships are still poorly understood. Knowledge of the evolutionary history of a group is necessary to test hypotheses of character evolution. Prior phylogenetic analyses of the family Lampyridae have been limited in terms of taxon sampling as well as restricted to either morphological or molecular data. Here, we present the first approach using both an extensive molecular matrix and a robust morphological matrix to reconstruct the lampyrid phylogeny. We then use this phylogeny to assess the hypothesis that adult use of bioluminescence occurred after the origin of Lampyridae.

All subfamilies except for Lampyrinae are recovered as monophyletic, and the ancestral state of adult bioluminescence is suggested to be non-bioluminescent.

INTRODUCTION

Fireflies (Coleoptera: Lampyridae Latreille, 1817) are some of the most captivating organisms on earth due to their fascinating bioluminescent signaling behavior and their close proximity to humans. Lampyridae is a cosmopolitan family composed of nine subfamilies, ~83 genera, and ~2,000 species, with the majority of diversity found in the tropical regions of the world (Branham, 2010). Because most fireflies are so charismatic, there is a large body of research dealing with life history and especially their bioluminescent behavior (Buck 1937; Buschman 1977; Lall *et al.* 2000; Lloyd 1971; Sagegami-Oba *et al.* 2007). It is well known that many firefly species that are luminous as adults have species specific flash patterns, and in some genera, specimens are difficult to impossible to identify to species without knowledge of their flash pattern. Given this level of morphological similarity, it is not surprising that phylogenetic relationships among fireflies have remained controversial. Furthermore, fireflies and their relatives have been shown to contain high levels of morphological convergence. Like many other insect groups, the uncertainty of firefly relationships extends from the species level up to the level of family, as multiple taxa have been repeatedly placed in and then removed from the family Lampyridae. These taxa often possess unique combinations of morphological characters that has made their taxonomic placement challenging (Branham 2010: Branham and Wenzel 2001; McDermott 1964: Crowson 1974). Discovering the patterns of these evolutionary relationships is necessary to extend studies of character evolution in a group that has such an interesting and charismatic method of communicating with bioluminescence.

Several authors have reconstructed the phylogeny of Lampyridae and allied taxa, and with each study discrepancies in relationships have been recovered. McDermott, one of the eminent workers in Lampyridae, stated that his 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, this classification should not be misconstrued as representing phylogenetic relationships (McDermott 1964). Olivier recognized nine subfamilies based mostly on head and antennal morphology in his initial classification of the family (Olivier 1907, 1910). Later, Green instituted a tribal classification specific to the subfamily Lampyrinae, which McDermott then applied to the whole Lampyridae (Green 1948, 1959; McDermott 1964). In his classification, McDermott recognized seven subfamilies with sixteen tribes/subtribes (McDermott 1966). Next, Crowson revisited the classification of Lampyridae with a system that set aside the tribal classifications of McDermott and Green, and recognized eight subfamilies (Crowson 1972). All of these studies were based solely on morphological analyses.

Suzuki was the first to put the classification into a phylogenetic framework based on molecular data. However, his study was limited in that it focused primarily on the Japanese fauna (Suzuki 1997). The lack of taxonomic coverage was increased in the morphological analyses of Branham and Wenzel (2001,2003). Branham and Wenzel recovered Lampyridae in a trichotomy with Rhagophthalmidae, which is also bioluminescent, and another clade consisting of several other cantheroid families. Several genera which were historically moved in and out of Lampyridae, (e.g. *Harmatelia*, *Drilaster,* and *Pterotus*), were again moved out of the family and into Elateroidea *incertae sedis* by these authors. Branham and Wenzel recovered only two subfamilies, Luciolinae and Photurinae, as monophyletic. Rhagophthalmidae was recovered as sister to Lampyridae (Branham and Wenzel 2003). In 2007, Stanger-Hall *et al.* performed a phylogenetic analysis on several North American taxa based on three genetic markers. They recovered both *Pterotus* and *Rhagophthalmus* within the Lampyridae. Stanger-Hall *et al.* did however point out that with the inclusion of more taxa and/or sequence information, these taxa

could move to a basal relationship sister to the rest of Lampyridae (Stanger-Hall *et al.* 2007). In 2008, Jeng reconstructed a phylogeny based on a morphological analysis that included the largest and broadest ingroup sampling to date representing ~80% of lampyrid genera, as well as representatives from nine other closely-related families. In this analysis, *Pterotus* was recovered as the basal genus within Lampyridae, while Rhagophthalmidae+Phengodidae+Telegeusidae was recovered in a clade sister to Lampyridae. Jeng reclassified the Lampyridae to include nine subfamilies, eight of which were recovered as monophyletic in his analysis. The only subfamily *not* rendered monophyletic was Ototretinae, with respect to the 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 + the subfamily Ototretadrilinae (Jeng 2008). Each of these studies differed in their methods, taxon sampling, and data sources, but one thing is constant: the classification of Lampyridae changes. Given these different hypotheses of lampyrid relationships, it is difficult to trace the evolution of interesting features found in this lineage of important biological systems such as gains and losses of adult bioluminescence.

Fireflies are one of the best-known producers of bioluminescence in the terrestrial world. Among Hexapoda, bioluminescence is only found in four orders (*e.g.* Collembola, Blattodea, Diptera, and Coleoptera). Within Coleoptera, bioluminescence occurs in the cantheroid families Elateridae, Rhagophthalmidae, Phengodidae, Lampyridae, as well as in the distantly related Staphylinidae (Lloyd 1978; Costa *et al.* 1986; Branham & Wenzel 2001; Sagegami-Oba *et al.* 2007). All beetles that bioluminesce do so through the same two-step process involving the chemical compound luciferin and a number of structurally similar luciferases (Wilson & Hastings 2013).

Bioluminescence in fireflies has been demonstrated to serve two primary purposes: aposematic warning and sexual communication. All fireflies are bioluminescent as larvae, and this condition is hypothesized to represent the first and earliest origin of bioluminescence in Lampyridae (see Branham & Wenzel 2003 for discussion). As lampyrid larvae are not sexually mature, this signal cannot be used in a courtship context (Crowson 1972). The behavioral context of larval bioluminescent emissions, *i.e.* glowing more brightly when harassed, *etc*. is consistent with an aposematic signal (Sivinksi 1981). Furthermore, fireflies do contain defensive steroids (lucibufagins) (Eisner *et al.* 1978) and it has been demonstrated that predators can learn to associate a glow with bad taste (Underwood *et al.* 1997; De Cock & Matthysen 1999). Bioluminescence is hypothesized to then have been co-opted in the adult life stage, where it now serves as signaling sexual communication while it simultaneously still serves an aposematic function in some adult fireflies (Lloyd 1989; Branham & Wenzel 2003; Moosman *et al.* 2009; Faust 2010). Branham and Wenzel (2003) hypothesize the ancestral state of adult communication in lampyrids to be pheromone only, and that adult bioluminescence was gained and lost at least four times each. Under the assumption that losses of bioluminescence are more likely than gains, Stanger-Hall *et al.* suggested the ancestral state of adult communication to be bioluminescence with one gain and nine subsequent losses. However, if this assumption is not valid, their data suggest the most parsimonious explanation agrees with Branham and Wenzel(see above, Stanger-Hall *et al.* 2007).

In an analysis based on larval morphology, Potatskaja found evidence for separate origins of bioluminescence in Phengodidae and Lampyridae (Potatskaja 1986). Branham and Wenzel further supported this conclusion based on analysis of male morphology (Branham & Wenzel 2001). Sagegami-Oba *et al.* suggested two independent derivations of bioluminescence in

Elateridae and Lampyridae + Phengodidae based on a study of cantharoid beetles (Sagegami-Oba *et al.* 2007). Kundrata *et al.* suggested the independent evolution of bioluminescence in Lampyridae, Elateridae, and a clade comprised of Omalisidae, Phengodidae, and Rhagophthalmidae (Kundrata *et al.* 2014).

The goal of the present study is to reconstruct lampyrid phylogeny using both molecular and morphological data. This is the first time that both data types will be used in a phylogenetic reconstruction. We will use these data to address three goals: (1) the classification of Lampyridae; (2) the ancestral state of adult use of bioluminescence; & (3) how many times throughout the evolution of Lampyridae use of adult bioluminescence has been gained and lost.

MATERIALS AND METHODS

Our taxon sampling included 51 lampyrid specimens collected mostly from North and South America. To these 51 taxa, 59 additional taxa (16S, 18S, & COI) were included from GenBank (see Table 1) for a total of 110 ingroup taxa. This taxon sampling includes six of the nine subfamilies and 36 of the 83 genera with a worldwide distribution. We were unable to sample from the small, geographically restricted Psilocladinae, Amydetinae, or the Cheguevarinae.

There has been debate over the sister group to the Lampyridae (see above). Branham and Wenzel (2001) recovered Rhagophthalmidae in a polytomy with several other families and the Lampyridae. In 2007, Stanger-Hall *et al.* alluded to a sister relationship with Lycidae. However, recent wide scale molecular analysis of the Series Elateriformia has recovered Cantharidae as sister to Lampyridae, with that clade sister to a clade comprising Elateridae, Phengodidae,

Drilidae and Omalisidae (Bocakova *et al.* 2007). Based on these analyses and the rarity of drilids and omalisids, we have chosen two cantharid, a lycid, a rhagophthalmid, a phengodid, and two elaterid species for our outgroup.

DNA extraction and amplification

Specimens were stored in 95% ethanol at -80 °C. Muscle tissue was removed from one metacoxa for each specimen. The rest of the body was preserved in 95% ethanol at -80 °C as a voucher specimen. DNA was extracted using the Qiagen DNAeasy extraction kit. Portions of three mitochondrial (12S, 16S, Cytochrome oxidase I) and three nuclear (18S, 28S, Wingless) genes were amplified using polymerase chain reaction (for primers and PCR conditions see Table 2).

Morphological dataset

A morphological matrix of 410 male characters (*e.g.* Antennae, head, photic organ, genetalia, *etc.*) was adapted from Jeng 2008. Because Jeng's species-level coding overlapped little with our taxon sampling, we re-coded his matrix to the genus-group level by consolidating all species scorings across a genus into a single genus-group scoring. Any characters that were polymorphic were coded as missing. This re-coding was then adopted for all taxa in our dataset.

Sequence alignment

Sequences were aligned according to two approaches: (1) a progressive alignment strategy using MAFFT v 7.017 (Katoh *et al.* 2002) under the L-INS-i strategy implemented in Geneious v 7.1.7 and (2) the consistency alignment method T-Coffee, specifically the meta-mode M-Coffee using default parameters on the T-Coffee webserver http://www.tcoffee.org (Notredame *et al.* 2000). All gene alignments for each strategy were concatenated using Geneious for downstream phylogenetic analysis.

Phylogenetic methods

For Maximum likelihood (ML) and Bayesian analyses, alignments were analyzed in PartionFinder v1.1.1 (Lanfear *et al.* 2012) to find suitable models of evolution (see Table 3). The morphological partition was run under the MK model (Lewis 2001). Trees were visualized in Figtree v. 1.4 (http://tree.bio.ed.ac.uk/software/figtree/) and tree figures were constructed in Adobe Illustrator CS5.

Parsimony analysis

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 calculated in TNT.

Maximum likelihood analysis

Maximum likelihood analyses were carried out using GARLI v.2.01 (Zwickl 2006). Default parameters were used with searchreps set to four. Analyses were re-run until at least half of the 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).

Ancestral State Reconstruction:

Adult bioluminescence was reconstructed onto the ML tree using the ancestral state reconstruction package in Mesquite (Maddison & Maddison 2011) under both a parsimony and ML framework. Bioluminescence was coded as follows: 0= Adult bioluminescence absent; 1= Adult bioluminescence present (male and/or female;). A specimen was coded as bioluminescent if it had a photic organ as an adult. We did not score additional flash pattern due to the lack of

empirical data for the majority of firefly species. Data on the presence or absence of bioluminescence/photic organs is widely available, both in the literature and through direct observation.

RESULTS

Alignment: The MAFFT alignment resulted in a total alignment length of 5,076 base pairs while the T-Coffee alignment had a total length of 5,159 bp. (See Table 4 for individual gene lengths).

Phylogenetic relationships

Parsimony: There were minor differences in topology and score between the MAFFT length: 12,128; full dataset length: 14,267) analysis and the T-Coffee (molecular score: 12,090; full dataset score: 14,234) analysis in either dataset (molecular/molecular+morphology). In the molecular only dataset Lampyridae was recovered as monophyletic with Luciolinae sister to the rest of Lampyridae with moderate support in both MAFFT and T-Coffee analyses (BtS 73 and 71 respectively). Pterotinae was recovered as sister to Ototretinae with low support (BtS <50) in the MAFFT analysis, and in an unresolved trichotomy with the (Cyphonocerinae+(Photurinae+Lampyrinae)) clade. This last clade was recovered in both analyses with low support. However, the clade Photurinae+Lampyrinae was highly supported (BtS 85 for MAFFT & 95 for T-Coffee) in both cases. When morphology was added to the molecular dataset, the resulting topologies were not congruent, however the were identical in terms of MAFFT vs. T-Coffee. In these analyses Lampyridae was recovered as monophyletic with Ototretinae sister to all other Lampyridae with very high support (BtS 99 in both cases). Photurinae is again recovered sister to Lampyrinae with moderate to high support (BtS: 76 for

MAFFT & 83 for T-Coffee). While consistent, the other subfamilial relationships were not recovered with even moderate support. In all analyses Lampyrinae was recovered paraphyletic with several taxa (*Vesta* sp., *Phausis reticulata*, & *Lamprigera* sp.; see below for discussion) grouping with or sister to other subfamilies. Only in the purely molecular dataset was Photurinae also recovered as paraphyletic. This was due to the inclusion of *Vesta* sp., which in the molecular+morphology dataset was recovered as sister to Photurinae. The monophyly of Ototretinae was always well supported (average BtS 98.7). The support for the monophyly of Luciolinae and Cyphonocerinae was low (>70 for both) in the molecular dataset, but high when morphology was added (BtS 75 and 100 respectively. Excluding the problem taxa above, the monophyly of Photurinae (average BtS 95) and the remaining Lampyrinae (average BtS 91.5) was high. For simplicity's sake, in our discussion we will refer only to the MAFFT full dataset topology (Fig. 1). All other topologies are provided in Appendix 1 for reference.

Maximum Likelihood: Owing to the length of time and computing limitations of GARLI and to facilitate discussion, the following support values were collected from a RAxML analysis of 1000 bootstrap replications, and placed on the GARLI topology. We do not expect these values to change significantly when GARLI bootstrap analyses are finished. This also means that support values for our full dataset analyses are not available; the discussion and results of these topologies is forthcoming.

The topologies for both the MAFFT (lnL: -56,713.0001) and the T-Coffee (lnL: -56,718.5354) analyses differed only in the placement of Pterotinae and Cyphonocerinae. In the MAFFT analysis Cyphonocerinae is recovered sister to Luciolinae (BtS 81), with this clade sister to Pterotinae (BtS <50). In the T-Coffee analysis Pterotinae is recovered as sister to Luciolinae. In both analyses two major clades are recovered: (Ototretinae+ the previously mentioned clade;

BtS 85 in MAFFT & 70 in T-Coffee) and (Photurinae+Lampyrinae; BtS 100 & 99). Similar to our parsimony molecular analyses, Lucioliane, Photuriane and Lampyrinae are rendered paraphyletic due to lampyrine taxa grouping with other subfamilies (*Lamprigera* sp. with Luciolinae and *Vesta* sp. with Photurinae). The Ototretinae (BtS 100), Cyphonocerinae (BtS 96), Photurinae (BtS 100), and Lampyrinae (BtS 100) clades are highly supported. Again for simplicity and consistency, only the MAFFT molecular topology will be discussed further (Fig. 2). The T-Coffee topology is provided in Appendix 1 as are the full dataset topologies.

Ancestral State Reconstruction:

Bioluminescence in the adult life stage was mapped across all topologies under both parsimony and likelihood frameworks (Appendix 1). The most parsimonious (least number of steps) results were found on the MAFFT full dataset topology (parsimony; Fig. 1). The most parsimonious reconstruction required 6 steps (four gains and two losses or three of each), and was ambiguous as to the ancestral state of adult bioluminescence in the lampyrids (Fig. 3). This ambiguity was due to the presence of adult bioluminescence in two of our outgroup taxa (*Rhagophthalmus ohbai* & *Zarhipis* sp.).

DISCUSSION

Owing to the extreme similarity in both topology and parsimony/likelihood scores between our T-Coffee and MAFFT datasets, in our discussion we will only be referring to the MAFFT datasets.

In terms of subfamilial relationships, two patterns are recovered. In our parsimony (MP) analyses (both molecular only and the full dataset), each subfamily is recovered as sister to the

rest of the subfamilies in a pectinate relationship with very poor support. In our likelihood (ML) analyses two major clades are recovered, Ototretinae, Pterotinae, Cyphonocerinae & Luciolinae and Photurinae & Lampyrinae, with high support for each clade (BtS 85 & 100 respectively).

In our molecular analysis, Lampyrinae and Photurinae were consistently recovered as polyphyletic, while with the addition of morphology, only Lampyrinae was recovered as polyphyletic. The change in monophyly of Photurinae is due to *Vesta* sp. When only the molecular data are considered, *Vesta* is recovered within the photurine clade associated with *Pyrogaster* and *Bicellonycha* with very high support (Bts 89 in MP; 100 in ML). When the morphological data are added, *Vesta* is recovered as sister to Photurinae with little to no support. Jeng (2008) recovered the genus *Vesta* as paraphyletic after analyzing only the morphological data. Jeng (2008) also recovered some *Vesta* species in a polytomy that included Photurinae and Lampyrinae. Given this analysis by Jeng, and considering that our morphological partition is from his analysis, it is not surprising that our *Vesta* would prove difficult to place. It seems clear that a re-examination of the genus *Vesta* is as its current assemblage of species might not represent a natural group. At times, *Vesta* has also been classified within the Amydetinae and an effort should be made to include representatives from this subfamily (McDermott 1966).

Lampyrinae is also rendered paraphyletic by the recovery of *Lamprigera* as sister to Luciolinae, although this relationship is only moderately supported (BtS 79) in one ML analysis. This relationship was recovered in all analyses, except when the morphological dataset was analyzed under a parsimony framework. In this MP analysis, *Lamprigera* is recovered as sister to the clade comprising Photurinae and the majority of Lampyrinae (although with very low support). *Lamprigera* has historically been recovered in various relationships, especially with regards to two other lampyrine genera: *Phausis* and *Lamprohiza*. In our molecular analyses these

genera are found spread out through the topology. When morphological data are added, these three taxa seem to be recovered closer together. Jeng (2008) found significant morphological similarities between these three genera but also found that they differed from the Lampyrinae by the unmodified mandibles, dorsal abdominal spiracles and a symmetrical aedeagal sheath with which they are usually classified (Jeng 2008). Based on the molecular data, and even including the morphological data, it would seem these taxa do not belong with the Lampyrinae. Further analyses, especially morphological analyses are needed to determine the position of these three taxa with regards to the rest of the lampyrid phylogeny. It is worth noting that *Phausis* is always recovered as sister to the Photurinae + Lampyrinae with high support (average BtS 90).

Also interesting is the relationship of *Ellychnia* with *Photinus.* Previous analyses have suggested that a revision of *Ellychnia* may be needed, and that it may in fact be a highly derived lineage of *Photinus* (Stanger-Hall 2007; Lewis & Cratsley 2008). While our topologies support a close association between the two genera, we do not find strong support for the hypothesis that *Ellychnia* is a derived lineage within *Photinus.* In our molecular analyses, we do recover *Ellychnia* within the *Photinus* clade. However there is no support except at the node subtending the entire clade (BtS 100; Fig. 2). When morphology is analyzed as well, *Ellychnia* is recovered as sister to *Photinus* (BtS 72). While there is some molecular evidence for the hypothesis, when analyzed in conjunction with morphology, there is more support for a sister relationship between *Ellychnia* and *Photinus* than for a relationship where *Ellychnia* is a highly derived *Photinus* clade.

Adult Bioluminescence

The ancestral state reconstruction was mapped across all topologies, however only the most parsimonious (the full dataset under MP) is included here (Fig. 3; see Appendix 1 for the

other topologies). This reconstruction includes six total steps in the evolution of adult bioluminescence. While there were at least two gains and three losses, the analysis remained ambiguous in terms of the ancestral state of adult use of bioluminescence in Lampyridae. This is due to the fact that two exemplars in our outgroup, *Rhagophthalmus ohbai* and *Zarhipis* sp., have adult bioluminescence. As stated above, the familial relationships among these families remains in question. If Rhagophthalmidae and Phengodidae are indeed sister to Lampyridae, then it seems that adult bioluminescence evolved before the origin of Lampyridae, and perhaps before the origin of this clade. This evolutionary pattern is in contrast to most analyses, which suggest that adult bioluminescence was not present ancestrally (Susuki 1997; Branham and Wenzel 2003; Jeng 2008). A reconstruction under a ML framework would slightly favor the results of previous authors, as the proportional likelihood that the ancestor of Lampyridae was bioluminescent is only .3566.

CONCLUSIONS

Lampyridae, including Pterotinae and Ototretinae is recovered as monophyletic with high support. With few exceptions, all subfamilies except Lampyrinae are recovered as monophyletic, however there is still room for debate as to the relationships of these subfamilies. Further analysis including all subfamilies is needed. We have also identified the need for further studies regarding *Phausis, Lamprigera, Lamprohiza, Vesta, Ellychnia,* and *Photinus.*

We find little-moderate support for the derivation of adult bioluminescence after the origin of Lampyridae. However, this finding is dependent on further study at the family level.

ACKNOWLEDGMENTS

We would like to thank the members of the Branham lab (Gainesville, FL), the Whiting and Bybee labs (Provo, UT), as well as Jim Lloyd (Gainesville, FL) and Tara Martin (Provo, UT) for specimen donation. We thank all undergraduates of the Bybee lab group for help with data generation, as well as the BYU DNA Sequencing center. We thank Perry Wood and Joey Mugleston for assisitance with data analysis. We want to thank BYU for access to the Fulton Supercomputer. We also thank Haley Wightman and Anton Suvorov (BYU) and the Whiting and Bybee lab groups for reviewing previous versions of this manuscript. We especially thank Nathan Lord (BYU) for extensive help with revisions to the manuscript. This study was supported in part by a BYU Graduate Student Fellowship (GJM), as well as grants from the National Science Foundation (SMB; IOS-1265714) and the American Philosophical Society (SMB).

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CHAPTER 2 TABLES

Table 1. Genbank accession numbers.

Family	Subfamily	Species	18S	16S	COI
Lampyridae	Lampyrinae	*Aspisoma sp. 2*	EU009248	EU009285	EU009322
Lampyridae	Ototretinae	*Brachylampis blaisdelli*	EU009230	EU009267	EU009304
Lampyridae	Ototretinae	*Ceylandidrilus sp.*	DQ100524	DQ198682	DQ198605
Lampyridae	Luciolinae	*Curtos costipennis*	AB298848	AB671250	AB671258
Lampyridae	Luciolinae	*Curtos okinawanus*	AB298849	AB671252	AB671262
Lampyridae	Luciolinae	*Curtos sp.*	DQ100513	DQ198671	DQ198594
Lampyridae	Cyphonocerinae	*Cyphonocerus ruficollis*	DQ100512	DQ198670	DQ198593
Lampyridae	Lampyrinae	*Diaphanes formosus*	EU009243	EU009280	EU009317
Lampyridae	Ototretinae	*Drilaster axillaris*	AB298853	AB436506	AB608756
Lampyridae	Ototretinae	*Drilaster borneensis*	DQ100522	DQ198680	DQ198603
Lampyridae	Ototretinae	*Drilaster sp.*	DQ100517	DQ198675	DQ198598
Lampyridae	Lampyrinae	*Ellychnia californica*	EU009218	EU009255	EU009292
Lampyridae	Lampyrinae	*Ellychnia corrusca*	EU009225	EU009262	EU009299
Lampyridae	Ototretinae	*Flabellotreta obscuricollis*	DQ100519	DQ198677	DQ198600
Lampyridae	Ototretinae	*Flabellotreta sp.*	DQ100520	DQ198678	DQ198601
Lampyridae	Lampyrinae	*Lamprohiza splendidula*	EU009245	EU009282	EU009319
Lampyridae	Lampyrinae	*Lucidina biplagiata*	AB298844	AB009922	
Lampyridae	Luciolinae	*Luciola cruciata*		AB009904	AF360953
Lampyridae	Luciolinae	*Luciola filiformis yayeyamana*	AB298850	AB436493	
Lampyridae	Luciolinae	*Luciola italica*		AB436494	
Lampyridae	Luciolinae	*Luciola kuroiwae*		AB009907	
Lampyridae	Luciolinae	*Luciola lateralis*	AB298851	AB009906	
Lampyridae	Luciolinae	*Luciola ovalis*		DQ371179	
Lampyridae	Luciolinae	*Luciola parvula*	AB298852	AB436504.1	AB608763
Lampyridae	Luciolinae	*Luciola sp.*	EU009244	EU009281	EU009318
Lampyridae	Lampyrinae	*Lychnuris formosana*	EU009242	EU009279	EU009316
Lampyridae	Lampyrinae	*Micronaspis floridana*	EU009240	EU009277	EU009314
Lampyridae	Lampyrinae	*Microphotus angustus*	EU009227	EU009264	EU009301
Lampyridae	Lampyrinae	*Paraphausis eximia*	EU009223	EU009260	EU009297
Lampyridae	Lampyrinae	*Phausis reticulata*	EU009237	EU009274	EU009311
Lampyridae	Lampyrinae	*Phosphaenus hemipterus*	EU009246	EU009283	EU009320
Lampyridae	Lampyrinae	*Photinus australis*	EU009224	EU009261	EU009298
Lampyridae	Lampyrinae	*Photinus floridanus*	EU009232	EU009269	EU009306
Lampyridae	Lampyrinae	*Photinus punctulatus*	EU009238	EU009275	EU009312
Lampyridae	Lampyrinae	*Photinus tanytoxis*	EU009241	EU009278	EU009315
Lampyridae	Photurinae	*Photuris aff. lucicrescens*	EU009216	EU009253	EU009290
Lampyridae	Photurinae	*Photuris congener*		EU301845	
Lampyridae	Photurinae	*Photuris pennsylvanica*	PPU65129		AY165656
Lampyridae	Photurinae	*Photuris quadrifulgens*	EU009236	EU009273	EU009310

Lampyridae	Photurinae	*Photuris tremulans*	EU009234	EU009271	EU009308
Lampyridae	Lampyrinae	*Pleotomodes needhami*	EU009231	EU009268	EU009305
Lampyridae	Lampyrinae	*Pleotomus pallens*	EU009217	EU009254	EU009291
Lampyridae	Cyphonocerinae	*Pollaclasis bifaria*	EU009221	EU009258	EU009295
Lampyridae	Luciolinae	*Prisolycus sangulatus*		AB009925	
Lampyridae	Pterotinae	*Pterotus obscuripennis*	EU009229	EU009266	EU009303
Lampyridae	Lampyrinae	*Pyractomena angulata*	EU009233	EU009270	EU009307
Lampyridae	Lampyrinae	*Pyractomena borealis*	EU009222	EU009259	EU009296
Lampyridae	Lampyrinae	*Pyractomena palustris*	EU009235	EU009272	EU009309
Lampyridae	Lampyrinae	*Pyrocoelia abdominalis*		AB009921	AB608766
Lampyridae	Lampyrinae	*Pyrocoelia amplissima*		DQ371190	
Lampyridae	Lampyrinae	*Pyrocoelia discicollis*		AB436511	AB608768
Lampyridae	Lampyrinae	*Pyrocoelia fumosa*	AB298846	AB436510	AB608769
Lampyridae	Lampyrinae	*Pyrocoelia rufa*		AB009913	
Lampyridae	Lampyrinae	*Pyropyga decipiens*	EU009226	EU009263	EU009300
Lampyridae	Lampyrinae	*Vesta sp.*	DQ100511	DQ198669	DQ198592
Elateridae		*Oxynopterus sp.*	HQ333800	HQ333710	HQ333982
Lycidae		*Plateros sp.*	DQ181109	FJ390407	FJ390409
Rhagophthalmidae		*Rhagophthalmus ohbai*	AB298864.1	AB009931.1	AB608775.1

Table 2. (a) Primer sequences.

Gene	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 *et al.* 2001
	3DR	5'- TAG TTC ACC ATC TTT CGG GTC -3'	Belshaw et al. 2001
COI	J-2195	5'- TTG ATT TTT TGG TCA TCC AGA AGT -3'	Simon *et al.* 1994
	PAT	5'- TCC AAT GCA CTA ATC TGC GAT ATT A -3'	Simon *et al.* 1994
Wingless	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 2. (b) Amplification profiles

	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 3. Models of evolution and partitioning schemes for MAFFT and T-Coffee datasets.

	MAFFT		T-Coffee	
	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	TrNef + Γ	2	TrNef + Γ + I	2
28S	GTR + Γ	3	GTR + Γ	3
COI pos1	GTR + Γ +I	4	GTR + Γ +I	4
COI pos2	GTR + Γ +I	5	TVM + Γ +I	5
COI pos3	TVM + Γ	6	TVM + Γ	6
WNT pos1	SYM + Γ	7	GTR + Γ	7
WNT pos2	TrNef + Γ	2	GTR + Γ	7
WNT pos3	HKY + Γ	8	HKY + Γ	8

Table 4. Breakdown by gene (bp) of MAFFT and T-Coffee alignments.

	MAFFT	T-Coffee
12S	366	376
16S	545	559
18S	996	1,000
28S	1,222	1,274
COI	1,497	1,497
WNT	450	453
Total	5,076	5,159

CHAPTER 2 FIGURES

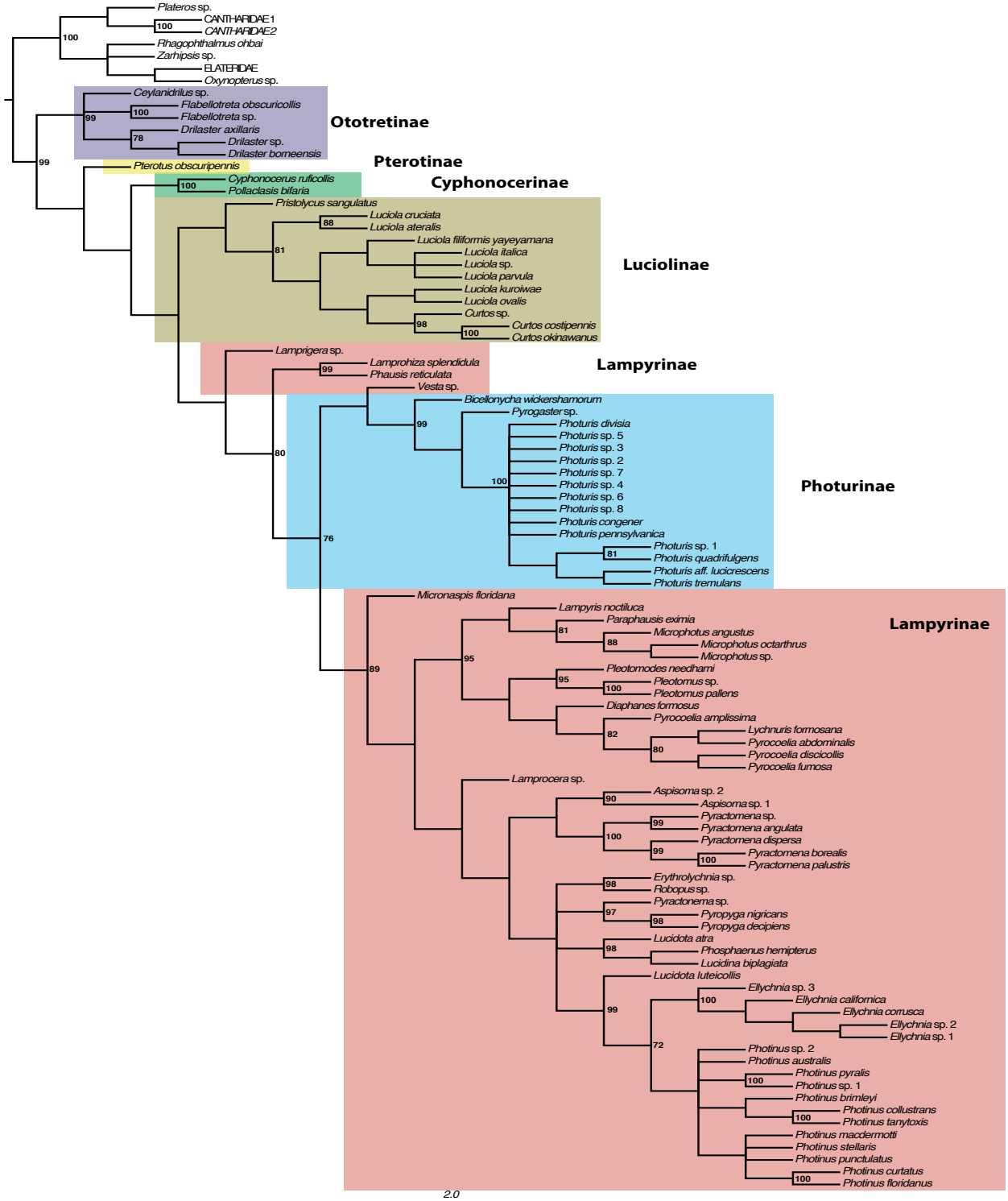


Figure 1. Maximum Parsimony reconstruction (Nelsen consensus of 540 trees, 14,267 steps) based on full molecular and morphological dataset. Molecular dataset aligned in MAFFT. Numbers at nodes indicate bootstrap support. Only those nodes with support above 70 labeled.

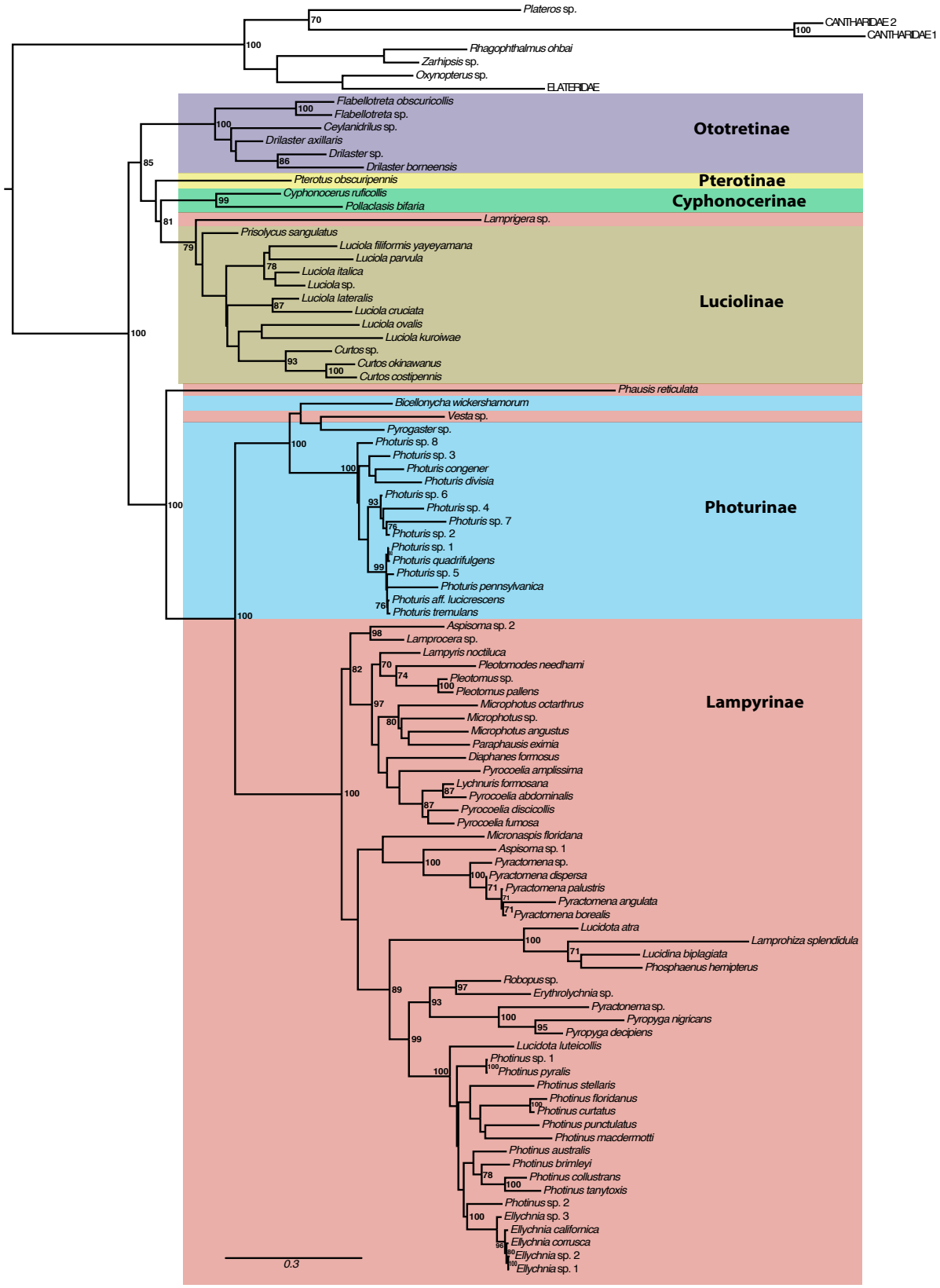


Figure 2. Maximum Likelihood reconstruction (lnL: -56,713.0001) based on MAFFT aligned molecular dataset.

Numbers at nodes indicate bootstrap support. Only those nodes with support above 70 labeled.

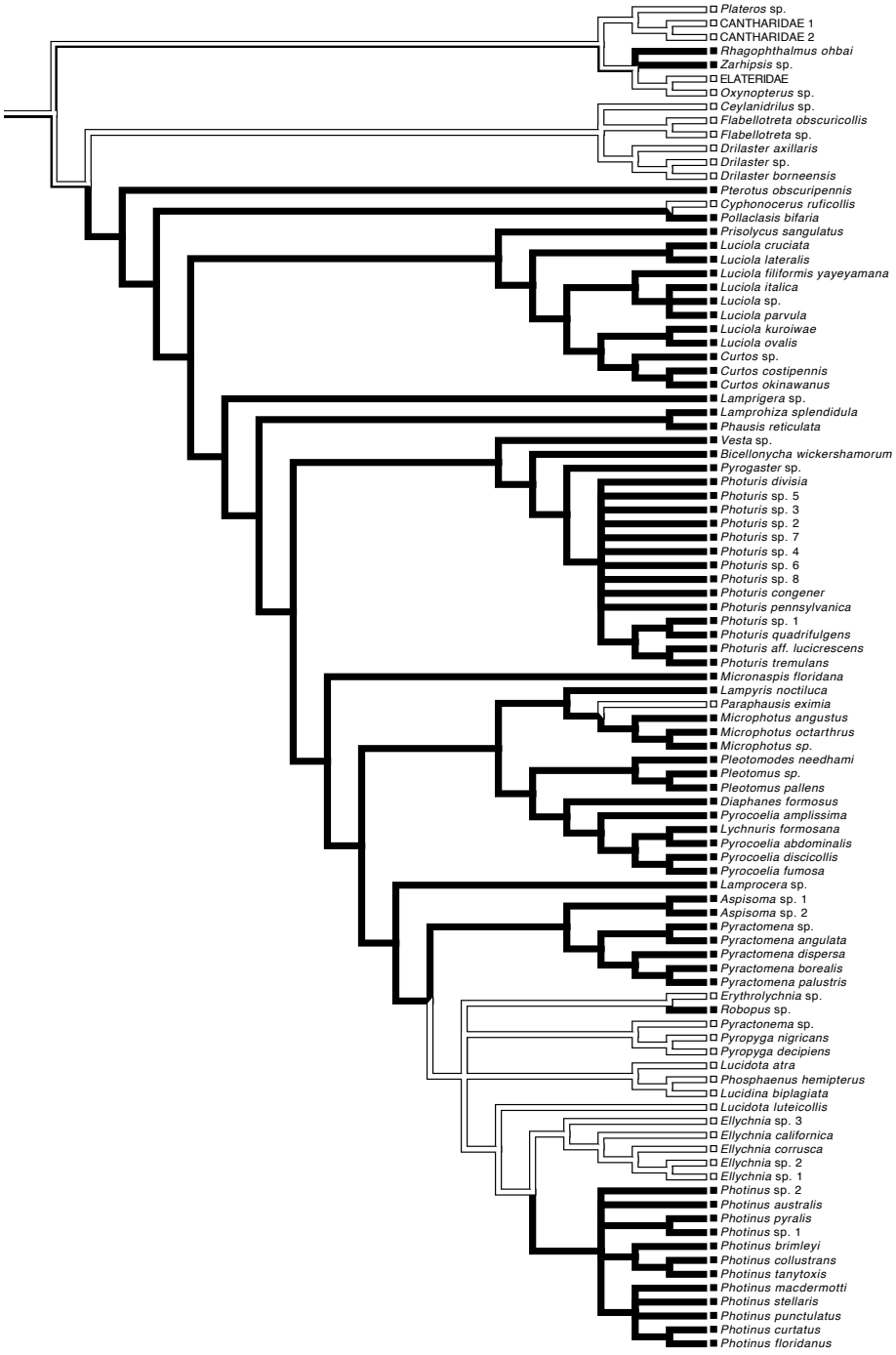
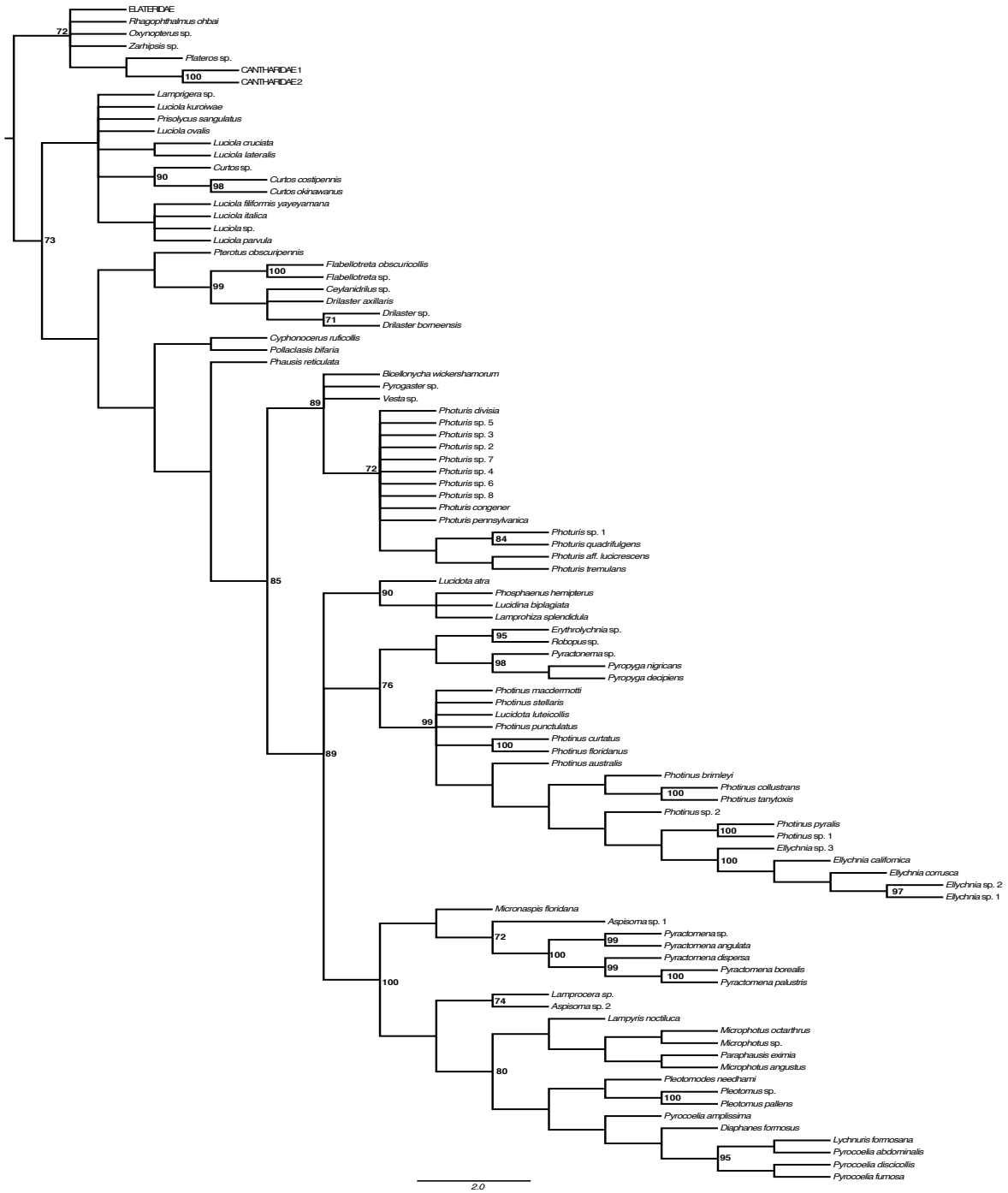


Figure 3. Ancestral State Reconstruction of adult bioluminescence mapped onto the MP tree including the MAFFT aligned full dataset. Black bars indicate presence of bioluminesce in the adult life stage, white bars indicate absence.

Appendix 1

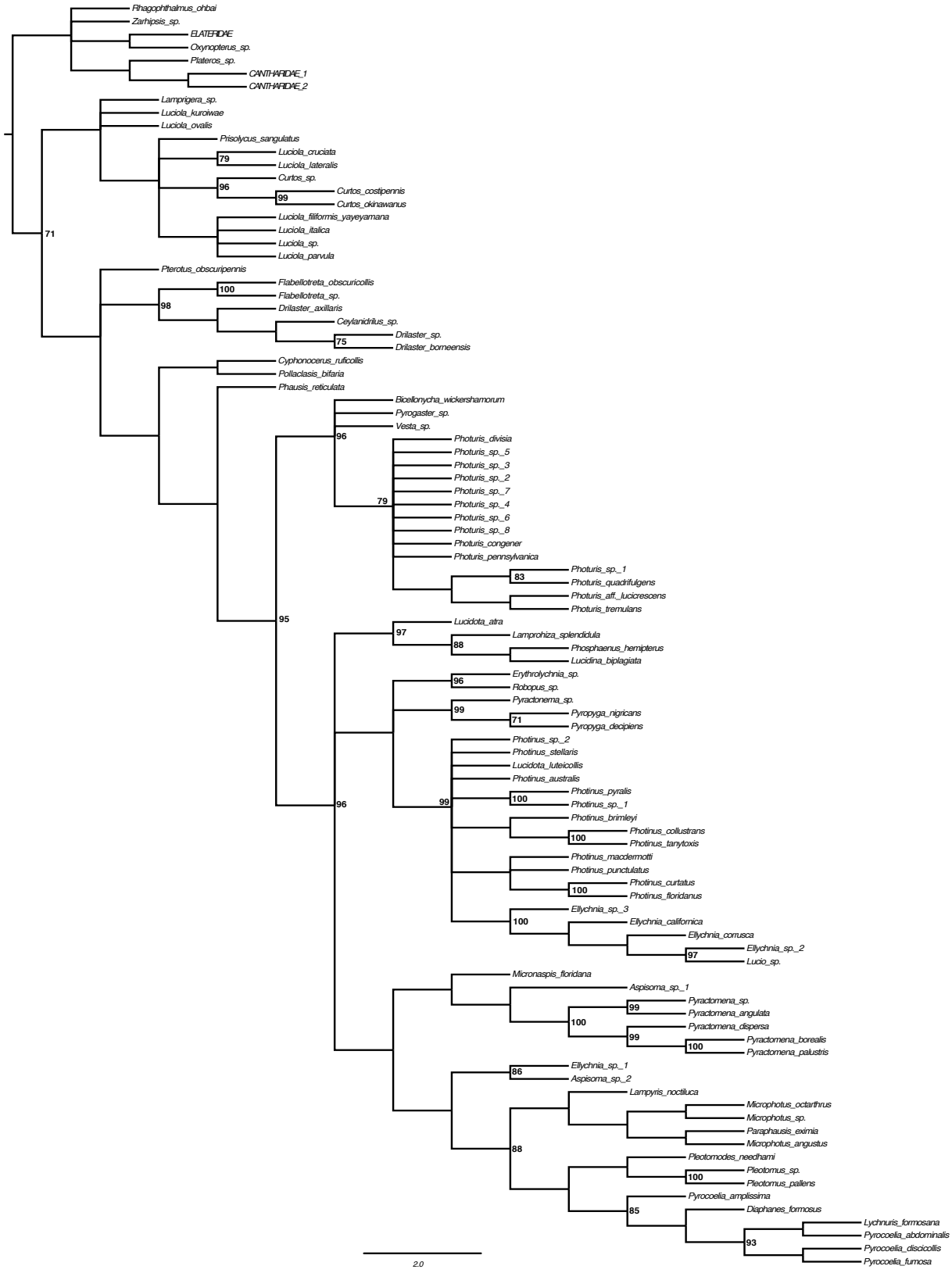
Supplemental material for Chapter 2.

Alternative phylogenetic topologies (*e.g.* T-Coffee):

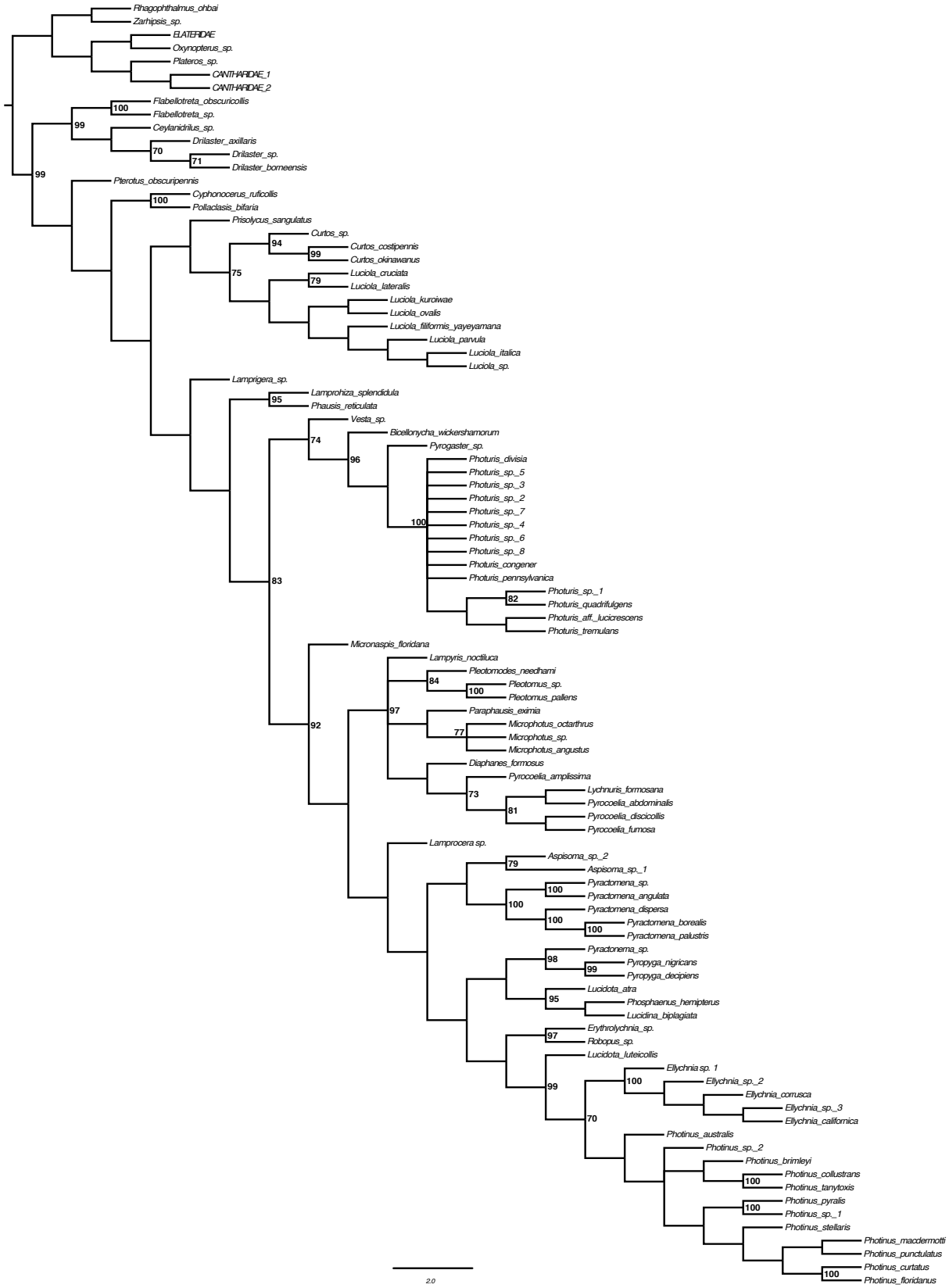


MAFFT; Molecular dataset; MP; 629 trees; score: 12,128

T-Coffee; Molecular dataset; ML; Score: -56,718.5354

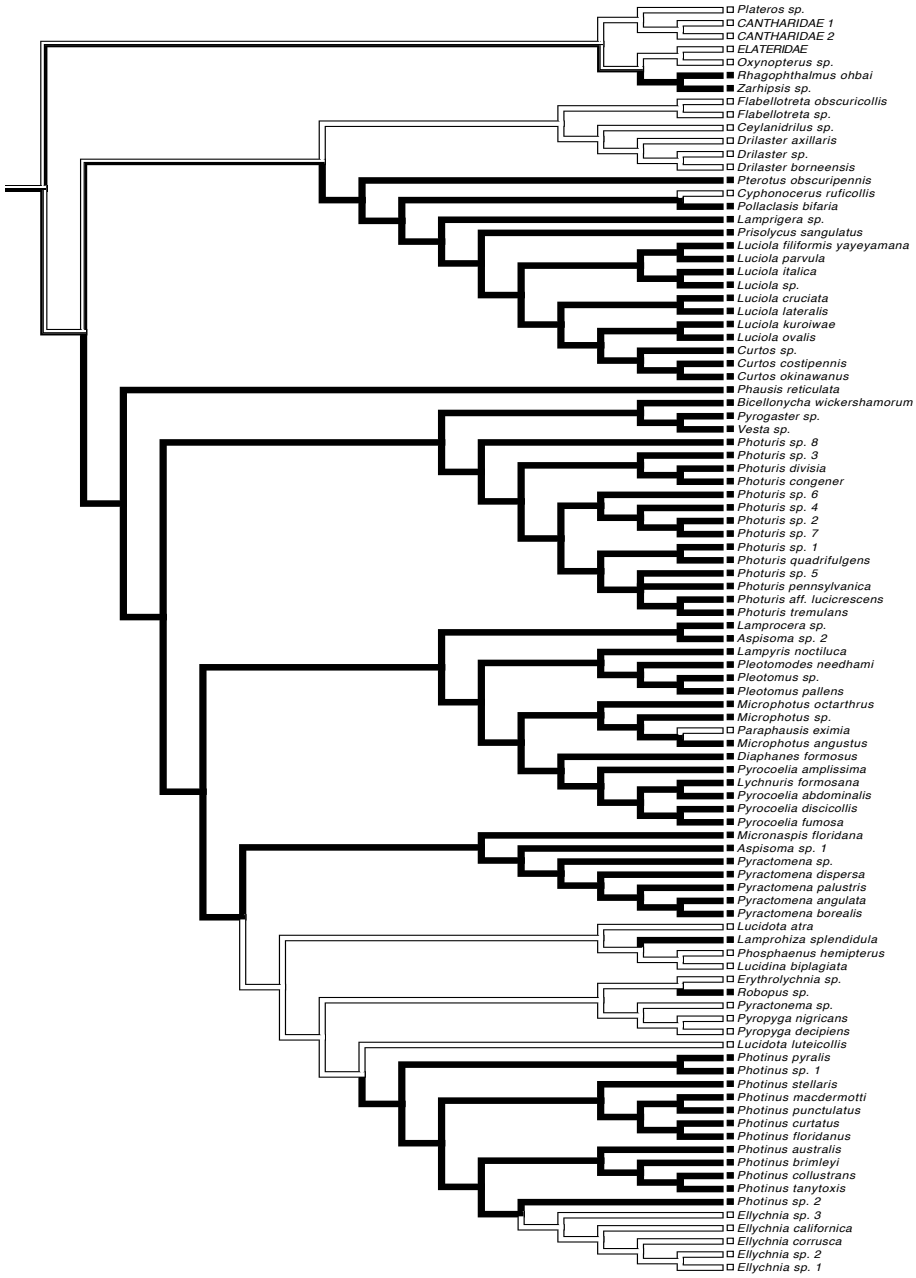


T-Coffee; Molecular dataset; MP; 714 trees; Score: 12,090

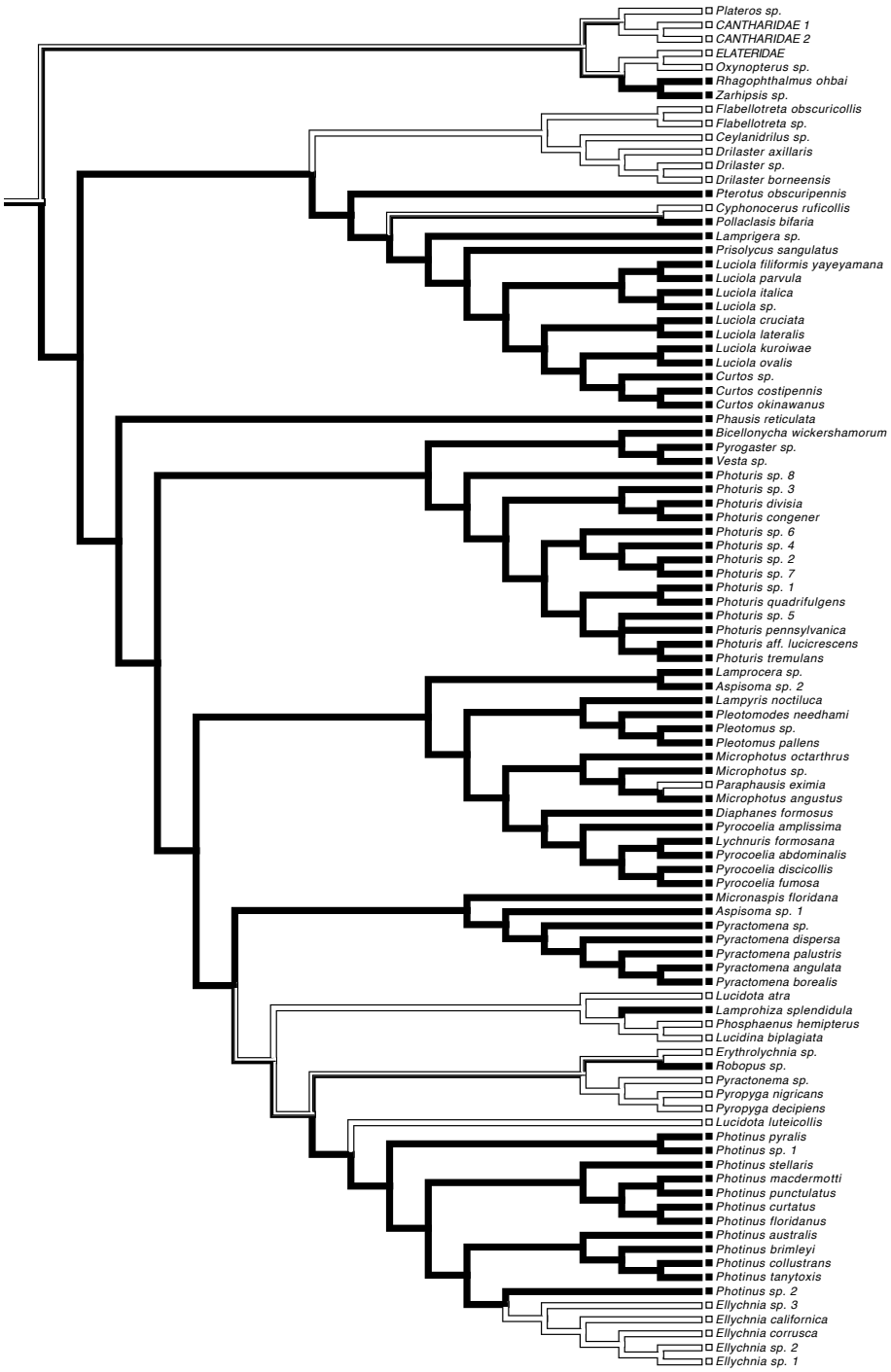


T-Coffee; Morphological dataset; MP; 90 trees; Score: 14,234

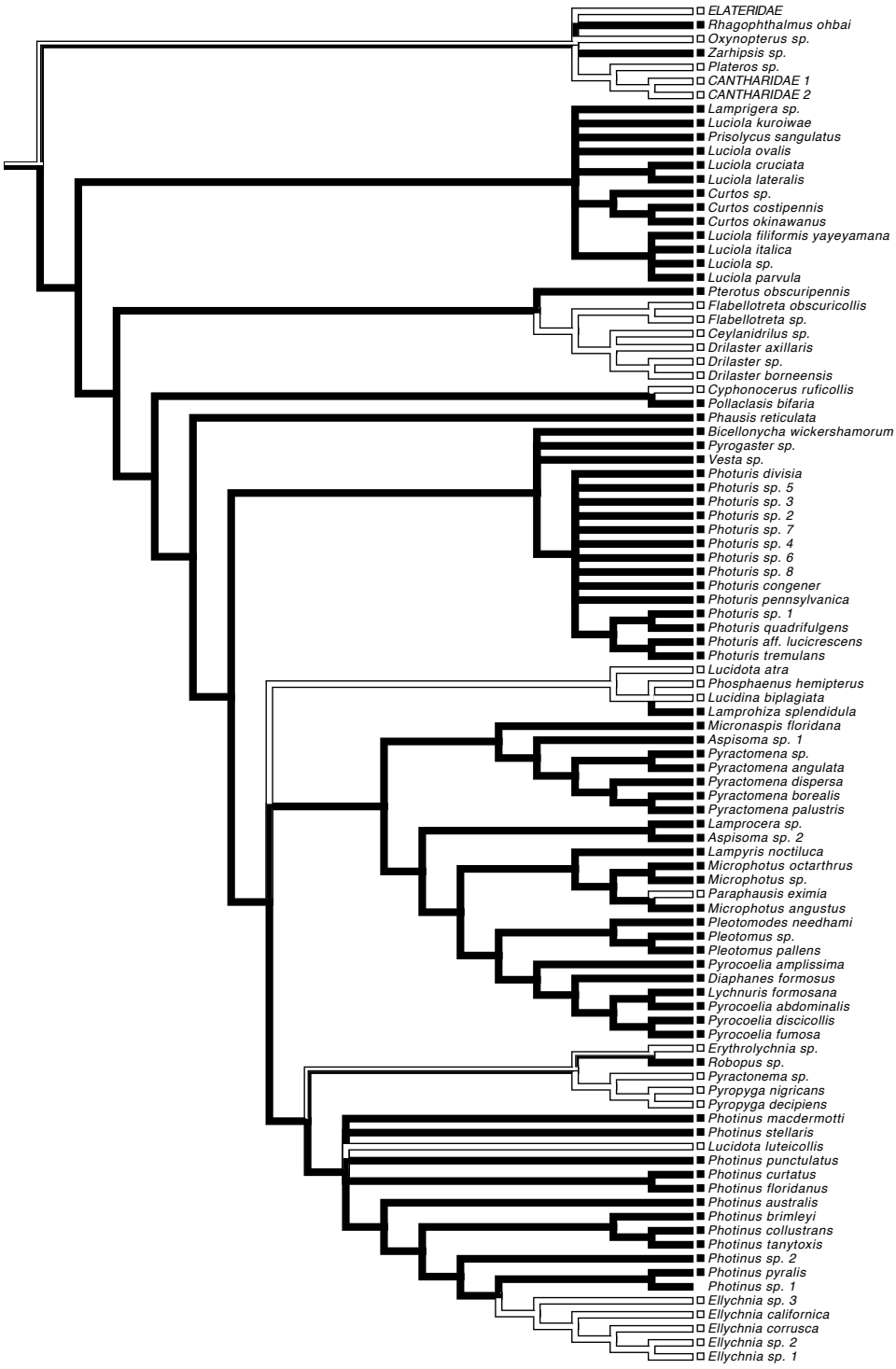
Alternative ancestral state reconstructions:



Ancestral state reconstruction, parsimony framework on MAFFT, Molec, ML.



Ancestral state reconstruction, likelihood framework on MAFFT, Molec, ML.



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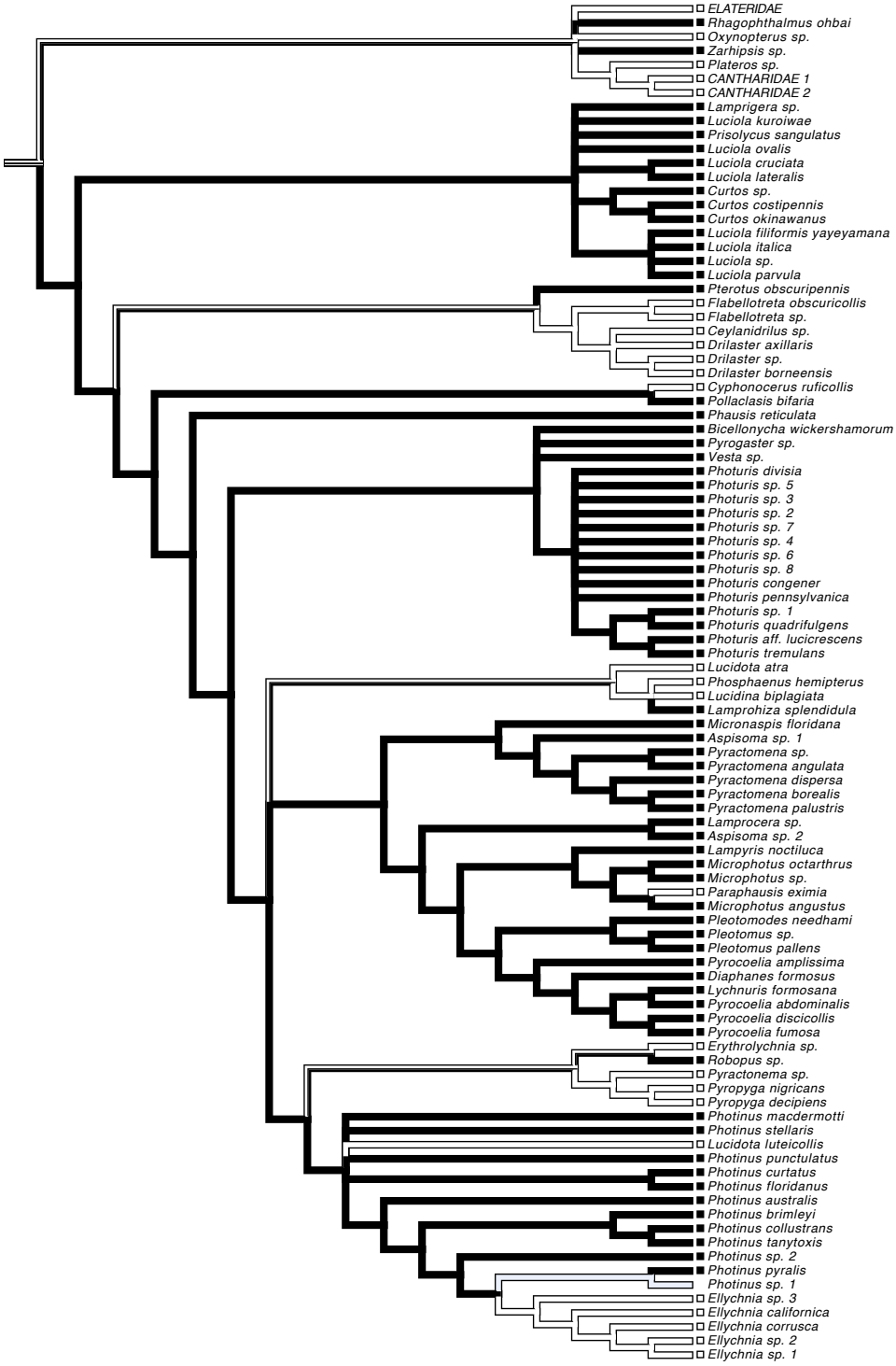

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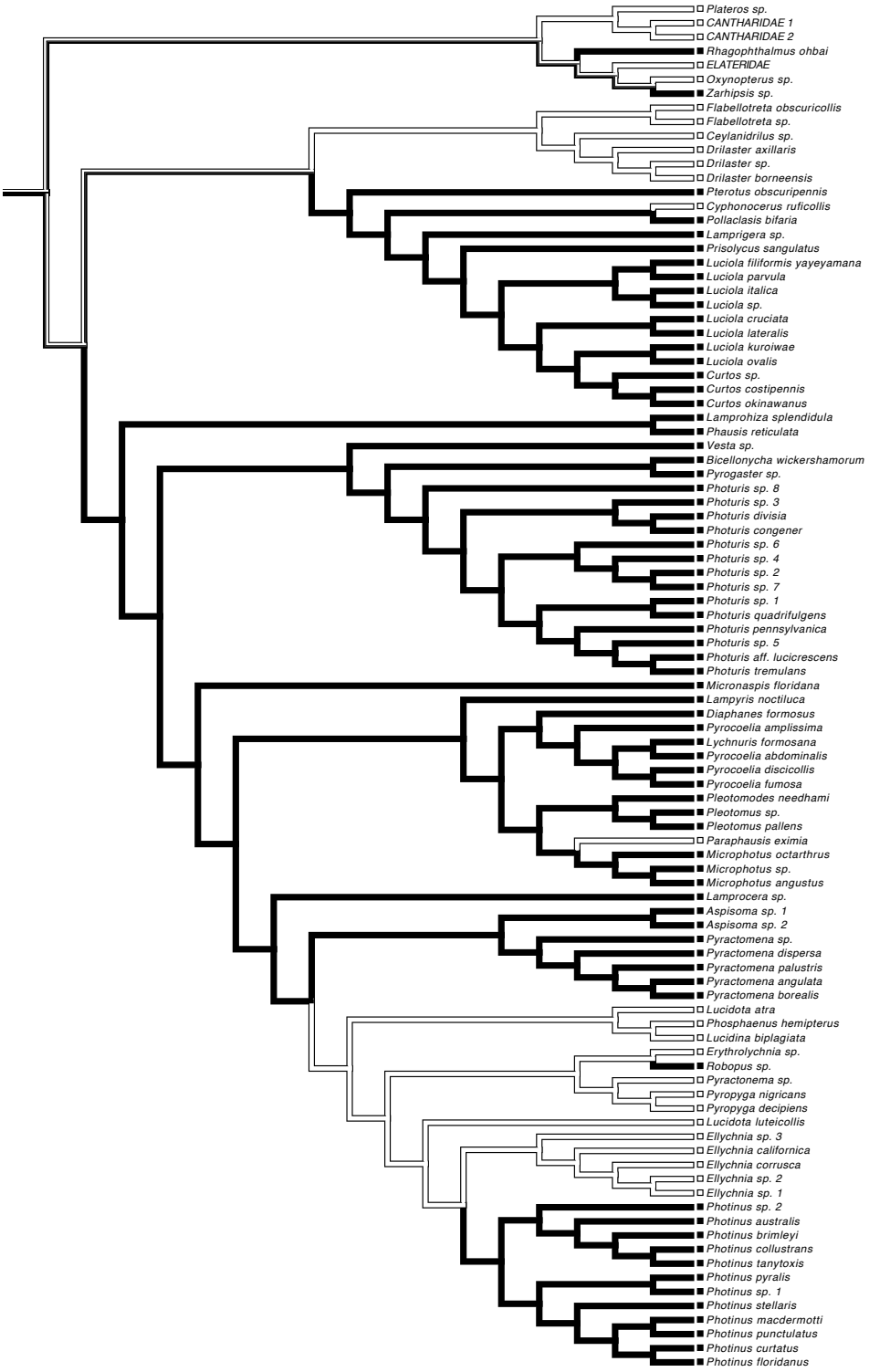
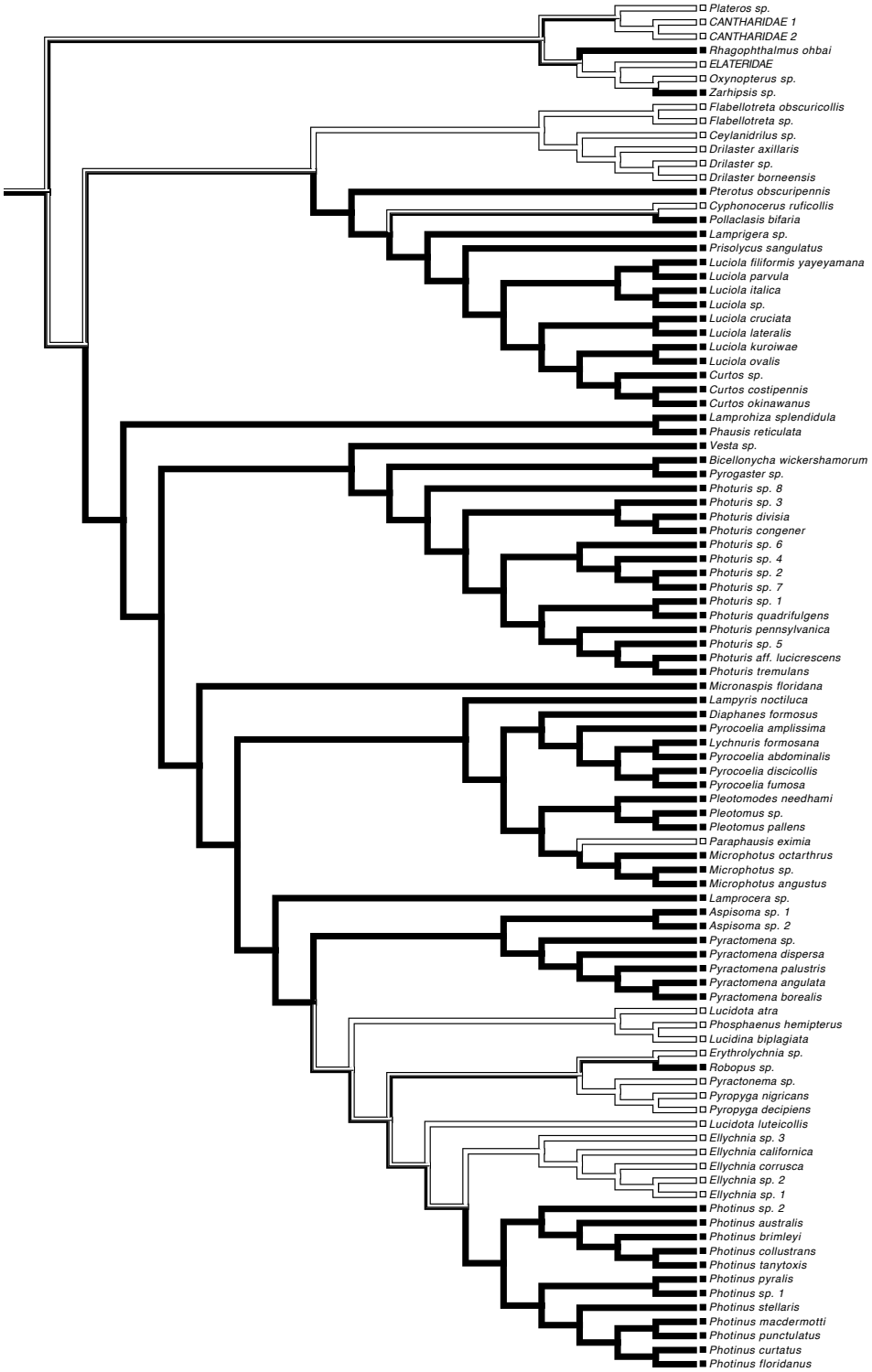
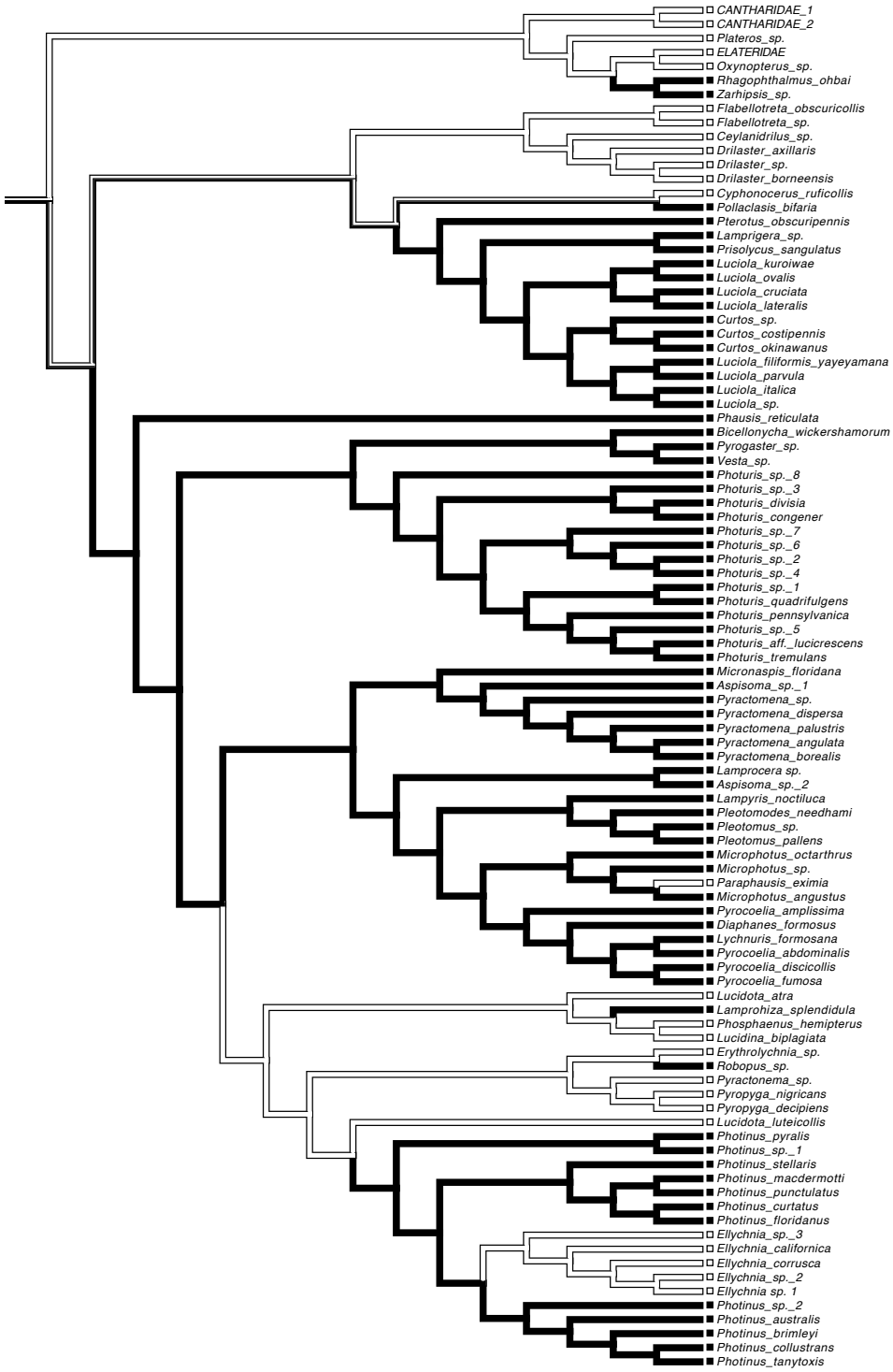


Ancestral state reconstruction, parsimony framework on MAFFT, Morph, ML.

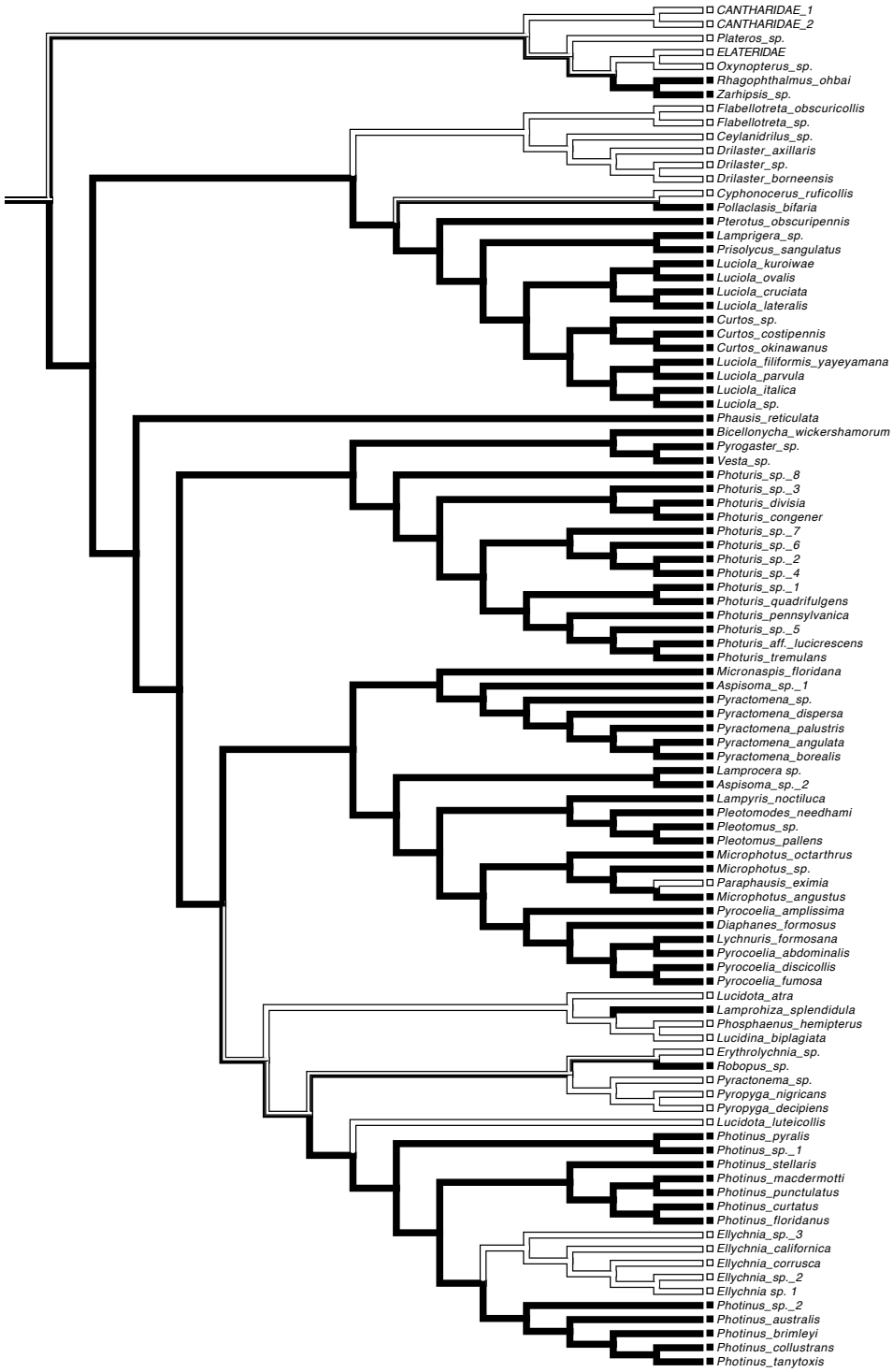


Ancestral state reconstruction, likelihood framework on MAFFT, Morph, ML.

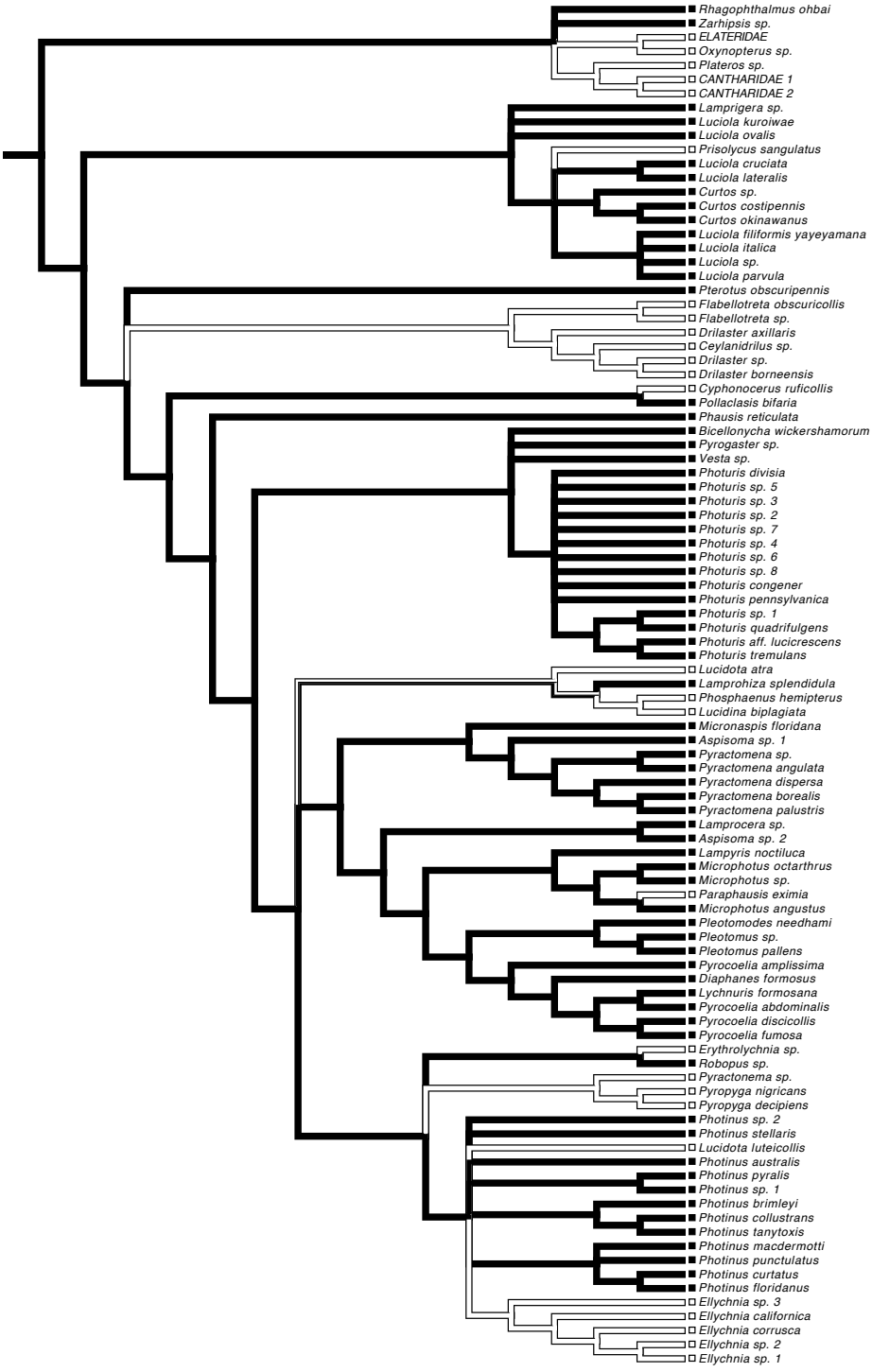
Ancestral state reconstruction, parsimony framework on MAFFT, Morph, MP.



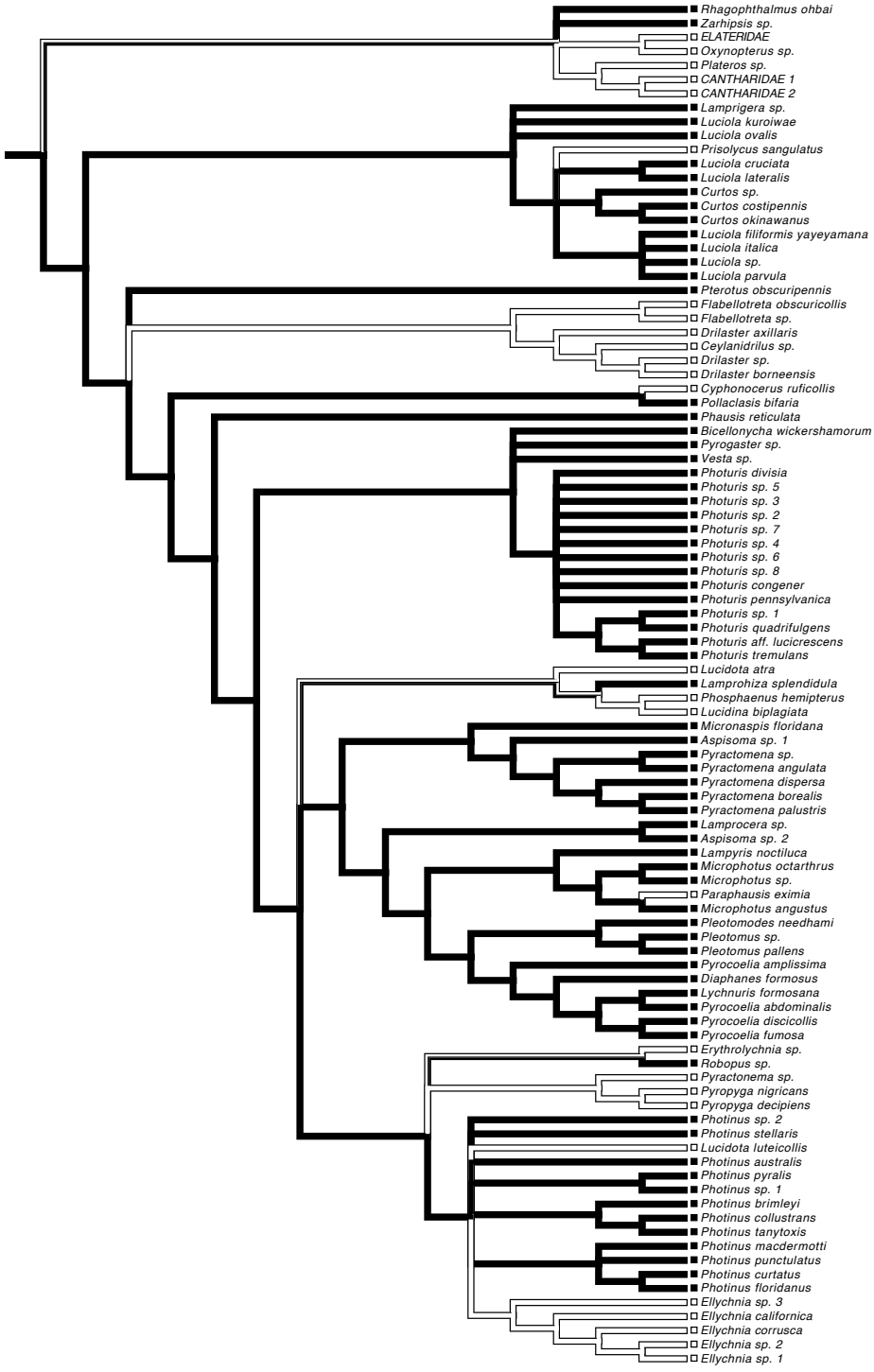
Ancestral state reconstruction, parsimony framework on T-Coffee, Molec, ML.



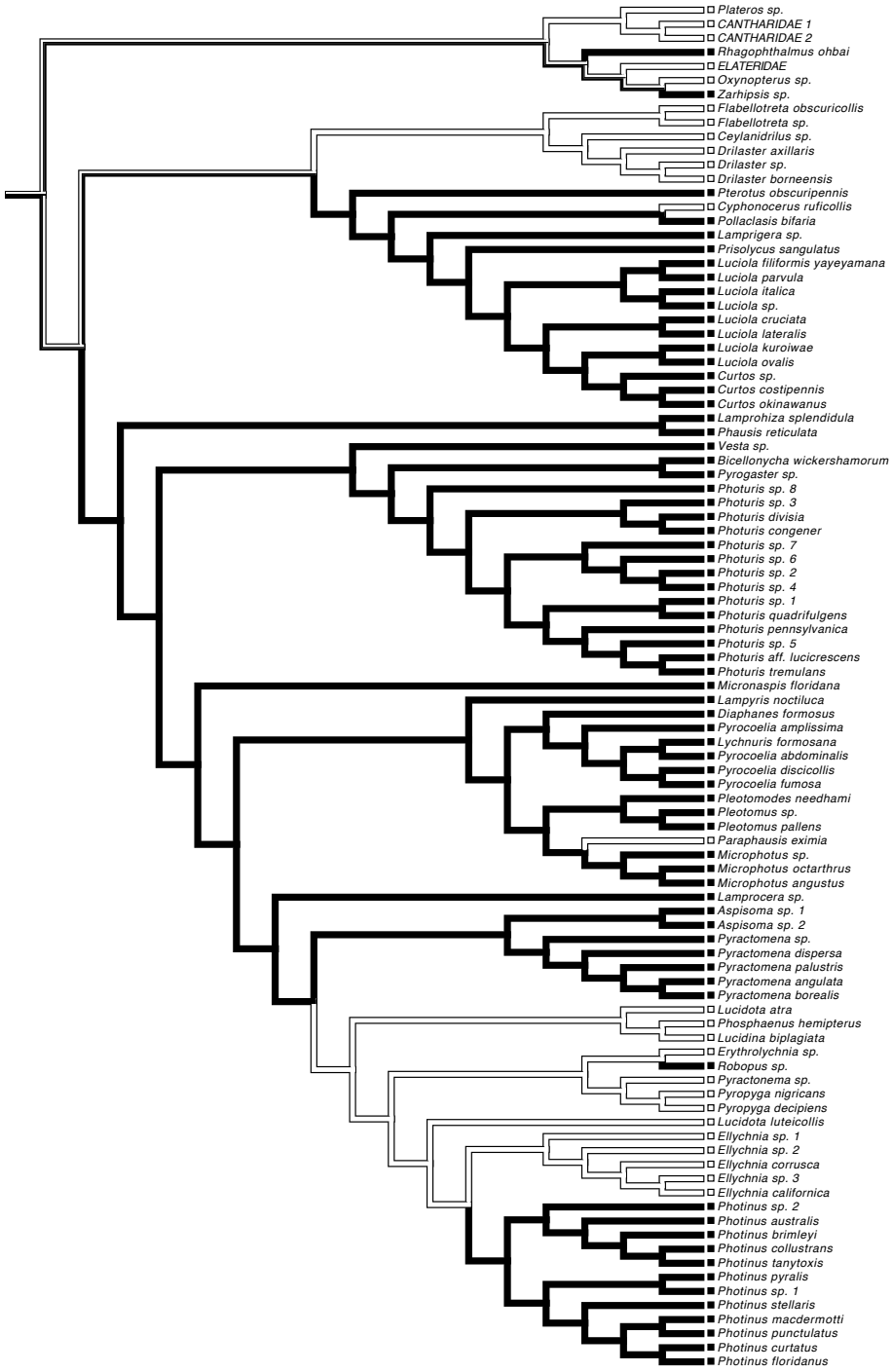
Ancestral state reconstruction, likelihood framework on T-Coffee, Molec, ML.



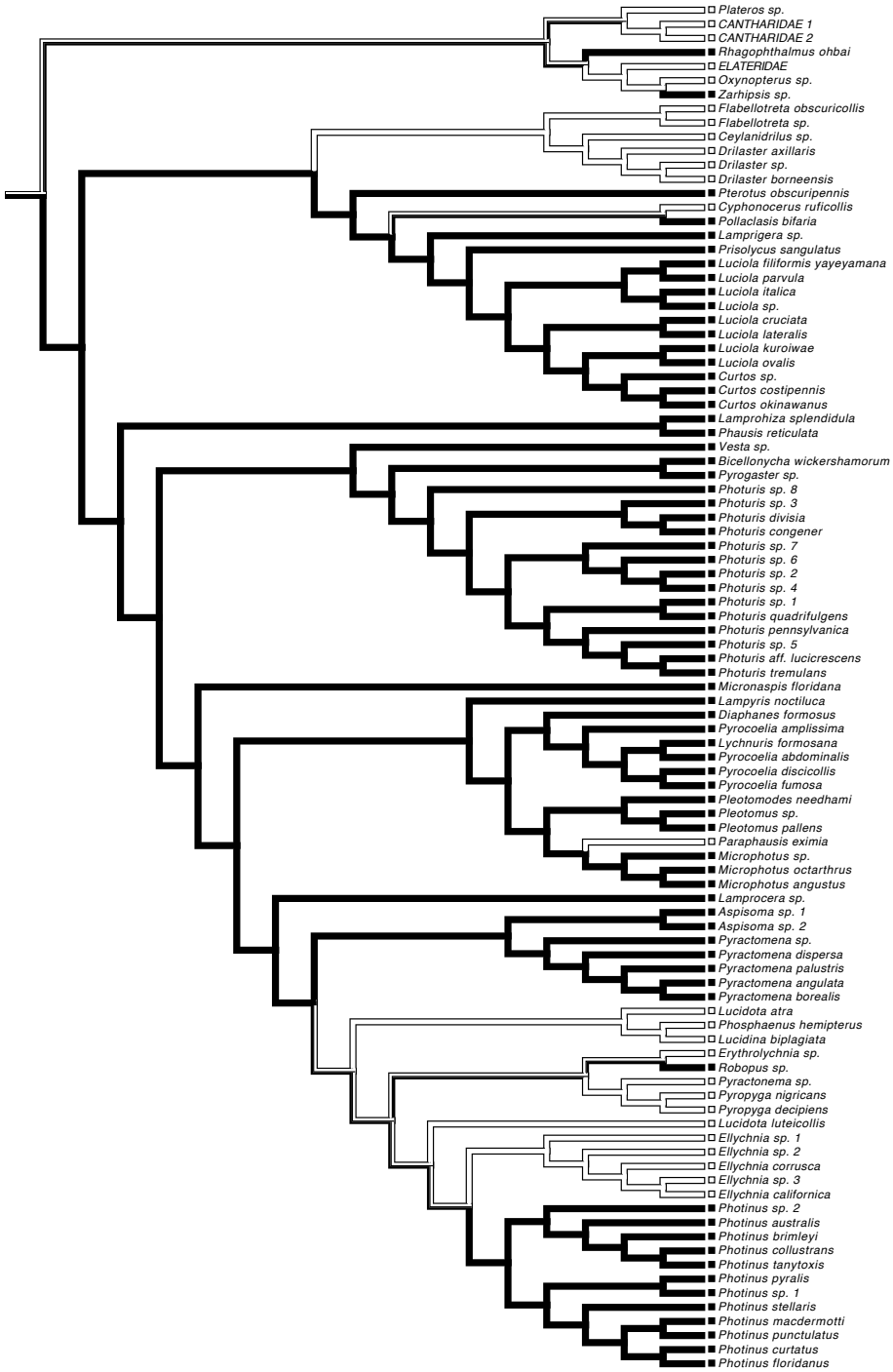
Ancestral state reconstruction, parsimony framework on T-Coffee, Molec, MP.



Ancestral state reconstruction, likelihood framework on T-Coffee, Molec, MP.



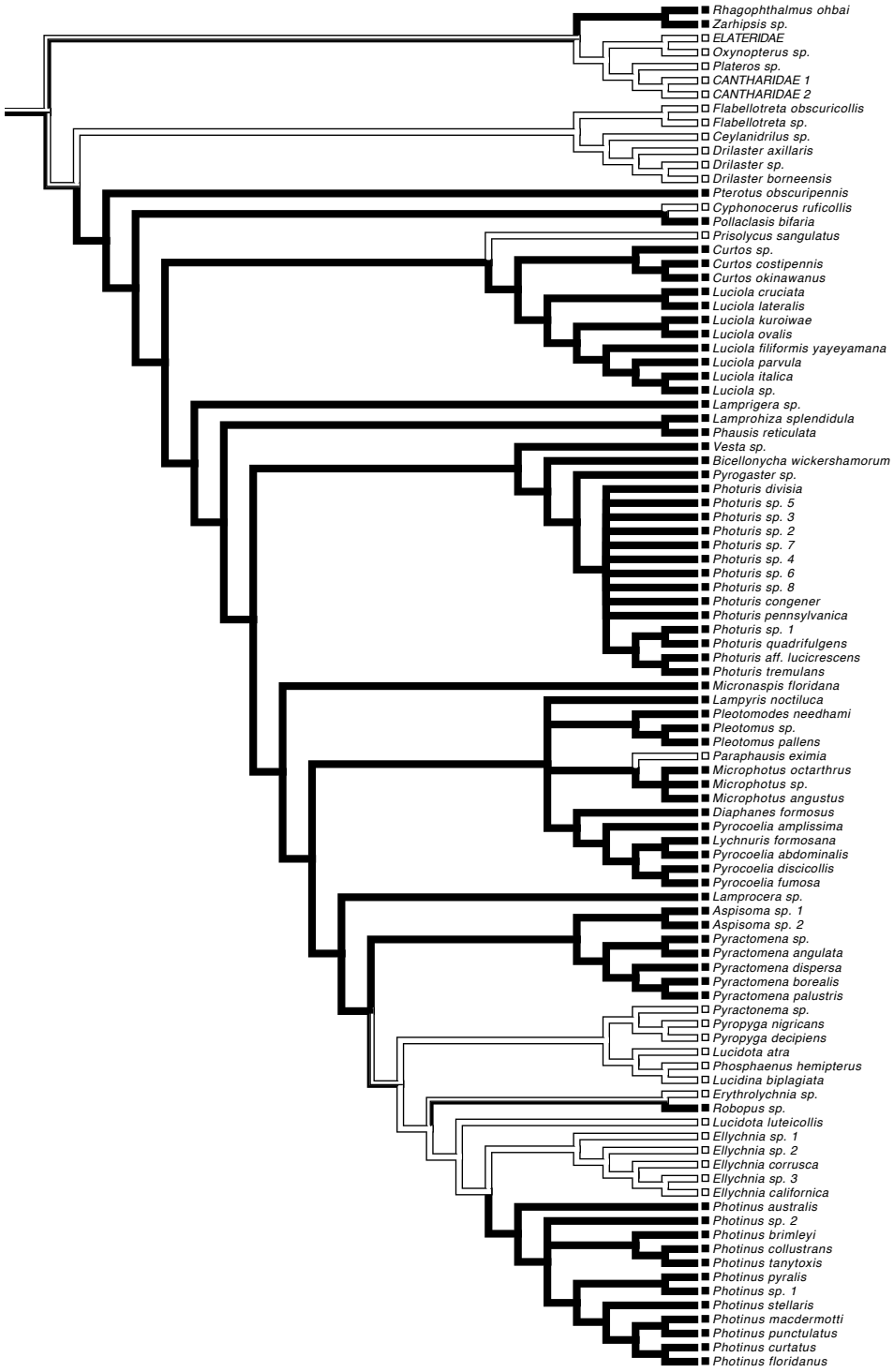
Ancestral state reconstruction, parsimony framework on T-Coffee, Morph, ML.



Ancestral state reconstruction, likelihood framework on T-Coffee, Morph, ML.

Ancestral state reconstruction, likelihood framework on T-Coffee, Morph, ML.

Ancestral state reconstruction, parsimony framework on T-Coffee, Morph, MP.



Ancestral state reconstruction, likelihood framework on T-Coffee, Morph, MP.