**Evolution of Opsin Genes in Caddisflies (Insecta: Trichoptera)**

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**Abstract:**

Insects have evolved complex and diverse visual systems controlled by light-sensing protein molecules called opsins, which couple with a chromatophore to form photopigments. Insect photopigments group into three major gene families based on wavelength sensitivity: long wavelength (LW), short wavelength (SW), and ultraviolet wavelength (UV). Here, we identified 124 opsin copies from whole genome assemblies across 25 species of caddisfly (Insecta: Trichoptera). We discovered LW opsins to have most diversity across species and found that the LW opsins form two separate clades in the opsin gene tree. Conversely, we observed a loss of the SW opsin in half of the species of Trichoptera in this study, which might be associated with the fact that caddisflies are active during low-light conditions. Lastly, we found a single copy of the UV opsin in all of the species in this study, with one exception: *Athripsodes cinereus* has two copies of the UV opsin and resides within a clade of caddisflies with colorful wing patterns.

**Keywords:** Caddisflies, evolution, opsins, visual systems, Trichoptera, insects

**Significance:**

While opsins in some insect groups have been well-characterized, opsins have never been studied across caddisflies. Our findings provide insight into the diversity of opsins in caddisflies and form a basis for further research into the evolutionary drivers and complex visual systems in Trichoptera.

**Introduction:**

Within the visual system, the ability to perceive light is critical and plays an essential role in the life histories of insects including finding food, avoiding predators, and selecting a mate (van der Kooi et al., 2021). Light perception occurs within three different types of visual organs in insects: the stemmata of larvae and the ocelli and compound eyes of adults (Guignard et al., 2022; van der Kooi et al., 2021). Upon light absorption, photoreceptors within the eyes—composed of an opsin protein and chromatophore—change their configuration from a resting state to a signaling state, thereby indicating a physiological response (Shichida & Matsuyama, 2009). In insects, visual opsins form three major gene clades based on their peak wavelength sensitivity, including long wavelength opsins (LW, 500–600 nm), short wavelength opsins (SW, 400–500 nm), and ultraviolet wavelength opsins (UV, 300–400 nm) (Feuda et al., 2016; Lord et al., 2016, van der Kooi et al., 2021). In many insect groups, an additional opsin type, Rhodopsin 7 (RH7), is present. RH7 opsins, which are not used in color vision, do not have a known function in most insect groups but were found to be involved in circadian rhythms in *Drosophila* (Ni et al., 2017).

While insects typically possess one or more copies of each opsin type, multiple cases of gene duplications and losses have been observed throughout opsin evolution (Briscoe et al., 2010; Feuda et al., 2016; French et al., 2015, Futahashi et al., 2015; Guignard et al., 2022; Lord et al., 2016; Mulhair et al., 2023; Sharkey et al., 2017; Sondhi et al., 2021; Suvorov et al., 2017). These duplications, and subsequent diversification, of visual opsin genes are the primary mechanisms of evolution that allow for greater visual capacity and flexibility (Suvorov et al., 2017) and are usually linked to a particular life history strategy, living environment, or light condition (Guignard et al., 2022). For example, in dragonflies (Insecta: Odonata), numerous

duplication events, resulting in up to 33 visual opsin genes in some species, have been observed. This is likely related to their advanced flying capabilities and prominent eyes (Futahashi et al., 2015). Conversely, there has been a loss of the SW opsin gene observed in the American cockroach (*Periplaneta americana*; Insecta:Dictyoptera) (French et al., 2015) and in all studied species of Neuropteroidea, likely due to having nocturnal ancestors (Lord et al., 2016; Sharkey et al., 2017; Sharkey et al., 2023). Additionally, in butterflies and moths (Insecta: Lepidoptera), diurnal species were found to possess significantly more duplications than nocturnal species. Together, these results indicate that light conditions may be drivers of opsin evolution (Sondhi et al., 2021). While the evolution of opsins has been relatively well studied in some insect orders, opsin genes in caddisflies (Insecta: Trichoptera) have only been characterized in a single species, *Limnephilus lunatus* as part of a broader comparative study across insects (Guignard et al., 2022).

Caddisflies are a highly diverse order of aquatic insects. As eggs, larvae, and pupae, they mainly inhabit benthic habitats of freshwater rivers, streams, ponds, and lakes, but as adults, they occupy terrestrial environments (Morse et al., 2019). There are two suborders of Trichoptera characterized by habitat, morphology, and silk use: Annulipalpia (retreat-making) and Integripalpia (cocoon- and case-making) (Thomas et al., 2020). Adult caddisflies resemble small moths; however, the wings and bodies of most species are covered in small hairs instead of scales (Holzenthal et al., 2007). In some families of caddisflies, distinct colors and patterns on the wings can be identifying features to distinguish different species of caddisflies, as in butterflies and moths (Holzenthal et al., 2007). While most caddisflies possess wing hairs and patterns that are somber shades of brown, yellow, or gray, a few species have brightly colored wings with red, orange, green, or silver regions (Fig. 1). The wing color in some of these species

is due to the development of hairs into scales (Holzenthal et al., 2007). Additionally, adult caddisflies possess varying eye sizes, some much larger than others (Fig. 1). Presumably, such varied environments and wing colorations require a plastic and diverse visual system. To gain an understanding of opsin evolution in caddisflies, we analyzed the occurrence and phylogenetic relationships of opsin genes across 25 caddisfly genomes, including five newly assembled genomes and 20 publicly available genome assemblies (Frandsen et al., 2019; Frandsen et al., 2023; Heckenhauer et al., 2019; Heckenhauer et al., 2022; Heckenhauer et al., 2023; Kawahara et al., 2022; Luo et al., 2018; Olsen et al., 2021; Ríos-Touma et al., 2021, The Darwin Tree of Life Project Consortium, 2022), representing the major evolutionary lineages within the order.

**Results:**

We found a total of 124 caddisfly opsins in this study. Of these sequences, 61 were LW opsins, 12 were SW opsins, 26 were UV opsins, and 25 were RH7 opsins (Fig. 2). These opsins were found across 25 genomes with representatives in all major lineages of Trichoptera (Fig. 3). The number of opsins found within each species ranged from three opsins to as many as nine opsins. These opsins formed four distinct clades in the maximum likelihood gene tree corresponding to the LW, SW, UV, and RH7 opsin groups (Fig. 2). Within these clades, the arrangements of suborders were maintained, but occasionally interspecific relationships of opsin sequences did not match those in the species tree.

The LW opsins formed two distinct clades in the opsin gene tree, a phenomenon observed in other insect orders (Feuda et al., 2016; Futahashi et al., 2015; Guignard et al., 2022; Lord et al., 2016; Mulhair et al. 2023; Sharkey et al., 2017; Sharkey et al., 2023; Sondhi et al., 2021, Suvorov et al., 2017). We labeled the larger group LW1 and the smaller one LW2, with

letters (e.g. a, b, c, etc.) distinguishing multiple copies within a species. All species had at least one LW opsin in the LW1 clade, while only 14 species had an additional LW2 opsin. The 14 species with the LW2 opsin were distributed across both suborders (Fig. S1). In species with multiple copies of the LW opsin, the duplicates were almost always on the same contig in the genome assembly, regardless of whether they were in the same clade (LW1 vs LW2) in the gene tree (Table S1).

In the opsin gene tree, we observed a few instances of clusters of opsins from the same species. For example, *Agrypnia vestita* was the only species with two copies of the LW2 opsin, and both copies grouped next to each other in the gene tree. Similarly, in *Athripsodes cinereus*, the species with the highest number of LW opsins, the LW1b, LW1c, and LW1d opsins all clustered together in the tree. *Athripsodes cinereus* was also the only species found to have two copies of the UV opsin and we found that the duplicate copies were adjacent to each other on the same contig in the genome assembly. After alignment, we found 11 amino acid substitutions between the two copies (Fig. S2), but they were still clustered together in the phylogeny (Fig. 2).

The number of opsin copies in each species was highly variable across the phylogeny and between suborders. Within the seven species of fixed-retreat makers (Fig. 3: Clade 1), opsin diversity was more uniform; all species had one copy of the UV and RH7 opsins, two or three copies of the LW opsin, and a single SW opsin was found in all except *Plectrocnemia conspersa.* The number of opsins recovered within Integripalpia (Fig. 3: Clade 2), however, was more variable. For example, the number of LW opsins among species of Integripalpia ranged from one to five copies. Furthermore, we recovered all three visual opsins in four of the seven species within “basal Integripalpia” (Fig. 3: Clade 2-a). The other three species, all from the family Rhyacophilidae, were found to have lost the SW opsin. Conversely, in the tube-case making

Integripalpia (Fig. 3: Clade 2-b), the SW opsin was lost in all but two species, *Nectopsyche paramo* and *Athripsodes cinereus*, which both belong to the family Leptoceridae.

**Discussion:**

We identified 124 opsin sequences across 25 caddisfly genomes and determined the phylogenetic relationships of opsins in Trichoptera. We discovered variety in the number of opsin genes across species, particularly in the LW and SW opsins. Our results suggest that caddisflies have evolved a diverse range of opsins, likely driven by life history strategies and ambient light conditions as has been hypothesized in other insect orders (Briscoe et al., 2010; Feuda et al., 2016; French et al., 2015, Futahashi et al., 2015; Guignard et al., 2022; Lord et al., 2016; Mulhair et al., 2023; Sharkey et al., 2017; Sondhi et al., 2021; Suvorov et al., 2017).

It is important to note that, in this study, we relied on previous research to verify that these genes correspond to insect opsins (Guignard et al., 2022). Therefore, we have confidence that the genes found in this study are in fact opsin genes within these genomes. However, we cannot confidently say when, where, or if these genes are expressed. Further studies using transcriptomic analysis in caddisfly opsins can address this limitation.

The distribution of opsins within Annulipalpia, the fixed-retreat makers, was relatively uniform. Interestingly, their ecological distributions are also less varied; most species inhabit fast-moving streams. While there were three species with a duplication of a LW opsin gene, the only loss we observed was the SW opsin gene in *Plectrocnemia conspersa*. The genome assembly of *Plectrocnemia conspersa* was high quality, indicating that this is likely a true loss (Table 1). Interestingly, *Plectrocnemia conspersa* also had a LW duplication, suggesting more dynamic opsin evolution in this species compared with other retreat makers.

The SW opsin gene was also lost in Integripalpia within the basal cocoon-making Rhyacophilidae and within the majority of the clade of tube-case makers (Fig. 3 Clade 2-b). Loss of the SW opsin in other groups such as the American cockroach (French et al., 2015) and Neuropteroidea (Lord et al., 2016; Sharkey et al., 2017; Sharkey et al., 2023) is hypothesized to be associated with the low-light environments in which the ancestors of these insects lived. Caddisflies are primarily crepuscular and thus are most active during low-light conditions, which may be related to the loss of the SW opsin in Rhyacophilidae and in many tube-case making caddisflies.

Interesting, in contrast to most species within the tube-case makers, we were able to find a copy of the SW opsin in both *Nectopsyche paramo* and *Athripsodes cinereus.* The latter was also the only species found with two copies of the UV opsin and the highest number of LW opsins. Three out of the five copies of the LW opsin in this species were clustered together in the gene tree and were adjacent to each other on the same contig in the genome, indicating a possible species-specific tandem duplication event. This is also true for both copies of the UV opsin in this species. *Nectopsyche paramo* and *Athripsodes cinereus* both belong to the family Leptoceridae, a family with long adult antennae and many genera with intricate wing patterns and brightly colored or iridescent wing hairs and scales (Holzenthal et al., 2007). This is true for *Athripsodes cinereus*, which has wings with patches of cream-colored hairs (Fig. 1A), and *Nectopsyche paramo*, which has spotted wings (Fig. 1B). The high number of opsins and presence of the SW opsins could be related to the variety in wing coloration and patterns in some species of Leptoceridae (Fig. 1). Future work should combine more sampling within this interesting area of the caddisfly phylogeny with gene expression and physiological data to better

model the visual system of Trichoptera and to test hypotheses related to color vision and opsin diversity.

Here, we conducted the first comprehensive study of visual opsins in Trichoptera. We found that caddisflies have evolved a diverse range of opsins across species. The species with the highest diversity of opsins were derived from the group with the most colorful and intricately patterned wings, the Leptoceridae. The evolution of opsins in caddisflies also may have been driven by life-history strategies and the low light conditions during which caddisflies are active. The findings of this study provide a basis for future research on the diverse and complex visual systems in Trichoptera.

**Materials and Methods:**

Genome sequences for 25 species, covering all the major lineages of Trichoptera, were assessed. Among these, five were newly assembled genomes, and 20 were publicly available genome assemblies downloaded from GenBank (Benson et al., 2013). Using the 1000 opsins found by Guignard et al. (2022), we performed a tBLASTn search of opsin sequences against each caddisfly genome. BLAST hits with an e-value $< 10^{-40}$ were kept. The resulting opsin sequences were extracted from their corresponding genomes using the Python package Pyfaidx (Shirley et al., 2015). We noticed many instances in which multiple queries of different opsin types hit to the same locations in the genomes. In this case, when extracting the sequence from the genome, the widest window from each contig was extracted, and the gene was later classified phylogenetically. Next, to verify the sequences we extracted, we performed gene prediction using AUGUSTUS v3.4.0 (Stanke, et al. 2006) followed by a BLASTp search against the online NCBI databases, keeping only sequences similar to other insect opsins. Lastly, we manually

checked the annotations in Geneious Prime 2023.0.4 (https://www.geneious.com), using outgroup sequences as a guide, to ensure that the entire gene was correctly annotated. We included opsin sequences from a variety of orders for outgroup comparison: Lepidoptera (*Danaus plexippus, Heliconius melpomene, Manduca sexta, Papilio xuthus*), Odonata (*Sympetrum frequens)*, Diptera (*Drosophila melanogaster*), and Coleoptera (*Tribolium castaneum*), all accessed through GenBank (Benson et al., 2013).

*Opsin gene tree reconstruction*

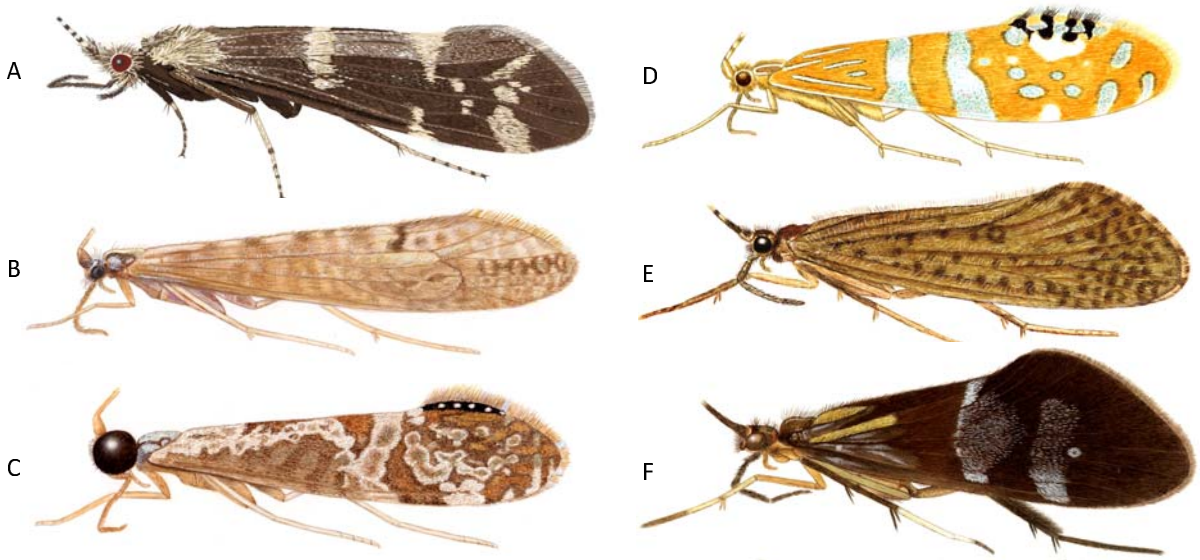
To determine phylogenetic relationships among opsin genes, we first aligned the caddisfly and outgroup opsin sequences using MAFFT v7.487 (Katoh & Standley, 2013). Phylogenetic reconstruction was performed using IQ-TREE v2.0.3 (Nguyen et al., 2015) with default parameters. The resulting trees were viewed in FigTree v1.4.4 (Rambaut, 2018). To classify which opsin group each sequence belonged to, we labeled them based on where they grouped in relation to the outgroup sequences. The branches in the tree were color-coded according to opsin type; LW opsin branches were colored green, UV opsin branches were colored purple, SW opsin branches were colored blue, and RH7 branches were colored black. Outgroup opsin branches were colored in lighter shades of the same colors.

*Species tree and bar plot*

We created the species phylogeny using the relationships inferred by Thomas et al. (2020). To summarize the number of opsins in each species, we wrote a custom R script that calculated the totals and plotted them using ggplot2 v.3.3.5 (Wickham, 2016). The species in the bar plot were ordered according to the species phylogeny.

**Figure 1**

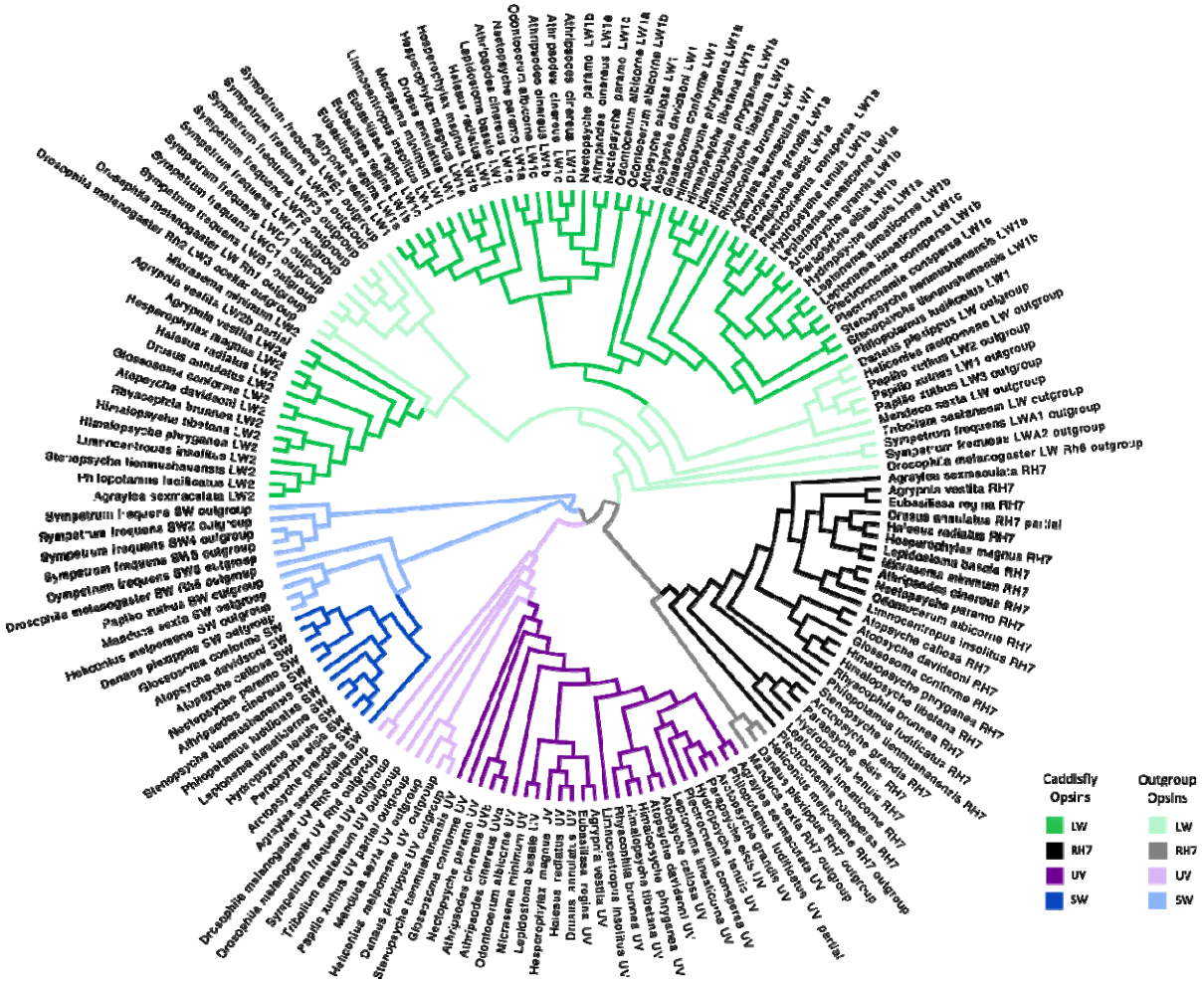
*Adult Caddisfly Illustrations*




*Note:* Adult caddisfly illustrations showing varying eye sizes and diverse wing colorations and patterns. (A) *Athripsodes cinereus* (Leptoceridae); (B) *Nectopsyche paramo* (Leptoceridae); (C) *Nectopsyche nigricapilla* (Leptoceridae); (D) *Nectopsyche ortizi* (Leptoceridae); (E) *Banyallarga vicaria* (Calamoceratidae); (F) *Phylloicus abdominalis* (Calamoceratidae). Illustrations by Julie Martinez and Ralph Holzenthal.

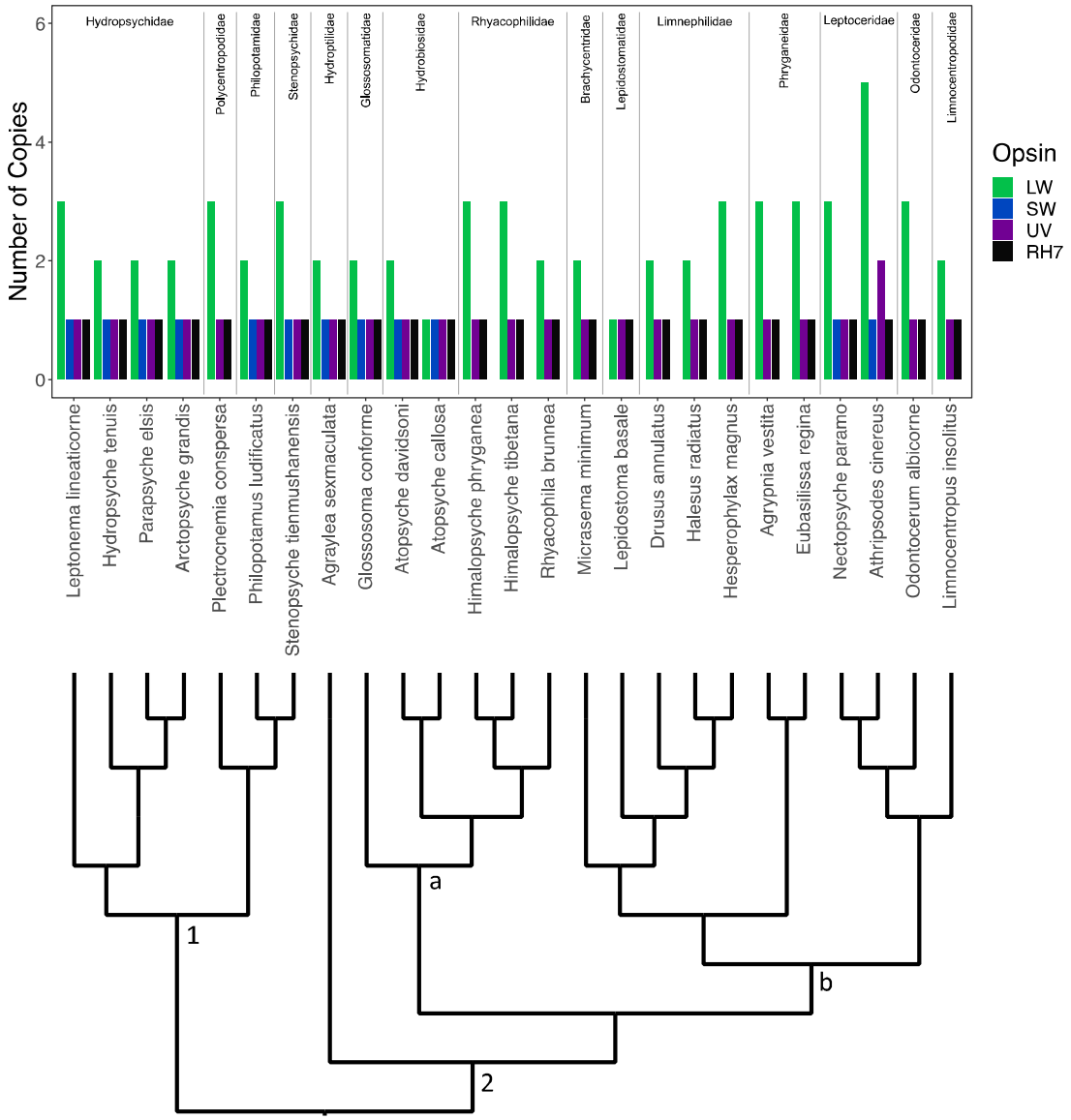
**Figure 2**

*Opsin Gene Tree*




*Note:* Maximum likelihood tree from caddisfly opsin DNA sequences found in this study along with various outgroup sequences. Branches are colored by opsin type, with caddisfly opsins shown in darker colors and outgroup opsins depicted in lighter shades. Letters at the end of the node labels (e.g., LW1a, LW1b, etc) denote multiple copies of that opsin type in a species.

**Figure 3**




*Note:* Bar plot of the number of opsin copies in each caddisfly genome. The bars are colored by opsin type. The species are ordered by the species phylogeny indicated below the bar plot. Families are labeled above the bar plot. Suborders are labeled within the phylogeny as follows: (1) Annulipalpia—retreat-makers; (2) Integripalpia—cocoon- and tube case-makers; (a) (and *Agraylea sexmaculata*) basal Integripalpia—cocoon-makers; (b) tube case making Integripalpia.

**Table 1**

*Genome Assemblies*

| Species | Accession number | Publication | Contig N50 (bp) | Compleasm (BUSCO) |
|---|---|---|---|---|
| *Leptonema lineaticorne* | GCA_024500535.1 | Heckenhauer et al. 2023 | 14,931,587 | C:98.30%[S:97.83%,D:0.47%], F:0.66%,I:0.00%,M:1.04% |
| *Hydropsyche tenuis* | GCA_009617725.1 | Heckenhauer et al. 2019 | 2,190,134 | C:97.93%[S:97.65%,D:0.28%], F:0.89%,I:0.00%,M:1.18% |
| *Parapsyche elsis* | GCA_022651745.1 | Frandsen et al. 2019, Heckenhauer et al. 2022 | 5,591,679 | C:96.99%[S:96.80%,D:0.19%], F:0.80%,I:0.00%,M:2.21% |
| *Arctopsyche grandis* | GCA_029955255.1 | Frandsen et al. 2023 | 6,470,670 | C:98.36%[S:95.39%,D:2.97%], F:0.71%,I:0.00%,M:0.94% |
| *Plectrocnemia conspersa* | Available on FigShare | New | 32,103,979 | C:97.74%[S:97.32%,D:0.42%], F:0.61%,I:0.00%,M:1.65% |
| *Philopotamus ludificatus* | GCA_022495035.1 | Heckenhauer et al. 2022 | 35,449 | C:94.63%[S:93.31%,D:1.32%], F:3.15%,I:0.05%,M:2.17% |
| *Stenopsyche tienmushanensis* | GCA_008973525.1 | Luo et al. 2018 | 1,296,863 | C:97.50%[S:95.15%,D:2.35%], F:1.04%,I:0.00%,M:1.46% |
| *Agraylea sexmaculata* | GCA_022606485.1 | Heckenhauer et al. 2022 | 86,524 | C:95.67%[S:90.96%,D:4.71%], F:1.04%,I:0.00%,M:3.30% |
| *Glossosoma conforme* | GCA_022606575.1 | Heckenhauer et al. 2022 | 2,212,131 | C:93.13%[S:92.47%,D:0.66%], F:0.66%,I:0.00%,M:6.21% |
| *Atopsyche davidsoni* | GCA_022113835.1 | Ríos-Touma et al. 2021 | 14,095,054 | C:98.68%[S:98.35%,D:0.33%], F:0.61%,I:0.00%,M:0.71% |
| *Atopsyche callosa* | Available on FigShare | New | 25,586,909 | C:98.54%[S:96.70%,D:1.84%], F:0.66%,I:0.00%,M:0.80% |
| *Himalopsyche phryganea* | GCA_022494535.1 | Heckenhauer et al. 2019 | 4,634,010 | C:98.21%[S:97.83%,D:0.38%], F:0.71%,I:0.00%,M:1.08% |
| *Himalopsyche tibetana* | GCA_030503985.1 | Heckenhauer et al. 2022 | 28,889,006 | C:98.91%[S:98.35%,D:0.56%], F:0.56%,I:0.00%,M:0.52% |
| *Rhyacophila brunnea* | Available on FigShare | New | 1,306,779 | C:97.74%[S:94.35%,D:3.39%], F:1.22%,I:0.00%,M:1.04% |
| *Micrasema minimum* | GCA_022494985.1 | Heckenhauer et al. 2019 | 69,526 | C:66.34%[S:66.20%,D:0.14%], F:7.25%,I:0.00%,M:26.41% |
| *Lepidostoma basale* | GCA_022606425.2 | Heckenhauer et al. 2019 | 1,001,566 | C:98.16%[S:97.55%,D:0.61%], F:0.85%,I:0.05%,M:0.94% |
| *Drusus annulatus* | GCA_022651775.1 | Heckenhauer et al. 2019 | 1,032,046 | C:97.92%[S:97.50%,D:0.42%], F:1.08%,I:0.00%,M:0.99% |
| *Halesus radiatus* | GCA_022606495.2 | Heckenhauer et al. 2019 | 124,280 | C:91.20%[S:89.69%,D:1.51%], F:4.38%,I:0.05%,M:4.38% |
| *Hesperophylax magnus* | GCA_026573805.1 | Frandsen et al. 2023 | 11,205,906 | C:98.68%[S:96.56%,D:2.12%], F:0.61%,I:0.00%,M:0.71% |
| *Agrypnia vestita* | GCA_016648135.1 | Olsen et al. 2021 | 111,757 | C:91.25%[S:83.10%,D:8.15%], F:4.14%,I:0.09%,M:4.52% |
| *Eubasilissa regina* | GCA_022840565.1 | Kawahara et al. 2022 | 29,378,647 | C:98.73%[S:96.28%,D:2.45%], F:0.80%,I:0.00%,M:0.47% |

| | | | | |
|---|---|---|---|---|
| *Nectopsyche paramo* | JAWQED000000000 | New | 1,090,281 | C:97.31%[S:95.24%,D:2.07%], F:0.80%,I:0.00%,M:1.88% |
| *Athripsodes cinereus* | GCA_947579605.1 | Darwin Tree of Life Project | 948,465 | C:97.22%[S:96.61%,D:0.61%], F:1.08%,I:0.00%,M:1.69% |
| *Odontocerum albicorne* | GCA_949825065.1 | Darwin Tree of Life Project | 387,033 | C:97.22%[S:96.75%,D:0.47%], F:1.18%,I:0.00%,M:1.60% |
| *Limnocentropus insolitus* | Available on FigShare | New | 33,230,923 | C:98.87%[S:98.26%,D:0.61%], F:0.56%,I:0.00%,M:0.56% |

*Note:* Locations and quality scores of genome assemblies for each species. Compleasm (BUSCO) was run with the Endopterygota OrthoDB v.10 gene set and the results categories are as follows: C: complete; S: single; D: duplicated; F: fragmented subclass 1 (only a portion of the gene is present in the assembly); I: fragmented subclass 2 (different sections of the gene align to different locations in the assembly); M: missing.

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Conceptualization: A.P. and P.B.F. Formal analysis: A.P., J.H., E.R.G., and P.B.F. Resources: J.H., B.R.-T., R.B.K., and P.B.F. Data curation: A.P., J.H., and E.R.G. Writing-original draft

prep: A.P. Writing-review and editing: all authors. Visualization: A.P., and R.W.H. Supervision: P.B.F. Funding acquisition: A.P., J.H., S.U.P., B.R.-T., and P.B.F.

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