Eyespots originated multiple times independently across the Lepidoptera

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Abstract

Eyespot color patterns often function as a defense against predators and in mate choice. In Nymphalid butterflies, eyespots have a single evolutionary origin close to the base of this clade, but eyespots are also present in many other lepidopteran lineages and may have multiple independent origins. Here we use phylogenetic comparative methods to investigate the evolution of eyespots across a multi-superfamily phylogeny of Lepidoptera, and to pinpoint lineages in which eyespots likely originated independently. We find a total of 28 separate origins of *Discal* eyespots (in the discal wing region) and 19 separate origins of *Marginal* eyespots (in the marginal wing region), including four separate instances where eyespots were preserved in most extant representatives of a species radiation. The first two eyespot radiations we observed are in the Nymphalidae, with a *Marginal* eyespot radiation occurring before a *Discal* one. While the remaining two eyespot radiations were observed in the Saturniidae, occurring in a reverse fashion, where a *Discal* eyespot radiation preceded a *Marginal* eyespot radiation. Even though eyespots do not appear to be homologous across Lepidoptera they may share a homologous gene-regulatory network. Our phylogenetic inference provides a roadmap for future developmental and functional studies addressing this hypothesis. This study therefore has implications for our understanding of the evolution of serial homologues and of convergent evolution of visual signals in insects.

Introduction

Lepidopteran wing color patterns have been a source of fascination and human inspiration for centuries (Hogue, 1987). While some of the classic early research focused on the adaptive role of general wing coloration, such as “industrial melanism” in moths (Kettlewell, 1973), other research

focused on the role of localized color patterns, such as eyespots (Blest, 1957). Eyespots consist of more than one concentric ring of contrasting colored scales, often mimicking a vertebrate eye (Oliver et al., 2014; Labanderia et al., 2016). These visually striking color patterns play roles in predator deterrence, predator deflection, and mate selection (Breuker & Brakefield 2002; Stevens, 2005; Robertson and Monteiro 2005; Vallin et al. 2007; Kodandaramaiah, 2011; Merilaita et al., 2011; Prudic et al. 2012; Kodandaramaiah et al., 2013; Prudic et al., 2015; Mukherjee & Kodandaramaiah, 2015; Huq et al., 2019; Halali et al., 2019). Eyespots are thus adaptive, having evolved in several clades of Lepidoptera, but their evolutionary history remains poorly understood.

Research on eyespot evolution to date has focused on nymphalid butterflies. Here eyespots develop in between veins at the wing margins (Fig. 1a-b). Such *Marginal* eyespots evolved once in Nymphalidae, shortly after the origin of the clade (Oliver et al., 2012 & 2014), and are therefore considered homologous. Several moth lineages (e.g., Semanturidae, Saturniidae, Cambridae) also exhibit *Marginal* eyespots, but their evolutionary history has not been investigated. In contrast to *Marginal* eyespots, *Discal* eyespots are centered on cross veins or placed within the discal cell region (Otaki., 2020; Fig. 1c-d). Across Lepidoptera, species may have *Marginal* eyespots, *Discal* eyespots, or eyespots located on both wing regions (Fig. 2). Because these distinct wing regions express different genes during development (Banerjee et al., 2023), the evolution of *Marginal* and *Discal* eyespots might have occurred independently in each region (Fig. 1E). Alternatively, eyespots might have first evolved on one wing region, and later evolved on the other region, with the order of these regions potentially varying if there are multiple independent eyespot origins (Fig. 1e-g). By investigating the evolution of eyespots across the Lepidoptera we aim to discover whether *Marginal* and *Discal* eyespots are homologous or convergently evolved across the Lepidoptera.

Here we used modern phylogenetic methods to investigate the evolutionary origins of *Discal* and *Marginal* eyespot across Lepidoptera. We first inferred a species-level phylogeny for Lepidoptera, sampling up to 27 (5-24 fragments per species) molecular sequences in over 715 species. Our

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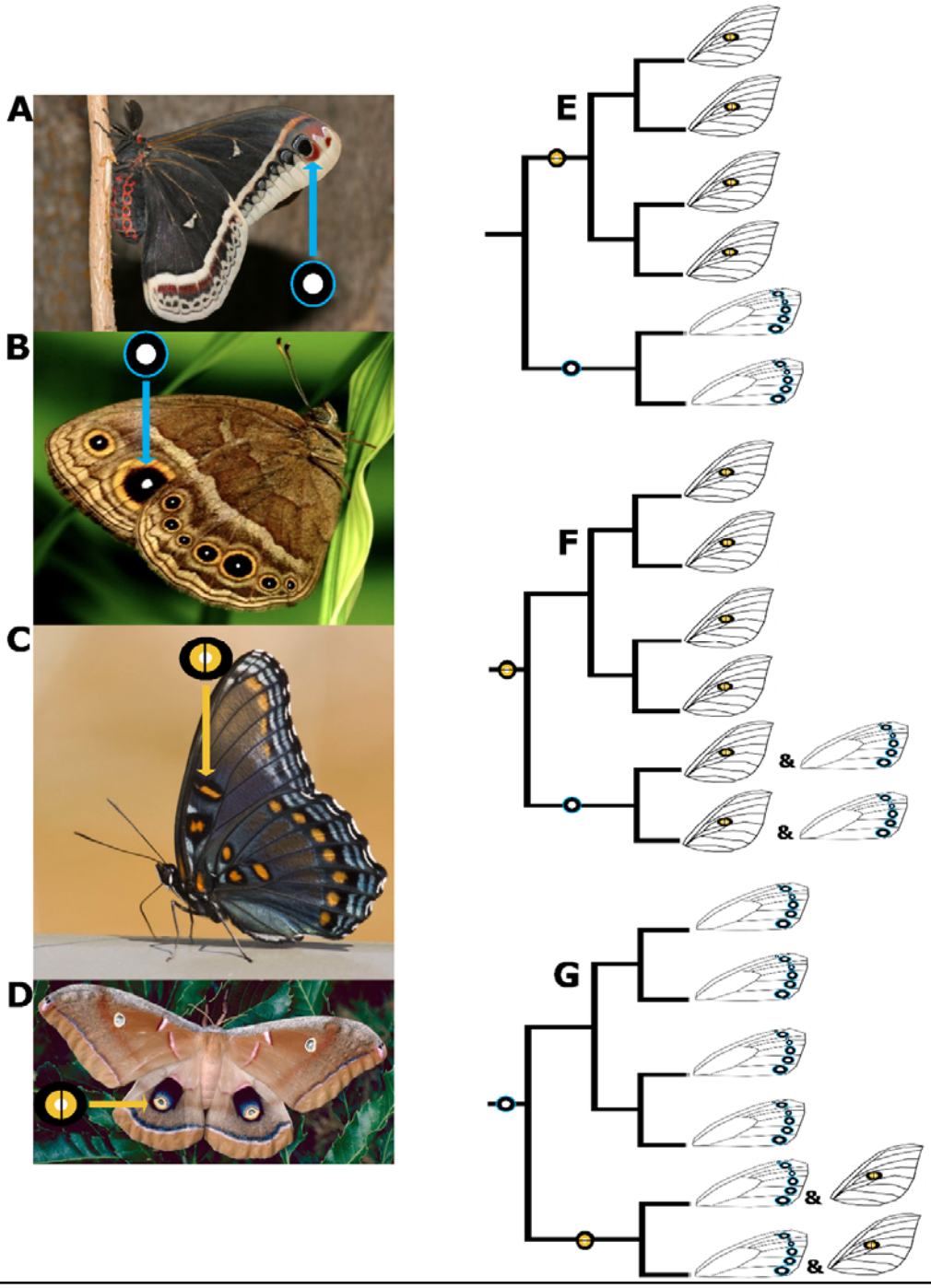


Figure 1 A/B/C/D/E/F/G. The regions of the wing for this study were partitioned into the following two zones, marginal and discal. marginal eyespots are eyespots found between butterfly wing veins, this is demonstrated by A & B as a real-world examples and E-G in our theoretical examples while discal eyespots are eyespots found in the anterior region of the wing (demonstrated by C, D and E-G respectively). Species: A = *Eupakardia calleta*, B = *Bicyclus anynana,* C = *Limenitis arthemis* & D = *Antheraea polyphemus.* Figure 1 E-G. Theoretical models of eyespot evolution taken into consideration by our model. In E it is theorised that Discal eyespots evolved first and marginal eyespots second. In F it is theorised that marginal eyespots evolved first, and discal eyespots evolved second. In G, it is theorised that marginal and discal eyespots evolve separately. It was decided that all three of these models is equally plausible with no prior assumptions made.

eyespot radiation. Colours in the key represent the corresponding super families in Figure 5.

phylogeny covers 90% of Lepidoptera families, allowing comparative inferences of eyespot evolution in moths and butterflies. We then modeled evolutionary origins and losses of eyespots and inferred ancestral states across the phylogeny. For each possible subtree with an ancestral eyespot origin, we implemented a model comparison approach, based on marginal likelihood estimation, to quantify support for eyespot homology among extant taxa. These analyses suggested differently ordered sequences of eyespot evolution in the two main eyes-spot bearing radiations, the silkmoths (Saturniidae) and the brush-footed butterflies (Nymphalidae). Our results demonstrate that eyespots in the Lepidoptera have evolved multiple times, and that *Discal* and *Marginal* eyespots have evolved in different temporal sequences in the main clades where they radiated (Nymphalidae and Saturniidae). We also find that eyespots have evolved in a further 10 superfamilies and numerous families across the Lepidopteran family tree. Finally, we find that eyespots across the lepidoptera are more commonly observed in the *Discal* region (28 occurrences) than the *Marginal* region (19 occurrences) which was unexpected as most eyespot research is focused on *Marginal* eyespots found in the Nymphalidae.

Materials and methods

Molecular data collection for phylogenetic tree construction

DNA sequence data for 645 species of Lepidoptera (moths and butterflies) and seven species of Trichoptera (caddisflies, outgroup) were kindly provided by Professor Emeritus Charles Mitter (University of Maryland, Table S1 & S2). Additional sequences for 70 species of Saturniidae (silkmoths) were downloaded from NCBI (GenBank). The full dataset was composed of 27 protein-coding genes (Table S2), with 5-24 fragments available across all species. Our taxonomic sampling includes ~68% (90/133, Van Nieukerken et al., 2011) of all families and ~65% (28/43, Van Nieukerken et al., 2011) of all superfamilies in the order Lepidoptera. This study focuses on the suborder Ditrysia, which comprises ~98% of currently described lepidopterans. Of these, ~90% of families (90/100, Reiger et al., 2009) and ~93-96% (28/29-30) of superfamilies (Van Nieukerken et al., 2011; Heikkla et

al., 2015; Mitter et al., 2017) are represented. We chose the Trichoptera for our outgroup because they are the closest extant relatives to the Lepidoptera (Mey et al., 2017), but distant enough to provide ingroup monophyly.

Sequence alignment and phylogenetic inference

DNA sequences were aligned via MAFFT v 7.490 (Katoh & Standley, 2013). A global alignment strategy with iterative refinement (G-INS-i) was employed to maximize alignment accuracy based on weighted sum-of-pairs and a consistency scores. The length of the final alignment, consisting of all 27 concatenated gene fragments, was 22,643 bp.

IQ-TREE v 1.6.12 (Nguyen et al., 2015, Kalyaanamoorthy et al., 2017 & Hoang et al., 2018) was used for phylogenetic inference under maximum likelihood. All genes were subject to a single substitution process automatically set by IQ-TREE,using ModelFinder (Kalyaanamoorthy et al., 2017). Substitutions followed a generalized time reversible (GTR) model, with estimated base frequencies. Rate variation among sites followed a gamma distribution containing 10 categories. Node support was estimated using ultra-fast bootstrap in UFBoot (Hoang et al., 2018) for 100,000 iterations. UFBoot is an efficient approximation to the traditional bootstrap method that is particularly well-suited for large datasets such as ours. After pruning the outgroup, our maximum likelihood tree was transformed into an ultrametric tree, with branch lengths scaled to time, using the ape package v 5.7-1 (Paradis & Schliep, 2019) in R v 4.3.0 (R Core Team 2023). For branch scaling, we used an age of ~290 Ma for the most recent common ancestor of Ditrysia, following the most recent dated phylogeny of Lepidoptera (Kawahara et al., 2019).

Image data collection

We scored images of each of the 715 species in our phylogeny for presence or absence of eyespot patterns (in any wing surface or sex). An eyespot pattern was identified as present only if it met three criteria: 1) it had at least two concentric rings of distinct colors, 2) the rings were circular or oblong in

shape, and 3) the color inside the internal ring differed from the color on the outside of both rings (Fig. 3). To explore the sensitivity of our results to these conservative criteria, we repeated our main comparative analyses under a more relaxed definition of eyespots, where criterion 2 was extended to non-circular/oblong shapes (e.g., triangles, rectangles, teardrops, see Fig. S1 for examples). Eyespots were further classified based on their locations on the wing surface. *Discal* eyespots straddle the cross veins in the discal cell region or are found within and around the discal cell (Otaki et al., 2020 & Figure 1), whereas *Marginal* eyespots are found in between longitudinal veins, at the wing margins and within the discal cell of the wing (Fig. 1a-d).

Figure 3. A visual guide demonstrating the difference between eyespots (A), a ring formation (B), spots (C) and an eyespot like pattern (D). Species: *Pyrrhia adela* (A), Junonia almana (B), Pieris canidia (C) and Attacus atlas (D).

We first queried a wide range of online databases for images of the type specimen of each of the sampled species (Table S3, Supplementary data file 1). If not available, images of other (non-type) specimens were collected from the same databases with a preference for museum specimens over other specimens. In cases where sequence data was assigned to a particular subspecies, we prioritized images of the same taxonomic rank when available. Ninety-six species included in our data set lacked publicly available and reliably identified images. We photographed 81 of these

126 species at the Mcguire Center for Lepidoptera (MGCL, Florida Museum of Natural History, 3215 Hull
127 Rd, Gainesville, Fl 32611), using a Cannon D50 DSLR camera. We were unable to obtain images for 15
128 species (2%), which are treated as missing data in all comparative analyses.

129 Modelling the origin and loss of eyespots in Lepidoptera

130 RevBayes v 1.2.1 (Höhna et al., 2016) was used in this and subsequent analyses to model eyespot
131 evolution across Lepidoptera. RevBayes is an open-source software package designed for Bayesian
132 phylogenetic inference. It allows users to build probabilistic graphical models using an interactive
133 model-specification language.

134 Eyespots were modelled as a discrete trait with two states (presence/absence) with unequal
135 transition rates drawn from identical exponential priors. The rate parameters of these priors were set
136 to reflect an expectation of 10 events (10 eyespot gains and 10 eyespot losses) along the tree. Root
137 state frequencies were in turn drawn from a Dirichlet prior, assuming equal probability of presence
138 or absence of eyespots at the origin. Eyespot evolution was modelled separately using three
139 datasets: eyespot presence/absence irrespective of eyespot location on the wing, presence/absence
140 of *Marginal* eyespots, and presence/absence of *Discal* eyespots. For all three datasets, eyespots
141 could be located on any wing surface and either sex.

142 Each model was run for 100,000 iterations with an initial burn-in of 10,000, and tuning parameter
143 proposals every 1,000 iterations on two independent chains. Joint conditional ancestral states were
144 sampled every 100 iterations and plotted using RevGadgets v 1.1.1 (Tribble et al., 2023) in R. We
145 evaluated the convergence and stationarity of the MCMC chains for each model using the R package
146 Convenience v 1.0.0 (Fanreti et al., 2013)

147 Testing eyespot homology in selected clades

148 We next implemented a model testing approach to investigate eyespot homology among extant
149 species of Lepidoptera. We identified all subclades including three or more taxa bearing eyespots,

regardless of their location. If the most recent ancestor of these clades also displayed eyespots, it is likely that eyespots in extant taxa are homologous. To test this hypothesis, we extracted all subclades from the complete ultrametric phylogeny and applied in each case two alternative versions of the discrete-trait model described above. In the first version (hereafter the multiple-origin model), we set the root frequency of eyespot presence to zero, effectively constraining the common ancestor of the subclade to lack eyespots. In the second version (the common-ancestor model), we instead enforced a common ancestor with eyespots by setting the root frequency of eyespot presence to one. We then compared the marginal likelihood (ML) of these alternative models using log 10 of the raw Bayes factor using thresholds based on Jeffreys (1998). In our study, results above positive 0.1 were considered to show some support for an eyespot originating at a specific node. Results above 0.5 were considered to have strong support, results above 1.0 were considered to have very strong support and results which were positive numbers but below 0.1 were considered to demonstrate very weak support which wasn't worth mentioning. Finally negative results were considered to represent no support for an eyespot origin at a given node. These thresholds for the support of eyespots being present at a particular node were chosen due to the high number of negative values we obtained in our study (values above -1.0) which is indicative of a lack of support for a single origin of eyespots.

ML estimates gauge the fit of a model, including its priors, to the data, in this case, the phylogeny and presence or absence of eyespots in extant taxa. Because the two models being compared differ only in their prior assumptions about the character state at the root, their direct comparison serves as a statistical test of the support for eyespot presence in the most recent common ancestor of the clade. ML was estimated via the use of two sampling methods, steppingstone sampling (SS) and path sampling (PS, Lartillot and Philippe, 2006, Fan et al., 2011 & Xie et al., 2011). For both methods the power posterior analysis was split into 50 intervals between the prior and posterior and was run for 5,000 iterations with a burn-in of 5,000 generations. We repeated ML approximations for each subclade and eyespot dataset and confirmed that both PS and SS estimates were stable (i.e. differing

by no more than 0.5 between independent runs of each set of power posteriors). The results of these analyses were summarized and plotted onto the phylogeny using the R packages phytools v1.9-16 (Revell, 2012), ggplot2 v3.4.3 (Wickham et al., 2016) and ggtree v3.9.0 (Yu et al., 2017 & 2018, Yu, 2020 & 2022 & Xu et al., 2022), followed by the online tool ITOL (2023).

Results

Tree topology

The phylogeny constructed for this study contains 28 superfamilies, each containing 1 – 128 species in our samples (median = 14). The full list of superfamilies as well as the number of species representatives for each superfamily are outlined in Table S1 (Supplementary tables and figures). The topology of our tree is largely congruent with previous phylogenies (Heikkil□ et al., 2015; Mitter et al., 2017; Kawahara et al., 2019). Topological differences observed between this study and previous ones involve species whose placement is phylogenetically uncertain (*incertae cedis*) or superfamilies previously shown to require reclassification (Tineoidea or the Cossioidea-Sesioidea complex for example; Mutanen et al., 2010; Bazinet et al., 2013; Reiger et al., 2013; Heikkil□ et al., 2015 Reiger et al., 2015A; Mitter et al., 2017; Appendix 1). These differences in topology are unlikely to have a significant impact on the main findings of this work, as they primarily nested within large clades entirely lacking eyespots. A summary and discussion of novel species relationships identified in this work can be found in the Supplementary Information (Appendix 1).

Ancestral state reconstructions and evolutionary rates

Our three ancestral state reconstructions (*Both*, *Marginal* and *Discal*) indicate that the common ancestor, at the root of Lepidoptera did not have any eyespots (Fig. 4, Figs. S16 & S17). Eyespots thus evolved multiple times in the history of Lepidoptera (Figs. 4 & 5). Nonetheless, eyespots may often be evolutionarily short lived. Our results show a higher rate of eyespot losses than eyespot gains regardless of eyespot type. The *Both* eyespot model (Fig. 4), *Marginal* eyespot model and *discal*

200 eyespot model all showing a higher rate of eyespot losses than gains (Fig. 4 & Fig. S16 & S17). To
201 explore the sensitivity of these results, we repeated all analyses under a more relaxed definition of
202 eyespots, where we also included non-circular/oblong shapes as eyespots. These results were
203 qualitatively similar to the results in our main analysis and are therefore presented in the
204 supplementary tables and figures section (Fig. S18).

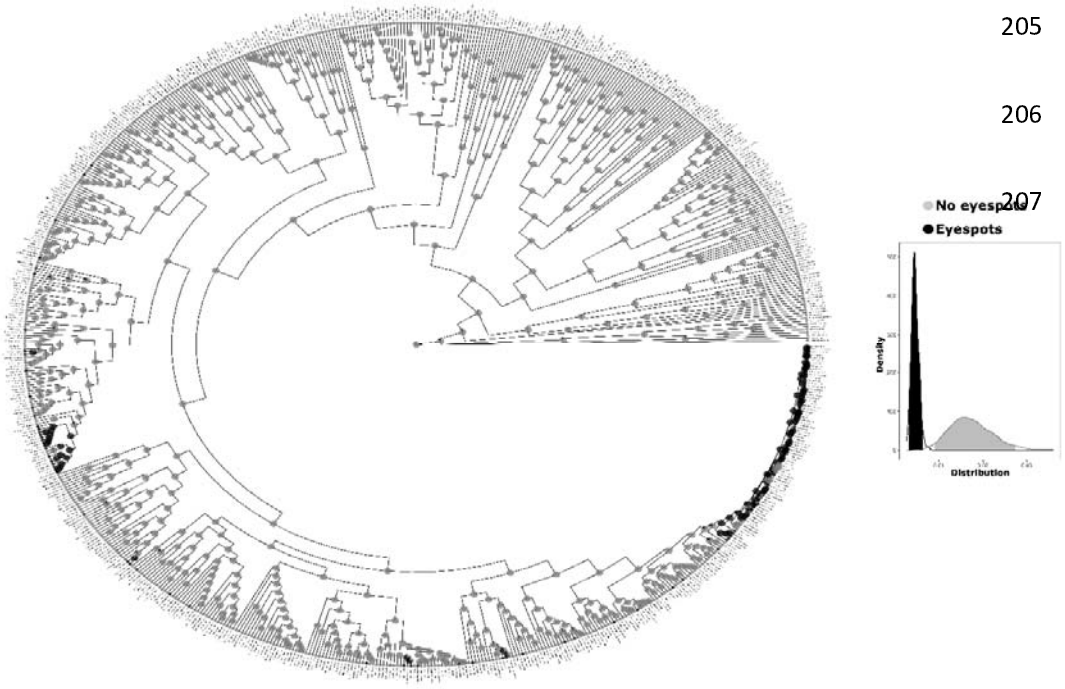


Figure 4. A) Phylogeny of the Lepidoptera with ancestral state reconstructions of what the ancestral state of each node represented by a Pi Chart. B). A smooth plot showing the density and distribution of each phenotype. When grouped together, *both* eyespot types in general appear to be found at high densities but with limited distribution across the phylogeny tree. Indicating eyespot radiations are present but not common throughout all sampled Lepidoptera. We can see from the smooth plot that the rates of gain and loss are not equal. With a higher loss than gain being observed for *both* eyespot types.

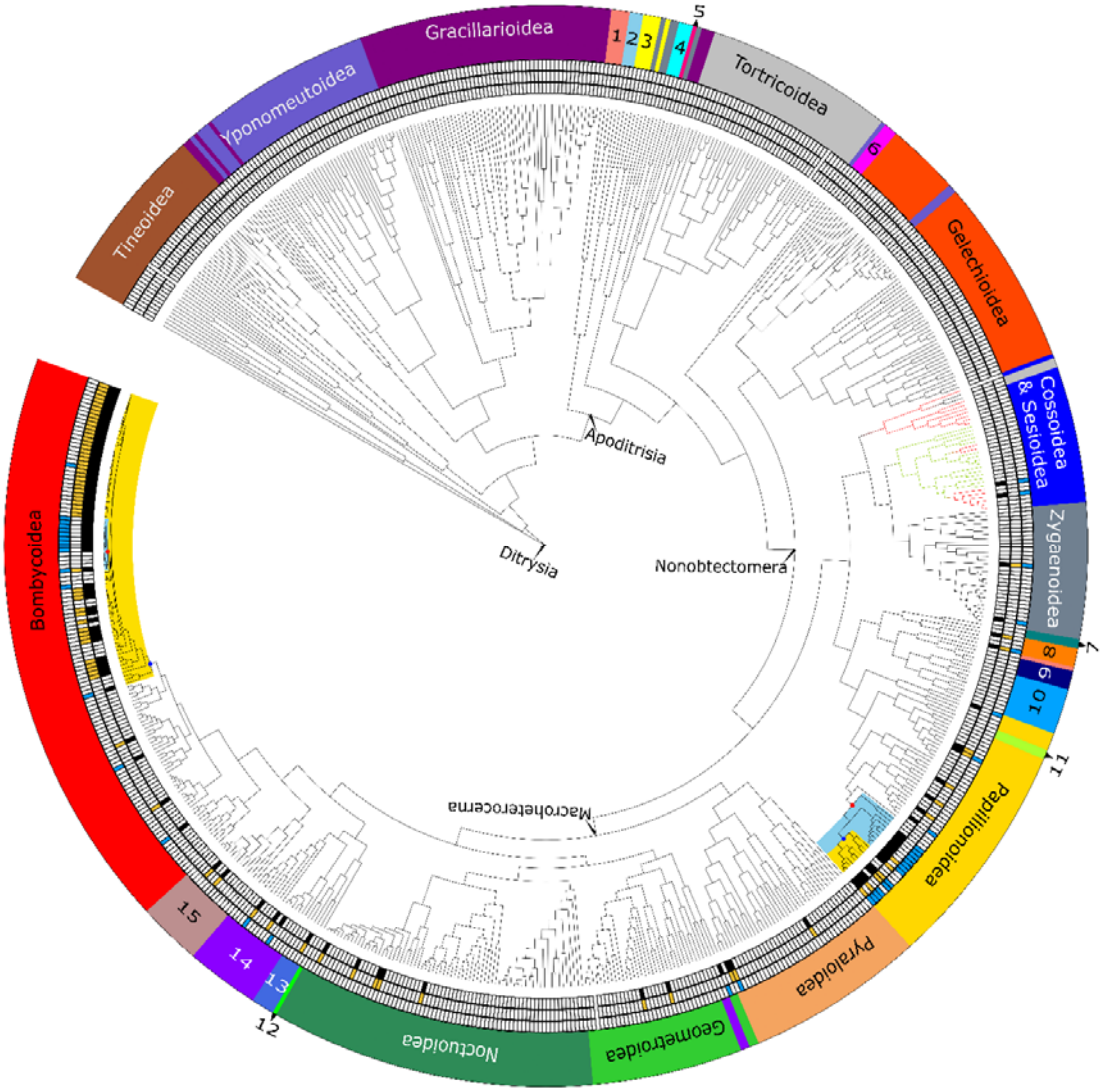
208 Our ancestral state reconstructions are consistent with 28 independent Discal eyespot gains, and 19

209 independent Marginal eyespot gains across 12 superfamilies of Lepidoptera represented in this tree

210 (Figs. 4, 5, S16 & S17). In contrast, we did not find any eyespots in specimens sampled from 16

211 superfamilies: Cimelioidea, Thyridoidea, Copromorphoidea, Hyblaeoidea, Sesioidea, Gelechioidea,

212 Epermenioidea, Tortricoidea, Galacticoidea, Immoidea, Choreutoidea, Urodoidea, Pterophoroidea,



213 Gracillarioidea, Yponomeutoidea and Tineoidea). Most of the eyespots found in the remaining 12

214 superfamilies occur in the single species/genera sampled and are not present in sister lineages on

215 the tree. There are, however, two major independent radiations of both Marginal and Discal

216 eyespots found in the families Nymphalidae and Saturniidae, where both eyespot types were

217 retained in multiple members of these families after they originated (Fig. 5, Figs. S10 & S15).

Figure 5. Phylogeny of the Lepidoptera comprising 715 species from 28 superfamilies. Superfamilies which are too small to be written are numbered as the following 1 = Pterophoroidea .2 = Urodoidea .3 =Choreutoidea 4 = Immoidea 5 = Galacticoidea .6= Epermenoidea .7 = Hyblaeoidea .8 = Calliduloidea .9 = Copomorphoidea .10 = Thyridoidea .11 = Hedyloidea .12 = Cimelioidea .13 = Mimallonoidea .14 = Drepanoidea .15 = Lasiocampoidea. Eyespot radiations are highlighted in blue (marginal) and yellow (discal). The presence/absence of the eyespot for each individual species is marked by a yellow or blue square as well as a black square indicating the presence or absence of the trait. The origin of the eyespot radiation is also placed on the tree as either a red (*marginal*) or dark blue (*discal* dot). SS and PS values for the ancestral state reconstructions are available in Table 1.

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Homology of eyespot radiations

To further investigate the ancestral nodes of independent eyespot origins, as suggested by our ancestral state reconstructions above, we contrasted the fit of a common-ancestor model *vs* a multiple-origin model, for the ancestor of each putative eyespot radiation. Our results using the combined eyespot data (i.e. all eyespots regardless of location), supported our earlier findings of eyespot bearing ancestors in Nymphalidae and Saturniidae (Table 1). By contrasting the results of analyses on the *Marginal* data and the *Discal* data, we inferred which eyespot location likely evolved first in each radiation (Fig. 1e-g). We were surprised to find that the area of the wing where eyespots first appeared was reversed between these two main clades. In nymphalids, the first eyespots appeared along the margin and were followed by *Discal* eyespots (Figs. 5, S10 & S15). The opposite was observed in saturniids, where *Discal* eyespots originated first followed by *Marginal* eyespots (Fig. 5. Figs. S10 & S15). *Discal* eyespots likely originated in the most recent common ancestor of all saturniids, while *Marginal* eyespots evolved within the Attacini tribe of the subfamily Saturniinae. In nymphalids, *Marginal* eyespots first evolved at the base of the sister lineage to the Libytheinae, and Discal eyespots followed in ancestors of Heliconiine and Nymphaline. We found that the Bayes Factor (BF) between our two sapling methods (PS & SS) was largely consistent between runs for the same taxa (Table 1). When the circular criteria for eyespot shape was relaxed (Fig. S18), we found some evidence for a more ancestral origin of Discal eyespots in the Saturniidae. The BF for this more relaxed model is available in the supplementary information (Fig. S18).

Table 1. Log 10 of the raw Bayes factor for each of our eyespot radiations identified in our tree. Values for both PS and SS sampling methods are provided.

Taxa (Family)	*Discal* PS	*Discal* SS	*Marginal* PS	*Marginal* SS	*Both* PS	*Both* SS
Saturniidae	0.146	0.145	0.110	0.104	0.247	0.253
Nymphalidae	0.127	0.126	0.131	0.126	0.150	0.132

To investigate the 27 other Discal and 18 other Marginal eyespot occurrences which were not part of any eyespot radiation (Fig. 5), we investigated the occurrence of eyespots in up to 3 closely related species. These closely related species did not feature on our tree and were limited from the genus to the family level (Fig. S19). We found that many clades with eyespots that were represented by a

243 single species on our tree (Fig. 5) had several closely related species also baring eyespots (Fig. S19).
244 This suggests that these multiple clades represent separate, independent eyespot radiations (Fig. 5 &
245 Fig. S19).

246 **Discussion**

247 Our results show that eyespot evolution in Lepidoptera is diverse and complex. The majority of
248 eyespot research, up to now, has been conducted in a single clade (Nymphalidae), but here we
249 demonstrate that eyespots have evolved multiple times independently across the Lepidoptera,
250 including in many poorly known moth clades (Fig. 4 & Fig. 5). In our phylogeny, these clades are often
251 represented by a single species. However, we documented eyespots in close relatives to each of
252 these lineages (Fig. S19), suggesting eyespots have been preserved across additional radiations.
253 Future phylogenetic studies, with a denser taxonomic sampling, will be required to characterize the
254 evolutionary history of eyespots in these lesser-known clades. Nonetheless, our study strongly
255 suggests that eyespots are not homologous across the entire Lepidoptera.

256 The evolution of Marginal eyespots in the Nymphalidae has been studied before and we report
257 similar findings to these previous studies. Our findings align more closely with the 'early' model of
258 eyespot evolution first proposed by Oliver et al. (2012 & 2014), as opposed to the 'late' model that
259 became the preferred model (Oliver et al. 2014). In Both our analysis, and in the 'early' model,
260 eyespots were coded as being present or absent anywhere on the wing, whereas the 'late' model
261 preferred by Oliver et al., (2014) had increased resolution by scoring the presence of eyespots in
262 specific wing sectors. This late model brought the origin of eyespots to a node above the one
263 discovered here, to the base of the lineage that is sister to the Danainae. Despite minor conflict as to
264 when exactly *Marginal* eyespots first evolved, this and previous studies agree on a single and
265 relatively early origin of Marginalthese eyespots in Nymphalidae.

266 Nymphalid *Discal* eyespots and their evolutionary history have not been investigated before. Here
267 we presented a first estimate of *Discal* eyespot evolution in the nymphalidae, as Oliver et al., (2012 &

2014) did not study these *Discal* eyespots. *Discal* nymphalid eyespots appear to have evolved in two separate clades and are not homologous across all species in the family Nymphalidae. Firstly, in the closely related Nymphalidae subclades Heliconiine (Limenitidinae- *Limenitis arthemis*) and Nymphaline (Apaturinae-*Asterocampa celtis*, Biblidinae-*Hamadryas arinome*, Melitaeini-P*hyciodes phaon* & Nymphalini-*Vanessa carye*), we find that the *Discal* eyespot evolved once and then radiated across several groups, making discal eyespots homologus across the Heliconiine and Nymphalini (Fig. 5 & Fig S10). The second instance of discal eyespot evolution which is not homologus to the other Nymphalidae is *Neope goschkevitschii* (Satryinae, Nymphalidae. Figs. 2 & 5 & Figs. S10 & S19). *Neope sp*. and closely related species within the Satryinae were found to have *Discal* eyespot patterns (Fig. S19), upon our investigation of closely related species not featured on the tree and may represent a unique *Discal* eyespot radiation within the Satyrinae (Fig. S19).

We provided the first examination of the evolution of eyespots (*Discal* and *Marginal*) in the Saturniidae and demonstrate that *Discal* eyespots in the Saturniidae are homologus while *Marginal* eyespots are not. We found that *Discal* eyespots likely originated in the most recent common ancestor of all Saturniids, with *Discal* eyespots being present in 62% of the represented clades within the family. Making discal eyespots homologus across the family Saturniidae. *Marginal* eyespots, on the other hand, have evolved and radiated within the Attacini tribe of the subfamily Saturniinae. The *Marginal* eyespot radiation observed within the Attacini includes species of the genus *Samia, Callosamia, Epiphora & Hyalophora*. We also observed independent origins of *Marginal* eyespots within the Saturniidae in three additional taxonomic groups, meaning that marginal eyespots in the Saturniidae are not homologus. Two of the three representatives of these groups in our phylogeny (*Copaxa multifenestrata*-tribe Saturniinae & subfamily Saturniinae and *Asthenidia transversaria*-subfamily Oxytenninae) were found to have close relatives with Marginal eyespots suggesting individual eyespot radiations within these taxa (Fig. 5 & Figs. S15A, S15C & S19). One species, *Eupackardia calleta* (tribe Attacini, subfamily Saturniinae) is monotypic at the genus level and its closest relatives are other Saturniidae of the tribe Attacini. Despite being in the Attacini, this species

294 is not picked up in our analysis as being part of the *Marginal* eyespot radiation associated with the
295 other Attacini in our study SS Log10 of BF = -0.123, PS Log 10 of BF = -0.130) (Fig. 5, Fig. S15A &
296 S15C). We therefore conclude that the marginal eyespot in this species likely evolved independently
297 to its congeners in the Attacini.

298 Our analyses revealed that eyespots appeared after each other (and in a different order in butterflies
299 and silk-moths). *Marginal* eyespots in Nymphalidae are considered serially homologous (Monteiro et
300 al., 2007 & Hombría 2011) but they appeared in a particular sequence on the wings. They first
301 originated in ventral hindwings, and millions of years later they appeared in forewings and dorsal
302 surfaces (Oliver 2014; Schachat et al 2015). *Discal* eyespots may be yet another instance of a serial
303 homolog, with a more distinct and central location on the wing. By statistically demonstrating that
304 eyespots, as a complex derived trait, can evolve in different locations on the wing in a different
305 sequence, we open the door to more in-depth developmental level studies that investigate how each
306 type of eyespot differentiates on the wing.

307 Although we demonstrate that nymphalid and saturniid eyespots evolved in lineages which are
308 currently understood to be ~110 million years apart (Kawahara et al., 2019) and are not
309 evolutionarily homologous, it is still possible that eyespots in these two superfamilies share the same
310 gene regulatory network (GRN). Previous research by Murugesan et al. (2022) found that an
311 appendage gene regulatory network was co-opted to build *Marginal* eyespots in *Bicyclus anynana*, a
312 nymphalid butterfly. We suggest that the co-option of the same GRN could have happened more
313 than once across the Lepidoptera. To test this, it will be important to characterize the *Marginal* and
314 *Discal* eyespot GRN in moth lineages and/or the *Discal* eyespot GRN in butterfly lineages, at the level
315 of gene expression. Early immunochemistry work in two saturniid species detected the presence of
316 two (nymphalid) eyespot marker proteins, Distal-less and Engrailed, in the moth *Discal* eyespots
317 (Monteiro et al. 2006). Stronger evidence for the use of the same appendage GRN in these Discal
318 moth eyespots may need to come from CRISPR knockouts of cis-regulatory elements belonging to

common eyespot and appendage genes, showing that Both appendages and eyespots are affected (Murugesan et al. 2022).

Finally, while the focus of this work is on eyespot evolution, this phylogeny also provides evidence supporting Both new and already established relationships among Lepidopteran lineages, including at the superfamily level (Appendix 1). These insights can fuel future systematic research and further comparative work on how wing color patterns, and other adaptive traits, evolve in the Lepidoptera.

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