

1 **Eyespots originated multiple times independently across the Lepidoptera**

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6 **Abstract**

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

23 **Introduction**

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

27 focused on the role of localized color patterns, such as eyespots (Blest, 1957). Eyespots consist of
28 more than one concentric ring of contrasting colored scales, often mimicking a vertebrate eye (Oliver
29 et al., 2014; Labandera et al., 2016). These visually striking color patterns play roles in predator
30 deterrence, predator deflection, and mate selection (Breuker & Brakefield 2002; Stevens, 2005;
31 Robertson and Monteiro 2005; Vallin et al. 2007; Kodandaramaiah, 2011; Merilaita et al., 2011;
32 Prudic et al. 2012; Kodandaramaiah et al., 2013; Prudic et al., 2015; Mukherjee & Kodandaramaiah,
33 2015; Huq et al., 2019; Halali et al., 2019). Eyespots are thus adaptive, having evolved in several
34 clades of Lepidoptera, but their evolutionary history remains poorly understood.

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

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

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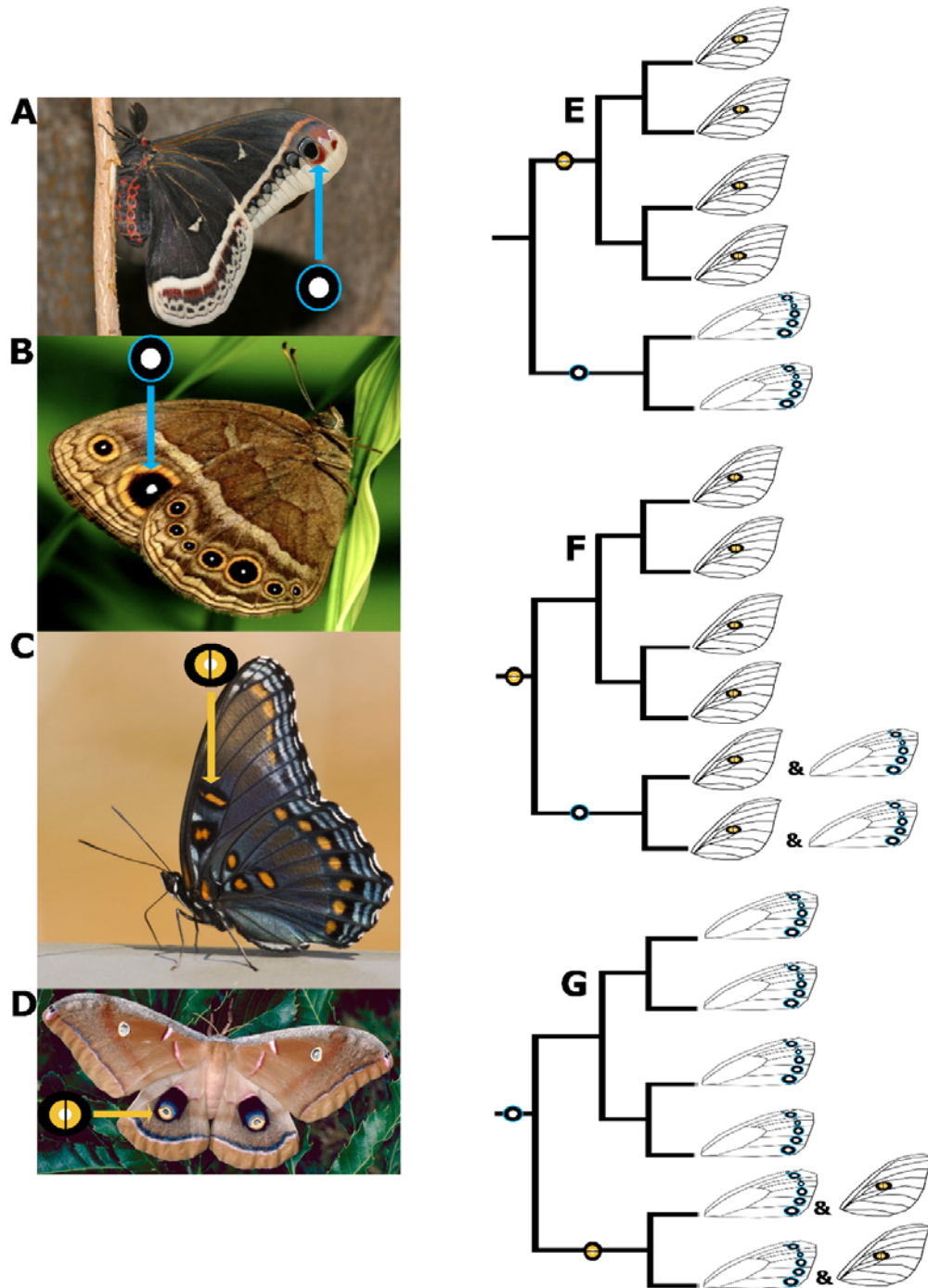


Figure 1 A/B/C/D/E/F/G. The regions of the wing for this study were partitioned into the following two zones, marginal and discal. marginal eyespots are eyespots found between butterfly wing veins, this is demonstrated by A & B as a real-world examples and E-G in our theoretical examples while discal 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.

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

68 **Materials and methods**

69 Molecular data collection for phylogenetic tree construction

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

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

82 Sequence alignment and phylogenetic inference

83 DNA sequences were aligned via MAFFT v 7.4.90 (Katoh & Standley, 2013). A global alignment
84 strategy with iterative refinement (G-INS-i) was employed to maximize alignment accuracy based on
85 weighted sum-of-pairs and a consistency scores. The length of the final alignment, consisting of all 27
86 concatenated gene fragments, was 22,643 bp.

87 IQ-TREE v 1.6.12 (Nguyen et al., 2015, Kalyaanamoorthy et al., 2017 & Hoang et al., 2018) was used
88 for phylogenetic inference under maximum likelihood. All genes were subject to a single substitution
89 process automatically set by IQ-TREE, using ModelFinder (Kalyaanamoorthy et al., 2017).

90 Substitutions followed a generalized time reversible (GTR) model, with estimated base frequencies.
91 Rate variation among sites followed a gamma distribution containing 10 categories. Node support
92 was estimated using ultra-fast bootstrap in UFBoot (Hoang et al., 2018) for 100,000 iterations.

93 UFBoot is an efficient approximation to the traditional bootstrap method that is particularly well-
94 suited for large datasets such as ours. After pruning the outgroup, our maximum likelihood tree was
95 transformed into an ultrametric tree, with branch lengths scaled to time, using the ape package v
96 5.7-1 (Paradis & Schliep, 2019) in R v 4.3.0 (R Core Team 2023). For branch scaling, we used an age of
97 ~290 Ma for the most recent common ancestor of Ditrysia, following the most recent dated
98 phylogeny of Lepidoptera (Kawahara et al., 2019).

99 Image data collection

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

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



119 **Figure 3.** A visual guide demonstrating the difference between eyespots (A), a ring
120 formation (B), spots (C) and an eyespot like pattern (D). Species: *Pyrrhia adela* (A), *Junonia*
121 *almana* (B), *Pieris canidia* (C) and *Attacus atlas* (D).
122

120 We first queried a wide range of online databases for images of the type specimen of each of the
121 sampled species (Table S3, Supplementary data file 1). If not available, images of other (non-type)
122 specimens were collected from the same databases with a preference for museum specimens over
123 other specimens. In cases where sequence data was assigned to a particular subspecies, we
124 prioritized images of the same taxonomic rank when available. Ninety-six species included in our
125 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 Canon 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 *Disca* 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,

150 regardless of their location. If the most recent ancestor of these clades also displayed eyespots, it is
151 likely that eyespots in extant taxa are homologous. To test this hypothesis, we extracted all subclades
152 from the complete ultrametric phylogeny and applied in each case two alternative versions of the
153 discrete-trait model described above. In the first version (hereafter the multiple-origin model), we
154 set the root frequency of eyespot presence to zero, effectively constraining the common ancestor of
155 the subclade to lack eyespots. In the second version (the common-ancestor model), we instead
156 enforced a common ancestor with eyespots by setting the root frequency of eyespot presence to
157 one. We then compared the marginal likelihood (ML) of these alternative models using log 10 of the
158 raw Bayes factor using thresholds based on Jeffreys (1998). In our study, results above positive 0.1
159 were considered to show some support for an eyespot originating at a specific node. Results above
160 0.5 were considered to have strong support, results above 1.0 were considered to have very strong
161 support and results which were positive numbers but below 0.1 were considered to demonstrate
162 very weak support which wasn't worth mentioning. Finally negative results were considered to
163 represent no support for an eyespot origin at a given node. These thresholds for the support of
164 eyespots being present at a particular node were chosen due to the high number of negative values
165 we obtained in our study (values above -1.0) which is indicative of a lack of support for a single origin
166 of eyespots.

167 ML estimates gauge the fit of a model, including its priors, to the data, in this case, the phylogeny
168 and presence or absence of eyespots in extant taxa. Because the two models being compared differ
169 only in their prior assumptions about the character state at the root, their direct comparison serves
170 as a statistical test of the support for eyespot presence in the most recent common ancestor of the
171 clade. ML was estimated via the use of two sampling methods, steppingstone sampling (SS) and path
172 sampling (PS, Lartillot and Philippe, 2006, Fan et al., 2011 & Xie et al., 2011). For both methods the
173 power posterior analysis was split into 50 intervals between the prior and posterior and was run for
174 5,000 iterations with a burn-in of 5,000 generations. We repeated ML approximations for each
175 subclade and eyespot dataset and confirmed that both PS and SS estimates were stable (i.e. differing

176 by no more than 0.5 between independent runs of each set of power posteriors). The results of
177 these analyses were summarized and plotted onto the phylogeny using the R packages phytools v1.9-
178 16 (Revell, 2012), ggplot2 v3.4.3 (Wickham et al., 2016) and ggtree v3.9.0 (Yu et al., 2017 & 2018, Yu,
179 2020 & 2022 & Xu et al., 2022), followed by the online tool ITOL (2023).

180 **Results**

181 Tree topology

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

194 Ancestral state reconstructions and evolutionary rates

195 Our three ancestral state reconstructions (*Both*, *Marginal* and *Discal*) indicate that the common
196 ancestor, at the root of Lepidoptera did not have any eyespots (Fig. 4, Figs. S16 & S17). Eyespots thus
197 evolved multiple times in the history of Lepidoptera (Figs. 4 & 5). Nonetheless, eyespots may often
198 be evolutionarily short lived. Our results show a higher rate of eyespot losses than eyespot gains
199 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).

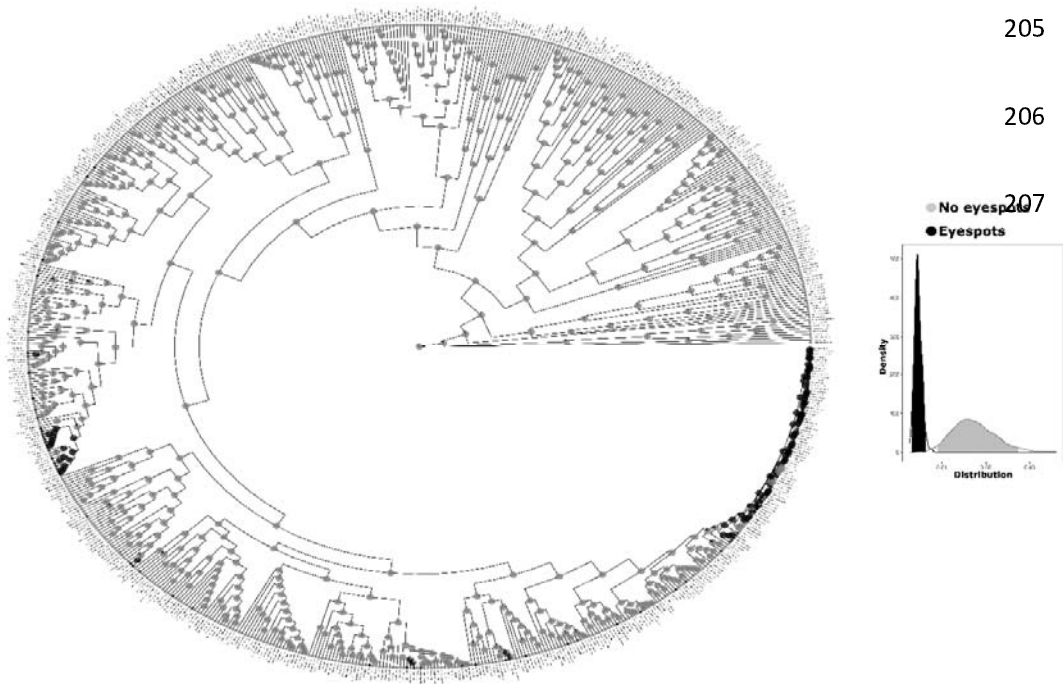
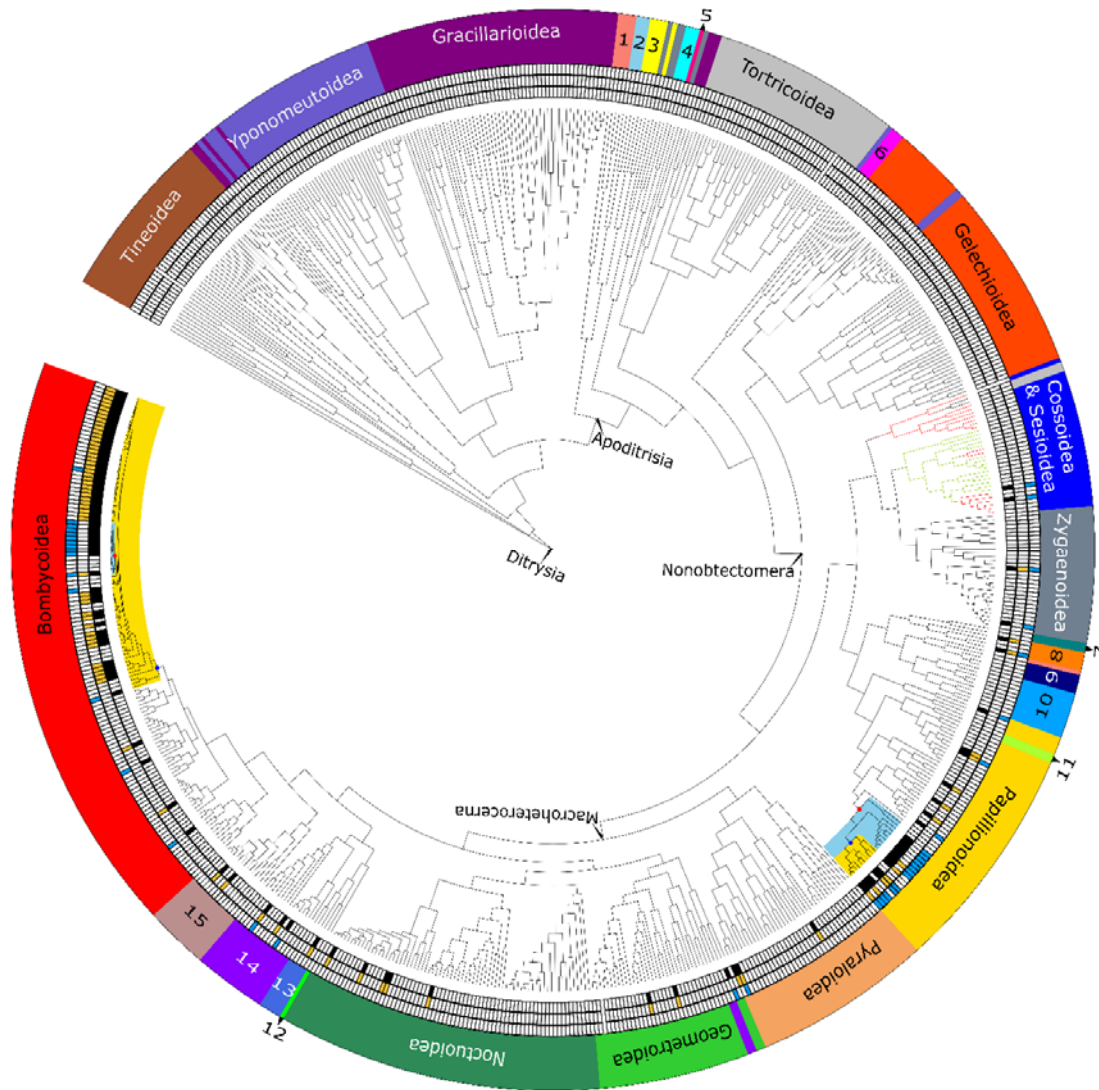


Figure 4. A) Phylogeny of the Lepidoptera with ancestral state reconstructions of what the ancestral state of each node represented by a Pi Chart. B) A smooth plot showing the density and distribution of each phenotype. When grouped together, *both* eyespot types in general appear to be found at high 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, Sesiioidea, Gelechioidea,
 212 Epermenioidea, Tortricoidea, Galacticoidea, Immoidea, Choreutoidea, Urodoidea, Pterophoroidea,



213 **Figure 5.** Phylogeny of the Lepidoptera comprising 715 species from 28 superfamilies. 12
 214 superfamilies (Epermenioidea, Yponomeutoidea and Tineoidea) miss of the eyespot trait and the remaining 12
 215 superfamilies which are too small to be written are numbered as the following 1 = Pterophoroidea, 2 = Urodoidea, 3 = Choreutoidea, 4 = Immoidea, 5 = Galacticoidea, 6 =
 216 Epermenioidea, 7 = Hyblaeoidea, 8 = Calliduloidea, 9 = Copomorpoidea, 10 = Thyridoidea, 11 =
 217 Hedylidae, 12 = Cimelioidea, 13 = Minnalloidea, 14 = Drepanoidea, 15 = Lasiaramphoidea.
 Eyespot radiations are highlighted in blue (marginal) and yellow (discal). The presence/absence
 eyespot presence in 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
 retained in the members of these families they originated (Fig. 5, Figs. S16 & S17). Yes
 for the ancestral state reconstructions are available in Table 1.

218

219

220 Homology of eyespot radiations

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

239 To investigate the 27 other *Discal* and 18 other *Marginal* eyespot occurrences which were not part of
240 any eyespot radiation (Fig. 5), we investigated the occurrence of eyespots in up to 3 closely related
241 species. These closely related species did not feature on our tree and were limited from the genus to
242 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 bearing 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 Marginal these eyespots in Nymphalidae.

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

268 2014) did not study these *Discal* eyespots. *Discal* nymphalid eyespots appear to have evolved in two
269 separate clades and are not homologous across all species in the family Nymphalidae. Firstly, in the
270 closely related Nymphalidae subclades Heliconiine (Limenitidinae- *Limenitis arthemis*) and
271 Nymphaline (Apaturinae-*Asterocampa celtis*, Biblidinae-*Hamadryas arinome*, Melitaeini-*Phyciodes*
272 *phaon* & Nymphalini-*Vanessa carye*), we find that the *Discal* eyespot evolved once and then radiated
273 across several groups, making discal eyespots homologous across the Heliconiine and Nymphalini (Fig.
274 5 & Fig S10). The second instance of discal eyespot evolution which is not homologous to the other
275 Nymphalidae is *Neope goschkevitschii* (Satryinae, Nymphalidae. Figs. 2 & 5 & Figs. S10 & S19). *Neope*
276 *sp.* and closely related species within the Satryinae were found to have *Discal* eyespot patterns (Fig.
277 S19), upon our investigation of closely related species not featured on the tree and may represent a
278 unique *Discal* eyespot radiation within the Satryinae (Fig. S19).

279 We provided the first examination of the evolution of eyespots (*Discal* and *Marginal*) in the
280 Saturniidae and demonstrate that *Discal* eyespots in the Saturniidae are homologous while *Marginal*
281 eyespots are not. We found that *Discal* eyespots likely originated in the most recent common
282 ancestor of all Saturniids, with *Discal* eyespots being present in 62% of the represented clades within
283 the family. Making discal eyespots homologous across the family Saturniidae. *Marginal* eyespots, on
284 the other hand, have evolved and radiated within the Attacini tribe of the subfamily Saturniinae. The
285 *Marginal* eyespot radiation observed within the Attacini includes species of the genus *Samia*,
286 *Callosamia*, *Epiphora* & *Hyalophora*. We also observed independent origins of *Marginal* eyespots
287 within the Saturniidae in three additional taxonomic groups, meaning that marginal eyespots in the
288 Saturniidae are not homologous. Two of the three representatives of these groups in our phylogeny
289 (*Copaxa multifenestrata*-tribe Saturniinae & subfamily Saturniinae and *Asthenidia transversaria*-
290 subfamily Oxyteninae) were found to have close relatives with *Marginal* eyespots suggesting
291 individual eyespot radiations within these taxa (Fig. 5 & Figs. S15A, S15C & S19). One species,
292 *Eupackardia calleta* (tribe Attacini, subfamily Saturniinae) is monotypic at the genus level and its
293 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 Log₁₀ 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

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

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

325 **Acknowledgements:** We thank Dr Mark Willmott and Prof Akito Kawahara (MGCL) for hosting B.H. at
326 the MGCL and facilitating access to the collections; Prof emeritus Charles Mitter (University of
327 Maryland) for graciously sending us molecular sequence data for most specimens reanalyzed here;
328 Monteiro lab members for lively discussion, and Mr David Teo Yan Hua for expertise in PC
329 engineering and technological assistance. B.H was supported by a National Research Foundation
330 (NRF) Graduate Fellowship; B.W. was funded by an International Postdoc Grant from the Swedish
331 Research Council (VR; grant no. 2019-06444); and research was supported by a NRF Investigatorship
332 award (NRF-NRFI05-2019-0006) to A.M.

333 **Conflict of interest:** The authors declare no conflict of interests.

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