Eyespots originated multiple times independently across the Lepidoptera

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Abstract

Eyespot colour patterns function as an effective defence against predators and have evolved numerous times independently across animals. 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 30 separate origins of Discal eyespots (in the discal wing region) and 21 separate origins of Marginal eyespots (in the marginal wing region), a type of serial homologue. In four instances eyespots were preserved in most extant representatives of a subsequent species radiation. In one such instance, in the Nymphalidae, we found a Marginal eyespot common ancestor predating the origin of a Discal eyespot baring ancestor, while in the Saturniidae we observed the opposite. We conclude that eyespots do not appear to be homologous across the Lepidoptera. However, our phylogenetic inference provides a roadmap for future developmental and functional studies that can address whether discal and marginal eyespots share a homologous gene-regulatory network. This study, therefore, has implications for our understanding of the evolution of serial homologues and of convergent evolution of visual signals in insects.

Introduction

Adaptive traits in animals such as protective colour patterns have long been a source of interest and research (Tylor, 1886; Poulton, 1890; Beddard 1895). Stripes, spots and blotches can all function as protective colour patterns (Stevens, 2007; Caro et al., 2016; Mizuno et al., 2024), but the ocellus, or eyespot, which is characterized by the presence of one or more concentric rings of contrasting colours, is among one of the most researched protective patterns. Eyespots are found across a wide variety of vertebrate and invertebrate species (Edmunds, 1976; Meadows, 1993; Loyau et al., 2007; He et al., 2010; Wong & Marek, 2019; Hernández-Palma et al., 2023) and represent a textbook example of evolutionary convergence in response to selection by visual predators (Stevens et al., 2008; Oliver et al., 2012; Cunha et al., 2023).

In Lepidoptera (butterflies and moths), eyespots serve a diversity of roles and develop in a diversity of locations in the body of larvae and/or wings of adults. In both larvae and adults, eyespots are used in predator deterrence and in attack deflection, but in adults they also function in courtship and mate selection (Breuker & Brakefield 2002; Robertson and Monteiro 2005; Vallin et al. 2007; Kodandaramaiah, 2011; Merilaita et al., 2011; Prudic et al. 2012; Kodandaramaiah et al., 2013; Hossie et al., 2014; Prudic et al., 2015; Mukherjee & Kodandaramaiah, 2015; Huq et al., 2019; Halali et al., 2019; Crees et al., 2021).

In adult wings, eyespots can be found on different regions and will be named, hereafter, as Marginal and Discal eyespots. Marginal eyespots develop close to the wing margin in between veins (Fig. 1A-B), whereas Discal eyespots are found more centrally located on the wing, either straddling the cross vein in the discal cell region or within the discal cell (Fig. 1 C-D; Otaki et al., 2020). Lepidopterans can have one or both types of eyespots on their wings. Marginal eyespots are often found in more than one unit on the wing margins and are considered serial homologs, whereas Discal eyespots are often found as single units. It is unclear whether Discal eyespots are serial homologs to Marginal eyespots, i.e., it is unclear whether these two eyespot types are using the same gene-regulatory network (Monteiro et al. 2006).

The evolutionary history of Discal and Marginal eyespots across the Lepidoptera is also still largely unknown, as most research on eyespot evolution has focused on Marginal eyespots of brush-footed butterflies. This clade of butterflies (Nymphalidae), which contains ~6,400 described species, accounts for only ~3.3% of lepidopteran diversity (~180,000 total described Lepidoptera; Brakefield, 2003; Heikkilä et al., 2015; Monteiro, 2015; Bhardwaj et al., 2020; Chazot et al., 2021). Phylogenetic comparative methods estimated that Marginal eyespots evolved once in the Nymphalidae, shortly after the origin of the clade (Oliver et al., 2012; Oliver et al., 2014). Thus, all Marginal eyespots in extant nymphalids are thought to be homologous. Nonetheless, Marginal eyespots are found in taxa across the entire order (e.g., Semanturidae, Saturniidae, Cambridae), and thus it is likely that these colour patterns have evolved independently in multiple lineages of this diverse insect clade.

Discal eyespots have also not yet been studied in a phylogenetic context but are widespread in lepidopterans. Because the two wing regions express different genes during development (Banerjee et al., 2023), Marginal and Discal eyespots might have evolved largely independently of each other. If so, we should expect the two eyespot forms to occur primarily in different lineages (Fig. 1E). Alternatively, eyespots might have first evolved on one wing region, and later gained a novel domain

72 of expression on the other region, with the order of these regions potentially varying (Fig. 1F-G).
73 Here, we aim to investigate the evolutionary history of lepidopteran eyespots beyond Nymphalidae,
74 to understand the diversity of paths that resulted in the morphological convergence of this complex
75 colour pattern.

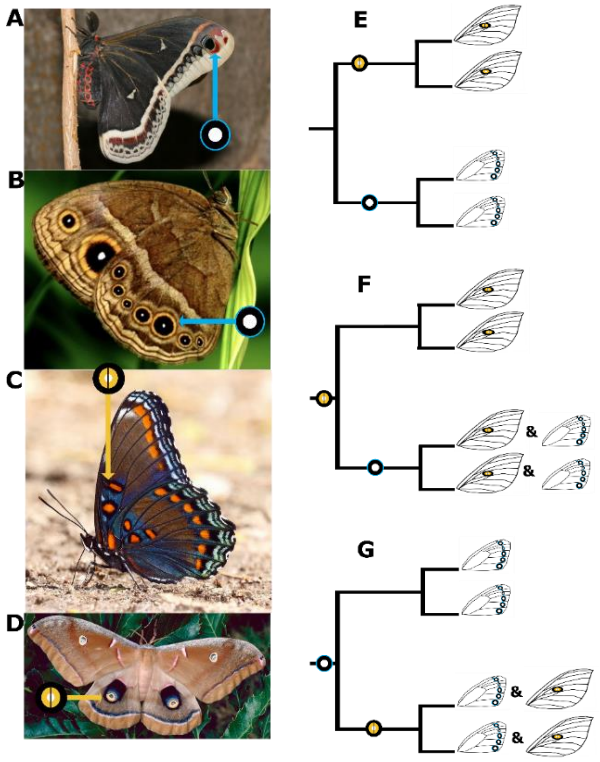

Figure 1. Lepidopteran eyespots have evolved in different wing regions: **A-B** the *Marginal* region, between veins and **C-D** the *Discal* region, straddling the cross vein in the discal cell or found within the cell. **E** These two eyespot types may have evolved independently of each other, and thus be primarily found in different clades. Alternatively, eyespots may first evolve in one region and then acquire a novel expression domain in a different wing region. In the latter scenario, **F** *Discal* eyespots may evolve first followed by *Marginal* eyespots, or **G** Marginal eyespots may enable the subsequent evolution of *Discal* eyespots. Photographs: **A** = *Eupakardia calleta* (Nicky Davis, WildUtah.US.), **B** = *Bicyclus anynana* (Antonia Monteiro, National University of Singapore), **C** = *Limenitis arthemis* (Rawpixel, Rawpixel.com), **D** = *Antheraea polyphemus* (Lacy L. Hyche, Auburn University, Bugwood.org).

76
77 We used modern phylogenetic methods to investigate the evolutionary origins of Discal and
78 Marginal eyespot across Lepidoptera. We first inferred a species-level phylogeny for Lepidoptera,
79 sampling 5 to 27 molecular sequences per species across 715 taxa. Our phylogeny covers 65% of all
80 superfamilies in the order Lepidoptera, allowing comparative inferences of eyespot evolution in
81 moths and butterflies. We then modelled evolutionary origins and losses of eyespots and inferred
82 ancestral states across the phylogeny. Additionally, for each subtree including multiple (two or
83 more) eyespot-bearing taxa, we implemented a model comparison approach, based on marginal
84 likelihood estimation, to quantify support for an ancestral origin of eyespots in the most recent
85 common ancestor of the subtree. If an ancestral origin of eyespots was supported, we tentatively
86 concluded that eyespots in all extant taxa of the subtree are likely homologous. These putative

ancestral eyespot origins were then compared with the ancestral state estimates above, to evaluate the robustness of our results to tree-wide *versus* subtree approaches.

Our results support the hypothesis that eyespots in Lepidoptera have evolved multiple times independently. Our data also supports the hypothesis that Discal and Marginal eyespots have evolved in different temporal sequences in the two main clades where they radiated (Nymphalidae and Saturniidae). We find that both Marginal and Discal eyespots have evolved in a further 10 superfamilies and numerous families across the Lepidopteran tree. Finally, we find that eyespots are more commonly observed in the Discal wing region (30 independent origins) than the Marginal region (21 independent origins). Together, our results suggest that eyespot evolution is commonplace in Lepidoptera. Our study provides a phylogenetic roadmap for the future sampling of independent lineages to test whether all these eyespots share a common gene-regulatory network.

Results

Tree topology

While large-scale multi-superfamily phylogenetic trees of Lepidoptera have been inferred in several recent studies (Heikkilä et al., 2015; Mitter et al., 2017; Kawahara et al., 2019), these studies focused on resolving deep ancestral nodes within Lepidoptera and sampled taxa accordingly. We aimed, instead, to maximize taxonomic coverage across the entire clade to enable large-scale comparative analyses of eyespot evolution, so started by reconstructing a new phylogeny of Lepidoptera. We focused on Ditrysia, a natural clade that includes 99% of extant species (Heikkilä et al., 2015) and omitted more basal branching taxa due to inexistent images. We sampled 5 to 27 gene fragments per species across 715 taxa and conducted maximum likelihood phylogenetic inference using a generalized time reversible (GTR) substitution model with gamma-distributed site heterogeneity upon the concatenated alignment. Omission or inclusion of taxa varied across different studies. As our goal was to include as many superfamilies as possible, our study kept all taxonomically identified species (Fig. 2), including those which are currently considered to be *incertae sedis* (Uncertain taxonomic placement) on the lepidopteran tree of life (Heikkilä et al., 2015; Mitter et al., 2017; Kawahara et al., 2019). Our inferred tree topology is largely congruent with previous phylogenies (Heikkilä 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 (i.e. Tineoidea or the Cossioidea-Sesioidea complex among others; Mutanen

et al., 2010; Bazinet et al., 2013; Reiger et al., 2013; Heikkilä et al., 2015 Reiger et al., 2015A; Mitter et al., 2017; Supplementary information 1). These differences in topology are unlikely to have a

Figure 2. All eyespot occurrences are visually represented in this figure. Species marked with a * are representatives for their specific (Saturniidae or Nymphalidae) taxonomic groups, sharing an eyespot baring common ancestor(s). Colours in the Key represent the corresponding super families in Fig. 3.

significant impact on the main findings of this work, as they are primarily nested within large clades entirely lacking eyespots. A summary and discussion of novel taxonomic relationships identified in this work can be found in Supplementary Information (Supplementary information).

Ancestral state reconstructions and evolutionary rates

To investigate the historical evolution of eyespots across Dytrisia, we obtained photographs of as many species as possible across the phylogeny (only 4 species had missing images) and scored presence or absence of Discal and/or Marginal eyespots anywhere on the wings of either sex (Fig. 3). We found that 41 species have Marginal eyespots, 81 species have Discal eyespots, and 12 species have both eyespot pattern type on their wings across our data set. Eyespots are absent outside the Nonobectometra (Tineoidea - Epermenoidea) and are also not observed in the Nonobectrometran Gelechioidea (Fig. 3).

Then, we modelled eyespot evolution as a discrete trait with two alternative states, presence or absence, regardless of eyespot location on the wings, using RevBayes (v1.21, Höhna et al., 2019). We monitored joint conditional ancestral states in two independent MCMC simulations and used stochastic character mapping (Frayman and Höhna, 2019 in Revbayes v1.2.1) to estimate the number of eyespot gains and losses across the Ditrysia tree. We then repeated these analyses for Marginal and Discal eyespots separately, to determine if these distinct eyespot types have radiated in different clades and in different temporal sequences.

Our three ancestral state reconstructions (for the two eyespot types combined, for Marginal, and for Discal eyespots) indicated that both the most recent common ancestor (MRCA) of all Ditrysia as well as the MRCA of all Nonobectrometra (including and excluding the Gelenchoidea) did not have eyespots (Figs. 4, S1, S2). Eyespots, regardless of where they occur, most likely evolved multiple times independently. We see this through a high number of potential eyespot origins across the Lepidoptera (Table 1). Eyespots also appear to be evolutionarily short lived, with a higher rate of losses than origins, regardless of eyespot type (Figs. 4, S1, S2).

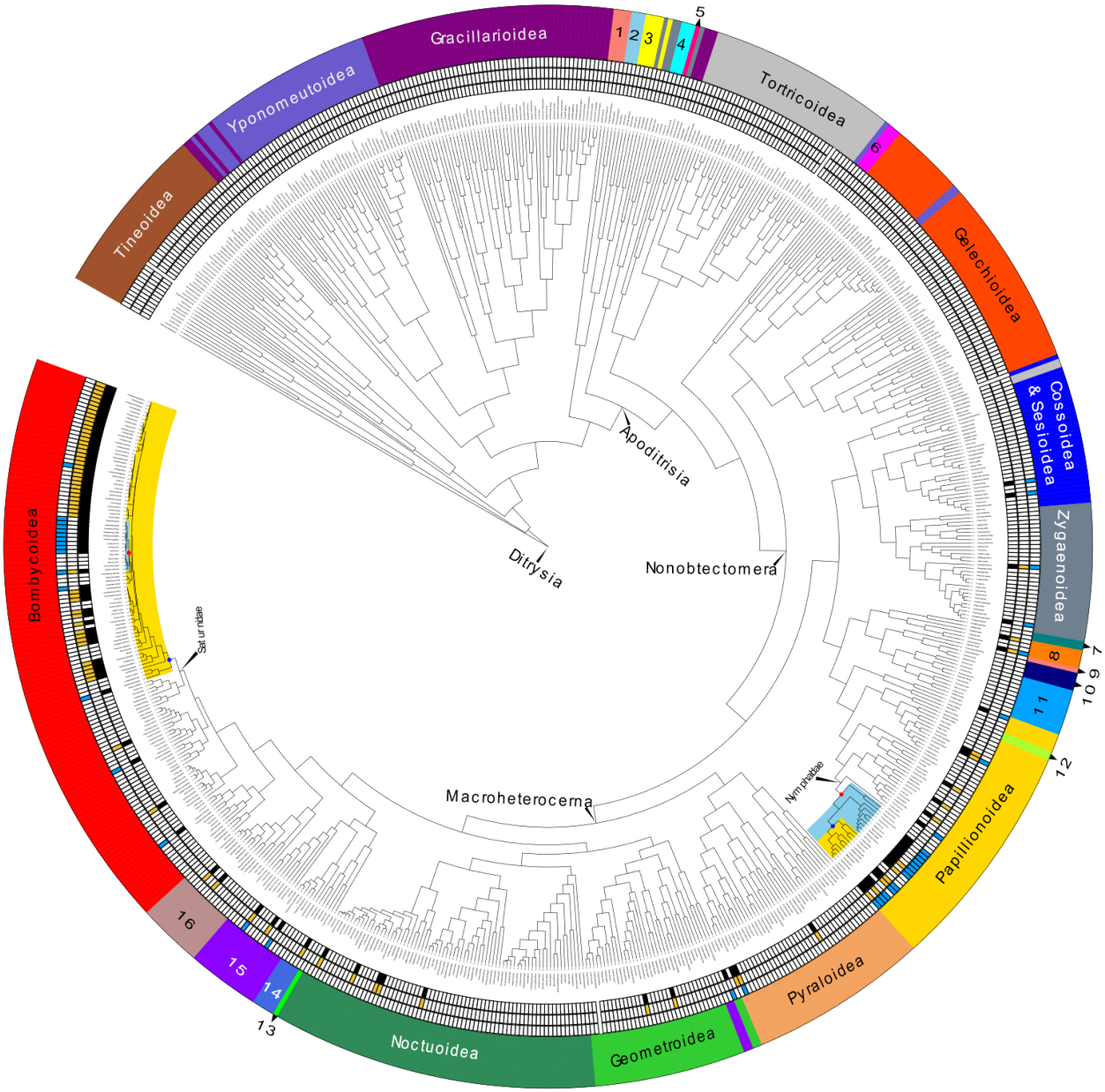

Figure 3. Phylogeny of the Lepidoptera compromising 715 species from 28 superfamilies. Superfamily names which are too small to be written on the figure are numbered as follows: 1 = Pterophoridea .2 = Uroidea .3 =Choreutoidea 4. = Immoidea 5. = Galacticoidea .6= Epermenoidea .7 = Hyblaeoidea .8 = Carposinididae .9 = Calliduloidea .10 = Copomorphoidea .11 = Thyridoidea .12 = Hedyloidea .13 = Cimelioidea .14 = Minmalloidea .15 = Drepanoidea .16 = Lasiocampoidea. Species with an eyespot baring common ancestor are highlighted in blue (Marginal) and yellow (Discal). The presence/absence of eyespots of each type for each species is marked at the tips of the tree by a yellow or blue rectangle, as well as a black square indicating the presence or absence of either eyespot type. The origins of each eyespot baring common ancestor, based on a marginal likelihood model comparison approach are shown on ancestral nodes as either red dots (Marginal eyespots) or dark blue dots (Discal eyespots).

Eyespot-bearing species are distributed across 11 superfamilies, but in most cases, eyespots occur in only one genus or a few genera, within a superfamily. Because taxonomic sampling remains sparse (22.60% of genera on average across all superfamilies in this study), it is currently difficult to discern how phylogenetically widespread eyespots are in most of these superfamilies. There are, however,

two major independent occurrences of Marginal and Discal eyespots sharing a single common ancestor for multiple species in the families Nymphalidae and Saturniidae, where both eyespot types were retained in multiple lineages after their origin (Figs. 3, 4, S1, S2).

Table 1. Number of independent eyespot origins based on stochastic character maps from discrete-trait models of the evolution of any eyespot type (irrespective of location), Discal eyespots, and Marginal eyespots. For each model we report the median number of origins across the posterior, and the 95% highest posterior density (HPD) interval.

	U95% HPD interval
Discal	[26-35] (Median: 30)
Marginal	[20-23] (Median 21)
Any eyespot type	[33-43] (Median 38)

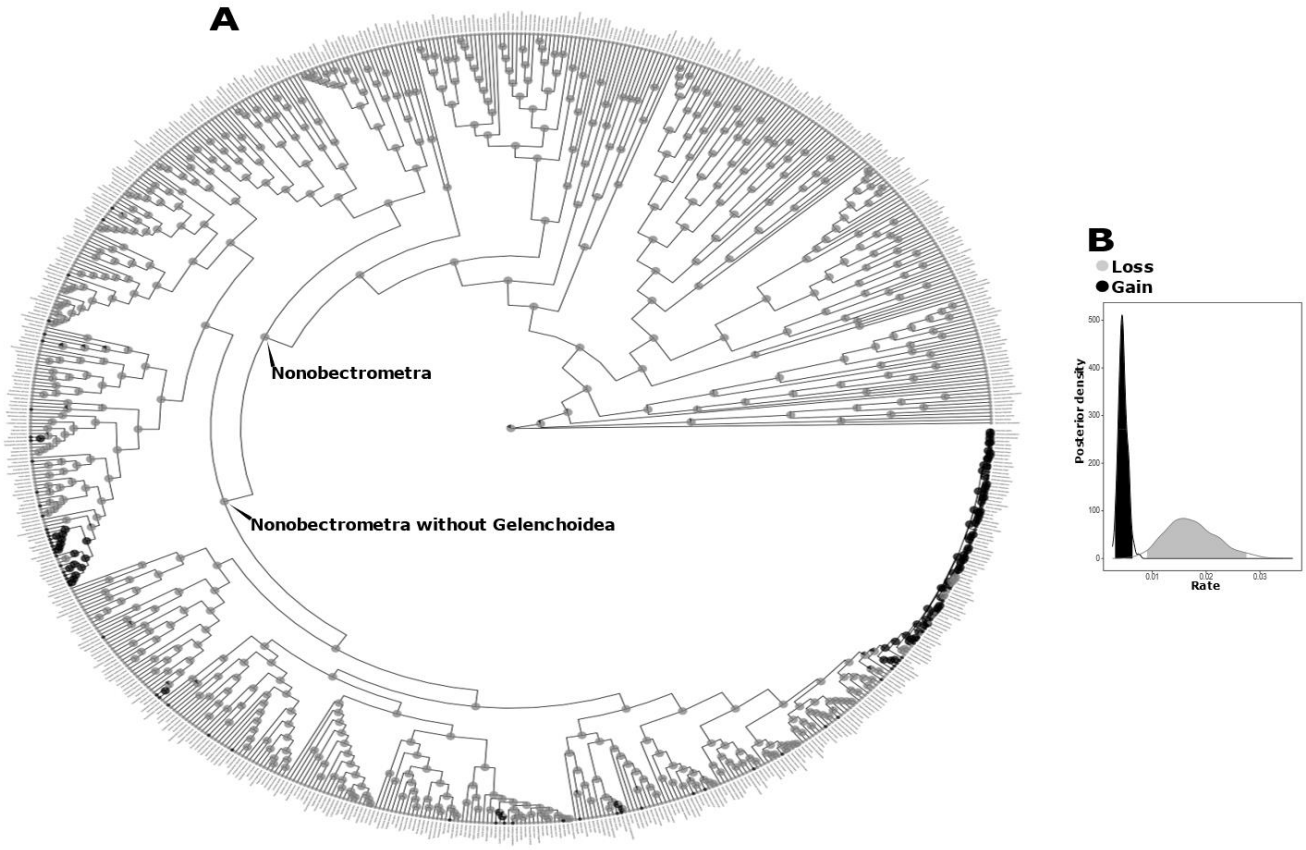

Figure 4. Eyespot ancestral state reconstruction and transition rates in Lepidoptera for Marginal and Discal eyespots combined. We modelled eyespot evolution as a discrete trait with two alternative states (presence or absence) regardless of marginal or discal location. **A)** Phylogeny of the Lepidoptera with ancestral state reconstructions. Pies indicate the posterior probability of eyespot presence for each internal node. The posterior probability of eyespot presence at the root of Lepidoptera was 0.191. The posterior probability of an eyespot at the base of the Nonobectometra was 0 and finally the posterior probability of an eyespot within the nonobectometra excluding the Gelenchoidea but including all present-day eyespot species is <0.0001. **B)** Posterior distribution of rate estimates for eyespot gain and eyespot loss across Lepidoptera. Higher rate values (i.e. a more rightward distribution) indicate a shorter waiting time for an evolutionary transition (eyespot gain or loss). For any given rate value, a higher posterior density indicates that the rate has high probability, given the model and data. Separate analyses for Marginal and Discal eyespots are shown in the SM (Figs. S1, S2).

Homology of eyespots with a single common ancestor – common ancestor model vs the multiple origins model

To validate ancestral state reconstructions pointing to independent eyespot origins, we conducted a separate test of eyespot homology for each subclade of our Ditrysia tree including at least two eyespot-bearing taxa (Fig. S3). These tests contrasted the hypothesis of a single ancestral origin of eyespots in the most recent common ancestor of the subclade (hereafter the 'common ancestor' model) against the hypothesis of two or more independent eyespot origins within the subclade (hereafter the 'multiple origins' model). Support for the 'common ancestor' model would be suggestive of eyespot homology across all extant species in the subclade, especially if congruent with the results of tree-wide ancestral state reconstructions (Fig. 4), which allowed for eyespot loss and regain.

The 'common ancestor' and 'multiple origins' models were parametrized as our eyespot evolution models used for ancestral state reconstructions above (see also Methods), except for one key difference. We specified alternative priors on the root state frequencies to enforce certainty of an eyespot presence at the root of each subclade in the 'common ancestor' model, and to enforce certainty of a common ancestor without eyespots in the 'multiple origins' model. For each subclade, we then estimated the marginal likelihoods of these two models and compared them using Bayes factors. Marginal likelihood estimates indicate the likelihood of a model, including all parameters and priors, given the data. By comparing alternative models that differ only by their prior beliefs on the character state at the root (presence or absence of eyespots), we thus tested if the assumption of eyespot homology across extant taxa is more likely, given the phylogeny and character data, than the assumption of independent eyespot origins.

We found that Discal eyespots are homologous within the Saturniidae and Marginal eyespots are homologous within the Nymphalidae, according to our SS (Stepping Stone) and PS (Path Sampling) values (Table 2). The model comparison approach based on all eyespots, Marginal and Discal, revealed two internal nodes with positive albeit weak support for the 'common ancestor' model (Fig. 3, Table 2). These nodes are positioned close to the base of the MRCA of the Saturniidae and Nymphalidae families with only the most basal groups excluded in both cases. This result is consistent with our tree-wide ancestral state reconstructions (Fig. 4). When the circular criteria for eyespot shape was changed (made more inclusive) to include non-circular shapes (Fig. S4), we found weak but positive evidence for a more ancestral origin of Discal eyespots in moths, in the common ancestor of Saturniidae and with the inclusion of some Bombycidae, which traditionally are

200 considered monophyletic but are paraphyletic in our analysis (BF = 0.144, Fig. S4). We found no
201 change in the results for Marginal eyespots when making this circular criterion more inclusive, as we
202 found no new marginal eyespot occurrences.
203

Table 2. Log 10 of the raw Bayes factors (log_{10} BF) of comparisons between the 'common ancestor' and the 'multiple-origins' models of eyespot evolution. Values can vary from -1 to +1. For each subclade with two or more eyespot-bearing taxa, we estimated the marginal likelihood of each of these alternative models and compared them via log_{10} BF. We consider log_{10} BF > 0.1 as support for the hypothesis of eyespot homology across a subclade. Here, we show subclades with weak but positive evidence for the 'common ancestor' model (log_{10} BF between 0.1 – 0.5), and Bayes Factors based on marginal likelihood estimates using the stepping stone algorithm. All other subclades examined, either had support for the 'multiple origins' model (log_{10} BF < -0.1), or showed no positive evidence for either model (log_{10} BF between -0.1 – 0.1). Results based on marginal likelihood approximation via the path sampling algorithm are qualitatively similar (Table S4). The specific nodes where the 'common ancestor' model was supported for Marginal or Discal eyespots are highlighted in Figs. S1, S2, respectively.

Clade (*subfamily where applicable)	Eyespot type	log_{10} BF for common ancestor
Saturniidae	All	0.247
Nymphalidae	All	0.150
Saturniidae	Discal	0.146
Nymphalidae (Nymphalini*)	Discal	0.126
Saturniidae (Attacini*)	Marginal	0.110
Nymphalidae	Marginal	0.131

204
205 Marginal eyespots, however, evolved more than once within the Saturniidae and are not
206 homologous. Separate analyses for Discal and Marginal eyespots suggested that the MRCA of the
207 Saturniidae first evolved Discal eyespots, followed by a later origin of Marginal eyespots in multiple
208 species in the Attacini tribe of the subfamily Saturniinae (Fig. 3, Table 2), in some species of the tribe
209 Saturniini (subfamily Saturniinae), and in the subfamily Oxytenninae (Fig. 3, S6, S7). Our analyses,
210 however, did not support Marginal eyespot homology across these three groups, (all common
211 ancestors of Attacini and Saturniinae, and Saturniinae and Oxytenninae have BF = -0.94592)
212 suggesting that Marginal eyespots evolved independently within the Saturniidae.
213
214 In the Nymphalidae, Marginal eyespots are homologous, whereas Discal eyespots appear to have
215 evolved two times independently (Fig. 3, Fig. S5A, Table 2). Discal eyespots evolved in the ancestors
216 of Heliconiine and Nymphaline whose Discal eyespots are likely homologous (Table 2), and in the

217 subfamily Satyrinae (Fig. S5A). The data remained unchanged when we extended Discal and
218 Marginal eyespot shape criterions to allow for non-circular eyespots. Together, our ancestral state
219 reconstructs, and model-comparison approach point at two taxonomic groups containing eyespot-
220 bearing lineages with contrasting evolutionary histories: the Saturniidae, where Discal eyespots
221 evolved first and were followed by a few origins of Marginal eyespots, and the Nymphalidae
222 butterflies, where Marginal eyespots evolved first and were followed by two independent origins of
223 Discal eyespots (Fig. 3, S1, S2).
224
225 Correlated evolution of Marginal and Discal eyespots
226
227 Our finding of two subclades, Nymphalidae and Saturniidae, with both Marginal and Discal eyespots
228 arising in different temporal orders (Fig. 3) led to the hypothesis that eyespots may initially evolve
229 on a single wing domain (Marginal or Discal), but once the genetic machinery for eyespot
230 development becomes established, eyespot evolution on a different wing domain is facilitated.
231 Critically, this novel-domain hypothesis is compatible with either wing region hosting the most
232 ancestral eyespot, but it requires evidence that the ancestral origin facilitated eyespot evolution in
233 novel domains, as would be expected if the eyespot gene-regulatory network is redeployed in a
234 different group of cells as opposed to evolving anew.
235
236 We addressed this hypothesis using a correlated evolution model, wherein the rate of eyespot
237 evolution on each wing region depends on whether eyespots are already present on the alternative
238 region. To test for correlated evolution of eyespots between wing regions (Marginal and Discal), we
239 modelled a reversible jump mixture distribution, sampling the posterior probabilities of correlated vs
240 independent eyespot evolution, and assuming equal prior probabilities for both scenarios. If
241 correlated evolution is supported, origin of novel Marginal and Discal eyespots should be
242 accelerated when eyespots already exist in the alternative wing domain, whereas no such
243 acceleration should be present if eyespot gains are independent. We tested this hypothesis in the
244 Papilionoidea (containing Nymphalidae) and Bombycoidea (containing Saturniidae), as both clades
245 encompass multiple putative origins of both Discal and Marginal eyespots (Figs S1 & S2), and
246 therefore enable estimation of evolutionary rates on each eyespot domain.
247
248 Our results demonstrated that Discal and Marginal eyespots are highly unlikely to be evolutionarily
249 correlated (Figs. S8). Specifically, we see a posterior probability of 36.3% for papilionid discal
250 eyespots and a posterior probability of 36.4% for papilionoid marginal eyespots to evolve in
251 correlation with each other. In Bombycoidea, we observed a posterior probability of 46.38% for

252 discal and 56.14% for marginal eyespots to evolve in correlation with each other (Fig. S8C). Both
253 probabilities are too low (<95%) to ascertain with certainty whether these eyespot domains evolved
254 independently or not. These results indicate that with the present data, the reversible jump model is
255 ambiguous between the two evolutionary scenarios. Consistently, the rate of Discal eyespot origins
256 in Papilionoidea is similar for lineages with and without pre-existing Marginal eyespots, and the rate
257 of Marginal eyespot origins in Bombycoidea is similar for lineages with and without pre-existing
258 Discal eyespots (Fig. S8BC). While we found no unequivocal support for correlated evolution
259 between Discal and Marginal eyespots in either Papilionoidea and Bombycoidea, modelling the joint
260 evolution of both eyespot types resulted in inferred ancestral origins that were largely consistent
261 with our previous model comparison approach and previous tree-wide ancestral state
262 reconstructions of individual eyespot domains (Figs. 3, S9.
263
264 Other smaller eyespot observations
265
266 Our stochastic character mapping analyses suggested that both Discal and Marginal eyespots have
267 evolved multiple times in Dytrisia (Table 1), with only a few of these origins occurring in
268 Nymphalidae and Saturniidae. The remaining origins of Discal and Marginal eyespots, occurred in
269 sparsely sampled families (superfamilies), namely : Brahmaeidae (Bombycoidea), Sphingidae
270 (Bombycoidea), Carthaeidae (Bombycoidea), Cossidae (Cossioidea-Sesioidea), Castniidae
271 (Cossioidea-Sesioidea), Drepanidae (Drepanoidea), Geometridae (Geometroidea), Sematuridae
272 (Geometroidea), Hesperiidae (Papilionoidea), Lycaenidae (Papilionoidea), Papilionidae
273 (Papilionoidea), Pieridae (Papilionoidea), Riodinidae (Papilionoidea), Lasiocampidae
274 (Lasiocampoidea), Mimallonidae (Mimallonoidea), Erebidae (Noctuoidea), Noctuidae (Noctuoidea),
275 Crambidae (Pyraloidea) and Limacodidae (Zygaenoidea, Figs 2, 3, S4, Table S5). These families
276 represent potential subjects for future comparative studies if phylogenies are or become available,
277 and eyespots are found in more than a handful of species.
278
279 To obtain a preliminary picture of whether eyespots are widespread within these clades, we
280 searched image databases for close relatives of eyespot-bearing taxa within these families and
281 recorded presence or absence of eyespots not included in our Ditrysia phylogeny. We looked at
282 every image available of species within these families and found that 59% of the species with
283 eyespots represented in Fig. 3 have one or more congeners (species with which they share a genus)
284 also with eyespots. Of the remaining species that did not have congeners with eyespots, 32% were
285 part of a subfamily, and 3% part of a family, with eyespot-bearing species, and 6% of species (three
286 taxa) did not have any congener, subfamily, or family member with eyespots. This could be due to

lack of image data (*Pterodecta felderi* and *Helicomitra pulchra*) or due to these clades being monotypic to the family level (*Carthaea saturnioides*). A visual summary of the species found on our tree with eyespots and up to 3 close relatives also supporting eyespots is provided in Fig. S10 and Table S5.

Discussion

Our results show that eyespot evolution in Lepidoptera is diverse and complex. Most eyespot research up to now has been conducted in a single clade (Nymphalidae), but here we demonstrate that eyespots have evolved multiple times independently across the Lepidoptera, including in many poorly known moth clades (Figs. 2, 3). Our stochastic character mapping analysis demonstrated that the median number of discal eyespot origins on our tree is 30 and the median number of marginal eyespots is 21 (Table 1). Most of the clades with eyespots on our phylogeny have low taxonomic coverage, but we documented eyespots in close relatives of each of these lineages (Fig. S7), suggesting a less “tippy” distribution than that documented in Fig. 3. Future phylogenetic studies, with a denser taxonomic sampling, will be required to characterize the evolutionary history of eyespots in these lesser-known clades. Nonetheless, our study strongly suggests that eyespots are not homologous across the order Lepidoptera, i.e., the MRCA of all Lepidoptera with eyespots did not have eyespots. We find that this result is robust to alternative analytical approaches as both ancestral state reconstructions (from tree-wide eyespot evolution models), and comparisons of different root-state assumptions within subclades, point at independent eyespot origins in the two main clades where these traits are known to be widely distributed, the Brush-footed butterflies (Nymphalidae) and the saturniid moths.

Here we provided the first evidence that Discal eyespots are homologous in the Saturniidae but not so in the Nymphalidae. Discal eyespots, present in 62% of the represented clades within the family Saturniidae in this study, are likely to have originated at or near the base of the family (Figs. 3, 4, S6, S9B). This result stands despite the large diversity of eyespot complexity, colour and size observed across the Saturniidae (D’Abrera, 1995;1998;2012, Figs. S10). All three analyses (common ancestor vs multiple origins, ancestral state reconstruction and correlated evolution), support a single Discal eyespot common ancestor. The evolutionary history of nymphalid Discal eyespots had not been investigated before and our analyses point to two separate origins within this clade.

Marginal eyespots are homologous within the Nymphalidae but not so in the Saturniidae. The evolution of Marginal eyespots in the Nymphalidae has been studied before and we report similar findings to these previous studies. We report a single origin for these eyespots that aligns with both the 'early' model of eyespot evolution first proposed by Oliver et al. (2012), and the 'late' model proposed later by Oliver et al. (2014). These two models merely differed in pinpointing the clade where eyespots first originated, due to a different way of scoring eyespots on the wing. In the 'early' model, eyespots were scored as being present or absent anywhere on the wing, whereas in the 'late' model eyespots were scored for specific wing sectors. In our analyses, both results were observed. The early model was supported by our common ancestor vs multiple origins model comparisons (Fig. 2, Table 2), while the late model was supported by our ancestral state reconstructions (Figs. 3, S2, S3). The current study, as well as these previous studies, therefore, agree on a single and relatively early origin of Marginal eyespots within Nymphalidae. Marginal eyespots in the Saturniidae, however, are not homologous, as we observed four independent origins within this monophyletic clade. Marginal eyespots evolved and radiated within the Attacini tribe of the subfamily Saturniinae which includes species of the genus *Samia, Callosamia* and *Hyalophora,* as well as *Epiphora*. Marginal eyespots evolved separately in species from the genus *Copaxa, Eupakardia* (monotypic) and *Asthenidia*.

Other than what is seen in the Families Nymphalidae and Saturniidae we observe a further 28 Discal and 19 Marginal eyespot occurrences close to the tips of our tree. Some of these groups are taxonomically unique e.g., the bombycoid moth *Cathaea saturnoides* (Walker, 1858) which is monotypic to the level of the family and supports a single large Discal eyespot on each wing surface. Other species represent more speciose groups such as moths from the Geometrid family Semanturiidae, or butterflies from the genus *Parnassius* which support interesting and unique Marginal eyespots.

Marginal and Discal eyespots in both the Bombycoidea and the Papilionoidea appear to have evolved independently from each other. While our taxon sampling for both clades is small, we did not find support for the hypothesis that the evolution of one eyespot type promoted the evolution of the other (Figs. S9). Our analyses, however, revealed that eyespots within those clades appeared in a specific order. Marginal eyespots appeared first in Papilionoidea, followed by Discal eyespots, whereas the reverse was observed in Bombycoidea.

Despite the independent origin of eyespots in the Papilionoidea and Bombycoidea, two superfamilies currently understood to be ~110 million years apart (Kawahara et al., 2019), it is still possible that these (and other) Lepidopteran eyespots share the same gene regulatory network (GRN). Previous research by Murugesan et al. (2022) found that an appendage gene regulatory network was co-opted to build Marginal eyespots in *Bicyclus anynana*, a nymphalid butterfly. It is possible that the same GRN was co-opted more than once across the Lepidoptera. To test this hypothesis, it will be important to molecularly characterize Marginal and Discal eyespot GRN in moth lineages as well as Discal eyespots in nymphalid butterflies. Early immunochemistry work in two saturniid species detected the presence of the two eyespot marker proteins previously identified in nymphalids, Distal-less and Engrailed (Monteiro et al. 2006). Stronger evidence for the use of the same appendage GRN in these Discal moth eyespots may need to come from CRISPR knockouts of cis-regulatory elements belonging to common eyespot and appendage genes, showing that both traits are affected (Murugesan et al. 2022). Demonstrating that all eyespots use a single GRN, or, alternatively, that different "eyespot" GRNs can evolve using different genes and/or cis-regulatory elements is a highly interesting question for future research. This line of research would help us understand molecular mechanisms of convergence of complex phenotypic traits.

From an ecological perspective, the origin of eyespots may be linked to the evolution of body and/or wing size as eyespots evolved only in the larger members of Dytrisa. This hypothesis is further strengthened by the occurrence of eyespots in large non Ditrysian primitive Lepidoptera, such as *Zelotypia stacyi* (Scott, 1869)*, a* Hepialidae, which was not part of this study. This species has large Discal eyespots, as well as a large body and wing size (Grehan & Mielke 2018).

The large number of independent origins for eyespots suggests that these traits can evolve readily, and especially in large wings, perhaps in a quantitative and gradual way. Previous genetic studies showed that eyespot number in *Bicyclus anynana* hindwings was regulated by a variety of QTLs around many different genes (Rivera-Colon et al. 2020). This suggests that eyespots differentiate following some threshold response to quantitative genetic variation. In addition, previous reaction-diffusion models also showed that eyespot centres could form on the wing by tweaks in a variety of different parameters (Nihout 1990; Connahs et al. 2019). It is possible that larger wing sector size, as well as multiple other genetic parameters, facilitate the stable expression of *Distal-less* in the middle of the wing sector, an essential top regulator of eyespots (Connahs et al. 2019; Matsuoka et al. 2022). Given that the expression of *Distal-less* is linked to the co-option of the appendage GRN (Murugesan et al. 2022), if *Distal-less* becomes stably expressed in a wing sector, it is possible that

387 the same appendage GRN gets co-opted every time eyespots independently evolve on the wings of
388 Lepidoptera. This idea, along with our hypothesis that body size is linked to eyespot development
389 will require further testing.
390
391 **Concluding remarks**
392
393 While the focus of this work is on eyespot evolution, the phylogeny presented here provides
394 evidence supporting both new and already established relationships among lepidopteran lineages, at
395 the superfamily and potentially family level (see supplementary information). These insights can fuel
396 future systematic research and further comparative work on how wing colour patterns, and other
397 adaptive traits, evolve in Lepidoptera.
398
399 **Materials and methods**
400
401 Molecular data
402
403 DNA sequence data for 645 species of Lepidoptera (moths and butterflies) and seven species of
404 Trichoptera (caddisflies, outgroup) were kindly provided by Professor Emeritus Charles Mitter
405 (University of Maryland, Table S1). Additional sequences for 70 species of Saturniidae (giant silk
406 moths) were downloaded from NCBI (GenBank). The full dataset was composed of 27 protein-coding
407 genes (Table S2), with 5-24 fragments available across all species. Our taxonomic sampling includes
408 ~68% (90/133, Van Nieukerken et al., 2011) of all families and ~65% (28/43, Van Nieukerken et al.,
409 2011) of all superfamilies in the order Lepidoptera. This study focuses on the suborder Ditrysia,
410 which comprises ~99% of currently described lepidopterans. Of these, ~90% of families (90/100,
411 Reiger et al., 2009) and ~93-96% (28/29-30) of superfamilies (Van Nieukerken et al., 2011; Heikkilä et
412 al., 2015; Mitter et al., 2017) are represented. For each superfamily, 1 – 128 species were sampled
413 (median = 14). The full list of superfamilies as well as the number of species representatives for each
414 superfamily are outlined in Table S1. We chose the Trichoptera for our outgroup because they are
415 the closest extant relatives to the Lepidoptera (Mey et al., 2017), but distant enough to provide
416 ingroup monophyly.
417
418 Sequence alignment and phylogenetic inference
419
420 DNA sequences were aligned via MAFFT v7.490 (Katoh & Standley, 2013). We used a global
421 alignment strategy with iterative refinement (G-INS-i) to maximize alignment accuracy based on
422 weighted sum-of-pairs and a consistency score. The length of the final alignment, consisting of all 27
423 concatenated gene fragments, was 22,643 bp.
424

Phylogenetic inference under maximum likelihood was done with IQ-TREE v1.6.12 (Nguyen et al., 2015, Kalyaanamoorthy et al., 2017; Hoang et al., 2018). 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 alternative to the traditional bootstrap method and it 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 v5.7-1 (Paradis & Schliep, 2019) in R v4.3.0 (R Core Team 2023). We assumed an age of ~290 Ma for the most recent common ancestor of Ditrysia (the root node of our tree), based on 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 all wing surfaces and in both sexes). Colour patterns were identified as eyespots if they met the following criterion: Patterns contained a circular or oblong spot encircled by one or more concentric rings of a distinct colour that also contrasted with the overall background colour of the wing (Fig. 1). To explore the sensitivity of our results to this conservative criterion, we repeated our main comparative analyses under a more inclusive definition of eyespots, where non-circular/oblong shapes (e.g., triangles, rectangles, teardrops, Fig. S10) encircled by another ring of Colour were also considered. Eyespots were further classified into Marginal or Discal, based on their locations on the wing surface (Figs. 1).

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). 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. 96 species included in our dataset lacked publicly available and/or reliably identified images of any form. We photographed 81 of these species at the Mcguire Center for Lepidoptera (MGCL, Florida Museum of Natural History, 3215 Hull Rd, Gainesville, Fl 32611), using a Cannon D50 DSLR camera. We were unable to obtain images for 4 species (0.56%), which are treated as missing data in all comparative analyses.

Modelling the origin and loss of eyespots in Lepidoptera

To model eyespot evolution across Lepidoptera, we used RevBayes v1.2.1 (Höhna et al., 2016). RevBayes is an open-source software designed for Bayesian phylogenetic inference. It allows users to build probabilistic graphical models using an interactive model-specification language. Eyespots were modelled as a discrete trait with two states (presence/absence). Eyespot states had unequal transition rates, drawn from identical exponential priors. The rate parameters of these priors were set to reflect an expectation of 10 events (10 eyespot gains and 10 eyespot losses) along the tree. Root state frequencies were in turn drawn from a Dirichlet prior, assuming equal probabilities of presence or absence of eyespots at the origin of the tree. Eyespot evolution was modelled separately using three datasets: eyespot presence/absence irrespective of eyespot location on the wing; presence/absence of Marginal eyespots; and presence/absence of Discal eyespots. For all three datasets, eyespots could be located on any wing surface (dorsal/ventral surfaces of fore or hindwings) and in either sex.

Each model was run for 100,000 iterations after an initial burn-in of 10,000, and tuning parameter proposals every 1,000 iterations on two independent chains. We evaluated the convergence and stationarity of the MCMC chains for each model using the R package *Convenience* v1.0.0 (Fanreti et al., 2013). Joint conditional ancestral states were sampled every 100 iterations and plotted using RevGadgets v1.1.1 (Tribble et al., 2023), *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). Posterior probability data for specific nodes was obtained using the R package Treeio (Wang et al., 2020). in R. Stochastic character histories (in Revbayes 1.2.1, Frayman and Höhna, 2019) were sampled every 100 iterations and summarized by computing the median number of transitions (eyespots gains and losses) and corresponding 95% highest posterior density (HPD) intervals. ITOL was used to enhance the aesthetics of phylogenetic figures (ITOL, 2023).

<u>Testing eyespot homology in selected clades</u>

We next implemented a model testing approach to investigate eyespot homology among extant species of Lepidoptera. We identified all subclades including two or more taxa bearing eyespots, regardless of eyespot location. If the most recent common ancestor (MRCA) of these clades also displayed eyespots, it is likely that eyespots in extant taxa are homologous — i.e. inherited from their common ancestor. To test this hypothesis, we extracted all the above-mentioned subclades (Fig. 5 for schematic example) from the complete ultrametric phylogeny (Fig. 2), and for each subclade applied two alternative versions of the discrete-trait model described previously. In the first version (hereafter the ‘multiple origins’ model), we constrained the root state to eyespot

497 absence, by assigning a fully informative prior to the root state parameter. In the second version
498 (the common ancestor model), we instead enforced a MRCA with eyespots by setting the root
499 frequency of eyespot presence to one.
500
501 We subsequently computed the marginal likelihood of the two alternative models for each subclade
502 and for our entire phylogeny. A marginal likelihood indicates the fit of a model, averaged across its
503 entire parameter space, to a data set. Marginal likelihood estimation is routinely used in
504 phylogenetic analysis, for instance, in the selection of appropriate nucleotide substitution models
505 and for species delimitation applications (Oaks et. Al 2019). Unlike other model comparison
506 approaches, marginal likelihoods are weighted by the prior, as prior beliefs are bound to influence
507 model fit (Xie et al. 2011). Here, the two models being compared (the 'multiple origins' model and
508 the 'common ancestor' model) differ only by the prior controlling the character state at the root.
509 Their direct comparison thus serves as a statistical test for the presence of eyespots in the most
510 recent common ancestor (MRCA) of each subclade with eyespots. Marginal likelihood was estimated
511 via two sampling methods, steppingstone sampling (SS) and path sampling (PS) (Lartillot and
512 Philippe, 2006, Fan et al., 2011; Xie et al., 2011). For both methods the power posterior analysis was
513 split into 50 intervals between the prior and posterior and was run for 5,000 iterations with a burn-in
514 of 5,000 generations. We repeated all marginal likelihood approximations and confirmed that both
515 PS and SS estimates were stable (i.e. differing by no more than 0.5 between independent runs).
516
517 Marginal likelihood estimates were then used to calculate the log 10 of the raw Bayes Factor ($\log_{10}$
518 BF) between the 'multiple origins' and 'common ancestor' models. Positive $\log_{10}$BF values support
519 the 'common ancestor' model and were interpreted as follows: < 0.1 = weak evidence not worth
520 mentioning, 0.1 – 0.5 weak evidence but worth mentioning, 0.5 – 1.0 strong evidence, and > 1.0 very
521 strong evidence. Negative values were in turn interpreted as support for the multiple origins model.
522 The main results of these analyses were summarized on the tree using ITOL (2023) after initial
523 visualization in R using *phytools* v1.9-16 (Revell, 2012), *ggplot2* v3.4.3 (Wickham et al., 2016) and
524 *ggtree* v3.9.0 (Yu et al., 2017 & 2018, Yu, 2020 & 2022; Xu et al., 2022).
525
526 <u>Correlated evolution of Marginal and Discal eyespots</u>
527
528 We then focused on two clades (Papilionoidea and Bombycoidea), in which both Marginal and Discal
529 eyespots have evolved in distinctly ordered sequences (see Results). These findings suggested that
530 an ancestral eyespot origin on one wing region (Marginal or Discal) could facilitate the subsequent
531 evolution of eyespots on the alternative region, by redeploying the ancestral eyespot gene-

532 regulatory network on a novel tissue domain. We tested this hypothesis using a correlated evolution
533 analysis for each clade, where the probability of gaining Marginal or Discal eyespots can depend on
534 whether the alternative eyespot type is already present. To do this, we modelled a reversible jump
535 mixture distribution, which samples the posterior probabilities of parameter spaces for correlated
536 and independent eyespot evolution models. Support for the correlated evolution model in the
537 posterior distribution of the reversible jump parameter would indicate that the evolution of one
538 eyespot types depends on the presence of the other and could then be followed up with inspection
539 of transition rates estimates to determine if ancestral eyespot expression facilitates the evolution of
540 new eyespot domains. Alternatively, support for the independent model would suggest that Discal
541 and Marginal are similarly likely to evolve whether the alternative eyespot type is present or absent.
542 As in our previous analyses, we modelled all transition rates (eyespots gains and losses on each wing
543 region) as drawn from identical exponential priors, Revbayes v1.2.4 (Höhna et al., 2016) was used to
544 run the comparative evolution analysis. The rate parameter of the exponential priors corresponded
545 to a mean of 10 transitions across the tree. Root frequencies were drawn from a flat Dirichlet
546 distribution. The models were run for 10,000,000 iterations, with a pre-burn-in of 25% and tuning
547 parameter proposals every 5,000 iterations on two independent chains. Joint conditional ancestral
548 states were sampled every 10,000 iterations and plotted using *RevGadgets* v1.1.1 (Tribble et al.,
549 2023) in R. We evaluated the convergence and stationarity of the MCMC chains for each model using
550 the R package *Convenience* V1.0.0 (Fanreti et al., 2013).
551
552 Identifying potential eyespot pattern common ancestors
553
554 Our initial image data collection included only species with available sequence data, resulting in
555 several taxa (19 families in 11 superfamilies) with a single eyespot (Discal and/or Marginal) bearing
556 taxon. To determine if these taxa belong to larger a single common ancestor of eyespot-bearing
557 species, warranting future research, we investigated the phenotype of all species currently classed in
558 the same family as one of the species on our tree (via images available online) which were part of
559 the 28 discal and/or 19 other marginal eyespot occurrences identified by our stochastic mapping
560 analysis. Whether species closely related to taxa represented in our tree (Fig. 2) have a Discal and or
561 Marginal eyespot pattern. Due to the large number of species and the variation in number of species
562 with eyespots across the 19 different families (11 superfamilies) we identified as having eyespots,
563 we summarize our findings in both table and figure form with up to 3 species per family (Fig. S10,
564 Table S5).
565
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