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A molecular phylogeny of *Eumorpha* (Lepidoptera: Sphingidae) and the evolution of anti-predator larval eyespots

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Abstract. Many insects possess conspicuous external circular ring markings that resemble the eye of a vertebrate. These 'eyespots' typically function to startle or otherwise deter predators, but few studies have examined how eyespots have evolved. We study the evolution of the posterior larval eyespot in the charismatic New World hawkmoth genus *Eumorpha*. While *Eumorpha* has a range of posterior larval eyespot shapes and sizes, little is known of how this trait has evolved because phylogenetic relationships of *Eumorpha* remain largely unknown. In this study, we included 62 individuals from 23 of 26 described *Eumorpha* species, and sequenced four genes (*CAD*, *EF-1α*, *Wingless* and *COI*), totaling 3773 base pairs. Maximum likelihood and Bayesian phylogenetic methods produced largely congruent trees with well-supported relationships. Our analyses reveal that *Eumorpha* probably had an ancestor with a posterior larval eyespot and that the eyespot was subsequently lost in at least three lineages. *Eumorpha* appears to have originated in Central and South America and expanded its distribution to North America.

Introduction

Many insects possess conspicuous external circular rings, or 'eyespot' markings. Eyespots are believed to have evolved to startle, intimidate or misdirect a predator's strike, although they might also function in sexual selection and species recognition (Edmunds, 1974; Stevens, 2005; Kodandaramaiah, 2011). Eyespots on adult Lepidoptera often function to direct predator attack towards less vital regions (Kodandaramaiah, 2011). For caterpillars, however, there is little advantage to directing attack towards body parts bearing eyespots (Janzen *et al.*, 2010) because caterpillar body regions are less expendable. Caterpillars bear eyespots as a means to startle or otherwise intimidate predators (Janzen *et al.*, 2010; Hossie & Sherratt, 2013), and insect-eating birds are less likely to attack caterpillars with eyespots (Hossie & Sherratt, 2012, 2013). Most studies of Lepidoptera eyespots have focused on the development, ecology and genetics of eyespot marks on the wings of a few model butterfly species (e.g. *Bicyclus anayana, Junonia almana*; Saenko *et al.*, 2008; Beldade *et al.*, 2009; Oliver *et al.*, 2009, 2012; Otaki, 2011; Prudic *et al.*, 2011; Monteiro *et al.*, 2013). Little phylogenetic information has been incorporated into these studies (but see Oliver *et al.*, 2009) and the need for empirical evolutionary data in understanding eyespot evolution has been emphasized (French & Brakefield, 2004; Monteiro, 2008; Kodandaramaiah, 2009).

In this study, we examine the evolution of larval eyespots in the hawkmoth genus *Eumorpha* Hübner. Hawkmoth caterpillars are well known as 'hornworms', because the larvae of many species have a dorso-central horn-like protuberance on the eighth abdominal segment. The horn generally becomes shorter with each successive moult, and in *Eumorpha,* it becomes reduced to a glossy flat tubercle or disappears as the larva develops (Kitching & Cadiou, 2000). This tubercle can appear

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as an eyespot in some species and is morphologically different from that of the anterior eyespots of pigmented cuticle of other hawkmoth genera, such as *Erinnyis* Hübner, *Hemeroplanes* Hübner, *Madoryx* Boisduval and *Xylophanes* Hübner (Curio, 1965; Janzen *et al.*, 2010; Hossie *et al.*, 2013).

Eumorpha contains 26 described species that are distributed widely across temperate and tropical regions of the Americas (Kitching *et al.*, 2011). The genus is ideal to study the evolution of eyespot patterns as they have a diversity of different eyespot morphology and behaviours associated with them. Some species have a conspicuous posterior eyespot, others have an eyespot that is reduced or missing, and at least two species can make the eyespot 'blink', much like that of a vertebrate eye (Hossie *et al.*, 2013). Furthermore, few studies have comprehensively examined the evolutionary relationships of species within a hawkmoth genus. Most phylogenetic studies on hawkmoths have focused mainly on relationships between subfamilies, tribes and genera (Kawahara *et al.*, 2009), or relationships between Sphingidae and related families (Regier *et al.*, 2008; Zwick *et al.*, 2011; Breinholt & Kawahara, 2013). Here, we construct the first molecular phylogeny of *Eumorpha* using multiple genes, trace the evolution of the posterior larval eyespot, and conduct a preliminary study of species' contemporary biogeography.

Materials and methods

Taxon sampling

We collected DNA sequence data from 62 individuals from 23 species of *Eumorpha* and eight outgroups. Tissue samples of five species were obtained from the University of Maryland, College Park, MD, and tissues of two species were acquired from the cryogenic collection at the American Museum of Natural History (AMNH), New York (Table S1). We selected the macroglossine species *Aleuron chloroptera* (Perty), *Amphion floridensis* Clark, *Enyo ocypete* (Linnaeus), *Euproserpinus phaeton* Grote & Robinson, *Pachygonidia subhamata* (Walker), *Proserpinus terlooii* Edwards, *Sphecodina abbottii* (Swainson) and *Unzela japix* (Cramer) as outgroups because they were placed as the closest relatives to *Eumorpha* in the most comprehensive phylogeny of hawkmoths thus far (Kawahara *et al.*, 2009). Larvae of *Proserpinus* Hübner and *Sphecodina* Blanchard also have a posterior larval eyespot (Pittaway, 1997-2006; Wagner, 2005; Pittaway & Kitching, 2006). The inclusion of species from these genera allows for a more complete evaluation of the evolution of the posterior larval eyespot.

DNA extraction, polymerase chain reaction (PCR) and sequence alignment

Genomic DNA was extracted from one leg of each specimen using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's standard protocol. We sequenced four genes. Their primers (forward and reverse) and amplicon lengths were as follows: *CAD* (CADm5F/CADm1mR, 984 bp), elongation factor-1α (*EF-1*α; ef44/efrcM4, 989 bp), *Wingless* (Wg1aF/Wg2aR, 362 bp) and *COI* (LepF1/LepR1 and Jerry/Pat, 1438 bp). The concatenated alignment totaled 3773 bp. Further details on protocols for amplification of these genes are described in Kawahara *et al.* (2013). Sequencing was conducted at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida, Gainesville, FL. We also obtained 40 COI sequences of *Eumorpha* from BOLD (http://www.boldsystems.org) and GenBank (Benson *et al.*, 2005), and these sequences were included in the study. Sequence chromatograms were examined and edited in GENEIOUS PRO 5.5.6 (http://www.geneious.com/). All loci were aligned separately with default parameters using the G-INS-i alignment strategy in MAFFT (Katoh, 2013). G-INS-i was used because the data matrix could be readily aligned (Katoh & Toh, 2008). We first created single gene matrices and then concatenated all genes into a single matrix in GENEIOUS. GenBank accession numbers for all sequence data are listed in Table S1, and the entire aligned dataset is available via the Dryad Digital Repository (http://www.datadryad.org; doi:10.5061/dryad.3gg62).

Phylogenetic analysis

We conducted phylogenetic analyses on each gene matrix and on the concatenated dataset. To assess the effect of taxon sampling and missing data on the overall topology, we constructed two datasets. Dataset 1 included data of all four genes from 12 *Eumorpha* species and an additional eight outgroups. Dataset 2 included all sequences in dataset 1 plus 40 COI sequences. For both datasets, maximum likelihood (ML) analyses were conducted in RAXML 7.3.2 (Stamatakis, 2006), and PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012) was used to select the best-fit partitioning scheme and model of molecular evolution according to the Akaike information criterion (AIC) (Akaike, 1973). We conducted 1000 ML tree searches starting from parsimony trees (using the '–f d' option in RAXML) and 1000 bootstrap replicates (implementing the '–f a' option).

Bayesian phylogenetic analyses were conducted on both datasets with MRBAYES 3.2 (Ronquist *et al.*, 2012). We ran two independent Markov chain Monte Carlo (MCMC) runs starting from a random starting tree with three hot chains and one cold chain, each using default priors sampling every 1000 generations for a total of 20 million generations. We partitioned the data and applied the best substitution model according to the PARTITIONFINDER results and unlinked the variables statefreq, revmat, shape and pinvar. To monitor the convergence of the MCMC runs, we used TRACER 1.5 (Rambaut & Drummond, 2007) and examined whether the split frequencies between runs had fallen below 0.01. We also removed trees prior to chain convergence, which was estimated as the first 25% of trees.

Evolution of the larval posterior eyespot

Ancestral character reconstruction of posterior larval eyespots was performed on the most likely RAXML tree and 1000 trees

from the posterior distribution of the MRBAYES analysis using BAYESTRAITS (Pagel *et al.*, 2004) implementing the ML multistate module. We used the Perl script bayestraitswrap.pl (Sklenarova *et al.*, 2013) to define nodes on the best ML tree and output a tree with each node labelled (Figure S1). We coded larval posterior eyespots as a three-state character. The states were as follows: 0, lacking eyespots entirely; 1, possessing a tubercle that resembles a very reduced posterior eyespot; 2, having a large distinct posterior eyespot (Table S2). Several species [*Eumorpha analis* (Rothschild & Jordan), *Eumorpha fasciatus* (Sulzer), *Eumorpha megaeacus* (Hübner), and *Eumorpha satellitia* (Linnaeus)] have caterpillar morphs that differ slightly in the eyespot trait and therefore were coded as variable. Species for which we did not have any larval eyespot data were coded as unknown. Three genera that were chosen as outgroups (*Pachygonidia*, *Proserpinus*, *Sphecodina*) include species with a larval anal eyespot, and *Pachygonidia* and *Proserpinus* include species that lack an eyespot. While we could code species in these genera as variable, such a coding scheme would be misleading for our study because the species sampled in these genera do not have larvae with multiple eyespot morphs. Our results could also be misled if we ignore species in these genera that were not sampled in this study. Therefore, we ran additional BAYESTRAITS analyses with alternative coding schemes for the two genera (*Pachygonidia* and *Proserpinus*) that have known alternative states. The four schemes and their character states (in parentheses) were as follows: Scheme 1, *Pachygonidia* (2) and *Proserpinus* (2); Scheme 2, *Pachygonidia* (0) and *Proserpinus* (0); Scheme 3, *Pachygonidia* (2) and *Proserpinus* (0); and Scheme 4, *Pachygonidia* (0) and *Proserpinus* (2) (Table S3). *Sphecodina abbottii* was coded only as state 2 because all species in this genus have a posterior eyespot.

To test whether *Eumorpha* caterpillars with an eyespot form a monophyletic group, we compared the topology of the ML tree with that of an alternative topology in which we forced species that have a larval eyespot (all species coded as '1' or '2') to be monophyletic. We conducted 200 RAXML tree searches (using the '−f d' option) with this constraint and an approximately unbiased (AU) test (Shimodaira, 2002) was conducted in CONSEL (Shimodaira & Hasegawa, 2001).

Biogeography

We estimated ancestral biogeographical regions in RASP 2.1a (Yu *et al.*, 2011). We re-rooted 1000 trees from the MRBAYES post burn-in tree distribution using PHYUTILITY (Smith & Dunn, 2008). Trees were re-rooted with *Aleuron chloroptera* + *Unzela japix*, following the topology of Kawahara *et al.* (2009). MRBAYES trees were re-rooted because RASP requires rooted input trees. These trees were imported into RASP and we ran 10 chains, sampling every 1000th generation for a total of five million generations. The first three million generations were discarded as burn-in and the outgroup option was set to wide. We coded each taxon in the dataset as present or absent for each of the eight biogeographical regions as defined by Morrone (2006) (Table S2). We also conducted a binary trait correlation analysis in BAYESTRAITS (Pagel *et al.*, 2004) to compare the presence of an eyespot with each species' distribution. Eyespots were coded as binary (presence or absence) and the distribution of each species was treated as tropical or temperate (below or above 23.5° latitude). For species with a distribution that crosses this latitude, we examined the most northern and southern points of its distribution and coded the species based on whether the majority of its geographical distribution (by area) was present above or below this line.

Results

PARTITIONFINDER split both datasets into seven partitions [COI-nt1 (GTR + I + G), CAD-nt2 COI-nt2 EF1α-nt2 (F81 + I + G), COI-nt3 (GTR + G), EF-1α-nt3 (HKY + I), CAD-nt3 (HKY + G), CAD-nt1 EF-1α-nt1 WG-nt1 WG-nt2 (GTR + G), WG-nt3 (GTR + I + G)]. When analysed independently, all genes but CAD recovered *Eumorpha* as monophyletic, but bootstrap support (BP) values were generally lower than branches in trees generated from datasets 1 and 2. In the *COI* gene tree, clades I, IV and V were recovered with bootstrap values higher than 76% but clades III, VI and VII were recovered with low (< 64%) bootstrap support (Figure S2). Clades I, III and IV were reasonably well supported (≥ 76% BP) in the *CAD*, *EF-1α* and *wingless* gene trees (Figures S3–S5).

Maximum likelihood and Bayesian analyses from dataset 1 resulted in a well-supported tree with > 85% BP and posterior probabilities (PPs) above 0.86 for all branches in the ingroup (Fig. 1, black lines). There were no topological conflicts between trees based on the two phylogenetic methods or datasets. However, clade II, which was strongly supported in trees generated form dataset 1, was recovered with weak support in the Bayesian analysis of dataset 2 (Fig. 2). The ML analysis of dataset 2 resulted in a tree with many poorly supported branches, especially for deeper divergences, but bootstrap support for relationships at the tips of the tree was often high (Fig. 1).

Branch supports were high (> 85% BP and > 0.95 PP) for clades I, IV, V and VII in all trees resulting from dataset 1 but lower in trees generated from dataset 2 (Fig. 1). Some consistent, strongly supported (> 85% BP and > 0.99 PP) relationships found in all trees were: (i) the monophyly of *Eumorpha*; (ii) the sister group relationship of *Eumorpha caprionnieri* (Boisduval) and *Eumorpha phorbas*; and (iii) the sister group relationship of *E. fasciatus* and *E. megaeacus.*

All ancestral-state analyses on the best ML topology recovered the highest probability for the ancestor of *Eumorpha* to have had a posterior larval eyespot that evolved before the basal split of the genus {[$P(0) \leq 0.12$, $P(1) \leq 0.19$, $P(2) \geq 0.68$]; Fig. 1, Table S3}. In addition, ancestral state reconstructions on the majority (97.5%, 3890/3988) of trees from the MRBAYES posterior distribution also support the conclusion that the ancestor of *Eumorpha* had an eyespot (Tables S4–S7). The 98 ancestral state reconstructions which did not assign the highest probability to state 2 was restricted to coding scheme 1 (*Pachygonidia* and *Proserpinus* coded as 2; 88 trees) and scheme 4 (*Pachygonidia* coded as 0 and *Proserpinus* coded as 2; 10 trees) and all of these

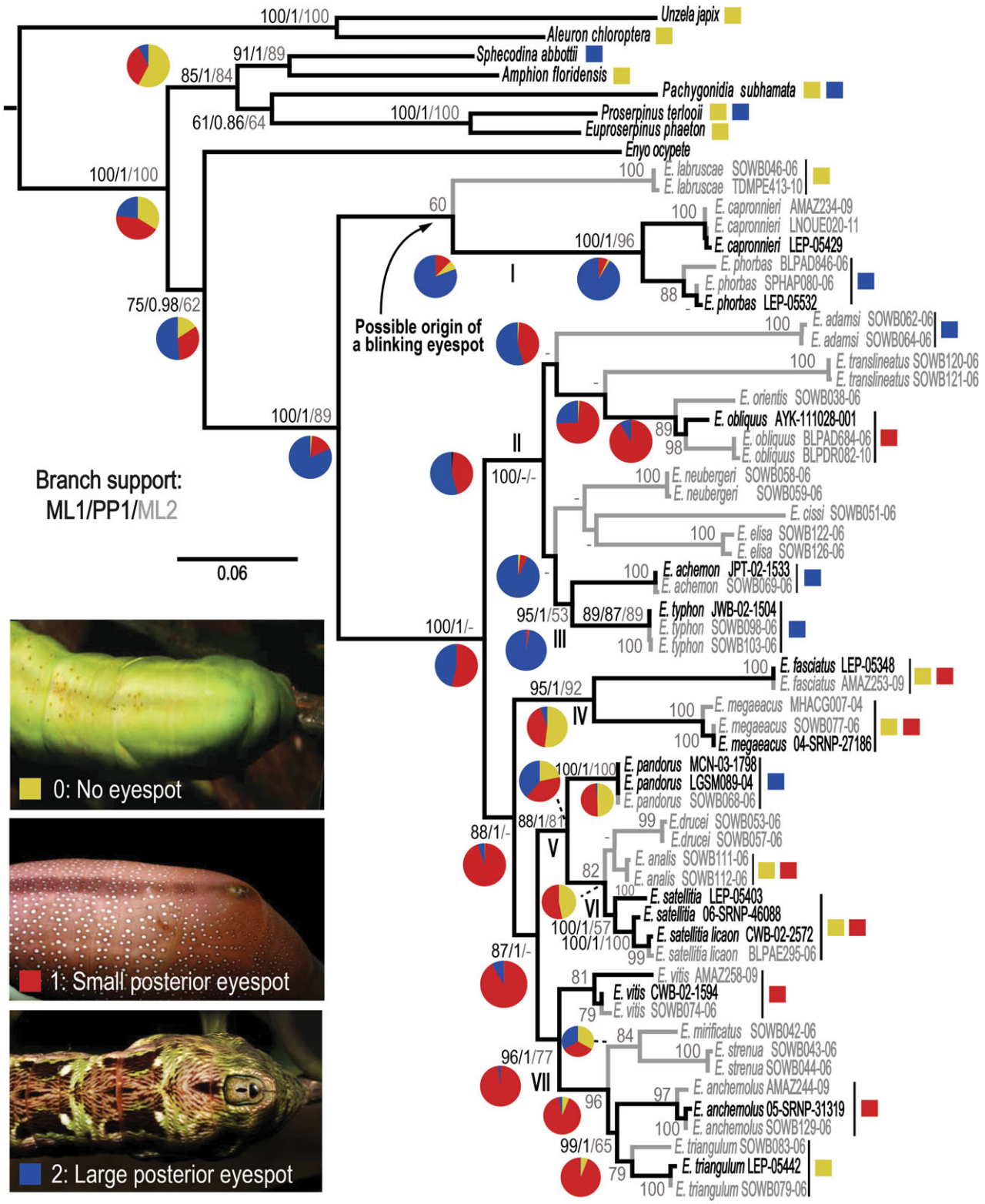

Fig. 1. Maximum likelihood (ML) tree of *Eumorpha* and the evolution of larval eyespots. Branches and taxa coloured black show results from dataset 1, while those in grey show results from dataset 2. Clades referred to in the text are labelled I–VII. Pies show the probability of larval eyespot type at each node (ML approach). Larval images are those of: *Eumorpha triangulum* (0), *Eumorpha satellitia* (1) and *Eumorpha phorbas* (2).

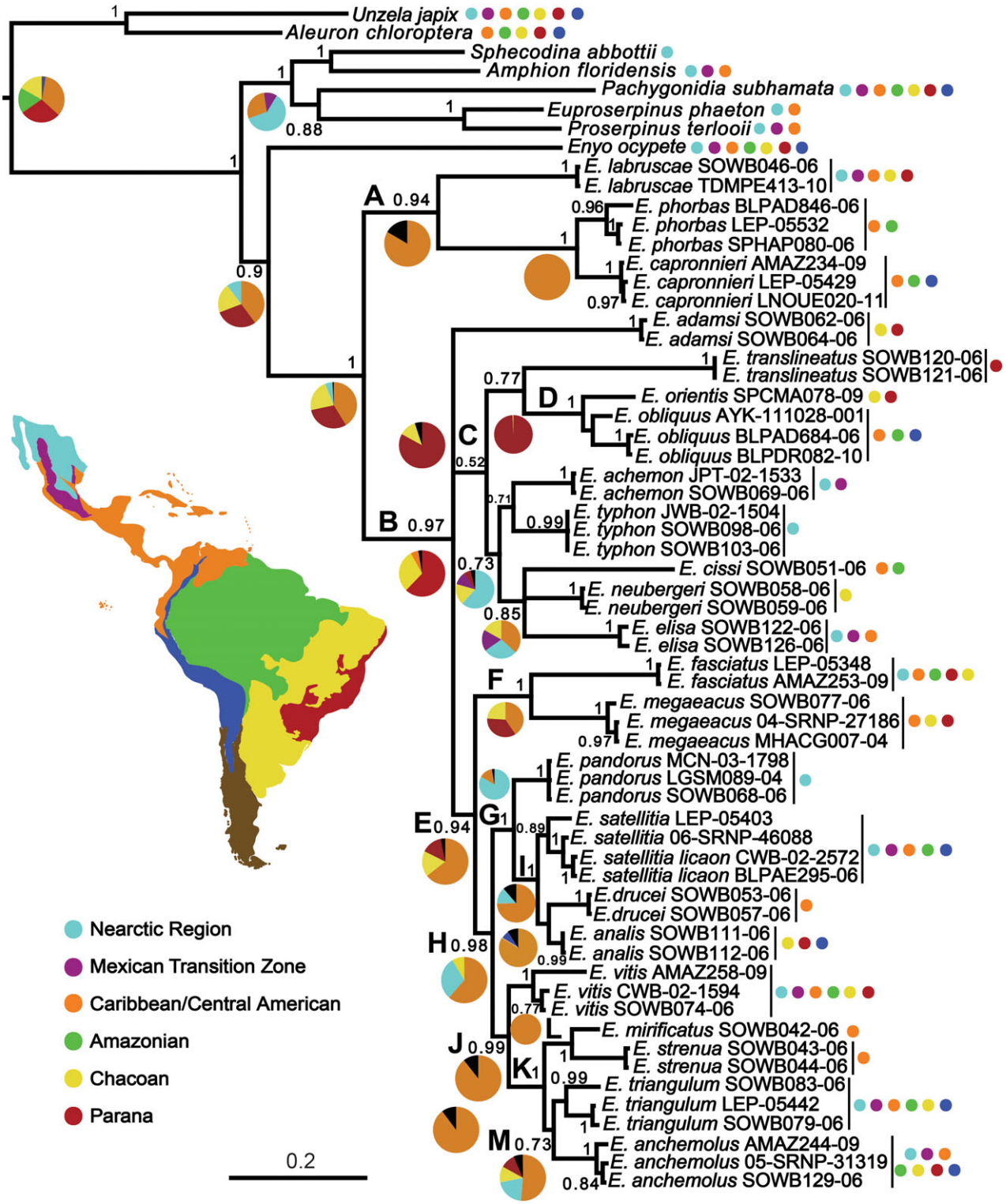

Fig. 2. Bayesian majority-rule consensus tree (dataset 2) showing probabilities of ancestral areas on each node. Colour dots to the right of each taxon code indicate the species' known current geographical distribution. Geographical regions refer to those defined by Morrone (2006).

placed the highest probability that the ancestor of *Eumorpha* had a reduced eyespot (Tables S4 and S7). At least three groups have lost the eyespot, mainly clades IV, VI and VII (Fig. 1). The AU test indicates that the best ML tree ($-\ln L = 16\,045.74$) was significantly better ($P = 7e{-}05$) than the best tree that constrained the monophyly of eyespot-bearing species ($-\ln L = 16653.0$).

Biogeographical analyses placed the highest probability on *Eumorpha* originating in Central America (42.8%) but also give significant proportion of the probability to South America (Parana 29.9%, Chacoan 20.4%) (Fig. 2). Clade A also has a high probability of a Central American origin (83%). Clade B has the highest probability (61%) for a South American origin and there appear to be three subsequent shifts to North America (clades C, G and M). Trait correlations in BAYESTRAITS between the presence of a larval posterior eyespot and distribution were not significant (*BayesFactor* < 1).

Discussion

The present study provides a phylogenetic, biogeographical and larval eyespot analysis of a diverse group of charismatic Neotropical hawkmoths. We sampled multiple specimens of each species when possible, and our results indicate that, based on the taxa included, the genus and all species are monophyletic. The outgroups chosen for the present study were those that were previously known to be closely related to *Eumorpha* (Kawahara *et al.*, 2009). One limitation of that study (and also the present study) is that neither could test whether *Eumorpha* belongs in the tribe Philampelini along with the Hawaiian endemic genus *Tinostoma* Rothschild & Jordan as proposed by Kitching & Cadiou (2000). Unfortunately, *Tinostoma* is known from less than 20 specimens (Heddle *et al.*, 2000) and the placement of *Eumorpha* and *Tinostoma* in the Philampelini remains uncertain.

Tree topologies from the ML and Bayesian analyses were largely congruent. Dataset 1 generally had higher branch support than dataset 2. This result is expected because the *COI* gene represented only a small fraction of the total number of base pairs in the concatenated dataset and the *COI* gene tree had poor branch support when analysed alone (Figure S1). Furthermore, lower bootstrap support for dataset 2 is perhaps due to weak phylogenetic signal from the *COI* barcoding region or the large amount of missing data that were added for the 40 taxa only represented by *COI*.

The evolution of the posterior larval eyespot in *Eumorpha* reveals a complex pattern of transitions between eyespot gains and losses. The ancestor of *Eumorpha* appears to have had an eyespot, which was subsequently lost in three lineages (clades IV, VI, VII; Fig. 1). Constraining the presence of a larval posterior eyespot to a single origin is significantly worse than when they are not (based on AU test results). This further supports the conclusion that the larval eyespot phenotype was lost multiple times.

To address the question of whether the presence of an eyespot is correlated with a particular geographical region, we conducted a correlation analysis in BAYESTRAITS. We predicted that eyespots should be more apparent in tropical regions, because bird-eating predators (e.g., snakes) are more abundant at lower latitudes (Remsen, 1991). Results from our preliminary trait correlation analyses indicate that eyespot presence is not strongly correlated with latitude. In fact, many species at lower latitudes lack an eyespot entirely, just as is the case with thousands of other species of caterpillars. This perhaps indicates that the protective value of eyespots does not depend strongly on sympatry with their putative models (Janzen *et al.*, 2010; Pfennig & Mullen, 2010). The eyespot is equally maintained in many lineages that have radiated to temperate regions simply from the lack of strong opposing selection (Janzen *et al.*, 2010). Some *Eumorpha* species with larvae that lack a posterior eyespot (e.g. *E. fasciatus*, *E. megaeacus, E. satellitia*, *E. triangulum*) have a behavioural response to perceived threats, whereby the larva pulls its head into its thorax and rears up (thereby enlarging the anterior body segments and mimicking a snake's threat and hunting posture), possibly as a means to intimidate or startle predators (see Janzen & Hallwachs, 2014). This defensive posture probably confers protection from insect-eating birds (Hossie & Sherratt, 2013, 2014).

While the present study provides the first formal evaluation of the evolution of anti-predatory larval eyespots, more data are clearly needed because our dataset has many ecological character states that are missing. Some species of *Eumorpha* have a large geographical distribution, and some are known to have polymorphic larvae. Future studies should incorporate additional behavioural data to further elucidate the complex history of behavioural evolution in hawkmoths.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12111

Figure S1. Relationships of *Eumorpha* and outgroups from the ML tree showing node numbers that refer to those in the ancestral state analysis.

Figure S2. ML *COI* gene tree with bootstrap values on braches. A dash indicates a bootstrap value less than 60%. Clades referred to in the text are labelled in roman numerals.

Figure S3. ML *EF-1α* gene tree with bootstrap values on braches. A dash indicates a bootstrap value less than 60%. Clades referred to in the text are labelled in roman numerals.

Figure S4. ML *wingless* gene tree with bootstrap values on braches. A dash represents bootstrap values below 60%. Clades referred to in the text are labelled in roman numerals.

Figure S5. ML *CAD* gene tree with bootstrap values on braches. A dash indicates a bootstrap value less than 60%. Clades referred to in the text are labelled in roman numerals.

Table S1. Taxa and samples in this study, including locality and accession numbers. An asterisk before an accession number indicates that it is from the Boldsystems v3 (http://www.boldsystems.org) DNA barcode database.

Table S2. Character states for posterior larval eyespot and geographical distribution. Posterior larval eyespot: 0, absent; 1, very reduced; 2. distinct. Distributions: A, Nearctic; B, Mexican Transition Zone; C, Caribbean/Central American; D, Amazonian; E, Chacoan; F, Parana; G, South American Transition Zone; H, Andean; ?, unknown, coded as missing.

Table S3. Results of BAYESTRAITS ML analyses with different possible coding schemes for taxa *Pachygonidia subhamata* and *Proserpinus terlooii* on the best ML tree. Node 57, highlighted in red, is the MRCA of *Eumorpha.*

Table S4. Results of BAYESTRAITS ML analyses using scheme 1.

Table S5. Results of BAYESTRAITS ML analyses using scheme 2.

Table S6. Results of BAYESTRAITS ML analyses using scheme 3.

Table S7. Results of BAYESTRAITS ML analyses using scheme 4.

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