Evolutionary history of the recruitment of conserved developmental genes in association to the formation and diversification of a novel trait

Leila T Shirai, Suzanne V Saenko, Roberto A Keller, Maria A Jerónimo, Paul M Brakefield, Henri Descimon, Niklas Wahlberg and Patrícia Beldade

Abstract

Background: The origin and modification of novel traits are important aspects of biological diversification. Studies combining concepts and approaches of developmental genetics and evolutionary biology have uncovered many examples of the recruitment, or co-option, of genes conserved across lineages for the formation of novel, lineage-restricted traits. However, little is known about the evolutionary history of the recruitment of those genes, and of the relationship between them - for example, whether the co-option involves whole or parts of existing networks, or whether it occurs by redeployment of individual genes with de novo rewiring. We use a model novel trait, color pattern elements on butterfly wings called eyespots, to explore these questions. Eyespots have greatly diversified under natural and sexual selection, and their formation involves genetic circuitries shared across insects.

Results: We investigated the evolutionary history of the recruitment and co-recruitment of four conserved transcription regulators to the larval wing disc region where circular pattern elements develop. The co-localization of Antennapedia, Notch, Distal-less, and Spalt with presumptive (eye)spot organizers was examined in 13 butterfly species, providing the largest comparative dataset available for the system. We found variation between families, between subfamilies, and between tribes. Phylogenetic reconstructions by parsimony and maximum likelihood methods revealed an unambiguous evolutionary history only for Antennapedia, with a resolved single origin of eyespot-associated expression, and many homoplastic events for Notch, Distal-less, and Spalt. The flexibility in the (co-)recruitment of the targeted genes includes cases where different gene combinations are associated with morphologically similar eyespots, as well as cases where identical protein combinations are associated with very different phenotypes.

Conclusions: The evolutionary history of gene (co-)recruitment is consistent with both divergence from a recruited putative ancestral network, and with independent co-option of individual genes. The diversity in the combinations of genes expressed in association with eyespot formation does not parallel diversity in characteristics of the adult phenotype. We discuss these results in the context of inferring homology. Our study underscores the importance of widening the representation of phylogenetic, morphological, and genetic diversity in order to establish general principles about the mechanisms behind the evolution of novel traits.
Background
The origin and diversification of novel traits are central and longstanding issues in evolutionary biology [1]. Evolutionary novelties are lineage-restricted traits often associated with new adaptive functions [1,2]. Compelling examples include angiosperm flowers, beetle horns, bird feathers, and butterfly wing color patterns. Studies in evolutionary developmental biology have shown that the origin of novel traits often involves the recruitment, or co-option, of conserved genetic circuitries. This idea is captured in the expression “teaching old genes new tricks” [3], used to explain the genetic mechanisms through which novel traits arise.

The “new tricks” learnt by the “old genes” can involve different, non-mutually exclusive mechanisms (see [4-8]), such as the acquisition of novel expression domains (e.g. the Hox gene Antennapedia in butterfly eyespots [9]), of novel regulators (e.g. homothorax in beetle horns [10]), and of novel downstream targets (e.g. Engrailed regulation of yellow in Drosophila wing spots [11]). Despite the growing body of knowledge on the redeployment of shared genes for the development of lineage-restricted traits, key questions remain unanswered. For example, are entire pathways recruited as a whole or are individual genes co-opted and re-wired de novo [12]? How do recruited or rebuilt pathways diversify along with trait diversification? Widening the representation of both phylogenetic and morphological diversity, together with focus on genetic networks rather than single genes, will be crucial to solving these issues (see [13]). In this study, we provide a taxonomically and genetically wide survey of a model evolutionary novelty, butterfly eyespots, to investigate the origin and diversification of the genetic circuitry associated to its development.

Eyespots are wing pattern elements composed of concentric rings of different colors, found in several lepidopteran species. They are involved in mate choice [14,15] and predator avoidance [16,17], and their diversification is shaped by natural and sexual selection (see [18]). Eyespots are one of the distinct types of pattern elements recognized in the “Nymphalid Groundplan” [19-21]. Based on morphology and position of pattern elements, this Groundplan summarizes homologies across butterflies from the family Nymphalidae [22]. Series of eyespots, or border ocelli, run marginally along the antero-posterior wing axis of most nymphalids, sometimes showing dramatic variation both within and between species (e.g. in the color and the number of different rings [20,23]). At the same time, non-nymphalid species (for example, of the family Papilionidae) can also have circular pattern elements whose morphology resembles that of nymphalid eyespots to different extents [23-25], even when not in equivalent positions of the wing (cf. the conserved venation pattern). In order to cover the diversity in morphology and in position of eyespots s.s. (i.e., border ocelli) and eyespot-like circular pattern elements - hereafter referred to as “(eye) spots” to encompass all diversity, we assayed a number of species across three butterfly families. This broad phylogenetic coverage of phenotypic diversity is presented along with data on the putative genetic circuitry associated to early eyespot specification.

Butterfly eyespots provide a good illustration of the recruitment of genetic circuitry implicated in developmental processes shared by all insects for the formation of novel traits. This includes commonalities between eyespot development (exclusive of butterflies) and processes such as embryonic development [26,27], appendage formation [28,29], and wound healing [27,30] (conserved across insects). The colored rings that make up eyespots are sequentially formed in pupal wings [31,32], around organizing centers which are themselves specified earlier in larval wing discs (reviewed in [33]). Recently, examination of the expression of conserved genes Antennapedia (Antp), Notch (N), and Distal-less (Dll) during the initial stages of organizer establishment revealed intriguing differences among lineages within nymphalids [9,34]. However, the lack of gene expression data outside this clade prevented the reconstruction of the evolutionary history of the recruitment of those genes for expression in larval eyespot fields. Here, we increased the taxonomic sampling by including representatives of an additional nymphalid clade and two non-nymphalid families. We also examined the expression of another transcription factor in the presumptive organizer, Spalt (Sal) [30], in all species sampled. Phylogenetic analysis of this comprehensive dataset revealed great flexibility in which genes (and combinations of genes) are expressed in association with this novel trait in different lineages.

Results and Discussion
To investigate the evolutionary history of the co-option of conserved genes to the location of a developing novel trait, we analyzed expression patterns in larval wings of multiple species in different butterfly families. We targeted four genes involved in transcription regulation: transcription factors Antp, Dll, and Sal, and the transmembrane receptor N. The latter, when bound to its ligands (Delta/Serrate/LAG-2 family of proteins), releases an Intracellular domain that regulates gene expression when associated to DNA-binding CSL proteins [35]. The expression patterns of Antp, N, and Dll were previously analyzed across all stages of last-instar larval wings in nymphalids of subfamilies Nymphalinae
and Satyrinae [9]. In this study, we added the expression analysis of Sal for those same species (Figure 1), and extended the phylogenetic sampling for all four genes to an outgroup comprised of another nymphaeid subfamily (Danainae) and two other butterfly families (Pieridae and Papilionidae; Figure 2). Based on the complete dataset for all four proteins in the 13 representative species (Figure 3), we investigated the evolutionary history of the recruitment of these genes. We mapped the localization of transcription regulators in presumptive eyespot centers onto the species tree, and performed ancestral character reconstructions using both parsimony and maximum likelihood (ML) methods (Figure 4). The species chosen in this study represent diversity in (eye)spot morphology and position on the wing (cf. the conserved venation pattern), allowing for discussions about the inference of homology (Figure 5).

**Taxonomically wide sampling of genes expressed in the developing eyespot field**

In a recent study, we showed that the homeobox transcription factor Antp is found in the presumptive eyespot organizers before Dll and N [9], both of which had, in turn, been characterized as the earliest gene to be expressed in those cells [25,36]. Antp was found exclusively in eyespot centers, whereas N and Dll were also detected in other cells of the wing disc of different butterfly species [9]. Here we add the analysis of expression

![Figure 1 Localization of transcription factor Sal in presumptive nymphaeid eyespot organizers](image-url)
of another conserved transcription factor previously associated to eyespot development in selected species [30], Sal. We found Sal protein in late larval wings at around the same developmental stage as N [9,25], at the location of future organizers and at the intervein region, consistent with what has been described for Junonia coenia [37]. In total, we found the transcription factor Sal in the location of border ocelli pattern elements in five out of ten nymphalid species, in both Nymphalinae and Satyrinae subfamilies (Figure 1).
Antp expression during early organizer establishment clearly distinguished Satyrinae and Nymphalinae clades, being present only in the former [9]. In contrast, N and Dll showed no clear dichotomy between those clades, being expressed in association to most, but not all, developing organizers [9]. Our new data on Sal show that its expression is also variable within nymphalids, in a pattern which does not follow that of the other genes (see discussion about gene co-recruitment below) nor that of any particular aspect of eyespot morphology, such as the size, color, shape, or number of rings (see Figure 3).

To infer the evolutionary history of gene recruitment to presumptive eyespot centers, we examined the expression of the four selected genes in a more distantly related nymphalid (Danaus plexippus) and in three non-nymphalid species (Pieris rapae, Parnassius apollo, and Papilio machaon). The monarch butterfly, D. plexippus, has series of white spots along the antero-posterior margin of its wings. These appear as multiple single-color spots on each wing compartment bordered by veins, instead of one single element with multiple concentric rings as is characteristic of nymphalid border ocelli (Figures 2 and 3). These single-color spots are generally not

---

Figure 3 Summary of expression data for the four developmental genes and adult (eyespot) traits. Complete dataset of the four transcription regulators targeted in this study for all 13 species coded as the presence (black) or absence (white) of expression (Lasiommata megera’s Sal expression could not be determined, grey box). Data for Nymphalidae Antp, N, and Dll expression were obtained from [9]. Sal expression for Nymphalidae is presented in Figure 1, and expression of all genes for outgroup species in Figure 2. Representative (eyespot) spots are illustrated on the right of the species name, and their phenotype is coded for characteristic aspects of Nymphalid border ocelli: 1) “concentric rings” relates to the occurrence of multiple concentric rings (black), of non-concentric rings (grey), and of a single spot (white); and 2) “position” relates to (eyespot) localization in the distal region of the wing cf. the Nymphalid Groundplan (black), versus in other regions (white). Notice that C. memnon bears eyespots in both positions (see Figure 5).
considered homologous to border ocelli [36], even though wing patterns of the Danainae subfamily can be described in terms of the Nymphalid Groundplan [20]. On the other hand, many non-nymphalid species have diverse types of spot-like elements that diverge to different degrees from typical eyespots both in morphology (e.g. in the number and color of rings) and position; illustrated here by *P. rapae*'s single black spot, *P. machaon*'s quasi-concentric rings, and *P. apollo*'s concentric rings around a white center (Figures 2 and 3) [23]. Whether (see [24] for Papilionidae) or not (see [20, 24, 30] for Pieridae) these circular pattern elements are homologous to nymphalid border ocelli is unclear. Moreover, little is known about which developmental processes and genes underlie the formation of these different types of patterns. Here we show that none of four transcription regulators associated to eyespot organizers in nymphalids localizes to the regions of the presumptive eyespot-like elements in the outgroup species (Figure 2). Also, with the exception of Dll for *P. rapae*, we could not detect any of those proteins at the intervein region, where N and Dll are found in some butterfly species [25, 30].

The absence of all four transcription regulators analyzed from the position of presumptive eyespots in the outgroup species suggests that different mechanisms might be at play in the formation of their spots, as previously suggested for *P. rapae* [30]. Possible scenarios include that 1) the same genes are associated with presumptive organizers but at a stage other than the last larval instar which we analyzed, when nymphalids specify their organizers [33], or 2) other genes are

**Figure 4 Ancestral reconstruction of protein recruitment to presumptive eyespot center.** Parsimony (A) and maximum likelihood (B) reconstructions of the evolutionary history of the recruitment of Antp, N, Dll, and Sal for expression at the putative (eye)spot organizers. (A) Two equally parsimonious scenarios with different optimizations are shown: ACCTRAN favoring reversals (top), and DELTRAN favoring parallelisms (bottom). Hash marks represent gain and × loss of expression. (B) The estimated probabilities for each protein at the presumptive (eye)spot centers is represented by piecharts at ancestral nodes: from 100% probability (black) to 0% (white); probabilities not determined in grey. Divergence times (bottom) are shown in Million years ago (Ma) [42, 43].
showed an unambiguous evolutionary history only for Ancestral character reconstructions with both methods. Mapping this information onto the species tree allows for the inference of the evolution of eyespot formation, their localization patterns, and the expression of Antp (Figure 4), found at the location of presumptive eyespot organizers of satyrines but not nymphalines [9]. Our sampling of outgroup species supports that the novel Antp expression is in fact exclusive to satyrines and originated in the common ancestor of the group (Figure 4).

Ancestral reconstructions of the recruitment of other three transcription regulators resulted in an evolutionary history that is less clear. There are two equally parsimonious scenarios of losses (Figure 4A top) and gains (Figure 4A bottom) of eyespot-related expression for each of those genes, with many instances of homoplastic events. This ambiguity is mainly due to the character states of Caligo memnon (absence of N, Dll, and Sal) and Melitaea cinxia (absence of N and Sal) in relation to all other members of their respective subfamilies (presence of N, Dll, and Sal). Given the phylogenetic positions of these species, it is not possible to recover a single scenario for the recruitment of the three transcription regulators to the presumptive eyespot organizers. Worthy of special attention is the case of the satyrine C. memnon, in which only Antp is expressed in the area of presumptive eyespot centers (Figure 3). The forewing eyespot of this species is composed of rings of different colors and placed at the typical location of nymphalid Groundplan’s border ocelli (Figure 1). According to the parsimony reconstructions, either C. memnon represents a secondary loss of N and Dll expression (Figure 4A, upper tree), or the absence of expression of these genes, together with that of Sal, is the ancestral state for satyrines (Figure 4A, lower tree). Even though our results do not favor one parsimony reconstruction over the other, the multiple origins or occasional losses of each character state compel us to speculate on the mechanisms by which gene recruitment evolves. For example, how would C. memnon have lost expression of both Dll and Sal (Figure 4A top)? Alternatively, how would J. coenia and A. io convergently have gained expression of N and Sal (Figure 4A bottom)? The level of homoplasy found in both parsimony reconstructions might indicate that gene recruitment is a flexible process, whose origin and evolution possibly require minimal changes at key nodes of conserved developmental networks (see [44]). Nonetheless, the expression patterns found in C. memnon - a member of the tribe Brassolini, which diverged from the remaining members of the clade (tribe Satyrini) some 60 Million years ago (Ma, Figure 4A and [41]) - uncover variation in which transcription regulators are associated to eyespot organizer regions at the level of tribes.

The ambiguity between the parsimony reconstructions is also reflected in the ancestral state inference obtained with the ML analysis, which estimates with equal probability the presence and absence for each of the three
proteins in the eyespot field at the ancestral node of Nymphalidae (Figure 4B). The variable expression found for these genes could only be revealed by having a taxonomically and genetically wide sampling such as we have (see [13]). However, and even though this is the largest comparative study of gene expression patterns in butterfly wing discs available, the evolutionary history of N, Dll, and Sal expression in eyespot organizer regions requires examination of further species, especially of different subfamilies.

Evolutionary history of gene co-recruitment

So far, we have analyzed differences in the expression of individual genes in the presumptive (eye)spot centers, and shown that it varies substantially, even within butterfly subfamilies. When we consider the evolutionary history of two or more of the targeted genes together (see Figure 3), we cannot see a consistent co-expression history, which would possibly be indicative of co-recruitment. The only consistent patterns we found were that whenever there is N expression, Dll is also present (but not the other way around, see M. cinxia in Figure 3) and, whenever there is Sal expression, N is also present (but not the other way around, see M. galathea in Figure 3). Pairwise comparisons of evolutionary histories, as analyzed by BayesTraits (see Methods section), showed significant correlations for the recruitment of N-, Dll and N-, Sal (Likelihood Ratios of 11.84 and 11.40, respectively, each with $P = 0.02$ for the $\chi^2$ test).

The four proteins targeted in this study are known to interact in other developmental contexts. For example, Antp activation of N signaling induces Dll expression and produces ectopic legs in Drosophila melanogaster heads [45]. Earlier in D. melanogaster development, Antp promotes the mesothoracic identity of the embryo by repressing Sal expression [46,47]. In the presumptive nymphalid eyespot organizers, different combinations of those proteins are found in different species (Figure 3): 1) Antp + N + Dll + Sal, as in Heteropsis iboina, Bicyclus anynana, and Pararge aegeria, 2) N + Dll without Antp, as in J. coenia and Aglais io, and 3) Antp without any of the other three proteins, as in C. mennon. These different combinations are consistent with either of two scenarios: different proteins were recruited individually to the eyespot field and possibly re-wired de novo, or an ancestral network was co-opted and then diversified independently in several lineages (see [12]) possibly involving “partial co-option” (as suggested for abdominal appendages of septid flies [48], and for beetle horns [10]).

Another example of co-option of key genes in butterfly eyespots relates to the recruitment of Hedgehog (Hh) [29]. The co-option of Hh was suggested as having led to novel expression patterns of its downstream targets Patched (Ptc), Cubitus interruptus (Ci), and Engrailed (En) in butterfly wing discs [3]. An important finding, however, was that although Ci and En are expressed in the presumptive eyespots of J. coenia and B. anynana’s larval wing discs [29], expression of Hh and its receptor Ptc were never found in B. anynana [9]. In other words, shared downstream targets of the Hh signaling pathway are found in presumptive eyespot centers with and without the upstream signal (see also [34]). The differences between those two laboratory models, together with the variation in gene combinations found here for a large number of species, reiterate the suggestion that gene recruitment and co-recruitment is a flexible process. Flexibility in the (co-)recruitment of conserved genes has been found for a few other model novel traits [e.g. 10,48,49], but we do not yet know whether it is more probable to gain or to lose expression, whether it depends on particular properties of developmental networks (see [44]), nor which are the more general constraints underlying genetic co-option and its evolution.

Variation in gene expression and in adult phenotype

The great flexibility found in the (co-)recruitment of the four proteins analyzed to the eyespot fields possibly reflects variation in eyespot development. In examining how these putative recruited or rebuild pathways relate to trait diversification, we observed that the variation of individual or groups of genes targeted in this study does not correlate to any particular aspect of (eye)spot morphology (e.g. presence and number of concentric rings) or position (e.g. distally located, as is characteristic of nymphalid border ocelli, or in other regions of the wing, see Figure 3). While (eye)spot position is likely established in larval wings where organizing centers are specified [38,50], the color and size of the rings produced around organizers are determined later, in pupal wings [31,32]. A comparative study of transcription factor localization in eyespot fields at this later stage has also reported great flexibility in the association between combinations of transcription factors and the color of nymphalid eyespot rings [31].

When looking at the association between circular pattern elements and the proteins putatively associated with their development, we observed that eyespot morphology, position, and underlying gene expression include three types of potentially conflicting messages (Figure 5). First, eyespots with very similar morphologies and located at the same position in the wing can be found with different combinations of proteins (e.g. J. coenia versus B. anynana, Figure 5A). Second, very different eyespot morphologies are found even when the same genes are expressed at the same position in the developing wing (e.g. J. coenia versus A. io, Figure 5A). Third, similar eyespot morphologies with the same gene
expression are also found in spots at different positions (e.g. the marginal eyespot in the forewing of C. menon, presumably corresponding to the border ocelli in the Nymphalid Groundplan, versus the more proximal spot on its hindwing, Figure 5B).

Inferring homology depends on establishing phenotypic criteria (like shared morphology and position [51]) that ideally are matched by developmental criteria (such as shared ontogeny and underlying genetic basis) [38,49-55]. Despite the sometimes extreme differences in morphology (e.g. the number, size, and color of concentric rings in J. coenia versus A. io, Figure 5A), all nymphalid eyespots along the distal half of the wing are considered homologous [20,22-25,30,31]. Our data showed that the putative genetic circuitry of nymphalid eyespot organizer specification is highly variable, reflecting that “homologous structures need not be controlled by homologous genes” [55]. There are other examples of homologous characters that diverge in their development or underlying genetics (reviewed in [53]) and show discontinuity in homology inference at different levels of biological organization [51]. This discontinuity is explained by what has been called phenogenetic drift, that is, the “drift in the relationship between genotypes and a given phenotype” [54] (also referred as developmental system drift [56]). At the same time, disparate eyespot phenotypes within nymphalids (including morphology and position) can be found associated to the expression of the same set of proteins in the larval eyespot field (Figure 5). A similar result has been reported for later stages of eyespot development in pupal wings, where the same transcription factors were found in presumably non-homologous spots (at different positions in the wing) of a nymphalid and a saturniid moth [30]. The pattern elements in lepidopteran wings are a good illustration that phenotypic diversity is not necessarily followed by equivalent levels of genetic diversity [10], being sometimes more and sometimes less variable than the underlying patterns of gene expression.

**Conclusions**

Our analysis of the evolutionary history of transcription regulators localization in the (eye)spot fields in larval wings of a variety of butterfly species has revealed substantial variation in the expression of N, Dll, and Sal within nymphalids. It also established a single origin of Antp expression at the presumptive organizer in the common ancestor of the Satyrinae clade. Ancestral reconstructions by parsimony and ML methods for all proteins, together with the lack of phylogenetic evidence for their co-recruitment, revealed ambiguity consistent with both divergence of a co-opted network or independent recruitment of individual genes. The variation found from ancient lineage divergences (among families) to more recent ones (among tribes) shows that the evolution of gene expression associated to the development of this novel trait is highly flexible. Additionally, different butterfly clades (i.e. Papilionidae, Pieridae, and Nymphalidae) seem to be using different mechanisms to specify the circular patterns on their wings. Butterfly eyespots illustrate that phenotypic similarity is not necessarily paralleled by similarity in which genes are expressed in association with trait development. Conversely, distantly related species might use orthologous genes to produce non-homologous circular pattern elements on their wings. The differences found between phenotypic and genetic evidence underscore the importance of covering phylogenetic diversity in relation to multiple components of potentially co-opted networks to understand the origin and diversification of novel traits.

**Methods**

**Biological material**

Thirteen species of three butterfly families were assayed in this study. The nymphalid data on Antp, N, and Dll was obtained from [9] (see reference for details of origin and maintenance of larvae). Additional species, stained for all genes, were obtained from the Lagartagis Butterfly House (Lisbon, Portugal) or field caught and kept as follows: D. plexippus (room temperature, and natural light (L) and dark (D) cycle, fed on milkweed), P. rapae (18/23°C at 6D:18 L, fed on cabbage), P. apollo (27°C at 12D:12 L, fed on stonecrop), and P. machaon (27°C at 12D:12 L, fed on fennel). The staging of larval wing development of all families was done following the tracheal extension into the vein lacunae (cf. [37]).

**Immunohistochemistry**

Immunostainings were performed as in [9] using different staged wing discs covering the entire last larval instar. Right fore- and hindwings from single individuals were stained with anti-Antp and anti-Sal antibody, and left fore- and hindwings were stained with anti-N and anti-Dll antibodies. Antibodies have been shown to be cross reactive across insect orders (e.g. [57-59]) and arthropods (e.g. [60]). The monoclonal mouse anti-Antp 4 C3 [57] (1:50 dilution) and anti-N C17.9 C6 [58] (1:5 dilution) were obtained from the Developmental Studies Hybridoma Bank. The polyclonal rabbit anti-Dll [60] (1:200 dilution), rabbit anti-Sal [59] (1:500 dilution), and guinea pig anti-Sal GP66-2 (1:1000 dilution, used for P. rapae) were provided by other labs. Alexa Fluor 488 anti-mouse, Texas Red anti-rabbit, and Alexa Fluor 594 anti-guinea pig (Molecular Probes) were used as secondary antibodies (1:200 dilution). Images were collected on a BioRad MRC 1024 or a Zeiss Imager M1 laser scanning confocal microscope.
Ancestral character reconstruction and correlation of protein recruitment history

(Eye)spot centers have been shown experimentally to have organizing properties in selected nymphalid lab models [39,40]. We documented localization of the study proteins at the wing regions corresponding to the developing (eye)spot fields, for those and other species (cf. larval venation patterns and (eye)spot location on adult wings). The presence (1) or absence (0) of circular expression patterns at this location was scored for \textit{Antp}, \textit{N}, \textit{Dll}, and \textit{Sal} (Figure 3). Reconstruction of ancestral states was done using parsimony and ML methods. Parsimony reconstructs the evolutionary history by minimizing the number of evolutionary transitions (from absence to presence of expression, and vice-versa), favoring reversals (ACCTRAN) or parallelisms (DELTRAN) when two equally parsimonious scenarios exist. Parsimony analyses were performed in WinClada [61] using ACCTRAN and DELTRAN tracing options to examine alternative scenarios in the case of ambiguous optimizations. ML estimates the probability of ancestral states given a model of evolution and takes into consideration the age of divergence between clades. Characters were traced onto a phylogenetic tree generated for the species included in this study. The tree topology used for the character mapping and illustrated in all figures is based on [41] for the family Nymphalidae, and on [42,43] for the superfamily Papilionoidea. Branch length estimates were calculated as described in [43]. ML reconstructions were performed in Mesquite 2.74 [62] choosing the Mk1 model [63].

To assess whether there is significant correlation between evolutionary histories of pair of genes, pairwise Likelihood Ratio Tests were performed comparing the likelihood of an independent versus Likelihood Ratio Tests were performed comparing the likelihood of an independent versus

\[ 2^{[64]} \], using the branch length estimates and character coding as above. The likelihood ratio was calculated as 2 [log-likelihood (Dependent Model) - log-likelihood (Independent Model)], and is expected to follow a $\chi^2$ distribution with four degrees of freedom [64,65].

Acknowledgements

We would like to thank colleagues who provided butterfly larvae (Arnaud Martin, Ben Kubbinga, Frederik H. Nijhout, Maaike de Jong, Maria João Verdasca, Marjo Saastamoinen, Melanie Gibbs, Oskar Brattström, and Robert Reed), and the Dil and Sal antibodies (labs of Sean B. Carroll, Rosa Barrio, and Antónia Monteiro), as well as two anonymous reviewers for helpful suggestions. This work was supported by funds to PB from the Portuguese Foundation of Science and Technology, FCT (PTDC/BIA-BEC/098808/2008), the Dutch Science Organization, NWO (VIDI 86408010 and ASPASIA 015.005.002), and an installation grant from the Oeiras municipal council. FCT fellowships also supported LTS (SFHR/BDE/51180/2010), MAJ (SFHR/BDE/73658/2010), and RAK (SFHR/BPD/65529/2009). NW would like to acknowledge the Academy of Finland for funding.

Author details

1Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, P-2780-156 Oeiras, Portugal. 2Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands. 3Université de Provence, 3 Place Victor Hugo, F-13331, Marseille Cedex 03, France. 4Department of Biology, University of Turku, 2004 Turku, Finland. 5Department of Genetics and Evolution, University of Geneva, Quai Ernest-Ansermet 30, 1211 Geneva, Switzerland. 6Department of Zoology, Cambridge University, Downing Street, Cambridge CB2 3EJ, UK. 72 Boulevard Rougemont, F-13012 Marseille, France.

Authors’ contributions

LTS coordinated and co-wrote the manuscript. SVS collected the bulk of the expression data, and MAJ collected individuals and data for \textit{Piers}. PMB and HD provided Papilio and Parnassius larvae, and participated in early discussions about comparative analysis of eyespot development. LTS, RAK, and NW performed the phylogenetic analyses. SVS and PB designed the study. PB coordinated the study and co-wrote the manuscript. All authors contributed to the analyses, and revised and approved the final manuscript.

Received: 21 November 2011 Accepted: 15 February 2012

References

1. Müller GB, Wagner GP: Novelty in evolution: restructuring the concept. Annu Rev Ecol Syst 1991, 22:229-256.
2. Pigliucci M: What, if anything, is an evolutionary novelty? Philos Sci 2008, 75:887-898.
3. True JR, Carroll SB: Gene co-option in physiological and morphological evolution. Annu Rev Cell Dev Biol 2002, 18:53-80.
4. Alonso CR, Wilkins AS: What, if anything, is an evolutionary novelty? Philos Sci 2008, 75:887-898.
5. Wagner A: Gene duplications, robustness and evolutionary innovations. Bioessays 2008, 30:367-373.
6. Sæenko SV, Marialva MS, Beldade P: Involvement of the conserved Hox gene Antennapedia in the development and evolution of a novel trait. BMC Ecol Evo 2011, 29.
7. Prud’homme B, Wittkopp PJ, Kassner VA, Carroll SB: Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in \textit{Dropophilia}. Nature 2005, 433:481-487.
8. Monteiro A, Podiash A: Wings, horns, and butterfly eyespots: how do complex traits evolve? PLoS Biol 2009, 7:209-216.
9. Kopp A: Metamodells and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evolution 2009, 63:2771-2789.
10. Costanzo K, Monteiro A: The use of chemical and visual cues in female choice in the butterfly \textit{Bicyclus anynana}. P Roy Soc London B Bio 2007, 274:849-851.
11. Kopp A, Metamodells and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evolution 2009, 63:2771-2789.
12. Rokas A, Holland PW: Evidence for the recent origin of the C. elegans genome. Nature 2003, 422:791-797.
13. Novick PA, Sur M, Stock JB: Mitotic recombination in C. elegans. Nature 1982, 298:59-65.
14. Costa J, Monteiro A: The use of chemical and visual cues in female choice in the butterfly \textit{Bicyclus anynana}. P Roy Soc London B Bio 2007, 274:849-851.
15. Costanzo K, Monteiro A: The use of chemical and visual cues in female choice in the butterfly \textit{Bicyclus anynana}. P Roy Soc London B Bio 2007, 274:849-851.
16. Kodandaramaiah U: The evolutionary significance of butterfly eyespots. Behav Ecol 2011, 22:1264-1271.
17. Stevens M: The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. Biol Rev Camb Phil 2005, 80:573-588.
18. Oliver JC, Robertson KA, Monteiro A: Accommodating natural and sexual selection in butterfly wing pattern evolution. P Roy Soc London B Bio 2009, 276:2369-2375.
19. Belgrade P, Brakefield PM: The genetics and evo-devo of butterfly wing patterns. Nat Rev Genet 2002, 3:442-452.
20. Nijhout HF: The development and evolution of butterfly wing patterns. Washington: Smithsonian Institution Press; 1991.
21. Schwanwitzsch B: Two schemes of the wing-pattern of butterflies. Zoomorphology 1929, 14:36-58.
22. Nijhout HF: Elements of butterfly wing patterns. J Exp Zool B 2001, 291:213-225.
23. Nijhout HF: A comprehensive model for colour pattern formation in butterflies. P Roy Soc London B Bio 1993, 253:81-113.
24. Martin A, Reed RD: wingless and aristaless define a developmental ground plan for moth and butterfly wing pattern evolution. Mol Biol Evol 2010, 27:2864-2878.
25. Reed RD, Serfas MS: Butterfly wing pattern evolution is associated with changes in a Notch/Distal-less temporal pattern formation process. Curr Biol 2004, 14:1159-1166.
26. Saenko SV, Brakefield PM, Belgrade P: Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty. BMC Evol Biol 2010, 8:111.
27. Saenko SV, French V, Brakefield PM, Belgrade P: Conserved developmental processes and the formation of evolutionary novelties: examples from butterfly wings. Philos T Roy Soc B 2006, 361:1549-1555.
28. Carroll SB, Gates J, Scott MP, Carroll SB: Pattern formation and eye-spot determination in butterfly wings. Science 1994, 265:109-114.
29. Keys DN, Lewis DL, Selegue JE, Pearson BJ, Goodrich LV, Johnson RL, Gates J, Scott MP, Carroll SB: Recruitment of a hedgehog regulatory circuit in butterfly eye-spot evolution. Science 1999, 283:532-534.
30. Monteiro A, Glaser G, Stockslager S, Glansdorp N, Ramos D: Comparative insights into questions of lepidopteran wing pattern homology. BMC Dev Biol 2006, 6:52.
31. Brunetti CR, Selegue JE, Monteiro A, French V, Brakefield PM, Carroll SB: The generation and diversification of butterfly eye-spot color patterns. Curr Biol 2011, 21:1578-1585.
32. Wittkopp PJ, Belgrade P: Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. Semin Cell Dev Biol 2009, 20:65-71.
33. Belgrade P, Saenko SV: Evolutionary and developmental genetics of butterfly wing patterns: focus on Bicyclus anynana eyespots. In Molecular Biology and Genetics of the Lepidoptera. Contemporary Topics in Entomology Series. Edited by: Goldsmith M, Marer F, Boca Raton: CRC Press; 2009:89-104.
34. Hombría JC: Butterfly eye-spot serial homology: enter the Hox genes. BMC Biol 2010, 8:1578-1585.
35. Kimble J, Simpson P: The LIN-12/Notch signalling pathway and its regulation. Annu Rev Cell Dev Biol 1997, 13:333-361.
36. Brakefield PM, Gates J, Keys D, Nesbitke E, Wijngaarden PJ, Monteiro A, French V, Carroll SB: Development, plasticity and evolution of butterfly eye-spot patterning. Nature 1996, 384:236-242.
37. Reed RD, Chen PH, Nijhout HF: Cryptic variation in butterfly eye-spot development: the importance of sample size in gene expression studies. Evol Dev 2007, 9:2-9.
38. Belgrade P, Brakefield PM: Concerted evolution and developmental integration in modular butterfly wing patterns. Evol Dev 2003, 5:169-179.
39. French V, Brakefield PM: Eye-spot development on butterfly wings: the focal signal. Dev Biol 1995, 168:112-123.
40. Nijhout HF: Pattern formation on lepidopteran wings: determination of an eye-spot. Dev Biol 1980, 80:267-274.
41. Wahlberg N, Leneveu J, Kodandaramaiah U, Peña C, Nylin S, Freitas AVL, Brower AV: Lepidopteran butterflies diversify following near demise at the Cretaceous/Tertiary boundary. P Roy Soc London B Bio 2009, 276:4295-4302.
42. Wahlberg N, Braby MF, Brower AV, de Jong R, Lee MM, Nylin S, Pierce NE, Sperling FA, Villa R, Warren AD, Zhukov E: Synergistic effects of combining morphological and molecular data in resolving the phylogeny of butterflies and skippers. P Roy Soc London B Bio 2005, 272:1577-1586.
43. Heinikia M, Kaila L, Mutanen M, Peña C, Wahlberg N: Cretaceous origin and repeated tertiary diversification of the redefined butterflies. P Roy Soc London B Bio 2012, 279:1009-1009.
44. Nahmad M, Glass L, Abouheif E: The dynamics of developmental system drift in the gene network underlying wing polyphenism in ants: a mathematical model. Evol Dev 2008, 10:360-374.
45. Kurata S, Go MI, Artavanis-Tsakonas S, Gehring WJ: Notch signaling and the determination of appendage identity. PNAS 2000, 97:2117-2122.
46. Kühnlein RP, Frommer G, Friedman M, Gonzalez-Gaitan M, Weber A, Wagner-Bernholz JF, Gehring WJ, Jackle H, Schuh R: spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo. Embio J 1994, 13:168-179.
47. Wagner-Bernholz JF, Wilson C, Gibson G, Schuh R, Gehring WJ: Identification of target genes of the homeotic gene Antennapedia by enhancer detection. Gene 1991, 92:2467-2480.
48. Bowsher JH, Nijhout HF: Partial co-option of the appendage patterning pathway in the development of abdominal appendages in the sepsid fly Thermia biloba. Dev Genes Ev 2009, 29:577-587.
49. Toromaya Y, Arakane Y, Kramer KJ, Denell RE: Repeated co-options of evenskelton formation during wing-to-elyontr evolution in beetles. Curr Biol 2009, 19:2057-2065.
50. Allen CE, Belgrade P, Zwaan BJ, Brakefield PM: Differences in the selection response of serially repeated color pattern characters: standing variation, development, and evolution. BMC Evol Biol 2008, 8:94.
51. Abouheif E: Developmental genetics and homology: a hierarchical approach. Trends Ecol Evol 1997, 12:405-408.
52. Wagner GP: The biological homology concept. Annu Rev Ecol Syst 1989, 20:51-69.
53. Young RL, Wagner GP: Why ontogenetic homology criteria can be misleading: lessons from digit identity transformations. J Exp Zool B 2011, 316B:165-170.
54. West KM, Fullerton SM: Phenogenetic drift and the evolution of genotype-phenotype relationships. Theor Popul Biol 2000, 57:187-195.
55. de Beer SG: Homology: an unsolved problem Oxford: Oxford University Press; 1971.
56. True JR, Haag ES: Developmental system drift and flexibility in evolutionary trajectories. Evol Dev 2002, 4:109-119.
57. Hayward DC, Patel NH, Rehm EJ, Goodman CS, Ball EE: Sequence and expression of grasshopper Antennapedia comparison to Drosophila. Dev Biol 1995, 172:452-465.
58. Fehon RG, Kooh PJ, Rebay I, Artavanis-Tsakonas S: Antennapedia: Two EGF-homologous genes in Drosophila. Cell 1990, 61:223-234.
59. de Celis JF, Barrio R, Kafatos FC: The spalt/spalt-related gene complex and its function during sensory organ development in the Drosophila thorax. Development. 1999, 126:2653-2662.
60. Panganiban G, Sebring A, Nagy L, Carroll S: The development of crustacean limbs and the evolution of arthropods. Science 1995, 270:1363-1366.
61. Nixson KC: WinClada ver. 1.00.08,[http://www.cladistics.com/].
62. Maddison WP, Maddison DR: Mesquite: a modular system for evolutionary analysis. Version 2.0,[http://mesquiteproject.org].
63. Lewis PO: A likelihood approach to estimating phylogeny from discrete morphological character data. Syst Biol 2001, 50:913-925.
64. Pagel M: Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. P Roy Soc London B Bio 1994, 255:37-45.
65. Barker D, Pagel M: Predicting functional gene links from phylogenetic-statistical analyses of whole genomes. PLoS Comp Biol 2005, 1:e3.
66. Pagel M: BayesTraits,[http://www.evolution.rdg.ac.uk] .