Ancient Wings: animating the evolution of butterfly wing patterns

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Abstract

Character optimization methods can be used to reconstruct ancestral states at the internal nodes of phylogenetic trees. However, seldom are these ancestral states visualized collectively. *Ancient Wings* is a computer program that provides a novel method of visualizing the evolution of several morphological traits simultaneously. It allows users to visualize how the ventral hindwing pattern of 54 butterflies in the genus *Bicyclus* may have changed over time. By clicking on each of the nodes within the evolutionary tree, the user can see an animation of how wing size, eyespot size, and eyespot position relative the wing margin, have putatively evolved as a collective whole. *Ancient Wings* may be used as a pedagogical device as well as a research tool for hypothesis-generation in the fields of evolutionary, ecological, and developmental biology.
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1. Introduction

Character state reconstruction on phylogenetic trees has been used to recreate individual character states (Felsenstein, 1985; Maddison and Maddison, 1992) and to reconstruct ancestral proteins (Chang and Donoghue, 2000; Chang et al., 2002), but seldom is it used to recreate a whole integrated suite of traits simultaneously. We created a computer program, *Ancient Wings*, to illustrate a novel method to visualize the evolution of suites of morphological traits.

We chose to examine and animate the evolution of the hindwing patterns of the butterfly genus *Bicyclus*, found in sub-Saharan Africa (Condamin, 1973). Out of a total of 80 *Bicyclus* species, 54 *Bicyclus* species and 6 outgroup species belonging to related genera, were used to build a rooted molecular phylogeny for the genus (Monteiro and Pierce, 2001). This genus has been studied intensively with regard to wing pattern plasticity (Brakefield and Reitsma, 1991; Roskam and Brakefield, 1996), and the genetic and developmental underpinnings of eyespot formation (French and Brakefield, 1995; Monteiro et al., 1994, 1997, 2003; Beldade et al., 2002a,b).

Reconstruction of the putative ancestral wing patterns and animation of these over the phylogenetic tree provides a visual aid and a hypothesis generating tool through which the mechanisms and selective agents regarding wing pattern evolution can be investigated.

2. Materials and method

Wing pattern data were collected from digital photographs of 253 females of 54 species of *Bicyclus* butterflies. Males and females have nearly identical pat-

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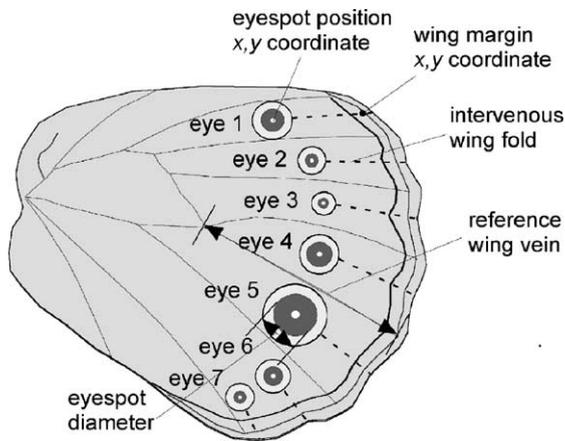*E-mail address:* monteiro@buffalo.edu (A. Monteiro).

Fig. 1. *Bicyclus* ventral hindwing measurements used in the calculations of ancestral wing patterns.

terns but females are usually lighter in coloration and the patterns are more contrasting against the background coloration. The photographs were taken from specimens collected in Africa (Monteiro and Pierce, 2001), from specimens illustrated in the monograph of the genus (Condamin, 1973), and from the extensive collection of *Bicyclus* found at the Royal Museum for Central Africa, Tervuren, Belgium. The digital images were analyzed with *Object-Image* 2.08 (Vischer et al., 1994).

Eyespot diameter measurements were collected by measuring the black central disc parallel to the wing veins from the seven hindwing eyespots from five females of each species (for five species we collected data from only four specimens, for two species we collected data from two specimens, and for two species from three and one specimen, respectively), and their average values and standard errors were calculated (see Fig. 1). The size of an eyespot's outer gold ring was extrapolated using a constant ratio (the gold and black colored portion of an eyespot can also evolve across the genus *Bicyclus* but this process was not modeled here; Condamin, 1973; Monteiro et al., 1997).

Eyespot positional information was collected using measurements from a single female from each of the 54 species examined in this study. We took the *x* and *y* coordinates for the center of each of the seven eyespots, as well as the coordinates of two points along one of the longitudinal wing veins (the one posterior

to the fourth eyespot; Fig. 1). This vein was used to align the eyespot patterns to the same relative *x*–*y* coordinates. The *x*–*y* coordinate at the left end of this vein was used to anchor all eyespot position coordinates across species. The *x*–*y* coordinate at the right end of the vein was used to rotate all the position coordinates to the same angle. These calculations were performed using Perl.

Wing margin positional information was collected by using measurements from the same single representative of each of the 54 species mentioned earlier. We took the *x* and *y* coordinates for the wing margin at the intersection with the intervenous fold for each of the seven eyespots (Fig. 1). The coordinate points along the reference wing vein were used again to anchor and rotate the wing margin coordinates.

We used a phylogenetic tree based on molecular sequence information as the basis for character reconstruction of internal nodes. This phylogeny was based on a maximum parsimony reconstruction of DNA sequence divergence of one nuclear gene (*Elongation factor 1α*), and two mitochondrial genes (*Cytochrome Oxidase I and II*; COI and COII, respectively) and is the only molecular phylogeny for the genus proposed to date (Monteiro and Pierce, 2001). There was strong bootstrap support all of the tip clades in the tree, excluding the clade containing *B. ena*, *B. technatis*, and *B. vansoni*, and the clade of *B. ignobilus* and *B. pavonis*, where long branch attraction may have brought these species together (Felsenstein, 1978). A few of the more basal nodes also lacked strong bootstrap support (see Monteiro and Pierce, 2001).

The eyespot sizes, *x* and *y* coordinates of the eyespot centers, and the *x* and *y* coordinates of the wing margins, were each run separately through *COMPARE* 4.4, a program that can estimate the ancestral traits of a group of taxa given a phylogenetic tree (Martins, 2001). The program uses Phylogenetic Generalized Least Squares (PGLS) with a linear model to estimate ancestral traits (Martins and Hansen, 1997). The PGLS method of ancestor reconstruction used the branch lengths of the phylogenetic tree (the maximum parsimony estimates of the total number of molecular changes from all the sequence data combined, from Fig. 5 in Monteiro and Pierce, 2001), along with the trait data for the tip nodes, to create weighted averages for the ancestral nodes. The calculations also

take into account inter-specific variation when this data is provided.

The ancestral wing pattern calculations for each of the internal nodes were done using a Brownian motion model of evolution in which phenotypic changes accumulate in a random fashion at constant rates. Evolutionary mechanisms that fit a Brownian motion model include random genetic drift (with or without mutation), strong stabilizing selection with randomly changing optima, and directional selection with random fluctuations in direction (Felsenstein, 1988). This model explicitly assumes that weaker stabilizing selection on the traits under evaluation has not occurred. These are obviously strong assumptions regarding the mode of evolution of these characters.

The animation program was created using *Macromedia Flash MX*, an authoring system with a built-in programming system language called Actionscript which is ideal for creating animations and interactive programs for use over the World Wide Web. We worked on a Power Mac G4 with OS X. *Ancient Wings* takes advantage of the graphical and object-oriented nature of *Flash*'s system to allow the programmer to easily manipulate and move on-screen elements. This was well-suited for allowing the visualization of how the eyespots have putatively changed size and position over time. The program runs on Netscape or Internet Explorer browsers with a *Flash Player 6* plug-in.

The tree topology used in the final computer program was time calibrated with COI assuming a rate of 2% sequence divergence between pairs of sequences for every million years of evolutionary separation (Desalle et al., 1987; Brower, 1994; Monteiro and Pierce, 2001). Time calibrating the tree with COII (Monteiro and Pierce, 2001) gave somewhat different estimates that diverged considerably for the more basal nodes (sometimes as much as by 5 million years). These time estimates should be taken with caution and were only provided for a very approximate estimate of the relative age of the different clades.

All phylogenetic and trait data were hard-coded into the computer program. The algorithm for the animation of trait evolution followed the same assumption used in calculating the ancestral nodes, i.e. that all evolutionary change occurred at a constant rate throughout the tree. Animation from node to node was accomplished by dividing the estimated trait differences between the two nodes by the branch length

between them. Branch length was determined based on the distance between the node-to-node screen coordinates based on the clock-constrained COI distances corrected with a general time reversible model, a γ distribution of rate heterogeneity across sites, a proportion of invariable sites and unequal base frequencies (see Monteiro and Pierce, 2001). Note that in order to display morphological change through time, the branch lengths displayed by the animation were not the same as those used to estimate the ancestral nodes with *COMPARE* (see Section 3 below). The program then created a smooth, constant, gradient so that traits change at a constant rate from node to node.
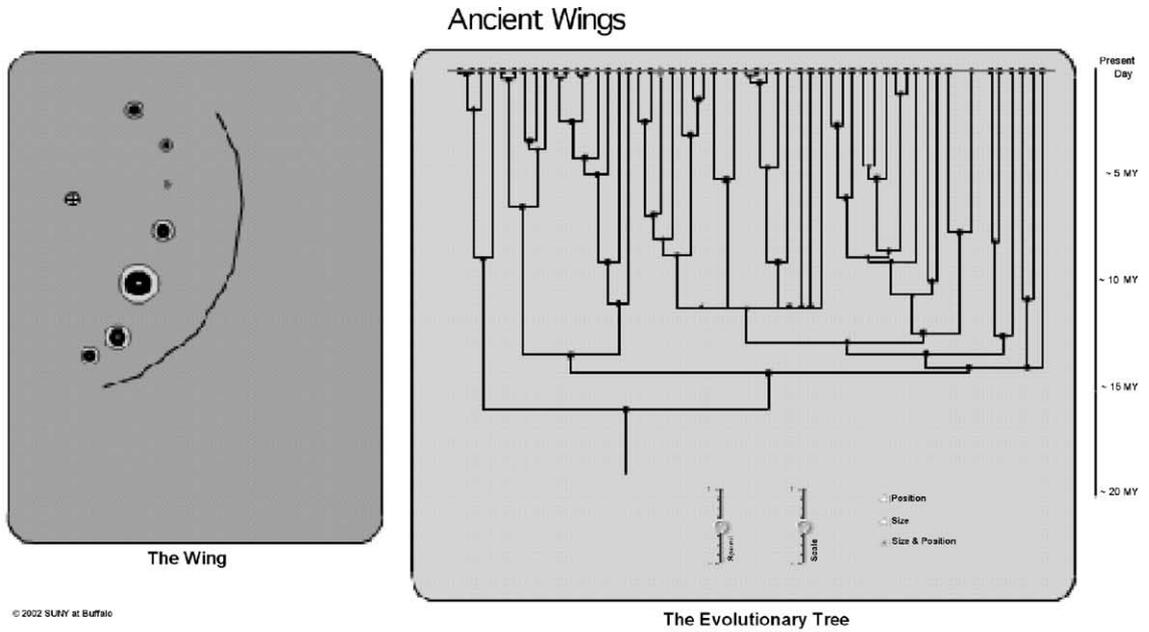
3. Results and discussion

The *Flash* animation, *Ancient Wings*, may be accessed at http://www.acsu.buffalo.edu/~monteiro/ancientwings/. The source code and *Flash* file for *Ancient Wings* is available without restriction at http://www.acsu.buffalo.edu/~monteiro/ancientwings/ancientwings.fla. A guide to the evolutionary tree, with the names of the tip nodes and the numbering scheme of the internal nodes as well as an *Excel* data file may also be downloaded from the same site (see Fig. 2).

Ancient Wings can be useful as a teaching device to allow students to see how putative wing patterns of a genus of butterflies have changed over evolutionary history. However, in addition to the educational value of *Ancient Wings*, the program also has value for researchers as an hypothesis-generation tool.
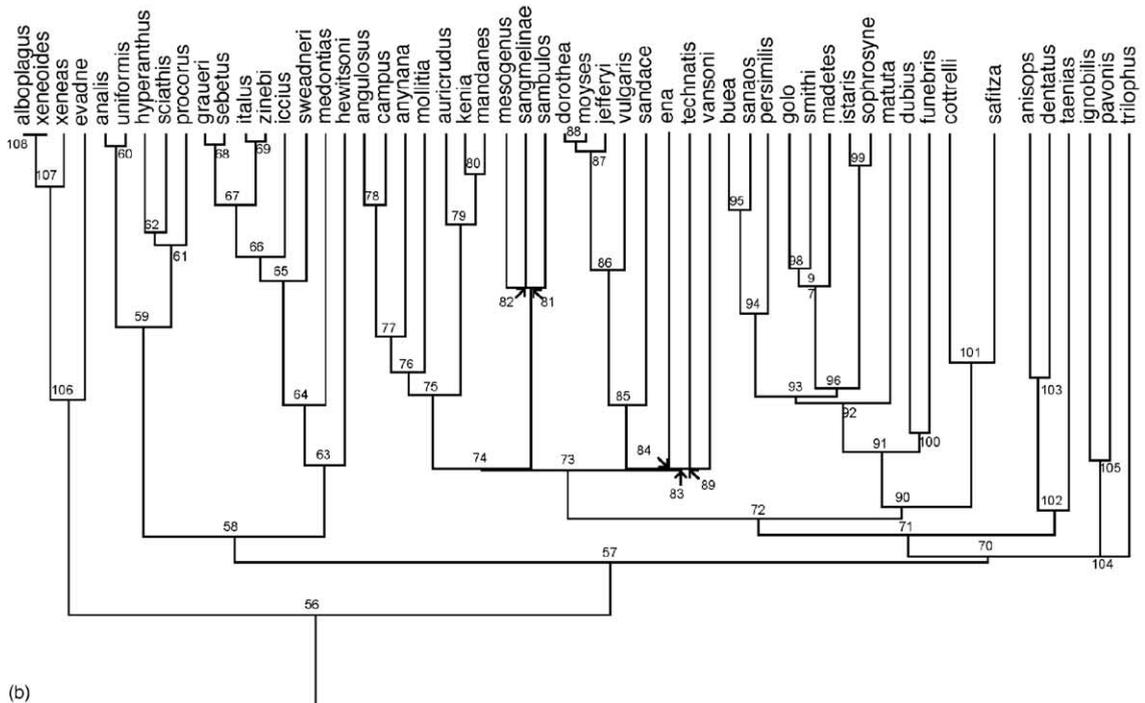
Due to the clear visualization of the calculated ancestral wing pattern data, a large amount of information can be absorbed by the user at a single glance. This allows the researcher to identify patterns within the data and formulate hypotheses. These hypotheses can then be tested both by further rigorous mathematical analysis of the data or by the design of further experiments and studies.

One of the assumptions behind the reconstruction of the ancestral wing patterns is that eyespot morphology is evolving at a constant rate by random drift, by strong stabilizing selection with randomly changing optima, or by directional selection with random fluctuations in direction (Felsenstein, 1988). We also assumed that the length of the molecular branch (amount of molec-

Ancient Wings

(a)

(b)

Fig. 2. (a) A screenshot from *Ancient Wings*. (b) Phylogenetic tree supplementary diagram, indicating the numbering and labeling system of the nodes used in the accompanying Excel data spreadsheet available online at http://www.acsu.buffalo.edu/~monteiro/ancientwings/.

ular evolution) is correlated with the amount of morphological evolution along the same branch. This last assumption has been supported in several other studies where branch lengths from independently derived molecular and morphological phylogenies were found to correlate (Omland, 1997). In the final animation of the program, however, we enforced a molecular clock and modified the branch lengths in order to portray evolution of the wing patterns against absolute time. This clock correction has the effect of slowing down or speeding up the rates of morphological change along subsets of the branches.

A recent paper showed that relatively small differences in ventral eyespots among two recently diverged species of Lycaenid butterflies played an important role in mate recognition (Fordyce et al., 2002). This indicated that small differences in morphology can be detected by the butterfly's visual system and be used in sexual selection or species recognition. In *Bicyclus*, although it has been shown that dorsal eyespot size may play a role in sexual selection (Breuker and Brakefield, 2002), no such effect has yet been shown for the ventral eyespots. It is also not clear what subtle differences in eyespot patterns may play in the interaction with predators. The actual selective forces driving the evolution of ventral wing patterns in the genus *Bicyclus* are largely unknown and thereby the reconstruction of ancestral traits is only valid as long as the assumption of constant rates of morphological evolution is valid.

Recent experimental work on one of the species of the genus, *Bicyclus anynana*, has showed that there are little constraints posed by the developmental system in obtaining a rapid response to artificial selection on eyespot size (Beldade et al., 2002b). In particular, selecting for opposing eyespot sizes on the same wing surface was accomplished with relative ease. One may ask whether if the selection experiments were performed with any of the other species in the genus, with distinct evolutionary histories the same result would hold. For instance, *Ancient Wings* can help us identify two species in the phylogeny that have a very different history of eyespot size evolution. In *B. anynana*, for instance, eyespot number 3 is usually smaller than eyespot 4 on the hindwing. Similar relative sizes are also found in the two closest relatives to *B. anynana* (*B. campus* and *B. angulosus*), and the eyespot size animation from the most recent common ancestor

(MRCA) to each of these three tip species (at around 7 million years ago) shows little morphological evolution in these two eyespots. In contrast, in the lineage leading to *B. sciathis*, there is rapid evolution of the relative sizes of eyespots 3 and 4 from a similarly aged ancestor. In this ancestor, eyespot 4 has a similar size to eyespot 3 but becomes much smaller relative to eyespot 3 in present day *B. sciathis* populations. Does this different evolutionary history influence present day levels of genetic variation and the matrix of genetic correlations for the sizes of these two eyespots as well as the relative ease that these two eyespots can be "uncoupled" from each other by present day artificial or natural selection? Does the developmental system retain some "memory" of recent events of morphological evolution? Will antagonistic selection on the sizes of eyespots 3 and 4 in *B. sciathis* be able to retrace the steps back to the MRCA in a much faster way than a similar selection on eyespots in *B. anynana* where there is no recent history of antagonistic size variation? Two other species that replicate a recent history of rapid evolution or stasis (from a 5 million year ancestor) involving eyespots 3 and 4, are *B. smithi* and *B. golo* (or *B. smithi* and *B. madetes*), respectively. These groups of paired species can be brought into the lab and used to test the role of recent evolutionary history on the speed of response to present day selection.

Another field of inquiry suggested by *Ancient Wings* is that of biogeography. Due to the visualization of changes in wing types unfolding alongside a timeline in the program, biogeographic studies can be examined. For example, the origin of certain taxa with distinct traits (such as larger wing size or missing eyespots) may be correlated with certain points in time. By conducting an analysis of the climatic changes occurring at these times, various hypotheses of the functional significance of wing traits can be examined.

The topic of functional integration may also be investigated. We observed how the eyespots change in size and position relative to each other and relative to the wing margin, and remarked that despite substantial changes in eyespot size, eyespots always tend to "dodge" each other by coordinated changes in position and/or size of neighbors. This avoids the merging of adjacent eyespots and produces consistent circular shapes. Mimicking circular "vertebrate eyes" may be important for deflecting the attacks of predators and the subsequent survival of these butterflies (Brakefield,

1996). This functional integration hypothesis awaits more rigorous testing. Alternatively, patterns of eyespot covariation may reflect underlying developmental compartments of the wing (Monteiro et al., 2003).

In addition, *Ancient Wings* can be used to analyze correlations between eyespot position and wing size. For example, Nijhout (1991, 2001) proposed that the dimensions of the wing section bordered by veins, where the eyespots develop, may determine the position of the eyespot's center (the focus) relative to the margin. If wing cells change shape or size, dependent morphogenetic processes of focus differentiation such as the putative mechanism of lateral inhibition of two diffusable interacting substances secreted by the wing veins, may change accordingly (Nijhout, 1991). This hypothesis may be investigated using *Ancient Wings* as a preliminary exploratory tool.

A fundamental assumption in the calculation of the ancestral wing patterns is that eyespot size has evolved gradually (at a constant rate). This may not have been the case. For instance, mutations of large phenotypic effect are known to occur sporadically in lab populations of one of the species, *Bicyclus anynana*, either introducing or removing fully developed eyespots, or shifting eyespots along the margin in an abrupt fashion (McMillan et al., 2002; Beldade and Brakefield, 2002; Monteiro et al., 2003). Presumably, *Ancient Wings* can be used to suggest in which lineages a gradual versus a punctuational type change has occurred on an evolutionary time-scale. Instances where the speed of the animation of certain eyespots (the rate of phenotypic change) is accelerated relative to the other eyespots could indicate that a linear model of ancestor reconstruction is trying to accommodate what is actually a much faster, non-gradual, process of evolutionary change (see Martins, 1994). In other words, by forcing the phenotypic difference between nodes to be divided equally by the time available between nodes, a mutation of large phenotypic effect (saltational evolution) would become represented in the animation as a rapid gradual change in morphology, standing out against the slower gradual animation of the other characters.

Ancient Wings, through its novel visualization method of evolutionary reconstruction, may be used both in the classroom and in the laboratory, both encouraging students to learn more about evolution and stimulating researchers to look at data in new ways. Its main strength lies in a continuous rendering of suites of ancestral character states that are displayed simultaneously. Hopefully, *Ancient Wings*, as a demonstration of this concept, will prompt others to bring evolutionary data to life.

References

Beldade, P., Brakefield, P.M., 2002. The genetics and evo devo of butterfly wing patterns. Nat. Rev. Genet. 3, 442–452.

Beldade, P., Brakefield, P.M., Long, A.D., 2002a. Contribution of distal-less to quantitative variation in butterfly eyespots. Nature 415, 315–317.

Beldade, P., Koops, K., Brakefield, P.M., 2002b. Developmental constraints versus flexibility in morphological evolution. Nature 416, 844–847.

Brakefield, P.M., 1996. Seasonal polyphenism in butterflies and natural selection. Trends Ecol. Evol. 11, 275–277.

Brakefield, P.M., Reitsma, N., 1991. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. Ecol. Entomol. 16, 291–303.

Breuker, C.J., Brakefield, P.M., 2002. Female choice depends on size but not symmetry of dorsal eyespots in the butterfly *Bicyclus anynana*. Proc. R. Soc. Lond. B 269, 1233–1239.

Brower, A.V.Z., 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. U.S.A. 91, 6491–6495.

Chang, B.S., Donoghue, M.J., 2000. Recreating ancestral proteins. TREE 15, 109–114.

Chang, B.S., Jonsson, K., Kazmi, M.A., Donoghue, M.J., Sakmar, T.P., 2002. Recreating a functional ancestral archosaur visual pigment. Mol. Biol. Evol. 19, 1483–1489.

Condamin, M., 1973. Monographie du genre *Bicyclus* (Lepidoptera, Satyridae). IFAN, Dakar.

Desalle, R., Freedman, T., Prager, E.M., Wilson, A.C., 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J. Mol. Evol. 26, 157–164.

Felsenstein, J., 1978. Cases in which parsimony and compatibility methods will be positively misleading. Syst. Zool. 27, 401–410.

Felsenstein, J., 1985. Phylogenies and the comparative method. Am. Nat. 125, 1–15.

Felsenstein, J., 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. 22, 521–565.

Fordyce, J.A., Nice, C.C., Forister, M.L., Shapiro, A.M., 2002. The significance of wing pattern diversity in the Lycaenidae:

mate discrimination by two recently diverged species. J. Evol. Biol. 15, 871–879.

French, V., Brakefield, P.M., 1995. Eyespot development on butterfly wings: the focal signal. Dev. Biol. 168, 112–123.

Maddison, W.P., Maddison, D.R., 1992. MacClade. Sinauer, Sunderland, MA.

Martins, E.P., 1994. Estimating the rate of phenotypic evolution from comparative data. Am. Nat. 144, 193–209.

Martins, E.P., 2001. Compare: computer programs for the statistical analysis of comparative data. Distributed by the author via the WWW at http://compare.bio.indiana.edu/, Bloomington, IN.

Martins, E.P., Hansen, T.F., 1997. Phylogenies and the comparative method: a general approach to incorporating phylogenetic information into the analysis of interspecific data. Am. Nat. 149, 646–667.

McMillan, W.O., Monteiro, A., Kapan, D.D., 2002. Development and evolution on the wing. Trends Ecol. Evol. 17, 125–133.

Monteiro, A., Pierce, N.E., 2001. Phylogeny of *Bicyclus* (Lepidoptera: Nymphalidae) inferred from COI, COII and EF-1a gene sequences. Mol. Phylogen. Evol. 18, 264–281.

Monteiro, A.F., Brakefield, P.M., French, V., 1994. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly *Bicyclus anynana*. Evolution 48, 1147–1157.

Monteiro, A., Brakefield, P.M., French, V., 1997. The genetics and development of an eyespot pattern in the butterfly *Bicyclus anynana*: response to selection for eyespot shape. Genetics 146, 287–294.

Monteiro, A., Prijs, J., Bax, M., Hakkaart, T., Brakefield, P.M., 2003. Mutants highlight the modular control of butterfly eyespot patterns. Evol. Dev. 5, 180–187.

Nijhout, H.F., 1991. The Development and Evolution of Butterfly Wing Patterns. Smithsonian Institution Press, Washington.

Nijhout, H.F., 2001. Elements of butterfly wing patterns. J. Exp. Zool. 291, 213–225.

Omland, K.E., 1997. Correlated rates of molecular and morphological evolution. Evolution 51, 1381–1393.

Roskam, J.C., Brakefield, P.M., 1996. A comparison of temperature-induced polyphenism in African *Bicyclus* butterflies from a savannah-rainforest ecotone. Evolution 50, 2360–2372.

Vischer, N.O.E., Huls, P.G., Woldringh, C.L., 1994. Object-image: an interactive image analysis program using structured point collection. Binary (Bioline) 6.