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Generating phenotypic variation: prospects from "evo-devo" research on *Bicyclus anynana* wing patterns

Patrícia Beldade,[a,b,*] Paul M. Brakefield,[b] and Anthony D. Long[a]

[a]Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525, USA
[b]Institute of Biology, University of Leiden, P.O. Box 9516, 2300 RA Leiden, The Netherlands
*Author for correspondence (email: pbeldade@uci.edu)

SUMMARY Understanding the generation of phenotypic variation is an important challenge for modern evolutionary biology, and butterfly wing patterns are an exciting system that can shed some light on this issue. Here, we report on recent advances in the genetics of *Bicyclus anynana* butterflies. This system provides the potential for a fully integrated study of the evolutionary and developmental processes underlying diversity in morphology.

DISSECTING THE MECHANISTIC BASIS OF PHENOTYPIC VARIATION

Heritable phenotypic variation is the raw material for evolution by natural selection, and understanding its generation is an important challenge in contemporary evolutionary biology (Stern 2000; Corley 2002). Furthermore, because genotypic and phenotypic variation are a universal characteristic of all living organisms, its study is central to all areas of biological research. Variation exists for most traits, in most organisms, including such complex traits as learning ability (Mery and Kawecki 2002), behavior (Mori 1999; Toma et al. 2002), and lifespan (Zwaan et al. 1995; Perls et al. 2002; Murphy et al. 2003), and many traits of medical (Glazier et al. 2002; Balmain et al. 2003; Botstein and Risch 2003) and economic (Wang et al. 1999; Andersson and Georges 2004) importance. An understanding of any biological process at whatever level of organization (from molecular–cellular level to the ecosystem) will be incomplete without knowledge of the mechanisms that account for variation intrinsic to the process. Conceptual and technological advances from the last decade have brought us to a position where the tools for a powerful dissection of the genetic basis of complex traits are now readily available (Black et al. 2001; Anderson and Ingham 2003), and are being used in many different systems.

Studies on insects have greatly contributed to such advances and to current knowledge of evolutionary developmental biology and morphological variation (Brakefield et al. 2003; Heckel 2003). Especially valuable have been studies on *Drosophila melanogaster* flies where the genetic dissection of variation across and within species has been attempted for different discrete and quantitative phenotypes, including bristle number (Mackay and Langley 1990; Lai et al. 1994; Long et al. 1995, 1996, 1998; Lyman and Mackay 1998), pattern of larval trichomes (Sucena and Stern 2000; Sucena et al. 2003), body pigmentation (Kopp et al. 2000; Wittkopp et al. 2003a, b), body size (Bochdanovits et al. 2003), ovariole number (Wayne et al. 2001; Wayne and McIntyre 2002), and eye-roughening (Dworkin et al. 2003). Despite the great potential of this model organism and its very sophisticated genetic resources, studies in other systems are essential not only to confirm the generality of the findings for *D. melanogaster* but also to fill important gaps in them (e.g., there is little known about the ecology of natural populations of fruitflies, and character manipulative experiments [cf. Brakefield and French 1995; French and Brakefield 1995] are difficult because of their small size). Exciting recent examples looking at the genetic basis of morphological variation in natural populations of nonmodel vertebrate organisms include eye regression in cave fish (Jeffery et al. 2003), melanism in pocket mice (Nachman et al. 2003), and pelvic reduction in threespine sticklebacks (Shapiro et al. 2004). However, such vertebrate systems remain difficult to transfer into the laboratory for extensive breeding programs. Here, we highlight past and ongoing research being carried out on the wing patterns of *Bicyclus anynana* butterflies, to illustrate the wide potential of this system for a comprehensive study of morphological variation, from genetics to phenotypes, via development and physiology, to ecology.

BUTTERFLY WING PATTERNS AS MODELS

Butterfly wing patterns are ideally suited to study the reciprocal interactions between evolutionary and developmental processes that shape variation in morphology (Nijhout 1991; Beldade and Brakefield 2002; McMillan

et al. 2002). There is great diversity in butterfly wing patterns with spectacular variation both across and within species. These patterns are interesting from an evolutionary point of view because they often have a known adaptive value as, for instance, in predator avoidance (Lyytinen et al. 2003; Brakefield and Frankino 2005) and mate recognition (Jiggins et al. 2001). Furthermore, our understanding of wing pattern development is expanding, with studies being carried out at different levels. We know about the genetic pathways that are involved in producing particular pattern elements (Carroll et al. 1994; Brakefield et al. 1996; Galant et al. 1998; Keys et al. 1999; Brunetti et al. 2001), about the cellular interactions at the basis of pattern formation (Nijhout 1980; Brakefield and French 1995; French and Brakefield 1995), about the physiological basis of pattern variants (Koch et al. 1996, 2003; Brakefield et al. 1998), and about the biochemical pathways (Koch et al. 2000a,b) that lead to the production of the pigments that are eventually deposited in the monochromatic-scale cells that cover butterfly wings.

The tropical Nymphalid *B. anynana* has been established as a laboratory organism and the patterns on its wings have been the focus of pivotal research in evo-devo (Beldade and Brakefield 2002). Lab studies have unveiled the existence of much genetic variation in wing patterns (Fig. 1), including standing quantitative variation that has enabled gradual responses to artificial selection (Figs. 1, B and F, and 2A), and spontaneous mutations of dramatic effect on wing patterns (Fig. 1, C and G). This species also shows seasonal variation in wing patterns resulting from hormonal-mediated plasticity in relation to environmental temperature during development (Koch et al. 1996; Brakefield and French 1999; Zijlstra et al. 2004). Furthermore, there is pattern variation among the ca. 80 *Bicyclus* species (Fig. 1, D and H), and both morphological (Condamin 1973) and molecular (Monteiro and Pierce 2001) phylogenies are available that can be used as a solid framework for comparative studies. This system provides an ideal opportunity for examining different types of variation, and comparing the developmental and genetic processes that underlie variation within species with those responsible for across-specific differences for developmentally dissectable traits of ecological relevance (Brakefield et al. 2003). There are few experimental systems where evo-devo can be studied in such an integrated manner.

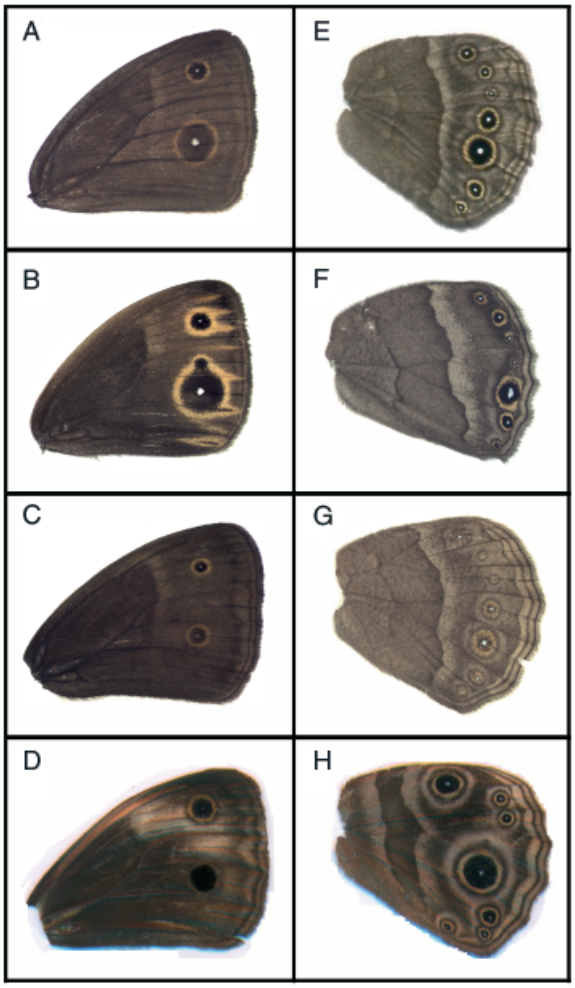

Fig. 1. Examples of different types of variation in *Bicyclus* wing patterns. (A–D) Dorsal surface of forewing, (E–H) ventral surface of hindwing. (A and E) "Wild-type" phenotypes of *B. anynana*. (B and F) Phenotypes obtained from gradual artificial selection in laboratory *B. anynana* populations: (B) selection on spread of eyespot-specific colored scales along the veins to produce the "lines" phenotype, and (F) selection on the eyespots to move toward the wing margin producing the "position" phenotype. (C and G) Phenotypes due to single spontaneous mutations of large effect in *B. anynana*: (C) *Pminus* mutant with reduced size of the posterior eyespot, and (G) *Goldeneye* with altered eyespot color. (D and H) Phenotypes of other *Bicyclus* species: (D) *B. alpoplagus* with characteristic eyespot relative size and color composition, and (H) *B. ignobilis* with characteristic eyespot relative size and position (photos D and H courtesy of Antónia Monteiro).

EVO-DEVO STUDIES ON *B. ANYNANA* EYESPOTS

The most striking feature of the *B. anynana* wings are the eyespots, serially repeated pattern elements composed of concentric rings of different colors. Evolutionary biologists, on the one hand, have characterized the patterns of genetic variance and covariance underlying variation in eyespot phenotypes. Artificial selection in the lab has revealed the presence of genetic variation for different aspects of eyespot morphology including their size (Monteiro et al. 1994; Beldade et al. 2002b,c), color composition (Monteiro et al. 1997a), shape (Monteiro et al. 1997b), and position along the proximal–distal axis (Brakefield 1998), and described patterns of correlations across different wing pattern traits (Monteiro et al. 1997c; Brakefield 1998; Beldade and Brakefield 2003a). Developmental biologists, on the other hand, have analyzed

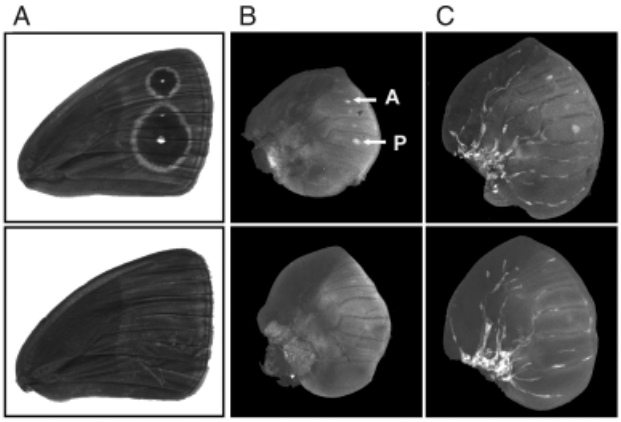

Fig. 2. Eyespot size and *engrailed* expression patterns in *Bicyclus anynana* selected lines. (A) Representative photos are shown of adult dorsal forewing surfaces from lines selected for larger (top) and smaller (bottom) eyespots. (B and C) Larval wing primordia stained against the En protein show quantitative differences in *en* expression associated with differences in adult eyespot size. Butterflies with large eyespots have larger and stronger areas of *en* expression on the location of the presumptive eyespot centers (brighter circles on wing regions indicated by white arrows on B, top; A for the anterior eyespot and P for the posterior). The differences in *en* expression between lines with large (top) and small (bottom) eyespots are clear both in early (before expansion of the trachea between dorsal and ventral wing surfaces, B) and mid (trachea already present in C) final instar larvae.

the expression of genes from *Drosophila* wing development on butterfly developing wings and identified a number of genetic pathways involved in eyespot formation. Genes such as *Distal-less* (*Dll*) (Carroll et al. 1994; Brakefield et al. 1996), *engrailed* (*en*) (Keys et al. 1999), *spalt* (Brunetti et al. 2001), and *Notch* (Reed and Serfas 2004) are expressed in association with the eyespot field in developing butterfly wings. These associations implicate the target genetic pathways in pattern formation, but tell us little about their contribution to pattern variation. To fully link the evolutionary and developmental studies mentioned above, we need to ask which of the genes (if any) within the implicated developmental pathways contribute to variation in phenotype. We have started to address this issue by implicating the transcription factor *Dll* as contributing to differences in eyespot size between *B. anynana* artificial selection lines (Beldade et al. 2002a). We found that lines with different adult eyespot sizes show quantitative differences in *Dll* expression in the presumptive eyespot area of developing wings, and that DNA sequence polymorphisms in *Dll* segregate with eyespot size phenotype in recombinants between the lines with divergent phenotypes, explaining up to 20% of the phenotypic difference between the selected lines (Beldade et al. 2002a).

Other developmental candidate genes remain to be tested and here we report on the first steps taken in that direction for the gene *en*. We looked at *en* expression patterns in final instar larvae from lines selected for large and small dorsal forewing eyespots (see Methods; Fig. 2). As previously described, *en* expression is restricted to the posterior compartment of the wing, and is seen at higher levels in association with the eyespot field (Keys et al. 1999). Furthermore, we found quantitative differences in *en* expression associated with differences in adult eyespot size (Fig. 2). This result, however, falls short of implicating *en* as contributing to variation in eyespot size, as differences in *en* expression might be because of DNA polymorphisms in *en* itself (*cis*) or in upstream regulators of *en* expression (*trans*) (candidate regulators in Keys et al. 1999). To distinguish between these alternatives, one needs to investigate whether *en* genotype and eyespot size phenotype co-segregate in experimental crosses involving the large- and small-eyespot selection lines (cf. Beldade et al. 2002a).

Knowledge about *D. melanogaster* wing development has undoubtedly greatly furthered our understanding of butterfly eyespot formation, and has provided a number of candidate genes for contributing to formation of, and variation in, this trait. However powerful this approach is, it is limited in that it does not generate candidates outside known *Drosophila* wing genes. Yet, because Lepidoptera and Diptera are so highly diverged and butterfly and fruitfly wings are so different (e.g., butterflies have an extra pair of wings, and colored scales covering their wings), it seems likely that not all the genes involved in butterfly wing pattern formation will be genes known from *Drosophila* wing development. Indeed, we have recently found that among the genes expressed in *B. anynana* developing wings (see Methods) are three genes involved in *Drosophila* eye pigmentation: *Henna* (*Hn*), *vermilion* (*v*), and *ruby* (*rb*) (Fig. 3A). This more unbiased approach suggests candidate genes typically not associated with *Drosophila* wing development, and that, in this case, are possibly involved in producing the color pigments on *B. anynana* wing scales (Koch 1991; Koch et al. 2000a).

NEW DEVELOPMENTS IN *B. ANYNANA* GENETICS

A number of genetic and genomic tools are currently being developed for *B. anynana* that can greatly further the potential of this butterfly as a target system for research on the genetic and developmental basis of morphological variation and its evolution.

The construction of Bacterial Artificial Chromosome (BAC) libraries for a number of Lepidopteran species, including *B. anynana*, is currently underway (http://www.genome.gov/Pages/Research/Sequencing/BACLibrary/LepidopteraBAC.pdf). This will be a useful resource not only for advancing the genetic studies of each of the target species but also for comparative studies of genome evolution in the Lepidoptera (butterflies and moths). This group contains over 150,000 species, including many of economic

A

Henna

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Bany:  1   LKAFGAGLLSSFGELQYCLSDKPELREXEPSVTGEQKYPITEYQPVYFVAQSFESAKEKM
Bmori: 119 LKAYGAGLLSSFGELQYCLSDKPELRELDPESTGGTKYPITEYQPVYFVANSFEDAQEKM
Dmel:  339 LKAYGAGLLSSYGELEYCLTDKPQLKDFEPEVTGVTKYPITQFQPLYYVADSFETAKEKT

Bany:  61  IKFAQTIPREFGVRYNPYTQSIDILDSPRQMTDLLKQIRTEM 102
Bmori: 179 IKLAQTIPRDLGVRYNPYTQSIDILDSARQIRVLMREVHQEM 220
Dmel:  399 IKFANSIPRPFGVRYNAYTQSVEVLDSKPQISNLMDNINSEF 440
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Ruby

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Bany:  1   NFADQDELVKLQLLSLAVKLSITQPD-TVPLSKYVLSLARYDASYDVRDRARMLRRFIDP
Dmel:  560 SFVDEQDVVKLQVLNLGVKLYLTNPEQTSLLCQYVFTLARYDPNYDVRDRARFLRQIIFP

Bany:  60  QQ--SGLLSQYAAQIFCPDKPKPTIQSNFKDRDQFTVGSLSQYIGSAAVGFRPLPQAPRA
Dmel:  620 ASGTSSVLSQHARQVFLASKPAPVPESKYRDGNNFQLGSLSHYLNMPAAGYKELPAFPVI

Bany:  118 PVGASQR 124
Dmel:  680 PPDSSVR 686
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Vermilion

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Bany:  29  LQGAIMITFYRDEPRFSQPHQLLTLLMDMDSLITKWRYNHVIMVQRMIGSQQLGTGGSSG
Bmori: 126 LQGAIMITFNRDEPRFSQPHQLLTLLMDIDSLITKWRYNHVIMVQRMIGSQQLGTGGSSG
Dmel:  273 LQGAIMITFYRDEPRFSQPHQLLTLLMDIDSLITKWRYNHVIMVQRMIGSQQLGTGGSSG

Bany:  89  YQYLRSTLSDRYKVFLDLFNLSTFLLPRSLIPPLDDGIKRDL 130
Bmori: 186 YQYLRSTLSD.............................. 195
Dmel:  333 YQYLRSTLSDRYKVFLDLFNLSTFLIPREAIPPLDETIRKKL 374
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B

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rb 1: AACTTTGCCGACCAGGACGAGCTAGTGAAGCTGCAACTGCTATCGTTAGCCGTCAAGCTG
rb 2: AACTTTGCCGACCAGGACGAGCTAGTGAAGCTGCAACTGCTATCGTTAGCCGTCAAGCTG 60

rb 1: TCCATAACCCAGCCGGACACGGTCCCGCTGAGCAAGTACGTGCTCTCGCTGGCGCGGTAC
rb 2: TCCATAACCCAGCCGGACACGGTCCCGCTGAGCAAGTACGTGCTCTCGCTGGCGCGGTAC 120

rb 1: GACGCGAGCTACGACGTGCGCGACAGAGCACGCATGCTCCGCCGGTTCATTGACCCGCAG
rb 2: GACGCGAGCTACGACGTGCGCGACAGAGCACGCATGCTCCGCCGGTTCATTGACCCGCAG 180

rb 1: CAGAGCGGCTTGCTCAGTCAGTACGCCGCGCAGATATTCTGCCCGGACAAACCGAAACCA
rb 2: CAGAGCGGCTTGCTCAGTCAGTACGCCGCGCAGATATTCTGCCCGGACAAACCGAAGCCA 240

rb 1: ACTATACAGAGCAATTTCAAAGACCGCGACCAGTTTACTGTGGGGTCGCTCTCACAGTAC
rb 2: ACTATACAGAGCAATTTCAAAGACCGCGACCAGTGTACTGTGGGGTCGCTCTCACAGTAC 300

rb 1: ATAGGTTCGGCAGCGGTGGGGTTCCGTCCCCTGCCTCAGGCCCCGCGCGCGCCCGTCGGA
rb 2: ATAGGTTCGGCAGCGGTGGGGTTCCGTCCCCTGCCTCAGGCCCCGCGCGCGCCCGTCGGA 360

rb 1: GCATCGCAGCGGGAGGCGGTCTCGGCCACGCTTGTCACACAAGTCACTGA
rb 2: GCATCGCAGCGGGAGGCGGTCTCGGCCACGCTTGTCACACAAGTCACTGA 410
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Fig. 3. Pigment synthesis genes expressed in *Bicyclus anynana* developing wings. (A) Sequence alignments of the *B. anynana* pigmentation proteins with the *Drosophila melanogaster* (Henna, GenBank AAF50517.1, 65% amino acid identity with *B. anynana*; Ruby, AAF45950.1, 47% identity; and Vermilion AAF47978.1, 75% identity) and *Bombyx mori* homologs (translation of Hn GenBank CK511723, 80% amino acid identity with *B. anynana*; and of v, CK508056, 74% identity). Gray-shaded amino acids are differences from the *B. anynana* sequence, dots represent unavailable sequence, and numbers show sequence position of amino acids. *B. anynana* partial protein sequences were obtained through translation of complementary DNA clones sequenced from the 3′ end (see Methods). (B) Alignment of two *B. anynana ruby* clones (GenBank AY766159 for rb1, and AY766160 for rb2) highlighting single nucleotide polymorphisms (SNPs) in gray. SNPs identified in this manner, using complementary DNA libraries made from a large number of outbred individuals (see Methods), will be used to build a high-density linkage map for *B. anynana*. As the per site heterozygosity in *B. anynana* is 0.5% (Beldade et al. 2002a), the majority of identified sequence differences should be true SNPs rather than sequencing errors (this will be explored in detail elsewhere).

importance (as agricultural pests, pollinators, and silk producers), but still has relatively few available genomic resources, despite recent developments for the silkmoth *Bombyx mori* (Nagaraju and Goldsmith 2002; Mita et al. 2003, 2004; Cheng et al. 2004; Xia et al. 2004). Genomic resources for different Lepidoptera species will enable the comparative analysis of phenomena that are particular to this group of insects, such as ZW sex determination, and holocentric chromosomes. The *B. anynana* BAC clones will furthermore be of great value to advancing our genetic dissection of pattern formation and diversification.

An extensive Expression Sequence Tags (EST) project is currently underway for *B. anynana* in order to identify new genes involved in wing pattern formation and variation. Using complementary DNA (cDNA) libraries built from different stages of developing wings (see Methods) biases gene discovery toward genes expressed in the relevant tissue (the wing), at the appropriate time (developmental stages when we know wing patterns are being specified; Brakefield et al. 1996; Koch et al. 2000b, 2003; Breuker and Brakefield 2003). Apart from being useful for identifying new *B. anynana* wing genes, these EST sequences can be the basis for the development of important genomic tools such as a linkage-map and DNA microarrays. EST redundancy enables identification of DNA sequence polymorphisms in wing genes (Fig. 3B), which can be used to build a high-density gene-based linkage map for this species (thus adding valuable "anchors" to the amplified fragment length polymorphism and microsatellite markers

already available). Such a map will be a fundamental step forward in the task of mapping wing pattern variation to gene regions, and simultaneously testing the contribution of a number of candidate loci to this variation.

cDNA clones can furthermore be spotted down to produce high-density gene arrays to look for differences in gene expression at different levels: (i) selection lines, mutant stocks, and species with phenotypes of interest, (ii) wet and dry seasonal forms (Brakefield and French 1999), (iii) different wing tissues (e.g., fore- and hindwing, wings at different developmental stages), (iv) different parts of the developing wings (e.g., eyespot-competent distal part vs. proximal part; eyespot presumptive focus vs. surrounding tissue; cf. French and Brakefield 1995), and (v) manipulated versus control wing discs (e.g., wing damage [Brakefield and French 1995] and transplanted tissue from an eyespot organizer or focus [French and Brakefield 1995], both of which characteristically result in the production of ectopic eyespots). These types of manipulative experiments are standard in the study of butterfly wing development and provide a valuable approach that is not readily available to Drosophilists. The size of the larvae and pupae of *B. anynana* makes organismal approaches to the study of development readily applicable (e.g., wing discs are much larger and thus easier to handle than those of *Drosophila*), while at the same time enabling the study of large laboratory and natural populations (an important limitation in vertebrate systems).

Another important recent advancement has been the development of germline transformation techniques for *B. anynana* (Marcus et al. 2004), to date the only butterfly where this is possible. Such techniques will be pivotal in testing the function of candidate genes and gene regions and their contribution to pattern development and evolution. Furthermore, they are an essential first step for the development of more sophisticated gene manipulation techniques already available in model systems (e.g., the powerful GAL4/UAS system [Duffy 2002] that enables temporal and spatially targeted gene expression in *D. melanogaster*).

PERSPECTIVES

The wing patterns of *B. anynana* butterflies provide an ideal opportunity to analyze different modes of phenotypic variation (intra- and interspecific, quantitative and mutants of large effect, and phenotypic plasticity) at different levels of biological organization (genetic pathways, cellular interactions, hormonal physiology, and ecological relevance). The genetic resources being developed for *B. anynana* will be used to gain an insight into important issues in the study of variation. What is the genetic basis of morphological variation of adaptive value? What are the specific genes that contribute to this variation? Are these the same genes that developmental, biochemical, and physiological studies have identified as being involved in trait formation (Beldade et al. 2002a)? Is phenotypic variation due to mutations that change coding or regulatory regions of these genes (Tautz 2000; Wray 2003; Genissel et al. 2004)? Do the same loci that harbor alleles of large effect responsible for extreme mutant phenotypes also harbor alleles of subtle effect that contribute to standing quantitative variation and response to artificial selection (Barton and Keightley 2002)? Are the same alleles identified using laboratory strains those that segregate in natural populations (Macdonald and Long 2004)? How genetically (and developmentally) diverged are different natural populations, that is, are similar phenotypes the result of the same, or of different processes (Jeffery et al. 2003)? What is the relationship between the genes that contribute to standing variation within populations and to differences between species (Orr 2001)?

Furthermore, studies of *B. anynana* wing patterns provide the opportunity to address other key issues in evolutionary developmental biology, including (i) the evolution of morphological innovations (Marshall et al. 1999; Arthur 2000) (such as the scale-covered wings, and the wing color patterns of butterflies) and the co-option of existing developmental pathways to produce such new phenotypes (Galant et al. 1998; Keys et al. 1999; Brunetti et al. 2001; True and Carroll 2002), (ii) modularity in development (Raff and Raff 2000) and how the developmental integration of traits might constrain or channel their evolutionary change (Beldade and Brakefield 2003b; Brakefield 2003), (iii) phenotypic plasticity and how the environment can influence development (Schlichting and Smith 2002; Brakefield and Frankino 2005), and (iv) the functional integration and concerted evolution of different aspects of morphology, such as butterfly wing patterns (Beldade and Brakefield 2002; McMillan et al. 2002) and butterfly color vision (Briscoe and Chittka 2001). The combination of different approaches to the study of different aspects of morphological character evolution in a single organism is extremely powerful and provides the opportunity for a truly integrated study of evolutionary developmental biology.

METHODS

Engrailed expression was studied in *B. anynana* butterflies from a pair of lines artificially selected for large and small anterior and posterior eyespots on the dorsal surface of the forewing (respectively, lines *AP1* and *ap1* from Beldade et al. 2002b). Wing discs from early and mid final-instar larvae were dissected out and stained with mouse Engrailed monoclonal antibody (Mab En4F11; Patel et al. 1989), and donkey anti-mouse fluorescein isothiocyanate secondary antibody (Jackson Laboratories, West Grove, PA, USA), following the protocol described in Brunetti et al. (2001). Images were collected on a Bio-Rad MRC 1024 ES laser confocal microscope (Bio-Rad, Hercules, CA, USA) using a $\times 5$ objective.

B. anynana homologs of *v*, *rb*, and *Hn* were cloned from cDNA libraries prepared from developing wings from a total of 188

laboratory outbred butterflies at different developmental stages (ranging from final instar-larvae to 72-h-old pupae). cDNA libraries were constructed from total RNA using the SMART cDNA kit and λTriplEx2.1 (Clontech, Palo Alto, CA, USA). From about 10,000 clones sequenced from the 3′ end using a modification of Clontech's protocol (Beldade, Rudd, Gruber, and Long, unpublished), we obtained one copy of the *B. anynana Henna* homolog (GenBank AY766157), one copy of *vermilion* (AY766158), and two copies of *ruby* (AY766159 and AY766160). We used ExPASy's translation tool (http://us.expasy.org/tools/dna.html) to obtain the translations of the nucleotide sequences and NCBI's BLAST tool (bl2seq; http://www.ncbi.nlm.nih.gov/BLAST/) to produce the protein alignments and calculate the percent of amino acid identity (Fig. 3).

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