

blood, and genetic distances from sequence data were consistent with RFLP analyses of purified mtDNA¹². We also scored length variation at 11 nuclear microsatellite loci using primers developed specifically for indigobirds²⁵.

Analyses

Phylogenetic relationships between mtDNA haplotypes were inferred using maximum parsimony as implemented in PAUP*²⁶. Measures of population differentiation (F_{ST}) were calculated using ARLEQUIN²⁷ and RSTCALC²⁸. To compare mtDNA haplotype frequencies between species, we used Φ_{ST} , an F_{ST} analogue that accounts for genetic distances between haplotypes. For microsatellites, we used R_{ST} , which accounts for differences in microsatellite repeat number under a stepwise mutation model. Standard F -statistics lead to identical conclusions. Significance was assessed using permutation procedures implemented in the respective programs. We excluded from analyses of population structure two individuals that may have been close genetic relatives of another individual in our sample, on the basis of shared location and mtDNA haplotype, and significantly greater microsatellite allele sharing than expected given population-level frequencies.

Significant genetic structure among indigobird species was not an artefact of geographic structure combined with sampling different species in somewhat different areas. Partial Mantel tests controlling for differences in allele frequency between species suggest no relationship between geographic distance and microsatellite allele sharing (West Africa: $r = -0.004$, $P = 0.34$; southern Africa: $r = 0.009$, $P = 0.21$). By contrast, allele sharing within species is greater than between species even when controlling for geographic distance between samples (West Africa: $r = -0.047$, $P < 0.001$; southern Africa: $r = -0.019$, $P = 0.052$; K.M.S. *et al.*, unpublished data).

To estimate the minimum number of host switches needed to explain the distribution of mtDNA haplotypes among indigobird species, the minimum number of steps in the multistate character 'host' was determined for the most parsimonious trees when 'host' was included as an additional character during tree search. To evaluate if the minimum number of host switches was smaller than expected under a null model of no association between host species and indigobird mtDNA haplotype, a null distribution was generated by reassigning individuals to hosts and determining the minimum number of host switches in 1016 replicate analyses.

To put the indigobird radiation in a broader phylogenetic context, we analysed mtDNA sequence data for representative indigobirds, other parasitic finches and their estrildid hosts. The phylogeny presented here (Fig. 3) is based on a maximum-likelihood analysis of 1,563 aligned positions, including the regions noted above plus half of the ND2 gene. Non-host estrildids were pruned from the tree, and branch lengths were estimated in PAUP*²⁶ under a GTR + I + Γ model of sequence evolution. We estimated relative divergence times using the Langley-Fitch method, as implemented in the program r8s²⁹, using a single calibration point of 20 million years for the divergence of parasitic and estrildid finches²⁰. Local molecular clocks were specified for *Vidua*, *Anomalospiza* and estrildids, respectively, to account for a faster rate of sequence evolution in parasitic finches²⁰.

Received 14 February; accepted 23 June 2003; doi:10.1038/nature01863.

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Supplementary Information accompanies the paper on www.nature.com/nature.

Acknowledgements For comments, logistical support, and assistance in the field and lab, we thank Laura Payne, Chris Balakrishnan, Clive Barlow, Adrian Craig, Nick Davies, Roger Fotoso, Kathy Groschupf, Janet Hinshaw, Mark Hopkins, Kit Hustler, Lacey Knowles, Kevin Njabo, Nedra Klein, David Mindell, and Bob Stjernstedt. Funding was provided by US National Science Foundation Grants to R.B.P. and M.D.S. and an Erwin Schrödinger Fellowship from the Austrian Science Fund for K.M.S.

Competing interests statement The authors declare that they have no competing financial interests.

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Genetic mechanisms and constraints governing the evolution of correlated traits in drosophilid flies

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 Some morphological traits differ greatly between related species, but it is not clear whether diversity evolves through changes in the same genes and whether similar, independent (that is, convergent) changes occur by the same mechanism^{1,2}. Pigmentation in fruitflies presents an attractive opportunity to explore these issues because pigmentation patterns are diverse, similar patterns have arisen in independent clades, and numerous genes governing their formation have been identified^{3–5} in *Drosophila melanogaster*. Here we show that both evolutionary diversification and convergence can be due to evolution at the same locus, by comparing abdominal pigmentation and trichome patterns and the expression of *Bric-à-brac2* (*Bab2*), which regulates both traits in *D. melanogaster*^{3,6}, in 13 species representing the major clades^{7,8} of the subfamily Drosophilinae. Modifications of *Bab2* expression are frequently correlated with diverse pigmentation and trichome patterns that evolved independently in multiple lineages. In a few species, *Bab2* expression is not correlated with changes in pigmentation but is correlated with a conserved pattern of trichomes, indicating that this locus can be circumvented to evolve new patterns when a correlated trait is under different constraints.

In *D. melanogaster*, the abdominal melanic pigmentation pattern comprises a banding pattern (found in most Drosophilinae), in which each abdominal segment bears a dark stripe, with a superimposed dimorphic pattern in which males are darkly pigmented on their terminal segments A5 and A6 (Fig. 1a). This dimorphic pattern is controlled by the products of the *bric-à-brac* (*bab*) locus³, which contains two largely redundant genes^{3,6}, *bric-à-brac1* (*bab1*) and *bric-à-brac2* (*bab2*) derived from a tandem duplication. Both genes are expressed sex-specifically and segment-specifically in

the pupal abdomen during epidermal development³, during which the Bab proteins act as repressors of pigmentation. It has been shown³ that dimorphic pigmentation associated with the posterior male-specific repression of *bab* evolved within the *D. melanogaster* species group (a group of closely related species within the subgenus *Sophophora*⁹; Fig. 2).

Because other species of the *Sophophora* subgenus are monomorphic with regard to pigmentation and *bab* regulation, as well as more distant species outside the *Sophophora*, we had no *a priori* expectation that *bab* had a role in pigmentation divergence in any group outside the *melanogaster* species group. We were therefore surprised to find that Bab2 expression is modulated in a variety of patterns in other members of the Drosophilinae (Fig. 1; see Fig. 2 for phylogeny). A survey of 13 species with conspicuous and diverse melanic pigmentation patterns representing the major clades of this subfamily^{7,8} revealed a spectrum of species-specific modulations of Bab2 expression in the dorsal epidermis of developing pupal abdomens (Fig. 2). Four modes of Bab2 regulation occur in the subfamily that are well correlated with adult patterns of pigmentation, including monomorphic posterior repression (*D. immigrans*, Fig. 1g), elevated expression along the dorsal midline (*D. funebris*, Fig. 1e; *D. immigrans*, Fig. 1h), and repression in territories along the dorsal midline (*D. tripunctata*, Fig. 1k; *D. duncani*, Fig. 1n). The male-specific posterior repression of Bab2 associated with pigmentation found in species of the subgenus *Sophophora* (*D. melanogaster*, Fig. 1b) has evolved repeatedly in the subfamily (*D. funebris*, Fig. 1d; *D. duncani*, Fig. 1m). Bab2 expression is further diversified by the combination of these four modes of regulation. For instance, the posterior repression of Bab2 can be dimorphic or not, and associated or not with modulation in expression along the midline (for example, *D. duncani* combines dimorphic and midline repression of Bab2).

The correlation between Bab2 expression and the repression of melanic pigmentation ranges from a very close correspondence between Bab2 expression and areas of no pigmentation in species such as *D. melanogaster* or *D. tripunctata* (Fig. 1a, b, i, j, k) to a partial correlation in other species in which Bab2 relates to some aspects of the pigmentation, such as *D. phalerata* (dimorphic posterior pigmentation, Fig. 2) but other regulators are necessary to explain the entire pattern (midline repression). In regions where Bab2 expression prefigures the absence of pigmentation, it is not strictly repressed in the prospective pigmented areas and activated elsewhere; rather, pigmentation seems to be repressed where Bab2 is expressed above a threshold (Fig. 1c, e, f, h, i, k, n). The widespread association of Bab2 expression with repression of pigmentation suggests that Bab2 functions as a regulator of pigmentation in most species and was expressed in the abdominal epidermis of a common ancestor.

Importantly, however, in 3 of 13 species no correlation exists between Bab2 expression and pigmentation. In *D. santomea* (Figs 2 and 3a), a close relative to *D. melanogaster*, both sexes are devoid of dark pigments. This is a recent loss of the melanic pattern, because its sister species *D. yakuba* is dimorphic¹⁰. One might expect Bab2 to be expressed uniformly throughout the abdomen in *D. santomea* to repress pigmentation, but that is not so. Bab2 is expressed in a dimorphic *melanogaster*-like pattern (Fig. 3a). The loss of pigmentation in *D. santomea* must therefore be due to evolution at other loci. The conservation of Bab2 expression is consistent with a recent genetic association study that ruled out the *bab* locus as contributing to differences in pigmentation between *D. yakuba* and *D. santomea*¹¹.

However, this raises the question of why Bab2 is regulated dimorphically in this species if it is not correlated with pigmentation. We found that another abdominal character, the pattern of trichomes, is perfectly correlated with Bab2 expression in *D. santomea* (Fig. 3a). Trichomes are small non-sensory hairs that cover the cuticle (Fig. 3b, inset). In *D. melanogaster*, *bab* function is

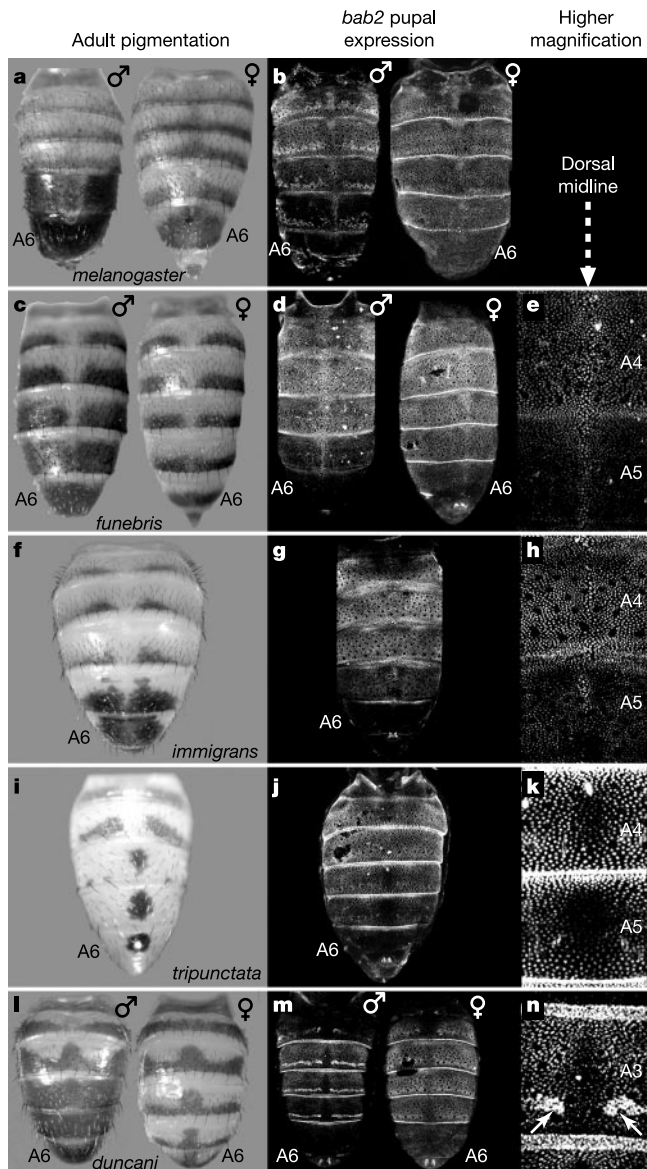


Figure 1 Modulation of Bab2 expression is correlated with diverse abdominal pigmentation patterns. **a, c, f, i, l**, Dorsal view of abdomens of *D. melanogaster* (**a**), *D. funebris* (**c**), *D. immigrans* (**f**), *D. tripunctata* (**i**) and *D. duncani* (**l**) adults. Pigmentation varies between species in the posterior segments, along the dorsal midline and between sexes. **b, d, g, j, m**, Bab2 expression in the developing adult epidermis of each species revealed by immunostaining is correlated with the repression of adult pigmentation. **e, h, k, n**, Higher-magnification views of Bab2 immunostainings along the dorsal midline (indicated by large arrow). Note that in the *D. tripunctata* adult, the spot on A4 is smaller than those on A5 and A6; this is related to the area and level of Bab2 repression. In *D. melanogaster* and *D. funebris*, the Bab2 protein concentration is somewhat lower but still detectable in A5 and A6 in the females, whereas it is faint in A5 and undetectable in A6 in the males. The small arrows in **n** point to a non-epidermal structure, the oenocytes, that express high levels of Bab2.

required for both pigmentation repression and trichome development³. The *melanogaster*-like pattern of Bab2 expression in *D. santomea* suggests that *bab* controls these traits independently and that their evolution can be uncoupled.

A second, illuminating example of a lack of correlation between Bab2 expression and pigmentation is found in *D. serrata*, another close relative of *D. melanogaster*. Pigmentation is dimorphic in some populations of this species¹², but the pattern is opposite to the *D. melanogaster* pattern: the male is pale and the female is darkly pigmented on A6 (Figs 2 and 3b). Despite this pattern reversal

between the sexes, we found a *melanogaster*-like Bab2 pattern in which posterior repression occurs in the male. This pattern is also correlated with the trichome distribution (Fig. 3b). We infer that Bab2 does not repress pigmentation in this species, which allows dark pigmentation of A6 in the female. The expanded posterior pigmentation in *D. serrata* females is a convergent pattern that must have evolved through a different genetic pathway, independent of *bab*, indicating that phenotypic convergence does not necessarily rely on the same genetic mechanism^{1,13}. We suggest that control of the trichome pattern by *bab2* has been maintained in *D. melanogaster*,

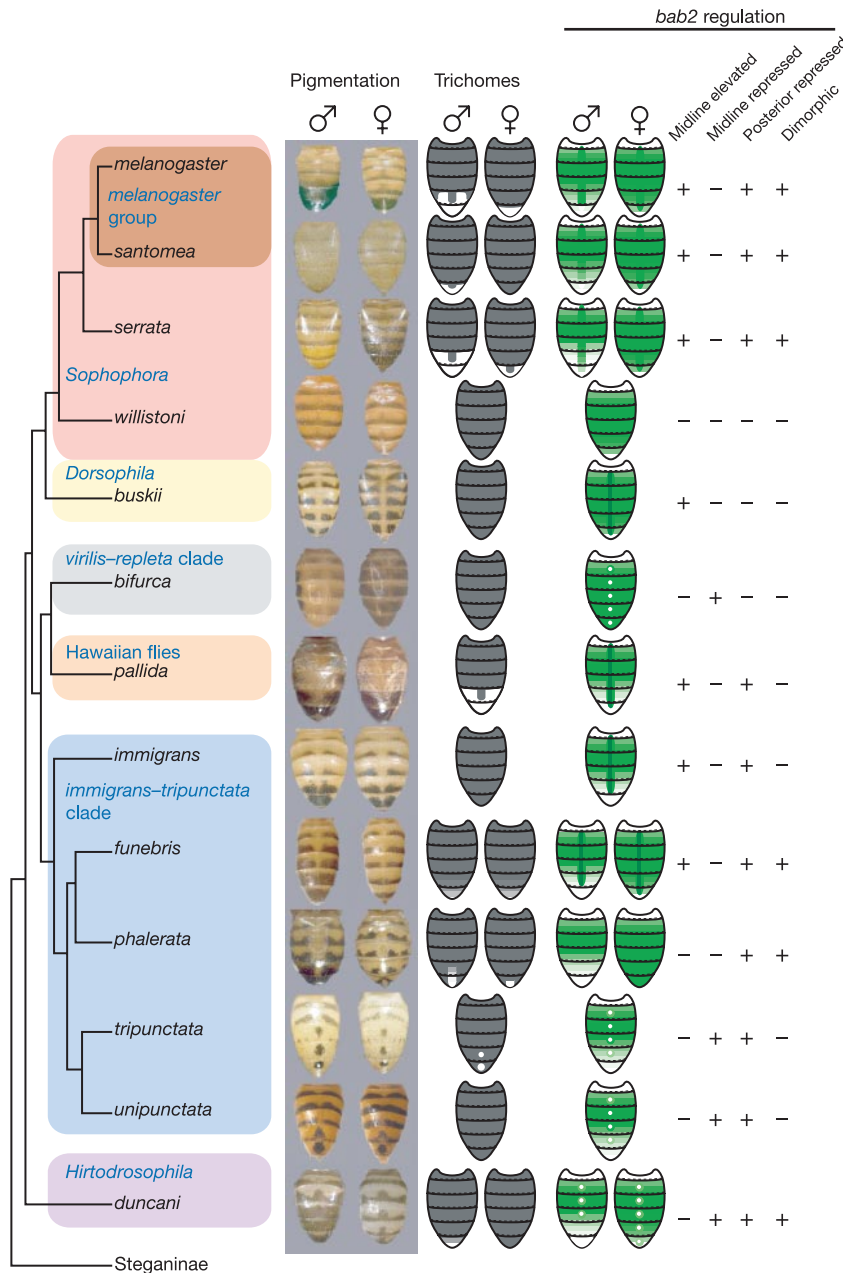


Figure 2 Modulation of Bab2 expression underlies the diversification and evolutionary convergence of cuticular traits throughout the Drosophilinae. Depicted are the phylogeny of the Drosophilinae, the variety of abdominal pigmentation patterns, Bab2 expression in respective species and sexes (green shading), the trichome patterns (grey shading) and the different modes of *bab2* regulation observed in each species (+ or - for presence or absence, respectively). Left, the phylogeny of the Drosophilidae shown here is adapted from ref. 8. There are conflicts in phylogenies of the family^{7,8,24} and none might reflect the actual evolutionary tree. We relied on the two largest phylogenies of the

family, one based on morphology⁷, the other on DNA sequences⁸, to reconstruct (using parsimony assumptions) the four changes observed in *bab2* regulation with MacClade version 4.03 (ref. 25) and found that characters have changed multiple times (13 changes in all for each phylogeny, but with different distributions). Note that trichomes are present on A1 in all species, although Bab2 is consistently repressed in this segment throughout the subfamily, except in some species that express Bab2 along the dorsal midline.

D. santomea and *D. serrata* because their absence from the terminal segments in males has some biological role.

The correlation between Bab2 expression and trichome patterns holds in many of the species we examined (9 of 13 species, for example *Scaptomyza pallida*, Fig. 3c; *D. tripunctata*, Fig. 3d), indicating that in most cases the two traits have evolved in concert, apparently under control of *bab*. However, there are a few exceptions such as *D. immigrans* (Fig. 3e) and *D. unipunctata* (Fig. 2), which are covered by trichomes in regions where Bab2 is strongly down-regulated, indicating that trichome pattern evolution can also be uncoupled from Bab2.

In species in which patterns have become uncoupled from Bab2 (*D. santomea* and *D. serrata*), it is reasonable to infer that each trait might be under selective pressures that are in conflict with respect to Bab2 function (for example, to become darker on A6 while preserving trichome formation on that segment). Alleles at other loci affecting one of the traits might be selected so that trait evolution might circumvent Bab2 function. In natural populations of *D. melanogaster*, variation at the *bab* locus is associated with

about 60% of the variation in female posterior pigmentation¹⁴, indicating that 40% of the variation is contributed by other loci. Such loci could evolve as major regulators of pigmentation if *bab* is constrained by a stable trichome pattern (as in *D. santomea* or *D. serrata*).

Our results indicate that Bab2 regulation has been modified repeatedly, leading to its modulation within the abdominal field, which seems to be the basis of much of the abdominal pattern diversification in this subfamily (Fig. 2). We suggest that changes *in cis* to *bab2*, in regulatory elements responding directly or indirectly to one or more body plan regulators (*AbdominalB* and *doublesex* for segment specificity or sex specificity³, possibly *pannier*¹⁵ or *decapentaplegic*¹⁶ for regulation along the dorsal midline), are responsible for the diversification of Bab2 expression. It is less likely that changes in the spatial deployment of these regulators account for differences in Bab2 patterns between species, because comparative data indicate that these regulators of body pattern are strongly constrained and that their expression or function is usually conserved at the family or order level^{17–19}. Isolation and analysis of *bab2* regulatory elements from different species will be required to determine how *bab* regulation has evolved.

Evolution of Bab2 expression underlies most cases of phenotypic convergence in our survey. Character reconstruction (legend to Fig. 2) shows that all of the regulatory changes in *bab2* have evolved more than once when we consider either of the two major phylogenies of the family^{7,8}. We suggest that convergence was achieved through similar, independent regulatory changes in *bab*. Other animal groups also exhibit melanic variation on one or a few basic themes, such as the spot patterns on the forewings of ladybirds. Alleles controlling many different patterns that occur in the 2-spot ladybird map to a single locus^{20,21}, indicating that different alleles represent regulatory variants and that similar ladybird patterns could arise through a common genetic mechanism. We suggest that the flexible modulation of a transcription factor that regulates colour patterns might be a general mechanism governing the diversity of body-colour patterns in related animal species. □

Methods

Breeding of flies

Flies were bred on cornmeal medium sometimes supplemented with banana and kept at 20 °C. Staging follows ref. 22 for *D. melanogaster*. A similar developmental stage was identified empirically for other species developing at various rates.

Immunocytochemistry

Epidermis from the dorsal abdomen of pupae were dissected (fat body was cleaned off) in PBS, and stained with an anti-Bab2 antibody³ and a fluorescein isothiocyanate-conjugated anti-rat IgG antibody (Jackson ImmunoResearch). Preparations were processed for confocal imaging on an Optiphot microscope (Nikon) equipped with a ×10 dry lens and a Bio-Rad 1024 system. In all species, Bab2 is expressed in the cells that prefigure the adult epidermis and the highest level of expression occurs at a stage equivalent to 38–42 h after puparium formation in *Drosophila melanogaster*²².

Cuticle preparation

Adult cuticles were cleared for 1 h in 10% KOH at 60 °C, washed in water and mounted flat in Hoyer's medium²³, then processed for dark-field imaging with a ×10 dry lens on a Zeiss Axiophot microscope equipped with a Kontron charge-coupled-device camera.

Received 26 March; accepted 14 May 2003; doi:10.1038/nature01787.

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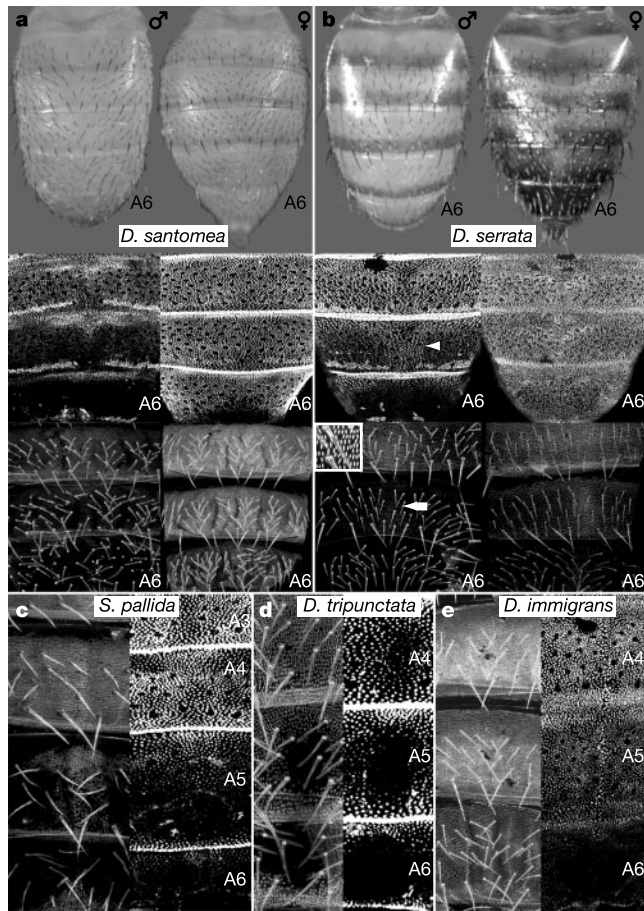


Figure 3 Bab2 expression is correlated with the distribution of cuticular trichomes. **a**, *D. santomea* adults are devoid of melanin pigment (top). Bab2 distribution (middle) is not correlated with repression of pigmentation, but is correlated with the presence of trichomes (bottom). Trichomes are small epidermal productions spread amid the sensory bristles (higher-magnification inset in **b**, bottom). **b**, *D. serrata* adults exhibit dimorphic melanin (top) but with a sexual pattern the reverse of *D. melanogaster*. Bab2 is expressed in a *melanogaster*-like pattern (middle) which is not correlated with pigmentation but prefigures the pattern of trichomes. Trichomes are absent from A6 and part of A5 in the males, but do form along the dorsal midline (arrow) where Bab2 expression is elevated (arrowhead). **c–e**, The correlation between trichomes (left panels) and Bab2 expression (right panels) is general in Drosophilinae as exemplified in *S. pallida* (**c**) and *D. tripunctata* (**d**). Occasionally, however, trichomes appear in areas where Bab2 is strongly repressed, as in the A6 segment in *D. immigrans* (**e**).

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Acknowledgements We thank C. Nelson for help with fly collection; D. Lachaise and P. O’Grady for species identification; T. Markow, L. Andrew (Tucson Stock Center), J. Coyne and D. Lachaise for providing fly stocks; F. Laski and D. Godt for the Bab2 antibody; V. Kassner for invaluable technical assistance; A. Rokas for help with character reconstruction; and B. Williams, A. Kopp, A. Rokas and C. Nelson for discussions on the project. N.G. has been funded by the Howard Hughes Medical Institute and the Philippe foundation and is supported by an EMBO long-term post-doctoral fellowship. The project was supported by the Howard Hughes Medical Institute (S.B.C.).

Competing interests statement The authors declare that they have no competing financial interests.

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Regulatory evolution of *shavenbaby/ovo* underlies multiple cases of morphological parallelism

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Cases of convergent evolution that involve changes in the same developmental pathway, called parallelism, provide evidence that a limited number of developmental changes are available to evolve a particular phenotype¹. To our knowledge, in no case are the genetic changes underlying morphological convergence understood. However, morphological convergence is not gener-

ally assumed to imply developmental parallelism². Here we investigate a case of convergence of larval morphology in insects and show that the loss of particular trichomes, observed in one species of the *Drosophila melanogaster* species group, has independently evolved multiple times in the distantly related *D. virilis* species group³. We present genetic and gene expression data showing that regulatory changes of the *shavenbaby/ovo* (*svb/ovo*) gene underlie all independent cases of this morphological convergence. Our results indicate that some developmental regulators might preferentially accumulate evolutionary changes and that morphological parallelism might therefore be more common than previously appreciated.

The pattern of microtrichiae (hereafter referred to as trichomes) on the ventral surface of first-instar larvae seems to be conserved across the genus *Drosophila*, whereas the dorsal and lateral surfaces have repeatedly evolved different patterns³. In most species, three kinds of trichome are produced in a specific pattern on the dorsal surface⁴. In a single species of the *D. melanogaster* subgroup, *D. sechellia*, many thin trichomes have been lost and the cells instead differentiate naked cuticle^{3,5}.

The patterning of dorsal trichomes is an interesting case of convergent morphological evolution, because four species from the *D. virilis* species group (*D. ezoana*, *D. borealis* eastern, *D. lacicola* and *D. montana*) also show evolutionary loss of the thin trichomes³ (Supplementary Fig. 1). All other species of the *D. virilis* species group that we have examined (*D. americana*, *D. borealis* western, *D. canadiana*, *D. flavomontana*, *D. kanekoi*, *D. littoralis*, *D. lummei*, *D. novamexicana* and *D. virilis*) and the more distantly related *D. arizonae* possess a lawn of trichomes similar to that observed in *D. melanogaster*. By mapping these phenotypes onto a recent molecular phylogeny of the *D. virilis* species group⁶, we can infer that at least three evolutionary transitions are required to explain the current distribution of trichome loss (Fig. 1). The convergence of trichome patterns in different fly lineages indicates that these changes might be driven by natural selection^{7,8}, although the selection pressure has not yet been identified. In addition, the evolutionary loss of trichomes in first-instar larvae mirrors an ontogenetic loss of the same trichomes in second-instar and third-instar larvae⁹ in all species we have examined, suggesting that these trichomes have a special function in first-instar larvae.

In theory, many genes might have evolved to alter the patterning of larval trichomes. For example, the *wingless* (*wg*) and *hedgehog* (*hh*) pathways and the *lines* gene are involved in patterning the trichomes on the dorsal epidermis^{4,10}. It might therefore be interesting to test whether evolution of patterning genes involved in segmentation can account for the evolution of trichome patterns. However, it has been shown³ that six genes involved in segmentation (*wg*, *gooseberry-distal*, *patched*, *engrailed*, *abdominal-A* and *hunchback*) are expressed identically in 12 species of the *D. virilis* species group, indicating that differences in trichome patterning might have evolved by changes in genes downstream of the segmentation pathway.

We have previously reported, after a full-genome genetic scan, that regulatory evolution at the *svb* gene accounts fully for the difference in trichome pattern between *D. sechellia* and other species of the *D. melanogaster* species group⁵. It has been shown¹¹ that the transcription factor Svb acts to switch cells between naked cuticle and the production of trichomes. In *D. melanogaster* embryos, *svb* is genetically required for trichome formation; when *svb* is expressed in a cell, that cell autonomously differentiates trichomes, whose morphology is determined by other patterning genes^{4,10}. Therefore *svb* integrates numerous sources of information (including the *wg*, *hh*, DER (for *Drosophila* epidermal growth factor receptor), homeotic and dorsal-ventral patterning systems) to specify the final pattern of trichomes.

To determine the genetic nature of the phenotypic differences observed in the *D. virilis* species group, we performed interspecific