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

Acknowledgements We thank M. Akam and R. Jenner for comments on the manuscript, I. Ruiz Trillo for sharing unpublished results, and the scientists of Kristineberg Marine Station for help in sample collection. We are grateful for support from the Wellcome Trust to M.J.T. and to D.T.J.L.

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

Correspondence and requests for materials should be addressed to M.J.T. (mt281@cam.ac.uk).
Newly determined sequences have been submitted to GenBank under accession numbers AY291292–AY291293.

Speciation by host switch in brood parasitic indigobirds

Michael D. Sorenson[1,2], Kristina M. Sefc[1] & Robert B. Payne[2,3]

[1]*Department of Biology, Boston University, Boston, Massachusetts 02215, USA*
[2]*Museum of Zoology and* [3]*Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan 48109, USA*

A growing body of empirical and theoretical work supports the plausibility of sympatric speciation[1–3], but there remain few examples in which all the essential components of the process are well understood. The African indigobirds *Vidua* spp. are host-specific brood parasites. Indigobird nestlings are reared along with host young, and mimic the mouth markings of their respective hosts[4–6]. As adults, male indigobirds mimic host song[4–7], whereas females use these songs to choose both their mates and the nests they parasitize[8]. These behavioural mechanisms promote the cohesion of indigobird populations associated with a given host species, and provide a mechanism for reproductive isolation after a new host is colonized. Here we show that all indigobird species are similar genetically, but are significantly differentiated in both mitochondrial haplotype and nuclear allele frequencies. These data support a model of recent sympatric speciation. In contrast to the cuckoo *Cuculus canorus*, in which only female lineages are faithful to specific hosts[9,10], host switches have led to speciation in indigobirds because both males and females imprint on their hosts[8,11].

The high degree of host specificity in indigobirds led previously to the suggestion that host–parasite associations in African finches were the product of a long history of co–speciation[4]. This model accounted for the remarkable mimicry of host mouth markings by the young parasites without requiring specialist parasites to have colonized hosts with different mouth markings. Genetic studies, however, indicate that indigobird species have a much more recent origin than their hosts[12,13]. Indeed, the lack of differentiation among indigobirds in mitochondrial DNA (mtDNA) restriction-fragment length polymorphism (RFLP) markers[12] is somewhat difficult to reconcile with their distinct behaviour[14] and morphology (Fig. 1). Behavioural imprinting in both males (song mimicry)[11] and females (mate choice, host choice)[8] suggests a mechanism for rapid sympatric speciation: indigobirds reared by a novel host species acquire the songs of that host and mate assortatively, resulting in immediate reproductive isolation after a new host is colonized. If this model is correct, the genetic similarity of indigobirds may be attributed to their recent origin from a common ancestor, but evidence of current reproductive isolation also is predicted. We tested this by comparing mitochondrial haplotype and nuclear microsatellite allele frequencies among seven indigobird species in West Africa (samples from Cameroon and Nigeria) and four species in southern Africa (samples from Zimbabwe, Zambia, Malawi and South Africa).

Figure 2 shows unrooted mtDNA haplotype trees for indigobirds. Species within each region share a set of closely related haplotypes, with overall diversity similar to that typically found within a single avian species. For example, a maximum divergence of 2.1% between

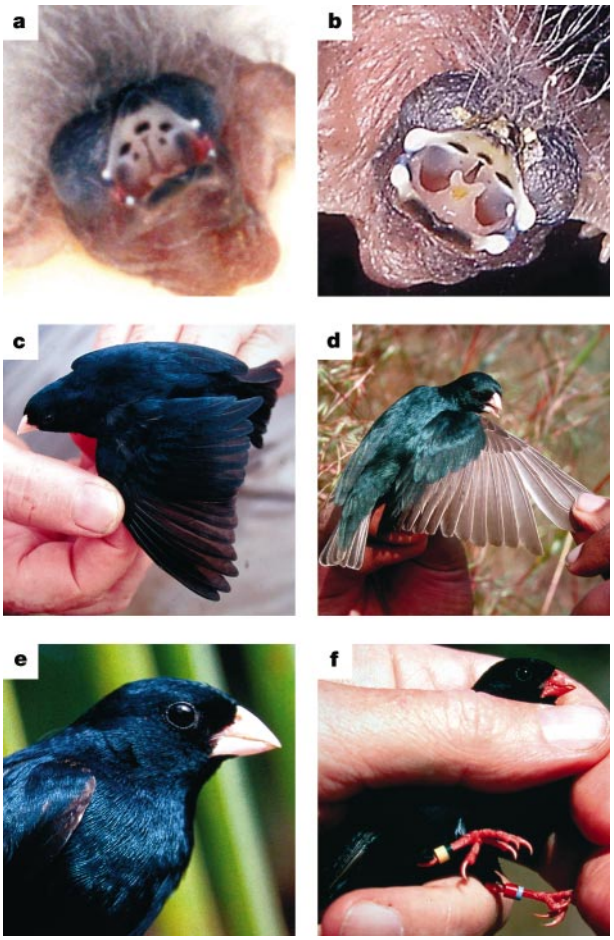

Figure 1 Examples of morphological variation between indigobird species. Nestling mouth markings in *V. camerunensis* (**a**) and *V. chalybeata* (**b**) mimic the young of their firefinch hosts, *L. rara* and *L. senegala*, respectively. Dark wing and plumage in *V. chalybeata* from West Africa (**c**). Pale wing and green plumage in *V. raricola* (**d**). White bill and blue plumage in *V. camerunensis* (**e**). Red bill and orange feet in *V. chalybeata* from southern Africa (**f**). See ref. 30 for a complete description of morphological differences between indigobird species.

western and southern indigobird haplotypes is less than that observed within two of their broadly distributed hosts, red-billed firefinch *Lagonosticta senegala* (2.5%) and African firefinch *L. rubricata* (3.9%) (see also Fig. 3). Nonetheless, haplotype frequencies differ significantly between indigobird species, particularly in West Africa (West Africa: $\Phi_{ST} = 0.41$, $P < 0.0001$; southern Africa: $\Phi_{ST} = 0.043$, $P = 0.0014$), providing strong evidence of current reproductive isolation[15]. Most pairwise comparisons between individual species were also significant (see Supplementary Information).

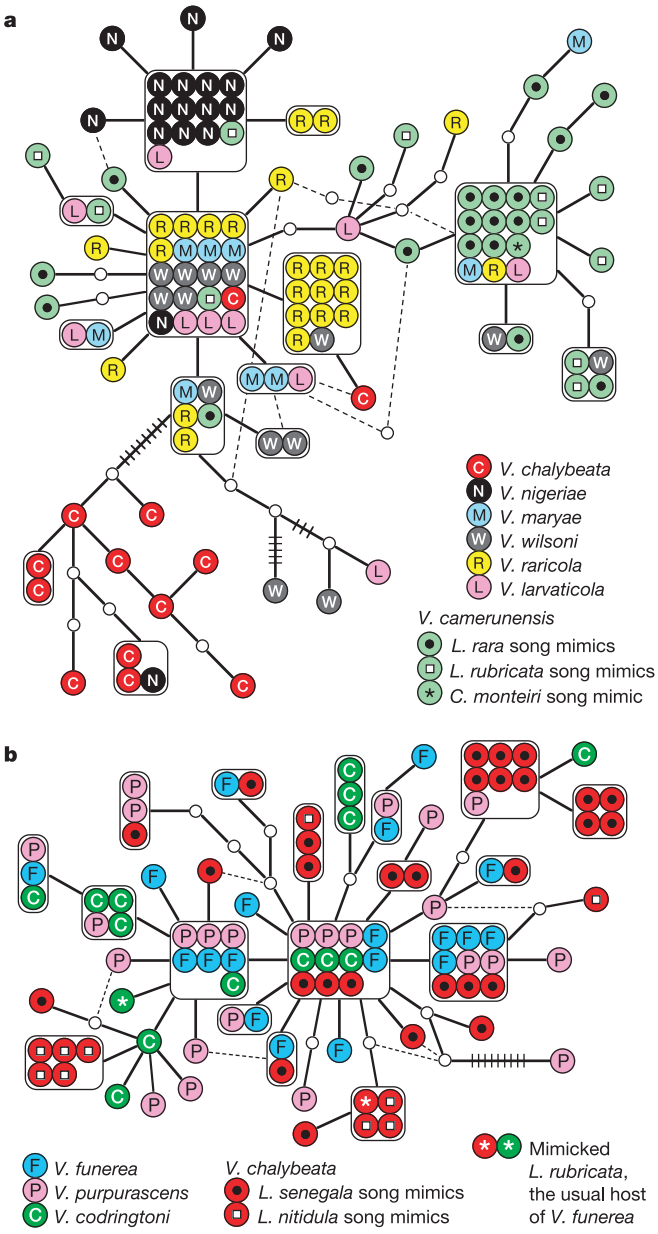

Figure 2 mtDNA haplotype trees for indigobirds. Unrooted phylogenetic trees are shown for 118 indigobirds representing seven morphologically distinct species from Cameroon and Nigeria (**a**), and 98 indigobirds representing four species from southern Africa (**b**). Each circle represents an individual bird. Individuals within a box share the same haplotype. Each line segment represents a single nucleotide substitution, except for branches with cross-marks, which indicate multiple steps. Dotted lines show alternative connections present in one or more of the 3,264 and 1,288 equally parsimonious trees for the two data sets, respectively. One haplotype was found in both regions: the *V. purpurascens* haplotype in the lower right of **b** is identical to the common *V. camerunensis* haplotype in **a**.

In each region, the most common haplotype is shared by all species, and many other haplotypes are derived from it by one or two mutations (Fig. 2). This suggests the retention of a common ancestral haplotype and is consistent with expectations for recent vicariant speciation[15]. In indigobirds, however, we suggest that speciation follows the colonization of new host species. If indigobird species retain multiple ancestral lineages, then multiple females must have colonized each new host. An indigobird population along the Zambezi river provides a potential example of this: some village indigobirds *V. chalybeata* in this region are associated with brown firefinch *L. nitidula* rather than their usual host, red-billed firefinch *L. senegala*[16]. Indigobirds associated with brown firefinch have four unrelated mtDNA haplotypes, a limited sub-sample of those present in southern birds (Fig. 2b).

Another test of genetic differentiation that emphasizes the colonization process is to treat 'host' as a character and determine the minimum number of changes (that is, host switches) required to explain the current distribution of mtDNA haplotypes. A minimum of 18 host switches is required in southern Africa, whereas at least 22 host switches are required in West Africa, significantly fewer than under a null hypothesis of no genetic structure (southern Africa: $P < 0.006$; West Africa: $P \ll 0.001$), but larger than the minimum possible value of one switch per host.

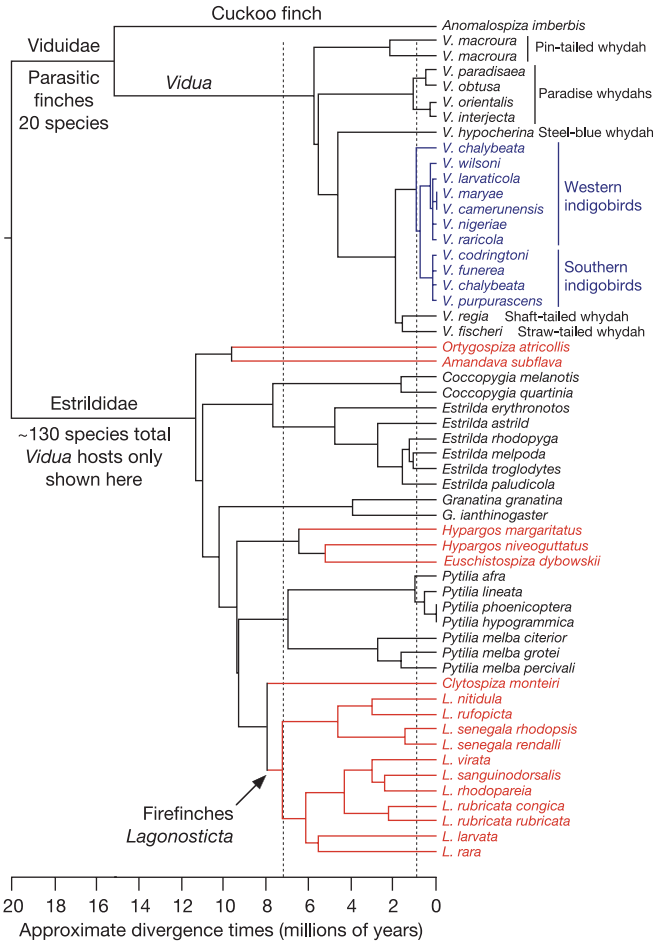

Figure 3 mtDNA phylogeny of brood parasitic finches and their estrildid finch host species. (The cuckoo finch is a parasite of several more distantly related warblers.) Indigobirds are shown in blue; firefinches and other indigobird hosts are shown in red. Other estrildids shown are hosts of the various whydahs. Dotted lines indicate the most recent mtDNA ancestor for indigobirds and firefinches, respectively. Absolute values of divergence times should be viewed as rough approximations at best, but relative times are directly comparable between host and parasitic lineages.

The same behavioural mechanisms involved in host colonization and speciation might also be responsible for occasional hybridization and introgression between established indigobird species. If a female parasitizes a host already associated with a different indigobird species, any surviving offspring will imprint on the alternative host and mate with individuals of the other parasitic species, resulting in hybridization in the second generation. A clear example of relatively recent introgression is the quailfinch indigobird *V. nigeriae* with a haplotype typical of *V. chalybeata* (Fig. 2a, bottom). This individual is the descendant of a *V. chalybeata* female that laid in a quailfinch *Ortygospiza atricapilla* nest, the usual host of *V. nigeriae*. This egg-laying 'mistake' must have occurred at least two and perhaps many more generations in the past, because the individual in question was morphologically *V. nigeriae* and not *V. chalybeata*.

In southern Africa, the mimicry songs of 490 male indigobirds were consistent with their morphology, whereas four males mimicked a species other than the usual host[14]. This provides a direct estimate of the proportion of young indigobirds that result from 'mislaid' eggs (~0.8%) and the potential rate of hybridization among established indigobird species. This is an impressively small number from a behavioural point of view, reflecting the importance of host imprinting in determining the egg-laying behaviour of female indigobirds, but is large by population genetic standards, where the diversifying effects of genetic drift may be counteracted by as few as one migrant per generation ($Nm = 1$)[17]. The above model of hybridization predicts no sex bias in 'gene flow' between species (because mislaid eggs produce both males and females), and therefore differentiation in both mitochondrial and nuclear DNA. Alternatively, frequent mating between male and female indigobirds of different song types would prevent differentiation in nuclear genetic markers even if females remain faithful to hosts in egg laying.

In contrast to recent results for cuckoo host races[9], nuclear microsatellite data provide evidence of significant genetic differentiation between indigobird species in each region, consistent with assortative mating of indigobirds reared by a particular host species. Overall R_{ST} values (see Methods) were significantly greater than expected under a null hypothesis of no genetic structure (southern Africa: $R_{ST} = 0.027$, $P < 0.0001$; West Africa: $R_{ST} = 0.034$, $P = 0.0015$). Some but not all pairwise comparisons between individual species also were significant (see Supplementary Information). Smaller values of R_{ST} (nuclear microsatellites) than Φ_{ST} (mtDNA) could be taken as evidence of sex-biased gene flow between species. In our view, this interpretation is not warranted, however, because (1) lower differentiation values are expected for highly variable microsatellite markers[18,19], and (2) the indigobird system is clearly not at equilibrium—following recent speciation, lineage sorting should proceed more rapidly for mtDNA than for nuclear loci given their different effective population sizes[15].

An alternative to our conclusion of recent speciation is a model in which indigobirds have had long and continuous associations with their current hosts but remain in a perpetual state of incomplete speciation due to gene flow. By impeding differentiation at neutral loci, ongoing gene flow could cause species to appear more recently diverged than they really are[1]. Both microsatellite data and nuclear intron sequences, however, are consistent with mtDNA in suggesting limited differentiation among all indigobirds, and an absence of the divergent lineages that would be expected at some loci if indigobirds had a more ancient history (see Supplementary Information). In addition, a phylogeny of parasitic finches indicates a recent divergence of southern and western indigobirds, and of indigobirds from their sister group, straw-tailed whydah *V. fischeri* plus shaft-tailed whydah *V. regia*, placing potential upper limits on the age of southern indigobird species and all indigobirds, respectively (Fig. 3). Finally, southern indigobirds show lower levels of genetic diversity for both mtDNA (Fig. 2) and microsatellites (see Supplementary Information), consistent with a recent origin from western ancestors (Fig. 3).

Absolute estimates of speciation times are complicated by a lack of relevant fossil information, which is useful for calibrating rates of mtDNA sequence evolution, and the potential for continuing gene flow between species. Using a rough estimate of 20 million years for the divergence of parasitic and estrildid finches[20], the common mitochondrial ancestor of all indigobirds occurred less than a million years ago, whereas the history of their primary hosts, the firefinches *Lagonostica* spp., is an order of magnitude longer (Fig. 3). Species divergence times, however, may be substantially more recent than the deepest divergence among extant mtDNA haplotypes[21]. Given an ancient origin of obligate parasitism in finches (Fig. 3), extant indigobirds may represent only the latest iteration of a dynamic process of host colonization, speciation and extinction in the parasitic lineage[20].

A remaining question is the evolution and maintenance of host-specific mouth mimicry in nestling indigobirds. Selection by host parents must be strong enough to generate this mimicry, but not so strong as to make occasional host switching impossible. A potential solution is that host discrimination varies with ambient food supply and/or the previous experience of individual hosts[22,23], such that selection on mouth markings varies over time or between host nests. Unfortunately, nothing is known of the genetics of mouth markings in indigobirds.

Indigobirds provide an example in vertebrates of sympatric speciation through host shifts, as has been suggested for phytophagous insects[2]. The behavioural mechanisms responsible for reproductive isolation in indigobirds have been tested experimentally and provide a clear explanation for rapid speciation[8,11]. Sexual selection in the form of female choice is an important component of this process. By mimicking host song, males advertise their success at having been reared by a particular host, whereas females acquire for their offspring compatible mouth mimicry genes by using song to choose their mates. In contrast to models in which divergent ecological selection precedes the evolution of assortative mating[3], behavioural imprinting sets the stage for sympatric speciation in indigobirds and facilitates the response to divergent selection on mouth patterns. Behavioural imprinting might also contribute to infrequent hybridization, but significant morphological and genetic differentiation among indigobird species indicates a strong degree of current reproductive isolation. □

Methods

Indigobird samples

During field work between 1991 and 2000, adult male indigobirds were recorded and then trapped using song playback. Adult males ($n = 190$) were identified to species on the basis of morphology and, secondarily, by host song mimicry. Indigobirds were collected from 30 separate locations (>5 km apart) in southern Africa and 23 locations in Cameroon and Nigeria, such that few closely related individuals are included in our sample (see also below). Along the Zambezi River in western Zambia, some *V. chalybeata* mimicked the songs of brown firefinch *Lagonosticta nitidula* rather than those of the usual host, red-billed firefinch *L. senegala*[16]. In Cameroon, *V. camerunensis* mimicked either African firefinch *L. rubricata* or black-bellied firefinch *L. rara*. In Nigeria, one male *V. camerunensis* mimicked brown twinspot *Clytospiza monteiri*. Finally, one male *V. codringtoni* and one male *V. chalybeata* sang the songs of *L. rubricata* rather than their usual hosts[12,14] (see Fig. 2). A smaller number of females ($n = 16$) and juveniles ($n = 10$) were identified to species on the basis of association with a given host species (for example, indigobird nestling observed with host) or association with male indigobirds of known species combined with consistent morphology (including mouth markings in juveniles). Samples for genetic analysis included feathers from birds that were individually marked and released, or muscle tissue from birds prepared as museum specimens. Further information on host-parasite associations and the criteria used to recognize indigobird species[24] is provided in Supplementary Information.

Genetic data

For each indigobird, we sequenced a 1,100-base-pair region of mtDNA that comprised most of ND6, tRNA-Glu and the 5′ half of the control region, using two overlapping primer pairs[16]. We are aware of potential problems caused by nuclear copies of mtDNA and are certain that limited genetic differentiation among indigobird species is not an artefact of this phenomenon. Extracts were from muscle tissue or feathers rather than from

blood, and genetic distances from sequence data were consistent with RFLP analyses of purified mtDNA[12]. We also scored length variation at 11 nuclear microsatellite loci using primers developed specifically for indigobirds[25].

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 ARLEQUIN[27] and RSTCALC[28]. 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 r8s[29], using a single calibration point of 20 million years for the divergence of parasitic and estrildid finches[20]. Local molecular clocks were specified for *Vidua*, *Anomalospiza* and estrildids, respectively, to account for a faster rate of sequence evolution in parasitic finches[20].

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 Fotso, 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.

Correspondence and requests for materials should be addressed to M.D.S. (msoren@bu.edu).
DNA sequence data have been deposited in GenBank under accession numbers AF090341, AF407021-AF407036, AF407107-AF407122, AY136611, AY322613-AY322837, AY323530-AY323607 and AY324232-AY324306.

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

Nicolas Gompel & Sean B. Carroll

Howard Hughes Medical Institute and Laboratory of Molecular Biology, University of Wisconsin, 1525 Linden Drive, Madison, Wisconsin 53706, USA

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.