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The genetic basis of adaptive pigmentation variation in *Drosophila melanogaster*

John E. Pool[1,2] and **Charles F. Aquadro**[1]

1*Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York, 14853*

Abstract

In a broad survey of *Drosophila melanogaster* population samples, levels of abdominal pigmentation were found to be highly variable and geographically differentiated. A strong positive correlation was found between dark pigmentation and high altitude, suggesting adaptation to specific environments. DNA sequence polymorphism at the candidate gene *ebony* revealed a clear association with the pigmentation of homozygous third chromosome lines. The darkest lines sequenced had nearly identical haplotypes spanning 14.5 kilobases upstream of the protein-coding exons of *ebony*. Thus, natural selection may have elevated the frequency of an allele that confers dark abdominal pigmentation by influencing the regulation of *ebony*.

Keywords

Ecological adaptation; abdominal pigmentation; *ebony*; partial selective sweep; *Drosophila melanogaster*; sub-Saharan populations

INTRODUCTION

As part of the interface between an organism and its environment, pigmentation holds considerable interest for the study of ecological adaptation. Within the genus *Drosophila*, pigmentation often varies greatly between species (e.g. Wittkopp *et al.* 2003a). For example, closely related species of the *D. dunni* subgroup show diverse pigmentation phenotypes which are more closely correlated with geographic location than with phylogeny (Hollocher *et al.* 2000). In contrast, most species of the *D. melanogaster* subgroup have similar pigmentation, although the lightly pigmented *D. santomea* is a notable exception (Lachaise *et al.* 2000).

Pigmentation can also vary within Drosophilid species. For example, “black” and “brown” morphs of *D. elegans* occur in different parts of this species’ range (Hirai and Kimura 1997). In *D. kikkawai* from Brazil, darker individuals were found in cooler seasons and habitats (da Costa *et al.* 2003). Yet in Brazilian samples of *D. polymorpha*, darker individuals were collected from warmer, more arid sites (Brisson *et al.* 2005). These contrasting results point out that a variety of factors may account for differences in pigmentation, including thermal regulation, desiccation resistance, resistance to parasitism, camouflage, ultraviolet protection, mating preferences, and neutral drift (reviewed in Wittkopp *et al.* 2003a).

Corresponding author: John E. Pool, University of Copenhagen, Universitetsparken 15, bygn. 10, 2100 Copenhagen, Denmark, Phone: +45 3532 1348, Fax: +45 3532 1281, e-mail: jpool@binf.ku.dk.
[2]Current address: Centre for Comparative Genomics, Institute of Biology, University of Copenhagen, Copenhagen, Denmark

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Natural variation in pigmentation has been documented for *D. melanogaster* as well. One polymorphic trait is the thoracic trident, a feature that may be absent entirely or present with varying levels of black pigment. Phenotypic plasticity in response to temperature has been observed for this trait, but there is also evidence for genetic polymorphism (David *et al.* 1985). Populations from Europe (David *et al.* 1985) and India (Munjal *et al.* 1997) show geographic clines for this trait, with flies from higher latitudes (and in the latter case, higher altitudes) having darker tridents. This pattern is consistent with the hypothesis that darker pigmentation aids thermal regulation of flies from colder environments by allowing them to absorb more heat from solar radiation.

Another polymorphic trait in *D. melanogaster* is female abdominal pigmentation. Female flies grown at lower temperatures have black pigment covering a greater portion of abdominal segments 5, 6, and 7 (David *et al.* 1990; Gibert *et al.* 1996). This phenotype also varies between lines from different geographic locations grown at the same temperature (Gibert *et al.* 1996; Gibert *et al.* 1998), suggesting a genetic effect. Kopp *et al.* (2003) detected an association between this trait and a QTL corresponding to the *bric a brac* locus.

The biochemical pathway underlying pigmentation in *D. melanogaster* has been partially elucidated, enabling a candidate gene approach to investigate the genetic basis of pigmentation differences. Wittkopp *et al.* (2002a) found that altered expression of the *ebony* and *yellow* genes produced a wide variety of pigmentation phenotypes in *D. melanogaster* (from nearly all yellow to nearly all black). Regulation of these genes is spatially correlated with wing spots in *D. biarmipes* (Wittkopp *et al.* 2002a), and a specific *cis*-regulatory change at *yellow* involved in the evolution of this spot was identified by Gompel *et al.* (2005). In another study, Wittkopp *et al.* (2002b) found that regulation of *yellow* contributed to divergent pigmentation phenotypes in three distantly-related Drosophilids (*D. melanogaster*, *D. subobscura*, and *D. virilis*). On the other hand, a QTL analysis of darkly pigmented *D. americana* and its lightly pigmented relative *D. novamexicana* suggested that *ebony* and at least three other genes, but not *yellow*, were involved in this pigmentation divergence (Wittkopp *et al.* 2003b).

The genic identity of *tan*, which catalyzes the reverse of the reaction performed by *ebony*, was only recently identified (True *et al.* 2005). Overexpression of *tan* or underexpression of *ebony* result in an excess of brown and black pigment, while the opposite changes produce pale yellow flies (Wittkopp *et al.* 2002b; True *et al.* 2005). Several other genes are known to affect the spatial pattern of abdominal pigmentation (Wittkopp *et al.* 2003a), including *optomotor-blind*, for which an association between haplotype and pigmentation was observed in *D. polymorpha* (Brisson *et al.* 2004).

Analysis of pigmentation differences between the very lightly pigmented *D. santomea* and its sister species, *D. yakuba*, did not reveal any QTL corresponding to *ebony* or *tan* (Carbone *et al.* 2005). While the fourth largest QTL might correspond to *yellow* or *optomotor-blind*, the three strongest QTL apparently reflect other pigmentation-related genes. Thus, while several genes may be considered strong candidates for pigmentation evolution, it is not possible to predict *a priori* which genes account for a particular phenotypic change.

In the present study, variation in pigmentation was documented within and between 25 population samples of *D. melanogaster*. All but four samples were from sub-Saharan Africa, which contains the ancestral range of the species (Lachaise *et al.* 1988; Pool and Aquadro 2006). Because the most dramatic pigmentation differences appeared to involve relative levels of yellow and brown pigment, *ebony* was investigated as a candidate gene. Genotype-phenotype associations and evidence for positive selection were assessed at this locus.

MATERIALS AND METHODS

Fly stocks studied

Isofemale lines examined for pigmentation were from population samples described by Pool and Aquadro (2006). Pigmentation was also scored for extracted (homozygous) X chromosome, 2nd chromosome, and 3rd chromosome lines derived from isofemale lines by a series of crosses with balancer stocks. Due to the crossing scheme, extracted autosome lines will have X chromosomes from the balancer stocks only (which are of cosmopolitan origin). For non-extracted autosomes in these lines, on average 25% of alleles will derive from the original isofemale line and 75% will be from the balancer stocks. The source populations of these homozygous lines were Cameroon (Mbengwi, Nkouondja, and Oku), Kenya (Malindi), Uganda (Namulonge) and Zimbabwe (Lake Kariba).

Scoring of pigmentation phenotypes

Flies were kept on standard yeast medium at 21°C, and transferred to new food weekly, until aged 30 to 33 days (initial observations had suggested that dark pigmentation might take some time to develop). One female fly from each line was anesthetized with carbon dioxide and photographed at 40x magnification using a Kodak DC290 camera. Exposure time, zoom width, and illumination level were kept constant.

Pigmentation was scored using Adobe Photoshop 8.0. For each female fly, the lightly pigmented (yellow or brown) portion of the fourth abdominal segment was examined from the right lateral perspective. This segment was selected because it was clearly visible in the photographs and black pigment always covered less than the posterior 50% of this segment. The specific location studied was near the anterior edge of the fourth segment, half-way between the dorsal midline and the tergite-sternite boundary (Figure 1). This location was selected because it was free from glare and fading in the photographs, was free of bristles, and displayed only the pigmentation of interest. Grayscale darkness (ranging from 0% to 100% in 1% units) was recorded within a selected square (250 × 250 pixels, or about 0.1 mm × 0.1 mm on an unmagnified fly). We did not estimate the portions of the fifth, sixth, and seventh abdominal segments covered by black pigment because these traits could not be scored accurately in the darkest individuals.

Crosses performed

Crosses involving four extracted 3rd chromosome lines (representing a variety of pigmentation intensities) were performed, and each of these lines was also crossed with a balancer stock (TM6) homozygous for the recessive e^1 mutant allele of *ebony*. The pigmentation of offspring was measured as described above.

Nucleotide variation and association-testing

Complete DNA sequences covering the *ebony* gene region were obtained for homozygous 3rd chromosome lines from the Kenya and Uganda population samples using standard protocols for DNA extraction, PCR amplification, and DNA sequencing. Sequence reads were aligned using Sequencher 4.2. Final alignments were constructed using MegAlign 5.08 and manually corrected. The published genome sequence of *D. sechellia* was used as an outgroup.

Summary statistics including Tajima's (1989) *D*, Fay and Wu's (2000) *H*, Z_{nS} (Kelly 1997), and the F_{st} of Hudson *et al.* (1992) were calculated using DnaSP 4.10 (Rozas *et al.* 2003). Simulations to test the significance of observed haplotype identity were performed using *ms* (Hudson 2002), using θ inferred from Kenya observed π and a conservative recombination rate of $\rho = 0.2$ / site (less than the 0.288 obtained from published estimates of *r* and N_e). Associations

between *ebony* polymorphism and pigmentation were tested using the permutation method of Doerge and Churchill (1996).

RESULTS

Geographic variation in female abdominal pigmentation

Abdominal pigmentation of female *D. melanogaster* was examined by quantifying the darkness of pigment within the yellow (or brown) section of the fourth abdominal segment (hereafter referred to as "A4 pigmentation"), as shown in Figure 1. Striking pigmentation differences were observed among the 356 individuals assayed. A4 pigmentation values, measured as percent grayscale darkness, averaged 65.9% (with a standard deviation of 6.0%) but ranged from 49% (a pale tan color) to 84% (dark brown; Figure 1).

Contrasting pigmentation was observed between populations with little or no genetic differentiation according to a recent study (Pool and Aquadro 2006). At the X-linked loci examined in that study, Uganda (mean A4 = 74.0%) was only modestly differentiated from the Kenya-M sample (mean A4 = 61.4%) and other eastern African samples. Likewise, no genetic structure was detected among any of the Cameroon samples, which had mean A4 values ranging from 64% to 72.1%. No relationship was found between genetic differentiation (F_{ST}) and pigmentation (difference in mean A4 values) in the full data or within sub-Saharan Africa (Mantel tests).

Notably, all 25 isofemale lines with A4 values above 75% were from sites above 1000 m in altitude. A strong positive correlation was observed between A4 pigmentation and altitude, whether population means ($R^2 = 0.433$; $P = 4.75 \times 10^{-4}$) or individual A4 values ($R^2 = 0.134$; $P = 9.09 \times 10^{-13}$) were considered. This correlation was stronger when only the 16 population samples from sub-Saharan Africa thought to be free from cosmopolitan admixture (Pool and Aquadro 2006) were considered, with $R^2 = 0.590$ and $P = 5.10 \times 10^{-4}$ for population means (Figure 2b) and $R^2 = 0.439$ and $P = 3.31 \times 10^{-13}$ for individual values.

A much weaker correlation was also observed between A4 pigmentation and latitude (degrees north or south of the equator) based on individual values ($R^2 = 0.037$; $P = 2.65 \times 10^{-4}$), but not population means ($R^2 = 0.121$; $P = 0.095$). This correlation was in the opposite direction expected from previous studies (darker flies at lower latitudes) and disappeared when latitude was compared against the residual values from the altitude regression ($R^2 = 0.003$; $P = 0.289$). Therefore, the latitude effect was due to higher elevation samples occurring at lower latitudes in our study. No correlation was found between longitude and pigmentation.

Genetic contributions to dark pigmentation

Pigmentation was also examined for extracted chromosome lines (which are homozygous for a specific chromosome from the original population sample). If extracted chromosome lines from darkly and lightly pigmented population samples are compared, a significant difference between the pigmentation scores of these samples may indicate that the extracted chromosome contributes to the pigmentation differences originally observed in the isofemale lines.

Significant differences between the A4 pigmentation distributions of extracted chromosomes from "dark" and "light" population samples were detected for the X chromosome and the 3rd chromosome, but not for the 2nd chromosome (Table 1). Therefore, the X and 3rd chromosomes may each contain one or more genetic factors responsible for dark pigmentation in high altitude sub-Saharan *D. melanogaster*.

Evidence for natural selection and genotype-phenotype associations at *ebony*

As a candidate gene on the third chromosome, *ebony* was selected for population genetic analysis and association testing. A contiguous block of 20.5 kb was sequenced in homozygous third chromosome lines from dark (Uganda) and light (Kenya) population samples. This region included the protein-coding exons of *ebony*, which span about 3 kb, along with 17.5 kb of upstream sequence (Figure 3).

Patterns of nucleotide variation are summarized in Table 2 for the *ebony* gene region, along with four X-linked, putatively neutral loci surveyed previously (Pool and Aquadro 2006). At the X-linked loci, the Kenya and Uganda samples yielded similar values for nucleotide diversity, Tajima's (1989) *D*,Fay and Wu's (2000) *H*, and Z_{nS} (Kelly 1997). At *ebony*, Uganda departed from these expectations with moderately reduced variation, a positive Tajima's *D* (which could indicate a loss of rare alleles), a negative Fay and Wu's *H* (indicating an elevated frequency of derived alleles), and a higher Z_{nS} (greater linkage disequilibrium). For the X-linked loci, F_{ST} (Hudson *et al.* 1992) values between these populations were fairly low (average F_{ST} = 0.031), indicating a modest level of differentiation. F_{ST} *P* values (calculated by permutation) were not significant for individual loci, but were marginally signifcant across all loci (P = 0.048). For the *ebony* region, genetic structure was pronounced (F_{ST} = 0.129) and significant (P = 0.001).

No pair of identical haplotypes were observed within either population at any of the X-linked loci. At *ebony*, the Uganda sample in particular showed long blocks of identical haplotypes across most of the sequenced region (Figure 3). In particular, the four most darkly pigmented lines were found to have very similar alleles across a 14.5 kb region. This haplotype identity was interrupted only by three unique substitutions, one unique 1 bp insertion, and three putative gene conversion events (tracts less than 250 bp long in which one of the four "dark alleles" differed from the others and resembled other alleles in the sample instead). The fifth darkest line (U73) also matched the haplotype of the darkest four lines within the 5.4 kb at the 5' end (with respect to *ebony*) of the 14.5 kb window mentioned above. Other lines in the Uganda sample also showed extended sequence identity, including U62 and U54, which were identical across 18.7 kb (Figure 3).

The presence and extent of these identical regions was surprising given that *ebony* is thought to experience a relatively high level of recombination (Kindahl 1994; Hey and Kliman 2002) in a genome where linkage disequilibrium normally decays within a few hundred bp, and also in light of previous data indicating that the Uganda sample is genetically diverse and has low linkage disequilibrium (Pool and Aquadro 2006). The significance of these observations was quantified by summing the lengths of all identical haplotype pairs greater than 1 kb within each population sample and comparing this total to that from simulated data (with $\rho = 0.2$ / site). For the Kenya sample, the observed haplotype identity (sum of identical pairs = 12,425 bp, or 1.35% of total pairwise lengths) was very close to simulated expectations (P = 0.522). For Uganda, observed haplotype identity (sum of identical pairs = 177,237 bp, or 15.7% of total pairwise lengths) was much greater than in the simulated data ($P < 0.0001$). Even if only the haplotype identity generated by the darkest four lines is considered, this sum (89,214 bp) exceeds the highest total observed among 10000 simulated replicates (70,886 bp).

The identical sequences shared by the darkest lines suggested a potential association between *ebony* genotype and pigmentation. This conclusion was supported by a non-parametric permutation-based association test (Doerge and Churchill 1996) that accounts for haplotype structure. Across the full sequenced region, a significant genotype/phenotype association was detected for the Uganda sample ($P < 0.001$) but not for the Kenya sample (P = 0.51). If the four darkest lines were excluded, no remaining association could be detected for the Uganda sample (P=0.557), possibly due to small sample size (n=7).

To investigate the mutation(s) near *ebony* responsible for dark pigmentation, candidate sites with a derived character unique to the darkest four (or five) lines were identified (Figure 3). The darkest four lines differed from all others by seven such mutations: six were between 1.8 and 5.1 kb upstream of the *ebony* promoter region, the other was within the intron separating the first (non-coding) exon of *ebony* from the remaining (protein-coding) exons. Three additional candidate sites differentiated the darkest five lines from all others.

Dominance and phenotype of *ebony* natural variants *versus* laboratory mutants

The classic *ebony* laboratory mutant, e^1, is a recessive allele producing dark pigmentation. Crosses performed between the TM6 balancer stock (homozygous for e^1) and four different homozygous third chromosome lines (K46, K60, U70, and U78) confirmed that e^1 behaves recessively (or nearly so) for the A4 pigmentation trait: for each cross, offspring had A4 pigmentation scores within 2% of the non-TM6 parent. In contrast, the natural dark variants tested (i.e. U78 and U70) appeared to show a pattern of partial dominance, with offspring of crosses between these lines and K60 or K46 displaying intermediate phenotypes.

The TM6 stock had only a moderately dark A4 pigmentation score (71). This result is compatible with previous observations that e^1 has a pronounced darkening effect on the thorax but has a lesser effect on the abdomen (Wittkopp *et al.* 2002a). Conversely, the natural variants examined in this study primarily influenced abdominal pigmentation, with relatively minor differences in thoracic pigmentation between flies with dramatically differing A4 pigmentation scores (Figure 1).

DISCUSSION

Geographic pattern and ecological basis of pigmentation differences

Geographic analysis of female abdominal pigmentation in *D. melanogaster* documented a diverse range of pigmentation intensities (Figure 1), and revealed the existence of darkly pigmented populations of *D. melanogaster* in sub-Saharan Africa (Figure 2a). Dark pigmentation was found exclusively at higher altitudes, and altitude accounted for 59% of pigmentation variation among populations within sub-Saharan Africa (Figure 2b). This result, together with the observation that pigmentation differentiation is unrelated to neutral genetic differentiation, suggested that dark pigmentation in *D. melanogaster* represents an adaptation to specific ecological conditions.

The “thermal budget hypothesis” that pigmentation differences help flies to maintain optimal body temperature in varying environments has been suggested to account for geographic variation of other pigmentation traits in *D. melanogaster* (e.g. David *et al.* 1985; Gibert *et al.* 2004). The strong association between dark pigmentation and high altitude would make sense in this context, given that montane environments tend to have cooler climates. However, three of the darker population samples studied come from altitudes of rougly 1200 m, and it is not clear whether the temperatures occurring at tropical sites of this altitude are cold enough to favor dark pigmentation. If not, the melanic phenotypes observed in these samples might result from gene flow with nearby populations from higher, cooler habitats. Or, factors other than temperature might favor dark pigmentation in these locations.

Given that populations from montane environments in sub-Saharan Africa show dark pigmentation, it is surprising that cosmopolitan populations from much colder climates (China and France) do not show similarly dark phenotypes. The pigmentation differences observed among cosmopolitan populations, though fairly minor, do seem to correlate with climate (with Israel and Tunisia more lightly pigmented than China and France), but even the darkest cosmopolitan sample (China) is significantly lighter than Uganda ($P < 0.0001$, Mann-Whitney

test). It is possible that other factors mitigate the potential advantage of dark pigmentation in temperate populations. Alternatively, the darkest alleles may have never reached temperate populations, or may have arrived only recently due to human transport.

Genetic basis of dark pigmentation

Patterns of nucleotide variation at *ebony* in the Uganda sample were consistent with the occurrence of a partial selective sweep, elevating the frequency of an allele associated with dark abdominal pigmentation. Haplotypes of the four darkest lines showed no evidence of crossing-over events within a 14.5 kb window (Figure 3), which is very improbable under neutrality for a highly recombining region. Instead, the long tracts of haplotype identity observed imply that strong selection has influenced allele frequencies at *ebony*. For comparison, a complete sweep affecting 14.5 kb with the level of polymorphism observed among the darkest four lines would yield an estimated selection coefficient of 1.04% (or 6.94% if polymorphism from putative gene conversion tracts is omitted; Maynard Smith and Haigh 1974).

If selection has strongly favored the “dark allele” in this population, it may seem surprising that this allele, and the dark pigmentation it generates, have not reached fixation. If the advantageous mutation is new, a selective sweep may still be in progress. However, given that identical haplotypes were also observed among less darkly pigmented lines in the Uganda sample, the selective dynamics of *ebony* alleles in this population might be complex. For example, the relative fitness of darker and lighter pigmentation could vary on a local geographic scale or fluctuate seasonally. Alternatively, the hitchhiking of *ebony* alleles conferring dark pigmentation might be influenced by the concurrent rise in frequency of dark pigmentation alleles at other loci. Lastly, in light of the partial dominance of natural dark variants of *ebony*, their intermediate frequency could reflect heterozygote advantage if the moderately dark phenotype generated by the combination of one dark allele and one average allele has the highest fitness.

Several potential candidate sites were identified as possible causative mutations for producing dark abdominal pigmentation (Figure 3). Additional functional and population genetic studies of this gene may lead to the identification of one or more specific nucleotide changes responsible for the pigmentation differences observed among homozygous 3rd chromosome lines. Also, further candidate gene or QTL studies may identify the X-linked contributor(s) to the dark abdomen phenotype.

All of the identified candidate sites potentially responsible for dark pigmentation were located upstream from the protein-coding exons of *ebony* (Figure 3). Thus, rather than a change in amino acid sequence (which might disrupt the other functions of *ebony*, such as visual perception), it seems most plausible that regulatory changes at *ebony* have given rise to divergent pigmentation phenotypes. Mutations at *ebony* that produce darkly pigmented flies are likely those that cause a quantitative reduction in the expression of this gene (e.g. Hovemann *et al.* 1998;Wittkopp *et al.* 2002b). In contrast to the e^1 laboratory mutant, the putative regulatory change affecting the natural dark pigmented lines appeared to have its strongest affect on abdominal pigmentation. Future functional studies based on this observation from natural variation could lead to the identification of tissue-specific regulatory elements that help govern the spatial expression pattern of *ebony*.

Summary and significance

Most studies of putatively adaptive traits in *D. melanogaster* have focused on cosmpolitan populations, which have less neutral variation than sub-Saharan populations (Begun and Aquadro 1993), but may face a different set of environmental challenges. However, eastern

African populations in particular are genetically diverse and have probably occupied diverse environments for a relatively long period of time (Pool and Aquadro 2006), so differential adaptation among these populations seems very plausible. This study supports the general utility of sub-Saharan *D. melanogaster* as a model for the study of adaptation and its genetic basis.

We have provided evidence that polymorphism at *ebony* is an important determinant of pigmentation in Uganda *D. melanogaster*, and that natural selection has increased the frequency of an allele with clear phenotypic effects. In view of what is known about pigmentation metabolism, one might have predicted that regulation of *ebony* could affect relative levels of brown versus yellow pigment. Although one can not always predict which genetic changes have produced a given phenotypic change, in this case prior knowledge of the biochemical pathway involved in pigment production has facilitated the identification of a gene implicated in local adapation.

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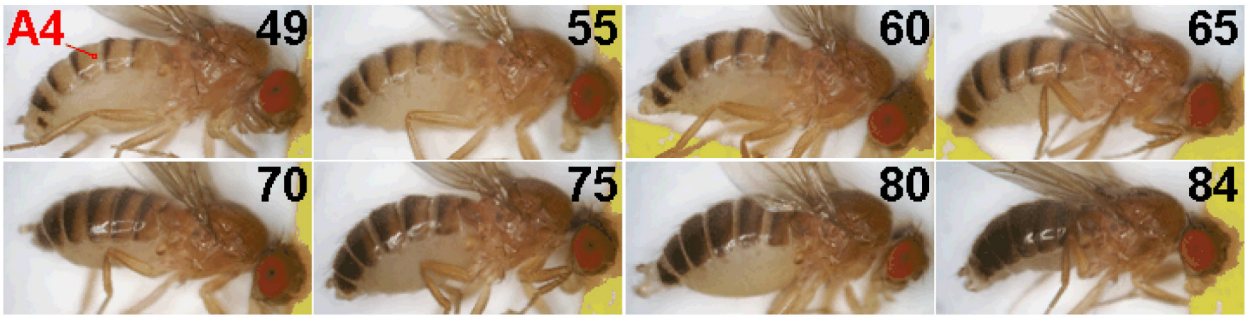

Figure 1. The diversity of female abdominal pigmentation observed in isofemale lines of *D. melanogaster* derived from natural populations

The upper left image depicts the specific location within the fourth abdominal segment where pigmentation was quantified (as grayscale darkness). The remaining images illustrate the range of A4 pigmentation values observed in our sample.

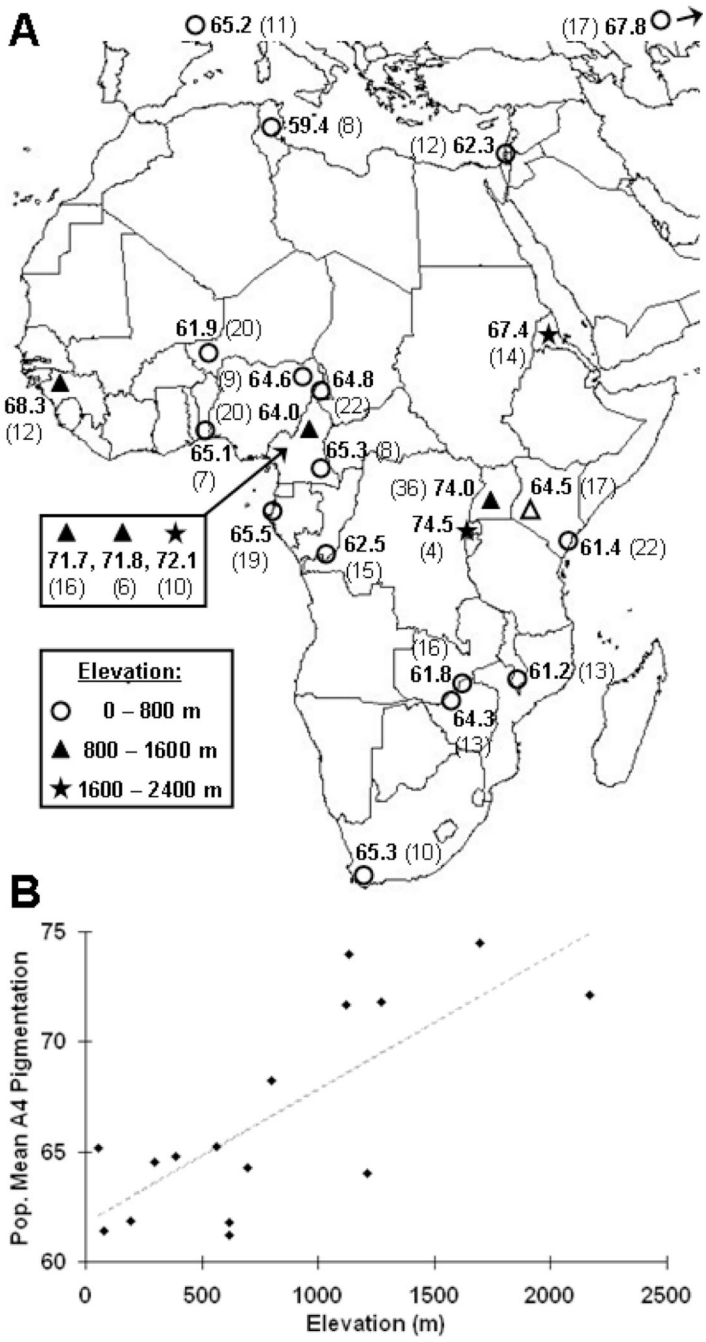

Figure 2. Geographic analysis of female abdominal pigmentation

(A) Location and mean A4 pigmentation for each population sample. Elevation range is indicated using the symbols listed above. The three samples from the western highlands of Cameroon are listed together and marked with an arrow. Kenya-N is marked with an open triangle – this sample was collected from multiple locations which range in altitude from 1070 to 2086 m. **(B)** The relationship between elevation and mean A4 pigmentation for 16 non-admixed sub-Saharan samples (Pool and Aquadro 2006). This correlation was found to be statistically significant ($P = 5.10 \times 10^{-4}$), with elevation accounting for the majority of variation in mean A4 pigmentation ($R^2 = 0.590$).

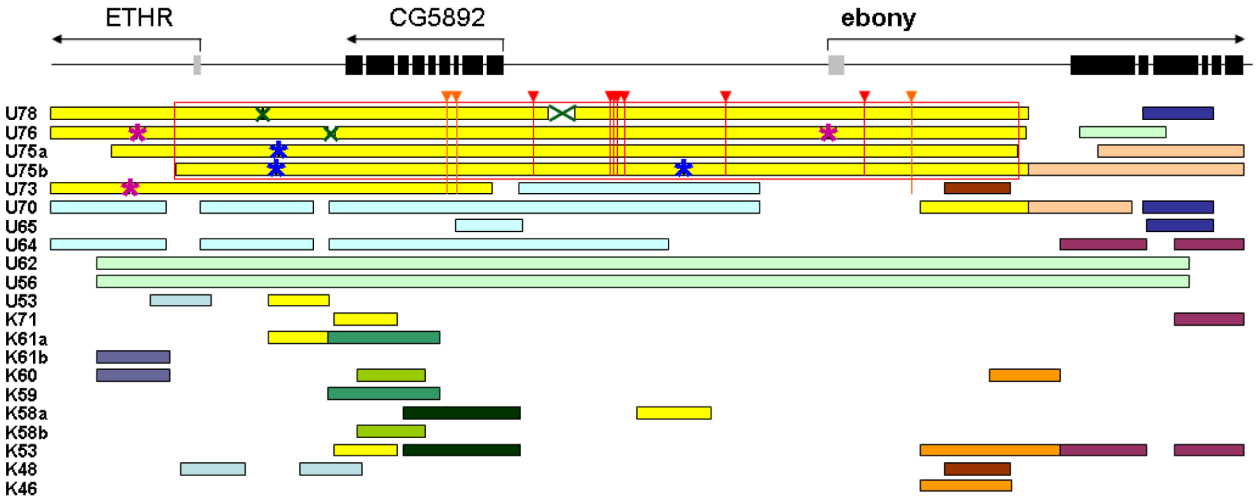

Figure 3. Gene features and haplotype identity in the *ebony* gene region

At the top, exons of *ebony* and neighboring genes are depicted (black exons are protein-coding, gray are non-coding). On the left, the sequenced lines are listed, each identified by its population (Uganda or Kenya) and its A4 pigmentation score. Identical haplotypes among two or more lines that extend more than 1 kb are illustrated with colored boxes. A red box indicates the 14.5 kb region of highly similar haplotypes among the darkest four lines. Green X's represent putative gene conversion events, blue asterisks represent unique substitutions, and purple asterisks represent unique insertions or deletions that interrupt sequence identity among the darkest lines. Vertical lines indicate derived substitutions unique to the darkest four (red) or darkest five (orange) lines.

Table 1

Pigmentation of homozygous chromosome lines

Homozyg. Chrom.	Dark Pop.	Light Pop.	Dark Pop. Isofem. A4	Light Pop. Isofem. A4	Dark Pop. H.C. Pigm.	Light Pop. H.C. Pigm.	M.W. P-value
X	C	K	71.8 (32)	61.4 (21)	69.0 (23)	63.1 (14)	0.003
2	U	Z	74.0 (36)	61.8 (16)	56.1 (15)	54.9 (8)	0.478
3	U	K	74.0 (36)	61.4 (21)	67.9 (11)	57.5 (10)	0.014

For each major chromosome, the A4 pigmentation scores of homozygous chromosome lines from populations that had shown either dark (Uganda, Cameroon) or light (Kenya, Zimbabwe) pigmentation of isofemale lines were compared. Numbers in parentheses indicate sample sizes. Mann-Whitney *P*-values less than 0.025 indicated a significant difference between the distributions of A4 pigmentation values from homozygous chromosome lines from the dark and light populations.

Table 2

Nucleotide variation in light and dark populations

Loci	Pop.	n	θ_s	π	Taj. *D*	F&W *H*	Z_{nS}	F_{st}
X	K	10	0.0131	0.0110	−0.79	0.69	0.149	0.0310
X	U	10	0.0130	0.0112	−0.72	1.42	0.175	*P*=0.048
e	K	10	0.0144	0.0127	−0.58	11.91	0.122	0.129
e	U	11	0.0093	0.0098	0.23	−21.15	0.238	*P*=0.0001

Population genetic summary statistics for the Kenya and Uganda populations for X-linked data (average of four loci totalling 3989 bp) and for the *ebony* gene region (19274 indel-free sites). Sample size (n), Watterson's estimator of theta based on the number of segregating sites (θs), nucleotide diversity (π), Tajima's *D*, Fay and Wu's *H*, the Z_{nS} measure of linkage disequilibrium, and F_{st} between samples were calculated.