Pigmentation scoring method for *Drosophila.*

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Abstract

Melanization in *Drosophila* is complex and varies from species to species. Several methods in practice today quantitate pigmentation in *Drosophila* species, but these existing methods are compromised at certain levels. The majority of drosophilid body tergites show a range of yellow to dark brown pigmentation as well as segment to segment variation, making it difficult to score correctly. We present a method which accounts for these ranges of pigmentation and segmental variability. This method is able to score melanization as well as the area for each abdominal tergite. This method is also applicable to score wing pigmentation features. We hope our method will inspire the fly community to attempt to score species with complex pigmentation patterns.

Introduction

Melanism is diverse across insect taxa and has been reported to be adaptive in numerous fitness related traits (Parkash *et al.*, 2009; Rajpurohit *et al.*, 2008; True, 2003; Wittkopp and Beldade, 2009). There are several techniques used to quantitate melanization, and they are complex and subjective. One such technique developed by David *et al.* (1985, 1990) scores body tergite pigmentation in cosmopolitan *Drosophila melanogaster*. Their method is based on manual scoring. The fly is positioned laterally under a dissecting microscope so the observer can see lateral to dorsal midline. Then a trained scorer assigns a pigmentation score for each segment ranging between 0-10 (0 for non-pigmented segment and 10 for completely pigmented segment). Even though their method shows high repeatable correlations among independent scorers, it requires a highly trained scorer. The method works well with species like *D. melanogaster* and *D. simulans*, which have easily visualized linear patterns across lateral views. Unfortunately other *Drosophila* species do not have such simple patterns.

To avoid these species-specific constraints, abdomen whole mounts spread on glass slides have been used to score pigmentation (Gibert *et al.*, 1998; Hollochor *et al.*, 2000). One particular method developed by Pool and Aquadro (2007) scores only a certain rectangular region in the fourth tergite. The scores are based on pixel density of the region selected. In cases where the total body pigmentation with respect to total surface area is needed for comparison, the Pool and Aquadro method cannot be used.

The methods described so far (David *et al.*, 1985, 1990; Hollochor *et al.*, 2000; Parkash and Munjal, 1999; Parkash *et al.*, 2008; Pool and Aquadro, 2007) either neglect the segment area or total body pigmentation. The existing methods are also inefficient in performing segment size corrections across the samples or populations, which is very important when looking at within species variations (Gibert *et al.*, 1998). There are several cases in *Drosophila* literature where total body area is equally important to segment pigmentation. Populations spread along latitudinal or altitudinal gradients vary in their body size as well as in total body pigmentation (Parkash and Munjal, 1999; Parkash *et al.*, 2008, 2009). In some species each body tergite shows a different kind of temperature reactivity (Gibert *et al.*, 1999).

Here we describe a method to determine pigmentation for a given body tergite, an entire dorsal surface, along with their total area measurements. The user can also target within a segment to obtain gray score and area measurements, while accounting for the variable shades of the segments.

Method

With forceps and a needle remove both wings and then detach the head and thorax from the abdomen. Place the abdomen on a glass slide, dorsal side up, and apply a drop of whole mount material covering the entire abdomen. Place a glass cover slip over the abdomen, allowing the cover slip to fall in the posterior to anterior direction. Press abdomen firmly until all the viscera squeeze out from the open abdominal cavity and all the segments spread smoothly parallel to the glass slide. You can place multiple abdomens on a single glass slide with practice. For wing spot melanin scoring, remove the wing, place it on a glass slide and follow the steps discussed above. Once the slides are dry they are ready to use for imaging. Images can be taken using a digital camera attached to microscope with 2.5× objective lens. On the same magnification an image of a reference scale coded glass slide is needed. Now go to 'ImageJ' software (http://rsbweb.nih.gov/ij/). Open scale image file first and select the distance between any two given points on the scale. Set those measurements as reference, open abdomen image file, and start selecting the segmental areas according to your research interest (Figure 1). When the area is selected in the image, the software simultaneously calculates the actual area and gray score quantitatively.

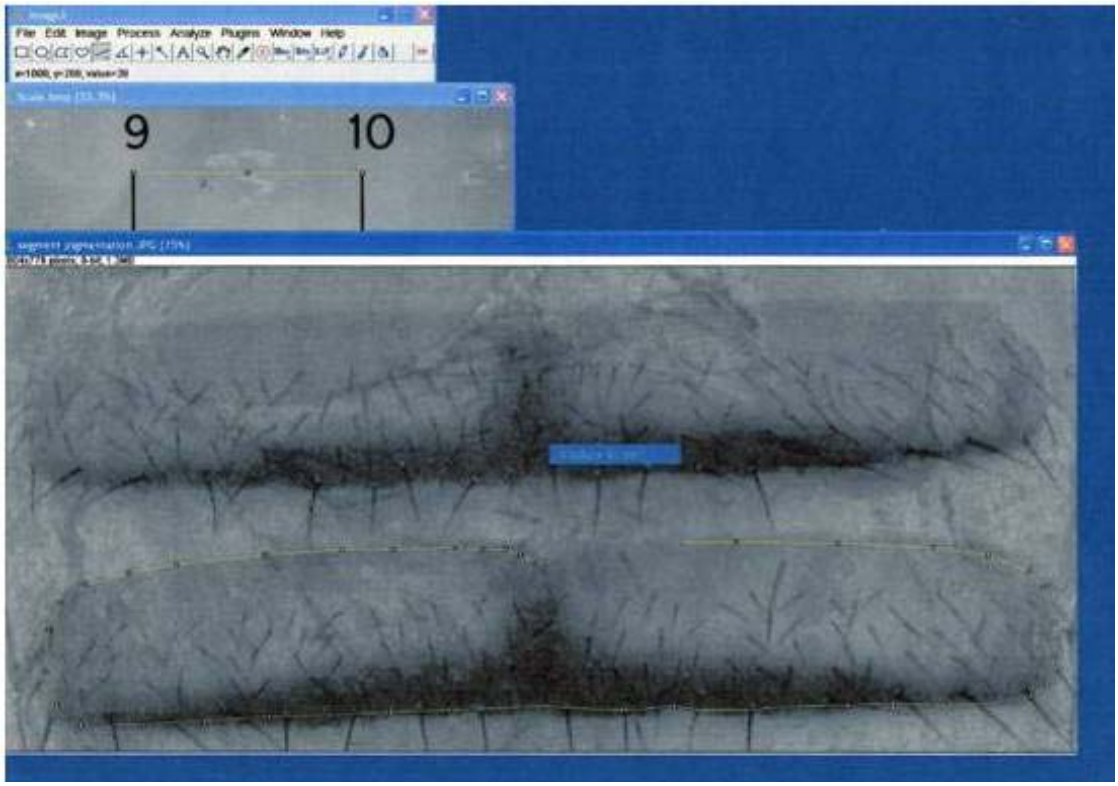

Figure 1. Image J working window and applets showing an area selection for 4th abdominal segment of *D. melanogaster* female (broader view of the actual image is shown in Figure 2a). Selected area is shown in yellow line.

Case Examples

In the Drosophilidae, pigmentation traits, including abdominal pigmentation, wing pigmentation, and sexual dimorphism, have evolved independently in several distantly related evolutionary lineages. Evolution of pigment patterns involves both divergent and convergent changes. In this section we will cover abdominal and wing pigmentation particularly where this technique could be applied for a variety of purposes.

Body tergite melanization

Abdominal melanization constitutes stripes of dark pigment at the posterior region of each abdominal segment; these stripes may be continuous or interrupted, or the pigmentation may appear as spots. Pigmentation patterns also vary between segments (Figures 2a and b). Thus, diverse patterns of pigmentation occur in a variety of evolutionary lineages within the Drosophilidae.

In cosmopolitan *D. melanogaster* each body tergite segment has a black melanin strip, which starts from the posterior region of every segment and covers none to the complete part of the segment. In *D. melanogaster* females, posterior body tergites are darker than their anterior segments (Figure 2a). The data presented in Table 1 show the gray score and area for each body tergite where only dark portion (in a segment) is selected (*D. melanogaster* female). The sum values for gray score and dorsal segmental area for all body tergites (1-7) have also been presented in the Table 1. In *D. melanogaster* females a total of 32.06% of abdominal segmental area is pigmented (example image, Figure 2a). Analyses made on image (shown in Figure 2a) conclude segment 4 as the largest and segment 6 as darkest segment (the lower the gray score, the darker the segment).

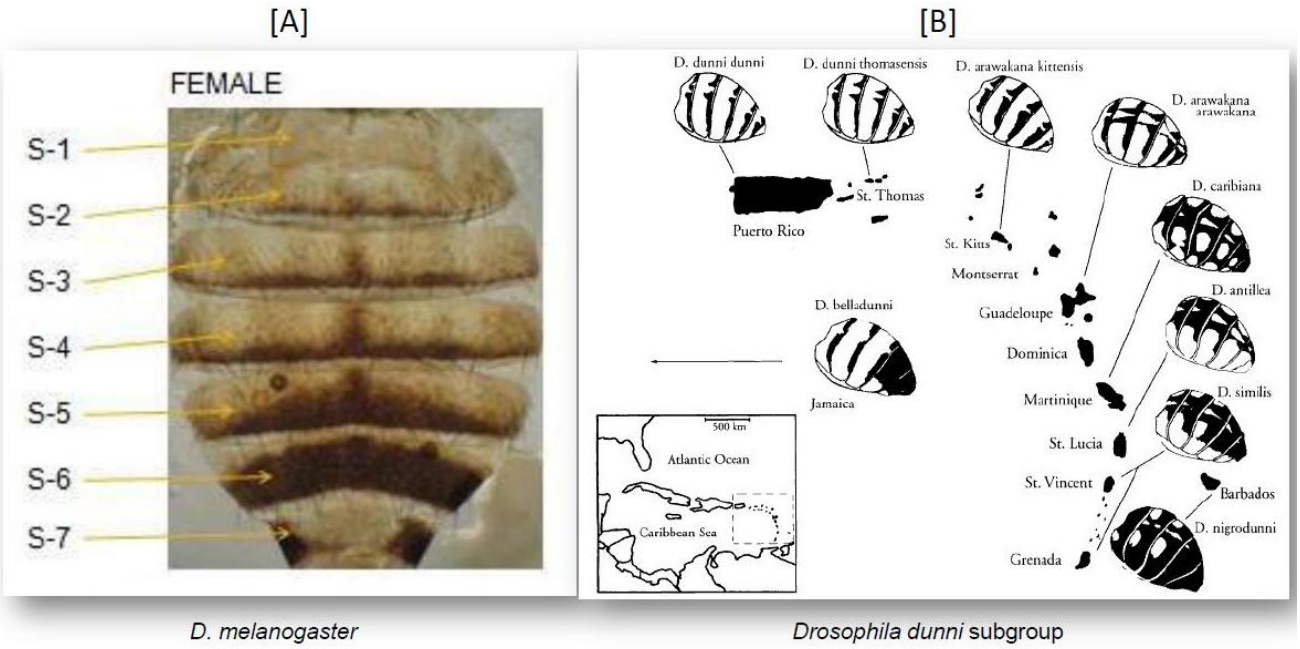

Figure 2. Abdominal pigmentation for females of *D. melanogaster* (A) and each species in the *Drosophila dunni* subgroup (B; adapted from Hollocher *et al.*, 2000).

Table 1. Data on abdominal segment area and abdominal pigmentation for *D. melanogaster* female. Scale of 10380 pixels/mm shown (known distance 0.1 mm). GS = Gray Score; A = Area (mm^2).

Segment	Parameter	Entire segment	Pigmented part	Non-pigmented part
1	GS	103.5	94.8	
	A	0.0015	0.0001	0.0013
2	GS	100.8	79.2	
	A	0.0028	0.0007	0.0020
3	GS	111.3	79.8	
	A	0.0042	0.0010	0.0031
4	GS	111.6	74.5	
	A	0.0048	0.0012	0.0031
5	GS	107.1	68.7	
	A	0.0039	0.0011	0.0027
6	GS	89.2	65.7	
	A	0.0033	0.0019	0.0013
7	GS	108.1	96.6596	
	A	0.0014	0.0006	0.0007
Total	GS	732.0	559.6	
	A	0.0216	0.0069	0.0141

Table 2. Data on total abdominal segmental surface area and their gray score in eight species of *D. dunni* species subgroup. Values are based on a hypothetical reference scale (81 mm) defined between tips of anterior and posterior abdominal region of *D. dunni dunni* female on the acquired image (Figure 2b; from Hollochor *et al.*, 2000). Scale of 9.37 pixels/mm is shown (known distance 81 mm). For David *et al.'s* (1985, 1990) method percent darkness was calculated after adding the values obtained for every segment (sum values are provided). TV = Trained Volunteer.

Species	Method Presented		Based on David *et al*. (1985, 1990) method		
	Gray Score	Area (mm^2)	Sum of body tergites (% Darkness)		
			TV-1	TV-2	TV-3
D. dunni dunni	173.9801	0.4963	20.00	08.57	22.85
D. dunni thomasensis	158.7276	0.4987	21.42	12.85	22.85
D. arawakana kittensis	151.1759	0.4848	28.57	20.00	27.14
D. arawakana arawakana	144.6642	0.5309	30.00	27.14	28.57
D. caribiana	142.9036	0.5838	55.71	54.28	58.57
D. antillea	105.7496	0.5242	25.71	25.71	35.71
D. similis	93.7803	0.5088	58.57	54.28	60.00
D. nigrodunni	50.3080	0.6087	78.57	78.57	75.71

In *D. dunni* subgroup species, the dark strip is not as regular as in the case of *D. melanogaster* females (Figure 2b, adapted from Hollocher *et al.*, 2000). The patterns are irregular and complex and are difficult to measure efficiently with routine available methods discussed in the introduction section. One could score the same parameters (as explained for *D. melanogaster*, Table 1) for the *D. dunni* subgroup or any other species with such complex patterns using our method. The selection scheme remains the same as explained in Figure 1. In Table 2 data on gray score and total abdominal

segmental area is given for eight species of *D. dunni* subgroup which demonstrates a striking variation for pigmentation across the islands of the Caribbean (Hollocher *et al.*, 2000). In this analysis (Figure 2b, Hollocher *et al.*, 2000), *D. nigrodunni* emerges as the darkest species (with lowest gray scores). One can also focus segment-wise gray score and area for *D. dunni* subgroup species (in Table 2, sum values for all segments are given) as presented for *D. melanogaster* in Table 1. Using this method a difference in gray score as minimum as 1.76 pixels/mm (which is statistically significant; data not shown) could be analyzed between *D. arawakana arawakana* and *D. caribiana* (Table 2).

Wing spot

Wing pigment spots occur in highly reproducible, species-specific, two-dimensional patterns, and their genetics and development are beginning to be understood (Edwards *et al.*, 2007). True *et al.* (2003) found that wing spot patterns have two main components: a vein-independent "prepattern" formed during wing development prior to eclosion, and vein-dependent melanization that forms after eclosion. These studies provide the platform required for understanding the evolution of complex pigment patterns in the *Drosophila* species where wing spots are present and their ecological significance is yet to be discovered. Analysis of pattern variation using color-coded overlays of wing photos suggested by Edwards *et al.* (2007) could be more extensively exploited and embedded in our method. We hope our method will inspire the fly community to attempt Hawaiian flies and other species with the same kind of wing-spot patterns to gain further molecular insights into morphological evolution which is deeply associated with behaviors like producing a camouflage or the role of wing-spot display during courtship in some species (*e.g.*, *D. elegans*) (Wray, 2006). The proximal and distal borders of spots (Figure 3) can vary independently, as shown by Edwards *et al.* (2007) in their Hawaiian drosophilids picture database. This suggests that wing patterning genes somehow exert a very flexible, fine-scale control over the pigmentation process. The intensity, size, and numbers of spots may vary among individuals and populations. These minor morphological deviations from spot to spot and species to species can be easily handled using our technique.

Analysis based on Figure 3 wing spots gray score measurements suggest that *D. planitibia* females are significantly different from *D. differens* females ($p < 0.05$) where they do not differ in the area ($P = 0.45$) covered by spots. No other available method can attempt measurements for such spot variation across *Drosophila* species.

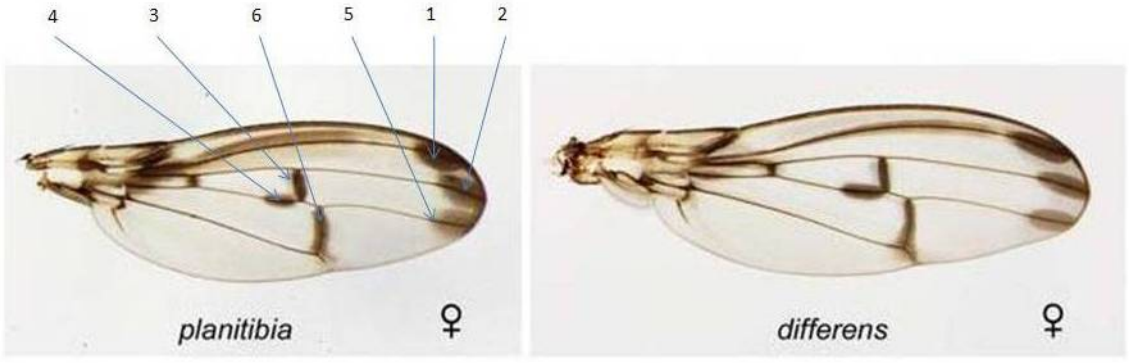

Figure 3. The planitibia subgroup of picture wing species from Hawaiian islands shows spot to spot variation in a single wing as well as species to species variations. Image was acquired from Edwards *et al.*, 2007.

Validation With Existing Methods

In order to validate our method with other available methods, we compared gray score data obtained for eight species of *D. dunni* subgroup (shown in Figure 2b; Hollocher *et al.*, 2000) with David *et al.*'s (1985, 1990) procedure. For this we trained three volunteers in both the techniques (David *et al.*'s, 1985, 1990; ours) and provided the hardcopy for David *et al.*'s method as well as the softcopy for our method of Figure 2b (from Hollochor *et al.*, 2000). The volunteers were asked to score pigmentation for eight species mentioned in Figure 2b. The data obtained from trained volunteers (TV-1, TV-2, and TV-3) are presented in Table 2. The gray score data using our method do not differ across users (Table 2; r = 0.98; authors of manuscript) but significantly differ across the users using David *et al.*'s method. This clearly indicates that user to user variation for assigning a score (0-10) for a given segment significantly affects the analysis. David *et al.*'s method works well (r = 0.97; between two trained scorers) with *D. melanogaster* where body tergite pigmentation is not as complicated as in other *Drosophila* species.

Materials Required

Here we list the materials required for the execution of the method: forceps, needles, glass cover slips, glass slides, dissecting microscope, mounting material, ImageJ software installed on a PC, a reference scale, and a microscope with camera attached.

Limitations

This method gives the user better results in yellow, brown, and black body surfaces, but it is less promising when one is looking at different species for comparisons which may have completely different patterns and colors. Secondly our method can better handle dorsal abdominal pigmentation and wing patterns.

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