The discrimination of sibling fruit fly species *Drosophila ananassae* and *D. malerkotliana* (Diptera, Drosophilidae) through wing traditional morphometry.

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Introduction

Drosophila (Sophophora) ananassae Doleschall 1858 and *D. (S.) malerkotliana* Parshad and Paika 1964 are widespread Drosophilidae fruit fly species (Bächli, 2014). *Drosophila malerkotliana* has two subspecies, *D. malerkotliana malerkotliana* and *D. malerkotliana pallens*, but the latter has been recorded only in Borneo and the Philippines (Bock and Wheeler, 1972). In Brazil, *D. ananassae* is particularly associated with human habitation (Pavan, 1959), whereas *D. malerkotliana* may be collected in forests (Sene *et al.,* 1980; Tidon and Sene, 1992; Martins, 2001), open vegetation (Tidon, 2006) or disturbed areas (Gottschalk *et al.,* 2007). As suggested by Tidon and Sene (1999), the few records of *D. ananassae* in Brazil may be the result of the difficulty of species identification rather than a low frequency of occurrence. Because the females of these species display very similar abdominal pigmentation patterns, they are indistinguishable by traditional means of external morphological analysis. In contrast, the males are distinguishable by differences in their abdomen color and the morphology and coloration of the sex comb teeth: *D. malerkotliana* males display a black abdomen and black sex comb teeth, as opposed to the yellow abdomen and yellowish and more numerous sex comb teeth of *D. ananassae* males.

The misidentification of sibling species may cause an underestimation of biodiversity or produce erroneous conclusions about species' life history, ecology, and genetics. In this context, morphometric analyses have been an efficient and sensitive method for discriminating between morphologically similar species by detecting small variations in individual morphology (Klaczko and Bitner-Mathé, 1990; Carreira *et al.,* 2006). Among the morphological traits of *Drosophila*, wings are particularly useful structures with a conserved venation pattern that allows the location of many well-defined landmarks and allows more accurate measurements than other body structures (Klingenberg, 2002; Houle *et al.,* 2003; Debat *et al.,* 2008). Although factors such as temperature, larval competition, sex, and karyotype influence wing morphology in Drosophilidae (De Moed *et al.,* 1997; Hatadani and Klackzo, 2008), studies have shown that wings strongly respond to natural selection, providing a good taxonomic marker and, therefore, identifying characteristics for sibling species (Macdonald, 2002; Moraes *et al.,* 2004ab; Franco *et al.,* 2006; Prado *et al.,* 2006; Lyra *et al.,* 2010).

The difficulty of identifying *D. ananassae* and *D. malerkotliana* females in taxonomic surveys motivated the current comparative study, which aimed to verify the use of traditional wing morphometry and of the costal index in the identification of these species and to evidence some aspects of the wings evolution of these species.

Material and Methods

Collection and establishment of isofemale lines

Drosophilidae were collected in the Brazilian Cerrado Biome (14°65'20"S; 57°43'37"W) with traps baited with banana and yeast (*Saccharomyces cerevisiae*) following the Tidon and Sene (1988) methodology. The captured flies were anesthetized and screened under a stereomicroscope (Tecnal-ME, SZ). Each *D. ananassae* and *D. malerkotliana* female was individually placed in a culture vial containing 3 mL of culture

medium prepared with 5% rye flour, 10% yeast, 10% sugar, and 1% agar, and maintained at 25ºC. Because the identification of the *D. ananassae* and *D. malerkotliana* females is imprecise, the male offspring were used for species determination. The F1 offspring of ten isofemale lines of each species were transferred to 30 mL of culture medium. The F2 individuals were preserved in 70% ethanol prior to their use in the wing morphometry analyses.

Wing landmarks measurements

Individual wings from ten males and ten females from the F2 generation of each isofemale line were mounted on a microscope slide. Images of the wings were captured with a digital camera (Dino-Eye) connected to an optical microscope (NIKON E200) at 40× magnification. Image-Pro Plus 4.5 software was used to measure the distance between landmarks. Nine measurements were taken between external landmarks (**ab**, **ac**, **ad**, **ae**, **af**, **bc**, **cd**, **de** and **ef**), and four measurements were taken between internal landmarks (**gh**, **hi**, **ij** and **jg**) (Figure 1).

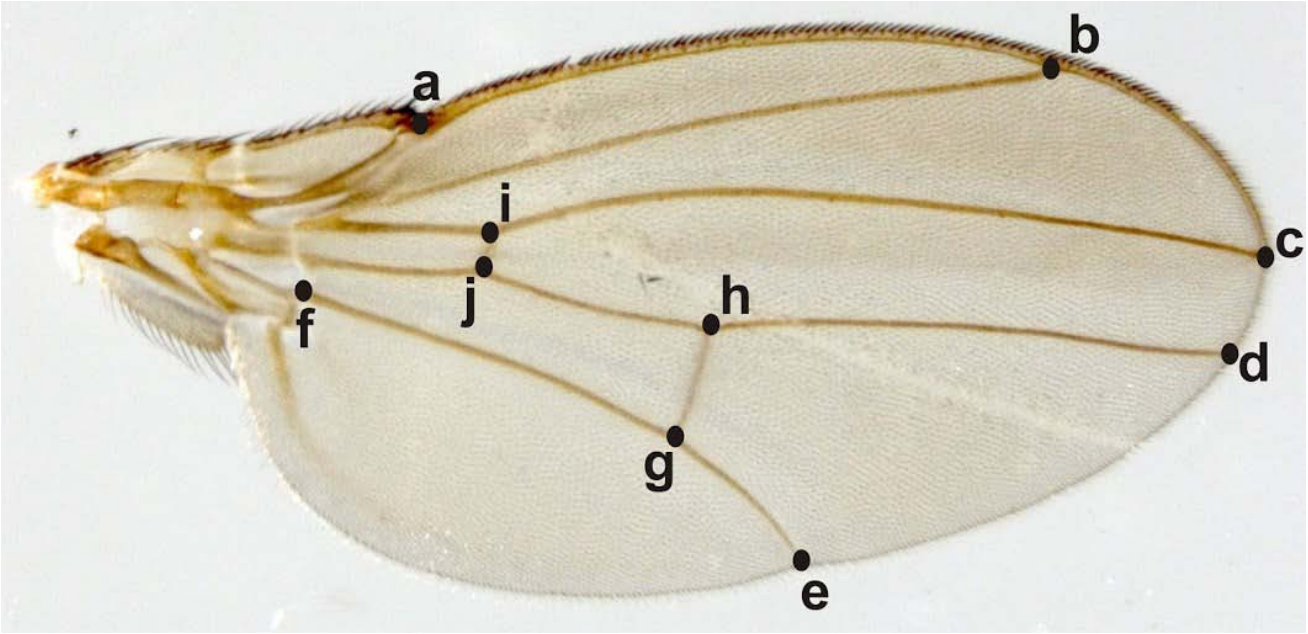

Figure 1. Wing landmarks.

Analysis of the measurements between landmarks

The wing measurements, after a logarithmic transformation, were analyzed with a principal component analysis (PCA) on the total variance-covariance matrix. To compare the wing size between species and sexes we conducted Mann-Whitney tests to verify the differences of the first PCA component. To detect any influence of the isofemale line heritability, Mann-Whitney tests were carried out using the PC1, PC2, and PC3 scores among the isofemale lines within each species and sex. A discriminant analysis was performed to evaluate the percentage of correct reclassifications of individuals by species and sex using the three first components obtained on the PCA. A Hotteling test was performed to evaluate the differences between the tested groups. The sexual dimorphism ratio (SD) was calculated to the average of each measurement (females/males).

The costal index (the ratio between the second and third section of the costal vein, **ab**/**bc**) was calculated for each individual, and the results were also compared using a Mann-Whitney tests between species and sex. We conducted a Spearman correlation test to evaluate the relationship of the costal index with PC1 (wing size). The Bonferroni correction was applied for all analyses, and the analyses were performed using PAST 1.82 software (Hammer *et al.*, 2001).

Table 1. Measurements between wing landmarks in mm (mean ± standard deviation) and sexual dimorphism ratio (SD) in *D. ananassae* and *D. malerkotliana*.

	D. ananassae			*D. malerkotliana*		
Landmarks	Male	Female	SD	Male	Female	SD
ab	2.07 ± 0.11	2.25 ± 0.12	1.09	1.81 ± 0.09	2.09 ± 0.10	1.15
ac	3.20 ± 0.18	3.51 ± 0.15	1.10	2.64 ± 0.12	3.00 ± 0.11	1.14
ad	3.15 ± 0.14	3.45 ± 0.14	1.10	2.58 ± 0.12	2.91 ± 0.14	1.13
ae	2.14 ± 0.10	2.31 ± 0.08	1.08	1.76 ± 0.08	1.96 ± 0.08	1.11
af	0.77 ± 0.06	0.84 ± 0.04	1.09	0.61 ± 0.03	0.69 ± 0.03	1.13
bc	1.39 ± 0.06	1.49 ± 0.06	1.07	1.06 ± 0.05	1.16 ± 0.04	1.09
cd	0.46 ± 0.02	0.48 ± 0.03	1.04	0.40 ± 0.02	0.42 ± 0.03	1.05
de	1.73 ± 0.09	1.91 ± 0.09	1.10	1.39 ± 0.07	1.58 ± 0.07	1.14
ef	2.08 ± 0.11	2.26 ± 0.08	1.09	1.69 ± 0.08	1.91 ± 0.08	1.13
gh	0.34 ± 0.02	0.37 ± 0.02	1.09	0.27 ± 0.02	0.30 ± 0.02	1.11
hi	0.88 ± 0.04	0.97 ± 0.04	1.10	0.67 ± 0.03	0.74 ± 0.03	1.10
ij	0.20 ± 0.17	0.19 ± 0.01	0.95	0.13 ± 0.01	0.14 ± 0.01	1.08
jg	0.96 ± 0.04	1.04 ± 0.04	1.08	0.72 ± 0.04	0.81 ± 0.03	1.13

Results

The averages of the measurements between the wing landmarks for each sex of *D. malerkotliana* and *D. ananassae* are displayed in Table 1.

The PCA identified three principal axes that explained 89.2% (PC1), 3.6% (PC2) and 1.7% (PC3) of the total variation in the data. The PC1 axis primarily represented the size of the wing and was highly correlated with all of the measurements (see scores for PC1 in Table 2). The PC2 and PC3 were more strongly correlation with the **ij** and **ab** measurements, respectively (Table 2 and Figure 2). Males of *D. malerkotliana* have more differentiated wing shape, while females of *D. malerkotliana* and males and females of *D. ananassae* have more similar PC2 and PC3 values.

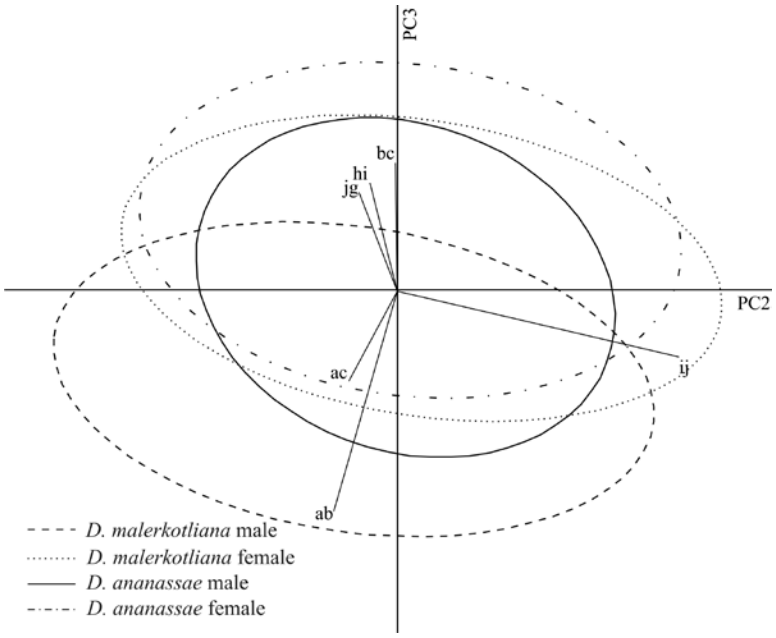

Figure 2.

The Mann-Whitney tests with the PC1 comparing species and sexes showed significant differences between all of the comparisons ($p < 0.0001$ for all pairwise comparisons), and *D. ananassae* has larger wings than *D. malerkotliana* and females have larger wings than males.

In the discriminant analysis, both species were 100% correctly reclassified ($p < 0.001$ for both sexes in the Hotteling tests) when the three first principal components were used. When only PC2 and PC3 were used, the success of the discriminant analysis decayed to 64.0% correctly reclassified

Table 2. Scores of the three first components of PCA conducted with the measurements between the wing landmarks.

Distances	PC1	PC2	PC3
ab	-0,18	0,21	-0,65
ac	-0,25	0,16	-0,27
ad	-0,26	0,12	-0,20
ae	-0,25	0,11	-0,10
af	-0,28	0,03	0,09
bc	-0,31	0,01	0,38
cd	-0,19	0,05	-0,01
de	-0,28	0,12	-0,17
ef	-0,26	0,15	-0,10
gh	-0,31	-0,01	0,18
hi	-0,33	0,09	0,32
ij	-0,33	-0,92	-0,20
jg	-0,33	0,13	0,29

Table 3. Costal index (mean ± standard deviation) of *D. ananassae* and *D. malerkotliana*.

Costal index	Male	Female
D. ananassae	1.49 ± 0.07	1.50 ± 0.07
D. malerkotliana	1.70 ± 0.08	1.79 ± 0.09

for males and 78.5% correctly reclassified for females ($p < 0.001$ for both in Hotteling tests).

Despite the morphometry analysis being quite reliable, we also investigated the possibility of using some measurement identified in the PCA as diagnostic. The measurement **ab** differed between females of *D. ananassae* and *D. malerkotliana*. This measurement comprises the costal index, commonly used as diagnostic character of species of *Drosophila*. The averages and standard deviations of the costal index are shown in Table 3. The costal index was also inversely proportional to the PC1 ($rs = -0.63$, $p < 0.0001$), showing that the index value was positively correlated with the wing size since all the measures have negative scores on PC1 (Table 2). *Drosophila malerkotliana* had higher values of costal index than *D. ananassae*, and this ratio was higher in the females of both species. The Mann-Whitney tests comparing the values of costal index between species and sexes showed differences between the species in both sexes and between males and females of *D. malerkotliana* ($p < 0.0001$ for all comparisons). The males and females of *D. ananassae* did not differ significantly ($p = 0.56$). In addition, the sexual dimorphism ratio (SD) in *D. malerkotliana* is higher for all measurements than in *D. ananassae* (Table 1).

The Mann-Whitney tests to assess the variation between isofemale lines in each studied species showed no significant differences in almost all of the comparisons. One isofemale line of *D. malerkotliana* showed significant differences from the other five lineages ($0.036 > p > 0.001$).

Discussion

Drosophila ananassae and *D. malerkotliana* females are morphologically very similar, and the distributions of these species in the tropical and equatorial regions overlap (Bächli 2014). The correct identification of these species is important due to their invasive character and because the *D. ananassae* species subgroup has been widely used as a model in population and evolutionary genetics (Matsuda *et al.*, 2009). The present study suggests that traditional wing morphometry could be used in the identification of *D. ananassae* and *D. malerkotliana*; once we noted the difference in wing size and shape, the discriminant analysis using PC1, 2, and 3 reclassified 100% of the flies correctly.

The principal source of wing variation between the sexes and species was size, as demonstrated by the PC1, which explained 89.2% of the variation. Consistent with previous studies, size was the principal variable character for wing sexual dimorphism (Gidaszewski *et al.*, 2009) among species (Moraes *et al.*, 2004ab) and even populations or linages (Bitner-Mathé and Klaczko, 1999, Hatadani and Klaczko, 2008).

In this study, we established ten isofemale lines of each species from recent samples. No differences in wing size and shape were observed between the lines (with one exception in *D. malerkotliana*), suggesting that our results are showing a lower intraspecies rather than interspecies variation. In general, *D. malerkotliana* have higher intraspecies variation, as evidenced by the larger ellipses in the PCA graphic and the lower correlation strengths of the measurements.

As expected, the wings of the females were larger than the wings of the males (Teder and Tammaru, 2005). These authors also verified that the sexual differences in size increased with the species' body size in several insect species; however, a higher wing size dimorphism was observed in *D. malerkotliana*, the smaller studied species. In general, sexual dimorphism in *D. malerkotliana* is more pronounced than in *D. ananassae*,

especially in the abdominal pigmentation, where males have almost all black tergites while females have yellow tergites.

Wing shape did not differ greatly between *D. ananassae* and *D. malerkotliana*, which supports the idea of canalization or stabilizing selection restricting wing shape divergence (Gilchrist and Partridge, 2001). Our results showed isometric growth of the measurements, even the **ij** and **ab**, the measurements that most correlated with PC2 and PC3. As observed by Hatadani and Klackzo (2008), the displacement of the landmarks **b**, **c**, and **d** is the main factor responsible for the shape variation of *D. mediopunctata* linage, and our results agree, in part, when **ab** and **bc** are the measurements most related to PC3; however, the **ij** measurement is also important for shape differentiation of *D. malerkotliana* and *D. ananassae* species.

Based on the foregoing, we calculate the costal index (**ab**/**bc**), a morphological taxonomic trait used in *Drosophila* species identification (Vilela 1983). The costal index differs significantly between *D. ananassae* and *D. malerkotliana*, supporting the use of the index in species identification. We propose costal index values of 1.79 and 1.50 for females of *D. malerkotliana* and *D. ananassae*, respectively, and 1.70 and 1.49 for *D. malerkotliana* and *D. ananassae* males, respectively. Although a previous study did not report differences in the costal index for individuals of both species collected in Kulu and Chandigarh, India (Parshad and Paika 1964), our study suggests that the costal index is relevant. Another important consideration of the present study was to establish the costal index for each gender. Finally, the morphological study of populations is important to aid in species identification, as stated by Parshad and Singh (1971), who reported a costal index of 1.46 for the *D. ananassae* population of the South Andamans, India, in contrast with the value of 1.54 established for the *D. ananassae* populations of Kulu and Chandigarh, India. However, for our proposal of identification of females of sibling species, the costal index and the wing size are informative characteristics for *D. ananassae* and *D. malerkotliana*.

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