Morphometric Differentiation of Fruit Fly Pest Species of the *Anastrepha fraterculus* Group (Diptera: Tephritidae)

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ABSTRACT Fruit flies (Tephritidae) include pests of quarantine importance, some of which belong to the genus *Anastrepha*. Some species in this group are difficult to identify. We tested the accuracy of morphometric techniques to distinguish three species of the *fraterculus* group (*A. fraterculus*, *A. obliqua*, and *A. sororcula*), using images of the aculeus and wing. The geometric morphometrics of the wings, using 17 landmarks, indicated differences in the wing shape of each species, separating them successfully into distinct groups. The conventional morphometrics of seven measurements of the aculeus tip, by linear discriminant analysis, also indicated differences in the species, separating them into three groups.

RESUMO As moscas-das-frutas (Tephritidae) são pragas quarentenárias, como algumas espécies do gênero *Anastrepha*. Algumas espécies desse grupo são de difícil identificação. A eficiência de técnicas de morfometria na classificação de três espécies do grupo *fraterculus* (*A. fraterculus*, *A. obliqua*, *A. sororcula*) foi testada, utilizando imagens do acúleo e da asa. A morfometria geométrica das asas, usando 17 marcos anatômicos, indicou que a forma das asas difere entre as três espécies, separando-as eficientemente em grupos. A morfometria multivariada de sete medidas do ápice do acúleo, por meio da análise discriminante linear (ADL), também indicou diferenças nas espécies, formando três grupos distintos.

KEY WORDS geometric morphometry, interspecific variation, conventional morphometry

Fruit flies are economically important pests worldwide. The genus *Anastrepha* is the most diverse, with >250 described species (Norrbom et al. 2012), but fewer than 10 species are of agricultural importance (Aluja 1994). Identification of *Anastrepha* species is based on differences in the shape of the aculeus, thoracic markings, wing pattern, and microtrichia. The genus has been divided into several species groups, including the *fraterculus* group. Several species of this group are identified by subtle morphological differences in the shape of the aculeus, and the limits between species are frequently difficult to recognize. Therefore, in addition to the external morphology, studies on molecular (Smith-Caldas et al. 2001), genetic (Selivon et al. 2005), and morphometric (Hernández-Ortiz et al. 2012) variation have also been carried out to clarify the identity of cryptic species.

Morphometric methods (conventional and geometric) have been used to assist in identifying many insect species (Daly 1985, Baylac et al. 2003). For example, in *Anastrepha*, conventional morphometry has been used to characterize populations of the *A. fraterculus* complex (Hernández-Ortiz et al. 2004, 2012; Selivon et al. 2005) and to assist the identification of species in the *fraterculus* group using aculeus measurements (Araujo and Zucchi 2006).

Species identification is crucial for the implementation of management and control programs and quarantine restrictions. In this study, we tested the accuracy of morphometric techniques for the identification of three major pest species of the *fraterculus* group: *Anastrepha fraterculus* (Wiedemann), *Anastrepha obliqua* (Macquart), and *Anastrepha sororcula* Zucchi, based on aculeus and wing images.

Materials and Methods

Fruit Flies. Female specimens of *A. fraterculus*, *A. obliqua*, and *A. sororcula* from the collection of the Instituto Biológico of São Paulo were studied. Specimens were collected in McPhail-type traps and were also reared from fruits. Individuals were identified using the aculeus shape, as described by White and Elson-Harris (1992) and Zucchi (2000). For each species, 50 individuals with aculei and wings in good condition were selected for analysis. Because the *fraterculus* complex comprises several cryptic species

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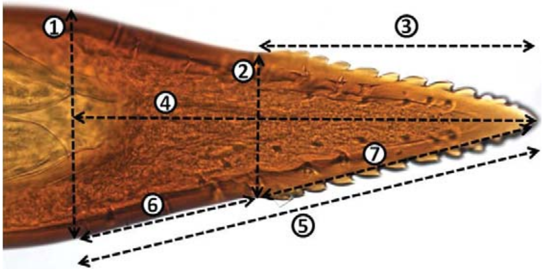

Fig. 1. Aculeus apex with measurements. 1 = width of aculeus tip at end of cloaca opening; 2 = width of aculeus at base of serrate part; 3 = length of serrate part; 4 = distance from cloaca opening to apical end of aculeus; 5 = lateral part, from the cloaca opening to the apical end; 6 = distance from cloaca opening to beginning of serrate part; and 7 = length of margin of aculeus tip from base of serrate part to apex. (Online figure in color.)

(Hernández-Ortiz et al. 2012), the name *A. fraterculus* is used here in its sensu lato.

Image Capture of Aculeus and Wings. Aculei were mounted on temporary microscope slides. The oviscape was dissected and treated in a solution of 10% KOH for 12 h. The aculeus was removed, placed ventral side up on a microscope slide with glycerin, and covered with a glass coverslip. The aculei were photographed with a Nikon DS-Fi1 camera (CCD, 5M, resolution 2560 × 1920) attached to a Nikon microscope (10× and 40× planachromatic objectives). After they were photographed, the aculei were stored in polyethylene tubes with glycerin.

The right wing of each specimen was dissected and mounted on a microscope slide with Euparal and covered with a glass coverslip. The slides were photographed with a Nikon DS-Fi1 camera (resolution 2560 × 1920) attached to a Nikon SMZ 1500 stereomicroscope (1.5× objective).

Morphometrics. For the conventional morphometrics, seven measurements of the aculeus apex were taken using the Motic Images Advanced 3.2 software: L1 = width of aculeus tip at end of cloaca opening; L2 = width of aculeus at base of serrate part; L3 = length of serrate part; L4 = distance from cloaca opening to apical end of aculeus; L5 = lateral part, from the cloaca opening to the apical end; L6 = distance from cloaca opening to beginning of serrate part; and L7 = length of margin of aculeus tip from base of serrate part to apex (Fig. 1). These measurements were used to calculate the mean and amplitude for each species, in the R software (R Development Core Team 2008). All the measurements were taken by P. P.

For the geometric morphometrics of the wings, 17 landmarks (Fig. 2) were scored for each wing, using TPSDig 2.1 (Rohlf 2006). We decomposed the form of all the landmark configurations into shape and size by means of geometric morphometrics (Bookstein 1991, 1996; Dryden and Mardia 1998). Size was measured as centroid size (CS), the square root of the sum of the squared distances of each landmark from the centroid. The centroid is the mean of all landmarks and can be interpreted as the gravity center of the landmark configuration (Zelditch 2004). To measure the shape, all configurations were scaled to unit CS, and superimposed by a generalized least squares (GLS) Procrustes procedure. A mean shape was calculated for all wings, and the differences between its landmarks and those of each specimen were the residuals of the GLS procedure. We used the Relative Warps, a principal components analysis on the residuals of GLS, as shape variables (Bookstein 1996). The last four axes are null, given the dimensionality lost in the Procrustes superimposition.

Analyses. To assess the variation in the three species studied, we carried out a multivariate analysis of variance for the aculei and the wings. To test the capacity to discriminate the species by aculeus measurements and wing shape, we performed separate linear discriminant analyses (LDA) for the aculeus measurements and for the wing-shape variables in combination with the CS. We also performed a leave-one-out cross-validation procedure, i.e., one specimen was not included in the calculations of the discriminant func-

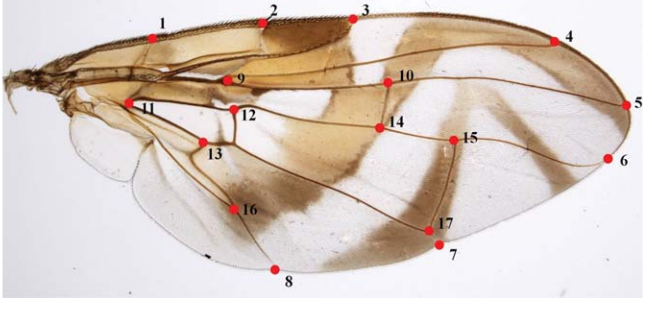

Fig. 2. Wing with landmarks. 1 = intersection of humeral (h) and costal veins (C); 2 = intersection of subcostal vein (Sc) with margin; 3 = intersection of vein R_1 with margin; 4 = intersection of veins R_{2+3} with margin; 5 = intersection of veins R_{4+5} with margin; 6 = intersection of vein M with apical margin; 7 = intersection of vein Cu_1 with apical margin; 8 = intersection of vein $A_1 + Cu_2$ with posterior margin; 9 = intersection of vein R_{2+3} and R_{4+5}; 10 = intersection of veins r-m and R_{4+5}; 11 = intersection of vein M and base of cell bm; 12 = intersection of veins M and m-cu; 13 = intersection of veins Cu_1 and Cu_2; 14 = intersection of veins M and r-m; 15 = intersection of veins M and DM-Cu; 16 = intersection of vein A_1 with apex of cell bcu; 17 = intersection of veins Cu_1 and DM-Cu. Wing vein terminology was based on White et al. (1999). (Online figure in color.)

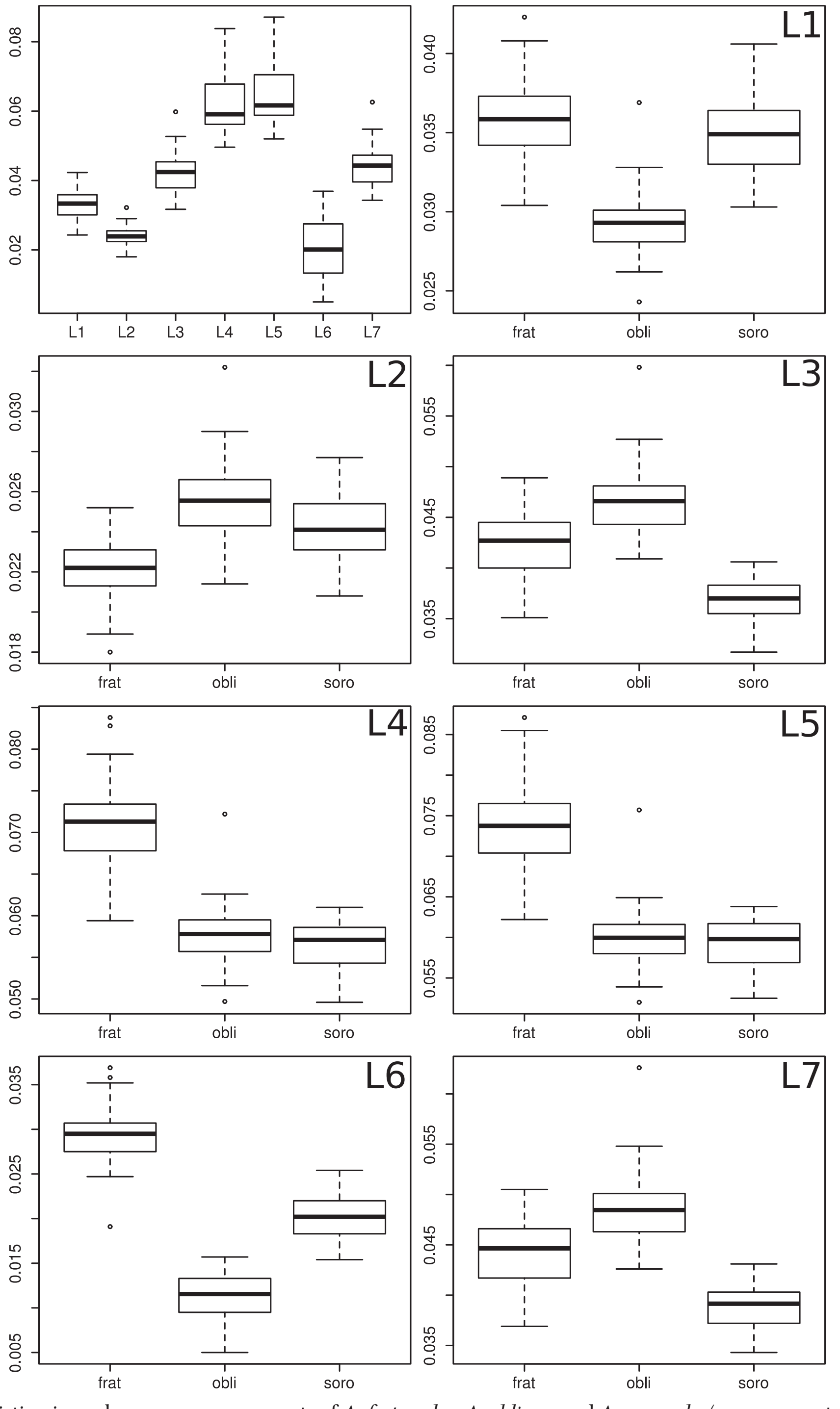

Fig. 3. Variation in aculeus apex measurements of *A. fraterculus*, *A. obliqua*, and *A. sororcula* (measurements as in Fig. 1).

tions; the specimen not used was then classified according to this function. As we produced an LDA for the shape variables of both structures, the results are directly comparable. All morphometric and statistical analyses were performed with the R software (R Development Core Team 2008).

Results

The aculeus tip measurements showed wide intraspecific and interspecific variation (Fig. 3). The aculeus tip lateral measurement L6 (distance from cloaca opening to beginning of serrate part) was the

Table 1. Identification of three *Anastrepha* species through LDA based on aculeus measurements, followed by leave-one-out cross-validation

Species	*A. fraterculus*	*A. obliqua*	*A. sororcula*	%	Total
A. fraterculus	48	0	2	96%	50
A. obliqua	0	50	0	100%	50
A. sororcula	0	0	50	100%	50

Rows show the actual identity of the individuals; columns show the identities indicated by the analysis.

measurement that best distinguished the three species. For *A. fraterculus*, the most variable aculeus tip measurements were L4 (distance from cloaca opening to the extreme apex) and L5 (serrate lateral part), and most clearly separated *A. fraterculus* from the other species. This result was expected, as these measurements are part of the characters used to identify *A. fraterculus.* Except for *A. sororcula,* almost all measurements had outliers, reflecting the intraspecific variation in the other two species.

The differences in the shape of the aculeus among the species were highly significant (Pillai = 1.641, $F_{14,284}$ = 92.6, $P < 0.001$), and multiple comparisons using Bonferroni correction showed that all pairs of species are significantly different. LDA of the conventional morphometric aculeus variables clustered the three species very successfully. Only 2 (1.31%) individuals were wrongly identified in the cross-validation test (Table 1). *A. obliqua* was isolated from other species on Axis 1, and *A. sororcula* and *A. fraterculus* were separated on Axis 2 (Fig. 4).

The geometric morphometric analysis showed significant differences in wing shape among the species (Pillai = 0.550, $F_{10,288}$ = 8.644, $P < 0.001$), and multiple comparisons using Bonferroni correction showed that all pairs of species are significantly different. All species were tightly clustered by means of LDA, forming three distinct groups (Fig. 5); only five (3.3%) individuals had identification errors in the cross-validation

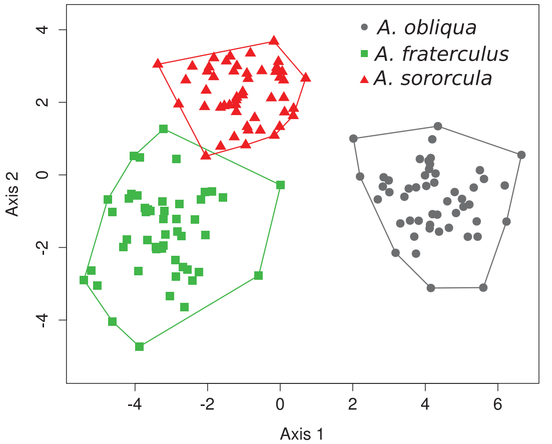

Fig. 4. Values on the first two axes of a linear discriminate analysis based on aculeus measurements, used to identify three *Anastrepha* species. (Online figure in color.)

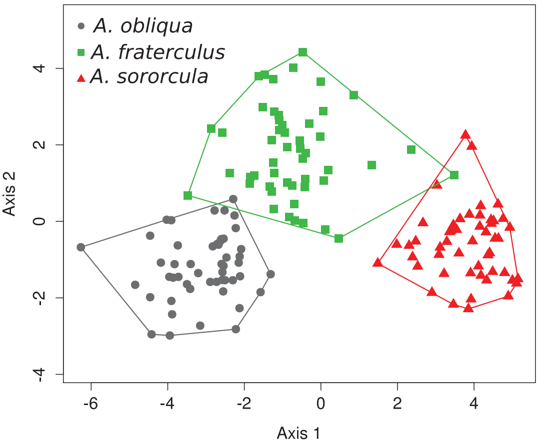

Fig. 5. Values on the first two axes of a linear discriminate analysis based on wing shape and size variables, used to identify three *Anastrepha* species. (Online figure in color.)

test (Table 2). Therefore, wing morphology separates these species quite clearly. The two axes that described the largest proportion of variation between species correspond to the posterior (Axis 1) and the distal (Axis 2) regions of the wing (Fig. 6).

Discussion

Geometric morphometrics has been used effectively in several genera of Tephritidae. For example, cryptic species such as *Rhagoletis pomonella* (Walsh, 1867) and *Rhagoletis zephyria* (Snow, 1894) can be separated based on wing shape (Yee et al. 2009). Several cryptic species of *Bactrocera* were also separated by means of geometric morphometrics (Schutze et al. 2012). Although it is an excellent tool to distinguish closely related species, geometric morphometry has not previously been applied to species of *Anastrepha*, except for a recent study on *A. pickeli* Lima, 1934, in which the wing shape varied significantly (Bomfim et al. 2011).

In this study, all species showed intraspecific variation, both in aculeus and wing measurements, with *A. sororcula* being the least and *A. fraterculus* the most heterogeneous. Probably the heterogeneity in *A. fraterculus* is due to the taxon being formed by a complex of cryptic species. However, the identification accuracies were high (98 and 96% for aculeus and wing, respectively). Wing pattern is used to identify

Table 2. Identification of three *Anastrepha* species based on linear discriminant analysis using wing shape and size variables, followed by leave-one-out cross-validation

Species	*A. fraterculus*	*A. obliqua*	*A. sororcula*	%	Total
A. fraterculus	47	1	2	94%	50
A. obliqua	1	49	0	98%	50
A. sororcula	1	0	49	98%	50

Lines show the actual identity of the individuals; columns show the identities indicated by the analysis.

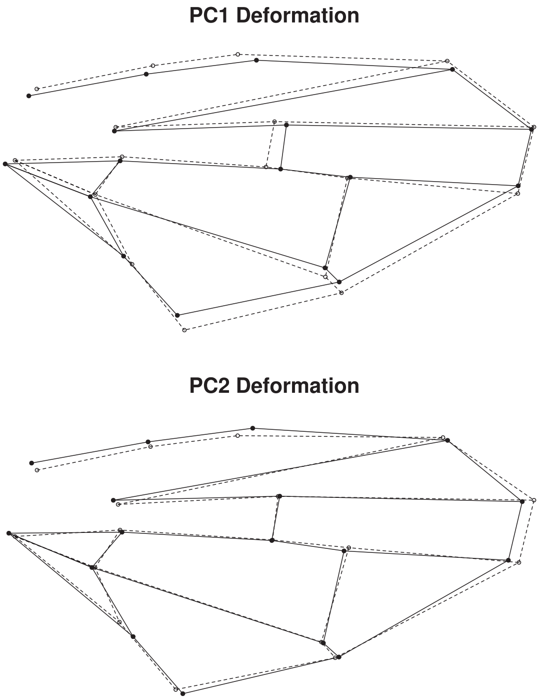

Fig. 6. Wing shape variation along each of the first two relative warps of three *Anastrepha* species. Dashed lines represent the shape at the minimum negative values; solid line represents the shape at the maximum values.

some species of *Anastrepha,* but is not reliable to separate species in the *fraterculus* group, and the 28 species of the group have not been taxonomically revised (Norrbom et al. 1999). However, our results showed that wing shape discriminated these three species of the *fraterculus* group with >96% accuracy.

Both geometric and conventional morphometrics separated the species successfully, but geometric morphometrics were more effective because this tool provides greater statistical power and allows the direct visualization of the geometric transformations of the objects (Rohlf and Marcus 1993). Wing images have many advantages over aculei: the preparation of wing microscope slides and image capture are far easier, cheaper, and require less technical training; wings have a venation system that allows the determination of precise landmarks; and both the female and male can be identified. However, to apply this method also with males, it will have to be validated with reared or genetically identified individuals. Given that these three species are pests of major importance, the techniques presented in this study, if extended to other species of *Anastrepha,* may conceivably be used by technical staff in the identification of fruit flies in quarantine and monitoring programs and in other applications. These techniques would be especially useful if implemented in a software package that acquires the wing shape variables automatically, allowing even nonexpert technical staff to identify economically important fruit flies. Such a tool is currently under development by our group. These techniques can help to resolve taxonomic uncertainties in the *fraterculus* complex. This is a large group of cryptic species, and several studies have used different approaches to identify them. Hernández-Ortiz et al. (2004, 2012) used conventional morphometrics of the mesonotum, wing, and aculeus, and his results suggested distinct entities. Also, through conventional morphometrics combined with molecular analyses, Selivon (2005) identified three species of the *fraterculus* group in Brazil.

The high accuracy of wing geometric morphometrics allowed us to identify with great accuracy these three pest species in the *A. fraterculus* group. The higher sensitivity and clear visualization of differences provided by geometric morphometrics are valuable tools in clarifying this large and cryptic complex. Moreover, this technique can be applied in automated identification techniques for these as well as other economically important species.

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