

# Discrimination of four *Culex* (*Culex*) species from the Neotropics based on geometric morphometrics

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**Abstract** Achieving correct identification of *Culex* species is difficult because many anatomical characters of larvae and females are polymorphic or overlap among distinct species. The overlapping is known to occur between *Culex bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*, from the subgenus *Culex*. The first three were incriminated in viruses' transmission in Argentina. The purpose was to distinguish between specimens of four species using geometric morphometric procedures. From field and entomological collections, 10 type I and 10 type II landmarks on the wings of females and the dorsomentum of larvae, respectively, were defined. The free morphometric software modules by J.P. Dujardin were used. Landmark coordinates were submitted to Procrustes and TPS analyses to generate size (centroid size, CS) and shape (partial warps, PW) variables. Size analysis was performed by nonparametric comparisons of CS measurements based on permutations, and shape, by submission of PW to discriminant analysis. Re-identification and identification of

external specimens were also realized. Wing and Dm shapes gave similar results. The individuals arranged into three groups coinciding with *Cx. bidens* + *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*. The latter two had a very different wing and Dm shapes. The accurate re-identification was greater than or equal to 77 % in all cases, and the identification of external specimens was achieved for all the species and both structures. Larval characters are more informative than female features. Geometric morphometrics, as a complementary tool, will facilitate identification in junction with others, such as DNA sequence analysis.

**Keywords** Taxonomy · Identification · Mosquito · Size · Shape

## Introduction

The genus *Culex* includes 768 species (Harbach 2013), and its classification is based primarily on anatomical characters of adults (Harbach et al. 2012). In the subgenus *Culex*, with 198 species, achieving accurate identification depends on a small number of morphological traits, chiefly of the male genitalia (Harbach et al. 2012). Anatomical characters of both fourth-instar larvae and females must be used with caution because most either are polymorphic or overlap among distinct species. The absence of clear distinctions between species creates problems for taxonomists, ecologists (Dobigny et al. 2002) and epidemiologists. Among other species problematic for identifying of the subgenus *Culex* in Argentina, *Cx. bidens* Dyar 1922, *Cx. interfor* Dyar 1928, *Cx. mollis* Dyar and Knab 1906 and *Cx. tatoi* Casal and García 1971 could be distinguished by some male genitalia traits. The first two species and the last two species are sister species, as shown in Laurito and Almirón

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(2013). *Culex bidens* and *Cx. interfor* differ from *Cx. mollis* and *Cx. tatoi* in that they lack the dorsal arm of the phallosome, and the ventral arm is curved laterally. *Culex bidens* could be distinguished by the presence of two teeth on the lateral arm and only one in *Cx. interfor*. *Culex mollis* and *Cx. tatoi*, the latter currently only recorded in Argentina, have the ventral arm of the phallosome T shaped. *Culex mollis* shows a setal pattern of the external tergal margin of the gonocoxite, which is not as strong as in *Cx. tatoi*. Finally, *Cx. tatoi* also has annulations on the apical third of the gonostylus.

Species of the subgenus *Culex* have been implicated in the transmission of several arboviruses in Argentina. *Culex bidens* in particular was suspected to be the vector of *Venezuelan Equine Encephalitis* virus (VEEV) during the 1988 epizootic (Sabattini et al. 1998). Strains of Río Negro virus of the VEEV complex were sequenced from a pool of *Cx. mollis* from S.M de Tucumán (Pisano et al. 2010), and *Cx. interfor* is considered to be a secondary vector of the *Saint Louis Equine Encephalitis* virus (Spinsanti et al. 2009) in Córdoba.

Geometric morphometrics is a new method for the study of shape variation (Rohlf 2002), with an increasing usage for solving taxonomic problems involving medically important insects (Dujardin et al. 2003; Jirakanjanakit et al. 2007, 2008; Demari-Silva et al. 2014). Advances over the traditional approach include the means of measuring the degree of differences between shapes by Procrustes distance, which allows shape space to be defined and characterized, and the development of specialized statistical methods (Rohlf 2002). Traditional morphometric approaches are based on standard multivariate analyses of arbitrary collections of distance measurements, ratios and angles. These variables represent only a part of the information that can be obtained from the relative positions of the landmarks on which these measurements are based. These methods ignore information about spatial relationships between the measured variables. Mathematically, shape has the properties of the landmarks that are invariant to the effects of size, location and orientation of an object (Rohlf 2002). It has been proven that geometric morphometrics is powerful enough to study variation among populations of a single species (Demari-Silva et al. 2014), species complexes (Dobigny et al. 2002; Gómez et al. 2013), interspecific hybridization (Monti et al. 1998), isofemale lines and distinct species (Jirakanjanakit et al. 2007, 2008; Börstler et al. 2014).

The purpose of this study was to distinguish between specimens of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* using geometric morphometric procedures. The anatomical structures used were the left wing of females and the right side of the dorsomentum (Dm) of the fourth-

instar larvae. The wing was chosen because of its two-dimensional form, homologous pattern of veins and ease of photography and location of landmarks (Baylac et al. 2003), as well as providing reliable information at different taxonomic categories for both traditional (Ruttner 1988) and geometric (Dujardin et al. 2003; Jirakanjanakit et al. 2007, 2008) morphometric approaches. Even though using geometric morphometrics with the Dm is novel, the structure has advantages similar to the wing.

## Materials and methods

### Insects

The mosquitoes studied are from Argentina, obtained from field and entomological collections. Because the available material includes pin-mounted adults, of the biological collections of the Centro de Estudios Parasitológicos y de Vectores (CEPAVE, CONICET-Universidad Nacional de La Plata, Argentina) and the Centro de Investigaciones Entomológicas (CIEC, Universidad Nacional de Córdoba, Argentina), wings were photographed without removing them from specimens. Larval exuviae were mounted on microscope slides in Canada balsam. Larvae were collected at field and individually reared in the laboratory until the adult stage. Therefore, from each larva it was possible to obtain its respective larval and pupal exuviae and the male or female that emerged. Females and fourth-instar larval exuviae were identified using published taxonomic keys (Darsie 1985; Forattini 2002). Male identification was based on the genitalia traits, which allowed an accurate species identification (Casal and García 1971; Harbach et al. 1986; Sallum et al. 1996). Male identification also helped to support female identification collected from the same breeding site. Until now, the species have never been found breeding simultaneously and together (even though they breed in pools at ground level). Sixty wings and 44 Dm were studied, as follows: *Cx. bidens* (15 wings and 11 Dm), *Cx. interfor* (19 wings and 18 Dm), *Cx. mollis* (8 wings and 8 Dm) and *Cx. tatoi* (18 wings and 7 Dm). Information on the collection and origin of the specimens is given in Table 1. Permission to visit collection sites was not required as the activities did not involve protected areas or endangered species.

Some specimens of collections were not perfectly preserved; wing dissection was not possible, and identifying females implied association with males from the same collection site to ensure identification. For the Dm, the reasons for the small sample size are similar to the wings and in some specimens other mouthparts covering the Dm precluded the taking of photographs.

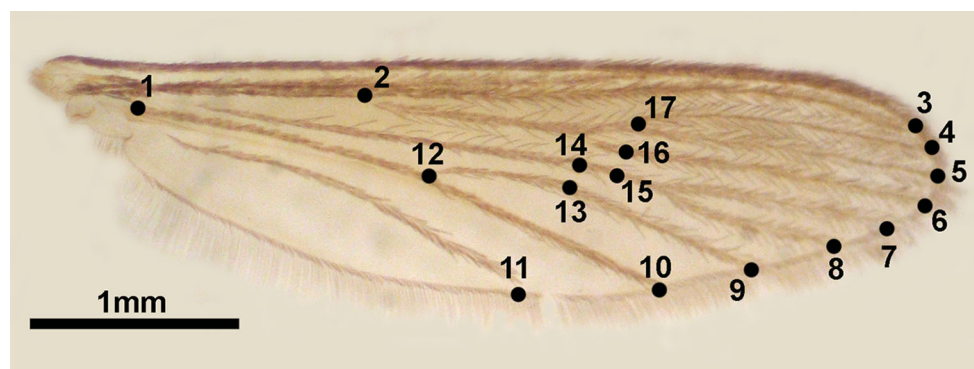
**Table 1** List of female specimens used to morphometric analysis, specimen numbers, localities, collection date, geographical coordinates, voucher location and structure

Species	Sample ID	Province	Locality	Date	Latitude	Longitude	Voucher location	Structure
<i>Culex bidens</i>	MLP0017	Cor	MC	Nov 2002	30°37'38.09"S	57°58'56.83"W	CEPAVE	W
<i>Culex bidens</i>	MLP0006	Cor	MC	Nov 2002	30°37'38.09"S	57°58'56.83"W	CEPAVE	W
<i>Culex bidens</i>	MLP0012	Cor	MC	Nov 2002	30°37'37"S	57°58'56.90"W	CEPAVE	W
<i>Culex bidens</i>	MLP0011	Cor	MC	Nov 2002	30°37'37"S	57°58'56.90"W	CEPAVE	W
<i>Culex bidens</i>	MLP0020	Cor	MC	Nov 2002	30°37'38.09"S	57°58'56.83"W	CEPAVE	W
<i>Culex bidens</i>	MLP0045	Mis	Pos	Dec 2005	27°25'19.95"S	55°57'38.63"W	CEPAVE	Dm
<i>Culex bidens</i>	M66-20	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex bidens</i>	M68-10	Cba	Coq	Feb 1987	31°22'28"S	64°36'33"W	CIEC	W/Dm
<i>Culex bidens</i>	M66-12	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex bidens</i>	M68-07	Cba	Coq	Feb 1987	31°22'28"S	64°36'33"W	CIEC	W/Dm
<i>Culex bidens</i>	M68-13	Cba	Coq	Feb 1987	31°22'28"S	64°36'33"W	CIEC	W/Dm
<i>Culex bidens</i>	M66-18	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex bidens</i>	M66-21	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex bidens</i>	M68-09	Cba	Coq	Feb 1987	31°22'28"S	64°36'33"W	CIEC	W/Dm
<i>Culex bidens</i>	M66-06	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex bidens</i>	M66-14	Cba	Coq	Feb 1987	31°22'34.4"S	64°35'34.7"W	CIEC	W/Dm
<i>Culex interfor</i>	MLP2052	ER	Con	Dec 2005	30°37'38.09"S	57°58'56.83"W	CEPAVE	W/Dm
<i>Culex interfor</i>	MLP2053	ER	Con	Dec 2005	30°37'38.09"S	57°58'56.83"W	CEPAVE	W/Dm
<i>Culex interfor</i>	S1-T1-05	Cba	AC	Nov 2004	30°54'S	62°18'W	CIEC	W/Dm
<i>Culex interfor</i>	S2-T4-14	Cba	AC	Jan 2006	30°14'22"S	62°01'12"W	CIEC	Dm
<i>Culex interfor</i>	S1-T1-04	Cba	AC	Nov 2004	30°54'S	62°18'W	CIEC	Dm
<i>Culex interfor</i>	S2-T4-01	Cba	AC	Jan 2006	30°14'22"S	62°01'12"W	CIEC	Dm
<i>Culex interfor</i>	S2-T4-09	Cba	AC	Jan 2006	30°14'22"S	62°01'12"W	CIEC	Dm
<i>Culex interfor</i>	S1-T1-15	Cba	AC	Nov 2004	30°54'S	62°18'W	CIEC	Dm
<i>Culex interfor</i>	M93-11	Cba	VMRS	Dec 1987	29°53'S	63°43'W	CIEC	W/Dm
<i>Culex interfor</i>	M93-23	Cba	VMRS	Dec 1987	29°53.02'10"S	63°43'02"W	CIEC	W/Dm
<i>Culex interfor</i>	M93-17	Cba	VMRS	Dec 1988	29°53'S	63°43'W	CIEC	W/Dm
<i>Culex interfor</i>	M93-05	Cba	VMRS	Dec 1987	29°53.02'10"S	63°43'02"W	CIEC	W
<i>Culex interfor</i>	M93-03	Cba	VMRS	Dec 1987	29°53.02'10"S	63°43'02"W	CIEC	W
<i>Culex interfor</i>	M93-06	Cba	VMRS	Dec 1988	29°53'S	63°43'W	CIEC	W
<i>Culex interfor</i>	M93-19	Cba	VMRS	Dec 1988	29°53'S	63°43'W	CIEC	W
<i>Culex interfor</i>	M93-20	Cba	VMRS	Dec 1988	29°53'S	63°43'W	CIEC	W
<i>Culex interfor</i>	LR-Ci15	LR	Ch	Feb 2008	30°22'S	66°19'W	CIEC	W/Dm
<i>Culex interfor</i>	LR-Ci08	LR	Ch	Feb 2008	30°22'S	66°19'W	CIEC	W/Dm
<i>Culex interfor</i>	LR-Ci07	LR	Ch	Feb 2008	30°22'S	66°19'W	CIEC	W/Dm
<i>Culex interfor</i>	LR-Ci12	LR	Ch	Feb 2008	30°22'S	66°19'W	CIEC	Dm
<i>Culex interfor</i>	SL-Ci07	SL	Can	Mar 2007	32°03'28"S	65°49'59"W	CIEC	W/Dm
<i>Culex interfor</i>	SL-Ci01	SL	Can	Mar 2007	32°03'28"S	65°49'59"W	CIEC	W/Dm
<i>Culex interfor</i>	SL-Ci04	SL	Can	Mar 2007	32°03'28"S	65°49'59"W	CIEC	W/Dm
<i>Culex interfor</i>	SL-Ci02	SL	Can	Mar 2007	32°03'28"S	65°49'59"W	CIEC	W
<i>Culex interfor</i>	SL-Ci05	SL	Can	Mar 2007	32°03'28"S	65°49'59"W	CIEC	W
<i>Culex mollis</i>	MLP0022	BA	PL	Jan 2003	34°47'8.92"S	58°0'57.35"W	CEPAVE	Dm
<i>Culex mollis</i>	Mi-Ci06	Mis	PI	Feb 2006	25°31'5"S	54°08'W	CIEC	W/Dm
<i>Culex mollis</i>	Mi-Ci04	Mis	PI	Feb 2006	25°38'29.2"S	54°34'06.5"W	CIEC	W/Dm
<i>Culex mollis</i>	Mi-Ci03	Mis	PI	Feb 2006	25°31'5"S	54°08'W	CIEC	W/Dm
<i>Culex mollis</i>	Mi-Ci09	Mis	PI	Feb 2006	25°31'5"S	54°08'W	CIEC	W/Dm
<i>Culex mollis</i>	Mi-Ci08	Mis	PI	Feb 2006	25°38'29.2"S	54°34'06.5"W	CIEC	W/Dm
<i>Culex mollis</i>	Mi-Ci12	Mis	PI	Feb 2006	25°38'29.2"S	54°34'06.5"W	CIEC	W

**Table 1** continued

Species	Sample ID	Province	Locality	Date	Latitude	Longitude	Voucher location	Structure
<i>Culex mollis</i>	M2-N05	CH	R	Jun 2001	27°27.2'12.09"S	58°58.7'13"W	CIEC	W/Dm
<i>Culex mollis</i>	M2-N02	CH	R	Jun 2001	27°27.4'05.6"S	58°59.6'19"W	CIEC	W/Dm
<i>Culex tatoi</i>	M8-N06	Cba	Cap	Mar 1994	31°21'29.23"S	64°13'31.31" W	CIEC	W/Dm
<i>Culex tatoi</i>	M9-N01	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W/Dm
<i>Culex tatoi</i>	M9-N12	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W/Dm
<i>Culex tatoi</i>	M8-N10	Cba	Cap	Mar 1994	31°21'29.23"S	64°13'31.31" W	CIEC	W/Dm
<i>Culex tatoi</i>	M8-N09	Cba	Cap	Mar 1994	31°21'29.23"S	64°13'31.31" W	CIEC	W/Dm
<i>Culex tatoi</i>	M5-N07	Cba	Cap	Feb 1997	31°24'6.61"S	64°12'10.64" W	CIEC	W/Dm
<i>Culex tatoi</i>	M9-N08	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W/Dm
<i>Culex tatoi</i>	M8-N15	Cba	Cap	Mar 1994	31°21'29.23"S	64°13'31.31" W	CIEC	W
<i>Culex tatoi</i>	M9-N11	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M5-N02	Cba	Cap	Feb 1997	31°24'6.61"S	64°12'10.64" W	CIEC	W
<i>Culex tatoi</i>	M9-N06	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M9-N05	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M5-N06	Cba	Cap	Feb 1997	31°24'6.61"S	64°12'10.64" W	CIEC	W
<i>Culex tatoi</i>	M5-N18	Cba	Cap	Feb 1997	31°24'6.61"S	64°12'10.64" W	CIEC	W
<i>Culex tatoi</i>	M9-N02	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M9-N03	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M9-N17	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W
<i>Culex tatoi</i>	M9-N20	Cba	Cap	Dec 1994	31°26'33.20"S	64°10'2.78" W	CIEC	W

AC Altos de Chipión, BA Buenos Aires, Can Candelaria, Cap Capital, Cba Córdoba, CEPAVE Centro de Estudios Parasitológicos y de Vectores, CH Chaco, Ch Chamental, CIEC Centro de Investigaciones Entomológicas de Córdoba, Con Concordia, Coq Cosquín, Cor Corrientes, Dec December, Dm dorsomentum, ER Entre Ríos, Feb February, Jan January, LR La Rioja, Mar March, MC Monte Caseros, Mis Misiones, Nov November, PI Puerto Iguazú, PL Punta Lara, Pos Posadas, R Resistencia, SL San Luis, VMRS Villa María del Río Seco, W wing



**Fig. 1** *Culex* sp. female left wing. The positions of the 17 landmarks are indicated with black circles. 1 1A origin, 2  $R_s$  termination, 3  $R_1$  termination, 4  $R_2$  termination, 5  $R_3$  termination, 6  $R_{4+5}$  termination, 7  $M_1$  termination, 8  $M_2$  termination, 9  $M_{3+4}$  termination, 10 CuP

termination, 11 1A termination, 12 CuA and mcu intersection, 13 mcu and  $M_{3+4}$  intersection, 14  $M_{3+4}$  origin, 15 M vein and rm intersection, 16 rm and  $R_{4+5}$  intersection, 17  $R_{2+3}$  bifurcation

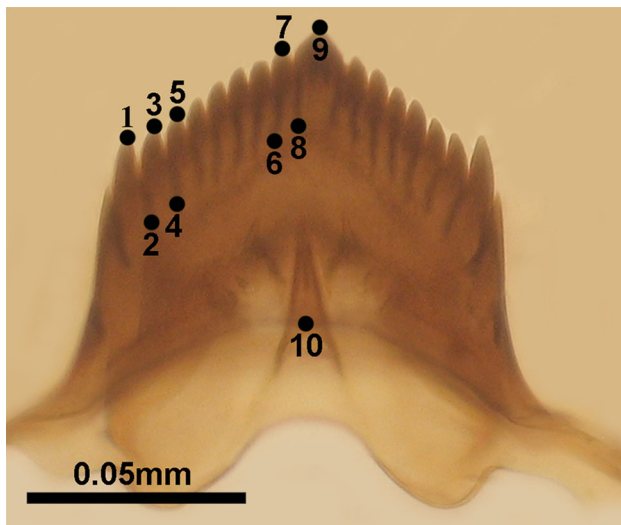
### Geometric morphometrics

The photographs of wings were taken with a Canon Power Shot A400 digital camera fitted to a Carl Zeiss Stemi 2000-C stereomicroscope, and an Olympus BX-40 phase contrast microscope was used to photograph the Dm. Seventeen type I landmarks on the dorsal surface of the

wing (Fig. 1) were defined as follows: 1—1A origin, 2— $R_s$  termination, 3— $R_1$  termination, 4— $R_2$  termination, 5— $R_3$  termination, 6— $R_{4+5}$  termination, 7— $M_1$  termination, 8— $M_2$  termination, 9— $M_{3+4}$  termination, 10—CuP termination, 11—1A termination, 12—CuA and mcu intersection, 13—mcu and  $M_{3+4}$  intersection, 14— $M_{3+4}$  origin, 15—M vein and rm intersection, 16—rm and  $R_{4+5}$  intersection and

17— $R_{2+3}$  bifurcation. Vein nomenclature is taken from Harbach (2013). Because the pictures were taken from intact specimens, and to ensure that the wings resembled to a two-dimensional surface, only the wings in which all landmarks could be focused on the same field were photographed. Due to the thickness of the veins, landmark location was standardized according to the method of Baylac et al. (2003), as follows: For two veins intersecting the landmark location was taken at the crossing center between both and for veins which terminate at wing margin, at the center of the vein apex. Ten type II landmarks of the Dm (Fig. 2) were defined (teeth are numbers with Roman numerals from the most lateral to the median/central tooth): 1—apex of tooth I, 2—most basal separation of teeth I and II, 3—apex of tooth II, 4—most basal separation of teeth II and III, 5—apex of tooth III, 6—most basal separation of teeth IV and V, 7—apex of tooth V, 8—most basal separation of tooth V and the central tooth (CT), 9—apex of the CT and 10—most basomedian point of the Dm. In spite of Dm is a two-dimensional structure, only Dms in which all landmarks could be focused on the same field were photographed. Landmark sizes in both figures are illustrative.

The free morphometric software modules (COO, MOG, VAR, PAD, COV) by J.P. Dujardin of the Institut de Recherche pour le Développement, available at <http://www.mpl.ird.fr/morphometrics>, were used. The coordinates of the landmarks were digitized by using the COO module to obtain geometric configuration matrices, each



**Fig. 2** *Culex* sp. larval dorsomentum. The positions of the 10 landmarks are indicated with black circles. 1 apex of tooth I, 2 most basal separation of teeth I and II, 3 apex of tooth II, 4 most basal separation of teeth II and III, 5 apex of tooth III, 6 most basal separation of teeth IV and V, 7 apex of tooth V, 8 most basal separation of tooth V and the central tooth (CT), 9 apex of the CT, 10 most basomedian point of the Dm

one corresponding to a single wing or Dm of a particular mosquito. With MOG module, each matrix was scaled, translated and rotated through the generalized Procrustes and TPS analyses (Rohlf and Slice 1990; Bookstein 1991; Rohlf and Marcus 1993) to generate both shape (partial warps, PW) and size (centroid size, CS) variables for the multivariate analyses. The average residual coordinates after Procrustes analysis were shown to illustrate areas that diverge between the species *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*.

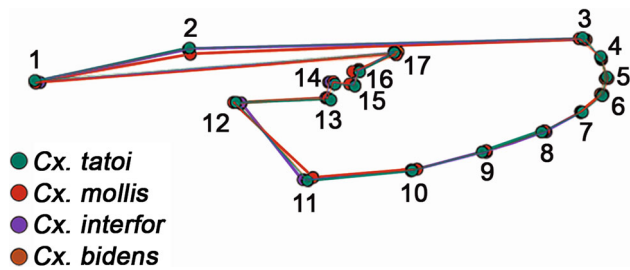
The size analysis of each structure of the species was performed by nonparametric comparisons of CS measurements based on permutation tests (Good 2006). The Qst value for the CS was also calculated; this variable divides the quantitative genetic variation analogously to the Fst for single genetic markers (Spitze 1993). Both nonparametric comparisons and Qst values were obtained using VAR module. To examine differences in wing and Dm shape between the species, the PWs were submitted to discriminant analysis (DA). The Mahalanobis distances obtained from the DA were used to evaluate how far the shape of each species is to the others and allowed the re-identification of the specimens previously used in the analysis. The landmark coordinates of other specimens not used in the analysis (external data) were entered in the discriminant function to obtain a tentative identification based on their position relative to the first two canonical factors. The Mahalanobis distances, DA, re-identification, identification of external specimens and statistical support were performed by using the PAD module.

Once the analyses were carried out, using COV module, the landmarks whose location had greater variation between the species were selected to redo the analyses because the sample size was small according to landmark number considered in the first instance. The complete landmark data set was used when the reduced data set was not great enough to characterized size and/or shape differences between the species. If the results remained unchanged with both landmark data sets, the reduced data set was preferred because it explained the greater shape variability of the structure. Structures of the wing and Dm were analyzed in parallel with the following organization: variability of landmarks, size and shape analyses, re-identification of specimens and identification of originally unidentified specimens (external).

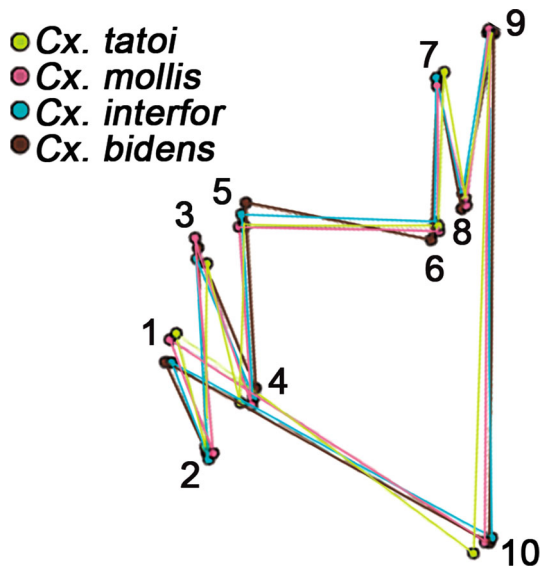
## Results

The wing landmarks whose location had greater variation between the species were: 1, 2, 3, 11, 12, 13, 14, 15, 16 and 17 (Fig. 3). Due to the fact that the outcome with the complete and reduced landmark data set remained





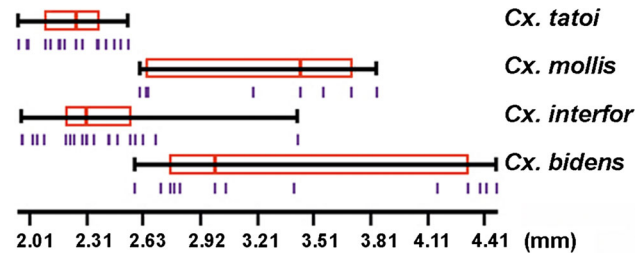
**Fig. 3** Average residual coordinates of the left wing of females of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*. Coordinates obtained after Procrustes analysis to the initial configurations and then averaged by species. Landmarks are numbered as in Fig. 1 and connected to display the wing geometry



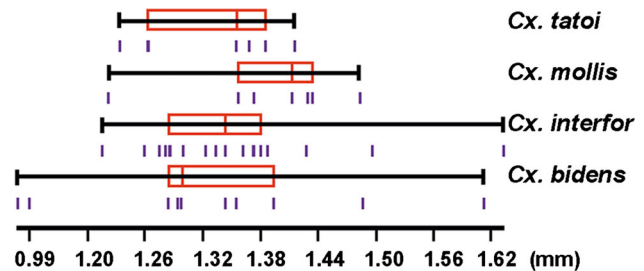
**Fig. 4** Average residual coordinates of the Dm of the fourth-instar larvae of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*. Coordinates obtained after Procrustes analysis to the initial configurations, and then averaged by species. Landmarks are numbered as in Fig. 2 and connected to display the Dm geometry

unchanged, the results obtained with the latter are shown. For the Dm, the reduced landmarks data set was compounded by the landmarks 1, 2, 4 and 10 (Fig. 4). Despite being the landmarks whose location had greater variation, these were insufficient to detect shape differences, whereby the complete data set was used.

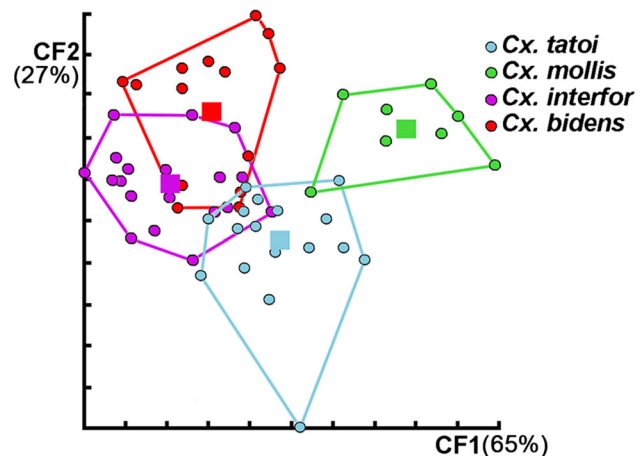
On average, the wings of *Cx. bidens* and *Cx. mollis* were significantly ( $P < 0.05$ ) larger than the wings of *Cx. interfor* and *Cx. tatoi* (Fig. 5). From this, nonsignificant differences were detected between the wings of *Cx. bidens*—*Cx. mollis* and *Cx. interfor*—*Cx. tatoi*. The Qst value (0.92) reflects a strong tendency to diversification between the two species pairs. Nonsignificant differences were found between the species relative to Dm size



**Fig. 5** Wing size analysis. Nonparametric comparisons of centroid size (CS) measurements (mm) based on permutations of the left wing of females of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* including median as a line across the middle of each box and the quartiles (25th and 75th percentiles). Vertical lines under the quartiles are specimens



**Fig. 6** Dorsomentum size analysis. Nonparametric comparisons of centroid size (CS) measurements (mm) based on permutations of the Dm of the fourth-instar larvae of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* including median as a line across the middle of each box and the quartiles (25th and 75th percentiles). Vertical lines under the quartiles are specimens



**Fig. 7** Discriminant analysis of wing shape. Distinctions between female specimens of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* based on wing shape. Polygons formed based on the projection of the individuals of each species onto the canonical factors 1 and 2. Contribution of each factor (CF1 and CF2) to the variation is indicated between brackets

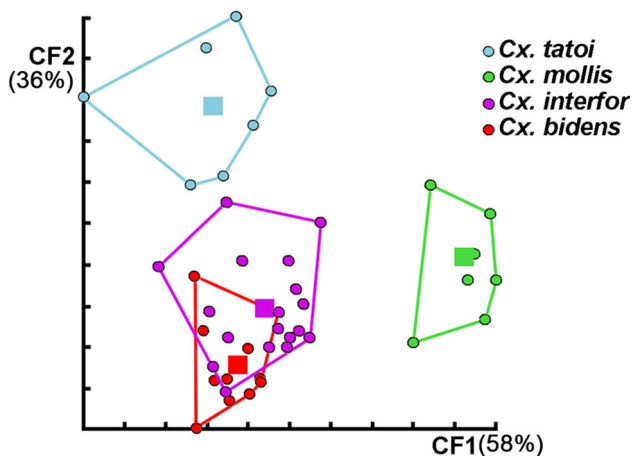
(Fig. 6). The Qst value (0.28) shows a strong tendency to homogeneity in Dm size between the species. Although polygons of all species share certain areas in Fig. 7,

**Table 2** Mahalanobis distances and  $P$  values obtained from the discriminant analysis carried out with the partial warps: left wing shape distinctions between specimens of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*; each comparison was tested for final significance using the Bonferroni test ( $P < 0.05$ )

	<i>Cx. interfor</i>	<i>Cx. mollis</i>	<i>Cx. tatoi</i>
<i>Cx. bidens</i>	1.97 (0.129)	4.01 (0.000)	2.80 (0.003)
<i>Cx. interfor</i>		4.75 (0.000)	2.59 (0.003)
<i>Cx. mollis</i>			3.41 (0.006)

**Table 3** Mahalanobis distances and  $P$  values obtained from the discriminant analysis carried out with the partial warps: Dm shape distinctions between the fourth-instar larvae of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*; each comparison was tested for final significance using the Bonferroni test ( $P < 0.05$ )

	<i>Cx. interfor</i>	<i>Cx. mollis</i>	<i>Cx. tatoi</i>
<i>Cx. bidens</i>	2.03 (0.417)	5.78 (0.000)	5.03 (0.000)
<i>Cx. interfor</i>		4.89 (0.001)	4.20 (0.000)
<i>Cx. mollis</i>			6.60 (0.000)



**Fig. 8** Discriminant analysis of dorsomentum. Distinctions between larval specimens of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* based on Dm shape. Polygons formed based on the projection of the individuals of each species onto the canonical factors 1 and 2. Contribution of each factor (CF1 and CF2) to the variation is indicated between brackets

significant differences ( $P < 0.05$ ) between wing shape were found except between *Cx. bidens* and *Cx. interfor* (Table 2). Two polygons so distinct illustrate the clear difference between the Dm shapes of *Cx. mollis* and *Cx. tatoi* and the overlapping areas of *Cx. bidens* and *Cx. interfor*, not significant enough to distinguish them (Table 3; Fig. 8).

Re-identification, based on the Mahalanobis distance of the mean wing conformation of a species and each specimen, was accurate (100 % of cases) in *Cx. bidens* and *Cx.*

*mollis*, 89 % in *Cx. interfor* and 94 % in *Cx. tatoi*. For the Dm, correct re-identification reached the following values: 100 % in *Cx. mollis* and *Cx. tatoi*, 81 % in *Cx. bidens* and 77 % in *Cx. interfor*.

Two external specimens were correctly identified as *Cx. tatoi* and *Cx. interfor* based on wing shape (the first two canonical factors). Accurate identification of two individuals as *Cx. mollis* and *Cx. tatoi* was also realized by the Dm data.

## Discussion

Achieving accurate identification of females of species of the subgenus *Culex* is problematic and depends on a small number of anatomical traits mainly restricted to male genitalia (Harbach et al. 2012). The unequivocal identification of species is essential for recognition of the vectors involved in the transmission of arboviruses. Size and shape analysis of structures such as the wings of adults and larval mouthparts, among others, may allow discovery of new useful features to distinguish morphologically similar species and resolve taxonomic problems (Dobigny et al. 2002; Gómez et al. 2013). Detecting shape differences at a general structural level or between two or more landmarks can be translated to traditional morphometric characters for use in taxonomic keys. Therefore, geometric morphometrics would be used to discover areas of variability, which searched exhaustively would be difficult to find. The purpose of this study was to distinguish between specimens of *Cx. bidens*, *Cx. interfor*, *Cx. mollis* and *Cx. tatoi* using geometric morphometric procedures.

Based on our data, both wing and Dm shapes gave similar results for the specimens of the four Neotropical species. The individuals were arranged into three groups coinciding with *Cx. bidens* + *Cx. interfor* (without differentiation), *Cx. mollis* and *Cx. tatoi*. *Culex bidens* and *Cx. interfor* have similar wing and Dm configurations, not allowing their differentiation. Partial warps of the wing show a significant organization of the three groups in spite of a slight intersection between them. Re-identification analyses based on wing shape reached high level of accuracy (more than 89 %). Among the most informative landmarks, 1, 2, 3, 11, 12, 13, 14, 15, 16 and 17, the relative position of the first two, associated with the origin of the anal vein (1A) and the termination of the radius sector vein ( $R_s$ ), and the 11th and 12th related to the termination of the anal vein (1A) and the intersection between cubitus anterior (CuA) and mediocubital crossvein (mCu) could be used to differentiate between the mentioned groups. Regarding the Dm shape, the polygons do not show intersection, allowing the differentiation of the three groups (*Cx. bidens* + *Cx. interfor*, *Cx. mollis* and *Cx. tatoi*). The

re-identification was also high (over than 77 %). The differentiation between the groups is based on the relative position of the landmarks 1, 2, 4 and 10, related the apex of tooth I, the separation of teeth I–II and II–III and the most basomedian point of the Dm. The differentiation could be obtained based on ratio or angle between the mentioned segments in each structure.

Wing size may contribute to diagnose *Cx. bidens* and *Cx. interfor*, but its suitability is limited to samples containing only these two species. Dorsomentum size could not discriminate among the four species. However, the size of structures may suffer environmental plasticity (Dujardin 2008) explained by temperature, altitude, breeding site conditions, among other factors, as documented for wings of *Anopheles funestus* Giles (Ayala et al. 2011) and larval head capsule of *An. merus* Donitz (Le Sueur and Sharp 1991); therefore, they should be used with caution. The use of wing size would be helpful if *Cx. bidens* and *Cx. interfor* were sympatric and the environmental and breeding conditions were still invariable in a certain range.

Geometric morphometric analyses revealed that individuals of the sister species, *Cx. mollis* and *Cx. tatoi*, can be distinguished based on wing and Dm shapes. In the subgenus *Culex*, many species could be identified, in addition to male genitalia traits, based on morphological characters of the fourth-instar larvae, as shown in Forattini (2002). Many phylogenetic relationships between subgenera of *Culex* have been inferred only from anatomical features of the larvae in general (Belkin 1962; Bram 1967; Danilov 1989) and larval mouthparts in particular (Navarro and Liria 2000), showing that this immature stage brings phylogenetic information as synapomorphic characters. Autapomorphic features are also common in the fourth-instar larvae, useful for diagnosis at different levels as species (Casal et al. 1966), subgenus (Rossi and Harbach 2008) and tribe (Harbach 2007), among others.

Specimens of *Cx. bidens* and *Cx. interfor* could be distinguished only by characters of the male genitalia. The high degree of overlap between wing and Dm shapes, morphological similarities in all stages (except male), close phylogenetic relationships (Laurito and Almirón 2013), the existence of anomalous male specimens of both species (unpublished data) and the fact that *Cx. interfor* is only recorded in Argentina lead us the question of the taxonomic status of these species. In Gómez et al. (2013), wing shape and two nuclear markers did not support the separation of the Albitarsis Complex members from Colombia, differences detected by barcode. As in the described situation for the Albitarsis Complex, the non-differentiation based on geometric morphometrics between *Cx. bidens* and *Cx. interfor*, in addition to data from other sources, could lead to the synonymy of the species or maybe, this tool is not powerful

enough to discriminate them. Besides morphological and morphometric data, molecular is necessary to evaluate the taxonomic status of *Cx. bidens* and *Cx. interfor*.

Our geometric morphometric analyses of four *Culex* (*Culex*) species from the Neotropics, despite being exploratory and with low prediction power because of the sample size, show a tendency toward distinction between a group which enclose *Cx. bidens* and *Cx. interfor* from the other the two species. The non-intersection between the polygons in the Dm shape analysis (except for *Cx. bidens* and *Cx. interfor*) shows that larval characters, particularly mouthparts, are more informative than female features. The use of geometric morphometric methods, as a complementary tool, will facilitate identification and the resolution of taxonomic problems, but it should be accomplished with large sample sizes in junction with other tools, such as DNA sequence analysis.

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