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## Comparative study of the phlebotomine sand fly species (Diptera: Psychodidae: Phlebotominae) of the genera *Nyssomyia* Barretto, 1962, *Bichromomyia* Artemiev, 1991, and *Migonemyia* Galati, 1995, vectors of American cutaneous leishmaniasis in Brazil

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### Abstract

Phlebotominae, a group of insects with great medical importance especially in Brazil, are responsible for transmitting causal agents of cutaneous and visceral leishmaniasis. In Brazil, the most important species of *Leishmania* Ross, 1903 are *L. (Viannia) braziliensis* Vianna, 1911, whose main vectors are *Nyssomyia intermedia* Lutz & Neiva, 1912, *Ny. neivai* Pinto, 1926, *Ny. whitmani* Antunes & Coutinho, 1939 and *Migonemyia migonei* França, 1920; and *L. (Leishmania) amazonensis* Lainson & Shaw, 1972, for which *Bichromomyia flaviscutellata* Mangabeira, 1942 is the main vector. The present study sought to investigate the morphological as well as geometrical and linear morphometric characteristics of these five sand flies in an attempt to cluster these species. Our aim was to reveal some of the characters that might help identify these phlebotomine species and also be useful in future phylogenetic studies. Comparative analyses by linear and geometric morphometric characters allowed us to distinguish the genera of these sand flies and assess the taxonomic position of *Ny. intermedia* and *Ny. neivai*, the so-called “cryptic species”. Significant differences were observed in several of the analyzed structures, including the centroid size of the wings and the ratio between the ejaculatory filament and its tip. Based on the linear morphometric analytical results, the size of the centroids of the wings and their shapes indicated that these three species of *Nyssomyia* are phenetically more similar to *Mg. migonei* (all vectors of *L. (V.) braziliensis*) than to *Bi. flaviscutellata* (vector of *L. (L.) amazonensis*). These results are in agreement with the division of the genera *Nyssomyia* and *Bichromomyia*.

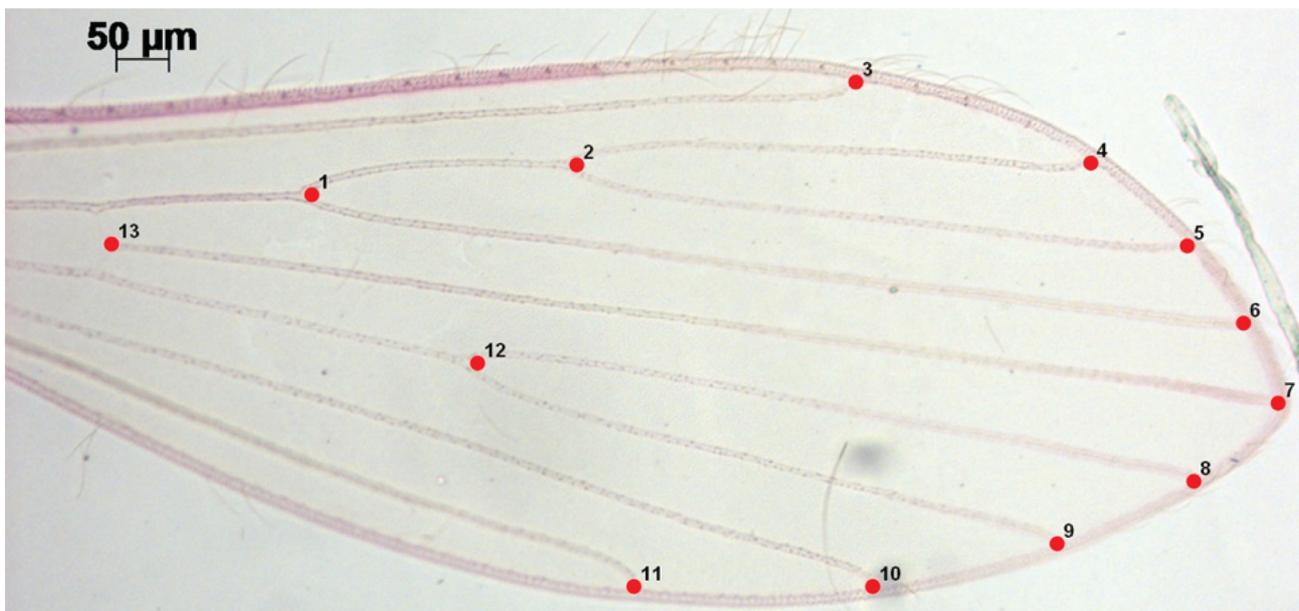
**Key words:** linear morphometric; geometric morphometric; morphology; discriminant analysis

### Introduction

Phlebotomine sand flies (Diptera, Psychodomorpha, Psychodidae) are of great medical importance due to their capacity to transmit etiologic agents such as protozoans of the genus *Leishmania* Ross, 1903, bacteria of the genus *Bartonella* Strong *et al.*, 1915, in addition to numerous arboviruses (Rangel & Lainson 2009). The most recent classification of New World Phlebotominae includes more than 500 species divided into two tribes: Hertigiini Abonnenc & Léger, 1976 and Phlebotomini Rondani, 1840. All the Brazilian species belong to four subtribes included in Phlebotomini: Brumptomyiina Artemiev, 1991, Sergentomyiina Artemiev, 1991, Lutzomyiina Abonnenc & Léger, 1976, and Psychodopygina Galati, 1995 (Galati 2003). Moreover, all the species that transmit *Leishmania* in Brazil are from the Lutzomyiina or Psychodopygina subtribes.

The principal sand fly species involved in the transmission of leishmania (i.e. species responsible for transmission in the majority of cutaneous leishmaniasis cases) include some species of the genus *Nyssomyia* Barretto, 1962 (Psychodopygina). Important vectors of *Leishmania* (*Viannia*) *braziliensis* Vianna, 1911 in Brazil are *Ny. neivai* Pinto, 1926 in the southeastern and southern regions, *Ny. intermedia* Lutz & Neiva, 1912 in the southeast and *Ny. whitmani* Antunes & Coutinho, 1939 in the south, midwest, northeast, and southeast of the country. *Migonemyia migonei* França, 1920 (Lutzomyiina) has been incriminated as a secondary *L. braziliensis* vector in many areas in the State of Ceará (where this sand fly was found naturally infected) and the State of Rio de Janeiro (Lainson & Shaw 2005; Brasil 2007; Rangel & Lainson 2009). *Bichromomyia flaviscutellata* Mangabeira, 1942, the main vector of *Leishmania* (*Leishmania*) *amazonensis* Lainson & Shaw, 1972, is widely distributed geographically, highly attracted to wild rodents and has already been found naturally infected with *L. (L.) amazonensis* (Shaw & Lainson 1972). These *Leishmania* cycles occur in primary and secondary forests, mostly in the Legal Amazon Region but also in the states of the northeastern (Bahia), midwestern (Goiás and Mato Grosso do Sul), southeastern (Minas Gerais, Rio de Janeiro and São Paulo), and southern (Paraná) regions of the country (Ashford 2000; Dorval *et al.* 2006; Brasil 2007; Azeredo-Coutinho *et al.* 2007; Rangel & Lainson 2009).

Considering the importance of these American cutaneous leishmaniasis (ACL) vectors, knowledge about their morphological and morphometric characteristics may indicate important diagnostic characters as subsidies for a future phylogenetic approach. In this context, this group of insects deserves more focused taxonomic studies on the sand flies associated with the transmission of *Leishmania* species belonging to the *Viannia* and *Leishmania* subgenera. Thus, our study sought to investigate the characteristics that could cluster some vectors of *L. (V.) braziliensis* and *L. (L.) amazonensis* according to morphological, linear, and geometric morphometric parameters. As vectors of *L. (V.) braziliensis*, three species of the genus *Nyssomyia*, *Ny. intermedia*, *Ny. neivai*, and *Ny. whitmani*, and one species of *Migonemyia*, *Mg. migonei*, and, as a vector of *L. (L.) amazonensis*, one species of the genus *Bichromomyia*, *Bi. flaviscutellata*, were analyzed. Essentially, we looked to pinpoint characters that would help identify these phlebotomine species.



**FIGURE 1.** Landmarks used for the geometric morphometric analysis of wings of the sandfly species.

## Materials and methods

*Taxonomic classification and abbreviations.* We followed the taxonomic classification proposed by Galati (1995), and the genus abbreviations recommended by Marcondes (2007).

*Sand fly capture and deposit locations of the specimens.* Sand fly captures were performed from 18:00h to 6:00h, using CDC light traps of the HP type (Pugedo *et al.* 2005) as well as Shannon traps (Shannon 1939) with the aid of a Castro aspirator (Castro 1937). The specimens were preserved in 70% ethanol for later processing. The

species and the locality of the captures were: *Ny. intermedia* (Jacarepaguá, RJ), *Ny. whitmani* (Ilhéus, BA, type locality), *Bi. flaviscutellata* (Bragança, PA), and *Mg. migonei* (Baturité, CE). Some other specimens held in the School of Public Health of the University of São Paulo collection were also examined: *Ny. neivai* (Eldorado, Itariri, Iporanga, and Pedro de Toledo, SP) and *Brumptomyia troglodytes* Lutz, 1922 (Iporanga, Barra Bonita, and Ariri, SP) for use as a control group to evaluate the accuracy of the discriminant analysis. *Bi. flaviscutellata*, *Ny. intermedia*, and *Ny. neivai* topotypes from the René Rachou Research Center of the Oswaldo Cruz Foundation were also analyzed.

*Processing and mounting of the material.* All the sand flies were processed via the methodology proposed by Forattini (1973) using 1% Fuchsin to stain the insects. All were mounted on slides with Eneçê medium (Cerqueira 1943). In all specimens, the right wing was detached from the body and longitudinally positioned for geometric and linear morphometric analyses. Character measurements were performed using AxioVision version 4.7 program by way of images captured with a light microscope (Zeiss Primo Star Brand) coupled with an AxioCam camera ICc1. All measurements were acquired in micrometers ( $\mu\text{m}$ ).

*Morphological analysis.* The morphological descriptions were based on 20 males and 20 females of each species in which a total of 35 characters of the head, thorax and abdomen, all vital for sand fly taxonomy, were examined. These characters are listed in Table 1 according to body segment. Frequency analysis of the characters was only performed among females of all six species due to the presence of characters with quantitative data. These characters were the numbers of lacinia teeth, cibarium teeth, and spermathecae rings (when present).

*Interspecific analysis of the linear morphometric data.* A total of 55 characters were measured in 20 males and 20 females of each species in that 10 were male-specific and 5, female-specific (Table 2). The mean measurement difference was evaluated by variance analysis (ANOVA) using a Tukey post-test for the parametric data and Kruskal-Wallis together with a Duns post-test for the non-parametric data (including the lengths of the head, labrum, labium, palpomere I, II, III, and IV, and the ratio between the length of the ejaculatory filaments and pump as well as the width of the common sperm duct of the spermathecae). Analyses were performed with the characters present in both sexes and with specific characters for the female and male terminalia. Structures such as the common duct of the spermathecae (absent in *Br. troglodytes*), length of the ejaculatory filament tip (an undifferentiated structure in *Mg. migonei* and *Br. troglodytes*) along with the ratio between the ejaculatory filament and its tip were analyzed separately since they are not found in all species.

*Discriminant analysis of the linear morphometric data.* We performed 3 different analyses (to evaluate the real contribution of each character to species differentiation) via the morphometric data to cluster the species. One was with the characters present in both sexes using 240 specimens and 38 characters, another was done with the shared characters plus the male specifics (120 specimens) using 48 characters, and the third analysis was with the shared characters plus the female specifics (120 specimens) for a total of 43 characters (Table 2). Regarding specific gender data, some of the characters not present in all the species were excluded from analysis. To interpret the data, we used Canonical varieties and discriminant function to evaluate the values of F and the Wilks' Lambda test in order to determine the characters that most influenced the discrimination of specific groups. All analyses were carried out via the SPSS version 20 program (IBM Corporation Software Group, Somers, NY, USA, 2011).

*Geometric morphometric analysis.* For these analyses, 13 landmarks (12 type I and one type 2) were used according to Bookstein's proposal (1991). The landmarks were established in the following wing positions shown in Figure 1. A total of 305 wings were evaluated (52 for each species, i.e., 26 males and 26 females), except for *Ny. neivai* (29 female wings and 23, male) and *Br. troglodytes* (17 female wings and 28, male) using optical microscopes with a 4x objective lens connected to an AxioCam ICc1. All the images were taken using AxioVision version 4.7 program (Carl Zeiss MicroImaging GmbH, Jena, TH, DE, 2008). To define the species clusters, topotypes of *Bi. flaviscutellata*, *Ny. intermedia*, and *Ny. neivai* were evaluated. TpsDig 2.17 (Rohlf, SUNY Stony Brook, Stony Brook, NY, USA, 2013) was used to set the wing landmarks.

Analyses were performed using Generalized Procrustes (AGP) to remove the effects of size and position, Principal Components (PCA) to explore the structure of the data, canonical variance analysis (CVA), and Mahalanobis distance to quantify the morphological differentiation of certain groups *a priori* via the MorphoJ version 1.04b program (Klingenberg 2011). The centroid size data were exported from MorphoJ and evaluated by ANOVA with a Tukey post-test in Graphpad Prism 5.0 (GraphPad Software, La Jolla, CA, USA, 2007).

**TABLE 1.** Morphological characters analyzed in accordance with the region of the sandfly body and sex.

	Morphological characters
Head	Presence/Absence of the interocular suture (F/M)
	Presence/Absence de labial furca (F/M)
	Presence/Absence of basal directed spur of the ascoids (F/M)
	Presence/Absence of basal directed spur of the ascoid in flagellomere I (F/M)
	Presence/Absence of basal directed spur of the ascoid in flagellomere II (F/M)
	Insertion of the ascoid in flagellomere I and II (F/M)
	Presence/Absence of papilla in flagellomere I, II and III (F/M)
	Presence/Absence and Position of the Newstead sensillae in palpomere III (F/M)
	Number of teeth rows in the maxillary lacinia (F)
	Number of teeth in the maxillary lacinia (F)
	Presence/Absence of sclerotized area in the cibarium chamber (F)
	Number of posterior teeth in the cibarium (F)
	Position of posterior teeth in the cibarium (F)
Thorax	Presence/Absence of setae in the anterior portion of the katepisternum (F/M)
	Presence/Absence of ventrocervical sensilla (F/M)
Female Abdomen	Aspect of the common duct of spermathecae
	Aspect of the individual ducts of spermathecae
	Aspect of the spermathecae body
	Presence/Absence and number of rings in the spermathecae
	Presence/Absence of capitulum in the spermathecae
	Angle of the insertion of the capitulum in relation to the spermathecae body
	Presence/Absence of sclerotized protuberance in the 9th tergum
Male Abdomen	Aspect of the apex of the lateral lobe
	Presence/Absence of processes in the paramere
	Presence/Absence of differentiated setae of paramere
	Aspect of the apex of the paramere
	Presence/Absence of setae tuft in the base of the gonocoxite
	Number of setae of the basal tuft of gonocoxite
	Presence/Absence of preapical perennial seta
	Number of spines in the gonostylus
	Position of the spines of gonostylus
	Form of the tips of the ejaculatory filaments
	Presence of long setae in the gonocoxite distal portion
Presence/Absence of sclerotized protuberance in the gonocoxite	

F = female; M = male

## Results

*Morphological analysis.* The maxillary lacinia of *Br. troglodytes*, *Mg. migonei*, and *Bi. flaviscutellata* presents only one row of teeth while the *Nyssomyia* species presents two. Consequently, these latter species have more teeth. The posterior teeth of cibarium are more numerous in the species of the genus *Nyssomyia* and less numerous in *Mg. migonei* and *Br. troglodytes*. Spermathecae rings were only present in *Bi. flaviscutellata* and in the species of the genus *Nyssomyia*. The number of rings varied widely among the species, being most numerous in *Ny. whitmani* and less so in *Ny. neivai* and *Bi. flaviscutellata*. All the data regarding variance and mode are listed in Table 3.

**TABLE 2.** Characters used in discriminant and interspecific analyses in accordance with sex.

Characters shared by both sexes	Specific Characters for Males (M) or Females (F)
Length of head	Length of gonocoxite (M)
Length of labrum	Width of gonocoxite (M)
Length of labium	Length of gonostylus (M)
Length of palpomere I, II, III, IV and V	Length of lateral lobe (M)
Length of clypeus	Width of lateral lobe (M)
Shortest distance between the eyes	Length of the piston (M)
Length of flagellomere I and II	Length of ejaculatory pump (M)
Length of R <sub>5</sub>	Length of ejaculatory filament (M)
Length of alpha	Length of tips of ejaculatory filament* (M)
Length of beta	Distance between the apical and the external superior spine (M)
Length of gamma	Ratio between the ejaculatory pump and filament** (M)
Length of delta	Length of spermathecael body (F)
Length of anterior, medial and posterior femur	Width of spermathecael body (F)
Length of anterior, medial and posterior tibia	Length of cercus (F)
Length of anterior, medial and posterior tarsus (tarsomeres I, II, III, IV and V)	Length of individual ducts (F)
	Width of individual ducts (F)

\* Character used only in the interspecific analysis

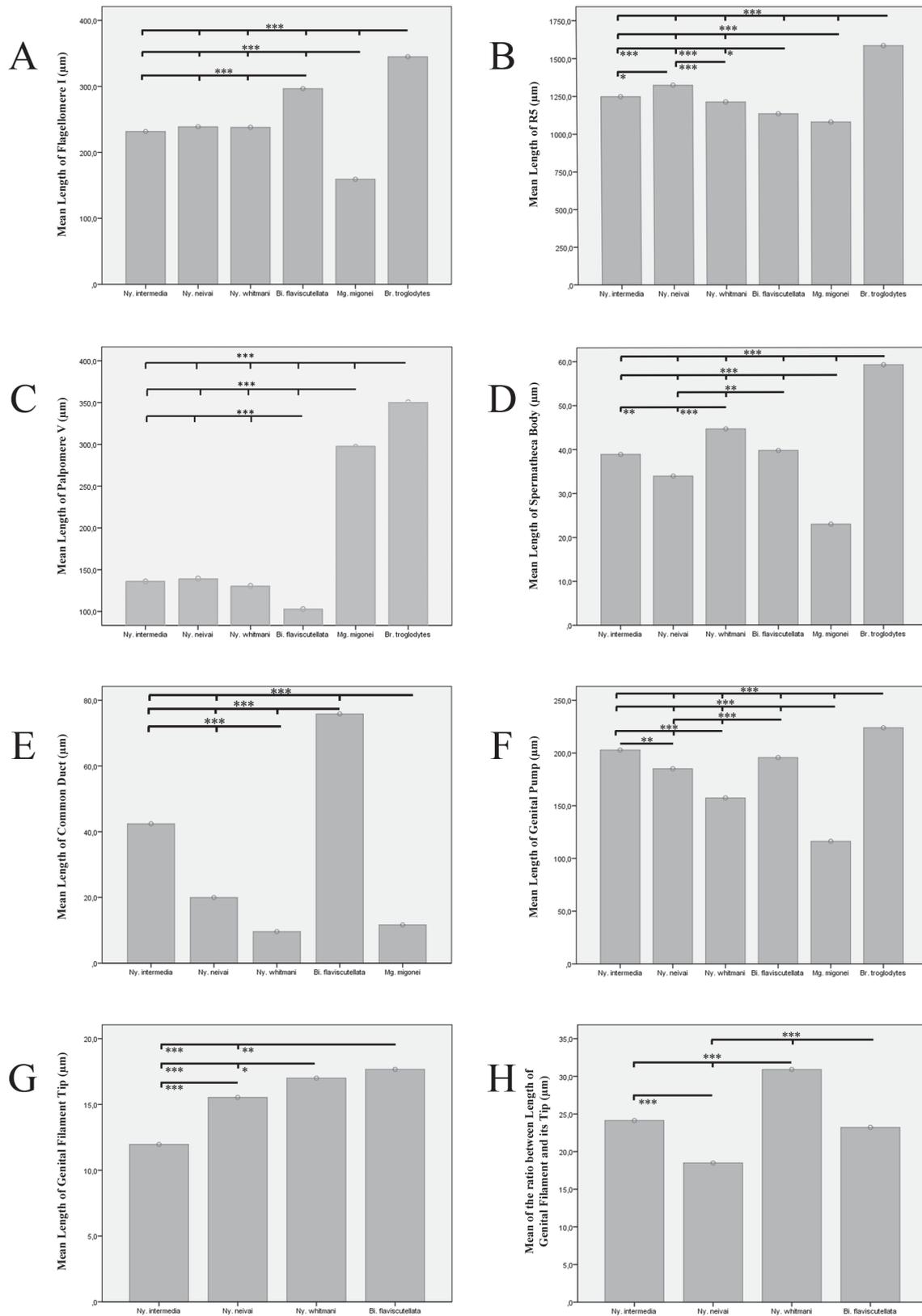
\*\* Character used only in the discriminant analysis

**TABLE 3.** Result of the morphological intraspecific analysis of females showing the variance and the mode of the characters analyzed.

	<i>Br. troglodytes</i>		<i>Mg. migonei</i>		<i>Bi. flaviscutellata</i>		<i>Ny. whitmani</i>		<i>Ny. neivai</i>		<i>Ny. intermedia</i>	
	Variance	Mode	Variance	Mode	Variance	Mode	Variance	Mode	Variance	Mode	Variance	Mode
Number of lacinia teeth	6 - 7	6 (90%)	3 - 5	4 (55%)	9 - 11	10 (55%)	15 - 21	20 (25%)	15 - 19	17 (35%)	12 - 19	17 (30%)
Number of posterior teeth of cibarium	15 - 20	18 - 19 (31,6%)	4	4 (100%)	6	6 (100%)	8 - 11	9 (45%)	8 - 12	10 (40%)	8 - 10	10 (40%)
Number of rings or annuli in the spermathecae	Absent		Absent		8 - 11	9 (40%)	13 - 17	16 (35%)	8 - 11	9 (50%)	10 - 13	11 (35%)

The following are all the unique characteristics found in *Br. troglodytes*: the presence of the interocular suture, proximal spur of the ascoid, papilla in flagellomere III, unpaired insertion of the ascoids, absence of the common duct of the spermathecae, the presence of two papillae in flagellomere I and five spines in the gonostylus in addition to the presence of ventro cervical sensilla. All the above are considered primitive conditions in sand flies (Galati 2003). Others, like the presence of long setae in the apical part of gonocoxites, the protuberance in the basal part of gonocoxites, and the presence of setae and process in paramere, were also found to be features unique to this species in comparison to the others.

Newstead's sensillae in palpomere III presented a similar insertion in the species of *Nyssomyia* and *Br. troglodytes*, with these structures concentrated in the medial part of palpomere III. In *Mg. migonei*, however, they were grouped together in the second third of the structure while, in *Bi. flaviscutellata*, they were evenly dispersed throughout. The ascoid apex extends to the level of pre-apical papilla in *Bi. flaviscutellata*. Just one ascoid exceeds the level of papilla in *Br. troglodytes* (due to the unpaired insertion of these structures).



**FIGURE 2.** Mean length of some important characters for the differentiation of the studied species. A—Antennomere I. B—R<sub>5</sub>, C—Palpomere V. D—Spermathecae Body. E—Common Duct. F—Ejaculatory Pump. G—Ejaculatory Filament Tip. H—Ratio between the Ejaculatory filament and its Tip. \*\*\*  $p \leq 0,001$ ; \*\*  $p \leq 0,01$ ; \*  $p \leq 0,05$ .

Characteristics of the spermathecae are important for group differentiation. *Mg. migonei* shows a smooth-walled spermathecae body, long individual ducts, and a short common duct. *Br. troglodytes* presents a striated spermathecae body, the absence of common duct together with very long, smooth individual ducts. Whereas species of the *Nyssomyia* genus and *Bi. flaviscutellata* show an annulated spermathecae body, the common duct in the latter species is poorly striated yet smooth-walled in *Nyssomyia*. The individual ducts are slightly striated in *Bi. flaviscutellata* and *Ny. intermedia* but smooth in the other species.

The final portion of the lateral lobe is tapered in *Br. troglodytes* and *Mg. migonei*, but oblong in other species. Likewise, parameres differ among the genera. The *Nyssomyia* species shows a similar structure with pronounced tapering in the apical third portion. On the other hand, in *Mg. migonei*, it is curved; in *Bi. flaviscutellata*, rhomboidal; and, in *Br. troglodytes*, club-shaped. Although the *Mg. migonei* gonocoxite basal tuft of setae has from 5-to-7 setae and *Br. troglodytes* has up to 70, the other species show no sign whatsoever of these characteristics. The tip of the ejaculatory filament does not present any difference in *Mg. migonei* or *Br. troglodytes*. In the other species, this particular structure differs in each species, as follows: in *Ny. intermedia*, it takes the form of a ladle; in *Ny. neivai*, it is spoonlike; in *Ny. whitmani*, bifurcated; and in *Bi. flaviscutellata*, it resembles an inverted "L". For the above reasons, this structure is of primary importance in male identification in these three *Nyssomyia* species.

*Interspecific analysis of the linear morphometric data.* Analysis of the characters present in both sexes showed some separation patterns in certain structures. The characteristics of the length of flagellomere I (Figure 2A) and II, of *gamma*, of the anterior femur, the medial and posterior tibia, and the anterior, medial, and posterior tarsomere I showed a cluster (no significant differences among structure measurements) of the three *Nyssomyia* species and significant differences in the *Mg. migonei*, *Bi. flaviscutellata*, and *Br. troglodytes* species in relation to the others. Another pattern was observed in  $R_5$  lengths (Figure 2B), the posterior femur, and the anterior, medial, and posterior tarsomere II, III, IV and V, whose measurements in *Mg. migonei*, *Bi. flaviscutellata*, *Br. troglodytes*, and *Ny. neivai* were significantly different from all the others.

The head lengths distinguished *Mg. migonei* from *Ny. intermedia* as well as *Br. troglodytes* from the others. Regarding the lengths of the *delta* (wing) and the anterior tibia, *Mg. migonei* and *Ny. neivai* presented significant differences from the others. Analysis of the palpomere V (Figure 2 C) showed that *Bi. flaviscutellata*, *Mg. migonei*, and *Br. troglodytes* presented significantly different measurements from the rest while *Bi. flaviscutellata* and *Br. troglodytes* were similar to each other. The length of the shortest distance between the eyes of *Bi. flaviscutellata* and *Mg. migonei* was different from the rest of the species whereas only the latter did not separate from *Ny. whitmani*. The length of *alpha* separated the species *Mg. migonei*, *Ny. neivai*, and *Br. troglodytes* from the others. In the *beta* analysis, the species of the genus *Nyssomyia* were not different from *Br. troglodytes*. However, *Ny. whitmani* separated from *Bi. flaviscutellata*.

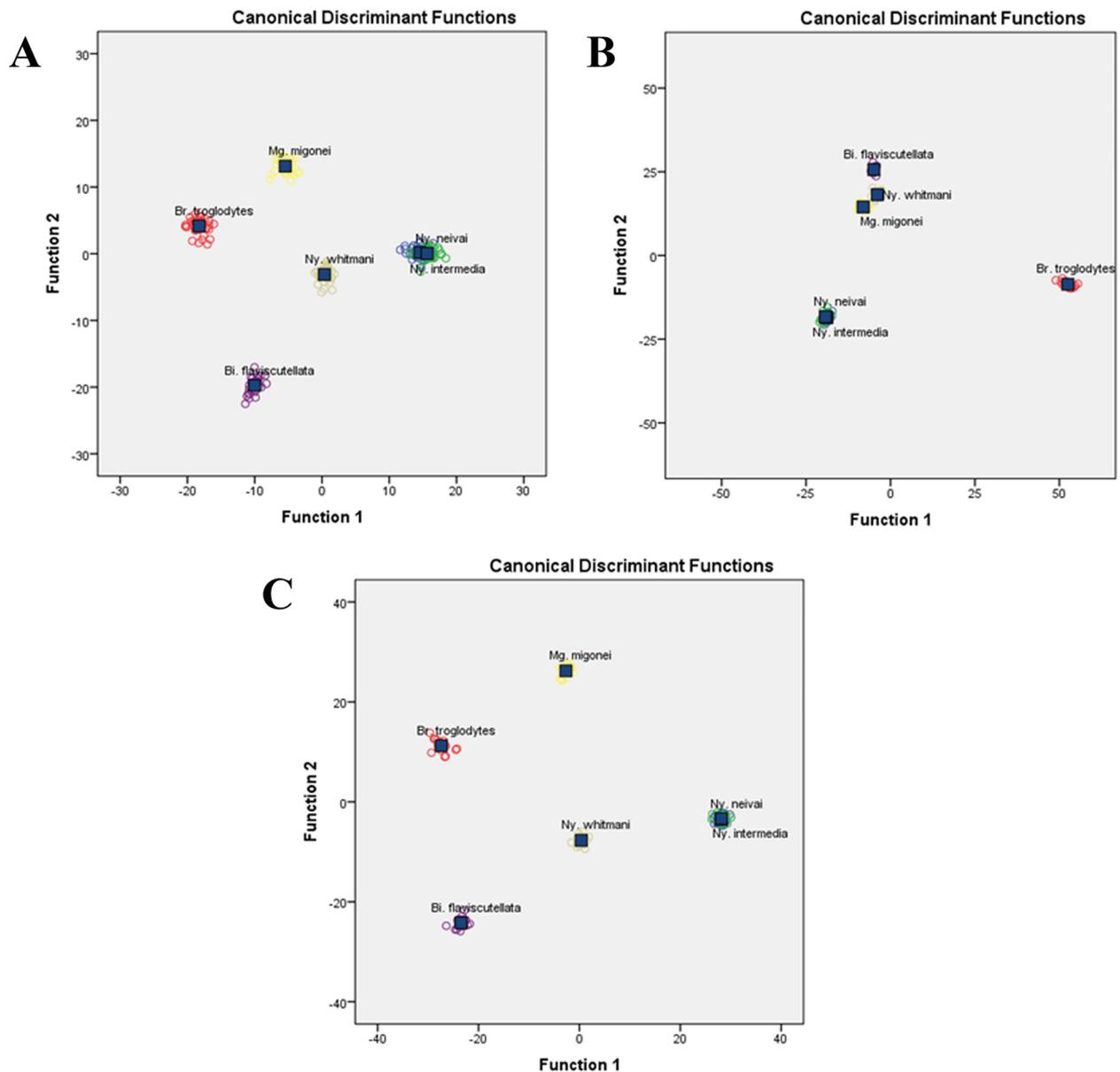
The most significant difference in the characteristics of the female abdomen was the length of the spermathecae body (Figure 2D), which varied in almost all species, except for *Ny. intermedia*, *Bi. flaviscutellata*, and *Ny. neivai*. In contrast, the width of the spermathecae body in the *Bi. flaviscutellata* and *Ny. neivai* species was the same. The cercus length in *Mg. migonei* and *Br. troglodytes* was significantly different from the others; but the width of the individual duct separated all the species. Its length differentiated the *Bi. flaviscutellata* and *Br. troglodytes* species from the others while not differentiating *Ny. neivai* from *Ny. intermedia* or *Ny. whitmani* from *Mg. migonei*. In analyzing the length (Figure 2E) and width of the common duct, the species *Br. troglodytes* was discarded due to the absence of this structure. As to the length of this character, all species varied on this point. Only *Ny. whitmani* and *Mg. migonei* did not separate from each other; and the width of *Bi. flaviscutellata* was significantly different from all the other species evaluated.

The characteristics of the male abdomen showed the following significant differences: the gonocoxite length was different in *Ny. whitmani* and *Br. troglodytes* when compared to all the other species. The width of this structure differentiated all the species but less so with respect to *Ny. neivai* from *Ny. whitmani* and *Mg. migonei*. The length of gonostylus showed no significant difference when comparing the *Mg. migonei* and *Bi. flaviscutellata* or *Nyssomyia* species. The length of the lateral lobe separated all the species except those of the *Nyssomyia* genus. Analyzing the lengths of the ejaculatory pump (Figure 2F) and the piston, almost all the species varied, apart from *Bi. flaviscutellata* and *Ny. intermedia*. As to the length of the ejaculatory filament, significant differences were found in all species but not in the case of *Ny. neivai* from *Ny. intermedia* or *Bi. flaviscutellata* from *Mg. migonei*.

The distance between the apical and external superior spine showed that *Ny. neivai* differed from *Ny.*

*intermedia*. The ratio between the filament and ejaculatory pump differentiated all the species, barring *Ny. intermedia* and *Ny. neivai*. After analyzing the length of the ejaculatory filament tip (Figure 2G) and the ratio between the ejaculatory filament and its tip (Figure 2H), the species *Mg. migonei* and *Br. troglodytes* were removed due to the absence of differentiation at the tip of the filament, making it impossible to measure. The results showed no significant differences except between *Ny. whitmani* and *Bi. flaviscutellata* regarding the first characteristic. *Ny. intermedia* and *Bi. flaviscutellata* were similar in the ratio between the ejaculatory filament and its tip.

*Discriminant analysis of the linear morphometric data.* The discriminant analysis of the characters shared by both sexes evaluated 228 specimens of the total 240 used. Twelve (5 % of the sample) were removed due to the absence of at least one variable, notably 11 specimens from *Bi. flaviscutellata* and one from *Br. troglodytes*. It was seen that the characters that most contributed to species differentiation (higher F values) were, respectively, the lengths of the anterior tarsomere I (F = 1124.48), palpomere V (1077.48), anterior tarsomere III (F = 699.66), anterior tarsomere II (F = 692.13), and medial tarsomere V (F = 684.91). All the above characters were considered highly significant at  $p < 0.001$ .



**FIGURE 3.** Canonical variates 1 e 2 showing the projection of the specimens. A—Using the shared characters of males and females. B—Using the shared characters and the specific for males. C—Using the shared characters and the specific for females.

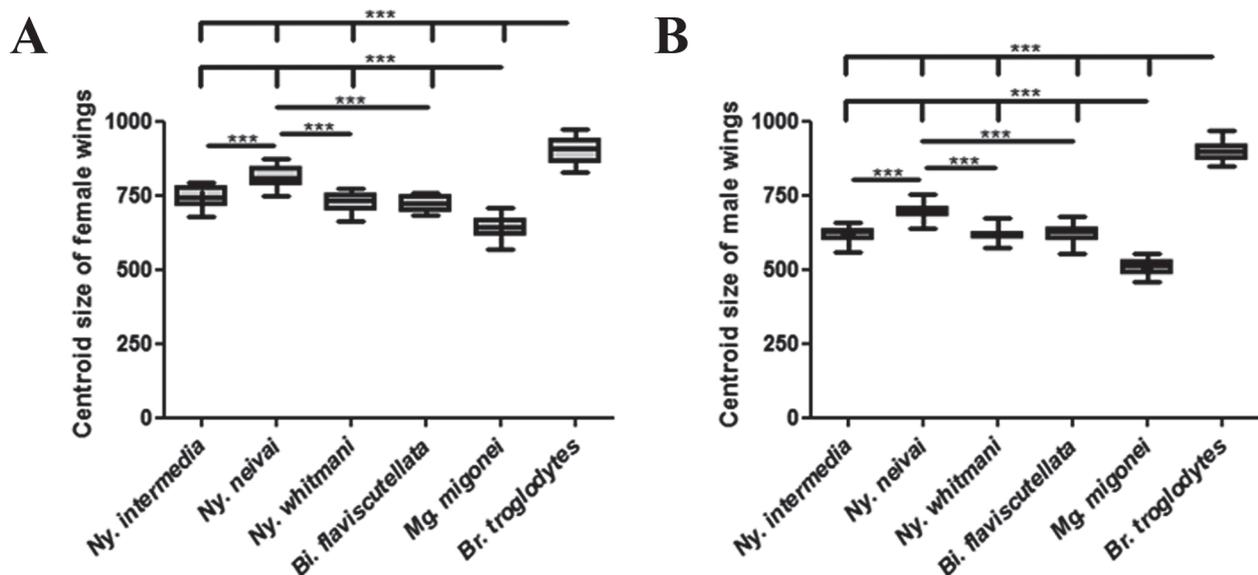


A total of five variables were produced consistent with the order of decreasing sample variation. It was shown that the first three variables were responsible for 98.6% of the sample variation. The Wilks' Lambda test demonstrated that these three variables had a p value equal to 0 (Figure 3A). The analysis of variables 1 and 2 showed that the *Nyssomyia* species were closer to each other. Conversely, specimens of *Ny. intermedia* and *Ny. neivai* seemed to overlap while *Bi. flaviscutellata* was more distant from *Nyssomyia* than *Mg. migonei*. Discriminant function analysis showed that the specimens pertaining to the species *Br. troglodytes*, *Mg. migonei*, and *Bi. flaviscutellata* were 100% correctly classified even after cross-validation. On the other hand, the species *Ny. intermedia*, *Ny. neivai*, and *Ny. whitmani* were, respectively, 95% (38/40 specimens), 87.5% (35/40), and 97.5% (39/40) correctly classified as were 90% (36/40 specimens), 75% (30/40), and 92.5% (37/40) of the above-mentioned species after cross-validation.

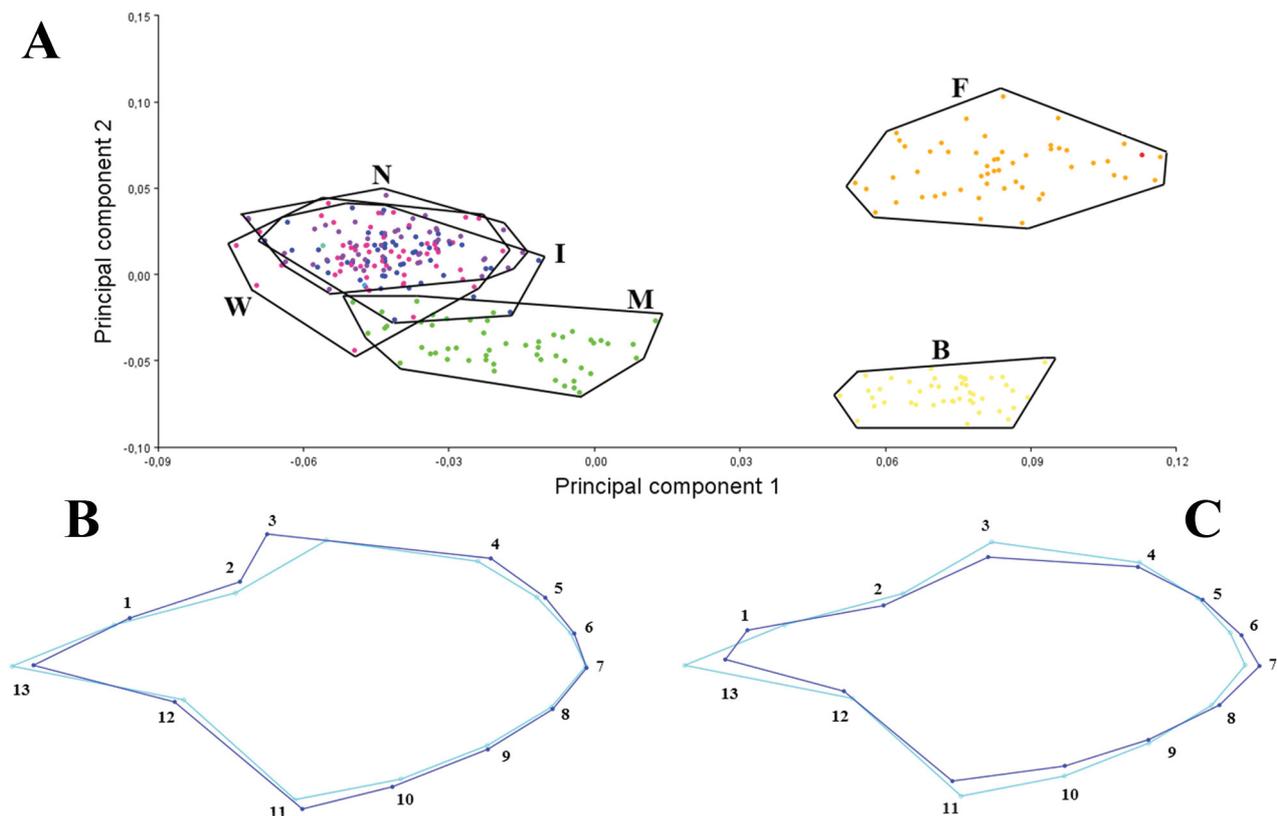
The discriminant male analysis evaluated 110 among 120 specimens from which 10 belonging to *Bi. flaviscutellata* were removed due to lack of information. The characteristics that most significantly contributed to species differentiation were: the distance between the apical and external superior spines ( $F = 3603.9$ ), the lengths of the gonostylus ( $F = 3225.9$ ), the gonocoxite ( $F = 2411.7$ ), and the labium ( $F = 1706.2$ ), along with the width of the gonocoxite ( $F = 1072.2$ ), demonstrating that it was the structure of the male abdomen that most importantly contributed to species differentiation.

Among the five generated variables, the first three represented a full 96.7% of sample variability, being highly significant ( $p = 0$ ) in the Wilks' Lambda test results. Analysis of these variables showed that there is an overlapping area between *Ny. neivai* and *Ny. intermedia* (Figure 3B). The classification of all the species was 100% correct even after cross-validation. Nonetheless, cross-validation of *Ny. intermedia* and *Ny. neivai* respectively showed a correct identification of 85% (17/20 specimens) and 90% (18/20).

Discriminant female analysis evaluated 114 among a total of 120 specimens. Six were removed due to the absence of information, four of which belonged to *Ny. neivai*, one to *Bi. flaviscutellata*, and one more to *Br. troglodytes*. The characters that most contributed to species differentiation were: the lengths of palpomere V ( $F = 866.6$ ), individual ducts ( $F = 794.8$ ), palpomere IV ( $F = 716.8$ ), the anterior tarsomere I ( $F = 597.6$ ), and, lastly, the width of individual ducts ( $F = 404.9$ ). For the five generated variables, the first three represented 97.2% of sample variability, which was highly significant ( $p = 0$ ) in the Wilks' Lambda test. The specimen distribution in the canonical variate analysis showed the same pattern as that of the shared characters due to the low variability in the specifically female characters (Figure 3C). One-hundred per cent of all the species were correctly classified including in cross-validation. Notwithstanding this figure, after cross-validation, *Ny. intermedia* and *Ny. neivai* were 85% (17/20 specimens) and 62.5% (10/16) correctly classified.



**FIGURE 4.** Graphic showing the difference between the centroid size of the wings of the sandfly species studied. A—Centroid size of the female wings. B—Centroid size of the male wings. (\*\*\*)  $p < 0.0001$ .



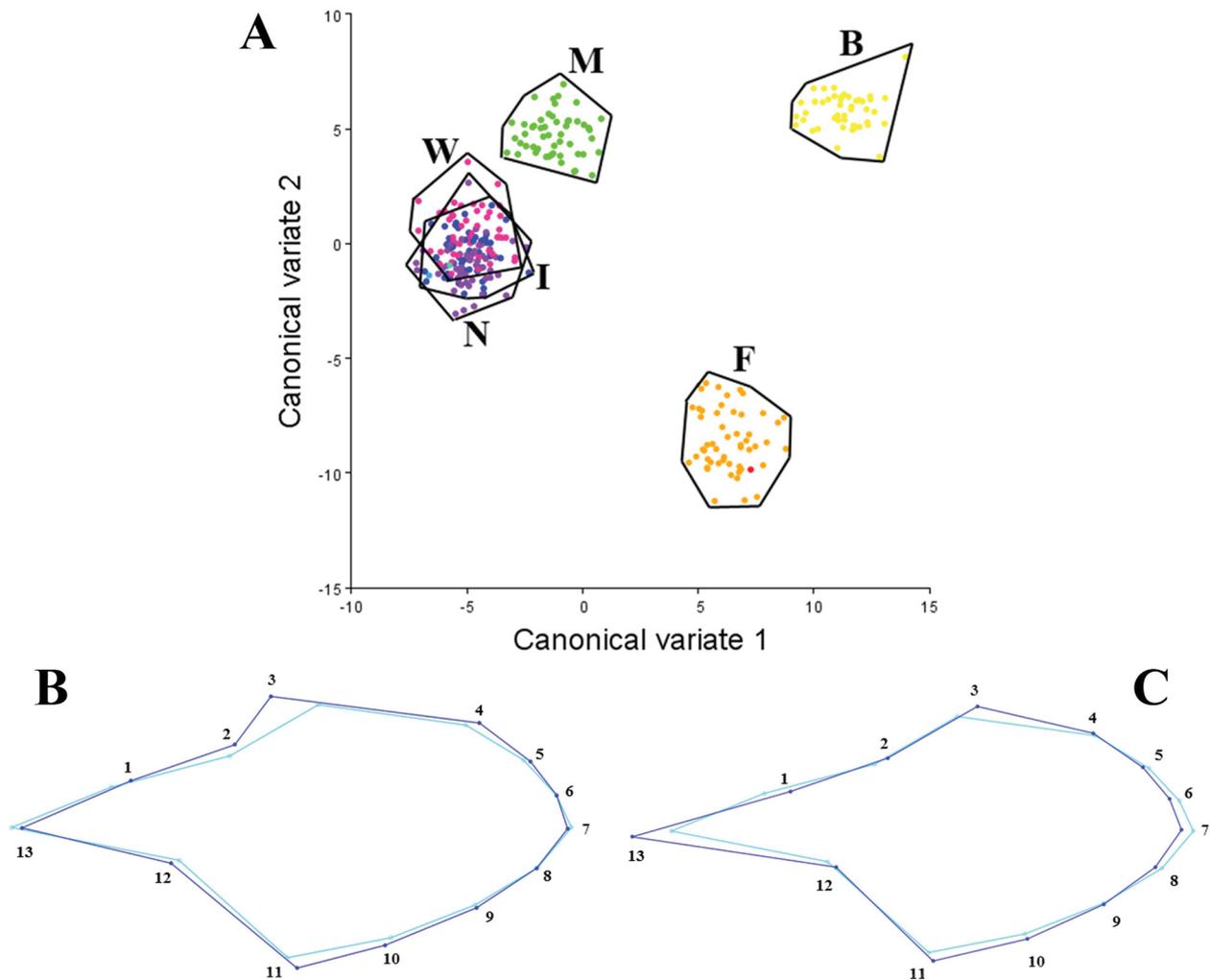
**FIGURE 5.** Analysis of the Principal Components 1 (46,4%) and 2 (27,8%). A—Graphic showing the projection of the specimens in PC1 and 2. B—Variation of the wings in the Principal Component 1, showing negative mean shapes (light-blue line: N,I,W,M) and positive (dark-blue line: F,B). C—Variation of the wings in the Principal Component 2 showing negative mean shapes (light-blue line: M,B) and positive (dark-blue line: I,W,N,F). B, *Br. troglodytes*; F, *Bi. flaviscutellata*; I, *Ny. intermedia*; M, *Mg. migonei*; N, *Ny. neivai*; W, *Ny. whitmani*.

*Geometric morphometric analysis.* Regarding centroid size, it was seen that the species with the most significant differences ( $p < 0.0001$ ) were *Br. troglodytes* in relation to all the other species and *Mg. migonei*. The former had the highest value and the latter, the lowest. Within the species of the genus *Nyssomyia*, *Ny. neivai* was found to be significantly different from the other two species (Figure 4A and B).

Twenty-two variables (principal components) from 13 landmarks in two dimensions (axis X and Y) were obtained in our research. The first three principal components were responsible for 85.16% of total variation (PC1 46.4%, PC2 27.8% and PC3 11%). PC1 revealed two distinct groups based on wing shape: the one with negative points was made up of the species of the genus *Nyssomyia* and *Mg. migonei*. The second group with the positive points consisted of *Bi. flaviscutellata* and *Br. troglodytes* (Figure 5B). PC2 showed a marked difference between these latter two species (Figure 5C) while PC3 displayed little variability.

Analyzing variation among the principal components (Figure 5A), it was evident that, in the first group, the wings presented a tendency (i.e., negative toward positive points) to enlarge the medial part, accompanied by a major displacement of landmark 3 to an anterior and more external part, which, as a result, displaced landmark 11 and decreased the anterior part of  $R_5$  (landmark 13) (Figure 5B). These modifications can be seen in Figures 5A and B, which show that the shapes were narrower in  $R_5$  and that the medial part of the wing was more elongated in the species of the genus *Nyssomyia* (light-blue line in Figure 5B) followed by an anterior gradual decrease in  $R_5$  and enlargement of the medial part of the wings, with the maximum of this differentiation occurring in *Br. troglodytes* and *Bi. flaviscutellata* (dark-blue line in Figure 5B).

In the second component, the variation occurred in landmark 13 together with a marked reduction in  $R_5$ , widespread displacement to the anterior part of landmark 1, and a narrowing of the width of the medial part of the wing (Figure 5C). Wing modification can be seen in Figure 5C, in which the wing shape represented by the light-blue line is the mean shape of the *Br. troglodytes* wing (most negative points). The dark-blue line represents the wing shapes more closely aligned with *Bi. flaviscutellata* (most positive points).



**FIGURE 6.** Analysis of the Canonical variates. A—Graphic showing the distribution of the specimens in CV1 and 2. B—Variation of the wings in the Canonical Variate 1, showing negative mean shapes (light-blue line) and positive (dark-blue line). C—Variation of the wings in the Canonical variate 2 showing negative mean shapes (light-blue line) and positive (dark-blue line). B, *Br. troglodytes*; F, *Bi. flaviscutellata*; I, *Ny. intermedia*; M, *Mg. migonei*; N, *Ny. neivai*; W, *Ny. whitmani*; Light green dot, topotype de *Ny. intermedia*; Light blue dot, topotype de *Ny. neivai*; Red dot, topotype de *Bi. flaviscutellata*.

Wing analysis in the six species resulted in five canonical variates. The first three corresponded to 98.67% of sample variability; and their respective contributions were 59.17% for canonical variate 1 (CV1), 33.38% for CV2, and 12.6% for CV3. Genera separation is displayed in CV1 and 2 from which we observed that the first variable separated all genera and the second more clearly defined the differences between *Bi. flaviscutellata* and *Br. troglodytes* (Figure 6A).

CV1 showed the variations observed in the narrower wings in the medial part in the specimens of the genus *Nyssomyia* (most negative points) up to the *Br. troglodytes* (most positive points) specimens with wider wings in the same location (Figure 6B). CV2 showed variations occurring in the specimens of *Bi. flaviscutellata*, which has a shorter  $R_5$  at the baseline and a more elongated apex, in contrast to specimens of *Mg. migonei* and *Br. troglodytes* that count on wider wings in the medial part and a more basally elongated  $R_5$  (Figure 6C). CV3 presented limited variability in the sample so that group separations were observed.

The topotypes of *Ny. intermedia*, *Ny. neivai*, and *Bi. flaviscutellata* were inserted in the area occupied by the specimens of the correlated species, confirming a correct species classification (Figure 6A).

The Mahalanobis distance, obtained by calculating the distance between the mean shapes of the groups involved generated subsequent to the discrimination process, indicated that *Br. troglodytes* was phenetically closer to *Mg. migonei* (13.79) than to the *Bi. flaviscutellata* (15.49) and *Nyssomyia* species. The *Nyssomyia* species were

also closer to *Mg. migonei* than to *Bi. flaviscutellata*. Very small distances were observed within the genus *Nyssomyia*, the shortest being between *Ny. intermedia* and *Ny. neivai* (1.64) and the longest, between *Ny. neivai* and *Ny. whitmani* (2.83).

Analysis of the discriminant function revealed 100% correct classification (CC) even after cross-validation (CV) at which time the wings of *Br. troglodytes*, *Bi. flaviscutellata*, and *Mg. migonei* were compared to those of the others species ( $p=0.0001$ ). For the three *Nyssomyia* species, the  $p$  values were identical even though this species showed a certain degree of misclassification, mainly between *Ny. intermedia* and *Ny. neivai*. These results also found that the wings of *Ny. whitmani* were more clearly differentiated than those of *Ny. neivai* (6/104 wrongly classified) and *Ny. intermedia* (11/104 wrongly classified). The last two species had a higher erroneously-allocated specimen rate in that 16.35% (17/104) of the specimens were misclassified, which increased to 27.88% (29/104) after cross-validation.

## Discussion

*Linear morphometric, morphological, and discriminant analyses.* In the present study, interspecific analyses showed that the size of some of the characters not widely used in species differentiation such as the length of  $R_5$  and other wing measurements together with a number of leg characters are capable of distinguishing some species. However, using leg structures to distinguish species is not always possible since these structures are often lost in laboratory processes.

The head structures that best differentiated species were the length of palpomere V due to the greater variation in length among genera. Palpomere V has consequently become an important character in defining genera, as can be seen in the present study. The length of the clypeus is an important character in identifying both *Bi. flaviscutellata*, which has an elongated head and a longer, narrower clypeus than the other species, and *Br. troglodytes* with the largest clypeus of all, reflective of the augmented size of the species of this genus.

With respect to female characteristics, the number of spermathecae rings in *Ny. intermedia* and *Ny. neivai* was similar while the number of rings in most of the specimens of the first species was higher compared to the second group. The findings in our study are in agreement with those of Marcondes (1996) and Andrade Filho *et al.* (2006), who demonstrated that the number of spermathecae rings is higher in *Ny. intermedia* than in *Ny. neivai*. For its part, *Bi. flaviscutellata* presented a number of annulations similar to what is found in *Ny. neivai* although the structural form and additional differences related to other characteristics easily separate these two species from one another. Moreover, *Ny. whitmani* presented a higher number of annulations, separating easily from the rest.

It is of interest that the length of the common duct differentiated all species, aside from *Ny. whitmani* and *Mg. migonei* in that both have very short ducts in relation to the other species. As such, *Bi. flaviscutellata* is the species with the longest duct followed by *Ny. intermedia* and *Ny. neivai*. The differences in sizes of the common duct were highlighted by Marcondes (1996) and Andrade Filho *et al.* (2006) as an important feature in distinguishing *Ny. intermedia* from *Ny. neivai*. This observation has also been made in our work in which the first species was shown to have a larger duct than the second.

The length of individual ducts is also important in distinguishing the species under study. *Bi. flaviscutellata*, for example, presented the shortest, and *Br. troglodytes*, the longest. *Ny. whitmani* has been found to have a much longer duct than the other two species of the genus *Nyssomyia*. By the same token, the size of the male ejaculatory filament in *Ny. whitmani* is also much longer than it is in the other two.

As to male characteristics, the ratio between the ejaculatory filament and its tip was significantly different between the species *Ny. intermedia* and *Ny. neivai*, thus adding one more species differentiation characteristic in male specimens. The length of the ejaculatory pump and piston, as well as the distance between the apical and the external superior spines, also aids in distinguishing these two species. It is noteworthy that this last character had not yet been described as a distinguishing characteristic between *Ny. intermedia* and *Ny. neivai*. Studies involving a larger number of specimens and sample populations need to be conducted to validate the actual differentiation capacity of this character in these two species.

Other morphological characters can also differentiate species and groups of species such as the number of teeth rows in the lacinia in that the genus *Nyssomyia* has two rows of teeth while the other species have only one and with fewer differentiated teeth. The presence of only one row of teeth appears to be an ancestral characteristic.

The insertion (presence) of posterior teeth in the cibarium along with the number of teeth are factors that highlight the differences among genera. *Br. troglodytes* features four teeth. The anterior ones, arranged in four columns, resemble the posterior teeth. In the *Nyssomyia* genus, there were from 8-to-19 teeth, 4 in *Mg. migonei*, and 6 in *Bi. flaviscutellata*, whose most unique feature is a sclerotized area in the cibarium chamber. A comparative study of various *Ny. neivai* and *Ny. intermedia* populations conducted by Andrade Filho *et al.* (2006) showed, in corroboration with our findings, that the number of teeth in the cibarium varies among different populations so that it cannot be considered an appropriate feature in determining species discrimination.

It is noteworthy that *Mg. migonei* has no ventrocervical sensillae. On the contrary, though, the species of the subgenus *Blancasmyia* Galati, 1995 (present in the *Migonemyia* genus), evidence this character, suggesting that the loss of this structure took place after separation from the group. We also observed that *Mg. migonei* specimens presented papilla in AV, a primitive characteristic that is no longer present in some populations of this species. The presence of a preapical perennial spiniform seta (which is a vestigial atrophied spine) was only seen in this particular species, revealing that it has maintained its plesiomorphic characteristics (Galati 2003).

Interestingly, in the discriminant analysis performed in males, the specific characteristics of this gender were more frequently present in the sample variation than in the female-specific ones. It should be stressed that an important differentiating character (length of the common duct) between *Ny. intermedia* and *Ny. neivai* was not calculated due to the absence of this structure in other species, which may have decreased the discrimination between them. Even in the absence of some characters, however, species separation is satisfactorily achieved via this technique.

In a study by Khalid *et al.* (2010) using discriminant analysis based on data obtained from morphometric measurements of 20 structures from three Old World species (*Phlebotomus duboscqi* Neveu-Lemaire, 1906, *P. papatasi* Scopoli, 1786, and *P. bergeroti* Parrot, 1934) belonging to the group of vectors of cutaneous leishmaniasis that has identification problems, particularly for females, it was possible to separate them significantly, notwithstanding a 100% correct classification rate after cross-validation. Data obtained in this study with *Ny. intermedia* and *Ny. neivai*, species that also have taxonomic identification problems, showed that these two species can be separated by way of this technique, demonstrating its validity as another important way to define cryptic species. However, it is necessary to carry out a more in-depth study including larger population samples of these two species.

Cazorla & Acosta (2003) did a comparative study among the following complex *cayennensis* species: *Micropygomyia yencanensis* Ortíz, 1965, *Mi. cayennensis cayennensis* Floch & Abonnenc, 1941 and *Mi. micropyga* Mangabeira, 1942. The authors were able to discriminate these three species with 100% accuracy using the canonical variate analysis. In turn, Dujardin & Le Pont (1999) evaluated some sub-species of the genus *Psychodopygus*, demonstrating that they were able to separate the species *Ps. carrerai thula* Young, 1979 from *Ps. carrerai carrerai* Barretto, 1946. Due to its similarity and occurrence in allopatry, the former had always been considered a sub-species of the latter.

We observed that uniting various techniques (integrative taxonomy) is vital to improving species identification and solving problems involving the cryptic species and the misidentification of certain groups of species. It was also seen that the specific characters from the male and female genitalia were most important in distinguishing species when compared to the characters shared by both sexes.

*Geometric morphometric technique.* Geometric morphometrics is a technique that has long been adopted in many different areas of study, including that of insects. However, among the few published papers on sand fly research, the majority use the above technique to distinguish populations of species and not the species itself. Analyses of geometric morphometric characters of the wings of *Ny. whitmani*, *Ny. intermedia*, *Ny. neivai*, *Mg. migonei*, *Bi. flaviscutellata*, and *Br. troglodytes* have provided an important contribution to the differentiation of these species, opening up new possibilities for the identification of taxonomic groups. In comparing the wing size of the species through centroid size, we found that *Br. troglodytes* had the largest wings and *Mg. migonei*, the smallest, indicative of a correlation between the wing and body sizes of the insect.

The main variation in PC1 showed that the shape of the wings was wider in the middle as exhibited in *Br. troglodytes*. It was also observed that the wing shape of the specimens of the genus *Nyssomyia* and *Mg. migonei* were similar to each other while those pertaining to *Bi. flaviscutellata* and *Br. troglodytes* were more dissimilar. PC2 found that, for the most part, modifications occurred on the very points that differentiated the wings of *Br. troglodytes* and *Bi. flaviscutellata*, which happened to be the most dissimilar regarding this particular component.

A study developed by Dujardin *et al.* (2003) using 6 anatomical landmarks on the wings to compare the species of *Ps. ayrozai* Barretto & Coutinho, 1940 and *Ps. geniculatus* Mangabeira, 1941 that originated in two different geographical areas described that the analysis of canonical variates was able to separate the species when CV1 was observed and separate the geographical regions when CV2 was observed. In contrast, a study done by De la Riva *et al.* (2001) with populations of species of *Lutzomyia longipalpis* Lutz & Neiva, 1912 using 5 anatomical landmarks on the wings showed an inability to separate populations from diverse geographical regions while, at the same time, managing to group these population into two groups, one with specimens of the most heterogeneous areas and the other with specimens from the more homogeneous areas. Prudhomme *et al.* (2012) compared the wings of *Phlebotomus papatasi* in different regions of Morocco (North and South) using 16 anatomical landmarks on the wings. Analysis of canonical variates and size of the centroid managed to separate the populations, suggesting that these differences may be due to environmental pressures as local populations are found in regions that include a variety of profiles.

In our study, the use of 13 anatomical landmarks on the wings allowed for discrimination at the genus level, at which point *Bichromomyia*, *Nyssomyia*, *Migonemyia*, and *Brumptomyia* presented significant differences in wing shapes, in corroboration with the classification proposed by Galati (1995). This differentiation was emphasized when comparing the canonical variates 1 and 2 in which the species of the genus *Nyssomyia* and *Mg. migonei* were more closely related to each other than to *Bi. flaviscutellata* and *Br. troglodytes*. It should be noted that the latter two species were only closer in the CV1 analysis. On the contrary, in CV2, *Mg. migonei* was closer to the species *Br. troglodytes*. Moreover, in CV2 (33.38% of the total variation), it was seen that the difference in this variable is related to increases in the  $R_5$  base (landmark 13) and the displacement of the apical part of the wings in landmarks 3 and 11, with bulging occurring in the same part. This variation is present in the wing shapes of *Mg. migonei* and *Br. troglodytes*.

Through the Mahalanobis distances, we discovered that *Br. troglodytes* is the most distant species in relation to the others, i.e., aside from *Mg. migonei*, whose wing shape is more similar to that of *Br. troglodytes*. *Bi. flaviscutellata* presents greater distances from the genus *Nyssomyia* than from *Mg. migonei*. The distance of *Bi. flaviscutellata* from the other species is due to a reduction in the  $R_5$  basal part, approaching the fork of  $R_{2+3+4}$  and a major displacement observed in the meeting of  $R_1$  with the margin of the wing, variations shown to be specific to this species.

When comparing the species of the genus *Nyssomyia* alone, we realized that the distances were very small, confirming the high degree of similarity found in these disparate species. The high degree of similarity in the wings of the genus *Nyssomyia* led to the misclassification of some specimens. These species appear to have speciated only recently in corroboration with studies describing the introgression of some genes in these three species (Marcondes 1996; Marcondes *et al.* 1997; Mazzoni *et al.* 2006).

## Conclusions

The analysis of morphometric data showed that the vector species of *L. (V.) braziliensis*, *Ny. intermedia*, *Ny. neivai*, *Ny. whitmani*, and *Mg. migonei* are phenetically closer to each other than they are to *Bi. flaviscutellata*, the *L. (L.) amazonensis* vector. In addition to the fact that morphological studies have significantly contributed over the years to correct species identification (Young & Duncan 1994), they have also been of great assistance in pursuing the phylogenetic studies of sand flies (Galati 2003). Furthermore, our linear morphometric studies revealed some important characters that have become crucial in species diagnoses such as the length of common ducts, the width of individual ducts, the ratio between the ejaculatory filament and its tip, and the distance between the apical and external superior spines of gonostylus.

Likewise, it has been demonstrated that geometric morphometrics, so underexplored in Phlebotominae studies, is an important additional tool for species diagnosis. The use of a character set to analyze morphology and morphometry (linear and geometric) has certainly provided a consistent basis for these studies. Our work has also demonstrated that geometrical morphometric wing characteristics are useful tools that are capable of identifying genera and species patterns in sand flies. Centroid size analysis of the wings showed significant differentiation between the species of *Ny. intermedia* and *Ny. neivai*, as further evidence of the separation of these two species. The use of linear and geometric morphometric characteristics in our work also made possible the separation of the

genera *Brumptomyia*, *Migonemyia*, *Bichromomyia*, and *Nyssomyia*, in support of the separation of the *Bichromomyia* and *Nyssomyia* genera proposed by Galati (2005).

Among all the characters observed in our linear morphometric evaluations, those that most significantly contributed to species differentiation were the lengths of the ejaculatory pump, body, and common ducts of the spermathecae. The ratio between the ejaculatory filament and its tip is a useful structure for species differentiation of *Ny. intermedia* and *Ny. neivai*. We also observed that the morphometric data of the male genitalia contributed additional information regarding species separation than the characters of the female genitalia.

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