doi: 10.5123/S2176-6223202601719

Study of *Anopheles nuneztovari* (Diptera: Culicidae) populations from the Brazilian Amazon using Wing Geometric Morphometrics

Estudo de populações de *Anopheles nuneztovari* (Diptera: Culicidae) na Amazônia brasileira utilizando Morfometria Geométrica de Asas

Sidney dos Reis Diniz[1,2], Marcia Moraes Martins dos Santos[2], Yan Carvalho da Silva[1,2], Thiago Vasconcelos dos Santos[1,2], Ana Cecília Feio dos Santos[2], Izis Mônica Carvalho Sucupira[1,2], Marinete Marins Póvoa[1,2]

[1] *Universidade Federal do Pará, Programa de Pós-Graduação em Biologia de Agentes Infecciosos e Parasitários, Belém, Pará, Brasil*
[2] *Instituto Evandro Chagas, Seção de Parasitologia, Ananindeua, Pará, Brasil*

ABSTRACT

OBJECTIVES: *Anopheles nuneztovari* is a complex of cryptic species that are difficult to distinguish or indistinguishable by morphological criteria, but exhibit relevant biological, ecological, and behavioral differences that may influence malaria transmission and vector control. This study aimed to investigate morphological characteristics that could differentiate species within the *Anopheles (Nyssorhynchus) nuneztovari* complex. METHODS: For this purpose, wing morphometric analyses were performed using the programs MorphoJ and PAST on females populations of *An. nuneztovari s.l.*, collected from two municipalities in Pará, two populations of males previously identified as *An. nuneztovari s.s* and *An. goeldii* and two external populations of females, *Aedes aegypti* and *An. darlingi*. RESULTS: Male differentiation and wing sexual dimorphism were observed among mosquitoes of the *An. nuneztovari* complex. Analysis of the centroid size by ANOVA and Tukey's test showed that the populations of males *An. goeldii* and *An. nuneztovari* s.s were significantly different between themselves and from all female populations. CONCLUSION: We demonstrated the usefulness of morphometric analyses in studies of medically important culicids and showed that this approach is efficient, fast, and less expensive than genetic characterization and can also be carried out in the field.

Keywords: Malaria, *Anopheles*, Classification, Biometry, Sexual Dimorphism, Wings.

RESUMO

OBJETIVOS: *Anopheles nuneztovari* constitui um complexo de espécies crípticas que são difíceis de distinguir ou indistinguíveis por critérios morfológicos, mas que apresentam diferenças biológicas, ecológicas e comportamentais relevantes, as quais podem influenciar a transmissão da malária e as estratégias de controle vetorial. Este estudo teve como objetivo investigar características morfológicas capazes de diferenciar espécies pertencentes ao complexo *Anopheles (Nyssorhynchus) nuneztovari*. MÉTODOS: Para esse propósito, foram realizadas análises morfométricas das asas utilizando os programas MorphoJ e PAST em populações de fêmeas de *An. nuneztovari s.l.*, coletadas em dois municípios do estado do Pará, duas populações de machos previamente identificadas como *An. nuneztovari s.s.* e *An. goeldii*, além de duas populações externas de fêmeas, *Aedes aegypti* e *An. darlingi*. RESULTADOS: Foi observada diferenciação entre machos e dimorfismo sexual das asas entre os mosquitos do complexo *An. nuneztovari*. A análise do tamanho do centróide por ANOVA e teste de Tukey demonstrou que as populações de machos de *An. goeldii* e *An. nuneztovari s.s.* diferiram significativamente entre si e também de todas as populações de fêmeas. CONCLUSÃO: Demonstramos a utilidade das análises morfométricas em estudos com culicídeos de importância médica e mostramos que essa abordagem é eficiente, rápida e menos onerosa do que a caracterização genética, podendo também ser realizada em campo.

Palavras-chave: Malária; *Anopheles*; Classificação; Biometria; Dimorfismo Sexual; Asass.

Correspondence / Correspondência:
Ana Cecília Feio dos Santos
Instituto Evandro Chagas/SVSA/MS, Seção de Parasitologia
Rodovia BR-316, km 7, s/n. Bairro: Levilândia. CEP: 67030-000 – Ananindeua, Pará, Brasil
E-mail: ceciliafeio@gmail.com

INTRODUCTION

Species of *Anopheles nuneztovari* complex are important malaria vectors in several countries in South America[1,2] and, according to studies, it is considered a local vector in some areas of the Brazilian Amazon[3–6]. The geographic distribution of this species is wide and can be found in Bolivia, Brazil (Amazon region), Colombia, Ecuador, Peru, French Guiana, Suriname, Venezuela, and also part of Panama in Central America[7–10].

An. nuneztovari s.l. Gabaldón, 1940 comprises four cryptic species that are difficult to distinguish or even indistinguishable based on external morphological criteria[7,11–13]. Some of these species exhibit biological, ecological, and behavioral differences that influence malaria transmission and vector control[14].

The species belonging to the *Anopheles (Nyssorhynchus) nuneztovari* complex are *Anopheles nuneztovari s.s* Gabaldón, 1940, *Anopheles goeldii* Rozeboom & Gabaldón, 1941, *Anopheles dunhami* Causey, 1945[15–18] and *Anopheles jamariensis*[19], formerly known as *Anopheles nuneztovari* A [17,18,20–23].

An. nuneztovari s.s, occurs in Colombia, Venezuela, and Brazil; *An. goeldii* and *An. jamariensis* in the Brazilian Amazon, and *An. dunhami*, in the Brazilian Amazon, Colombia and Peru[13,17,23–27].

Adult mosquitoes of *Anopheles nuneztovari* complex species can be identified using molecular methods and by the morphology of the male genitalia[6,13,17–19,23]. Geometric morphometry, a tool that has proven efficient for the identification of medically important mosquitoes, is fast and presents quality results[28–36]. Unlike classical morphometrics, which uses linear measurements (lengths, widths, and angles) in structures or parts of organisms for multivariate statistical analysis, geometric morphometrics uses Cartesian coordinates obtained from points distributed in a two- or three-dimensional plane, called homologous anatomical landmarks[37–39].

Anatomical landmarks can be categorized according to the degree of reliability regarding the homology of the points. They are classified into three types: type I (highly reliability) is used in bone structures or wing venation, type II (medium reliability) is used at the extremities of processes and type III (low reliability) is used for example at the top of a concavity[39–41]. The wing is the part of the mosquito's body most used in morphometric analysis studies because it has a rigid two-dimensional structure and ribs where anatomical landmarks can be easily positioned, reducing scanning errors[42,43].

Thus, this study aimed to use wing geometric morphometrics as an auxiliary tool for differentiating species within the *An. nuneztovari* complex, providing information that may contribute to control measures for these malaria-transmitting vectors, given that females of some species from this complex do not present proven vector competence[19,27].

MATERIAL AND METHODS

Adult female specimens were collected using protected human attraction capture methods in the municipality of Altamira-PA (S 03°12'12", W 52°12'23) and by animal attraction (cattle and buffalo) in the municipality of Peixe Boi – PA (S 01° 09' 56.6"; W 047° 17' 60.6") with the aid of an oral suction aspirator (Castro aspirator) and flashlight, and stored in containers.

The specimens were identified under a stereoscopic microscope using the identification keys proposed by Gorham *et al*[44], Consoli & Oliveira[9], and Forattini[45]. The wings of female mosquitoes identified as *An. nuneztovari s.l.* were removed and subjected to geometric morphometric analysis.

For the morphometric analysis, the wings of female mosquito from two populations of *An. nuneztovari s.l* were used: one collected in the municipality of Altamira and the other in the municipality of Peixe Boi. In addition, two male populations identified as *An. nuneztovari s.s.* and *An. goeldii* were obtained from larvae collected in the municipality of Altamira[23]. The species *An. dunhami* and *An. jamariensis* were not included in the study because they were not found in the two study areas. Wings of female mosquitoes from two additional species were also analyzed, *Aedes aegypti,* created in the entomology laboratory of the Arbovirology Section of the Evandro Chagas Institute and *An. darlingi* collected in the municipality of Peixe Boi (Table 1).

The preparation and assembly of the slides followed the protocol used in the Leishmaniasis Laboratory of the Evandro Chagas Institute (adapted), in which the pair of wings of each specimen was removed using a stereoscopic microscope with the aid of fine-tip tweezers and a stylet. The wing in the best physical condition, either right or left, from each specimen was used. The wings were then subjected to a dehydration process by passing them twice in absolute ethanol for 30 minutes. Afterwards, the wings were mounted between the slide and coverslip with a drop of Canada balsam in a horizontal position with the proximal region facing the left side of the slide. The slides were then labeled, photographed, and stored in a slide cabinet.

Table 1 – Mosquitoes populations and the number of specimens used in the present study

Population	Quantity (specimens)
An. nuneztovari s.l (female) - Altamira	50
An. nuneztovari s.l (female) - Peixe Boi	50
An. nuneztovari s.s. (male)	03
An. goeldii (male)	35
Aedes aegypti (female)	50
An. darlingi (female)	50

Wing images were captured using an Axiostar Plus stereoscopic microscope (ZEISS), equipped with an AxioCam CR3 (ZEISS®) camera, at 50x magnification with the help of the AxioVision version 4.7 program.

To acquire morphometric data, all images were digitized and transferred to the tpsUtil and tpsDig software programs from the TPS (Thin Plate Spline) series developed by Rohlf[38]. The tpsUtil program was used to organize, arrange, and manipulate the image lists and the tpsDig program was used to digitize the anatomical landmarks on each wing.

Thirteen type I anatomical landmarks were digitized on each wing for geometric morphometric analysis (Figure 1). These landmarks were selected based on the wing morphology described in studies on anophelines[33,46,47]. The order in which the landmarks were introduced was the same for all wings analyzed to establish the spatial homology of the structures.

After digitization the anatomical landmarks on the wings, the superposition method, namely Generalized Procrustes Analysis (GPA), was used to generate size and shape variables. For this analysis, Canonical Variables analysis (CVA) was then performed to assess shape variables between a priori defined groups using a multivariate data set. The morphospaces of each group were determined using ellipses generated with a 90% confidence interval. The Mahalanobis distances derived from shape variables were used to assess similarity among populations, as the greater the distance, the greater the difference in shape between groups or species[48]. A Mahalanobis distance matrix was constituted and used for the phylogenetic reconstruction of the groups, through the UPGMA method[49] (Unweighted Pair Group Method with Arithmetic Mean; available at http://genomes.urv.es /UPGMA/DendroUPGMA). Analyzes were performed using the MorphoJ version 1.04b program[50]. To verify differences in the size of the wing centroid and the construction of the violin-plot graph, analysis of variance (ANOVA) was performed, and the Tukey test was applied in the PAST version 2.17 program using data generated in the MorphoJ program.

RESULTS

Canonical Variable analysis showed that the first (VC1) and second (VC2) variables were responsible for 99.1% of the total variation in wing shape, highlighting the morphometric distinction among the study populations in the two-dimensional scatterplot.

The scatterplot (Figure 2) of populations in morphospace demonstrated the separation of the *Aedes aegypti* population (outgroup) from the target populations. No evident separation was observed among *An. darlingi, An. nuneztovari s.l.* populations from Peixe Boi and Altamira, however male populations of *An. nuneztovari s.s* and *An. goeldii* were distinct from the populations of *Aedes aegypti* and other populations of anophelines but showed little intersection between them. Specimens located outside the confidence ellipses were also observed.

In the dendrogram derived from Mahalanobis distances obtained from discriminant analyses among populations (Figure 3), a greater distance between males and females was observed, where the highest dissimilarity value was 35.32.

Among females, *A. aegypti* population was more distant from the other populations in the study, with a dissimilarity value ranging from 8.54 to 30.75, while the smallest morphological distance (1.06) was observed between the populations of *An. nuneztovari s.l.* from Altamira and Peixe Boi. The *An. darlingi* population is closer to other anopheline populations, when compared to the *A. aegypti* population, showing dissimilarity value close to 2. Male population *An. nuneztovari s.s* and *An. goeldii* showed great phenotypic distance, with a dissimilarity value of 11.31.

The result of centroid size analysis according to ANOVA and Tukey's test (Figure 4) showed that the populations of males *An. goeldii* and *An. nuneztovari s.s* were significantly different ($p<0.05$) between themselves and with all populations of females (*A. aegypti, An. darlingi, An. nuneztovari s.l* from Altamira, *An. nuneztovari s.l* from Peixe Boi).

Among the female populations, the size of the centroid of *A. aegypti* differed from that of *An. darlingi* and *An. nuneztovari s.l* from Peixe Boi, but not from that of *An. nuneztovari s.l* from Altamira.

When comparing female anopheline populations, no significant difference was observed in centroid wing size between *An. nuneztovari* s.l. populations from Altamira and Peixe Boi. However, both populations differed significantly from *An. darlingi*.

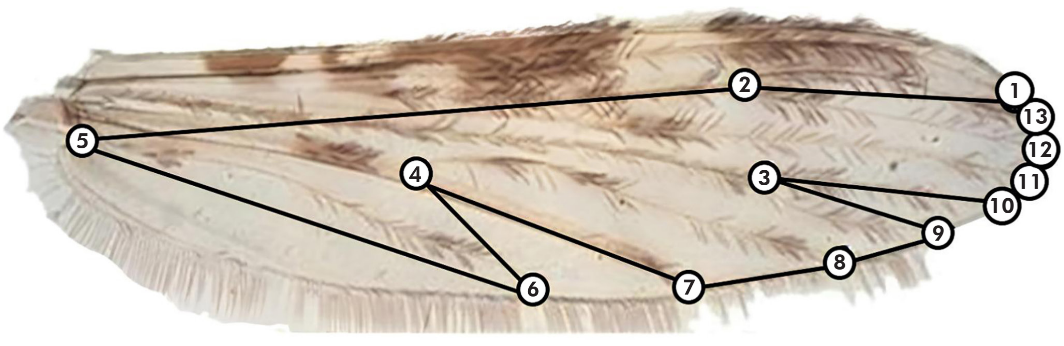

Figure 1 – Wing *Anopheles nuneztovari s.l.* with delimitation of 13 landmarks (white circles) and resultant shape (black lines) used for geometric morphometric analysis

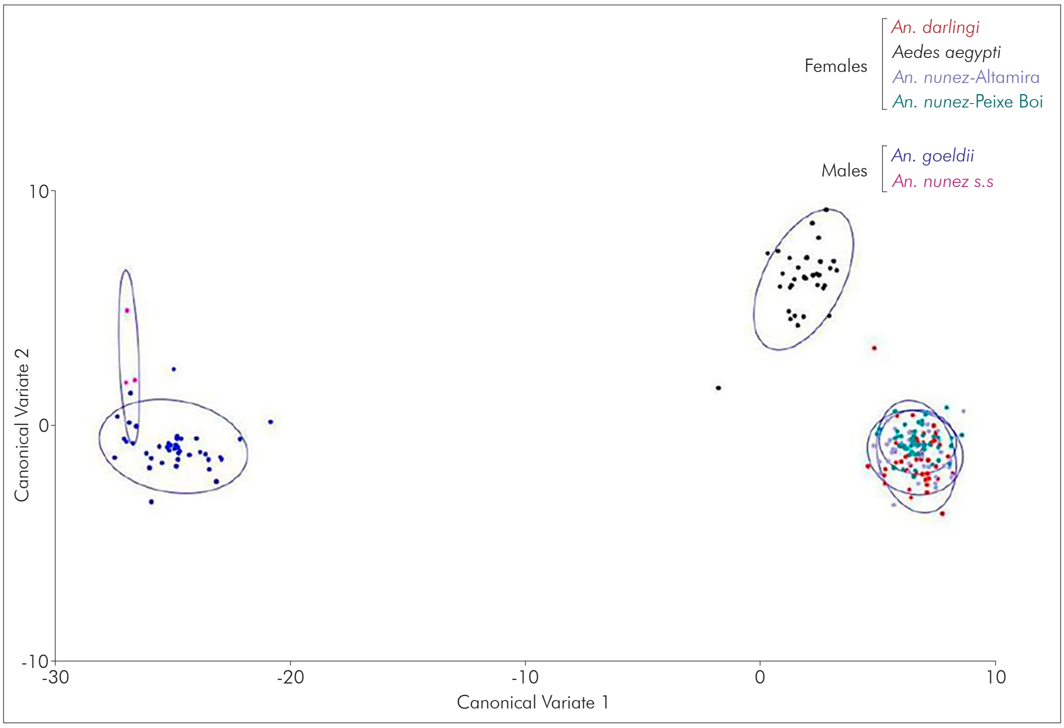

Figure 2 – Analysis of Canonical Variables using the shape of the wings of the studied populations. The colors represent populations and Ellipses, 90% confidence interval

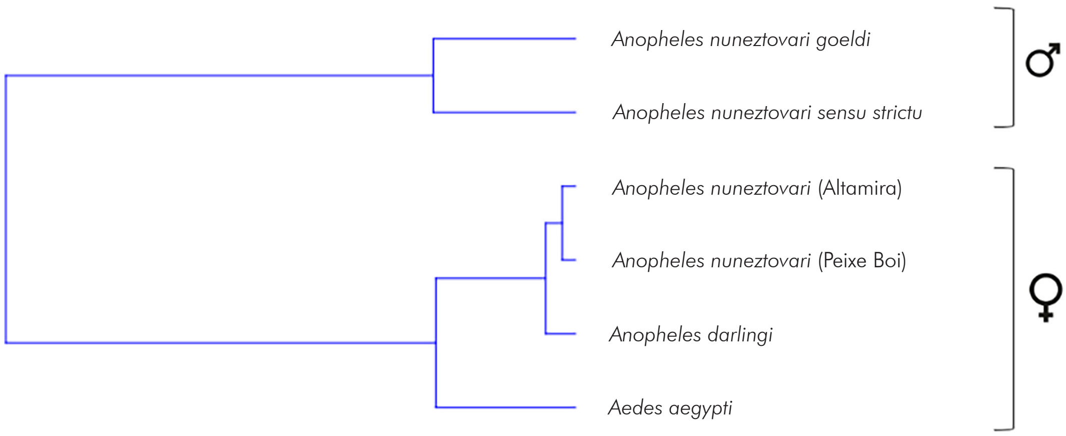

Figure 3 – Dendrogram of morphological similarity derived from Mahalanobis distances between study populations

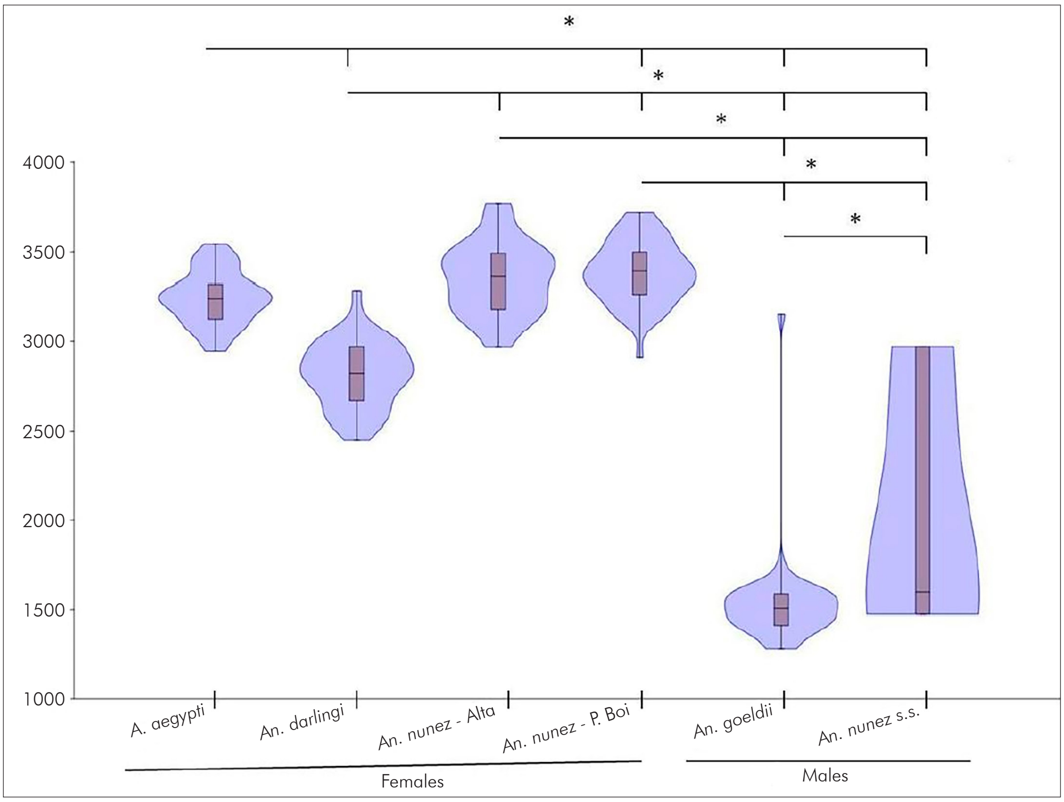

Figure 4 – Violin-plot/boxplot graph showing the comparison between the size of the centroids of the study populations (* = P < 0.05)

DISCUSSION

The analysis of interpopulation wing morphometry revealed clear differentiation among the studied populations at both genus and species levels, particularly in the canonical variable analysis. These variables distinguished the populations into distinct groups within the morphospace, whereas centroid size analysis indicated significant differences in size among populations. The latter result is similar to that of Wilke *et al*[32] and Lorenz & Suesdek[36], who managed to distinguish three genera of mosquitoes, *Aedes*, *Anopheles*, and *Culex*, down to the taxonomic classification levels of subgenus and species. It was also observed on the analysis of canonical variables some specimens outside the confidence ellipses, which in the future could be elucidated using a molecular approach, as these specimens may represent other species.

Interpopulation morphometric analyses among female anophelines belonging to the subgenus *Nyssorhynchus* showed that the population of *An. darlingi* differed from the populations of *An. nuneztovari s.l* from Altamira and Peixe Boi, with the distance from Mahalanobis to *An. darlingi* very close to both the population of *An. nuneztovari s.l* from Altamira (2.51) and from Peixe Boi (2.22). Furthermore, a significant difference was observed ($p<0.05$) in centroid size, highlighting the differentiation of these anopheline species.

Other authors found that wing geometric morphometrics is efficient in differentiating species of mosquitoes from the subgenus *Nyssorhynchus*[47–49], populations of mosquitoes from the subgenus *Kerteszia*[50,51] and culicid species belonging the subgenera *Stegomyia, Ochlerotatus, Kerteszia, Nyssorhynchus* and *Culex*[32].

The results of inter and intrapopulation analyses of *An. nuneztovari s.l* females from Altamira and Peixe Boi showed that showed no significant difference in size ($p=1.16$) and shape between these populations, as no segregation was observed in morphospace in the analysis of canonical variables and small Mahalanobis distance (1.06). However, between male populations of *An. nuneztovari s.s* and *An. goeldii*, a significant differences centroid size were detected ($p<0.05$) and difference in shape, evidenced in the segregation in the analysis of canonical variables and a Mahalanobis distance with a dissimilarity value of 11.31.

Female specimens of mosquitoes belonging to the *nuneztovari* complex are morphologically indistinguishable according to existing identification keys, however, males can be identified based on genital morphology[17,19,23]. Similar distinguishing characteristics have been reported in some species of mosquitoes of the *Culex* subgenus that are considered difficult to identify, as they do not present evident morphological differences and with distinctive characteristics restricted to the male genitalia[52].

Monteiro & Reis[39] proposed that the sample size should be at least four times greater than the number of anatomical landmarks, although this is not always feasible for all target populations because of the limited number of specimens collected in the field. However, the present study demonstrated that males of species from the *An. nuneztovari* complex can also be differentiated by wing geometric morphometrics despite sampling limitations. Among females of this complex, no difference in wing shape was observed, however, we cannot say that these females cannot be differentiated by wing geometric morphometry, as no other identification method such as by molecular biology was carried out to ensure that there are no different species within these population. Another hypothesis is that the males of this complex present greater variation in wing shape than females, enabling their differentiation. This hypothesis is relevant because similar patterns have already been observed in the filariasis vector mosquito species of the *Mansonia* subgenus[53].

Although this is the first study to use wing geometric morphometry to differentiate species within the *An. nuneztovari* complex, this technique has already been successfully applied to other cryptic mosquito species complexes[52,54] and other medically important insects such as sandflies[55,56] and triatomines[57,58].

Using the morphometric analysis, it was also possible to verify the sexual dimorphism in wing shape and size, that is, males and females of *An. nuneztovari* formed distinct groups without intersection in the morphospace of canonical variables, and the dendrogram derived from Mahalanobis distances showed a large distance between males and females with different dissimilarity values.

Centroid size analysis revealed a significant difference ($p<0.05$) between sexes, wings of females are larger than those of males. This difference in wing size was observed by other authors in mosquitoes of the genus *Culex*[59] and the genus *Aedes*[29,60–62].

Although distinction between male and female anophelines is commonly based on morphological characteristics of the antennae, palps, and genitalia[45] and this distinction is important for public health, our findings show that the analysis of the wings, and conditions of *An. nuneztovari,* also reveals sexually dimorphic characteristics. Wing shape is a sex-specific phenotypic trait among these anophelines. This result is in line with Virginio et al[63] who explained sexual dimorphism in several mosquito species of the genera *Aedes, Anopheles,* and *Culex*, as well as those by Manimegalai *et al*[64] in *Cx. quinquefasciatus* from India, Devicari *et al*[29] in *Aedes scapularis*, Vidal *et al*[30] in *Aedes albopictus*, Lorenz *et al*[65] *Anopheles cruzii*, and Christe *et al*[66] in *Aedes fluviatilis*.

Therefore, wing morphometry can provide useful information for assessing sexual dimorphism in medically important culicids, which is relevant because males and females perform distinct ecological roles and use their wings differently[45].

CONCLUSIONS

Wing geometric morphometry proved to be an effective approach for taxonomic differentiation and the evaluation of sexual dimorphism, and showed considerable potential for distinguishing male mosquitoes within the nuneztovari complex. The results indicate that wing shape may represent a valuable morphological marker for taxa that are difficult to identify based on conventional external morphology, especially cryptic species.

ACKNOWLEDGMENTS

The authors thank their colleagues from the Arbovirology, Leishmaniasis, and Malaria Entomology Laboratories of the Evandro Chagas Institute for their support and collaboration.

FUNDING

This study was financially supported by Norte Energia SA, Instituto Evandro Chagas, and CNPq (grant numbers 302292/2017-9 and 306420/2022-8).

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

MMP and TVS conceived and supervised the project. MMP, TVS and IMCS coordinated the study. SRD, MMMS and YCS helped in the execution of the study and data collection. SRD, MMMS, ACFS, IMCS, TVS and MMP contributed to data analysis. SRD, YCS and MMMS drafted the manuscript. MMP, TVS, ACFS and IMCS reviewed and edited the manuscript.

REFERENCES

1 Galbadón A, Aguilera C. Variaciones del color de los anofelinos venezuelanos de la subserie oswaldoi (Diptera, Culicidae). In: Proceedings of the 8th American Scientific Congress; 1940 May 10-18; Washington, EUA. Washington: Department of State; 1942. p. 389-409. Vol. III, Biological Sciences.

2 Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. Parasit Vectors. 2012 Apr 4:5:69.

3 Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, Rodrigues IB, Rafael MS. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. Am J Trop Med Hyg. 1998 Aug;59(2):325-35.

4 Tadei WP, Thatcher BD. Malaria vectors in the Brazilian Amazon: *Anopheles* of the subgenus *Nyssorhynchus*. Rev Inst Med Trop S Paulo. 2000 Apr;42(2):87-94.

5 Póvoa MM, Wirtz RA, Lacerda RNL, Miles MA, Warhurst D. Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. Mem Inst Oswaldo Cruz. 2001 Feb;96(2):179-84.

6 Sant'Ana DC, Bergo ES, Sallum MAM. *Anopheles goeldii* Rozeboom & Gabaldón (Diptera, Culicidae): a species of the Nuneztovari Complex of *Anopheles* Meigen. Rev Bras Entomol. 2015 Jan-Mar;59(1):68-76.

7 Kitzmiller JB, Kreutzer RD, Tallaferro E. Chromosomal differences in populations of *Anopheles nuneztovari*. Bull World Health Organ. 1973;48(4):435-45.

8 Faran ME. Mosquito studies (Diptera, Culicidae). XXXIV. A revision of the albimanus section of the subgenus *Nyssorhynchus* of *Anopheles*. Contrib Amer Ent Inst. 1980;15(7):1-215.

9 Consoli RAGB, Oliveira RL. Principais mosquitos de importância sanitária no Brasil. Rio de Janeiro: Fiocruz; 1994.

10 Manguin S, Carnevale P, Mouchet J, Coosemans M, Julvez J, Richard-Lenoble D, et al. Biodiversity of malaria in the world. Montrouge: J Libbey; 2008.

11 Faran ME. The importance of an integrated approach in solving a problem in mosquito systematics. Mosq Syst. 1979;11(4):280-88.

12 Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, et al. Cryptic species as a window on diversity and conservation. Trends Ecol Evol. 2007 Mar;22(3):148-55.

13 Scarpassa VM, Conn JE. Mitochondrial DNA detects a complex evolutionary history with pleistocene epoch divergence for the neotropical malaria vector *Anopheles nuneztovari* sensu lato. Am J Trop Med Hyg. 2011 Nov 1;85(5):857-67.

14 Collins FH, Paskewitz SM. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol Biol. 1996 Feb;5(1):1-9.

15 Scarpassa VM, Tadei WP, Suarez MF. Allozyme differentiation among allopatric populations of *Anopheles nuneztovari* (Diptera: Culicidae). Braz J Genet. 1996;19(2):265-9.

16 Scarpassa VM, Tadei WP, Suarez MF. Population structure and genetic divergence in *Anopheles nuneztovari* (Diptera: Culicidae) from Brazil and Colombia. Am J Trop Med Hyg. 1999 Jun;60(6):1010-8.

17 Calado DC, Foster PG, Bergo ES, Santos CLS, Galardo AKR, Sallum MAM. Resurrection of *Anopheles goeldii* from synonymy with *Anopheles nuneztovari* (Diptera, Culicidae) and a new record for *Anopheles dunhami* in the Brazilian Amazon. Mem Inst Oswaldo Cruz. 2008 Dec;103(8):791-9.

18 Foster PG, Bergo ES, Bourke BP, Oliveira TMP, Nagaki SS, Sant'Ana DC, et al. Phylogenetic analysis and DNA-based species confirmation in *Anopheles* (*Nyssorhynchus*). PLoS One. 2013 Feb 4;8(2):e54063.

19 Santana DC, Sallum MAM. A new species of the Nuneztovari Complex of *Nyssorhynchus* (Diptera: Culicidae) from the western Brazilian Amazon. Zootaxa. 2022 May 10;5134(2):275-85.

20 Conn J. A genetic study of the malaria vector *Anopheles nuneztovari* from western Venezuela. J Am Mosq Control Assoc. 1990 Sep;6(3):400-5.

21 Conn J, Puertas YR, Seawright JA. A new cytotype of *Anopheles nuneztovari* from western Venezuela and Colombia. J Am Mosq Control Assoc. 1993 Sep 1;9(3):294-301.

22 Foster PG, Oliveira TMP, Bergo ES, Conn JE, Sant'Ana DC, Nagaki SS, et al. Phylogeny of Anophelinae using mitochondrial protein coding genes. R Soc Open Sci. 2017 Nov 8;4(11):170758.

23 Santos MMM, Sucupira IMC, Santos TV, Santos ACF, Lacerda RNL, Póvoa MM. Morphological identification of species of the Nuneztovari Complex of *Anopheles* (Diptera: Culicidae) from an area affected by a Brazilian hydroelectric plant. Zootaxa. 2019 Mar 8;4565(2):zootaxa.4565.2.7.

24 Lounibos LP, Wilkerson RC, Conn JE, Hribar LJ, Fritz GN, Danoff-Burg JA. Morphological, Molecular, and Chromosomal Discrimination of Cryptic *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) from South America. J Med Entomol. 1998 Sep 1;35(5):830-8.

25 Ruiz F, Linton YM, Ponsonby DJ, Conn JE, Herrera M, Quiñones ML, et al. Molecular comparison of topotypic specimens confirms *Anopheles (Nyssorhynchus) dunhami* Causey (Diptera: Culicidae) in the Colombian Amazon. Mem Inst Oswaldo Cruz. 2010 Nov;105(7):899-903.

26 Moreno M, Saavedra MP, Bickersmith SA, Lainhart W, Tong C, Alava F, et al. Implications for changes in *Anopheles darlingi* biting behaviour in three communities in the peri-Iquitos region of Amazonian Peru. Malar J. 2015 Jul 30:14:290.

27 Prussing C, Bickersmith SA, Moreno M, Saavedra MP, Alava F, Sallum MAM, et al. *Nyssorhynchus dunhami*: bionomics and natural infection by *Plasmodium falciparum* and *P. vivax* in the Peruvian Amazon. Mem Inst Oswaldo Cruz. 2018 Dec 3;113(12):e180380.

28 Jirakanjanakit N, Dujardin JP. Discrimination of Aedes aegypti (Diptera: Culicidae) laboratory lines based on wing geometry. Southeast Asian J Trop Med Public Health. 2005 Jul;36(4):858-61.

29 Devicari M, Lopes AR, Suesdek L. Dimorfismo sexual alar em Aedes scapularis (Diptera: Culicidae). Biota Neotrop. 2011 Jun 13;11(2):165-9.

30 Vidal PO, Carvalho E, Suesdek L. Temporal variation of wing geometry in *Aedes albopictus*. Mem Inst Oswaldo Cruz. 2012 Dec;107(8):1030-4.

31 Demari-Silva B, Suesdek L, Sallum MAM, Marrelli MT. Wing geometry of *Culex coronator* (Diptera: Culicidae) from South and Southeast Brazil. Parasit Vectors. 2014 Apr 9:7:174.

32 Wilke ABB, Christe RO, Multini LC, Vidal PO, Wilk-da-Silva R, Carvalho GC, et al. Morphometric wing characters as a tool for mosquito identification. PLoS One. 2016 Aug 23;11(8):e0161643.

33 Gómez GF, Correa MM. Discrimination of Neotropical *Anopheles* species based on molecular and wing geometric morphometric traits. Infect Genet Evol. 2017 Oct:54:379-86.

34 Multini LC, Wilke ABB, Marrelli MT. Urbanization as a driver for temporal wing-shape variation in *Anopheles cruzii* (Diptera: Culicidae). Acta Trop. 2019 Feb:190:30-6.

35 Chaiphongpachara T, Laojun S. Variation over time in wing size and shape of the coastal malaria vector *Anopheles* (*Cellia*) *epiroticus* Linton and Harbach (Diptera: Culicidae) in Samut Songkhram, Thailand. J Adv Vet Anim Res. 2019 Jun;6(2):208-14.

36 Lorenz C, Suesdek L. The use of wing shape for characterising macroevolution in mosquitoes (Diptera: Culicidae). Infect Genet Evol. 2020 Jan:77:104052.

37 James Rohlf F, Marcus LF. A revolution morphometrics. Trends Ecol Evol. 1993 Apr;8(4):129-32.

38 James Rohlf F. Morphometrics. Annu Rev Ecol Syst. 1990 Nov;21:299-316.

39 Monteiro LR, Reis SF. Princípios de morfometria geométrica. Ribeirão Preto: Holos; 1999.

40 Bookstein FL. Morphometric tools for landmark data: geometry and biology. Cambridge (UK): Cambridge University Press; 1992.

41 Fornel R, Cordeiro-Estrela P. Morfometria geométrica e a quantificação da forma dos organismos. In: Marinho JR, Hepp LU, Fornel R, organizadores. Temas em biologia: edição comemorativa aos 20 anos do Curso de Ciências Biológicas e aos 5 anos do PPG-Ecologia da URI Campus de Erechim. Erechim: EDIFAPES; 2012. p. 101-20.

42 Dujardin JP. Modern morphometrics of medically important insects. In: Tibayrenc M, editor. Genetics and evolution of infectious diseases. Boston: Elsevier; 2011. Chapter 16, Modern morphometrics of medically important insects; p. 473-501.

43 Lorenz C, Almeida F, Almeida-Lopes F, Louise C, Pereira SN, Petersen V, et al. Geometric morphometrics in mosquitoes: what has been measured? Infect Genet Evol. 2017 Oct:54:205-15.

44 Gorham JR, Stojanovich CJ, Scott HG. Clave ilustrada para los mosquitos anofelinos de sudamerica occidental. Illustrated key to the anopheline morquitoes of western South America. Mosq Syst. 1973;5(2):97-156.

45 Forattini OP. Culicidologia médica. Vol. 2, identificação, biologia, epidemiologia. São Paulo: EDUSP; 2002.

46 Gómez G, Jaramillo L, Correa MM. Wing geometric morphometrics and molecular assessment of members in the Albitarsis Complex from Colombia. Mol Ecol Resour. 2013 Nov;13(6):1082-92.

47 Gómez GF, Márquez EJ, Gutiérrez LA, Conn JE, Correa MM. Geometric morphometric analysis of Colombian *Anopheles albimanus* (Diptera: Culicidae) reveals significant effect of environmental factors on wing traits and presence of a metapopulation. Acta Trop. 2014 Jul:135:75-85.

48 Lima Filho JM, Lessa R, Stosic B, Duarte Neto PJ, Vieira JW. Morphological discrimination in juveniles of two *Selene* Species (Teleostei: Carangidae) using truss net distances. Braz Arch Biol Technol. 2006 Mar;49(2):231-8.

49 Sneath PHA, Sokal RR. Numerical taxonomy: the principles and practice of numerical classification. San Francisco: W. H. Freeman; 1973. (A Series of books in biology).

50 Klingenberg CP. MorphoJ: an integrated software package for geometric morphometrics. Mol Ecol Resour. 2011 Mar;11(2):353-7.

51 Jaramillo N, Calle DA, Quiñones ML, Erazo HF. Discriminación por morfometría geométrica de once especies de *Anopheles* (*Nyssorhynchus*) presentes en Colombia. Biomedica. 2008;28(3):371-85.

52 Motoki MT, Suesdek L, Bergo ES, Sallum MAM. Wing geometry of *Anopheles darlingi* Root (Diptera: Culicidae) in five major Brazilian ecoregions. Infect Genet Evol. 2012 Aug;12(6):1246-52.

53 Lorenz C, Marques TC, Sallum MAM, Suesdek L. Morphometrical diagnosis of the malaria vectors *Anopheles cruzii*, *An. homunculus* and An. bellator. Parasit Vectors. 2012 Nov 13:5:257.

54 Lorenz C, Patané JSL, Suesdek L. Morphogenetic characterisation, date of divergence, and evolutionary relationships of malaria vectors *Anopheles cruzii* and *Anopheles homunculus*. Infect Genet Evol. 2015 Oct:35:144-52.

55 Börstler J, Lühken R, Rudolf M, Steinke S, Melaun C, Becker S, et al. The use of morphometric wing characters to discriminate female *Culex pipiens* and Culex torrentium. J Vector Ecol. 2014 May 12;39(1):204-12.

56 Ruangsittichai J, Apiwathnasorn C, Dujardin JP. Interspecific and sexual shape variation in the filariasis vectors *Mansonia dives* and *Ma. bonneae*. Infect Genet Evol. 2011 Dec;11(8):2089-94.

57 Bellin N, Calzolari M, Callegari E, Bonilauri P, Grisendi A, Dottori M, et al. Geometric morphometrics and machine learning as tools for the identification of sibling mosquito species of the Maculipennis complex (*Anopheles*). Infect Genet Evol. 2021 Nov:95:105034.

58 De La Riva J, Le Pont F, Ali V, Matias A, Mollinedo S, Dujardin JP. Wing geometry as a tool for studying the *Lutzomyia longipalpis* (Diptera: Psychodidae) complex. Mem Inst Oswaldo Cruz. 2001 Nov;96(8):1089-94.

59 Giordani BF, Andrade AJ, Galati EAB, Gurgel-Gonçalves R. The role of wing geometric morphometrics in the identification of sandflies within the subgenus *Lutzomyia*. Med Vet Entomol. 2017 Dec;31(4):373-80.

60 Villegas J, Feliciangeli MD, Dujardin JP. Wing shape divergence between *Rhodnius prolixus* from Cojedes (Venezuela) and *Rhodnius robustus* from Mérida (Venezuela). Infect Genet Evol. 2002 Dec;2(2):121-8.

61 Oliveira J, Marcet PL, Takiya DM, Mendonça VJ, Belintani T, Bargues MD, et al. Combined phylogenetic and morphometric information to delimit and unify the *Triatoma brasiliensis* species complex and the Brasiliensis subcomplex. Acta Trop. 2017 Jun:170:140-8.

62 Vidal PO, Peruzin MC, Suesdek L. Wing diagnostic characters for *Culex quinquefasciatus* and *Culex nigripalpus* (Diptera, Culicidae). Rev Bras Entomol. 2011 Mar;55(1):134-7.

63 Jirakanjanakit N, Leemingsawat S, Thongrungkiat S, Apiwathnasorn C, Singhaniyom S, Bellec C, et al. Influence of larval density or food variation on the geometry of the wing of *Aedes* (*Stegomyia*) *aegypti*. Trop Med Int Health. 2007 Nov 28;12(11):1354-60.

64 Henry A, Thongsripong P, Fonseca-Gonzalez I, Jaramillo-Ocampo N, Dujardin JP. Wing shape of dengue vectors from around the world. Infect Genet Evol. 2010 Mar;10(2):207-14.

65 Prudhomme J, Velo E, Bino S, Kadriaj P, Mersini K, Gunay F, et al. Altitudinal variations in wing morphology of *Aedes albopictus* (Diptera, Culicidae) in Albania, the region where it was first recorded in Europe. Parasite. 2019 Sep 6:26:55.

66 Virginio F, Oliveira Vidal P, Suesdek L. Wing sexual dimorphism of pathogen-vector culicids. Parasit Vectors. 2015 Mar 14:8:159.

67 Manimegalai K, Arunachalam M, Udayakumari R. Morphometric geometric study of wing shape in *Culex quinquefasciatus* Say (Diptera: Culicidae) from Tamil Nadu, India. J Threat Taxa. 2009 May 26;1(5):263-8.

68 Lorenz C, Marques TC, Sallum MAM, Suesdek L. Altitudinal population structure and microevolution of the malaria vector *Anopheles cruzii* (Diptera: Culicidae). Parasit Vectors. 2014 Dec 16:7:581.

69 Christe RO, Wilke ABB, Vidal PO, Marrelli MT. Wing sexual dimorphism in *Aedes fluviatilis* (Diptera: Culicidae). Infect Genet Evol. 2016 Nov:45:434-6.

Received / Recebido em: 22/5/2025
Accepted / Aceito em: 1/9/2025

Managing Editor / Editor Responsável:

Edivaldo Herculano Correa de Oliveira

How to cite this article / Como citar este artigo:

Santos MMM, Diniz SR, Silva YC, Santos TV, Santos ACF, Sucupira IMC, et al. Study of *Anopheles nuneztovari* (Diptera: Culicidae) populations from the Brazilian Amazon using wing Geometric Morphometrics. Rev Pan Amaz Saude. 2026;17:e202601719. Doi: https://doi.org/10.5123/S2176-6223202601719