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Discrimination of *Uranotaenia* species (Diptera: Culicidae) from Madagascar based on morphology and wing morphometric traits

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Abstract

The genus *Uranotaenia* (Diptera: Culicidae) has been well documented in Madagascar where it includes 73 species, 89.4% being endemic. However, one problem is that most species are morphologically similar in the adult stage. Here, 713 *Uranotaenia* specimens collected in the tropical forests of Anorana and Maromizaha between 2008 and 2014 were examined. Using the dichotomous keys for the *Uranotaenia* fauna of Madagascar published in 2004, three species were identified: *Uranotaenia neireti* (220), *Ur. alboabdominalis* (110) and *Ur. mayottensis* (28). The other specimens (355) were not identifiable and were classified as *Uranotaenia* sp1. Using wing morphometry, the four taxa were classified into four morphogroups. Within the *Uranotaenia* sp1 group, specimens from the Anorana forest and those from the Maromizaha forest overlapped. This result suggests that wing morphometric traits could be a good marker to distinguish *Uranotaenia* species in Madagascar.

Key words: Culicidae, *Uranotaenia neireti*, *Ur. alboabdominalis*, *Ur. mayottensis*, taxonomy, morphology, wing morphometry, Madagascar

Introduction

The first observation of the genus *Uranotaenia* in Madagascar dates back to 1920, when the presence of *Ur. neireti* Edwards, an endemic species, was reported by Edwards (1920). Subsequent field studies on mosquitoes in relation to malaria, filariasis and arbovirus control programs then led to the report of multiple new *Uranotaenia* species (17 new endemic species in Madagascar and two African species) (Doucet 1949a, 1949b, 1950, 1951a, 1951b; Grjebine & Lacan 1953). In 2004, a monograph of Afrotropical *Uranotaenia* included 46 new Malagasy species (da Cunha Ramos & Brunhes 2004), and in 2013 three additional new species were described from the country (Boussès & Brunhes 2013). In Madagascar, *Uranotaenia* is the mosquito genus that includes the largest number of species and has one of the highest rates of endemism (89%)—65 of the 73 *Uranotaenia* species that occur in the country are endemic (da Cunha Ramos & Brunhes 2004; Boussès *et al.* 2012; Tantely *et al.* 2016).

Currently, the identification of adult specimens of Malagasy *Uranotaenia* relies on use of the dichotomous identification keys provided by da Cunha Ramos & Brunhes (2004). During a previous entomological study in the Anjozorobe District, 643 *Uranotaenia* specimens were examined (Tantely *et al.* 2013). The results of that study revealed a high degree of morphological variation in *Uranotaenia* specimens. Separating or attributing some mor-

phological forms to a described species was difficult using the key. These observations highlighted an identification problem for species of *Uranotaenia* using the available morphology-based key, indicating the need for additional identification tools.

In recent times, a landmark-based geometric morphometric method was developed to analyse wings for the identification of Culicidae (Dujardin 2008). This method has been shown to be useful in distinguishing the genus *Uranotaenia* from other genera, including *Aedes*, *Anopheles*, *Armigeres*, *Coquillettia*, *Culex*, *Mansonia* and *Mimomyia* (Dujardin 2011), different species within a species group such as the *Culex univittatus* group (Boussès *et al.* 2012) and malaria vectors such as *Anopheles cruzii* Dyar & Knab, *An. homunculus* Komp and *An. bellator* Dyar & Knab (Lorenz *et al.* 2012). However, the method has not been used for separating different *Uranotaenia* species.

The present study aimed to determine whether wing morphometric analysis (Dujardin 2008) was suitable for distinguishing the adults of species of *Uranotaenia* given the presence of uncertain species.

Materials and methods

Mosquito collections

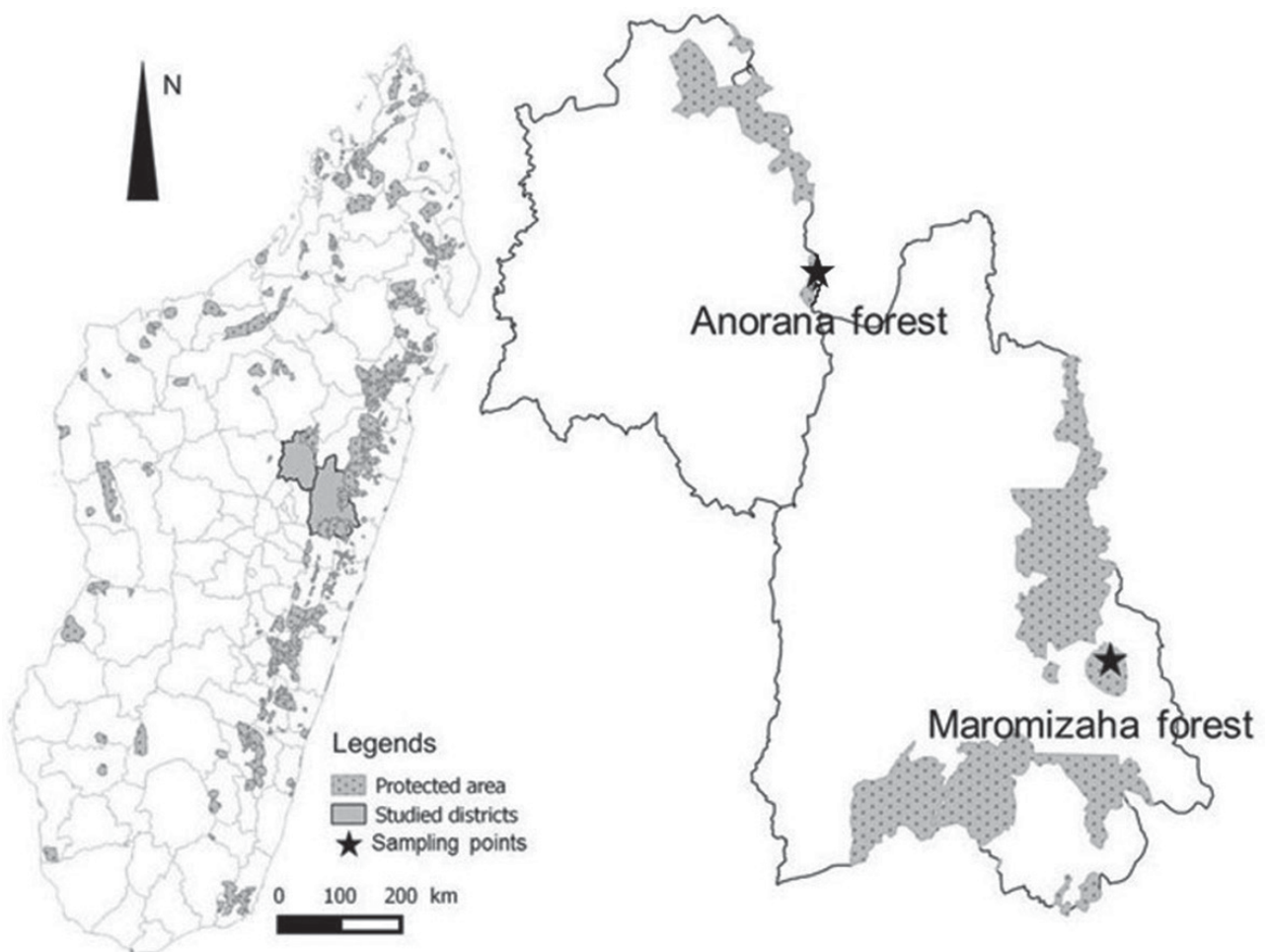


FIGURE 1. Distribution of Malagasy protected areas and location of the two study sites.

Adult *Uranotaenia* examined in this study were collected in the rainforest of Anorana between 2008 and 2010 (Tantely *et al.* 2013) and in the rainforest site of Maromizaha in November 2014 (Schmid *et al.* 2017) (Fig. 1). Adults from the Anorana rainforest were collected as a part of an entomological study on Rift Valley fever virus (Tantely *et al.* 2013). The rainforest of Anorana ($18^{\circ} 18' 44''$ S, $48^{\circ} 00' 98''$ E) is located 30 km northeast of Anjozorobe city at an altitude above 1,150 m (with a highest peak at 1,345 m) in the district of Anjozorobe, (Fig. 1). Adults from the Maromizaha rainforest were collected as a part of a study on vectors of avian malaria (Schmid *et al.* 2017). The

Maromizaha rainforest (18° 56' 49" S, 48° 27' 33" E) is located 24 km east of Moramanga city, at an altitude above 943 m (with a highest peak at 1,213 m) in the district of Moramanga (Fig. 1). Adult mosquitoes were collected using Centers for Disease Control and Prevention (CDC) light traps (BioQuip Products, Inc, Rancho Dominguez, CA) in both rainforests (Tantely *et al.* 2013; Schmid *et al.* 2017; Tantely *et al.* 2019). In the Anorana forest, 10–12 light traps were distributed along valley floors, slopes and ridges (Tantely *et al.* 2013). Mosquitoes were anesthetized with chloroform vapor, pooled in cryotubes according to species, sex and female status (fed or unfed), frozen in liquid nitrogen in the field and then stored at -80°C in the laboratory (Tantely *et al.* 2013). In the Maromizaha forest, four nights of capture were carried out during one week. Twelve traps were distributed along three sampling lines, including the valley floor, slope and ridge. Mosquitoes were stored separately in ELISA plate wells at ambient temperature both in the field and in the laboratory (Schmid *et al.* 2017).

Mosquito identification and description

The keys of da Cunha Ramos & Brunhes (2004) were used to identify the species of *Uranotaenia*. Adult males and females were mounted on card rectangles on insect pins for morphological description and/or stored individually in 1.5-ml dry Eppendorf tubes at ambient temperature pending further morphological and morphometric analyses of the wings. The terminology used for the description is that of Harbach & Knight (1980).

Morphometric analyses of wings

The two wings of each mosquito were dissected their bases, mounted on microscope slides in Euparal and covered with a cover slip. Wing images were captured with a Nikon Digital Sight Fi1 camera under a phase contrast microscope. For each wing, the Collecting Landmarks for Identification and Characterization (CLIC) program (<http://xyom-clic.eu/the-clic-package/>) was used to identify and mark 12 landmarks on the images according to Ayala *et al.* (2011) (Fig. 2). Modules of Dujardin (2008, 2011) were used to perform the morphometric analyses: “Collection of Coordinates” (COO) module to digitize the coordinates of the landmarks, “Morfometria Geometrica” (MOG) module to generate partial warps and centroid sizes, “Covariance” (COV) module to compute metric disparity and, “Asimetria” (ASI) module to calculate Procrustes distance, and “Permutaciones, Analisis Discriminante” (PAD) module to compute Mahalanobis distance. Procrustes distance is a measure of absolute shape difference (Dryden & Mardia 1998). Mahalanobis distance is a measure of distance relative to the variation in each direction of the multivariate space (Mardia *et al.* 1979). Wing size was analyzed using the isometric estimator “centroid size” (CS), which is defined as the square root of the sum of the squared distances between the center of the configuration of landmarks (Dujardin 2011). Two repeated measures of wing size were performed to avoid random measurement error (Ayala *et al.* 2011).

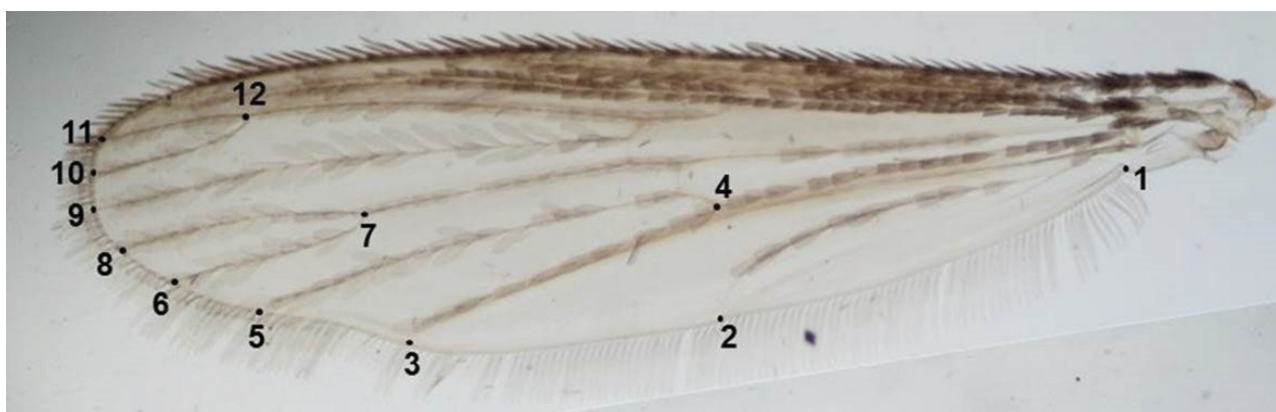


FIGURE 2. Twelve landmarks on the wing of the *Uranotaenia* specimens used in the morphometric analyses.

Statistical analysis

Wing shape differences between species were measured by multivariate analysis of variance (MANOVA) on partial warps on CLIC (<http://mome-clic.com/>). Wing size differences were investigated by applying an analysis of variance (ANOVA) to the centroid size in R version 2.10.1. (R Foundation for Statistical Computing; <http://www.r-project.org>).

Results

Morphological identifications

Of 358 male and female specimens collected in the Anorana forest, 220 were identified as *Ur. neireti*, 110 as *Ur. alboabdominalis* Theobald and 28 as *Ur. Mayottensis* da Cunha Ramos & Brunhes. Another 222 adults from that forest could not be identified to any described species using the dichotomous key of da Cunha Ramos & Brunhes (2004). These last specimens were then recognized as a single morphogroup based on the following morphological characteristics: postpronotum without scales, scutum and abdominal terga without bands or spots of white scales, two dark transverse bands on the thoracic pleura (Fig. 3A), tibia and tarsomeres of hindleg dark and aedeagus strongly toothed (Fig. 3B). This group was designated *Uranotaenia* sp1 as shown on the labels of specimens stored at the Institut Pasteur de Madagascar. In the Maromizaha forest, 133 adult specimens were identified as *Uranotaenia* sp1.

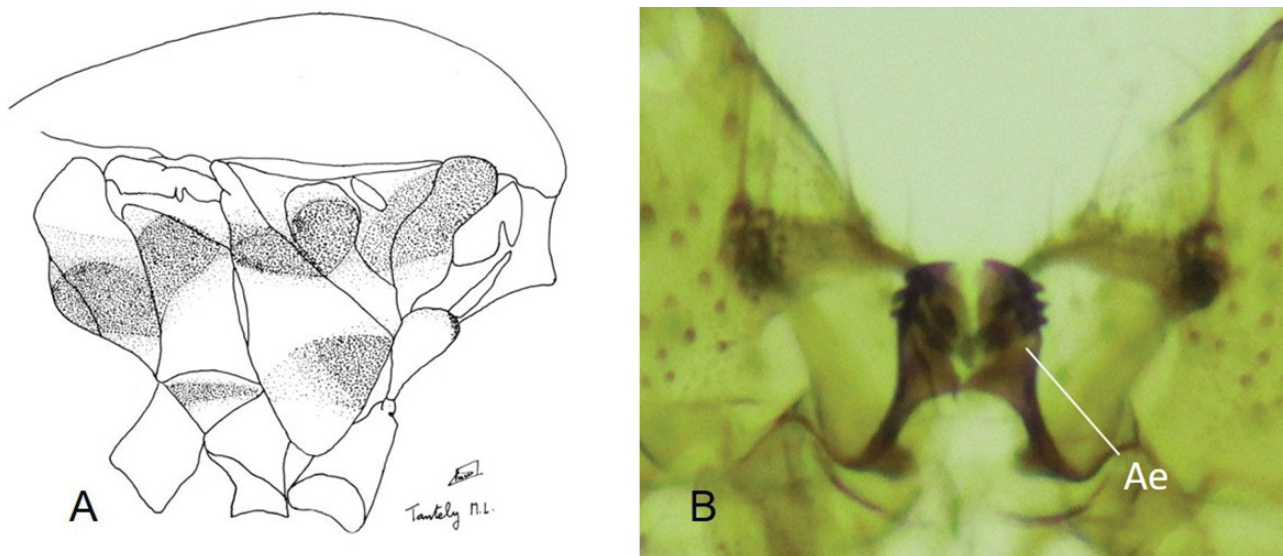


FIGURE 3. *Uranotaenia* sp1. (A) Lateral view of the thorax showing the two dark transverse bands; (B) male genitalia showing the strongly hooked aedeagus (Ae).

Wing morphometrics

A total of 137 *Uranotaenia* adult females were processed for wing morphometric analysis because they had intact wings. These included 34 *Ur. neireti*, 16 *Ur. alboabdominalis*, 49 *Uranotaenia* sp1 and seven *Ur. mayottensis* from the Anorana forest, and 31 *Uranotaenia* sp1 from the Maromizaha forest. The shifts of landmarks 4, 7, 8, 9, 10, 11, 12 observed after the pairwise comparison of species were the most useful for species identification. No marked displacement of all landmarks was observed between the *Uranotaenia* sp1 specimens from the Anorana and the Maromizaha forests (Fig. 4). Wing size differed significantly between the four species collected in the Anorana forest ($F = 89.67$, $df = 3$, $p < 0.001$). The comparison of the wing size of *Uranotaenia* sp1 from both study sites showed a significant difference ($F = 5558$, $df = 4$, $p < 0.001$) (Fig. 5). The canonical variate analysis for wing shape showed that all species and/or groups were completely isolated from each other in the morphospace, except for *Uranotaenia* sp1 from the Anorana and the Maromizaha forests that overlapped (Fig. 6). Significant differences in wing shape among the three identified species and *Uranotaenia* sp1 from the Anorana forest were highlighted through MANOVA (Wilk's $\lambda = 0.48$, $F = 2.58$, $p < 0.0001$). *Uranotaenia* sp1 specimens of the Anorana and the Maromizaha forests showed no significant difference (Wilk's $\lambda = 0.823$, $F = 1.56$, $p < 0.075$). While specimens from both forests showed considerable overlapping variation in wing measurements (Fig. 6), this did not coincide or overlap with measurements of *Ur. alboabdominalis*, the nearest relative in the group. Lower Mahalanobis and Procrustes distances were observed between *Uranotaenia* sp1 specimens from the Anorana and Maromizaha forests (Table 1).

● *Ur. neireti* ● *Ur. alboabdominalis* ● *Uranotaenia* sp1 ANR
 ● *Uranotaenia* sp1 MMZ ● *Ur. mayottensis*

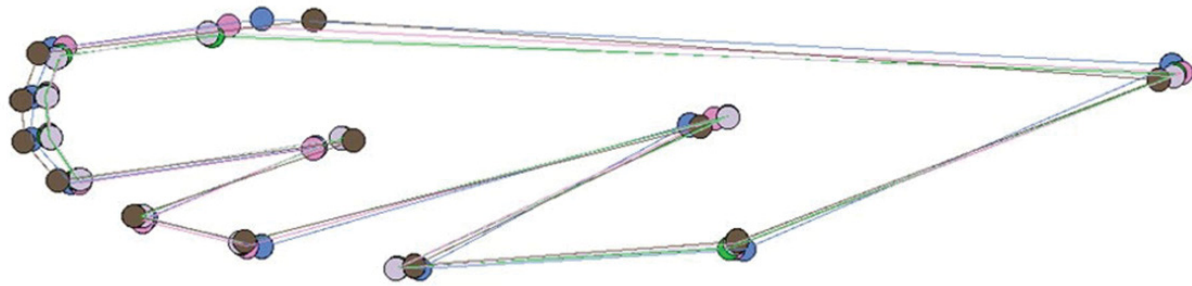


FIGURE 4. Mean coordinates of landmark points on the wings of *Uranotaenia* species. X = from -0.272 to 0.689; Y = from -0.109 to 0.098. ANR, Anorana forest; MMZ, Maromizaha forest.

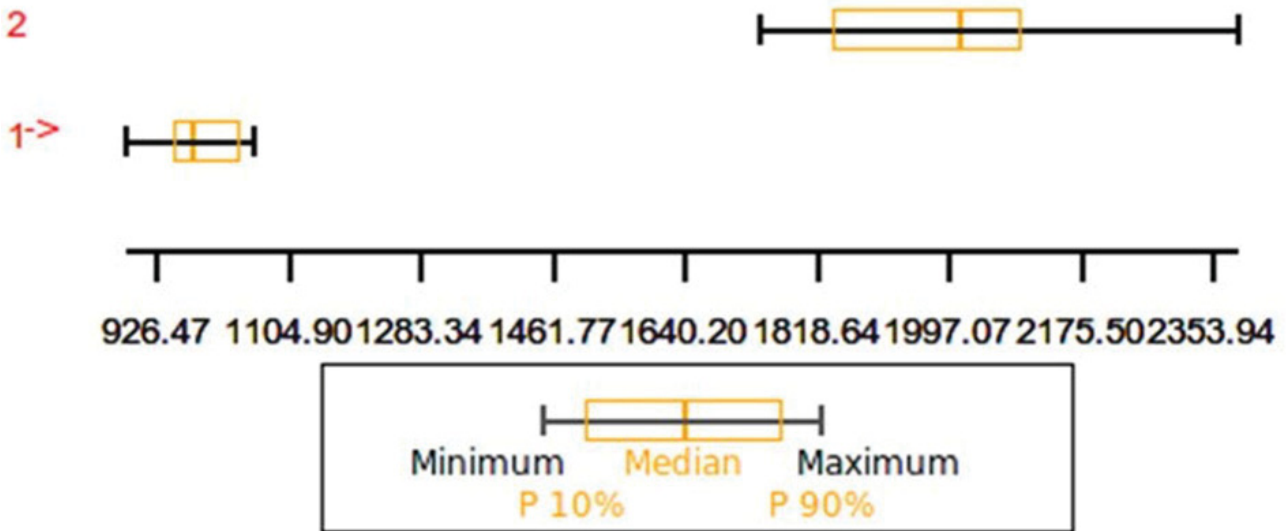


FIGURE 5. Variation of the centroid size of the wing for *Uranotaenia* sp1 from the Anorana forest (1) and the Maromizaha forest (2). Each box shows the group of median values separating the 10th and 90th quartiles.

TABLE 1. Relative wing shape distances of *Uranotaenia neireti* (1), *Ur. alboabdominalis* (2), *Uranotaenia* sp1 (3 and 4) and *Ur. mayottensis* (5). Above diagonal = Procrustes distances; below diagonal = Mahalanobis distances (from CVA computed from Procrustes shape data corrected for allometry). 1, 2, 3 and 5 are specimens from Anorana forest and 4 from Maromizaha forest.

	1	2	3	4	5
1		0.081	0.069	0.071	0.063
2	9.73		0.087	0.089	0.121
3	14.50	8.18		0.009	0.099
4	15.16	8.66	2.99		0.102
5	10.11	15.30	17.97	18.85	

● *Ur. neireti* (68) ● *Uranotaenia* sp1 ANR (98) ● *Ur. mayottensis* (14)
 ● *Ur. alboabdominalis* (32) ● *Uranotaenia* sp1 MMZ (62)

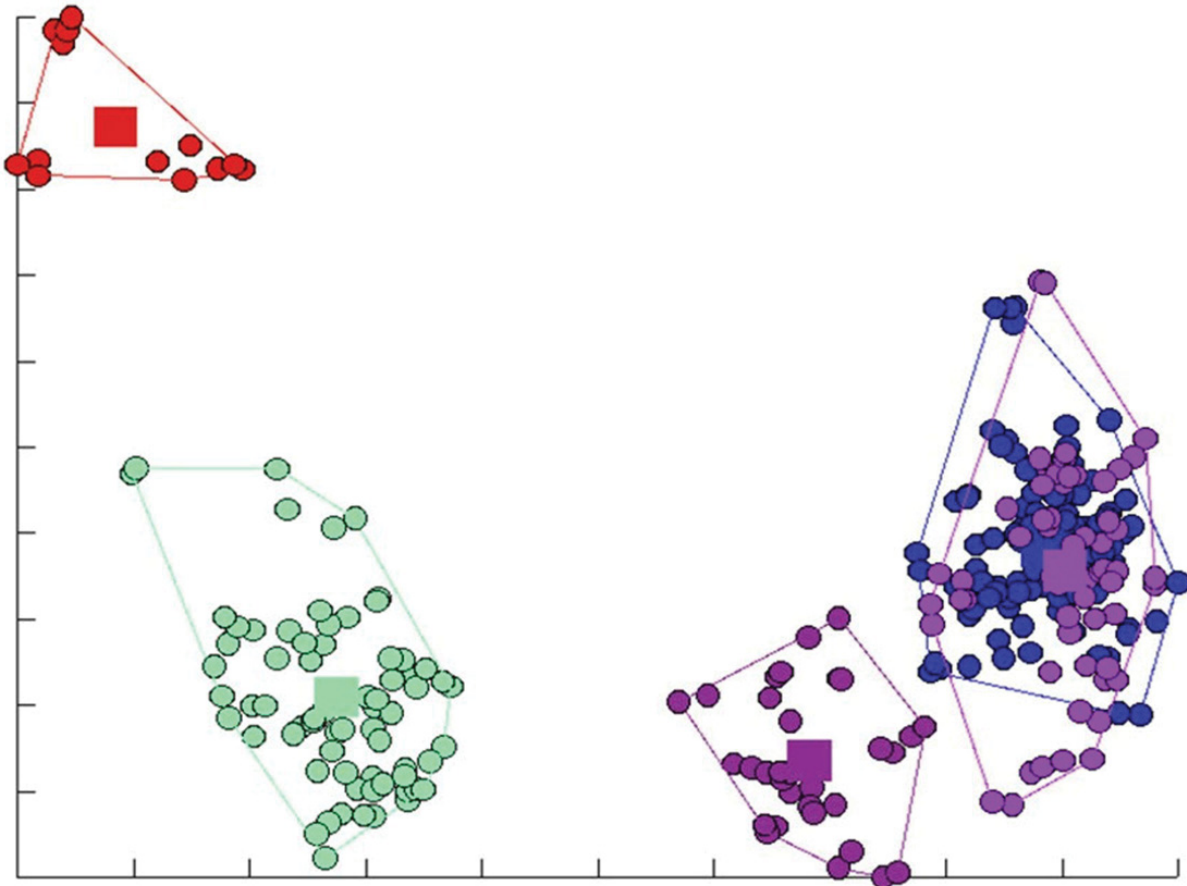


FIGURE 6. Scatter plots from the principle component analysis of morphometric data for wings of *Uranotaenia* species. Squares represent the mean centroid size for each species and group. X = from -0.075 to 0.037; Y = from -0.029 to 0.059.

Discussion

The inclusion of *Uranotaenia* sp1 in the subgenus *Uranotaenia* is justified by the following characteristics: absence of scales on alula, wings veins without white scales and the presence of a mesanapleural suture, as highlighted by da Cunha Ramos & Brunhes (2004). Among 21 species of the subgenus *Uranotaenia* known in Madagascar (da Cunha Ramos & Brunhes 2004), 16 were described only as adults. *Uranotaenia dumonti* (Doucet 1949b) and four species described by da Cunha Ramos & Brunhes (2004), i.e. *Ur. bidentata*, *Ur. hebrardi*, *Ur. joucour* and *Ur. roberti*, are only known from their larval stages. These observations might raise the question whether *Uranotaenia* sp1 might belong to one of these five species. Compared to *Ur. neireti*, *Ur. alboabdominalis* and *Ur. mayottensis* recorded in the districts of Moramanga and Anjozorobe (da Cunha Ramos & Brunhes 2004, Tantely *et al.* 2013), *Uranotaenia* sp1 is morphologically closer to *Ur. mayottensis*. However, it differs from this species in having two dark transverse bands on the thoracic pleura (Fig 3A) and a strongly toothed aedeagus (Fig. 3B). The aedeagus of *Ur. mayottensis* is adorned with three to five teeth (da Cunha Ramos & Brunhes 2004).

The present study confirms that the observation that wing morphometric variation is suitable for completing mosquito morphological identification using dichotomous keys by distinguishing morphologically similar species (Laurito *et al.* 2015). Moreover, this method has proved to be an extremely promising, cost-effective alternative to DNA sequencing (Börstler *et al.* 2014). Based on the subsequent morphometric analysis that clustered *Uranotaenia* sp1 as one group (Fig. 6), we consider with caution that this species is one species that is different at least from *Ur.*

neireti, *Ur. alboabdominalis*, and *Ur. mayottensis* previously recorded in the districts of Moramanga and Anjozorobe (da Cunha Ramos & Brunhes 2004, Tantely *et al.* 2013). The lower Mahalanobis and Procruste distances (Table 1) and the overlapping measurements (Fig. 6) of *Uranotaenia* sp1 from the Anorana and Maromizaha forests would indicate a conspecific status. Moreover, the use of only females for wing morphometric analysis would reduce the interpretation bias due to the sexual shape dimorphism as described in *Aedes albifasciatus* (Macquart) (not recorded in Madagascar) (Garzón & Schweigmann 2018). The significant variation in the size of the wing with no difference in wing shape of *Uranotaenia* sp1 may be due to the fact that wing size variation is associated with environmental affects, while wing shape variation may be explained by chromosomal polymorphism (Ayala *et al.* 2011).

Further study of the taxonomy of *Uranotaenia* sp1 is needed. This is essential as studies aimed at the prevention and control of vector-borne pathogens that cause diseases require a tool to correctly identify the Culicidae present in Madagascar. Indeed, *Uranotaenia* sp1 was recently found infected with avian haemosporidian parasites (*Plasmodium* and *Haemoproteus*) during our 2014 field work (Schmid *et al.* 2017). This species was found to be abundant in the rainforest of Maromizaha, mainly at the rainforest edge (Tantely *et al.* 2019), which is an ecozone (Despommier *et al.* 2006) between intact rainforest and the nearest human habitation (Tantely *et al.* 2019).

The results of this study show that wing morphometric tools are of use for distinguishing at least *Uranotaenia* species and to detect the occurrence of potentially novel species by resolving problems in morphological variation.

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