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Wing morphometrics of biting midges (Diptera: *Culicoides*) of veterinary importance in Madagascar

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ABSTRACT

Biting midges are vectors of arboviruses such as bluetongue virus, bovine ephemeral fever virus, Akabane virus, African horse sickness virus, epizootic haemorrhagic disease virus and Schmallenberg virus. Fast and accurate identification of biting midges is crucial in the study of *Culicoides*-borne diseases. Morphological identification of biting midges has revealed the presence of cryptic species. A total of 20 species are reported in Madagascar. In this study, we assessed wing morphometric analysis for identification of seven species namely *C. dubitatus* Kremer, Rebholtz-Hirtzel and Delécolle, *C. enderleini* Cornet and Brunhes, *C. kibatiensis* Goetghebuer, *C. miombo* Meiswinkel, *C. moreli* Clastrier, *C. nevillei* Cornet and Brunhes, and *C. zuluensis* de Meillon. *Culicoides enderleini*, *C. miombo*, *C. moreli*, *C. nevillei* and *C. zuluensis* are vectors diseases. A molecular approach, based on the cytochrome oxidase I gene (Cox1), was used for species delimitation. The molecular analysis presented seven different clades grouped two-by-two according to morphological characters. A total of 179 wing images were digitised. We found morphometric variation among seven species based on 11 landmarks and two outlines. Wing shape variation plots showed that species overlapped with species belonging to the same group. The cross-validation revealed a relatively high percentage of correct classification in most species, ranging from 91.3% to 100% for landmarks; 60% to 82.6% for outlines-1 and 77.1% to 91.3% for outlines-2. Our study suggests that wing geometric morphometric analysis is a robust tool for reliable “Moka Fohy” identification in Madagascar. This inexpensive and simple method is a precise supplement to morphological identification, with reaches the accuracy of Cox1 barcoding.

1. Introduction

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are small hematophagous flies. Currently, more 1400 species have been described worldwide (Borkent et al., 2022). While some *Culicoides* species do transmit human pathogens, including Oropouche virus and multiple filarial nematodes associated with mansonellosis (Mellor et al., 2000), *Culicoides* are primarily known for their role in transmitting viruses of veterinary importance (Mellor et al., 2000), such as Bluetongue virus (BTV), African horse sickness virus (AHSV) and epizootic haemorrhagic disease virus (EHDV).

AHSV is an arboviral disease endemic in Yemen and Sub-Saharan Africa, extending to South Africa. Outbreaks are also reported in the Maghreb, Spain, the Middle East and the Indian subcontinent (Onyango

et al., 2015; Assefa et al., 2022). Madagascar has been identified as a suitable niche for the extension of AHSV from 2020 to 2060 (Assefa et al., 2022). BTV is present on the African continent (Wilson and Mellor, 2009), including Madagascar Island (Andriamandimby et al., 2015). The geographical distribution of EHDV is less well document than that of AHSV and BTV. Moreover, its distribution seems to be similar to BTV and includes tropical and temperate regions throughout the world (Maclachlan et al., 2015).

Culicoides imicola Kieffer is considered the most important vector both of BTV (Mellor et al., 2000) and AHSV (Mellor and Hamblin, 2004) and possibly EHDV (Yadin et al., 2008). In Africa, some species of *Avaritia* Fox subgenus (*C. actoni*, *C. bolitinos*, *C. brevitarsis*, *C. imicola*, and *C. miombo*) could transmit AHSV and BTV (Meiswinkel et al., 2004; Venter et al., 2006; Diarra et al., 2018; Ciss et al., 2019). Some species of

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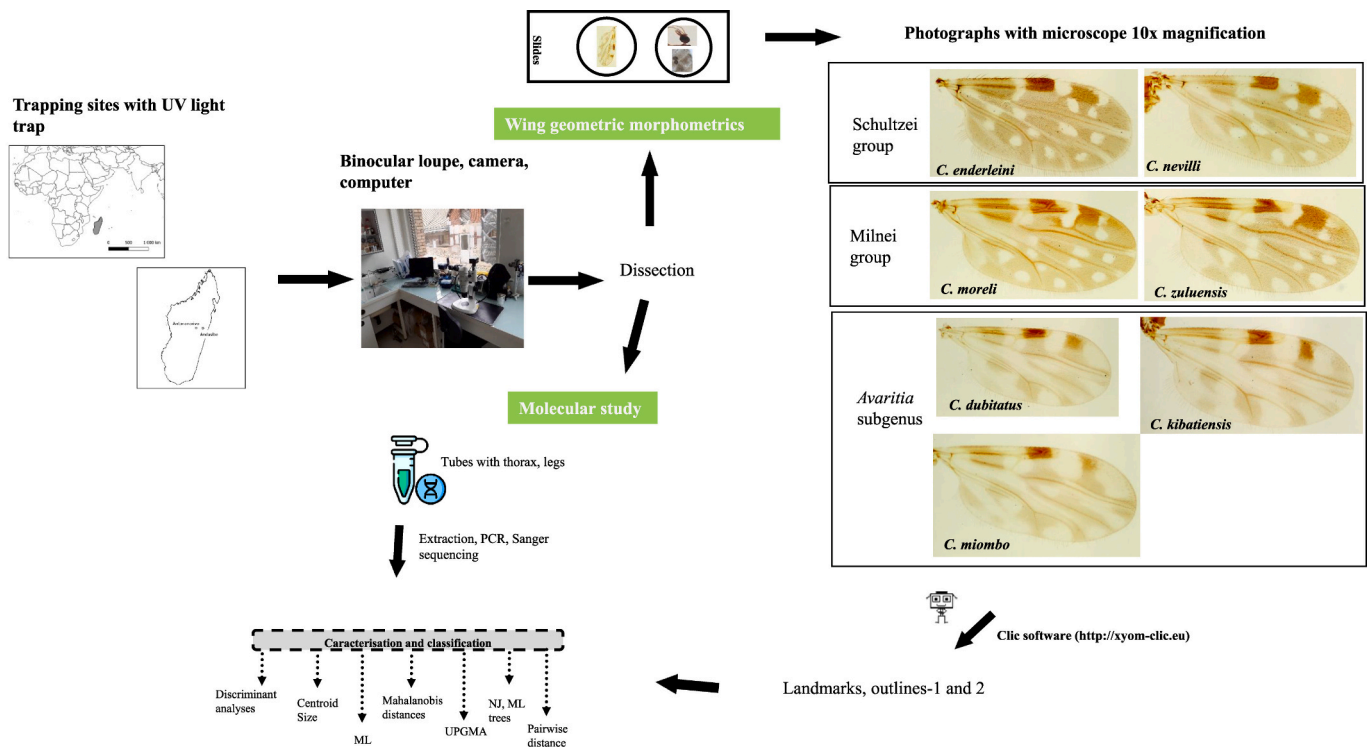


Fig. 1. Block diagram of the study.

the Schultzei complex (*C. oxystoma*, *C. kingi*, *C. enderleini*, and *C. nevillei*) are considered potential vectors or vectors of AHSV, BTV and EHDV (Mellor et al., 1984; Nevill et al., 1992; Meiswinkel et al., 2004; Venter et al., 2006; Diarra et al., 2018). Some species of the Milnei group (*C. austeni*, *C. milnei*, and *C. zuluensis*) are proven or suspected vectors of AHSV, BTV, parasites and several viruses (Meiswinkel et al., 2004; Borkent, 2005; Venter et al., 2006; Diarra et al., 2018).

There is potential for considerable confusion regarding the *Culicoides* genus because of both delimitation and identification of species, on the one hand, and utilisation of the terms such as 'species group' and 'species complex', on the other (Harrup et al., 2015). Moreover, species boundaries are complicated by large intra-specific variations (Bakhom et al., 2013; Desvars et al., 2015; Harrup et al., 2015). However, the determination of species is based on morphological characters; the method is time-consuming and requires taxonomic expertise. Alternative identification methodologies exist, such as molecular biology and matrix-assisted laser desorption/ionisation-time of flight MALDI-TOF analysis (Harrup et al., 2015; Rot et al., 2020). The conservation of specimens in alcohol strongly affects species identification by MALDI-TOF analysis (Benyahia et al., 2021). However, the benefit/cost comparison to molecular techniques has not yet been demonstrated (Harrup et al., 2015). Molecular identification approaches require a molecular laboratory, personnel and funding. Sequencing of the mitochondrial cytochrome oxidase subunit I (Cox1), referred to as DNA barcoding, has been proposed as a tool for rapid identification of species (Ander et al., 2013; Stur and Borkent, 2014). Furthermore, reference DNA libraries (GenBank) for species identification still do not cover all biting midges, with references to certain unknown specimens, called "*Culicoides* spp.". Wing geometric morphometric (WGM) analysis allows us to investigate phenotypic variations (shape and size) using the principles of geometry (Dujardin, 2008). This technique makes it possible to separate species such as Triatominae (Dujardin et al., 2017; Kamimura et al., 2020), mosquitos (Lorenz et al., 2017; Sauer et al., 2020), sandflies (Prudhomme et al., 2016; Sumruayphol et al., 2017) and biting midges (Harrup et al., 2015) at relatively low cost. This approach requires a stereomicroscope, a microscope with camera coupled to a

computer. To date, few wing morphometric analyses of biting midges have been published for European (Muñoz-Muñoz et al., 2011, 2014, 2016, 2021; Hajd-Henni et al., 2014, 2015, 2021) and African (Oke et al., 2019) *Culicoides* fauna.

Fast and accurate identification of biting midges is crucial in the study of *Culicoides*-borne diseases, particularly for the main species of veterinary interest. This is an essential prerequisite for the implementation of vector control methods. The Madagascan *Culicoides* fauna was unraveled during 1960s and 1970s. The number of known species is 20 (Wirth and Hubert, 1959; Kremer and Brunhes, 1972; De Meillon, 1961; Callot et al., 1968; Glick, 1990; Sebastiani et al., 2001; Meiswinkel and Paweska, 2003; Gomulski et al., 2005; Augot et al., 2013, 2017). The estimated number of species in Madagascar is 71 (Garros et al., 2019).

Here, we report on WGM analysis for identification of species of veterinary important, "Moka Fohy" in Madagascar; i.e., the Schultzei group (*C. nevillei* and *C. enderleini*), Milnei group (*C. moreli* and *C. zuluensis*) and *Avaritia* subgenus (*C. dubitatus*, *C. kibatiensis*, *C. miombo*). However, during the mounting process and/or the conservation of slides, wings could be damaged. As a result, the entire wing is not always visible. Our approach relies on the relative position of points (landmarks used as anatomical points on the entire wing) and cells (outlines with closed contours where landmarks are lacking) (Dujardin, 2008; Dujardin et al., 2014), according to Hadj-Henni et al., 2023. For comparison, we also used a molecular approach, based on the cytochrome oxidase I gene (Cox1), for species delimitation.

2. Materials and method

The workflow of the entire process is shown in Fig. 1.

2.1. Collection of *Culicoides* and identification

This study was the continuation of work done by Augot et al., 2013 and 2017. Briefly, biting midges were captured in the village of Andasibe near the Special Reserve of Analamazaotra in the Alaotra-Mangoro

Table 1

Description of the sampling in our study and number of species wings analyzed for the geometric morphometric analysis. Lm: landmarks; Outlines-1 localized on apex on the wing and outlines-2 corresponding to cell m4 on wing.

Subgenus/group	Species	Genbank-accession number (COI)	Number of wings by categories		
			Landmarks	Outlines-1	Outlines-2
<i>Avaritia</i>	<i>C. dubitatus</i>	KY707796-98, OR458393-405	32	33	35
	<i>C. kibatiensis</i>	KY707781, KY707799, OR458414-16	21	20	21
	<i>C. miombo</i>	KY707800, OR458417-19	19	20	20
<i>Milnei</i>	<i>C. moreli</i>	KY707804, OR458420-39	23	23	23
	<i>C. zuluensis</i>	KY707802-03, OR458444-49	35	36	36
<i>Schultzei</i>	<i>C. enderleini</i>	KF186429-31, OR458406-13	26	25	26
	<i>C. nevillei</i>	KF186428, OR458440-43	18	18	18

Region, Fivondronana of Moramanga (18°55'412" S and 48°24'913" E; altitude: 858 m a.s.l.). *Culicoides* specimens were identified to the species level using the literature for the Afrotropical region (Cornet et al., 1974 and Cornet and Bruhnes, 1994; Glick, 1990; Khamala and Kettle, 1971). The specimens ($n = 179$; Table 1) are mounted individually on slides with head, wings and spermathecae on microscope slides in Chloral gum or Euparal. The rest of the body (thorax, legs and the beginning of the abdomen) was used for DNA extraction.

2.2. Acquisition and analysis of molecular data

2.2.1. DNA extraction and PCR amplification

DNA was extracted from individual *Culicoides* using a QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Cox1 was amplified using the primers LepF/LepR and C1J1718/C1N2191 as indicated by Augot et al., 2013, 2017. Direct sequencing of both DNA strands was performed with the primers used for PCR. Alignments and phylogenetic analysis were conducted with MEGA 7 (Kumar et al., 2016). The DNA sequence-based analyses were performed using i) the Neighbour-Joining (NJ) method (Kimura-2 parameter), and ii) the Maximum Likelihood (ML) method (Hasegawa-Kishino-Yano-Tamura model), and 1000 bootstrap replicates were used to test the reliability of the constructed tree. The tree was rooted using the sequence of *Culicoides nubeculosus* (KJ624102) as an outgroup (Augot et al., 2017). For comparison with our fresh specimens, Cox1 sequences of *C. dubitatus* (KY707798), *C. enderleini* (HQ447064–066, KF186429–431, KF682471–479, KF682526–528, KJ186131,

KJ186132, KJ833692–714, MF399706–708, MF399733, MF399752–754, MF399755, MF399770, MF399775, MF399776, MF399688, MF399689, MH339999, MH340000, MN329609–611, MN329647, MN329653, MN329655, MN329656, MN329661, MN329662), *C. kibatiensis* (HQ447074, KJ186137, KY707799, MF399789, MK760183, MK760186, MK760187), *C. miombo* (KF417706, KY707800, MF399747, MF399768, MF399778, MK760192, MK760195, MK760199, MK760200), *C. moreli* (KP025646, KY707804, MF399714–717, MF399774, MF399781), *C. nevillei* (KF186428, KF682496, KF682497, KP025642, KP025643, MF399686, MF399687, MF399736, MF399696), and *C. zuluensis* (KY707802, KY707803, MF399690, MF399691, MF399695, MF399769, MF399793, MF399794) were included in our molecular analyses – in bold our previous studies, see above. No sequences of *C. dubitatus* outside Madagascar are available on GenBank. *Culicoides dubitatus*, *C. brosetti* and *C. trifasciellus* are sibling species (Kremer et al., 1975). Only sequences of *C. sp # 20*, sister species of *C. trifasciellus* (Bakhoun et al., 2017), are published and incorporated in our study (MF399739–MF399744).

2.3. Acquisition and statistical analysis of landmark data

2.3.1. Wing preparation

For the geometric analysis, right wings of females were photographed using an Olympus BX53 microscope equipped with an Olympus SC100 camera, under 10 X magnification. The differential directional asymmetric effects between left or right wing have been estimated at 1%

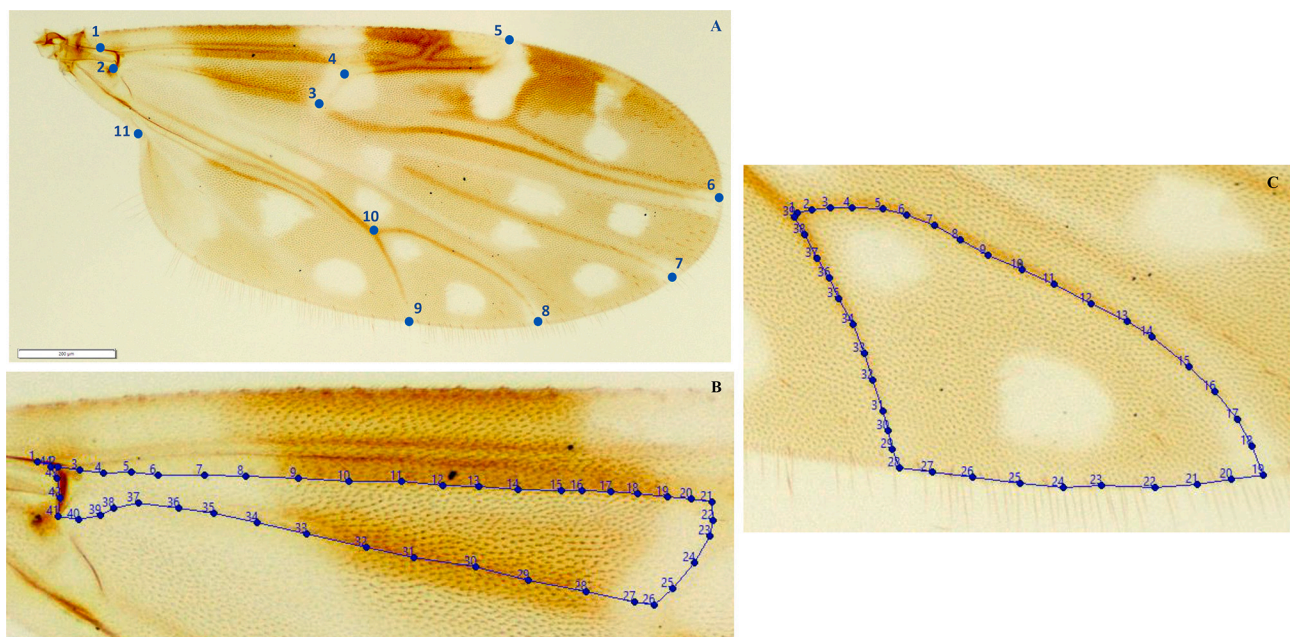


Fig. 2. Position of the 11 landmarks (A), outlines-1 (B) and outlines-2 (C) on *Culicoides* wings used for geometric morphometric analysis.

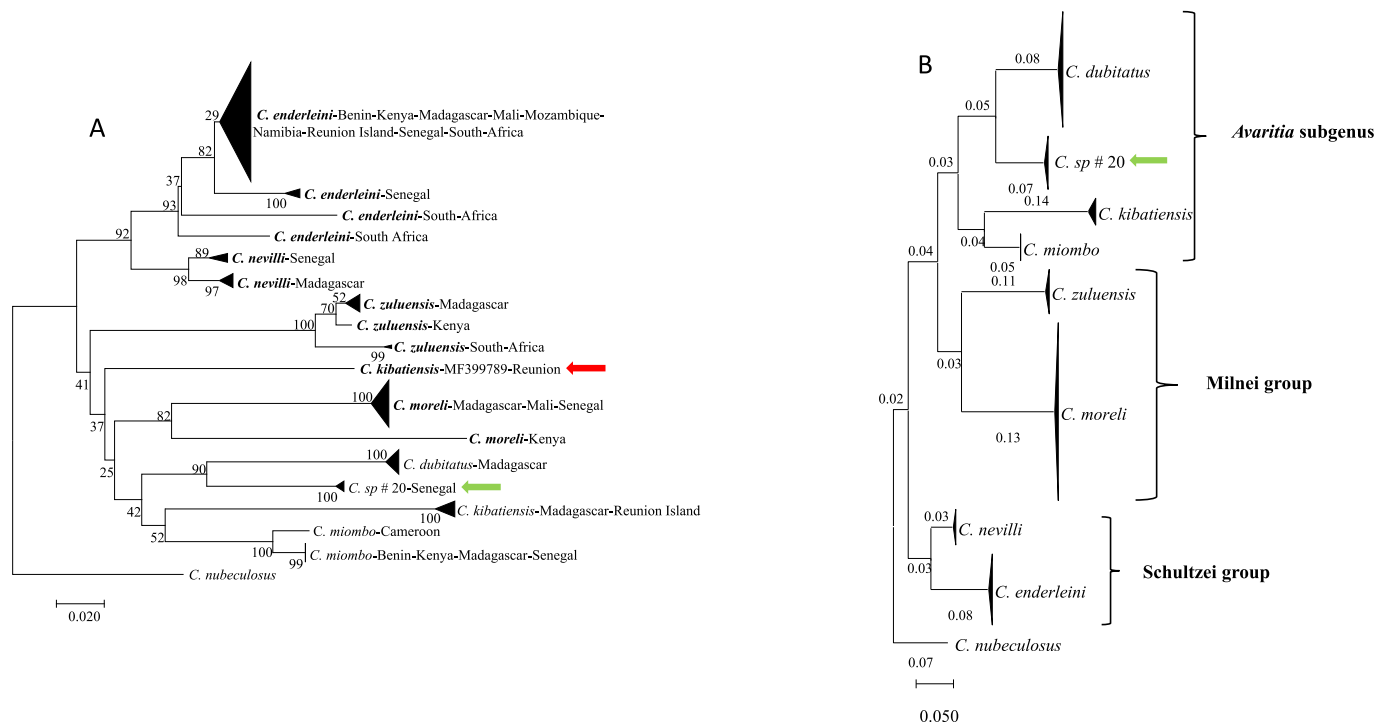


Fig. 3. Trees obtained from the analysis of seven Madagascar's *Culicoides* cytochrome oxidase I (COI) mitochondrial DNA using: i) the Neighbour-Joining method (A) on our samples and GenBank sequences and ii) the Maximum Likelihood on (B) on our samples and *C. sp # 20*. Bootstrap values are shown on nodes (1000 replicates).

or 2% of the interindividual variation (Klingenberg, 2015), which should not interfere with our comparisons based on one side of the biting midges. A total of 179 specimens were chosen for landmarks and outlines (Fig. 2). Additionally wings of the syntypes of *C. dubitatus* ($n = 4$) from Kremer et al. (1975) were included as a reference specimen. A total of 11 landmarks were selected, based on the ease with which they could be plotted across all *Culicoides* species (Hadj-Henni et al., 2014, 2015, 2021). We chose two outline sets (Fig. 2): the contour of the cell between the r-m cross vein, M, radiale and arculus (defined by landmarks 1, 2, 3 and 4), and the contour of cell m4 (defined by landmarks 8, 9 and 10), according to Hadj-Henni et al., 2023.

2.3.2. Morphometric analysis

Digitisation of anatomical landmarks and contours was performed using free CLIC software (ancestor of XYOM). Data analyses (characterisation and classification) were performed using XYOM online (<https://xyom.io>), according to Dujardin and Dujardin (2019).

The landmark configurations were scaled, translated and rotated against the consensus configuration using the GLS Procrustes superimposition method to remove all non-shape differences and to separate the size and shape components of the form (Rohlf and Slice, 1990; Bookstein, 1991). Wing size was estimated using the isometric estimator of the centroid size (CS), derived from data on coordinates (Bookstein, 1991). Statistical comparisons of the CS among the species were performed by one-way ANOVA and illustrated by colour quantile boxes. To evaluate the statistical significance of the one-way ANOVA, a non-parametric test (1000 runs) with Bonferroni correction was performed at p -values < 0.05 . To test the validity of global size (CS) for accurate species identification, we used a ML approach (Dujardin et al., 2017). The allometric effect (the effect of size on shape variation) was performed by linear regression of the first (shape derived) discriminant factor on the CS, and then estimated by determination of the coefficient r^2 (Chaiphongpachara et al., 2022). The wing shape variables were computed after Procrustes superimposition according to Generalised Procrustes Analysis (Rohlf, 1990). Their principal components were used as final shape variables. The visual comparison of shape changes

across species was provided by the superposition of the average wing of each species. The final wing shape variables (thus, excluding size) were used as input for discriminant analysis (DA), which were illustrated by the factor map. For statistical comparisons of wing shapes based on pairwise Mahalanobis distances between biting midge species, a non-parametric permutation test (1000 permutations) with Bonferroni correction was used at p -value < 0.05 . To estimate the potential of shape geometric morphometric (GM) analysis for species identification, the validated (cross-checked) classification test based on Mahalanobis distances (MD) was performed to verify the correct assignments. To illustrate morphological divergence between biting midge species, a hierarchical classification tree (Unweighted Pair Group Method with Arithmetic Mean algorithm – UPGMA) was built based on MD.

For outlines, three variables characterised the size: the square root area within the outlines, the perimeter, and the semi-major axis of the first ellipse. During the analyses, the half major axis of the first ellipse, which contained information about the perimeter, and the square root area of the contour (Dujardin and Dujardin, 2019) estimated the centroid size. Statistical comparison of size among groups was performed using a non-parametric ANOVA and illustrated by quantile boxes, giving the mean and the standard deviation of the global size. The non-parametric test was computed after 1000 permutations with Bonferroni correction at p -values < 0.05 . Finally, to test the validity of global size for accurate species identification, we used an ML approach. Allometry was evaluated using the linear determination coefficient after regressing the principal components of the normalised elliptic Fourier coefficients on the global size (Santillán-Guayasamín et al., 2017). For species discrimination based on shape, variables derived from the normalised elliptic Fourier coefficients were used for principal component analysis. After that, the results of principal components were used as the final shape variables. The shape divergence of each species was examined by (DA) on principal components of shape and illustrated by a factor map. The statistical significance of the corresponding MD was tested with non-parametric tests, using 1000 permutations and a Bonferroni correction with significance at p -values < 0.05 . Verification of the classification was performed on the shape employing MD. Cross-

Table 2Estimation of pairwise distance between species our Madagascar *Culicoides* specimens for the COI mtDNA domain.

	1	2	3	4	5	6	7	8
1. <i>C. dubitatus</i>		0.024	0.027	0.023	0.027	0.024	0.026	0.025
2. <i>C. enderleini</i>	0.205		0.026	0.024	0.027	0.018	0.024	0.023
3. <i>C. kibatiensis</i>	0.249	0.248		0.022	0.031	0.025	0.027	0.024
4. <i>C. miombo</i>	0.194	0.192	0.183		0.023	0.020	0.023	0.024
5. <i>C. moreli</i>	0.241	0.220	0.291	0.192		0.025	0.026	0.027
6. <i>C. nevillei</i>	0.209	0.124	0.228	0.152	0.205		0.025	0.020
7. <i>C. zuluensis</i>	0.234	0.205	0.257	0.198	0.228	0.224		0.024
8. <i>C. nubeculosus</i>	0.231	0.182	0.225	0.210	0.243	0.149	0.214	

Table 3

Mean wing centroid size (CS) of our samples.

Species	Landmarks				Outlines-1				Outlines-2			
	N	Mean (mm)	Variance	SD	N	Mean (mm)	Variance	SD	N	Mean (mm)	Variance	SD
<i>C. dubitatus</i>	32	1.0837	0.0041	0.0646	32	0.1371	0.0000	0.0097	35	0.1041	0.0000	0.0079
<i>C. enderleini</i>	26	1.2952	0.0047	0.0690	25	0.1544	0.0001	0.0107	26	0.1272	0.0000	0.0089
<i>C. kibatiensis</i>	21	1.5032	0.0063	0.0794	20	0.1964	0.0001	0.0116	21	0.1505	0.0001	0.0100
<i>C. miombo</i>	19	1.2657	0.0112	0.1062	20	0.1554	0.0001	0.0139	20	0.1170	0.0001	0.0120
<i>C. moreli</i>	23	1.6547	0.0097	0.0985	23	0.2140	0.0001	0.0139	23	0.1737	0.0001	0.0131
<i>C. nevillei</i>	18	1.2705	0.0045	0.0675	18	0.1524	0.0000	0.0093	18	0.1346	0.0000	0.0084
<i>C. zuluensis</i>	35	1.7205	0.0162	0.1273	35	0.2211	0.0003	0.0177	36	0.1819	0.0002	0.0170

N, number; SD, standard deviation.

validation classification over MD was performed with an ML approach and computed with 1000 permutations. To illustrate morphological divergence between biting midge species, a hierarchical classification tree (UPGMA) was built based on MD.

Finally, the mean validated reclassification scores our GM is interpreted following the benchmark scale proposed by Landis and Koch (1977): 41%–60%: Moderate, 61–80%: Substantial, and 81%–100%: Almost perfect.

3. Results

3.1. Molecular analysis

NJ was constructed, based on 386 bp, with *Culicoides* sequences obtained from GenBank and our data. ML tree was constructed with our sample and *C. sp # 20*. The membership of each sample in the various branches was strongly supported by bootstrap values (Fig. 3), with and without sequences from GenBank. The analysis also yielded moderate bootstrap support of 39% and 56% for clustering of the Schultzei and Milnei groups, respectively (Fig. 3). The species of *Avaritia* are clustered together (Fig. 3b). In contrast, with sequences in GenBank, the topology was somewhat similar (Fig. 3a). Our tree (Fig. 3a) shows several populations of *C. enderleini*, *C. nevillei* and *C. miombo*. The species of *Avaritia* (Fig. 3b) clustered in the same clade with two clades: on the one hand,

C. dubitatus and *C. sp # 20*, and on the other, *C. kibatiensis* with *C. miombo*. Surprisingly, a sequence of *C. kibatiensis* (MF-399789) did not cluster with other *C. kibatiensis* specimens.

The low intraspecific divergences are as follow: *C. dubitatus* (0.00), *C. enderleini* (0.00), *C. kibatiensis* (0.01), *C. miombo* (0.00), *C. moreli* (0.00), *C. nevillei* (0.00) and *C. zuluensis* (0.01). The interspecific K2P distances ranged from 0.124 to 0.257 (Table 2). Finally, the ML tree (Fig. 3c) shows three clades according to morphological characters. Lastly, *C. dubitatus* and *C. sp # 20* grouped together on the tree.

3.2. Classification on geometric morphometrics

A total of 179 wings were analysed (Table 1).

3.2.1. Size variation

Mean wing centroid size (CS) of our specimens ranged from 1.083 mm to 1.72 mm (Table 3) for landmarks; from 0.1371 mm to 0.2211 for outlines-1, and from 0.1041 to 0.1819 for outlines-2. The highest CS was observed for *C. zuluensis*, and the smallest for *C. dubitatus* (Fig. 4). Within three species groups, the CS only permitted us to separate species of subgenus *Avaritia* with landmarks (Table 4). In contrast, between species groups, CS permitted us to separate several species with three types of GM (Table 4; $p < 0.05$). However, validated classification based on ML among seven species revealed moderate results as follows: 46% for

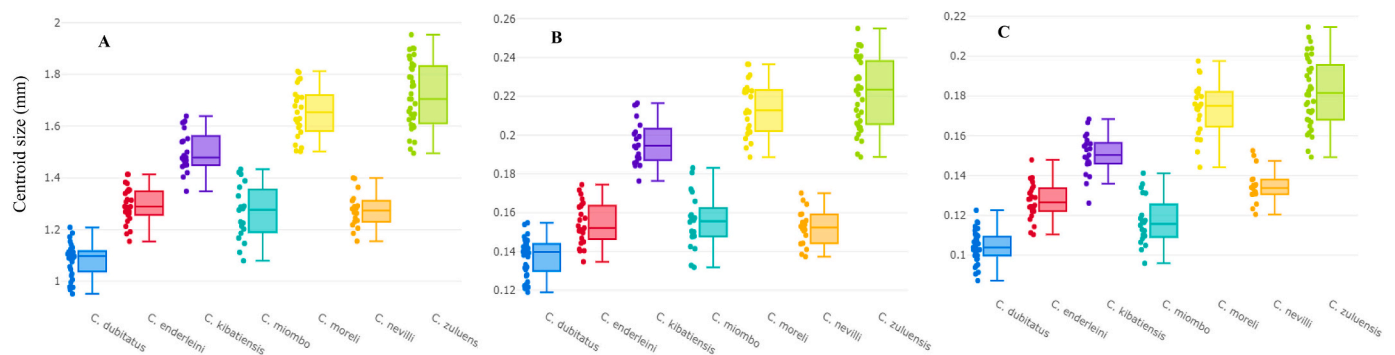


Fig. 4. Boxplot illustrating wing size (centroid size, CS) variation based on the landmarks (A), outlines-1 (B) and outlines-2 (C) sets of seven Madagascar's *Culicoides*. Expand: median, percentile and outliers.

Table 4
Non-parametric comparisons of global size estimations, 1000 permutations (*P*-values).

Choice of landmarks	Species	<i>C. dubitatus</i>	<i>C. enderleini</i>	<i>C. kibatiensis</i>	<i>C. miombo</i>	<i>C. moreli</i>	<i>C. nevilli</i>	<i>C. zuluensis</i>
Landmarks	<i>C. dubitatus</i>							
	<i>C. enderleini</i>	0.0010*						
	<i>C. kibatiensis</i>	0.0000*	0.0080*					
	<i>C. miombo</i>	0.0070*	0.6963	0.0010*				
	<i>C. moreli</i>	0.0000*	0.0000*	0.0450*	0.0000*			
	<i>C. nevilli</i>	0.0130*	0.8142	0.0020*	0.9221	0.000*		
	<i>C. zuluensis</i>	0.0000*	0.0000*	0.0000*	0.0000*	0.3287	0.0000*	
Outlines-1	<i>C. dubitatus</i>							
	<i>C. enderleini</i>	0.0579						
	<i>C. kibatiensis</i>	0.0000*	0.0000*					
	<i>C. miombo</i>	0.0719	0.9281	0.0000*				
	<i>C. moreli</i>	0.0000*	0.0000*	0.0939	0.0000*			
	<i>C. nevilli</i>	0.1399	0.8591	0.0000*	0.8022	0.0000*		
	<i>C. zuluensis</i>	0.0000*	0.0000*	0.0130*	0.0000*	0.4466	0.0000*	
Outlines-1	<i>C. dubitatus</i>							
	<i>C. enderleini</i>	0.0040*						
	<i>C. kibatiensis</i>	0.0000*	0.0000*					
	<i>C. miombo</i>	0.1489	0.2777	0.0000*				
	<i>C. moreli</i>	0.0000*	0.0000*	0.0130*	0.0000*			
	<i>C. nevilli</i>	0.0000*	0.4246	0.1149	0.0859	0.0000*		
	<i>C. zuluensis</i>	0.0000*	0.0000*	0.0000*	0.0000*	0.3307	0.0000*	

* : significatif at $p < 0.05$.

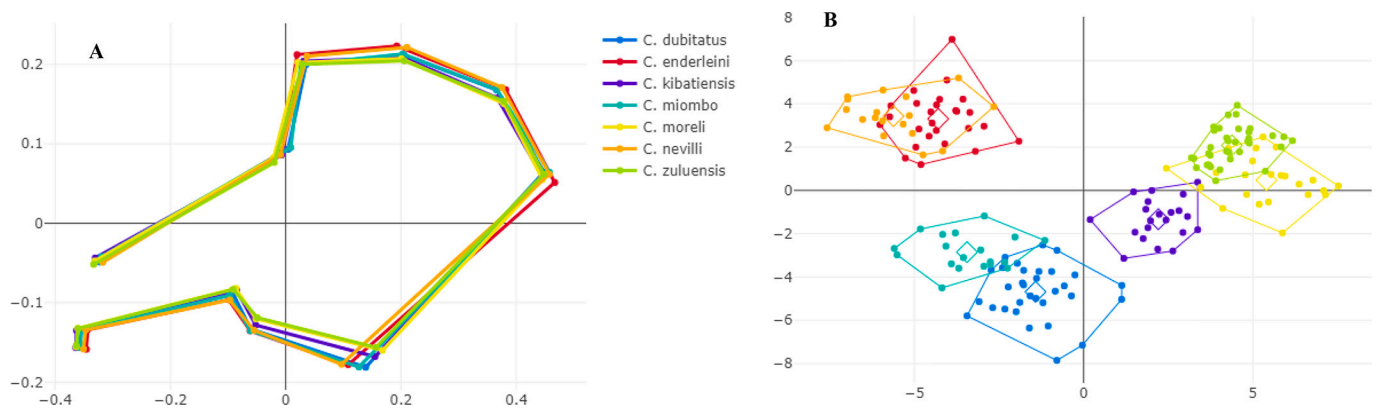


Fig. 5. Shape variation of *Culicoides* from Madagascar based on landmarks. Superposition of aligned mean configurations among the seven species (A). Factor map of the two discriminant factors (DFs) among seven species (B). Each point represents an individual. The horizontal axis is the first DF; the vertical axis is the second DF; their cumulated contributions reach 100% of the total variation.

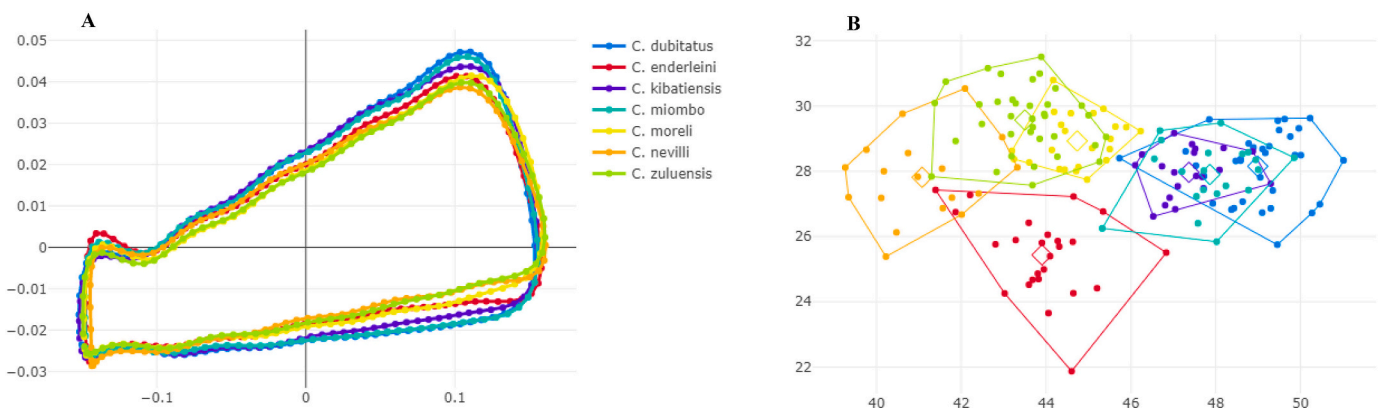


Fig. 6. Shape variation of *Culicoides* from Madagascar based on outlines-1. Superposition of aligned mean configurations among the seven species (A). Factor map of the two discriminant factors (DFs) among seven species (B). Each point represents an individual. The horizontal axis is the first DF; the vertical axis is the second DF; their cumulated contributions reach 100% of the total variation.

outlines-1, 49% for landmarks, and 54% for outlines-2.

3.2.2. Allometry

The first and second discriminant factors derived from the Procrustes residuals were still under the influence of size after regression on CS for

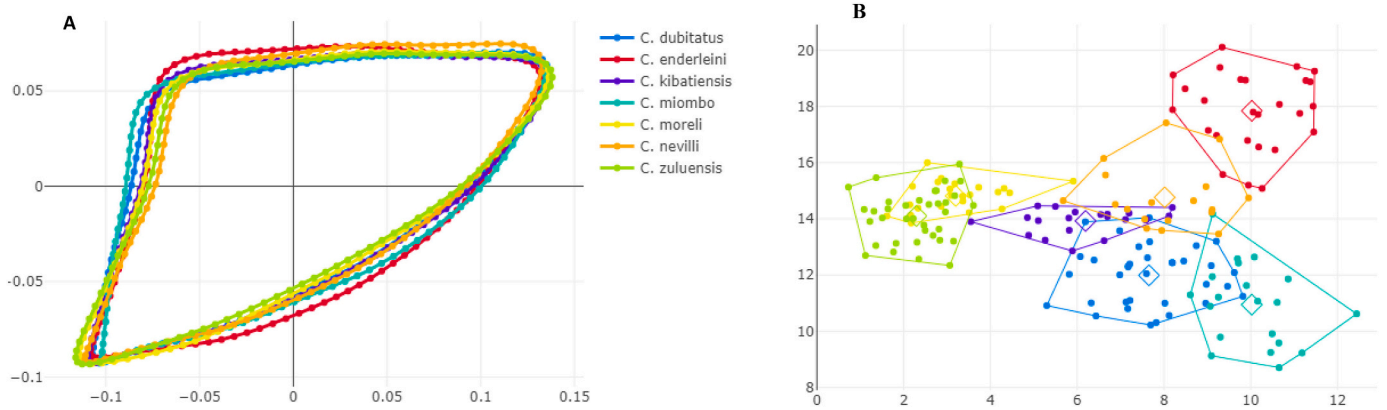


Fig. 7. Shape variation of *Culicoides* from Madagascar based on outlines-2. Superposition of aligned mean configurations among the seven species (A). Factor map of the two discriminant factors (DFs) among seven species (B). Each point represents an individual. The horizontal axis is the first DF; the vertical axis is the second DF; their cumulated contributions reach 100% of the total variation.

Table 5

Mahalanobis distances for landmarks, outlines-1 and outlines-2, with 1000 permutations (*P*-values).

Choice of landmarks	Species	<i>C. dubitatus</i>	<i>C. enderleini</i>	<i>C. kibatiensis</i>	<i>C. miombo</i>	<i>C. moreli</i>	<i>C. nevillei</i>	<i>C. zuluensis</i>
Landmarks	<i>C. dubitatus</i>	0						
	<i>C. enderleini</i>	9.238	0					
	<i>C. kibatiensis</i>	5.825	8.626	0				
	<i>C. miombo</i>	4.411	6.863	6.454	0			
	<i>C. moreli</i>	8.877	10.31	4.662	9.871	0		
	<i>C. nevillei</i>	9.609	6.468	10.15	8.404	12.37	0	
	<i>C. zuluensis</i>	9.053	9.669	5.03	9.66	3.708	10.49	0
Outlines-1	<i>C. dubitatus</i>	0						
	<i>C. enderleini</i>	5.944	0					
	<i>C. kibatiensis</i>	2.838	4.885	0				
	<i>C. miombo</i>	2.57	5.045	3.13	0			
	<i>C. moreli</i>	5.127	4.067	3.895	4.079	0		
	<i>C. nevillei</i>	8.1	4.613	6.714	7.267	5.114	0	
	<i>C. zuluensis</i>	5.794	4.216	4.814	5.076	2.916	3.924	0
Outlines-2	<i>C. dubitatus</i>	0						
	<i>C. enderleini</i>	6.484	0					
	<i>C. kibatiensis</i>	2.901	5.652	0				
	<i>C. miombo</i>	3.716	7.037	5.387	0			
	<i>C. moreli</i>	5.655	7.77	4.201	8.228	0		
	<i>C. nevillei</i>	4.545	5.513	4.44	5.994	6.403	0	
	<i>C. zuluensis</i>	6.114	8.679	4.236	8.488	3.249	6.709	0

* : significatif at $p < 0.05$

Table 6

Mean validated reclassification scores on landmarks and outlines approaches, of Madagascar species.

Species	Landmarks			Outlines-1			Outlines-2		
	n	Classification accuracy (%)	Level of strength	n	Classification accuracy (%)	Level of strength	n	Classification accuracy (%)	Level of strength
<i>C. dubitatus</i>	32 /32	100	Almost perfect	27/ 33	81.81	Almost perfect	27/ 35	77.14	Substantial
<i>C. enderleini</i>	25/ 26	96.15	Almost perfect	19/ 25	76	Substantial	23/ 26	88.46	Almost perfect
<i>C. kibatiensis</i>	19/ 21	90.47	Almost perfect	17/ 20	85	Almost perfect	17/ 21	80.95	Almost perfect
<i>C. miombo</i>	19/ 19	100	Almost perfect	12/ 20	60	Moderate	16/ 20	80	Substantial
<i>C. moreli</i>	21/ 23	91.30	Almost perfect	19/ 23	82.60	Almost perfect	21/ 23	91.30	Almost perfect
<i>C. nevillei</i>	18/ 18	100	Almost perfect	14/ 18	77.77	Substantial	16/ 18	88.88	Almost perfect
<i>C. zuluensis</i>	32/ 35	91.42	Almost perfect	30/ 36	83.33	Almost perfect	29/ 36	80.55	Almost perfect

landmarks ($r = 0.761$ (57.9%) and $r = 0.465$ (21.7%), for outlines-1 ($r = -0.340$ (11.6%) and $r = 0.450$ (20.3%)) and for outlines-2 ($r = -0.796$ (63.4%) and $r = 0.252$ (6.4%).

3.2.3. Shape variation

When superposing the mean wing shapes of Malgach's *Culicoides*, specific points are located according to the groups (Fig. 5a): landmarks 3

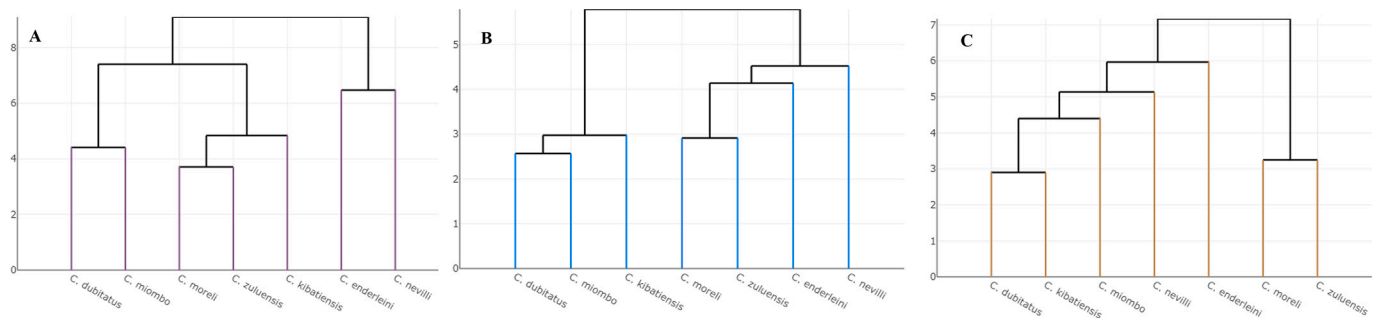


Fig. 8. Dendrogram trees based on Mahalanobis distances between seven *Culicoides* species from Madagascar: A) landmarks; B) outlines-1 located at the apex of the wing; C) outlines-2 located in cell m4 of the wing.

to 5 and 8 to 11 for the *Milnei* group; 1, 2, 5 to 9 for the *Schultzei* group and all landmarks for species of *Avaritia*. The examination of outlines-1 and outlines-2 of the seven species showed clear distinctions between groups, according to taxonomic classification (Fig. 6a, Fig. 7a).

Visualisation of the discriminant analysis (DA), based on shape divergence, is shown in Figs. 5b, 6b, and 7b. The landmarks-based analysis showed less overlapping than in the outlines analysis (Fig. 5b, 6b, and 7b). Despite some overlap, wing shape was significantly different among all seven species ($p < 0.05$, Table 5). The landmarks data allowed us to discriminate groups and species. The DA suggested a consistently better discrimination power of the landmark approach (Fig. 5b, 6b, and 7b). This was confirmed by the validated reclassification: higher correct assignment scores were obtained when performed on the landmarks than on the outlines-1 and outlines-2 data (Table 6).

The UPGMA trees based on Mahalanobis distances showed the proximity of wing shape for the two species in the *Moreli* group with the three GM approaches (Fig. 8a, b, and c). Phenotypic variability in wing shape indicated the closest relationship between the three species of *Avaritia* with outlines-1 and outlines-2 (Fig. 8b, c). In contrast, the position of the two species in the *Schultzei* group is not clear with outlines-1 and outlines-2 (Fig. 8b, c).

4. Discussion

This is the first geometric morphometric study on biting midges from Madagascar, here represented by species of veterinary importance: the *Milnei* group (*C. moreli* and *C. zuluensis*), *Schultzei* group (*C. enderleini* and *C. nevillei*) and subgenus *Avaritia* (*C. dubitatus*, *C. kibatiensis* and *C. miombo*).

DNA barcoding of the *Cox1* gene discriminated all morphologically determinate species (Fig. 2a). Our results reveal a low intraspecific level (ranging from 0% to 0.7%) and high interspecific genetic distance (ranging from 11.5% to 31.6%). DNA barcoding studies show an interspecific genetic distance ranging from 2.1% to 27.1%, and intraspecific distance ranging from 0% to 16.5% for *Culicoides* of America (Carvalho et al., 2022); from 13% to 31.3% and from 0% to 2% for *Culicoides* of Europe (Ander et al., 2013), and from 4.5% to 20.1% and from 0% to 10.7% for *Culicoides* of Africa (Bakhoun et al., 2018), respectively. A taxonomically universal threshold in the barcoding gap is impossible to define (Yassin et al., 2010) and the size of the barcoding gap strongly depends on taxonomic groups and practices (Candek and Kuntner, 2015). Interestingly, a reference *C. kibatiensis* sequence from GenBank (MF399789) did not group with the remaining *C. kibatiensis* sequences from this study (red arrow in Fig. 3) and is probably indicative of initial erroneous identification.

Our results indicate that these three groups were correctly distinguished by wing shape. When a lower hierarchical level (species) was analysed, WGM was also efficient, yielding high reclassification scores for most of the biting midges species analysed using landmark and outline approaches. Relationships between *C. moreli* and *C. zuluensis*

were well supported in our study. These species belong to the *Milnei* group (Cornet et al., 1974) and include 19 species (Borkent et al., 2022). Based on the wing morphological (Fig. 1), *C. moreli* presents two pale spots in Cua1 cell and tips of veins M1, M2, CuA1 are pale. *Culicoides zuluensis* does not have these morphological characters and owns a large pale spot in r-m crossvein and 1st costal cell. Similarly, relationships between *C. enderleini* and *C. nevillei* in the UPGMA tree were well supported. The *Schultzei* complex includes eight species with cryptic species (Cornet and Bruhnes, 1994). Regarding to wing morphological characters (Fig. 1), *C. nevillei* has a large pale spot in r-m crossvein. Tips CuA1 and CuA2 of *C. enderleini* are pale-bordered at the wing margin. Finally, *C. dubitatus*, *C. kibatiensis* and *C. miombo* were well separated; but the position of *C. kibatiensis* varied according to the WGM set. *Culicoides dubitatus* belong to the *Orientalis* complex (in Afrotropical region), while *C. miombo* belongs to the *Imicola* complex (in Afrotropical region) (Meiswinkel, 2004). The wing of *C. miombo* (Fig. 1) possesses a dark spot at an angle of anal cell and a pale spot rounded in the distal part of r3 cell. *Culicoides kibatiensis* presents a large pale spot including of the half part of the 2nd radial cell in r3 and 2 pale spots fused in distal part of anal cell. Moreover, 2 pale spots well defined are present in r3 without touch the wing margin. Finally, *C. dubitatus* has 2 pale spots in r3 and the area of pale costal spot is smaller than the dark costal spot or approximately equal. Previous studies emphasised a slightly higher scores with the outline technique than the other techniques to discriminate between groups (Dujardin et al., 2014; Kaba et al., 2017). In our study, landmarks present a high score compared with outlines-1 and outlines-2 (95.4% vs 78.9% vs 83.2%, respectively). Nevertheless, the reclassification score with outlines-2 is almost perfect for 5 species and substantial for 2 species (Table 6), according to the scale of Landis and Koch (1977). Outlines-1 shows a level of strength lower than outlines-2 (Table 6). Outlines-1 and outlines-2 allowed us to separate *C. circumscriptus* populations (Hadj-Henni et al., 2023). Therefore, the outlines technique can be applied in complement or as an alternative to the landmark-based approach, especially when the wing is damaged during the mounting process and/or the conservation of slides. Wing morphometrics was previously used by Oke et al. (2019), who were able to identify the species *C. enderleini*, *C. oxystoma*, *C. schultzei* and *C. subschultzei* using landmarks. Our study shows comparable scores with species of subgenera *Avaritia* and *Culicoides* from Europe obtained by the landmarks approach (Muñoz-Muñoz et al., 2011, 2014, 2016, 2021; Hajd-Henni et al., 2014, 2015, 2021).

In Madagascar, the number of known *Culicoides* species is 20 as follows: *C. africanus*, *C. bisolis*, *C. bolitinos*, *C. brosetti*, *C. distincipennis*, *C. dubitatus*, *C. enderleini*, *C. fulvithorax*, *C. grahamii*, *C. imicola* (= *C. pallidipennis*), *C. kibatiensis*, *C. madagascarensis*, *C. miombo*, *C. moreli*, *C. neavei*, *C. nevillei*, *C. rhizophorensis*, *C. schultzei*, *C. subflavesens*, and *C. zuluensis* (Wirth and Hubert, 1959; Kremer and Bruhnes, 1972; De Meillon, 1961; Callot et al., 1968; Glick, 1990; Sebastiani et al., 2001; Meiswinkel and Paweska, 2003; Gomulski et al., 2005; Augot et al., 2013, 2017). This list includes several sibling species: *C. miombo* and

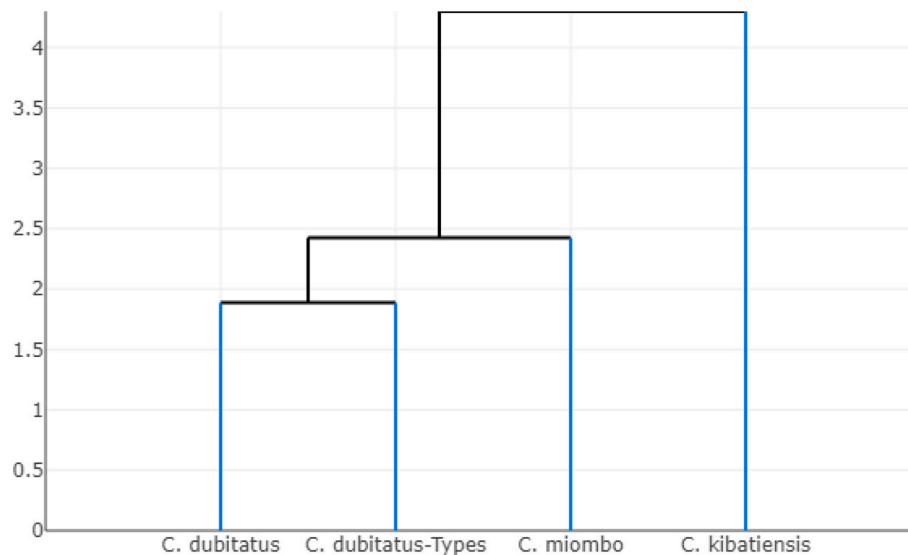


Fig. 9. Dendrogram trees based on Mahalanobis distances of landmark-based between *Culicoides dubitatus*, *C. dubitatus*-Types, *C. kibatiensis* and *C. miombo*.

C. imicola (Meiswinkel, 1991); *C. dubitatus*, *C. brosetti* and *C. trifasciellus* (Kremer et al., 1975). In Africa, a confused taxonomy is present between *C. brosetti*, *C. imicola* and *C. miombo* (Meiswinkel, 1991).

Based on fresh entomological studies, several *Culicoides* species have been collected in a neighbouring country of Madagascar: Mayotte (*C. bolitinos*, *C. distinctipennis*, *C. enderleini*, *C. imicola*, *C. kibatiensis*, *C. moreli*, *C. nevillei*, *C. rhizophorensis*, and *C. trifasciellus*) (Garros et al., 2019); Reunion Island (*C. bolitinos*, *C. enderleini*, *C. grahamii*, *C. imicola*, and *C. kibatiensis*) (Desvars et al., 2015; Grimaud et al., 2019). Moreover, *C. imicola* and *C. miombo* were collected in Madagascar (Mathieu et al., 2020). Finally, *C. zuluensis* has been reported in Ethiopia and South Africa (Riddin et al., 2019; Fetene et al., 2022).

A very confused taxonomy exists between *C. dubitatus*, *C. brosetti* and *C. trifasciellus*; these three species could be synonyms (Kremer et al., 1975). Currently, the three species are considered valid species (Borkent et al., 2022). Our colleagues, in their entomological inventories of Mayotte, reported only *C. trifasciellus* described in 1935 by Goetghebuer. Our *C. dubitatus* specimens were compared with type slides of Kremer et al. (1975). The CS are closed with a similar wing (Fig. 9). As a result, we consider that it is the same species with two populations (Angola and Madagascar). Interestingly, in our molecular analysis, *Culicoides* sp # 20 (green arrow in Fig. 3) captured in Senegal (Bakhoum et al., 2017) clustered on a sister branch of *C. dubitatus* (Fig. 3a and Fig. 3b). Moreover, relationships based on a mitochondrial gene (16 rDNA) and nuclear gene (28S rDNA) were strongly supported between *C. sp # 20* and *C. trifasciellus* (Bakhoum et al., 2017). So, *C. sp # 20* is a distinct species of *C. dubitatus*. Moreover, *C. sp # 20* is a distinct species of *C. trifasciellus*. Future investigations are needed to evaluate the species status of *C. dubitatus*, *C. trifasciellus* and *C. sp # 20* using WGM according to Hadj-Henni et al. (2015).

In conclusion, our results revealed that landmarks-based and outlines-1 and outlines-2 approaches can distinguish species within groups and could separate one group from another. The accuracy obtained by geometric wing morphometrics was comparable to the results of COI barcoding. Furthermore, the taxonomic confusion of sibling species (*Avaritia* subgenus) could be resolved by WGM and molecular approaches. WGM represents an effective technique, with a low-cost, to confirm species and to help future epidemiological investigations of vector-borne diseases in Madagascar.

Author contributions

LH-H, CM, and DA: conceptualisation. LH-H, CM, VL, and DA:

methodology, data curation, and writing-original draft preparation. DA: software. DA: validation and resources. LH-H, CM and DA: investigation and writing-review and editing. All authors contributed to the article and approved the submitted version.

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Data statement

Scientific data will be made available on request.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

All COI sequences of *C. dubitatus*, *C. enderleini*, *C. kibatiensis*, *C. miombo*, *C. moreli*, *C. nevillei* and *C. zuluensis* were submitted and available in the GenBank Database.

All scientific data will be made available on request.

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