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Wing morphology variations in *Culicoides circumscriptus* from France

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The biting midge *Culicoides circumscriptus* Kieffer, 1918 is a European widespread vector of avian malaria throughout the continent and is a possible vector of Akabane virus and Bluetongue virus. This species populates a wide range of environments in contrasting ecological settings often exposed to strong seasonal fluctuations. The main goals of this study were to investigate *C. circumscriptus* phenotypic variation at three departments in France (Corsica Island, Moselle and Var) and to determine if its phenotypes vary with the environment. *Culicoides circumscriptus* wing phenotypes were analyzed using a geometric morphometric approach based on anatomical landmarks and outlines of the wing. Dendogram trees based on landmarks and the outlines-2 set (cell m4) showed similar topologies and separated populations of *C. circumscriptus*. In contrast, another set of outlines-1 (covering the r-m cross vein, M, radiale and arculus) presented a different hierarchical clustering tree. The phenotypic variation observed in *C. circumscriptus* indicated that these populations are exposed to environmental and ecological pressures. Our results suggest the presence of phenotypic plasticity in this species.

KEYWORDS

geometric morphometrics, landmark, outline, *Culicoides*, wing, population

Highlights

- We applied a geometric morphometrics approach to *Culicoides* midge populations.
- This approach revealed phenotypic variation in *Culicoides circumscriptus.*
- Geometric morphometrics discriminates southern from northeastern French populations.
- Landmarks and outlines-covering the r-m cross vein, M, radiale and arculus; and cell m4 of wings gave similar results.

Introduction

Culicoides biting midges (Diptera: Ceratopogonidae) play a central role in the transmission of pathogens—including viruses, filarial nematodes, and protozoans—to humans, livestock, and wildlife (1, 2). In the past years, *Culicoides* species have been involved in the spread of three major arboviruses around the world: Bluetongue Virus (BTV), Schmallenberg Virus (SBV) and Oropouche Virus (ORV) (2, 3). In 2018, Yavru and others (4) detected BTV for the first time in field-collected

C. circumscriptus during an outbreak in Turkey. Recently, nucleic acid of Akabane virus and avian haemosporidian DNA have been detected in *C. circumscriptus, C. longipennis, C. schultzei* (5) and in *C. circumscriptus, C. impunctatus, C. kibunensis, C. paolae, C. pictipennis, C. punctatus* and *C. segnis* females (6, 7) respectively. Avian hemoprotozoa encompass different genera of blood parasites, including *Leucocytozoon, Haemoproteus* and *Plasmodium. Leucocytozoon caulleryi* and *Heamoproteus* spp. parasites are transmitted by *C. circumscriptus* (1, 8).

In Europe, *Culicoides* populates a wide range of environments in contrasting ecological settings often exposed to strong seasonal fluctuations.[1] The distribution, abundance and seasonal occurrence of biting midges is determined by the availability of moisture-rich habitats that are essential for the development of immature stage. The muds are associated with aquatic or semiaquatic habitats. The composition of these muds comes from animals and vegetal detritus (9–11). Most of *Culicoides* species include anautogenous adult females (9), requiring a blood meal to produce eggs. The frequency of feeding varies with species and meteorological conditions (12, 13); host availability plays an important role in the feeding behavior of biting midges in general. Currently, biology and ecology of *C. circumscriptus* remain poorly known. Previous studies based on mitochondrial markers indicated several populations of *C. circumscriptus* (14, 15). Natural populations of *C. circumscriptus* show morphological variability in antennal *sensilla coeloconia* (16) and wing patterns (17). *Culicoides circumscriptus* shows plasticity in the type of habitat occupied for larval development, and can include sand dunes, sewage channels (sites poor in oxygen), damp sites (without surface water), salt marshes, shady areas and most livestock farming areas (18–21). In France, *C. circumscriptus* is considered as a low abundance species (22). *Culicoides circumscriptus* is abundant on Corsica island, but not in other French regions (23). The optimum temperature for *C. circumscriptus* adults is 14°C (24). At a local scale, the distribution of vector species of pathogens can change according to environmental parameters and, in turn influence disease distribution. The question is whether there are any differences in terms of phenotypic or genetic features between southern and northern French populations of *C. circumscriptus*. Our study focuses on three departments in France: Corsica Island, Moselle (North-East) and Var (South-East). The trapping sites in the South of France (Corsica and Var) were set up near horse farms and facultative summer diapause occurs during the hot periods of the year. In Moselle site, insects were collected in salt marshes, which undergo large variations during the year, from flooding (during the winter and the spring) to drought (during the summer). During drought, no specimens can be caught (Augot et al., *comm. Pers.*). Under climate change, plastic responses involving diapause are often critical for population persistence, but key diapause responses under dry and hot conditions remain poorly understood. Thus, the beginning and the end of diapause may also play a role in the phenotypic differentiation observed in adults.

Wing geometric morphometrics (WGM) is a newly developed morphometric technique to investigate phenotypic variations (shapes and sizes) of organisms using the principles of geometry Dujardin, 2008 (25). WGM analyses can be conducted using landmarks, semi-landmarks or outline based methods (25, 26). The landmark-based approach used anatomical points (called "landmarks"); in general a small biological structure. These approaches compared the relative position of landmarks (size and shape) on several individuals. The outline-based is generally restricted to closed contours (called "outlines") where anatomical landmarks are lacking. The outline-based approach evaluated the size and the shape describing contours of forms (27). Insect wings are the most appropriate structures for geometric morphometric studies (28). WGM is largely developed in several vectors families like as Culicidae [see review of Lorentz et al. (29)] to explore intraspecific variations among mosquito populations or to research interspecific variation, to study in sexual dimorphism, plasticity and deviation, to detection of parasites and to characterize laboratory strain. This technique was used to study the intra specific variations in Glossinidae (30), Muscidae (31), Psychodiae (28, 32, 33), Reduviidae (34) and Tabanidae (35, 36) and to show inter specific variations in Muscidae (37). The landmark-based WGM analysis of *Culicoides* wings has proven to be a valuable tool for interspecific discrimination (38–42), *C. circumscriptus* intersexes specimens (43), sexual dimorphism (44) and geographic variations (45).

A better knowledge of *C. circumscriptus* is advisable because this species is involved in the transmission of pathogens. Here, we investigated the morphological variation of French populations on a quantitative basis. The main aim here is to assess the intraspecific phenotypic variability of *C. circumscriptus* at a population level using landmark and outlines based on WGM methods, and to evaluate the intrapopulation wing shape and size variabilities. More specifically, we compared the efficiency of anatomical landmarks and outlines of the wing to separate populations. This research will serve as a guideline for choosing the best WGM landmark set (s) for separating populations in the field. Moreover, for comparison, a molecular approach based on the DNA mitochondrial (mtDNA) cytochrome C oxidase I (Cox1) gene was used to distinguish between populations.

Materials and methods

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

Sample material

The three study sites were located in Porto-Vecchio (41°35′30″N; 9°16′49″E), Corsica (collected in July 2015), Marsal (48°47′24″N; 6°36′35″E), Moselle and Le Beausset (43°11′56″N; 5°48′12″E), Var (collected in June 2010; Figure 1; Table 1). In Var ($n = 24$) and Corsica ($n = 20$), insects were caught using UV light traps near horse farms; in Moselle ($n = 22$), specimens came from soil samples collected in a salt marsh (46) near cattle farms. Soil samples (water and underling soil) were collected haphazardly in salt marsh between March and June 2018 and 2019 according to *Culicoides* abundance (47). Soil samples placed into plastic buckets were stored in individual netted cage in the laboratory at 22°C (71.6°F). Tap water was added regularly to keep samples humid but not waterlogged (48). The adults that hatched rose toward the light and could thus be easily harvested with a mouth-operated aspirator or an Eppendorf tube. Emerging *Culicoides* were collected two or three times a week (48). Trapping and emerging adults were stored in 70% ethanol before mounted. All females were identified under the microscope according to morphological characters (49) and each individual specimen was mounted in Euparal® medium with head, wings and genitalia (50) (Figure 1).

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1 www.iikculicoides.net
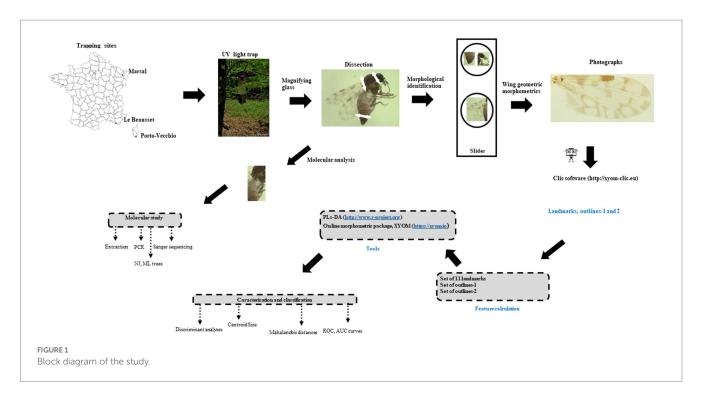
FIGURE 1
Block diagram of the study.

TABLE 1 Description of the sampling stations and number of *Culicoides circumscriptus* wings analyzed by site for the geometric morphometrics analysis.

Site	Coordinates		Biotope	Number of wings analyzed by category		
	North	East		Landmarks	Outlines-1	Outlines-2
Corsica	41°35′30″	9°16′49″	Rural area, near horse farms	20	20	20
Moselle	48°47′24″	6°36′35″	Salt marsh, rural area, near cattle farms	20	21	22
Var	43°11′56″	5°48′12″	Rural area, near horse farms	23	24	23

Acquisition and analysis of molecular data

DNA extraction, amplification and genotyping

Biting midge DNA was extracted from the thorax and legs (Figure 1) using the QIAmp DNA Mini kit (Qiagen, Germany) according to the manufacturer's recommendations (50). Polymerase chain reaction (PCR) amplification of the cytochrome oxidase subunit I (Cox1) gene region was conducted with the protocol published by Hadj-Henni et al. (41) using the primers C1J1718 (5′-GGAGGATTTGGAAATTGATTAGT-3′) and C1N2191 (5′-CAGGTAAAATTAAAATATAAACTTCTGG-3′) (51). The PCR products were visualized by gel electrophoresis in 1.5% agarose gel, stained with GelGreen (Biotium). All positive amplicons were Sanger sequenced (Genewiz, GmbH, https://www.genewiz.com).
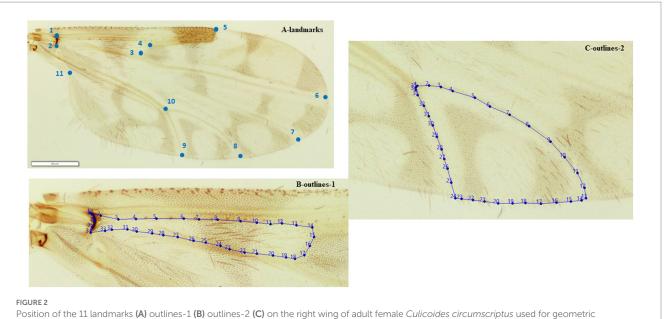
Phylogenetic analysis

Cleaned PCR products were sequenced by Genewiz, GmbH (www.GENEWIZ.com). Assembly of sequences were performed using the Pregap and Gap programs included in the Staden software package (52). Additionally, the Cox1 Genbank sequences of *C. circumscriptus* populations were also included in our molecular analyses (Supplementary Table 1). Alignments and phylogenetic analysis were conducted with MEGA 7 (53). Distance analysis was performed using the neighbor-joining (NJ) method (Kimura 2-parameter = K2P). Trees were constructed using the neighbor-joining (NJ) method (Kimura-2 parameter) and the maximum likelihood (ML) method (Hasegawa-Kishino-YanoTamura 3 model); 1,000 bootstrap replicates were used to test the robustness of the constructed trees. Trees were rooted using a sequence from *Culicoides nubeculosus* (KJ624102) as an outgroup (54).

Acquisition and statistical analysis of landmark data

Wing preparation

For the WGM analysis, the right wings from females were fixed on slides with Euparal® and flattened under cover slips (Figure 1). The differential directional asymmetric effects between left or right wing has been estimated at a 1% or 2% of the interindividual variation (55); which should not interfere with our comparisons based on one side of the biting midges. The wing samples were photographed using an Olympus BX53 microscope equipped with an Olympus SC100 camera, under 10 X magnification. A total of 66 specimens were chosen for plotting landmarks and outlines (Figure 2). A total of 11 landmarks were selected based on the ease with which they could

FIGURE 2
Position of the 11 landmarks **(A)** outlines-1 **(B)** outlines-2 **(C)** on the right wing of adult female *Culicoides circumscriptus* used for geometric morphometric analysis.

be plotted across all *Culicoides* species (39–41). We chose two outlines sets (Figure 2). The contour of the cell between the r-m cross vein, M, radiale and arculus (defined by the landmarks 1, 2, 3, and 4) and the contour of the cell m4 (defined by the landmarks 8, 9, and 10) were selected; for terminology, see (49, 56).

Morphometric analysis

Anatomical landmarks and outlines were plotted and data analyses and graphical outputs were performed using both the CLIC package and the recently available online morphometric package, XYOM[2] (57). The software aligned landmarks and outlines and then calculated the mean of the plotting. Landmarks were computed as orthogonal projections and compared together for every group. For outlines, an elliptic Fourier analysis was used to construct the shape variables, i.e., the normalized elliptic Fourier coefficients (NEF). Coordinates permit to characterize variables linked to the size and the shape separately.

For landmarks, wing size was estimated using the isometric estimator of the centroid size (CS) derived from data on coordinates (58). For outlines, three variables characterized the size: the square root area within the outlines, the perimeter, and the semi-major axis of the first ellipse. During the analyses, the centroid size was estimated by the half major axis of the first ellipse.

In both approaches, statistical comparisons of the CS among the species were performed by Oneway ANOVA and illustrated by quantile boxes. The CS difference was compared among species by a non-parametric test (1,000 runs) with Bonferroni correction at p-values <0.05. To test the validity of global size for accurate species identification, we used a maximum likelihood approach based validated reclassification approach (59). 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 the determination coefficient r^2 (36).

The visual comparison of shape changes across species was provided by the superposition of the average wing of each species. The generalized least squares Procrustes superimposition algorithm (60) was used to produce shape coordinates (partial warps), and the principal components (relative warps) (58) were used to compare samples (principal component and discriminant analyses). To assess the degree of similarity between biting midges, pairwise Mahalanobis distances between samples were calculated. To illustrate morphological divergence among populations, a hierarchical classification tree was built based on Mahalanobis distances.

Classification by machine learning

Principal component analysis (PCA) was used to explore the correlation between variables and machine-learning algorithms used to predict individual species based on variable values with the partial least squares discriminant analysis (PLS-DA) (41). Classical tools as ROC (receiver operating characteristic) curves, AUC (area under the curve), Kennard-Stone algorithm were used on our dataset to assess, optimize and predict the final models (41). Statistical analyses were performed using the R 3.6.0 Software (The R Foundation for Statistical Computing, http://www.r-project.org).

Results

Molecular analysis

Sequences obtained are available in GenBank under the following accession numbers: MW353288, MW353289, MW353291-96, MW353299-302, MW353304-08 and OQ711946-958.

ML and NJ trees were constructed, based on 401 bp, with and without *C. circumscriptus* sequences obtained from GenBank

2 https://xyom.io

(Figure 3). The trees showed the same topology: the Moselle specimens are separated from the Corsica and Var populations (Figures 3A,B). *Culicoides circumscriptus* populations from China, India and Switzerland clustered separately from another clade with *Culicoides* specimens from North Africa and Europe (Figure 3B).

The intraspecific K2P values for the three populations is as follow: for Corsica (0.020 ± 0.005), for Moselle (0.001) and for Var (0.014 ± 0.003). The pairwise distances between our samples ranged from 0.017 (±0.004) to 0.026 (±0.007). The distances between the other populations are given in Supplementary Table 1. *Culicoides circumscriptus* from Portugal present a high intraspecific variation (>0.19). Specimens are clustered separately from other populations on the ML tree (Figure 3B).

Classification on geometric morphometrics

Size variation

According to CS, the largest wing was found in a female from Moselle (landmark: 1.887 mm ± 0.096; outlines-1: 0.227 mm ± 0.012; outlines-2: 0.216 mm ± 0.000), whereas the smallest wing was found in Corsica (landmark: 1.363 mm ± 0.074; outlines-1: 0.161 mm ± 0.014; outlines-2: 0.148 mm ± 0.012; Figures 4A–C).

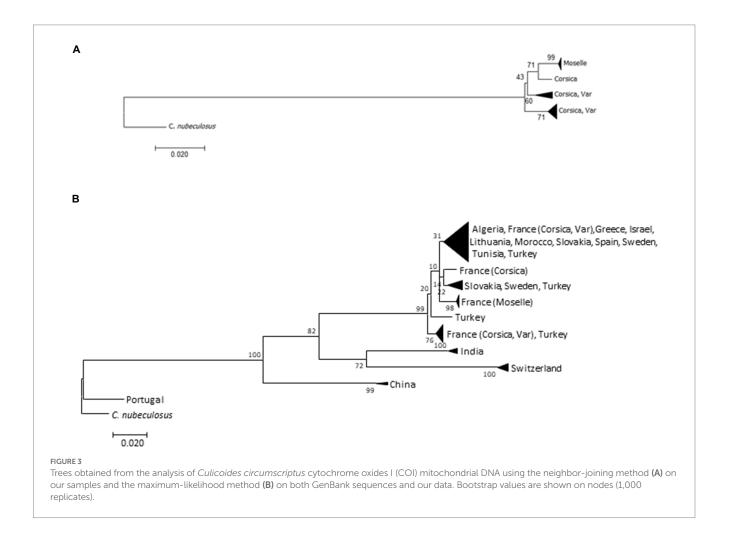The wing CS of *C. circumscriptus* differed significantly between all sites (Table 2) for landmarks and outlines-1 and outlines-2 ($p < 0.05$). The accuracy of the maximum likelihood validated size-based classification was very high for three landmarks types: 83% for landmarks (Corsica: 90%; Moselle: 85%; Var: 73.91%), 78% with outlines-1 (Corsica: 75%; Moselle: 90.47%; Var: 70.83%) and 82% for outlines-2 (Corsica: 75%; Moselle: 95.45%; Var: 73.91%).
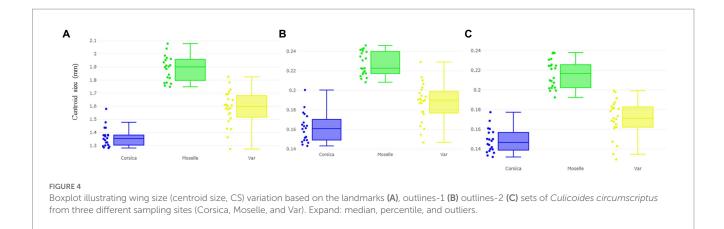
Allometry

The allometric effect of *C. circumscriptus* was very important. The first and second discriminant factors (DF) derived from the Procrustes residuals were still under the influence of size (70.1% and 0.8%, respectively) after regression on centroid size with landmarks, outlines-1 (44.9% and 7.3%, respectively) and for outlines-2 (52.9% and 0% respectively).

Shape variation

The visual comparisons of the mean anatomical landmark positions between populations revealed that the most visible landmark displacements were located in the upper and lower part of wing (landmarks 3, 4, 5, 7, 9, 8, 10, 11; Figure 5A). When superposing, the mean wing shape of *C. circumscriptus* between populations, the shape of Moselle appeared to be the most distinct one with outline-1 (Figure 6A) and to a lesser extent with outline-2 (Figure 7A). The discriminant analyses (DA) showed wing shape differentiation between sites and WGM approaches (Figures 5B, 6B, 7B). The DA

FIGURE 3
Trees obtained from the analysis of *Culicoides circumscriptus* cytochrome oxides I (COI) mitochondrial DNA using the neighbor-joining method (A) on our samples and the maximum-likelihood method (B) on both GenBank sequences and our data. Bootstrap values are shown on nodes (1,000 replicates).

FIGURE 4
Boxplot illustrating wing size (centroid size, CS) variation based on the landmarks **(A)**, outlines-1 **(B)** outlines-2 **(C)** sets of *Culicoides circumscriptus* from three different sampling sites (Corsica, Moselle, and Var). Expand: median, percentile, and outliers.

TABLE 2 Non-parametric comparisons of global size estimations, 1,000 permutations (*P*-values).

Choice of landmarks		Corsica	Moselle
Landmarks	Corsica		
	Moselle	0.000*	
	Var	0.000*	0.000*
Outlines-1	Corsica		
	Moselle	0.000*	
	Var	0.011*	0.000*
Outlines-2	Corsica		
	Moselle	0.000*	
	Var	0.034*	0.000*

*Significant ($p < 0.05$).

FIGURE 5
Shape variation of *Culicoides circumscriptus* based on landmarks. Superposition of the geometric morphometric set landmarks **(A)** in three geographically distant sites. Factor map of the two discriminant factors (DFs) among three sites **(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.

indicated that all populations of *C. circumscriptus* were faintly overlapped (Figures 5B, 6B, 7B).

The pairwise Mahalanobis distances based on shape, landmark and outlines-1 and outlines-2, were significant ($p < 0.05$, Table 3) between Corsica and Moselle. The shape is also significant different ($p < 0.05$, Table 3) between Moselle and Var with landmarks.

Accordingly, cross-validated classification scores of the Mahalanobis distances ranged from 26 to 85% and were highest in

Moselle for landmarks (85%; Table 3). The total performance scores were 57.14%, 52.31%, and 41.54% for landmarks, outlines-1 and outlines-2, respectively.

Dendogram trees based on Mahalanobis distances between female specimens, computed from shape variables, separated the three populations with two different topologies (Figure 8): one in which Corsica and Var populations were grouped on the same clade, separated from Moselle (landmarks and outlines-2), and one in which
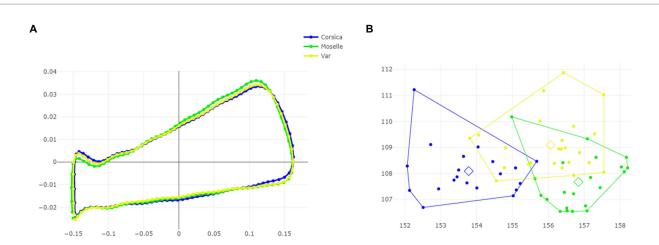
FIGURE 6
Shape variation of *Culicoides circumscriptus* based on outlines-1. Superposition of the geometric morphometric set landmarks **(A)** in three geographically distant sites. Factor map of the two discriminant factors (DFs) among three sites **(B)**. Each point represents an individual. The horizontal axis is the first DF; the vertical axis is the second DF; their cumulated contributions reach 100% of the total variation.

FIGURE 7
Shape variation of *Culicoides circumscriptus* based on outlines-2. Superposition of the geometric morphometric set landmarks **(A)** in three geographically distant sites. Factor map of the two discriminant factors (DFs) among three sites **(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.
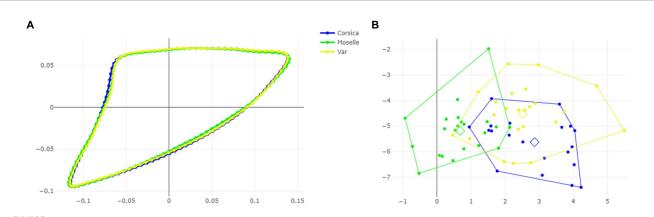
TABLE 3 Mean validated reclassification scores of *Culicoides circumscriptus* populations from three French sites according to geometric morphometrics (landmarks, outlines, Mahalanobis distances for landmarks, outlines-1 and outlines-2).

Geometric morphometrics set	Sites	*n*	Classification accuracy (%)	Mahalanobis distances	
				Corsica	Moselle
Landmarks	Corsica	10/20	50		
	Moselle	17/20	85	0*	
	Var	9/23	39.13	0.117	0*
Outlines-1	Corsica	12/20	60		
	Moselle	15/21	71.42	0*	
	Var	7/24	29.16	0.003*	0.287
Outlines-2	Corsica	6/20	30		
	Moselle	15/22	68.18	0.018*	
	Var	6/23	26.08	0.867	0.064

*Significant ($p < 0.05$).

Var and Moselle populations were grouped together, separated from Corsica (outlines-1).

Classification by machine learning on geometric morphometrics

We performed, with the PLS-DA classifier, a PCA on landmarks and outlines (Figure 9). The first two axes accounted for 44% and 30% of the variance for landmarks, 33% and 28% for outlines-1, 31% and 27% for outlines-2.

The tuning step of the number of components to select showed that 5 components were necessary to lower the balanced error rate with landmarks and outlines (-1 and-2; Supplementary Figure 1). For landmarks, the AUC values were 0.9802 ($p = 1.073$ e^{-09}) for Corsica, 0.9977 ($p = 2.621$ e^{-10}) for Moselle, and 0.9109 ($p = 6.801$ e^{-08}) for Var. For outlines-1, the AUC values were 0.9533 ($p = 6.668$ e^{-09}) for Corsica, 0.9903 ($p = 2.091$ e^{-10}) for Moselle, 0.9228 ($p = 1.561$ e^{-08}) for Var; for outlines-2, 0.9633 ($p = 3.085$ e^{-09}) for Corsica, 0.9958 ($p = 7.926$ e^{-11}) for Moselle, 0.9017 ($p = 1.020$ e^{-07}) for Var. A perfect AUC of 1.0 (Table 4) was obtained for Moselle and Corsica (for outlines-1).
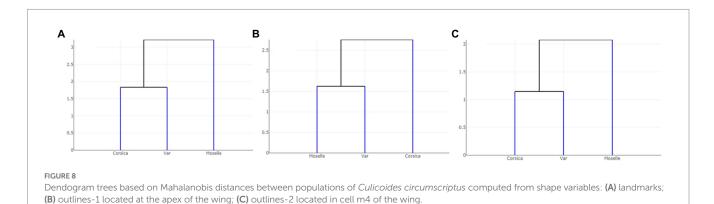
Discussion

This is the first WGM study on biting midges from France, here represented by three mainland and island geographic locations of *C. circumscriptus*.

By comparison of the wing based on results of the three GM methods, we found that they have the same appearance patterns for landmarks and outlines-2 (Figure 8). The outlines-1 set presented a different hierarchical clustering tree. Corresponding to the previous researches, the utilization of landmarks, semi-landmarks and outlines based on WGM show similar scores for separating species, including closely related or cryptic species (27, 61, 62). These morphometric approaches are an option to use for the species identification in studies on arthropods. The outlines-2 (cell m4) set has the advantage of being an easily recognized cell and only three landmarks delimit the contour. Moreover, the cell m4 is readily visible under a stereomicroscope and can be used directly on captured images for entomological surveillance, without mounted slides preparation. Future investigations are needed to evaluate outlines as a tool for discriminating among *Culicoides* species.

Our results show that the larger-winged population is distributed in Moselle. *Culicoides circumscriptus* exhibits changes in wing size (CS) across environments in France's departments (Figure 4; Table 1). In insects, the wing size difference (without excluding genetic differences) is probably influenced by environmental factors (25, 63) such as temperature, relative moisture and food availability (64–66). Our results clearly show that size can be used to separate *C. circumscriptus* populations. Villard et al. (47) suggested that the *Culicoides* life-cycle depends on climatic conditions; e.g., temperature (67) but not the photoperiod (68). The larvae stages play an important part of survive of *Culicoides* during the bad climatic conditions (larval in diapause or quiescence) (68). The large wings may be favorable for finding mates, food sources and adapting to specific environments (69). *Culicoides* species are generally smaller in warm climates and larger at higher altitudes (in colder environments) according to

FIGURE 8
Dendogram trees based on Mahalanobis distances between populations of *Culicoides circumscriptus* computed from shape variables: **(A)** landmarks; **(B)** outlines-1 located at the apex of the wing; **(C)** outlines-2 located in cell m4 of the wing.

FIGURE 9
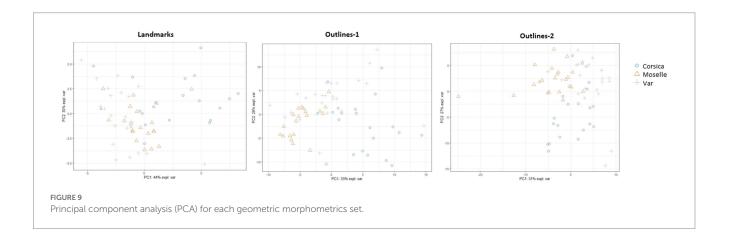Principal component analysis (PCA) for each geometric morphometrics set.

TABLE 4 Mean validated reclassification scores of *Culicoides circumscriptus* populations from three French sites according to classifier used (Partial Least squares discriminant—PLSDA).

Geometric morphometrics set	Sites	AUC (correctly classified rate)	*P*-value
Landmarks	Corsica	0.9965	$2.883e^{-10}$
	Moselle	1.0000	$2.164e^{-10}$
	Var	0.9467	$4.425e^{-09}$
Outlines-1	Corsica	1.0000	$1.595e^{-10}$
	Moselle	1.0000	$9.127e^{-11}$
	Var	0.9929	$4.319e^{-11}$
Outlines-2	Corsica	0.9989	$1.750e^{-10}$
	Moselle	1.0000	$5.474e^{-11}$
	Var	0.9917	$7.189e^{-11}$

P-value for landmarks, outlines-1 and outlines-2.

Bergmann's rules (70). The phenomics field (71), consists of acquiring high-dimensional phenotypic data on an organism-wide scale, and can be applied to *C. circumscriptus* using WGM.

Regarding wing shape variation, the divergence observed in Moselle site has two possible origins: the environmental effects, or the genetic drift due to geographic isolation. Moreover, the discriminant space base on shape was still affected by size variation (allometric). The influence is due mainly to the presence of a large population (Moselle, Figure 4) and it did not necessarily mean that shape variation was under the influence of environmental factors (25). Our results indicate that changes of wing shape between distant conspecific populations of *C. circumscriptus* are not a result of size variation and suggest that genetic differences may arise as species-specific adaptation to particular environments. An argument supporting that hypothesis in our sample is that, despite of significant WGM between geographic locations, there was molecular divergence between populations (Figure 3). Significantly, based on Cox1, the barcode gap between populations from Moselle and Corsica/Var is >2.5%; generally, a 2% gap is used to separate one species from the next (72). In contrast, Corsica and Var populations are grouped in different branch in our tree (Figure 3). The mtDNA is widely used for the molecular identification of species and to study their genetic diversity, including to population genetics (73). Few studies have investigated *Culicoides* sequences of Cox1 in several areas (14, 15, 74, 75). The intraspecific mtDNA genetic distances could vary considerably among species (72, 76–80) and it is difficult to give a DNA barcode distance threshold to species delimitation. Nevertheless, a high value of intraspecific distance could be indicative of the early stages of speciation. Our results show different *C. circumscriptus* populations according to morphological observations (16, 81) and molecular investigations (14, 15). Because environmental conditions in the Mediterranean Basin are comparable in terms of moisture and temperature, this similarity may account for the morphological and genetic similarity of populations captured in Var and Corsica, and the differences observed in the Moselle site.

A potential bias affecting our study could be the different collection procedures for the three populations: directly at the adult stages in Corsica and Var, after emergence in our laboratory in Moselle. Few studies have compared the phenotype between specimens collected in the field and laboratory. In mosquito, wings of wild strain of *Aedes albopictus* were significantly larger than those of the laboratory strain (82). In contrast, no information was reported between *Culex quinquefasciatus* specimens from a wild and laboratory (83). Finally, Morales et al. (65) emphasized the importance of the emerged period of *Aedes aegypti* eggs as a critical time for the size of future adults. Wild and colony fourth instar larvae (L4) of *C. sonorensis* differed in many standard metrics such as head length or width, but head ratios and pharyngeal armature measurements were comparable (84). Wild L4 appears longer than the colony. Moreover, under laboratory conditions, larval stage of *C. insignis* duration is ranged from 15.4 to 29.0 days and pupal stage ranged from 2.6 to 3.2 days (85). In our case, we have collected muds with pupae or L4 larvae stages. The emergence of the adults has been fast after the collect (<7 days) with a size bigger than biting midges collected using UV traps. Therefore, our specimens maintained during a short time in the lab, present the same parameters than wild specimens.

In conclusion, we demonstrated morphological variability in *C. circumscriptus* wing shape and size of specimens collected in southern and northeastern France. Our results support the use of WGM; landmarks and outlines-1 (covering the r-m cross vein, M, radiale and arculus) and outlines-2 (cell m4); for the morphological discrimination of populations. Differences in wing size and shape corresponded to differences in abiotic factors, and likely reflect adaptation to the environment and may furthermore affect the potential to act as vectors of disease. However, further studies on morphological differences are required to compare biting midges from different environments using standardized samples and to explore vector-borne disease transmission.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

LH-H, CM, and DA: conceptualization. LH-H, CM, MC, VL, and DA: methodology, data curation, and writing-original draft preparation. ZD and DA: software. DA: validation and resources. LH-H, CM, ZD, and DA: investigation and writing-review and

editing. All authors contributed to the article and approved the submitted version.

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Conflict of 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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1089772/full#supplementary-material

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