ACAROLOGY

Morphological characterization of ticks of the genus *Rhipicephalus* (*Boophilus*) based on geometric morphometry: the case of the Savannah District in Côte d'Ivoire

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Abstract

The introduction of the invasive tick *Rhipicephalus (Boophilus) microplus* in Côte d'Ivoire has led to a change in the population of ticks of the genus *Rhipicephalus (Boophilus)*. Thus, this study aims to characterize morphologically the tick species of the genus *Rhipicephalus (B.)* by geometric morphometry. It consisted of collecting all ticks from cattle in 74 farms in the Savanes District. The collected ticks were identified in the laboratory and, after identification, only non-gorged adult ticks of the genus *Rhipicephalus (B.)* were retained for morphometric characterisation. A total of 394 ticks were examined under a digital microscope, images were digitised using the online program XYOM and the data were analysed. This study revealed that males of each species have a smaller average size than females. It also showed a variation in mean size in all species except for the females of *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) geigyi* whose mean sizes did not differ. Geometric morphometry allowed the separation of *Rhipicephalus (B.) annulatus* males from other males. In females, *Rhipicephalus (B.) geigyi* and *Rhipicephalus (B.) microplus* were distinguished from each other and from other females.

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Introduction

The structuring of populations of different living organisms is an important factor in the evolution of genetic diversity within and between species (Conner and Hartl, 2004). The study of organism structuring is important for the proper management and control of these organisms. Many tools have been used to morphologically evaluate the structure of populations of living organisms. These tools include morphological (phenotypic) and molecular markers (Smýkal *et al*., 2008). Over the last two decades, a significant development of molecular markers such as microsatellites and SNPs (single-nucleotide polymorphism) has been observed, with their highly significant contributions in terms of population characterization (Avise, 2012). These markers are often expensive and require a long time to develop, which makes them difficult to use in routine (Schlötterer, 2004). The recent development of geometric morphometry tools may provide a cheaper alternative for studying population structure (Francoy *et al.*, 2011). It has been recognised that morpho-geometric variations can reveal genetic differences in organisms. Thus, geometric morphometry studies have made it possible to characterise several organisms (Diaha-Kouame *et al*., 2017; Bopo *et al*., 2018). In this context, tick species of the genus *Rhipicephalus (Boophilus)* present in cattle farms in northern Côte d'Ivoire constitute a very interesting model for the study of structuring using geometric morphometry.

The tick species of the genus *Rhipicephalus (B.)* that infest cattle in West Africa are *Rhipicephalus (B.) decoloratus*, *Rhipicephalus (B.) geigyi*, *Rhipicephalus (B.) annulatus*, and *Rhipicephalus (B.) microplus*. Among these species, the exotic tick *Rhipicephalus (B.) microplus* has expanded throughout West Africa, most recently in central Burkina Faso (Compaore *et al*., 2022). In Côte d'Ivoire, tick species of the genus *Rhipicephalus (B.)* are proliferating not only in the south but also in the north, which is the cattle-breeding area (Toure *et al*., 2014). In addition to the various cattle deaths and decreases in milk production recorded on farms, tick species of the genus *Rhipicephalus (B.)* are increasingly developing resistance to the usual acaricides (Achi *et al*., 2022). Also, it has been observed, a replacement of 96% of the autochthonous species of the genus *Rhipicephalus (B.)* (*Rhipicephalus (B.) decoloratus, Rhipicephalus (B.) geigyi* and *Rhipicephalus (B.) annulatus*) by the exotic tick *Rhipicephalus (B.) microplus* (Boka *et al*., 2017; Madder *et al.*, 2011). In addition, in a personal communication, Madder *et al.* (2012) stated that there are hybrids from crosses of *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) annulatus* and also of *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) decoloratus* in West Africa. Thus, there is a real problem of morphological structuring of the species of this genus. This study therefore aims to validate the application of geometric morphometry as a relevant tool for the morphological characterisation of tick species of the genus *Rhipicephalus (B.)*.

Materials and Methods

Study area

The study took place in the Savannah District located in the north of Côte d'Ivoire between 8° and 11° north latitude and between 4° and 7° south longitude. It is bordered to the north by the Republics of Mali and Burkina Faso, to the south by the Béré, Hambol and Worodougou Regions, to the east by the Boukani Region and to the west by the Kabadougou and Folon Regions. The climate is tropical Sudanian, marked by two alternating seasons: a dry season (November to April) and a rainy season (May to October). Rainfall varies between 1,000 and 1,200 mm per year and temperatures between 16 and 36°C (Le Guen, 2004). The vegetation is mainly composed of grassy and shrubby savannah.

Sampling and identification of ticks

Tick collections were carried out on cattle from August to December 2021, during the end of the rainy season and the beginning of the dry season. Cattle from 74 traditional farms were collected (Figure 1). In each selected pen of cattle, 15 cattle were held for 5 to 7 minutes. Ticks were collected from each animal and stored in 50 ml jars containing 70° ethanol. The geographical coordinates of the collection sites were recorded using a Global Positioning System (GPS) and the number assigned to the cattle, the name of the locality and the date of collection were recorded on each jar. Morphological identification of tick genera and species was done using the dichotomous identification keys of Arthur (1957), Bouattour (2002), Walker *et al.* (2003), by examination of specimens under a binocular magnifying glass of the brand Motic Digital microscope at 40× magnification, and with a digital microscope (model USB PCE-MM200), at 40× and 60× magnification connected to a laptop. For the present study, only tick species belonging to the genus *Rhipicephalus (B.)* were considered for further work.

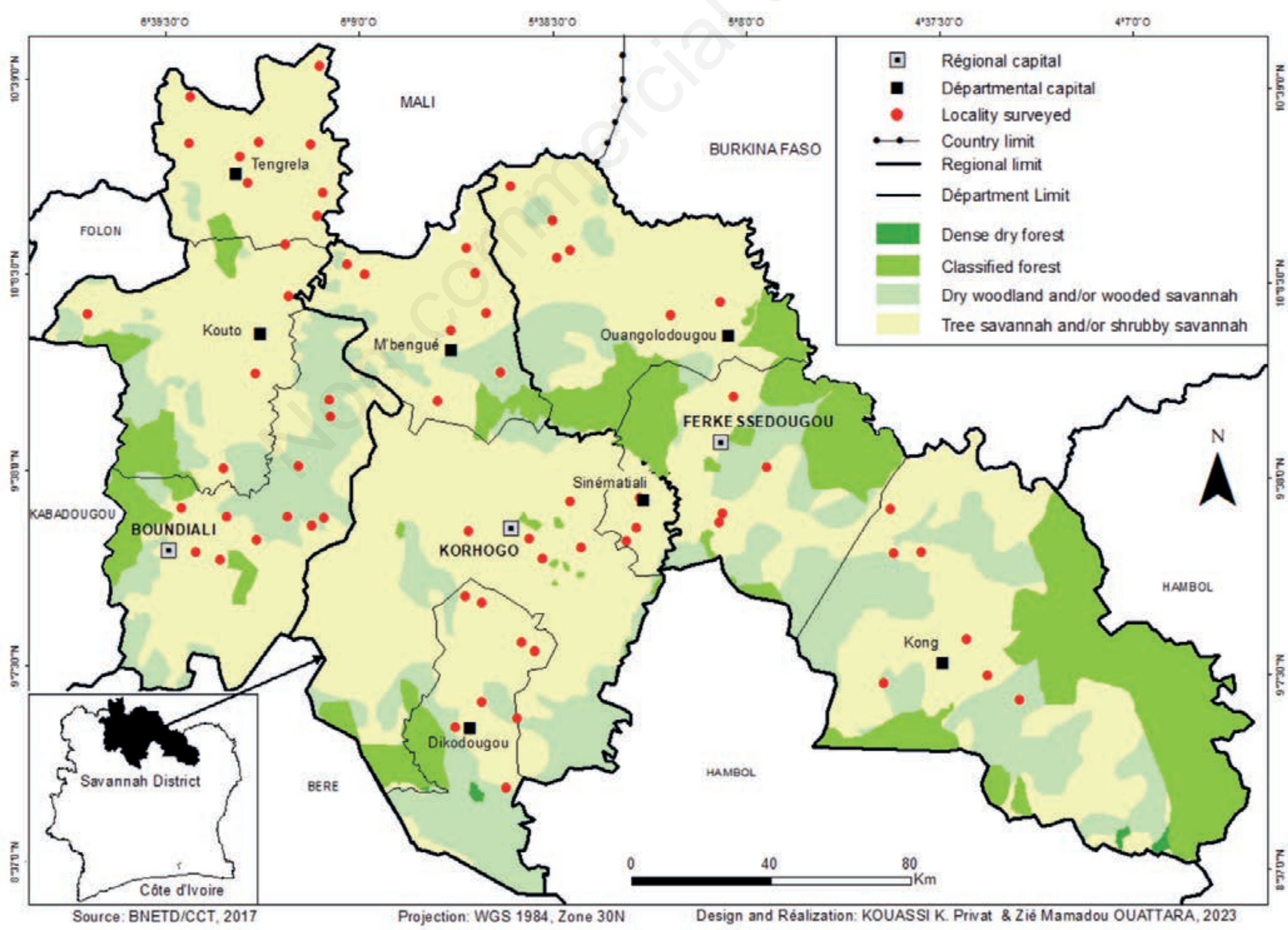

Figure 1. Map of the Savannah District showing tick collection sites.

Geometric morphometry analysis

Selection of specimens

This study was carried out on adult individuals, males and non-engorged females because the engorgement state can influence the morphological variation of ticks. Thus, a total of 355 individuals were considered, including: 58 males and 44 females of *Rhipicephalus (B.) microplus,* 59 males and 52 females of *Rhipicephalus (B.) decoloratus,* 30 males and 60 females of *Rhipicephalus (B.) annulatus* and 52 females of *Rhipicephalus (B.) geigyi*. No males of *Rhipicephalus (B.) geigyi* were identified during this study.

Mounting and scanning of ticks

Each tick specimen was positioned on graph paper for scanning. All individuals were scanned with the same resolution, 1600 dpi at the dimensions (0.30 cm length and 0.25 cm width) (Diaha-Kouame, 2017). Ticks were numerized using a digital microscope (PCE-MM 200) connected to a computer at the same scale and magnification (X 100). The scanning was carried out by the same operator in order to reduce any errors in the resolution of the tick images. (Kaba, 2014; Muñoz-Muñoz and Perpiñan, 2010). The image of each tick was saved in JPEG image file format.

Digitalisation

The method used for digitisation is the outline method. Outline point analysis is considered to be the first technique used in geometric morphometry. This method consisted of positioning more or less regular points around the whole tick and always in the same order around the specimens (Figure 2). Each point placed automatically generated x and y coordinates (Dujardin *et al*., 2010). These coordinates were converted into millimetres (mm). The connection of all the contour points forms a polygon that allows the shape variables to be obtained after superimposing each individual configuration. The digitization of the contour points was carried out on each of the digitized tick images by one and the same operator using the online program XYOM (Dujardin and Dujardin 2019).

Contour-based morphometry

The tick contour method was analyzed in order to separate the size variable from the shape. The coordinates of the pseudo-landmarks (outlines) were subjected to elliptic Fourier analysis (EFA) (Kuhl and Giardina, 1982). This method has been applied to arthropods with a low number of anatomical landmarks and also for the first time to tsetse fly pupae (Dujardin *et al.,* 2014; Dupaz *et al.,* 2016; Ta *et al.,* 2021). Fourier transforms allow to describe a periodic function by decomposing it into an infinite series of trigonometric functions of decreasing wavelength called harmonics defined by sine and cosine terms, themselves weighted by coefficients a_n and b_n (the Fourier coefficients). When the number of points around (harmonic) an object is high, this allows the contour of the object to be accurately described (Crampton, 1995; Ferson *et al.,* 1985). The size variables could be the perimeter of the contour or the square root of the area within the contour boundary (ARE). All these estimates are highly correlated, so we used the latter method (ARE).

Statistical analysis of data

Data analysis was performed using the online program XYOM (Dujardin and Dujardin, 2019) freely available at https://xyom.io. Mean values of tick centroid size between species were examined using non-parametric permutation tests (1000 rounds), with Bonferroni correction at $P<0.05$ (Bookstein, 1991). The difference in shape between each group was measured using discriminant analysis, illustrated by a factorial map. The reclassification matrices were obtained from the discriminant analyses performed on each population (male and female). Phenetic trees were constructed using Mahalanobis distances measured from one group to another with Bonferroni's sequence correction at $P<0.05$.

Results

Sexual dimorphism

The size study shows that there is a significant difference between the average size of the male and female of each tick species.

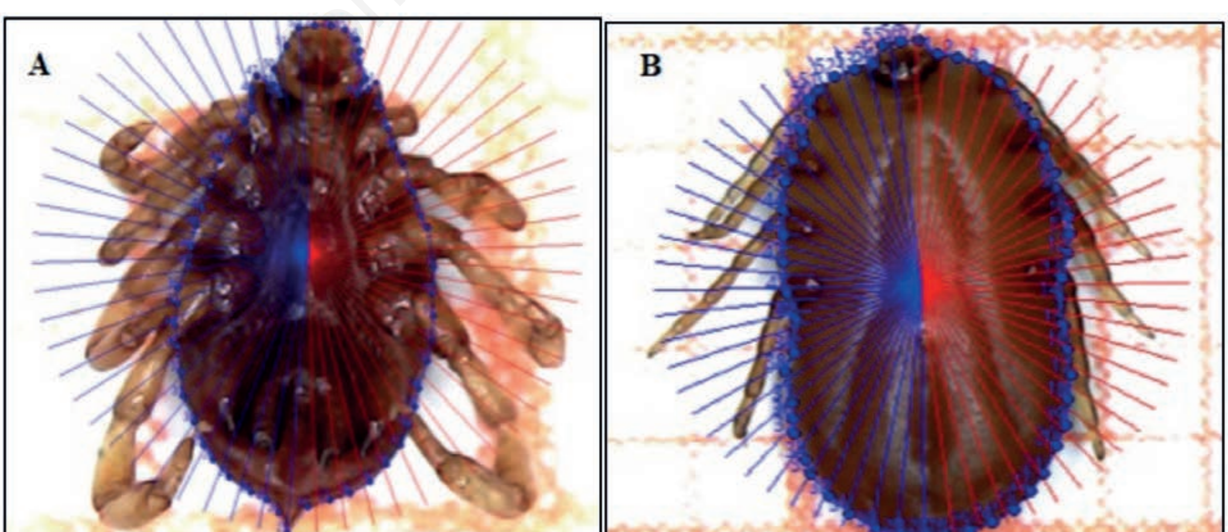

Figure 2. Digitalization using the outline technique on the ventral side of ticks: A) *Rhipicephalus* (*B.*) *microplus* male; B) *Rhipicephalus* (*B.*) *annulatus* female.

The males of *Rhipicephalus (B.) annulatus, Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) microplus* have significantly lower average size than the females (Table 1).

Size variation between tick species

In males, the pairwise comparison of mean size shows a significant difference among all species (*Rhipicephalus (B.) decoloratus, Rhipicephalus (B.) annulatus* and *Rhipicephalus (B.) microplus*) (Table 2). *Rhipicephalus (B.) microplus* males have a significantly larger mean size than *Rhipicephalus (B.) decoloratus* males which have a significantly larger mean size than *Rhipicephalus (B.) annulatus* males.

A pairwise comparison of the mean size of the four tick species in the female population shows that the mean size is significantly different between all species except between *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) geigyi*, which are not different. *Rhipicephalus (B.) annulatus* females have a significantly larger average size than females of other species. *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) geigyi* females have significantly identical mean sizes. Finally, *Rhipicephalus (B.) microplus* females have the smallest size among the females of the studied species (Table 2).

Variation in the shape of tick species: discriminant analysis

The factorial map analysis obtained at the level of male ticks shows a better distinction of *Rhipicephalus (B.) annulatus* from the other two species. However, it also shows an overlap of *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) microplus* (Figure 3A).

In females, the factor map shows that *Rhipicephalus (B.) geigyi* and *Rhipicephalus (B.) microplus* are distinct species from each other and also from *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) annulatus*. However, *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) annulatus* overlap, that is to say by superimposing one on the other (Figure 3B).

Valid reclassification based on Mahalanobis distances

The results of the reclassification show that all individuals are correctly reclassified in their population of origin with a good mean score for all species together: 74% in males and 76% in females (Table 3). In males, the single species show high reclassification scores: *Rhipicephalus (B.) annulatus* (93%), *Rhipicephalus (B.) microplus* (74%) and finally *Rhipicephalus (B.) decoloratus* (64%). In the female population, 100% *Rhipicephalus (B.) geigyi*, 79% *Rhipicephalus (B.) microplus,* 65% *Rhipicephalus (B.) decoloratus* and 63% *Rhipicephalus (B.) annulatus* are well classified (Table 3).

Phenetic trees

The phenetic tree constructed using Mahalanobis distances shows that the males of *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) microplus* are significantly close species to each other and to the males of *Rhipicephalus (B.) annulatus* (Figure 4A). In females, the phenetic tree reveals that *Rhipicephalus (B.) annulatus* and *Rhipicephalus (B.) decoloratus* are species significantly close to each other (Figure 4B). *Rhipicephalus (B.) decoloratus* females are more closely related to *Rhipicephalus (B.) microplus* females than to *Rhipicephalus (B.) geigyi* females.

Table 1. Size variation between sexes within each species.

Species	Males Mean±SD	Females Mean±SD	P-value	SIG
R. (B.) annulatus	2.31±0.04	6.10±0.78	0.001	***
R. (B.) decoloratus	2.47±0.29	4.56±0.85	0.001	***
R. (B.) microplus	2.51±0.29	2.93±0.23	0.030	***

***Means are statistically different at the α 5% threshold (P<0.05). *R.*, *Rhipicephalus*; *B.*, *Boophilus*; SD, standard deviation; SIG, significance.

Table 2. Comparison of size means between tick species.

Espèces	Male population			Females population		
	Mean±SD	P-value	SIG	Mean±SD	P-value	SIG
R. (B.) annulatus	2.31±0.04	0.0160	**	6.10±0.78	0.0001	***
R. (B.) decoloratus	2.47±0.29			4.56±0.85		
R. (B.) annulatus	2.31±0.04	0.0001	***	6.10±0.78	0.0001	***
R. (B.) microplus	2.51±0.29			2.93±0.23		
R. (B.) decoloratus	2.47±0.29	0.0001	***	4.56±0.85	0.0001	***
R. (B.) microplus	2.51±0.29			2.93±0.23		
R. (B.) annulatus	-	-	-	6.10±0.78	0.0001	***
R. (B.) geigyi				4.10±0.51		
R. (B.) decoloratus	-	-	-	4.56±0.85	0.0699	NS
R. (B.) geigyi				4.10±0.51		
R. (B.) microplus	-	-	-	2.93±0.23	0.0001	***
R. (B.) geigyi				4.10±0.51		

***Means are statistically different at the α 5% threshold (P<0.05). *R.*, *Rhipicephalus*; *B.*, *Boophilus*; SD, standard deviation; NS, not significant at the α 5% threshold (P<0.05); SIG, significance.

Table 3. Percentage of reclassification based on Mahalanobis distances of tick species of the genus *Rhipicephalus* (*B.*).

Espèces	% reclassification of males	% reclassification of females
R. (*B.*) *annulatus*	28/30 (93%)	38/60 (63%)
R. (*B.*) *decoloratus*	38/59 (64%)	34/52 (65%)
R. (*B.*) *geigyi*	--	52/52 (100%)
R. (*B.*) *microplus*	43/58 (74%)	35/44 (79%)
Total	109/147 (74%)	159/208 (76%)

R., *Rhipicephalus*; *B.*, *Boophilus*.

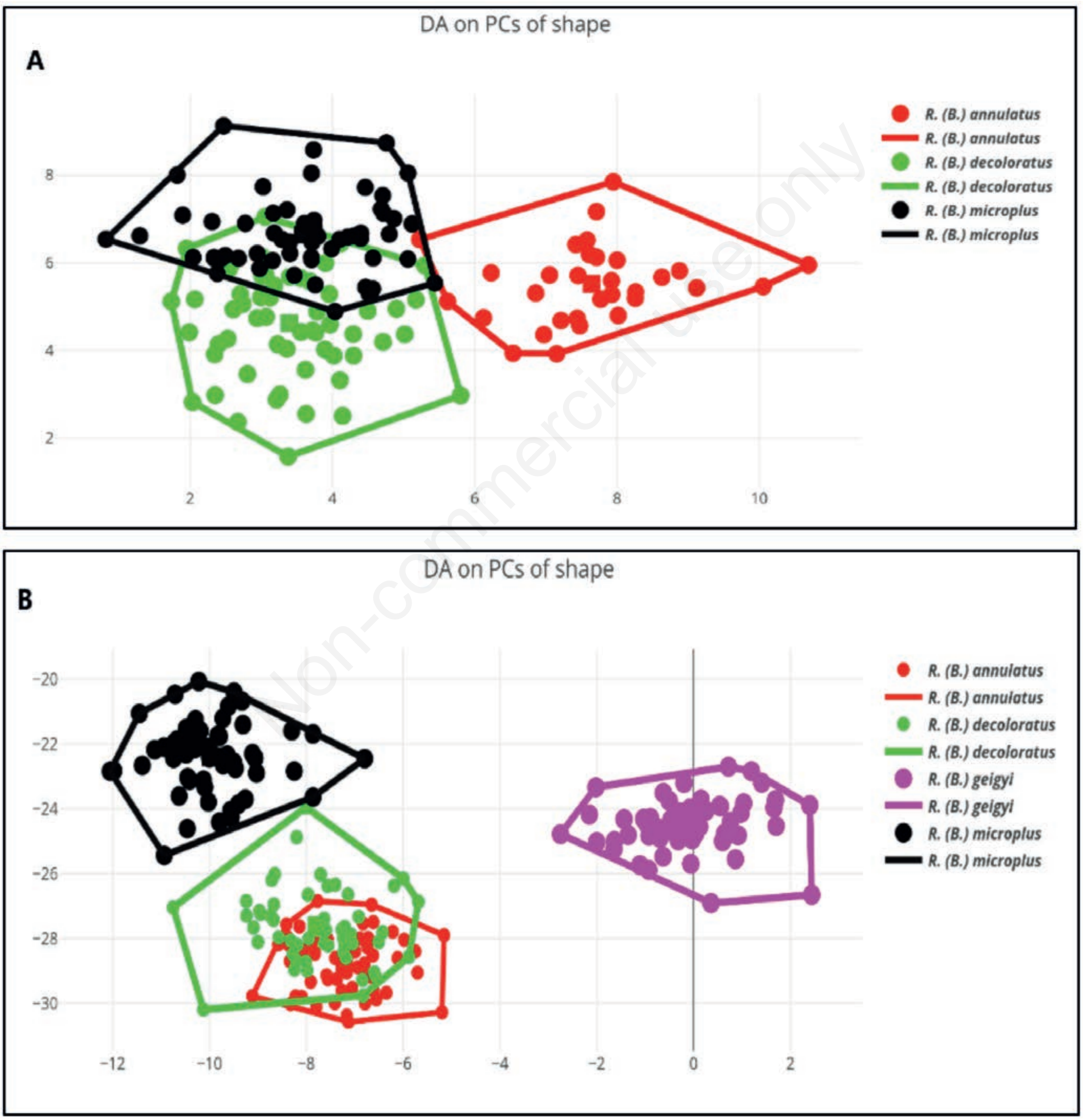

Figure 3. Factor maps of the discriminant analysis of the male and female population: A) males; B) females.

Discussion

This study presents the morphological differentiations of the four species of ticks of the genus *Rhipicephalus (B.)* encountered in the Savanes District. Described as a comparative biology approach by Adams *et al.* (2004) geometric morphometry revealed the presence of sexual dimorphism within each tick species, which is reflected in the variation in size of the two sexes (male and female). Males are significantly smaller ($P<0.05$) than females, as has been observed in *Amblyomma mixtum* ticks collected in Mexico (Dominguez *et al*., 2021). Thus, size can be selected as a morphological characteristic to distinguish males from females of three (3) species: *Rhipicephalus (B.) microplus, Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) annulatus* (Walker *et al.*, 2003). In addition to sexual dimorphism, the size of individuals of the different species varied significantly from one species to another in the two (2) populations (males and females) considered. With the exception of *Rhipicephalus (B.) geigyi* and *Rhipicephalus (B.) decoloratus* females, there was no significant difference in size ($P>0.05$). The tick species examined in this study were collected in the same agro-ecological zone. Thus, the variation in the sizes of these species is linked to variation in the genes responsible for size expression (Dujardin *et al*., 2014).

Size does not inform the shape of an organism (Diaha-Kouamé *et al*., 2017). For this reason, the present work made it possible to show the overall morphological variations (size and shape) of tick species of the genus *Rhipicephalus (B.)*. Tick shape was studied by means of discriminant analyses carried out on the two tick populations (males and females). The results show a correct distinction between male *Rhipicephalus (B.) annulatus* and other species, and also a good distinction between female *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) geigyi* and other females. Although, the discriminant analyses also reveal the overlap of certain male individuals of the species *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) decoloratus* and also the overlap of certain female individuals belonging to the species *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) annulatus*, the species reclassification matrices at the level of each population present very high reclassification percentages of 74% for males and 76% for females. The high reclassification percentages show that individuals from the two populations were morphologically well-identified according to species. It should be noted that the operations of digitizing and digitalizing the tick images were carried out by one and the same operator, as suggested by Dujardin and Dujardin (2019) in order to reduce errors during manipulation. Thus, the values of the reclassification matrices also reflect that operator errors that occurred during the digitization of the tick images were strongly reduced.

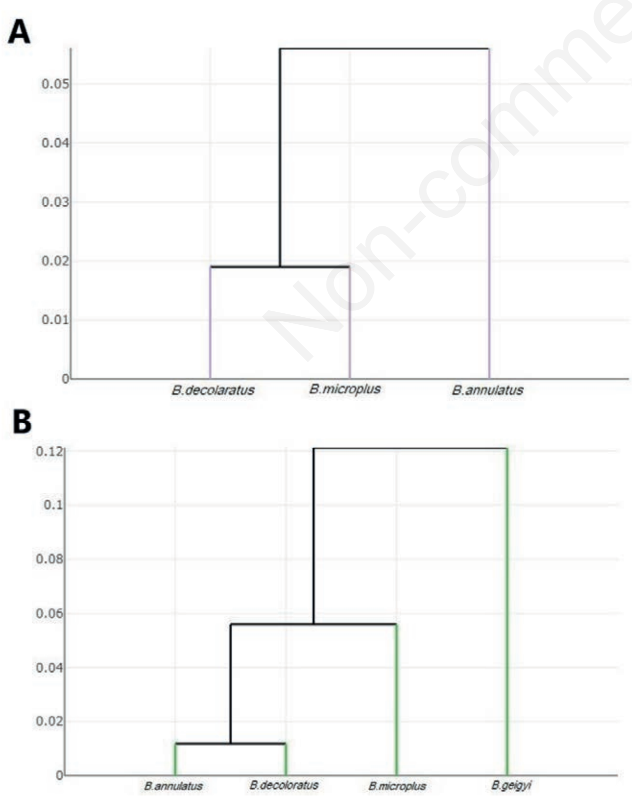

Figure 4. Phenetic tree of tick species based on Mahalanobis distances in females: A) males; B) females.

From all of the above, the overlap between individuals of different species in the male and female populations can be explained by the presence within the populations of hybrid individuals resulting from cross-breeding between these species. Thus, there could be male hybrids resulting from crosses between *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) microplus* and also female hybrids resulting from crosses between *Rhipicephalus (B.) decoloratus* and *Rhipicephalus (B.) annulatus*. Indeed, Kiffopan *et al.* (2019) encountered female hybrids resulting from crosses between *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) annulatus* in the Savanes District of Côte d'Ivoire. They suggested that the almost exclusive presence of female hybrid ticks may be due to the fact that male hybrid larvae are more sensitive to high temperatures and low hygrometry than females. The variability of hybrids in the tick population of the Savannah District could lead to inconsistencies in the morphological identification of tick species if the identification keys are not regularly updated (Lempereur *et al*., 2010).

The phenetic tree in males shows that *Rhipicephalus (B.) microplus* and *Rhipicephalus (B.) decoloratus* are descended from the same ancestor, which could be due to the presence of a caudal appendage in these two species. However, in the males of *Rhipicephalus (B.) annulatus,* the caudal appendage is absent. This organ is one of the morphological characteristics that distinguishes male *Rhipicephalus (B.) annulatus* from the males of other tick species of the genus *Rhipicephalus (B.)* (Berry, 2017). However, in females the phenetic tree shows that there is a strong resemblance in the shape of female *Rhipicephalus (B.) annulatus* and *Rhipicephalus (B.) decoloratus* ticks. This resemblance was mentioned by Walker *et al.* (2003) in the identification key.

Durango *et al.* (2020) employed geometric morphometry to investigate morphometric similarities among the *Rhipicephalus (B.) microplus* tick population in the north-western region of Colombia. However, geometric morphometry was used in this study to examine morphological differences between four tick species of the genus *Rhipicephalus (B.)* observed in Côte d'Ivoire. The results of this study must be confirmed by the molecular results because the results of the molecular study can sometimes be contradictory to the morphological results as reported by Yousseu *et al.* (2022).

Conclusions

This study investigated morphological variations within the genus *Rhipicephalus (B.)* using geometric morphometry. It was possible to study the shape separately from the size of the individuals. In males, a distinction between *Rhipicephalus (B.) annulatus* and an overlap between *Rhipicephalus (B.) decoloratus* and *Rhipicephalus*

(B.) microplus was observed. In females, however, there was an overlap between *Rhipicephalus (B.) annulatus* and *Rhipicephalus (B.) decoloratus.* Geometric morphometry thus opens up interesting prospects for studying the morphological variability of tick species. Consequently, for a better understanding of the results of geometric morphometry, it would be necessary to compare them with the results of molecular characterisation studies.

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