Chromosomal and environmental determinants of morphometric variation in natural populations of the malaria vector Anopheles funestus in Cameroon

Diego Ayala [a,*], Harling Caro-Riaño [a], Jean-Pierre Dujardin [a], Nil Rahola [a], Frederic Simard [a,b], Didier Fontenille [a]

[a] IRD, UMR 224 MIVEGEC/BEES, 911 Av Agropolis, 34394 Montpellier, France
[b] IRSS-DRO, BP 545, Bobo-Dioulasso, Burkina Faso

ABSTRACT

Anopheles funestus is one of the most proficient malaria vectors in the world, mainly because of its remarkable ability to populate a wide range of ecological settings across Africa. Its formidable environmental plasticity has been primarily associated to high amounts of genetic and inversion polymorphisms. However, very little is known about the morphological changes that this ecological adaptation entails. Here, we report on wing morphometric variations in karyotyped specimens of this species collected throughout a wide range of eco-geographical conditions in Cameroon (Central Africa). Our results revealed strong selection on mosquito wing traits. Variation of wing size was dependent on temperature and elevation ($p < 0.001$), while wing shape did not exhibit a specific environmental pattern. On the other hand, we observed a significant correlation of wing shape variation ($p < 0.001$), but not size ($p > 0.05$), with regard to karyotype. This pattern was maintained across different environmental conditions. In conclusion, our findings cast strong evidence that change in morphometric traits are under natural selection and contribute to local adaptation in *A. funestus* populations. Furthermore, the robust relation between chromosome polymorphisms and wing shape suggests new evolutionary hypotheses about the effect of chromosomal inversions on phenotypic variation in this malaria vector.

1. Introduction

One of the major factors which establish the ability of an insect to become an important vector for human diseases is its "anthropophilic" behavior, e.g. its preference for feeding human blood and/or exploiting man-made resting or breeding habitats. Hence, medically important insects, such as malaria vector mosquitoes, are exposed to control pressures and habitat modifications due to their close contact with human populations, revealing frequent genotypic and phenotypic variations (Dujardin, 2008). In the field, these variations can be based either on genetic divergence, direct environmental effects, or both. Thus, studies on phenotypic variation can provide relevant insights into the evolution of vector systems and help detect local populations with potentially important characters, which might affect disease transmission (Dujardin, 2008; Pigliucci, 2005). However, despite their importance, little is known about phenotypic variation and morphological plasticity in *Anopheles* species across the different habitats that they populate. This is an important limitation for studies aimed at exploring ecological adaptation in malaria vectors with direct consequences on disease epidemiology.

Morphometric traits have been employed to analyze adaptive variation in natural populations of animals and plants (Mayr, 1942). Patterns of morphological variation involving size or shape dimensions have been often interpreted with regard to their evolutionary importance (Pigliucci, 2005). In this sense, insect wings have been reported as an excellent model for studying morphological evolution in natural populations. Wing size is directly related to body size (Sokoloff, 1966) and there exists considerable evidence that size and shape are targets of natural selection (Soto et al., 2006). Moreover, they respond to environmental variation in complex ways, suggesting that the range of phenotypes produced by a particular genotype might be different depending on environmental conditions. As a result, morphological changes may be part of an adaptive response (Carreira et al., 2006; Weber, 1990). Consequently, investigations on morphological traits variation necessarily require to involve the simultaneous analysis of genetic and environmental factors, which somehow cause intra-specific variation and interspecific divergence (Mackay, 2004).

* Corresponding author. Tel.: +33 0 4 67 41 61 78; fax: +33 0 4 67 54 20 44.
E-mail addresses: diego.ayala.g@gmail.com (D. Ayala), harling.caro@gmail.com (H. Caro-Riaño), jean-pierre.dujardin@ird.fr (J.-P. Dujardin), nil.rahola@ird.fr (N. Rahola), frederic.simard@ird.fr (F. Simard), didier.fontenille@ird.fr (D. Fontenille).

Insect chromosomal polymorphism has been frequently associated with environmental adaptation (Coluzzi et al., 1979; Hoffmann et al., 2004; Krimbas and Powell, 1992; Krimbas, 1967). Natural populations of *Drosophila* and *Anopheles* species have recurrently shown clinal variation in some paracentric chromosomal inversions along latitudinal or altitudinal gradients (Balanya et al., 2003; Collinge et al., 2006; Hoffmann et al., 2004; Simard et al., 2009). Numerous chromosomal rearrangements have been linked to effects on *Drosophila* morphometric traits, establishing additional variation on which selection may be acting (Colombo et al., 2001, 2004; Orengo and Prevosti, 2002; Santos et al., 2004). Hence, the observations of adaptive environmental clines where chromosomal polymorphisms and morphometric traits running in parallel suggest that both might be related and subject to similar evolutionary forces (Orengo and Prevosti, 2002). In Africa, malaria transmission is primordially ensured by three anopheline species, *Anopheles gambiae*, *A. funestus* and *A. arabiensis*, which are widely distributed across sub-Saharan Africa. The ability of those malaria vectors to thrive the wide range of habitats present in Africa has been associated to the richness of chromosomal polymorphisms (Ayala et al., 2011; Coluzzi et al., 2002; Pombi et al., 2008). However, to date, no study has examined how this ecological plasticity has modeled their phenotypic traits, contributing to increasing their local population fitness.

In this study, we questioned the effect of chromosomal polymorphism and environmental conditions on phenotypic variation (wing size and shape) of the malaria mosquito, *A. funestus*. We hypothesized that, as observed in other insect species, environmental conditions and chromosomal background have an impact on morphometric traits, directly linked to local adaptation. Natural populations of adult females *A. funestus* were sampled across nine eco-geographical zones in Cameroon (Central Africa) displaying large variation in environmental conditions. First, we compared patterns of wing size and shape among ecological zones. Second, we related wing traits to environmental variables and we
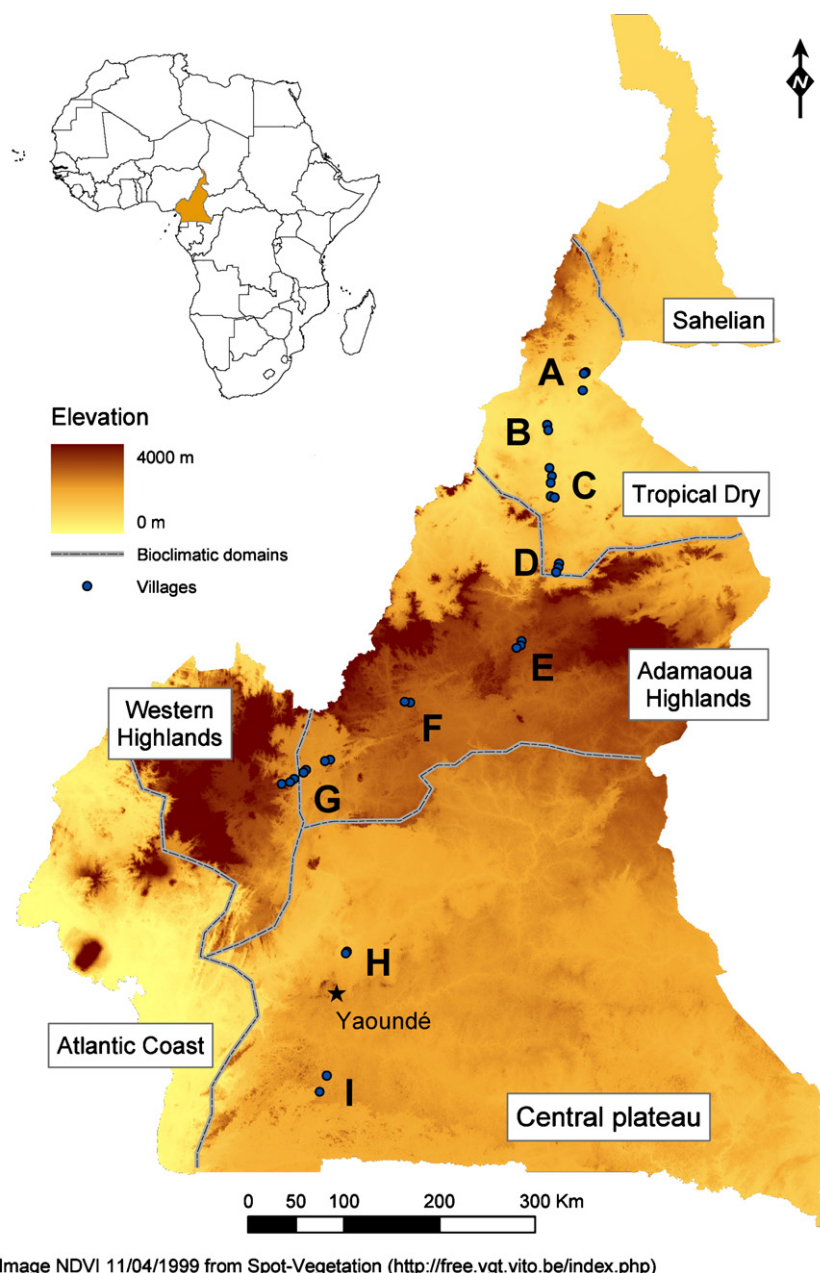

Fig. 1. Topographic map of Cameroon showing sampling zones (A–I) and villages in each zone (dots). Dotted lines delimit biogeographical domains (Olivry, 1986).

elucidated the contribution of each variable in morphometric variation. Finally, we explored the effect of chromosomal inversion polymorphisms on wing morphology. Our findings revealed significant effect of local environmental conditions on wing morphology. Chromosomal polymorphism was associated to wing shape variation across populations. These outcomes are discussed in a context of environmental adaptations and their impact on malaria epidemiology and vector control strategies.

2. Materials and methods

2.1. Study sites and mosquito sampling

Wing morphometric traits were assessed and compared in *A. funestus* mosquitoes collected in nine ecological zones belonging to five different bioclimatic domains of Cameroon (Olivry, 1986), encompassing most of the bio-geographic diversity of the country, ranging from the northern arid savannas to the evergreen rainforest in the south (Fig. 1, Table 1). To reduce possible local effects and increase the number of specimens, mosquitoes were collected from 2 to 7 villages in each zone (average distance between villages within zone = 7.83 km). Adult females *A. funestus* were captured by day-time spraying aerosols of pyrethroid insecticides inside human dwellings (Service, 1993). Anopheline mosquitoes were identified using morphological identification keys (Gillies and de Meillon, 1968). Ovaries from half-gravid *A. funestus* females were dissected and stored in Carnoy fixative solution (3 parts of 100% ethanol: 1 part glacial acetic acid by volume) for subsequent cytogenetic analysis. Carcasses were stored individually in labeled tubes containing a desiccant and kept at −20 °C.

2.2. Mosquito PCR identification and karyotyping

Genomic DNA was extracted from the body of adult mosquito females using the protocol described in Morlais et al. (2004). DNA was then resuspended in sterile water in individual tubes. Morphological identification of *A. funestus* s.s. (hereafter *A. funestus*) was confirmed by molecular identification (Cohuet et al., 2003; Koekemoer et al., 2002). Polytene chromosomes obtained from the ovaries of half-gravid females *A. funestus*, were squashed and stained according to standard protocols (della Torre, 1997). The preparations were examined under a phase-contrast microscope, and paracentric chromosomal inversions were scored according to the *A. funestus* cytological map (Guelbeogo et al., 2005; Sharakhov et al., 2004).

2.3. Environmental data

A set of seven eco-geographical variables (EGVs) was used to describe the average environmental conditions in each zone (source: *LocClim* database developed by the Food Agriculture Organization – FAO, http://www.fao.org/sd/2002/EN1203a_en.htm): elevation (in m), rainfall (in mm), temperature (in °C), evapotranspiration (in mm), relative humidity (water vapor pressure in %), mean number of hours of sunlight per day (h), and wind speed (in ms^{-1}). Climate data are yearly means, averaged over the past 30 years, obtained from interpolation of field stations data. Computational operations linked to geo-analysis requirements were performed using the software ArcGIS 8.3 (http://www.esri.com/software/arcgis/index.html).

2.4. Morphometric analysis

2.4.1. Sample processing

A digital image of each mosquito female wing, left and right (dorsal view) was taken through a binocular microscope (Leica MZ6). Both wings were removed and mounted on microscope slides under cover slips. Morphometric measurements were taken from both wings of each female, except when only one undamaged wing was available. Twelve morphometric measurements, as recommended by Bookstein (1991), were scored from the digital images of each wing (Fig. 2) using the free software COOw (http://www.mpl.ird.fr/morphometrics/). All measurements were taken by the same person for more consistency (Bookstein, 1991).

2.4.2. Repeatability

Random measurement error is common in morphometric analysis, and it can cause serious statistical problems (Arnqvist and Martensson, 1998). To detect this kind of error, we repeated measures of all individuals twice, and we quantified their repeatability by the ratio between the individual variance and the total variance. For this purpose, we used the free software VAR 1.4 (http://www.mpl.ird.fr/morphometrics/).

2.4.3. Size and shape

For comparing overall wing size between zones, we used the isometric estimator known as "centroid size" (CS) derived from coordinates data: it is defined as the square root of the sum of squared distances between the center of the configuration of landmarks and each individual landmark (Bookstein, 1991). Variation in shape was examined using geometric morphometrics based on generalized least squares Procrustes superim-

Table 1
Chromosomal inversion frequencies and mean wing size in *Anopheles funestus* mosquitoes sampled from a range of ecological zones in Cameroon.

Zone[a]	Bioclimatic domain[b]	Temperature[c] (°C)	Rainfall[c] (mm)	Elevation[c] (m)	Number of villages	Number of mosquitoes	Inversion frequency (%)			Wing size (mm)
							3Ra	3Rb	3La	
A	Tropical dry	27	878	249	3	27	0.0	0.0	0.0	2.66 ± 0.12
B	Tropical dry	28	989	284	2	33	1.5	0.0	3.0	2.67 ± 0.15
C	Tropical dry	26	1223	335	5	59	63.6	68.6	66.9	2.70 ± 0.16
D	Tropical dry	24	1348	590	3	24	87.5	97.9	95.8	2.76 ± 0.15
E	Adamaoua Highlands	23	1509	1073	3	21	76.2	73.8	100.0	2.90 ± 0.14
F	Adamaoua Highlands	23	1680	969	2	26	71.2	80.8	100.0	2.84 ± 0.12
G	Western Highlands	22	1874	779	7	22	63.6	72.7	100.0	2.93 ± 0.12
H	Central Plateau	25	1633	493	2	35	100.0	100.0	100.0	2.70 ± 0.13
I	Central Plateau	24	1853	708	2	18	100.0	100.0	100.0	2.63 ± 0.10

Source: Climatic data are yearly means averaged across the past 30 years. http://www.fao.org/sd/2002/EN1203a_en.htm.

[a] Zone denomination refers to Fig. 1.
[b] Bioclimatic domains are as defined by Olivry (1986).
[c] Climatic data are yearly means averaged across the past 30 years.