Chapter 8

Highlights on *Anopheles nili* and *Anopheles moucheti*, Malaria Vectors in Africa

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55153

1. Introduction

Anopheles nili Theobald 1904 and *An. moucheti* Evans 1925 are major human malaria vectors in forested and humid savannah areas of West and Central Africa [1]. Yet, they remain critically understudied and basic knowledge on their biology, ecology and genetics is crucially lacking [2]. To date, most studies of African malaria vectors have focused on *An. gambiae*, *An. arabiensis*, and *An. funestus*, in part, because molecular and cytogenetic tools for characterizing population structure, ecological adaptation, and taxonomic status of other species have been lacking until recently. Further, no laboratory colony is available for experimental work involving these neglected species. This gap in knowledge needs to be addressed for successful implementation of global strategies for malaria elimination and eradication in the Afrotropical region [3].

Recent studies of the ecological niche profile of major African malaria vectors demonstrated that the habitats of *An. gambiae*, *An. arabiensis*, and *An. funestus* have more overlap with each other than with the habitat of *An. nili* and *An. moucheti* [4-7]. This results in an unusual geographic distribution of *An. nili* and *An. moucheti* (Figure 1), revealing their crucial role in malaria transmission in forested and degraded forest areas of equatorial Africa [8-13]. Unique aspects of ecological adaptation and behaviour can, in part, explain the increased vectorial capacity of the species in these environments and might protect them from conventional vector control tools targeting highly endophilic and endophagic mosquito species [3, 14]. Moreover, the recent findings of circulation of *Plasmodium falciparum* along with other *Plasmodium* species in great apes and monkeys [15-17] raise concerns about pathogen transfer between humans and primates and further highlight the need to improve our knowledge of forest malaria vectors.

In this chapter, we review knowledge gained so far on mosquitoes from *An. moucheti* and closely related species, as well as the *An. nili* complex. We highlight specific bionomical,

ecological and genetic attributes that distinguish these species from the most well-known major African malaria vectors, providing opportunities for further research on neglected aspects of vector biology and control.

2. *Anopheles moucheti* and closely related species

Anopheles moucheti belongs to the series Myzomyia and closely resembles *Anopheles marshallii* Theobald of the Marshallii complex. This close morphological similarity resulted in *An. moucheti* being initially considered a variety of *An. marshallii* before it was raised to the rank of full species on the basis of morphological and bionomic differences [18]. However the taxonomic status of *An. moucheti* has been subject to several interpretations during the past decades. Based on morphological similarities between *An. bervoetsi* and *An. moucheti nigeriensis*, *Anopheles moucheti* was later considered by Brunhes *et al.* [19] as a group consisting of three morphological forms, namely *An. moucheti moucheti* (referred to as the type form), *An. moucheti bervoetsi* and *An. moucheti nigeriensis* distinguishable by slight morphological characters present at the adult and/or at the larval stages [2, 19, 20]. In their classification, Brunhes *et al*. [19] referred to *An. bervoetsi* as a subspecies of *An. moucheti* while they suggested to put in synonymy *An. m. nigeriensis* and the type form. Genetic analysis conducted subsequently provided evidences against any taxonomic value for this morphological classification [21-23]. Recent classification by Harbach [24] recognizes *An. moucheti* and *An. bervoetsi* as formal species while *An. m. nigeriensis* is considered as a morphological variant within *An. moucheti*.

Anopheles moucheti is widely distributed across West and Central Africa (Figure 2) whereas the two other taxa have only been reported so far from their type locality in Nigeria near Lagos (06°27′N; 03°24′E) for *An. moucheti nigeriensis* and in Tsakalakuku (06°34′S; 17°35′E) in the Democratic Republic of Congo (DRC) for *An. bervoetsi* [18].

Anopheles moucheti is among the most important human malaria vectors in the equatorial forest region of Africa, particularly in villages situated along slow moving rivers or streams where its larvae develop in and around floating vegetation and debris (Figure 3) [4, 5]. Larval collections to assess ecological factors influencing *An. moucheti* distribution across river networks in south Cameroun showed that *An. moucheti* larvae are frequently associated with lentic rivers, low temperatures and the abundance of aquatic vegetation at the edge of the river (Figure 4) [5]. Increased urbanization and deforestation as well as lower-scale landscape modification such as river banks cleaning for gardening and/or recreational purposes were shown to be highly detrimental to the species, fostering changes in the malaria vector system composition with a higher prevalence of *An. gambiae*, taking the lead over *An. moucheti* [9]. Insecticide susceptibility tests conducted on several populations from South Cameroon in 2007 indicated that *An. moucheti* is fully susceptible to DDT, permethrin and deltamethrin (Etang *et al.*, unpublished data).

In rural villages situated in deep forest areas, *An. moucheti* usually is the major vector of *Plasmodium*, and quite often the only one maintaining a high level of malaria endemicity in humans. Natural infection rates in the range 1–3% are commonly reported in wild females,

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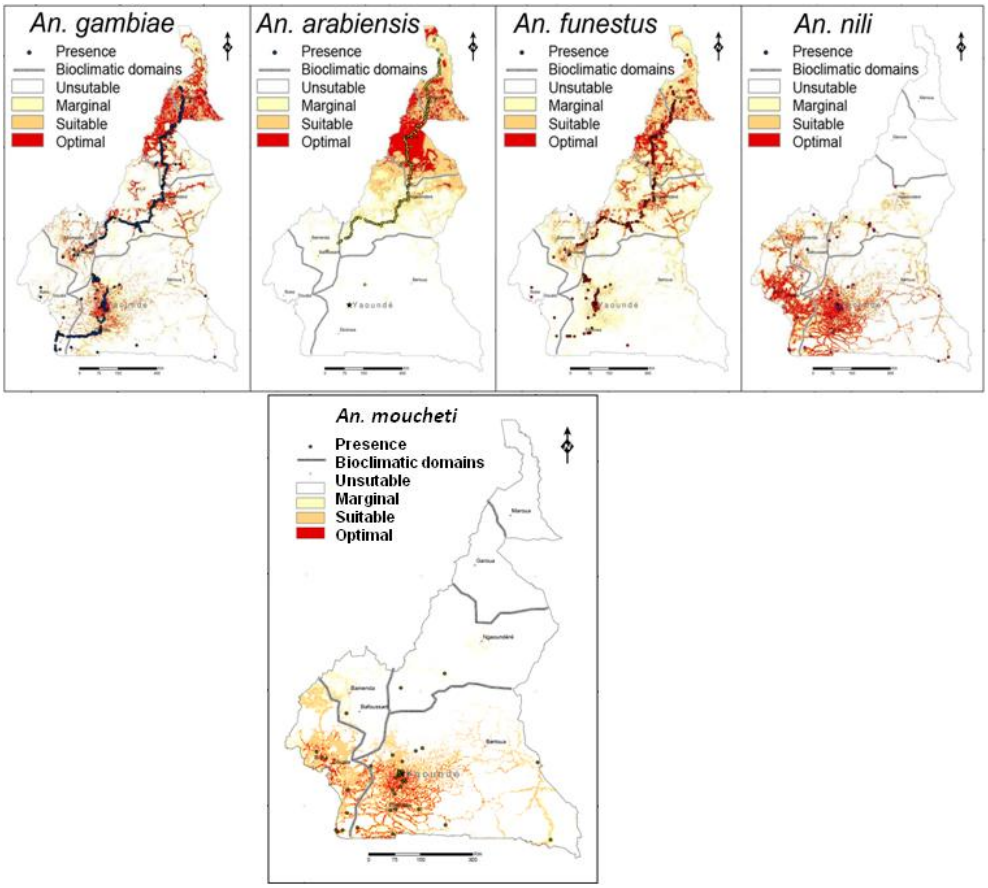

Figure 1. Habitat suitability maps for the five major malaria vectors in Cameroon. A/ *Anopheles gambiae*, *An. arabiensis*, *An. funestus*,, *An. nili*, *An. moucheti*. Different colors identify four classes of habitat quality including optimal (red), suitable (orange), marginal (yellow) and unsuitable habitat (white). Figure drawn from Ayala *et al.*, 2009 [4].

sustaining annual entomological inoculation rates (EIR) reaching up to 300 infective bites/human/year [27, 28]. As such, the species has been incriminated in malaria transmission in a number of countries in Central Africa, including Nigeria [29], Cameroon[28, 30], Gabon [31, 32], Equatorial Guinea [10, 11], Congo [18], the DRC [18] and Uganda [18]. In these settings, *An. moucheti* frequently bites indoors and high densities of blood-fed females can be collected resting indoors, over 95% of which had taken their blood meal on humans demonstrating strong anthropophily. However, high mosquito densities might also be collected far from any human settlements, indicating a probable zoophilic behaviour in some forest populations [33, 34].

Anopheles bervoetsi has only been reported so far from its type locality and surrounding villages in the DRC. Larvae are found in small rivers sheltered by forest galleries that wind through the valleys in a hilly landscape. Adults are highly anthropophilic and preferentially bite

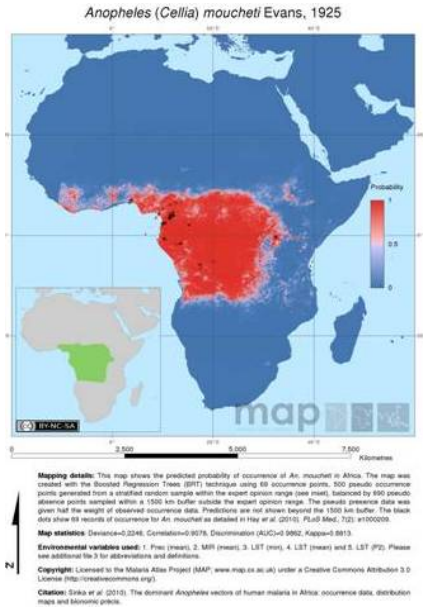

Figure 2. Map of the predicted probability of occurrence of *Anopheles moucheti* in Africa (redrawn from [25]). Black dots represent 69 records of occurrence for *An. moucheti* as described in Hay et al. [26].

Figure 3. A typical breeding site for *Anopheles moucheti* larvae along river Nyong in southern Cameroon.

outdoors. However, it can be collected biting and resting indoors when abundance is high at the end of the rainy season (Antonio-Nkondjio et al. unpublished data). Biting occurs at night with a peak of activity usually recorded in the second part of the night. A recent study reported three specimens found infected by *Plasmodium falciparum* out of 237 tested by ELISA, confirming its role in malaria parasites transmission [35].

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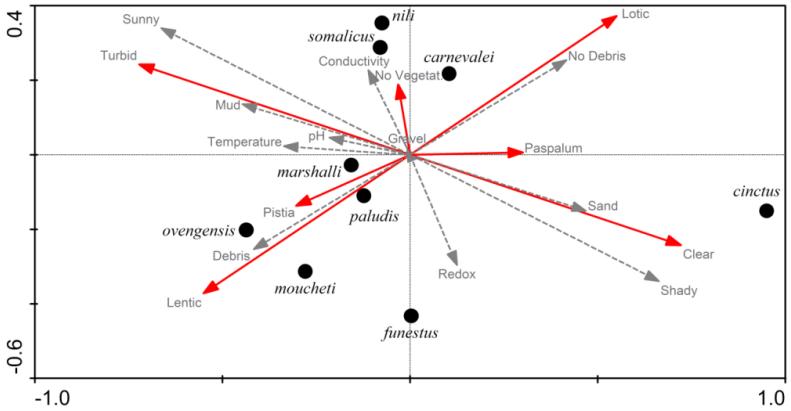

Figure 4. Canonical Correspondence Analysis (CCA) diagram showing the ordination of anopheline species along the first two axes and their correlation with environmental variables. The first axis is horizontal, second vertical. Direction and length of arrows shows the degree of correlation between mosquito larvae and the variables. Figure drawn from Antonio-Nkondjio *et al.*[5].

Anopheles m. nigeriensis is considered as a synonym to *An. moucheti*, due to the absence of reliable morphological differences at the adult and larval stages between the two morphs [19, 24]. Nothing is known of the species bionomics. The only report of its implication in malaria parasites transmission is from Baber and Olinger in 1931 ([18], *loc. cit.*) who reported 1 in 87 mosquitoes infected with sporozoites. Collections conducted in its type locality in 2005 reported few specimens (<10, Antonio-Nkondjio and Simard, unpublished data), probably reflecting habitat deterioration due to the expansion of the urban domain around Lagos.

From morphological analysis (Figure 5), it appears that the type form could display high morphological variation with variants similar to *An. m. nigeriensis* and *An. bervoetsi*. However, genetic investigations and the follow-up of morphological diversity in the progeny of field collected gravid females demonstrated that a single taxon was represented, at least in Cameroon [21]. Population genetic investigations using a set of ten microsatellite markers [36] further strengthened this view, revealing genetic homogeneity between natural populations of *An. moucheti* in South Cameroon and throughout Central Africa, including Uganda and the DRC [36, 37]. Studies comparing sequence variations in nuclear (rDNA Internal Transcribed Spacer 1, ITS2 and the D3 domain of the 28S ribosomal subunit) and mitochondrial (cytochrome b) DNA regions were also concordant, depicting a low level of genetic diversity and differentiation between specimens from Cameroon, Uganda and the DRC and confirming the high genetic homogeneity of *An. moucheti* populations throughout Central Africa [23]. However, when mosquito samples collected from the type localities of *An. bervoetsi* and *An. m. nigeriensis* were included in the analyses, sequence differences were detected between the three taxa, similar in degree to the differences found previously between sibling species within other anopheline groups or complexes [23]. An allele specific PCR assay based on sequence differences in the rDNA ITS1 region was developed to allow rapid identification of each of these three genetic lineages (Figure 6) [23]. Microsatellite analysis further demonstrated

significant genetic differentiation between *An. bervoetsi* populations form the DRC and *An. moucheti* populations from Cameroon, suggesting that they represent two different species [35]. In light of accumulating evidences (morphological, behavioral and genetic differences) this taxa was raised to the rank of full species and named *An. bervoetsi* [35] [24]. Yet the issue of the taxonomic status of *An. m. nigeriensis* remains unresolved. It might still be considered as a variant of *An. moucheti* to be further studied.

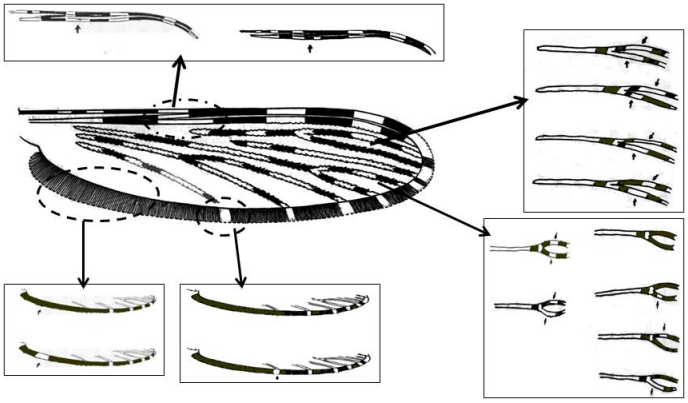

Figure 5. Morphological variations on the wing of *An. moucheti.*

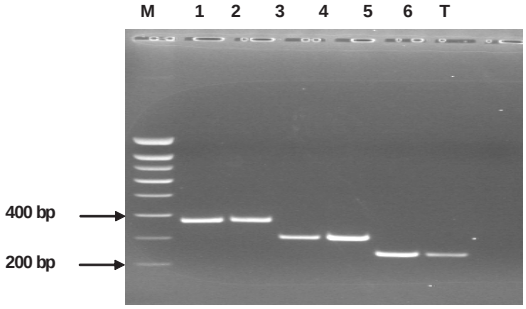

Figure 6. An agarose gel stained with ethidium bromide revealing size differences in the PCR amplification products discriminating *An. moucheti* and closely related species: *An. bervoetsi* (lanes 1 and 2), *An. moucheti* (lanes 3 and 4) and *An. m. nigeriensis* (lanes 5 and 6). Figure from Kengne *et al.*, 2007 [23]

3. Anopheles nili complex

Important morphological, ecological and behavioral differences among natural populations of *Anopheles nili* from sub-Saharan Africa suggested the existence of several taxonomic units

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and resulted in the description of four formal species, namely: *Anopheles nili sensu stricto*, *An. somalicus*, *An. carnevalei* and *An. ovengensis* [20, 21]. Morphologically, these four species are very close from one another, differing only through subtle morphological characters present at the adult and/or at the larval stages (Figure 7) [18, 38, 39]. Apart from *An. somalicus*, which is zoophilic and was never incriminated in human malaria transmission, the three other members of the complex are highly anthropophilic and are vectors of malaria.

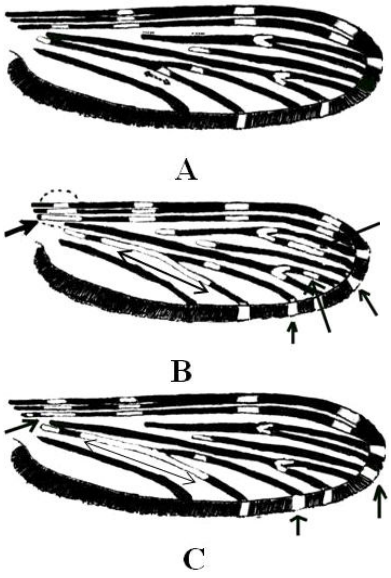

Figure 7. Morphological differences between members of the *An. nili* complex. A: wing of *An. nili* and *An. somalicus,* B: wing of *An. carnevalei,* C: wing of *An. ovengensis.*

Anopheles nili s.s. is among the most important malaria vectors in sub-Saharan Africa. It has a wide geographic distribution range spreading across most of West, Central and East Africa mainly populating humid savannas and degraded rainforest areas (Figure 8) [1, 4, 20, 40]. Larvae thrive at the sunny edge of fast running streams and rivers, where floating vegetation and debris provide suitable shelters (Figure 9) [32]. Forest populations are usually highly anthropophilic and feed regularly indoors whereas savanna populations are more exophilic and exophagic [12, 28]. Despite feeding preferentially on humans, this mosquito can be, at times highly zoophilic [41]. *Anopheles nili* is usually responsible for a high nuisance to humans in villages along rivers, and abundance rapidly decreases within a few kilometers from the breeding sites [42]. It is also present at the periphery of urban areas.

The prevalence of *Plasmodium* infections in wild females typically ranges between 1 and 3%and transmission rate reaching 200 infective bites/human/year have been reported in the literature for *An. nili* [12, 13, 28, 43]. Reports on its epidemiological role in East Africa however, are scarce, dating back to the 1970s [18, 44]. There is no published record available for insecticide

susceptibility in *An. nili* populations, although unpublished results from South Cameroon suggest full susceptibility to DDT and pyrethroids (permethrin and deltamethrin) using the diagnostic doses recommended for assessing *An. gambiae* populations (Etang *et al*., unpublished data). The analysis of key ecological factors associated with the distribution of An. nili larvae across 24 hydrographic networks in Cameroon showed that *An. nili* distribution conforms to that of a generalist species which is adapted in exploiting a variety of environmental conditions (Figure 4).

Anopheles carnevalei and *An. ovengensis* are mainly distributed in deep forest areas where they take over *An. nili s.s.* in this environment [4, 41]. *Anopheles carnevalei* has been reported so far only from Côte d'Ivoire, Cameroon and Equatorial Guinea [10, 11, 38]. It is rarely collected resting indoors and bites more frequently outdoors [12]. This mosquito is mostly zoophilic although it regularly feeds on humans in villages situated close to its breeding sites. Interestingly, although biting activity can be detected all night long, man-biting activity peaks early in the evening, between 6-7 PM, when inhabitants traditionally meet at the river for domestic and body care activities [12]. Studies conducted in Cameroon and Equatorial Guinea reported infection rates *circa* 1% in Cameroon [12, 28], raising up to 24% when using PCR-based protocols for parasite detection in specimens from Equatorial Guinea [10].

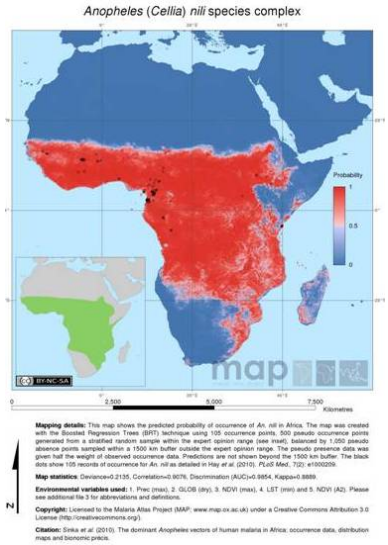

Figure 8. Map of the predicted probability of occurrence of *Anopheles nili* complex in Africa [25]. Black dots represent 105 records of occurrence for *An. nili* complex as described in Hay et al. [26].

Anopheles ovengensis, the most recently described species of the *An. nili* complex, is highly anthropophilic, and bites and rests frequently outdoors [39]. However, studies conducted in Equatorial Guinea reported high densities collected by window exit traps indicating some degree of endophagic and endophilic behavior [11]. *Anopheles ovengensis* usually displays high

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Figure 9. A typical breeding site for *An. nili* along the river Sanaga in South Cameroon.

Figure 10. A typical breeding site for *An. ovengensis* along river Njoh in South Cameroon (Photo: P Bousses, IRD/MIVEGEC).

biting rates for humans, ranging from 50 to 300 bites/man/night along rivers where its larvae develop (Figure 10). Infection rates by *P. falciparum* ranges between 0.4 to 4.4% in specimens from Cameroon [39] and in Equatorial Guinea [11]. Larvae are often found in sympatry with those of *An. moucheti* with whom it shares most of its distribution area. The distribution range of the species probably extends further East, throughout the Congolese forest basin but this has not been investigated yet.

Anopheles somalicus is strictly zoophilic. At the adult stage, *An. somalicus* closely resembles *An. nili* from which it can be morphologically separated at the larval stage only [18]. Adults are rarely recorded in villages although larvae are always found in sympatry with those of *An. nili* [5]. Nothing is known of its bionomics. According to Gillies and De Meillon [18] its distribution range includes Sierra Leone, Guinea, Burkina Faso, Ivory Coast, Cameroon, Somalia and Tanzania.

Genetic studies conducted on the *An. nili* complex using various molecular markers confirmed the high genetic heterogeneity among its members [2]. Multilocus enzyme analysis of the genetic variability detected species-specific alleles and large differences in shared allele frequencies among species of the complex collected in South Cameroon [45]. Analysis of sequence polymorphism in the rDNA ITS2 region estimated genetic distances in the range of 0.11-0.25 between the four species [46]. This heterogeneity in ITS2 DNA sequences was further used to develop a PCR-based protocol for molecular identification of the different species within the complex (Figure 11) [46]. These data provided support for the recent taxonomic classification within the *An. nili* complex [24].

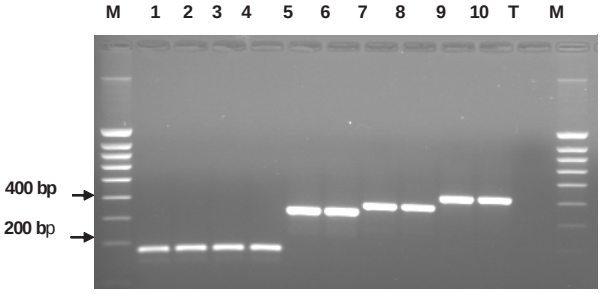

Figure 11. An agarose gel stained with ethidium bromide revealing size differences in the PCR amplification products discriminating between members of the *An. nili* complex: *An. nili* (lanes 1 to 4), *An. somalicus* (lanes 5 and 6), *An. ovengensis* (lanes 7 and 8) and *An. carnevalei* (lanes 9 and 10). Figure from Kengne *et al.*, 2003 [46].

Microsatellite loci were developed in 2003 to allow for more in-depth population genetics investigations [47]. A first comprehensive study explored the level of genetic variability and differentiation between nine populations of *An. nili* distributed in West and central Africa, including samples from Senegal, Ivory Coast, Burkina Faso, Nigeria, Cameroon and the DRC using a set of 11 microsatellite markers and sequence variation in four genes within the nuclear rDNA subunit (ITS2 and D3) and mtDNA (COII and ND4). High genetic homogeneity was revealed among *An. nili* populations distributed from Senegal to Cameroon, suggesting shallow population substructure throughout the humid savannas of West Africa, in agreement with a weak effect of geographic distance [48]. However, the population sampled in DRC was highly significantly differentiated from the core of West African populations (F_{ST}>0.118, P<0.001), and all individuals segregated into a single genetic cluster separated from all other West African populations in Bayesian cluster analysis (Figure 12). Sequence variation in mtDNA genes matched these results, whereas low polymorphism in rDNA genes prevented

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detection of any population substructure at this geographical scale in savannah populations [48]. Extensive allele sharing between populations and homogeneity across microsatellite loci in the level of genetic differentiation suggested that enhanced genetic drift in the DRC population, rather than selection was responsible for the observed pattern.

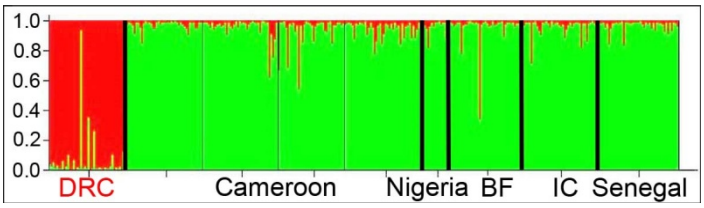

Figure 12. Bayesian genetic cluster analysis of microsatellite allele frequencies in *An. nili s.l.* populations. Genetic homogeneity within savannah populations of *An. nili s.s.* from West/Central Africa and high genetic drift in the DRC population.

In Cameroon, the pattern of genetic differentiation was explored among species within the *An. nili* complex and between populations of *An. nili* collected in different ecological settings including the deep evergreen forest, deforested areas and savannah areas. The average observed heterozygosity varied from 0.359 for *An. ovengensis* to 0.661 for *An. nili s.s.* and mean pairwise F_{ST} over all loci varied from 0.281 (between *An. nili* and *An. carnevalei*) to 0.416 (between *An. somalicus* and *An. ovengensis*) and were highly significant (P<0.0001) [45]. The limited number of loci which could readily amplify and the high proportion of loci departing from Hardy-Weinberg equilibrium in samples collected from the deep forest region suggested the presence of new taxonomic units in this area. Up to seven clusters could be identified in *An. nili* after processing Bayesian cluster analysis (Figure 13). Two of these clusters were specific for *An. nili* populations collected in the East Cameroon forest area, suggesting that *An. nili* from East Cameroon may consist of four new taxa. Data obtained from microsatellites analysis were consistent with the high genetic distance measured with rDNA and mtDNA genes [49].

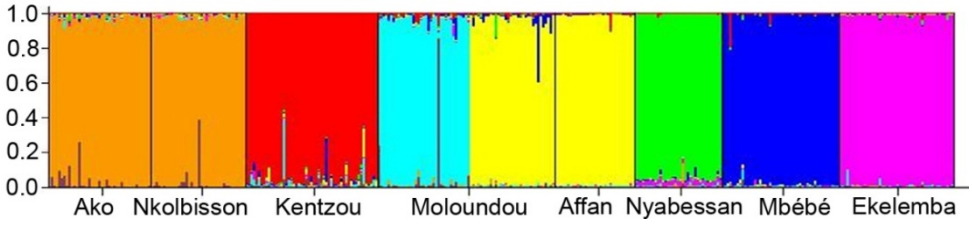

Figure 13. Bayesian genetic cluster analysis of microsatellite allele frequencies in *An. nili s.l.* populations. Genetic heterogeneity between forest populations of *An. nili s.l.* in South Cameroon showing genetic clustering of *An. carnevalei* (yellow), *An. ovengensis* (green), *An. somalicus* (dark blue) and the four genetic clusters suggesting further taxonomic subdivision within *An. nili s.s.* in this area.

Recently, cytogenetic analysis depicted a physical chromosome map for *An. nili* upon which nine microsatellite markers could be mapped (Figure 14) [50, 51]. Chromosomal arm homology with *An. gambiae* was assessed by fluorescent *in situ* hybridization of DNA probes which established that chromosomes X, 2R and 3R are homologous between the two species, while the 2L arm of *An. gambiae* corresponds to the 3L arm of *An. nili*, and vice versa [50]. Preliminary analysis of chromosomal polymorphism in natural *An. nili* populations from Burkina Faso and Cameroon demonstrated that two polymorphic inversions, named 2R*b* and 2R*c*, are often present simultaneously on the right arm of chromosome 2 [50, 51].

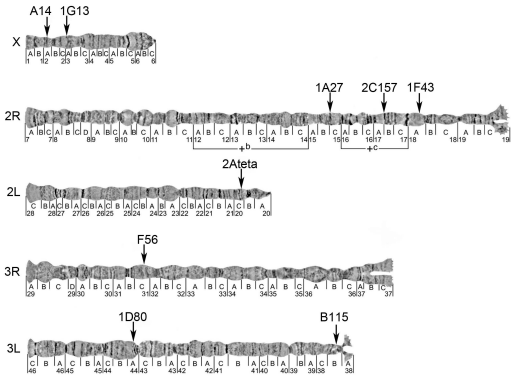

Figure 14. Physical chromosome map of *An. nili* showing the cytological location of the nine microsatellite markers mapped on polytene chromosomes (arrows). Two chromosomal inversions are indicated by brackets. Figure from Peery *et al.*, 2011 [51].

Frequencies of inverted and standard 2R*b* variants were almost equal in the savannah areas of Burkina Faso, albeit with strong deficit in heterozygotes (Fis=+0.603, P<0.0001). In forest areas of Cameroon, only the standard arrangement was found. It is postulated that this inversion may be involved in local ecological or behavioral adaptation in *An. nili* [50]. Inversion 2R*c* occurred at high frequency in Burkina Faso (83%) while its frequency was only 0.6% in samples from Cameroon, suggesting its involvement in ecogeographic cline from dry to more humid environments. Because *An. nili* is a forest-savannah transition species, polymorphic inversions could provide genetic plasticity that allowed its expansion into dry savannah and deforested areas of central Africa, where most of the human population is present. High frequencies of these inversions in savannah areas make them useful markers for studying ecological adaptations of this important vector.

4. Conclusion

Most of the work on malaria vectors has been conducted in the savannah environment, whereas principal vectors and their roles in malaria transmission in the immense African

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rainforest have barely been explored. Therefore, data are crucially lacking for a large part of Africa where malaria transmission is both intense and permanent throughout the year. Recent results demonstrated high levels of differentiation between populations/species of *An. moucheti* and the *An. nili* complex over short geographic distances within the forest block but not in the savannah. These data suggest that, unlike other major vectors, these mosquitoes originated and speciated in the equatorial forest. Because malaria elimination in forested areas is most difficult, detailed understanding of the genetic structure, gene flow, and species diversity of malaria vectors is important. Original information gained on the genetic structure of *An. moucheti* and *An. nili* can further be used to investigate genes for a signature of selection to uncover the genetic mechanisms of ecological adaptations, speciation, and susceptibility to *Plasmodium*, within a comparative framework that will use information available for other major human malaria vectors. Furthermore, because some species/populations within *An. moucheti* and the *An. nili* complex are highly exophagic/exophilic and can bite man as well as other vertebrates in remote areas, they are likely candidates for acting as bridge vectors, providing opportunities for wildlife pathogens to cause zoonosis in humans. These findings raise a concern in the light of recent reports confirming the circulation of various *Plasmodium* species, including strains of *P. falciparum*, in chimpanzees, gorillas, and guenons in the equatorial forest region [52].

Acknowledgements

Part of the work reported in this manuscript was supported by grants no. A00942, A20727, A60347 from the UNDP/World Bank/WHO Special programme for Research and Training in Tropical Diseases (TDR) to C.A.N, a Wellcome Trust Intermediate Fellowship in Public Health and Tropical Medecine (WTO86423MA) to CAN, the NIH grant R21 AI079350, the Pal+ programme from the French Ministry of Research and the French Institut de Recherche pour le Développement (IRD/MIVEGEC).

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