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Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations

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Abstract

Malaria transmission dynamics is highly variable throughout Africa: inoculation rates vary from almost null to more than a 1000 infective bites per year, transmission can occur throughout the year or only during a couple of months, and heterogeneities are also observed between years within the same locale. Depending on the area, as much as five different anophelines species can transmit parasites to the human population. Major vectors are *Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles funestus*, *Anopheles nili* and *Anopheles moucheti*. They all belong to species complexes or groups of closely related species that are very difficult to set apart on morphological grounds. Recent research on the bionomics, morphology and genetics of these mosquito species and populations produced innovative results. New species were described and straightforward molecular identification tools were implemented. We review here these recent findings and discuss research opportunities in light of recent advances in molecular entomology and genomics.

Keywords: *Anopheles*; Vector; Malaria; Africa; Polymerase chain reaction; Microsatellites

Résumé

En Afrique, la transmission du paludisme est extrêmement polymorphe. Selon les zones biogéographiques elle peut être saisonnière courte ou pérenne avec des taux d'inoculation variant de presque 0 à plus de 1000 par an. Jusqu'à cinq espèces d'anophèles peuvent être impliquées simultanément, ou en alternance au cours de l'année. Les vecteurs majeurs appartiennent tous à des complexes ou à des groupes d'espèces. Des recherches récentes sur la biologie, la morphologie et la

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génétique de ces anophèles ont permis de préciser la systématique, le rôle vecteur et la distribution des espèces du complexe *Anopheles gambiae*, des groupes *Anopheles funestus*, *Anopheles nili* et *Anopheles moucheti*. Des espèces nouvelles ont été mises en évidence. Des outils moléculaires d'identification ont été développés, et la structure génétique des populations a été étudiée. Cet article fait le point sur ces résultats récents et les perspectives ouvertes par l'accès à la séquence complète du génome d'*A. gambiae*.

Mots-clé: Anopheles; Vecteur; Paludisme; Afrique; Polymerase chain reaction; Microsatellites

Despite many efforts in basic and applied research, malaria remains, 120 years after *Plasmodium* discovery, one of the major public health problems, particularly in Africa. In the last century, hundreds of studies, often particularly exhaustive, demonstrated the huge variability of transmission patterns across Africa [1–4]:

- Entomological inoculation rates may vary from less than 0.01 to more than 1000 infective bites per man per year,
- Transmission can occur throughout the year or only during 2 or 3 months,
- High variations can be observed depending on the year, or between villages few kilometres apart.

Any strategy aiming at significantly reduce malaria burden in Africa will have to account for this heterogeneity. The whole issue of acquisition (or loss) of protective immunity in humans and its relevance for vaccine development, is indeed directly linked to transmission dynamics, i.e. temporal (seasonal) variations in parasite inoculation rates. The spread of drug resistance genes within and between parasite populations also is a function of transmission intensity, as genetic recombination between different *Plasmodium* strains will be favoured in high transmission intensity foci [5]. Thus, a clear and comprehensive understanding of malaria transmission dynamics is crucially needed in the context of malaria control strategies implementation and development. This would be achievable only through a thorough knowledge of the vectors involved, namely, anophelines mosquitoes. Moreover, vector control itself, whether based on traditional (insecticides and impregnated nets) or genetic methods (sterile males release or introduction of incompetent transgenic mosquitoes), is an important component of malaria control and research.

However, current knowledge of the vector system responsible for malaria transmission remains incomplete. In most locations throughout Africa, several vector species transmit malaria simultaneously, or replace each other seasonally. These vectors differ widely in their density and vector efficiency. Accurate species recognition is therefore required to identify vector species and implement suitable control measures, specifically and selectively directed towards the relevant targets. Moreover, because most mosquito phenotypic traits relevant to the disease epidemiology and/or control (such as feeding preference, susceptibility to infection by *Plasmodium* or insecticide resistance) are likely to be genetically encoded, intraspecific population structure needs to be known and gene flow between populations to be assessed. Recent developments in population genetics and molecular entomology have allowed significant progress in this view. For historical

and practical reasons, most studies have so far focused on *Anopheles gambiae*, the most notorious vector of human malaria in Africa, and almost all ongoing work targets this species complex, particularly since its genome sequence was published [6]. However, *A. gambiae* is not the only one vector in the field and malaria transmission is much more complicated than expected (and generally believed). Targeting only this species, whatever the method of control, is nonsense.

We will briefly describe several contrasting malaria transmission patterns observed in Africa and present up to date results on the bionomics and genetics of the four major African malaria vectors systems: the *Anopheles gambiae* complex and the *Anopheles funestus*, *Anopheles nili* and *Anopheles moucheti* species groups.

1. Malaria transmission

Depending on biogeographic areas and malaria transmission patterns, different epidemiological prototypes have been described in inter-tropical Africa.

1.1. Equatorial regions

It concerns forest and post-forest areas. For biological reasons, malaria transmission is not observed in deep forest: human populations are generally very scarce in such areas and malaria vectors usually develop only in deforested zones or along rivers. Elsewhere, malaria is stable, in the Macdonald sense [7], and transmission occurs throughout the year, although with seasonal variation. Annual entomological inoculation rates (thereafter referred to as EIR and defined as the number of infective bites per man per year) vary between 10 in rural, forested zones [8] and 1000 in densely populated, deforested areas [9]. Very often several vector species, including *A. gambiae*, *A. funestus*, *A. nili* and/or *A. moucheti* can transmit malaria together. Protective immunity against severe cases is generally acquired between 5 and 10 years of age.

1.2. Tropical regions

It concerns humid savannas areas. Transmission season (i.e. the rainy season) lasts about 6 months. Malaria is stable with EIR varying between 50 and 300 [10]. Major vector species are *A. gambiae*, *A. arabiensis*, *A. funestus* and *A. nili*. Protective immunity against severe cases is generally acquired between 5 and 10 years of age.

1.3. Dry tropical regions

It concerns dry savannas areas. The rainy season lasts 2–4 months. The stability of malaria depends on duration and intensity of transmission. EIR vary between 1 [11] to more than 100 [12]. Vectors are *A. gambiae*, *A. arabiensis* and *A. funestus*. Protective immunity against severe cases is generally acquired only in young adults.

1.4. Desert fringe and highlands

Malaria transmission is generally unstable, with EIR frequently under 1 and large annual variations, leading to low-level immunity in resident human populations and epidemic outbreaks of the disease. Vector species are *A. gambiae*, *A. arabiensis* and *A. funestus*, depending on locations [13].

1.5. Urban and other man-modified habitats

Man-made environmental modifications such as deforestation for urbanization or agriculture, flooding through dam construction and/or irrigation of arid lands have created new epidemiological prototypes. In urban centres, EIR vary from 0.01 to a few 10s [14] and rises to several hundreds in irrigated agricultural settings [15], depending on the bioclimatic region, type and intensity of agriculture, socio-economic conditions of locale communities, etc. Vector species typically encountered in these areas are *A. gambiae*, *A. arabiensis* or *A. funestus*.

1.6. Examples of malaria transmission complexity

The heterogeneity and complexity of malaria transmission is well illustrated in villages in Cameroon and in Senegal where in-depth longitudinal studies have been conducted.

The village of Simbock (300 inhabitants) is situated in an equatorial rural forest region of Cameroon, only 2 km from the capital city Yaoundé. A study was conducted from November 1998 to September 2000, using a standardised protocol for collecting and analysing mosquitoes [16]. Malaria vectors were *A. funestus*, *A. gambiae* s.s. (M and S forms), *A. moucheti* and *A. nili*. *A. moucheti* was the most abundant mosquito captured during the study, accounting for over 54% of total anophelines caught. The annual *Plasmodium falciparum* EIR measured by enzyme linked immunosorbent assay (ELISA, [17]) was 277 for the first year and 368 for the second year. *A. gambiae*, *A. funestus*, *A. moucheti* and *A. nili* were responsible for 23.8, 26.8, 39.2 and 10.2% of malaria transmission, respectively. As shown on Fig. 1 malaria transmission is perennial throughout the year, with high seasonal variation, in terms of intensity and implication of the different vector species.

The village of Dielmo (250 inhabitants) is situated in a dry tropical rural region of Senegal, on the marshy bank of a small permanent stream which permits the persistence of anophelines larval development sites all year round. A 3-years study was conducted from April 1992 to March 1995, using a standardised protocol [18]. Malaria vectors were *A. gambiae*, *A. arabiensis*, and *A. funestus*. The entomological inoculation rate for the three vectors varied greatly according to the month, with a peak of transmission during and at the end of the rainy seasons, from July to September (Fig. 2). *P. falciparum* EIR was 233, 79 and 135 for the first, the second and the third year, respectively. Great variations in the entomological components of transmission were observed, such as the human biting rate (HBR), the infection rate, as well as the number and relative proportion of the three vectors over the 3 years. The first year *A. funestus* had a much greater effect on transmission than *A. gambiae* and *A. arabiensis*. This is due to two factors: a higher HBR and a higher infection rate due to its longer life expectancy and its higher anthropophilic

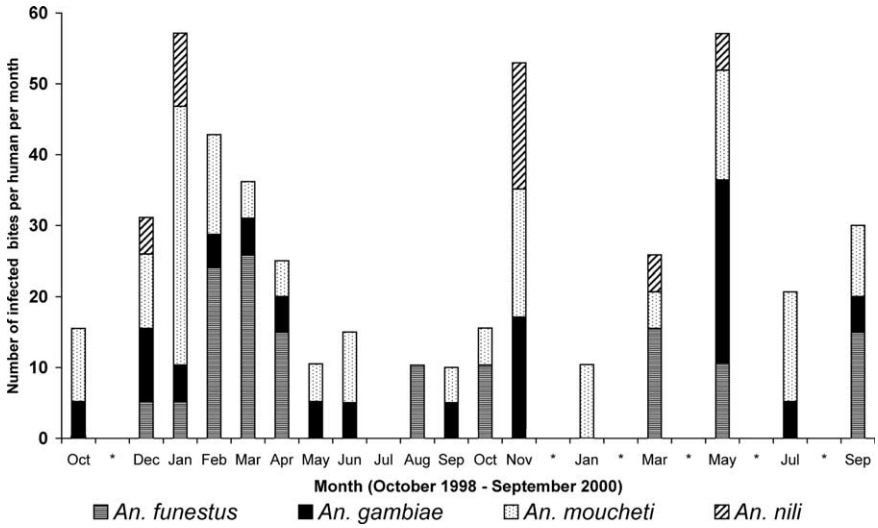

Fig. 1. Monthly entomological inoculation rate for each vector species in Simbock, Cameroon (from Ref. [16]).

rate (i.e. marked preference to feed on humans rather than other vertebrates). The role of *A. funestus* in transmission was particularly significant since it was the main vector during the dry season, therefore ensuring transmission throughout the year. It was responsible for 77, 26 and 34% of *P. falciparum* transmission for the first, the second and the third year, respectively. The second and the third year *A. arabiensis*, which is generally considered to be a less efficient vector, was the major vector due to its very high HBR and despite its low infection rate. *A. arabiensis* ensured, respectively, 8, 61 and 60% of *P. falciparum* transmission over the 3 years. Transmission by *A. gambiae* was the lowest. This species was responsible for only 15, 13 and 6% of the transmission for the first, the second and the third year, respectively.

2. Malaria vectors

The biology of the main African malaria vectors has been known in their broad lines for more than 50 years [7]. The description and identification of vector species was based on morphological characters, and sub-divisions called sub-species, form, variety, race, etc. have been described depending on distribution, biology, behavior, and slight morphological differences. As early as the beginning of the 20th century it became evident that in many cases, isolated genetic entities belonged to the same morphological 'species'. It is the definition of a species complex, following Mayr [19]: 'morphologically similar or identical natural populations that are reproductively isolated'. The two most famous examples are the *A. maculipennis* complex with at least nine species in Europe [20] and the *A. gambiae* complex with seven species in Africa. Very often, efficient malaria vectors and nonvectors species are found within the same complex. It is then crucial to be able to identify all these species properly for an accurate vector control.

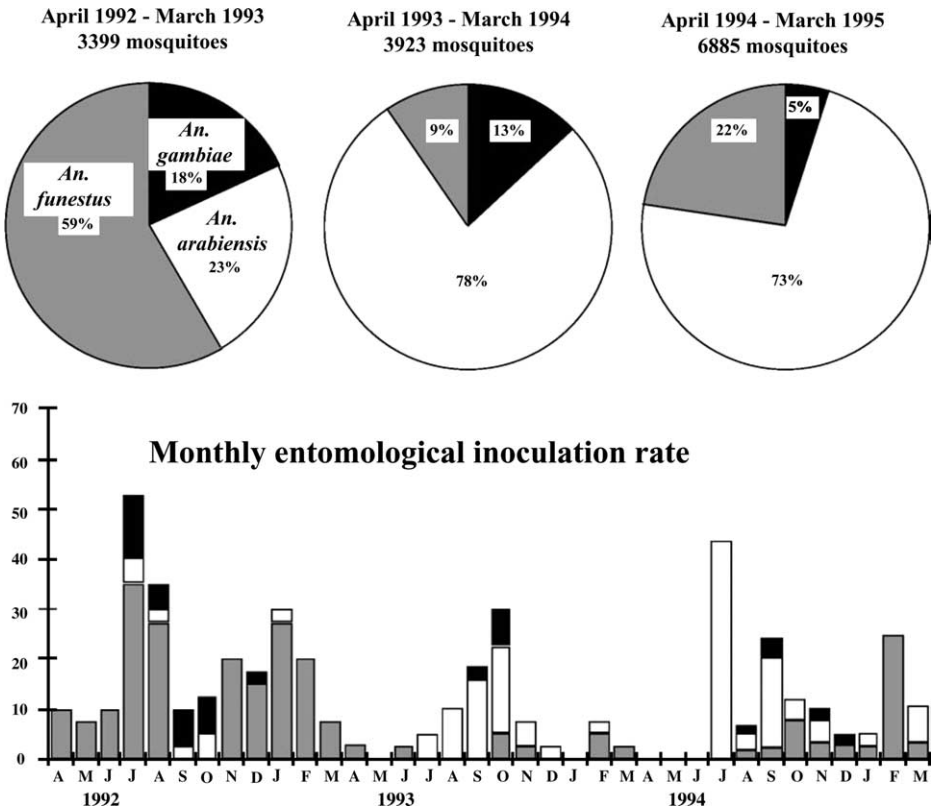

Fig. 2. Percentage of malaria vector species depending on the year and monthly entomological inoculation rate in Dielmo, Senegal (from Ref. [18]).

In Africa five different species are considered as major vectors: *A. gambiae*, *A. arabiensis*, *A. funestus*, *A. nili* and *A. moucheti*. At least eight or nine other species are also secondary or locally important vectors, such as *A. paludis* in Democratic Republic of Congo (DRC, a.k.a. Zaire) [21], *A. mascarensis* in some locations in South-East Madagascar, *A. hancocki* in Cameroon [22,23], *A. pharoensis* in Egypt [24], *A. melas* and *A. merus*, two halophilic mosquitoes from the *A. gambiae* complex, in some coastal regions of West [25,26] and East Africa/Madagascar [27], respectively.

2.1. The Anopheles gambiae complex

Initially regarded as a single species with ecological salt–water variants, the *Anopheles gambiae* complex has now been split into seven distinct species, including two of the most efficient human malaria vectors worldwide: *A. gambiae* sensu stricto and *A. arabiensis*.

Other recognised species of the complex are *A. melas*, *A. merus*, *A. bwambae*, *A. quadriannulatus* and *A. quadriannulatus* B, recently described from Ethiopia [28]. These five species have only limited or no role at all as malaria vectors, due to restricted geographic distribution and/or zoophily. *A. melas* and *A. merus* are salt–water species that

only develop in mangrove swamps along the West and East coast of Africa, respectively. *A. bwambae* is known from a single location in Uganda where its larvae develop in heavily mineralised water springs. Both species of *A. quadriannulatus* (still referred to as *A. quadriannulatus* A in southern Africa, and B in Ethiopia) are mostly zoophilic and therefore not involved in the transmission of human parasites. On the other hand, both *A. gambiae* and *A. arabiensis* have wide geographic distributions throughout sub-Saharan Africa and surrounding islands. They coexist widely over much of their range, although *A. gambiae* is usually predominant in humid environments while *A. arabiensis* is found in drier areas [29].

Both species appear highly dependent on humans for their feeding, resting and, to a certain extent, breeding habits [30–32].

Reproductive isolation between species of the *A. gambiae* complex was established through straightforward crossing experiments, leading in most cases to sterility of F1 (heterogametic) males with various degrees of abnormality of the reproductive system (ranging from complete atrophy of testes to partial spermatogenesis), sometimes associated with distorted sex ratio in the progeny [33]. The first recorded mass-cross between populations of *A. gambiae* was that of Muirhead–Thomson [34] between salt- and fresh-water 'populations' from Nigeria, leading to recognition of *A. melas* as a formal species. First evidence for genetic heterogeneity within fresh-water *A. gambiae* was obtained in 1956 by Davidson [35] during the course of a study on the mode of inheritance of dieldrine resistance that led to the split of *A. gambiae* A (later called *A. gambiae* s.s.) and B (later *A. arabiensis*). By 1964, five species were recognized (including *A. melas*, *A. merus* and *A. gambiae* sp.A, sp.B and sp.C $=$ *A. quadriannulatus*). *Anopheles bwambae* (formerly species D) was described in 1973 [36] and both species of *A. quadriannulatus* were finally split in 1998 [28].

However, all these species are morphologically identical (or nearly so) and no satisfactory morphological character has been found that allow reliable and reproducible identification of single specimens using ordinary taxonomic methods. Although meristic characters for separating the species at the population level have been demonstrated [37,38], compatible crosses with laboratory-reared reference 'mating types' was the only way for identification. The study of the banding pattern of giant polytene chromosomes, observable from ovarian nurse cells of adult females at their half-gravid stage (i.e. during blood digestion and egg maturation), provided the first diagnostic tool for accurate species identification within the *A. gambiae* complex [39]. Fixed paracentric inversions between members of the complex were evidenced and served for diagnosis but, the technique was limited to half-gravid female specimens. Following development of molecular biology and implementation of the polymerase chain reaction (PCR) technique in particular, a PCR-based diagnostic tool was designed on the basis of species-specific sequence differences in the ribosomal DNA intergenic spacer (rDNA-IGS) region [40]. This very convenient tool allows rapid and reproducible identification of field-caught specimens from both sexes, at all their developmental stage and/or gonotrophic state, and from very few starting material (such as a leg or a wing).

Although chromosomal differences between species are based on fixed paracentric inversions, further cytological studies in *A. gambiae* and *A. arabiensis* uncovered a complex system of polymorphic paracentric inversions leading to different chromosomal

arrangements [39]. Frequencies of alternative arrangements (i.e. karyotypes), especially involving inversions on chromosome 2, were shown to correlate with ecological factors such as the degree of aridity of the environment, suggesting an adaptive value for inversions [39,41–43]. Furthermore, extensive studies of karyotype distributions in natural *A. gambiae* populations often revealed strong and persistent deviations from Hardy–Weinberg equilibrium due to a deficit or even complete absence of certain heterokaryotypes. These results led to the designation, in West Africa, of five 'chromosomal forms' named under the nonLinnean nomenclature Forest, Savanna, Mopti, Bamako and Bissau [39,41,43]. Each form has been described by combinations of inversions on chromosome 2 and appears highly specialised in its habitat. The Forest form for example, is almost fixed for the standard arrangement (no inversion) on both arms of chromosome 2 and is found in humid forested areas, whereas the Mopti form, characterised by arrangement 2Rbc/u and 2La, is found in dry to arid savannas where it breeds all year long in irrigated fields.

Analysis of the rDNA-IGS region revealed fixed sequence differences between sympatric and synchronous Savanna/Bamako and Mopti populations in Mali and Burkina Faso [44,45], and led to the designation of two nonpanmictic molecular forms, named M and S [46]. Both molecular forms are found throughout West and Central Africa, while only the S form has been reported from East Africa and Madagascar [47]. All Mopti specimens identified so far belong to the M molecular form, however, outside Mali and Burkina Faso, the M form may exhibit chromosomal arrangements typical of the Bissau, Forest or Savanna forms. The S molecular form as well may carry standard chromosomes, indicative of the Forest form, or typical Savanna and Bamako karyotypes. In addition to the extreme scarcity of M/S hybrids reported from areas where both forms occur, evidences for reproductive isolation between molecular forms have accumulated to a point that incipient speciation is being suggested [47–49]. For example in south Cameroon, a population genetics study based on microsatellite DNA markers, demonstrated significant genetic differentiation between sympatric M and S populations, within the (standard) Forest chromosomal form of *A. gambiae* [49]. The biological significance of this genetic subdivision, its putative effect on vectorial capacity and its overall relevance for malaria transmission epidemiology and control are currently under investigation.

This broad area for future research will benefit from the recently available whole-genome sequence of *A. gambiae*, published in October 2002 [6]. Outstanding perspectives for both basic and applied research indeed led a consortium of laboratories to join efforts to achieve sequencing and annotation of the genome of this major pest species. A shotgun approach was used and resulted in complete assembly of 278 millions base pairs (c.a. 91% of the genome), organized in 303 scaffolds (fragments of continuous DNA sequences). Around 14,000 putative genes were estimated to occur throughout the sequences analyzed. Predicted proteins were classified according to protein domains and homologies into several functional categories, including gustatory or odor receptors, bloodmeal digestion and metabolism, immunity, insecticide resistance. Detailed study of the polymorphism of theses genes in natural populations using high throughput genotyping methods, comparative genomics and state-of-the-art bioinformatics tools will result in a better assessment of their phenotypic effect on the biology and role in malaria transmission of this mosquito, and will undoubtedly lead to the discovery of new targets for efficient,

selective and specific vector control to be implemented in the fields in combination with existing strategies.

2.2. The A. funestus group

A. funestus is widespread throughout sub-Saharan Africa and Madagascar. It is known since the 1930s that this group is composed of several species closely resembling each other, which can only be differentiated by very small morphological characters on their larvae or adults. *A. funestus*, *A. confusus*, *A. leesoni*, *A. rivulorum* and *A. brucei* can be identified at the larval stage, while species of the sub-group *funestus*—i.e. *A. funestus*, *A. parensis*, *A. aruni*, and *A. vaneedeni*—can be identified by small morphological differences observable at the adult stage only [31]. Their biology and their vectorial capacity are very different. With the exception of *A. funestus*, these species are mainly zoophilic. Human *Plasmodium* have only been found in *A. funestus*, which is a very efficient vector, and rarely in *A. rivulorum* in Tanzania [50]. Experimental transmission was obtained in *A. vaneedeni* [51]. In 2003, Cohuet et al. [52] have described a new taxon closely related to *A. rivulorum*, based on biological, morphological and genetic characters. The species, provisionally called '*A. rivulorum*-like', is present in Burkina Faso [53] and Cameroon, and is clearly different from South African *A. rivulorum*. This new taxon does not seem to play any role in malaria transmission.

Acurate species identification is thus highly relevant within this group, to avoid misidentification of the dangerous taxon, namely *A. funestus*. For example, in Tanzania and South Africa, indoor spraying was used to eliminate *A. funestus*. However, some specimens persisted suggesting failure of the control program. Subsequent careful identification revealed that these mosquitoes were in fact *A. parensis*, *A. rivolurum* or *A. vaneedeni*, which hardly ever transmit human *Plasmodium* [51]. Zoophilic and exophilic habits probably reduced exposure to insecticides in this case. More recently, *A. parensis* was almost the only one member of the *A. funestus* group found resting inside human dwellings in a village of Kenya [54].

Since 1998, different molecular biology techniques have been developed for species identification within the *A. funestus* group [55,56]. Subsequent methodological upgrades led to the implementation of a convenient multiplex PCR assay based on the selective amplification of species-specific ITS2 rDNA haplotypes [52,56]. This new tool now enables straightforward identification of six species within the *A. funestus* group.

The species *A. funestus* itself is very polymorphic, biologically and genetically. Cytogenetic studies conducted from Senegal to Madagascar, have shown that the species presents at least 11 paracentric chromosomal inversions on chromosomes 2 and 3 [57–60]. In Senegal, *A. funestus* populations with different chromosomal arrangements showed different anthropophilic rates [57] and in Burkina Faso, specimens with inverted karyotypes were found in higher frequencies in indoor, human-fed samples [61]. Inversions therefore, could be valuable markers of vector ability, because carriers of different chromosomal arrangements could be more or less prone to become infective. Huge Hardy–Weinberg disequilibrium and linkage disequilibrium between inversions observed in populations from Burkina Faso led Costantini et al. [61] to described two chromosomal forms that they called 'Kiribina' and 'Folonzo', based on the presence

and association of paracentric inversions. The strong lack of 'hybrid' heterokaryotypes in areas where both forms are present led these authors to hypothesize incipient speciation within *A. funestus*.

In Senegal, three chromosomal populations were recognized. In the village of Kouvar two of these forms are sympatric, and the very strong deficit of heterokaryotypes observed suggests, like in Burkina Faso, the presence of two genetically distinct populations [60]. In Cameroon, northernmost populations are related to the Kiribina form, and to the Folonzo form in the south. A cline of inversion frequencies is observed from the humid forest in the South to the dry savannas in the North, with strong heterozygote deficiency in areas where both forms occur. All these data suggest restricted gene flow between chromosomal forms of *A. funestus* (Cohuet et al. unpublished results).

However, on the other hand, several observations from Cameroon, Kenya [62], Angola [63] and Madagascar (Le Goff et al., unpublished results) detected no evidence for reproductive isolation between Folonzo and Kiribina, with heterokaryotypes observed at their expected frequencies in the population.

Recent development and use of microsatellite markers [64–66], which are supposed to be neutral, allowed to revisit the speciation hypothesis. Population genetics studies were conducted in Senegal and in Cameroon using a set of nine microsatellite markers spread over the entire genome of *A. funestus*. Results suggested that gene flow is permitted between chromosomal forms. No evidence for population subdivision was obtained in samples where strong deficits in heterokaryotypes were observed. Isolation by distance between geographical populations was nonetheless detected, confirming the ability of microsatellite markers to detect population subdivision (Cohuet et al. unpublished results).

These results strongly suggest that heterozygote deficits at chromosomal loci are mostly locus-specific and reflect some kind of environmental selection on the inversions themselves (or the genes they contain) rather than population subdivision or incipient speciation. In other words, gene flow and reproduction seem to occur between chromosomal forms of *A. funestus*, although specimens with hybrid karyotypes may be viable under certain ecological conditions only. However, too few data are available to date to draw any firm conclusion in this regard. Care should thus be taken to account for this high level of genetic and behavioural polymorphism when dealing with the species *A. funestus*.

2.3. The A. nili group

A. nili has a wide geographic distribution, spreading across most of tropical Africa [67]. Larvae of *A. nili* are typically found in vegetation or in dense shade along the edges of streams and large rivers. Extensive morphological, ecological, and ethological variations among *A. nili* populations have been reported by many authors [30,31,68,69] suggesting that *A. nili* is a group of species. Based on such observations, three species were described within this group: *A. nili* s.s., *A. somalicus*, and *A. carnevalei* [69]. Awono-Ambene et al. [70] recently described an additional species discovered from forested areas in southern Cameroon. This new species was called *A. ovengensis*, from its type locality.

A. nili has been reported throughout inter-tropical Africa, mainly in humid savannas areas. Sporozoite rates reaching 3% have been observed in *A. nili* and annual EIR over 100

were recorded [68]. A recent study conducted in a village in Eastern Senegal has shown that this species, although neglected until now, was responsible for 56 infected bites per human per year in this area [71]. *A. carnevalei* is known from Cameroon and Cote d'Ivoire only. However, there is no doubt that this species has a much larger distribution area in humid tropical and equatorial Africa. Females infected by *P. falciparum* were captured biting humans at night, demonstrating anthropophily and vector ability for this species. To date, very few data are available on the recently described *A. ovengensis*. Females have been captured biting humans with a HBR of 50–300 per night, alongside rivers in forested areas of South Cameroon. This species was captured very rarely resting inside houses, suggesting exophilic behaviour. Sporozoite rates between 0.4 and 1.9% have been recorded, demonstrating that it is a malaria vector. Almost nothing is known from *A. somalicus*, which seems to be exophilic and zoophilic, and thus not involved in human malaria transmission [72].

Distinction between members of the *A. nili* group is often difficult in the field, because of very slight diagnostic differences between species at the larval and/or adult stages. Morphological identification is made even more difficult when specimens are not well preserved. As a result, the distribution, the biology and the role in malaria transmission of each of these species is largely unknown.

To assess relevance of morphological characters as an accurate means for classification within the group *A. nili*, sequence variation in the rDNA ITS2 and D3 domains of the four species of this group was investigated [73]. Ribosomal DNA sequence analysis was in full agreement with morphological classification. Four different clusters, corresponding each to one species of the group, were obtained after analysis of both the rDNA ITS2 and D3 domains. Genetic distances between ITS2 consensus haplotypes for each of these four species were in the range 0.11–0.25, a value much higher than expected between populations within the same species [74], and similar to those observed within the *A. funestus* group [53,56], or between members of the North American *A. quadrimaculatus* complex [75]. Based on fixed nucleotide differences between ITS2 haplotypes, primers were designed to develop an allele specific PCR assay for rapid identification of species within the *A. nili* group [73]. This technique allows accurate identification of single mosquito specimens at all developmental stages, even from badly preserved adults or from larvae kept in alcohol. This innovative tool will undoubtedly reveal very useful to increase current knowledge on the distribution, biology, and role in transmission of the four species of the *A. nili* group in Africa.

2.4. The A. moucheti group

Mosquitoes from the *A. moucheti* group are forest mosquitoes, which larvae develop in slow moving streams and large rivers of Equatorial Africa, from Guinea to Uganda and the south of Sudan, even though this mosquito was also repeatedly found in Namibia [30].

This mosquito is a very efficient vector of *Plasmodium* with sporozoite rates up to 4%. In the forest regions, in villages of thousands of inhabitants, *A. moucheti* is quite often the major vector [16,76], and sometimes the only one, with an annual EIR reaching 300 [77]. However, very few studies have been carried out on *A. moucheti*, despite its

epidemiological importance as a malaria vector. Most studies and observations go back to the 1960s [67,78–81].

Morphological and behavioural variations observed among natural populations suggested that several taxa may belong to the *A. moucheti* group: *A. moucheti moucheti* sensu stricto, *A. moucheti nigeriensis* and *A. bervoetsi*, reported only from the Democratic Republic of Congo [30]. Brunhes et al. [82] considered that *A. moucheti moucheti* and *A. moucheti nigeriensis* are synonymous and that *A. bervoetsi* is a sub-species (i.e. a geographical population) of *A. moucheti*. Recent data from Cameroon based on isoenzyme markers and the study of inheritance of morphological characters in F1 progenies obtained from field collected females demonstrated that all three forms belonged to the same gene pool and can be considered as morphological variants of a single species, at least in this area [83].

However, despite results from Cameroon, taxonomic issues within the group *A. moucheti* remain poorly understood. We have compared ITS1, ITS2 and D3 sequences from the rDNA, as well as mitochondrial DNA Cytochrome b sequences from females captured in Cameroon, Uganda and Democratic Republic of Congo. Specimens from Cameroon showed a low level of nucleotide diversity, without any correlation with morphological patterns, emphasizing genetic homogeneity for this species in this region. Specimens from Uganda appeared very close genetically from Cameroonian samples ($d = 0.001$), despite high geographical distance between sampling sites. These results suggested that both populations belong to the same species. On the other hand, sequences from DRC were very different from those of Cameroon, with genetic distances reaching 0.15 for the ITS1 region, a value generally observed between established species. Moreover, preliminary results based on recently developed microsatellite markers [84] also showed huge differences between DRC and Cameroonian populations, suggesting that they may represent two different species. At this stage, an allele specific PCR assay has been implemented to allow rapid identification of each 'molecular form' of *A. moucheti*, explore their respective geographic distribution and assess their importance as malaria vectors throughout their range. Fine scale population genetics studies using microsatellite markers are actually ongoing to further question the issue of speciation within the *A. moucheti* group, and the cytogenetic map of *A. moucheti*'s chromosomes is being established.

3. Conclusions

The huge diversity of African ecosystems, and recent anthropic modifications they undergo, generate a large number of malaria figures to the point that each malaria situation may appear as unique. Systematics of malaria vectors reflects this diversity, and is obviously incidental to it. Comprehensive knowledge of behavioural and underlying genetic heterogeneities that exist within and among natural vector populations will thus benefit the whole area of malaria control and epidemiology. Molecular and genetic studies, as well as in depth monitoring of vectors biology, show that the situation is more complex than expected based solely on morphological observations. True cryptic species exist among *A. gambiae*, *A. nili* and *A. moucheti* complexes. *A. gambiae* populations are well

structured, with M and S incipient species between which gene flow is highly restricted. Chromosomal polymorphism within *A. gambiae* and *A. funestus* very likely reflects adaptive responses to various environments, but the exact role of inversions in determining vector ability and/or prompting speciation within vector species still requires further investigation.

Current ongoing studies try to answer to the very simple question: what's a malaria vector? If several parameters quantifying vectorial capacity of a mosquito population are well known and can be easily assessed through standardized experimental protocols, such as the antropophilic rate or life expectancy of locale vector populations, these are clearly not sufficient. Why and how a given mosquito transmits malaria parasites remains only superficially understood and research has now to shift from pure descriptive studies towards explorative monitoring and assessment of the mechanisms involved in *Plasmodium*/mosquito/human interactions.

The recent publication of the nearly complete genome sequence of *A. gambiae* together with ongoing developments in functional and comparative genomics should allow significant progress in our understanding of the mosquito-parasite and mosquito–human relationships [6].

Host seeking behaviour and immunity in mosquitoes are good examples of areas which can greatly benefit from recent advances in genomics.

Bloodmeal acquisition is the endpoint of a very complex cascade of metabolic and physiologic processes (activation, recognition, orientation, landing, probing, seeking), produced in response to different stimuli (odour, moisture, temperature, sound, etc.). All these responses have genetic bases. Search for conserved molecular signatures and/or orthologs of *Drosophila* genes in the *A. gambiae* genome sequence uncovered 79 putative odour receptors and 76 putative gustatory receptors genes [85]. The precise role of these G protein-coupled receptors (GPCRs) is not know yet, but they nonetheless represent very promising candidates for unravelling the complex mechanisms shaping feeding preferences and biting behaviour of this mosquito. New targets for innovative vector control are likely to be identified through extensive characterization of such effectors.

Insect immunity is fairly well documented [86–88]. It is an innate response that differs from the adaptive response of vertebrates, insects being incapable of mounting highly specific antibody responses or producing memory cells. In *A. gambiae*, as well as in other insects, immunity can be divided into cellular and humoral responses. Cellular immunity includes melanotic encapsulation and phagocytosis by hemocytes, while humoral immunity is related to the production of more or less specific antimicrobial peptides. Several lines of evidence suggest that malaria parasites are detected by the mosquito's immune surveillance system, including considerable numerical loss during parasite development in its host and transcriptional activation of immune response genes in the mosquito following infection [89–92]. Although *Plasmodium* development does not kill the mosquito host, depleting fitness effects of malaria infection have been suggested [93,94]. Moreover, inbred mosquito lines have been selected for refractoriness to *Plasmodium* development following challenge with malaria parasites [95,96] and naturally occurring refractory mosquitoes and/or gene alleles were observed in the fields [97,98]. All this body of knowledge suggests finely-tuned specific interactions between the parasite and its hosts, shaped by thousands of years of co-evolution.

A. gambiae immune response is a stepwise process that involves a number of effectors: recognition of the pathogen(s) by 'specific' receptors (i.e. pattern recognition receptors (PRRs)) triggers activation of signalling pathways and enzymes cascades that eventually lead to the killing of the pathogen by various mechanisms such as lysis mediated by antimicrobial peptides, encapsulation or phagocytosis [88]. New candidate genes encoding putative effectors involved in these processes are identified almost on a daily basis [99–103]. Development of molecular tools, availability of partial or complete genome sequences and expressed sequence tags (EST) collections, have greatly enhanced our ability to investigate mosquito–parasite interactions. DNA microarrays technologies, expression profiling analysis, high throughput genotyping methods and whole-genome comparison will help to decipher biological pathways associated with vector competence and to define the key aspects of the mosquito immune response.

These recent advances allow us to envision innovative and complementary vector control methods, which could reinforce current tools such as impregnated materials. However, considering the very high diversity of malaria transmission and vector populations in Africa, long-term field studies will be necessary before results of the post genomic area translate into field oriented strategies to be implemented in the 'real word'.

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