Chapter 7

Advances and Perspectives in the Study of the Malaria Mosquito *Anopheles funestus*

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Additional information is available at the end of the chapter

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1. Taxonomy, biology and distribution of the species within the Funestus Group

1.1. Introduction

Anopheles funestus Giles, 1900 is considered one of the most proficient malaria vectors worldwide [1]. It thrives in a wide range of habitats through the Afrotropical Region. Largely neglected with regard to its counterpart *Anopheles gambiae, An. funestus* cannot be ignored in any comprehensive control program aiming at the eradication of malaria from the African continent. Its transmission role goes beyond that of secondary vector, surpassing *An. gambiae* in many parts of Africa [2]. One of the main reasons of this inattention is the difficulty of adapting this species to standard insectary conditions, despite noteworthy molecular and epidemiological advances over the past three decades. Currently, substantial evidence shows that a group of species belongs to the taxon "*An. funestus*", with different morphological, behavioural and epidemiological characteristics.

1.2. The Funestus Group

The term "Funestus Group" was first coined in its strictest sense by Gillies and De Meillon [3] to designate a group of species morphologically close to *An. funestus*. Seventy years after the first description of *An. funestus sensu stricto* (hereafter *An. funestus*) by Giles in 1900, Mick Gillies and Botha De Meillon developed a new classification based on larva, pupa and adult stages. In fact, first suspicions of the existence of heterogeneity within *An. funestus* populations came from the early 1930's [4, 5]. They stated, based on larval studies, the presence of '*varieties*', most of them were subsequently recognized as species within the group. These species showed

minor or no morphological differences at adult stage. They were then classified under the Funestus Group and their recognition was based on the identification of eggs, larvae or pharyngeal armature [3]. However, in Southern and Eastern Africa, several populations of outdoors resting mosquitoes were distinguishable from *An. funestus* by small morphological characters at the adult stage, while the larva were indistinguishable. These taxonomical observations were later confirmed by cytogenetic studies as different species of *An. funestus* [6-8].

Given the laborious nature of morphological and cytogenetic techniques, several studies were undertaken for the research of simple and useful molecular identification tools [9-12]. These techniques have the advantage to be applicable to all developmental stages. On the basis of morphological [13, 14] and molecular studies [15, 16], the status and position of each species within the Funestus Group was revisited. It is now accepted that *An. funestus* belongs to a group composed of five subgroups of which 3 groups containing 13 species are present in the Afrotropical region (Table 1) [17].

		African species of the Funestus Group		
Subgroup	**Species**	**Geographical distribution**	**Host preference**	**Vector role**
Funestus	*An. funestus*	continental	anthropophilic	major
	An. funestus-like	local	unknown	unknown
	An. aruni	local	unknown	unknown
	An. confusus	regional	zoophilic	unknown
	An. parensis	regional	unknown	minor
	An. vaneedeni	local	unknown	unknown
	An. longipalpis type C	local	zoophilic	unknown
Minimus	*An. leesoni*	continental	zoophilic	minor
	An. longipalpis type A	local	zoophilic	unknown
Rivulorum	*An. rivulorum*	continental	zoophilic	minor
	An. rivulorum-like	local	unknown	unknown
	An. brucei	local	unknown	unknown
	An. fuscivenosus	local	unknown	unknown

Table 1. Summary of ecological characteristics of Funestus Group in Africa.

1.3. Geographical distribution

Among the species of the Funestus Group, *An. funestus*, *An. leesoni* and *An. rivulorum* exhibit the widest distribution. They are traditionally represented throughout the entire sub-Saharan Africa [1, 3]. Figure 1 presents the predicted distribution of these species [11, 12]. *Anopheles funestus* is found virtually all across the continent (Fig. 1A). Being predominantly a savannah mosquito [18], this malaria vector is present in many other areas, such as high altitude zones (900 m in Madagascar [19], 1400 m in Central Africa [20] and up to 2000 m in Kenya [21]) and forested areas of West and Central Africa [18, 22-25]. Moreover, it can inhabit extreme dry

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conditions in the Sahel, when suitable breeding place are available, such as human-made irrigation zones [26, 27]. On the other hand, *An. funestus* is scarce or completely absent along the coast [18]. *Anopheles funestus* disappeared from several parts of Africa after adverse climatic conditions (i.e recurrent droughts) and/or vector control programs [28]. Unfortunately, this mosquito gradually re-emerged once control measures stopped or suitable environmental conditions re-appeared [29-32], evidencing its extraordinary environmental plasticity and dispersion ability.

The other species of the group exhibit locally defined distribution (Fig. 1B, C). *Anopheles parensis*, *An. confusus* and *An. aruni* are localized in East Africa [33, 34]. In West and Central Africa, we find *An. rivulorum*-like and *An. brucei* [11, 12]. Finally, in Southern Africa, we find *An. vaneedeni, An. parensis* again, *An. fuscivenosus, An. funestus*-like and *An. longipalpis* types A (South Africa) and C (Zambia) [1, 35, 36]. Certainly, these records are based on sampling efforts, and we might expect changes in the number of species within the group as well in their distribution.

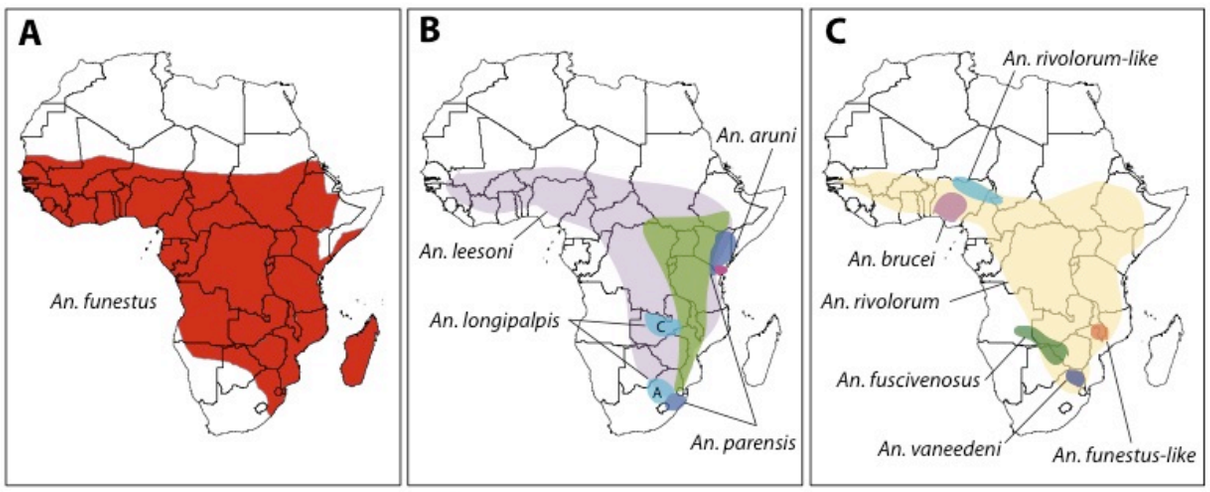

Figure 1. Distribution of the 13 species of the Funestus Group in Africa, A: *Anopheles funestus*, (modified from [37]); B: *An. leesoni*, *An. longipalpis* (type A and C), *An. aruni* and *An. parensis* (Courtesy of Dr. S. Manguin), C: *An. rivolorum, An. rivolorum-like, An. funestus-like, An. vaneedeni, An. fuscivenosus* and *An. brucei* (Courtesy of Dr. S. Manguin).

1.4. Breeding place

Anopheles funestus breeds in natural/artificial permanent and semi-permanent water bodies with floating or emerging vegetation. However, in areas with both vegetation types, this mosquito prefers the latter one [3]. Natural breeding occurs in edges of swamps, in weedy and grassy parts of rivers, streams, furrows, ditches and ponds. The presence of vegetation is crucial for mosquito breeding (Fig 2. A-C). Mainly because aquatic stages have a marked preference for shaded habitats and can barely survive in water bodies directly exposed to sunlight. Artificial breeding opportunities include rice fields, wells and domestic water-containers [3]. The main limiting factors to their development include salinity, extreme

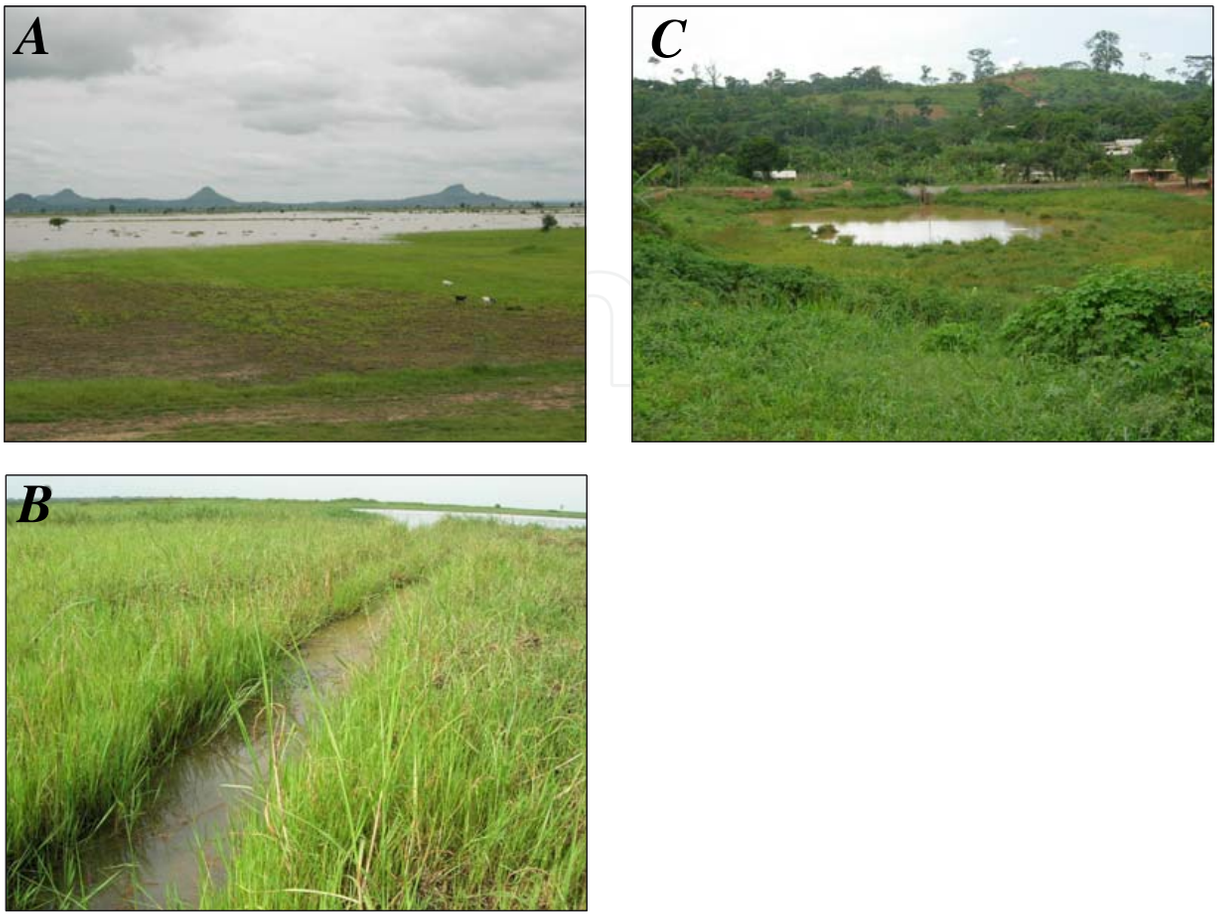

Figure 2. Breeding sites of *Anopheles funestus* (Photos D. Ayala, Cameroon). *A:* Pitoa (Cameroon) is situated in the northern dry savannah, close to a permanent human-made lake, which provides a year-round breeding site for *An. funestus*. *B:* Tibati (Cameroon) is located in the central highlands of the country. *Anopheles funestus* breeds year-round in the lake, which provides shaded areas thanks to the lake vegetation. *C:* Mfou (Cameroon) is situated in the southern rainforest, in the surroundings of Yaoundé. The artificial water-body provides an excellent breeding site for *An. funestus*, making it the major vector of the village.

temperatures and sometimes, heavy rains. For the other species within the Funestus Group, the biology of aquatic stages is poorly understood. The larva of *An. leesoni*, *An. rivulorum* and *An. vaneedeni* are often found in association with those of *An. funestus*. In Kenya, *An. rivulorum* replaced *An. funestus* in rice fields after indoor residual spraying [38]. The presence of vegetation appears to be essential too. These breeding sites are represented generally by slow-moving backwaters of grassy rivers and tide pools. In western Kenya, larva of *An. rivulorum* were recently found in hyacinth water protected by trees [39]. Similarly, *An. parensis* develops in permanent swamps and ponds between the reeds and the emergent vegetation. However, *An. parensis* is a species of stagnant water that has never been found in rivers. The larva were always collected in marshes, temporary and permanent ponds, among reeds and emerging vegetation [1, 3]. *Anopheles aruni* breeds in ponds, rice fields or ditches near human habitations. Larva of *An. brucei* were found in streams of forested river beds. A*nopheles confusus*, on the other hand, breeds in the vegetation of the edges of slow flowing rivers. *Anopheles longipalpis*

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prefers relatively calm water with abundant aquatic vegetation on the banks of fast-flowing rivers [3]. In many occasions, breeding places are very similar to *An. funestus*. Unfortunately, no information exists about breeding places for *An. fuscivenosus*, *An. rivulorum*-like and *An. funestus*-like [1, 3, 36, 40].

1.5. Resting behaviour and host feeding preference: Their impact on vector capacity

Despite the morphological similarities that exist between members of the group, these species show extreme behavioural differences that affect their vectorial capacities. To date, all malaria transmission studies have shown that *An. funestus* is the main malaria vector in the group, with infection rates up to 11% [41] and exceptionally 50% [42]. *Anopheles funestus* has late-night biting patterns, commonly between midnight and the early hours of the morning [22, 43, 44]. It is also the most endophilic and anthropophilic member of the Funestus Group [45-47]. In savanna areas where its breeding sites are rain-dependant, *An. funestus* follows in peak abundance its counterpart *An. gambiae*, therefore extending malaria transmission from the beginning to the first part of the dry season [48, 49]. Overall, *An. funestus* shows fairly consistent host feeding preferences (human) and resting behaviour (indoor) throughout its entire range. However, behavioural differences linked to chromosomal polymorphisms have been documented. For instance, Lochouarn *et al.* [50] reported a west-east gradient of human to animal biting preference, corresponding to chromosomal polymorphisms that also follow this cline. In Burkina Faso, different chromosomal inversion combinations (chromosomal forms, see below) were associated with different resting and biting activities [42]. These studies showed that carriers of inverted arrangements on the arm 2R and 3R feed predominantly on humans (anthropophilic) and rest inside dwellings, while the standard counterpart exhibit higher levels of zoophily and exophily (Guelbeogo, pers. Comm.). In Madagascar, the carriers of inverted arrangements 3Ra and 3Rb were less anthropophilic than carriers of standard arrangements [51]. In Senegal, the population of mosquitoes with inverted arrangements 3Ra and 3Rb was also more zoophilic. However, this heterogeneity in host preference might also be related to specific local conditions, such as host availability [52] or indoor microclimatic conditions (i.e. humidity).

The other species of the group are mainly zoophilic, but can occasionally feed on humans [3]. *Anopheles rivulorum* has been incriminated as a malaria vector in Tanzania [53]. Indeed, this species was found naturally infected by *Plasmodium falciparum.* However, this species is mainly zoophilic (77% animal hosts) and shows a lower longevity compared to *An. funestus*. Positive infected specimens of *An. rivulorum* were also observed in coastal Tanzania by Temu et al. [54]. This study also found positive specimens of *An. leesoni* and *An. parensis* to *P. falciparum*, suggesting a secondary role of these mosquitoes in malaria transmission. *Plasmodium falciparum* infected *An. parensis* specimens were also observed during an entomological study in South Africa using an Enzyme-Linked Immunosorbent Assay (ELISA) [55]. *Anopheles vaneedeni* feeds rarely on humans outdoors (1.22%). Although experimentally infected with *P. falciparum* in the laboratory, it has never been found involved in transmission in natural conditions [56]. *Anopheles longipalpis* has never been involved in malaria transmission [1, 3, 57]. In East Africa (Tanzania and Ethiopia), different

authors have reported human feeding behaviour of *An. longipalpis* from indoor and outdoor collections [58-60]. Recently, Kent et al., [57] reported that even when found in large numbers resting indoors together with *An. funestus* in Zambia, *An. longipalpis* remains predominantly zoophilic.

2. Insecticide susceptibility and vector control

Because of its highly anthropophilic and endophilic behaviour, *An. funestus* has been an "easy" target in malaria control programs (i.e. insecticide treated materials or indoor residual spraying). *Anopheles funestus* has developed insecticide resistance in many parts of the African continent [61-64]. To date, *An. funestus* has been shown resistant to pyrethroids, carbamates and DDT. The first documented reports on insecticide resistance in this malaria mosquito (mainly to BHC, dieldrin, and malathion) were in West Africa (Mali, Ghana, Benin), Central Africa (Cameroon) and East Africa (Kenya), following vector control programs [65-68]. Recent studies have shown that dieldrin resistance is still high in *An. funestus* populations from Burkina Faso, despite the fact that this insecticide is no longer used in public health [47]. In agreement with Burkina Faso results, Wondji et al. [69] documented *An. funestus* resistant populations to dieldrin in Cameroon due to the remaining presence of Rdl^R target-site mutation. With regard to pyrethroids, resistant *An. funestus* populations were first detected in Southern Africa, being at the origin of the malaria outbreaks in the late 1990's [31, 62]. Pyrethroid resistant populations for this mosquito were also reported in Ghana, West Africa, combined with carbamate resistance [70]. Altogether, it is now clearly established that *An. funestus* populations in Africa show resistance to at least the 4 insecticide classes recommended for vector control by WHO.

During the last decade, efforts have been made in order to unravel the molecular mechanisms involved in insecticide resistance. The mechanisms discovered involve insecticide detoxification by one or multiple metabolic pathways mediated by glutathione S-transferases (GST), monooxygenases and/or esterases [61, 71-73]. No evidence for the presence of L1014F *kdr* mutation or G119S *Ace-1* mutation has been detected in *An. funestus* [63, 64, 71, 72]. However, a multiple insecticide resistance profile has been recently observed in Benin [74]. Insecticide resistance is an threat to effective malaria control. With the advent of malaria control program through the use of LLINs (Long Lasting Insecticidal Nets) and IRS (Indoor Residual Spraying), the presence of insecticide resistant populations should be carefully monitored. It would improve the implementation and management of current and future malaria vector control programs in Africa. In this context, a novel approach using the pyrrole insecticide chlorfenapyr against pyrethroid resistant *An. funestus* populations has led to valuable results [75]. An important challenge for the study of molecular mechanisms of insecticide resistance is the development and maintenance of laboratory colonies. To date, only two colonies are currently maintained at insectarium conditions, coming from southern Africa [76], although, some progress has been made and new strains have been established in Burkina Faso (Sagnon *et al.*, pers. comm.).

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3. Molecular tools

3.1. Introduction

In 2002, the genome of *An. gambiae* s.s. was publicly released [77]. This event had a very large impact on the better understanding of the complexity of the malaria system. Furthermore, the publication of the *An. gambiae* genome brought with itself a rapid development of new genetic tools, from molecular markers (i.e. SNPs chips, microarrays, microsatellites, etc) to transgenic mosquitoes, for instance. To date, no other malaria mosquito genome has been released but progress has been made, and soon (2013), the release of several *Anopheles* genomes, including *An. funestus* [78], is expected.

Three inherent characteristics of *An. funestus*, have hampered the study of this mosquito at the molecular level. First, its "eternal" role as second important malaria vector. For decades, *An. funestus* has been neglected with regard to its well-studied congener *An. gambiae*. With virtually the same geographical distribution as *An. gambiae* across the African continent, *An. funestus* has been many times overruled because its mosaic-like presence (see previous section in this chapter). However, its major role in malaria transmission has been evidenced throughout the continent, surpassing in a number of locations *An. gambiae* and *An. arabiensis* [2] in many places. Second, the extreme difficulties to breed *An. funestus* in standard insectary conditions. To date, as mentioned earlier in this chapter, there exist only two colonies of *An. funestus* with published records: FANG and FUMOZ (and its pyrethroid resistance counterpart FUMOZ-R), originating from Angola and Mozambique, respectively [76, 79]. Both colonies have been recurrently used in insecticide resistance studies of *An. funestus* [74, 79, 80]. Indeed, it is one of these colonies (FUMOZ), which has been elected as reference *An. funestus* genome for sequencing [78]. Unfortunately and besides the numerous efforts in many parts of Africa, only one new colony has been colonized (Sagnon *et al.,* pers. comm.). Third, polytene chromosomes of this species exhibit a poor quality in comparison with *An. gambiae* [7]. The assembly of the *An. gambiae* genome was primarily based on techniques, which required the identification of probes through polytene chromosomes [77]. Although polytene chromosomes are readable, as several studies assert, however, the effort involved is very high and the rate of success, significantly lower.

Despite these challenges, and the lack of a publicly available *An. funestus* genome, several noteworthy molecular and genetic advances have been reached in this malaria mosquito during the last decade. These advances have been inspired by those previously achieved in *An. gambiae*. Particularly, we can distinguish two fields: molecular markers and expression profiling analysis.

3.2. Molecular markers

In the late 70's and beginning of the 80's, several studies revealed the importance of chromosomal inversions as genetic markers to differentiate species within the Funestus Group [6, 7]. These results mirrored those obtained in the *An. gambiae* complex [81, 82]. But, we had to wait until the end of the 90's and the past decade to settle the role of the chromosomal inversions

in local adaptation and speciation within *An. funestus* populations [42, 52, 83-86]. Despite its evident interest, the technical demands of traditional karyotype analysis, the low rate of success in chromosome preparations, and the sex- and stage-specific limitations, have hampered the proliferation of this kind of studies. Nowadays, the new advances in molecular karyotyping in *An. gambiae* (based on quick, low-cost and convenient PCR reactions) have relaunched an interest in this field [87, 88]. Together with new high-throughput technology, the *An. funestus* genome will undoubtedly open new possibilities to develop molecular karyotyping in this mosquito.

Chromosome	Locus	Accession number	Forward primer	Reverse primer	Allele size
Chr. X	FUNE	AY6009	GACCGGTTCTGGTATCGTC	ATCGAGTCACCCAATTCTCC	136–154
	FUNQ	AY6021	GCAAACTGCTAGTAAATGTTTCC	*ACACAACGCCACCACTATGA	84–98
Chr. 2	AFND6	AF171036	GCTTCTTCTCCCCTAATCTG	TCCTGCTTTTTAGTTTGTCG	184–212
	AFUB15	AY029722	GATGCCGGGAGTAATAGCAA	AGACAGCCCGTAGAACGGTA	155–191
	AFND2	AF171032	ATAAACCCGTCCATTCCCTT	CCTATGATTCGCTCCTGACA	131–151
	AFND32	AY291367	GAAGCATTTTGGGTTAGACTC	GCAGTTGTTTACCTTTCACTG	103–121
	AFUB14	AY029721	ATCAGTGCTCCTCCACATCC	CGTGGTTGGCAATGTTACTG	152–188
	AFND17	AF171047	AAAACGCCACAAAGAGCAC	CGGGTCAAATTCTACCGTAAG	129–157
	AFUB4	AY029711	CTATCAGCAGCCGCCACA	GATGCCGATGAGGAATGTTG	183–192
	AFUB25	AY029723	GTGGAAACGGTGGTACTGT	CGCCATGTAGCTAGGGTTTG	212–224
	AFUB10	AY029717	TGTCCATGTACAACCGCAAC	TTCTCCAGCATCATCAGCAC	195–210
	AFND37	AY291373	GATCGATACAATAAGTGTAGAAATAAT	TCACGATGTGCAACCTATAA	161–189
	AFUB30	AY029737	GCCAGTTTGCAGAACCAAAT	CTGCTGCTGATGTTGCTGAT	154–163
	AFUB7	AY029714	ATGGGACGATGGATTACCAA	GCCAGTTTGCAGAACCAAAT	220–223
	AFUB16	AY029723	CGTGGATGGCAATGTTACTG	TGCGACTTATCAGTGCTCCT	179–209
Chr. 3	AFND21	AF171051	CCGCACACCAACTTACACTC	TGGCGTGGGATTAAATAGG	96–104
	AFUB13		GACTTCCGCCACAGAACATC	CTCAGGCTCGCAGTAGGAGT	207–210
	AFND19	AF171049	CAGAACCACTTCGATTCAAC	CCTGCACTCAGAAACACAC	172–205
	FUND	AY6008	GCTAACTACTCCGAAGCGCT	GATCGCAAAACTTCCGGTT	145–177
	FUNI	AY6013	*GCAACTAAGCTGGGACAGGA	GCATCTAACCCTGCTGCTT	181–197
	AFND3	AF171033	ACGACTGTAACCACAACACC	TAGTAGCGAAGGCGAAAGAT	171–195
	FUNF	AY6010	CCTTCAGTTTCGATTGGCG	AATAAGATGCGACCGTGGC	104–118
	AFND10	AF171040	TTTTTTCTTCCCGTGTTGC	TACCATTTGATTACAGCGCC	114–146
	AFUB17	AY029724	GAAAACCGTACGAACGATGG	TGCGACAGTAGCACAGGGTA	187–196
	AFUB1	AY029708	CAGCAGCAGCAGCAACAG	GACGTTAGCATCTCCACCAG	266–269
	AFUB12	AY029719	TGGGGAACTGGTCGTTAGAG	CTGGTGATGGGATTGAGGAT	152–158
	FUNK	AY6015	GCGCTTCCGCAAACATAC	ACTCACACCCCATTCTTGTG	184–202
	263B12		AGTGCGTCAGAGTTTGAA	TCGATTGATGGCGATGATAA	230–242
	261H03		CGCTCAAACTGAAAGCGATA	GGATGCGGAGATGATGTTGT	208–220
	263A06		CGTTCGGTTTCGCTAACTGT	CGTTCTATTTCGGGGTGTGT	210–220
Unknown	AFUB21	AY029728	*AACGCAGCAGTGGAGAGAAT	AACACCAACCCTTGTTGTGC	224–230
	AFND30	AY291369	GCCAGTTTGCAGAACCAAAT	CTGCTGCTGATGTTGCTGAT	81–107

Table 2. Summary of microsatellite loci in *An. funestus* modified from Wondji et al. [89].

In *An. funestus*, several genes have been recurrently involved in genetic studies: three nuclear genes (ITS1, ITS2 and D3) and another three mitochondrial genes (COI, COII and ND5). Nuclear genes have been involved in species differentiation within the Funestus Group [15, 16], while mitochondrial genes revealed signatures of incipient speciation between populations of Burkina Faso [85]. Another kind of molecular markers, Single Nucleotide Polymorphisms (SNPs), have been recently developed in this malaria mosquito. Wondji et al. [79]

reported a genome-wide set of SNP markers from 50 genes. A total of 494 SNPs were identified, which were added to 15 SNPs previously discovered by analyzing sequence traces of 11 physically mapped DNA fragments of cytochrome P450s of *An. funestus*. However, to date, microsatellites are the most frequently employed molecular markers in *An. funestus* [89-92]. Seventy-five microsatellites have been developed, although, only 32 were successfully revisited by Wondji et al [89] (Table 2). They are widely distributed across the *An. funestus* genome. They have allowed the analysis of population genetic structure, gene flow and demographic events across Africa [93], from Senegal [40], Cameroon [83, 86], Kenya [94] to Madagascar [95], revealing important signatures of local adaptation, dispersion or speciation.

These molecular markers have been key in numerous advances. For instance, SNPs and microsatellites allowed to Wondji and co-workers to explore the genetic basis of insecticide resistance in this malaria vectors [79]. Several genes including the P450 cytochrome (CYP6P9a and CYP6P9b) were associated to DDT resistance by Quantitative Trait Loci (QTL) analysis using both markers [72]. The role of microsatellites in population genetic studies is discussed in other sections of this chapter (see below). Despite, we are still far from the molecular advances carried out on *An. gambiae*. For instance, in *An. funestus* 75 microsatellite loci have been identified, compared to 300 in *An. gambiae*. With regards to SNPs, 509 have been reported in *An. funestus* [79, 89], compared to 400,000 in *An. gambiae* [80].

3.3. Expression profiles

Considering the lack of *An. funestus* genome, transcriptome analysis appeared as a suitable alternative to whole genome sequencing. This technique is significantly cheaper and provides important information at the gene transcript level. Moreover, it provides valuable molecular tools for the analysis of gene expression evolution and comparative analysis among other Culicidae members, such as *An. gambiae*, *Ae. aegypti* or *Cx. pipiens*.

In 2007, Calvo et al., [96] investigated salivary gland genes from 916 cDNA clones coming from adult females. This study debuted the analysis of transcripts in this mosquito, providing important clues about the evolution of salivary gland proteins in blood feeding insects and Culicidae. In particular, a 30 KDa allergen family and several *mucins* were exclusively found in *Culicidae* when compared to *Aedes albopictus*, *Aedes aegypti* and *Culex pipiens quinquefasciatus*. Moreover, ten proteins and peptide families were only found in *Anopheles* when included in the analysis *An. gambiae*, *Anopheles stephensi* and *Anopheles darlingi*. Later, two new studies emerged with the aim to analyze the transcriptome evolution and differences in expression profile between insecticide susceptible and resistant phenotypes of *An. funestus*, respectively [80, 97]. While, Serazin et al. [97] used SANGER sequencing technology for this purpose, Gregory et al. [80] employed *de novo* expression profiling by 454 pyrosequencing. In general, these two studies were largely complementary and boosted the available genetic information in *An. funestus*. However, 454 pyrosequencing allowed parallel DNA sequencing and increased sequencing depth and genome coverage. For instance, Gregory et al. [80] improved the number of ESTs (Expressed Sequence Tags) from 2,846 [97] to 18,103 contigs. Regarding comparative analysis with other mosquitoes, both studies agreed on the fact that the highest similarity pattern remains with *An. gambiae*. Interestingly, the mean percentage of similarity differs

drastically between functional groups. Two groups of housekeeping functions show the highest amino acid sequence conservation: protein synthesis and degradation. On the other hand, three groups of interest patently showed very low similarity scores, suggesting accelerated rates of evolution. These three functional categories – salivary, immunity and extracellular structures – may be driven by environmental selection pressures. For instance, selective pressures imposed by parasites could explain both the highest genetic variability and the lowest conservation of immune genes between *An. funestus and An. gambiae*. Alternatively, *de novo* 454 sequencing offered the opportunity to identify new SNPs. In this sense, 31,000 potential SNPs were discovered over 4.579 Mb of sequence, meaning one SNP every 70 bp [80]. Thus, expression profile studies led to identify genes under selective pressures (i.e. insecticide resistance, immunity genes) and might generate new functional genomic tools (i.e. microarrays or SNP platforms) while we wait for future genomic sequencing of *An. funestus*.

4. Population genetic structure across Africa

4.1. Introduction

In malaria mosquitoes, population genetics have been revealed as an excellent tool for implementation of vector control programs. The study of gene flow among vector populations allows the analysis of mosquitoes' movement in natural populations, and therefore, how those populations are segregated. They can, for instance, assist to follow the expansion of genes of interest, such as those that confer insecticide resistance [98], or potentially help to introduce transgenic mosquitoes, refractory to parasite infection [99, 100]. On the other hand, these population genetic studies might be useful to investigate the genetic basis of speciation and/or local adaptation processes. They evidence a considerable importance in vector control measures [101].

The biology of *An. funestus* has supported several "*a priories*" about its population structure in natural conditions. As mentioned previously in this chapter, this malaria mosquito mainly breeds in permanent or semi-permanent water bodies, such as rice fields, swamps or artificial lakes, always linked to human presence (see above). Moreover, this mosquito has exhibited a very slow recolonization power of those areas treated with insecticide. Both characteristics have led to assume the population subdivision of *An. funestus*. In this section, we will discuss the population structure of this malaria vector across Africa as revealed by two types of markers: chromosomal inversions and molecular markers.

4.2. Cytogenetic studies

The study of chromosomal rearrangements – cytogenetics – of *An. funestus* debuted early in the 1980's [6, 7], preceded by the success of this kind of studies in its congener *An. gambiae* [81, 82, 102]. It allowed differentiating members of the Funestus Group, avoiding the challenging interpretation of taxo-morphological rules. Green & Hunt [7] and Green [6] showed differences in the chromosomal polymorphism within the species of the group. As in *An. gambiae*, several chromosomal inversions were species-specific, while other inversions were polymorphic in

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some species and fixed in others. Although, other cytogenetic studies appeared in the meantime, we had to wait until 2001 when Sharakhov et al. [103] finally established the chromosome map of this species (Fig. 2), based on comparisons to the *An. gambiae* map [102].

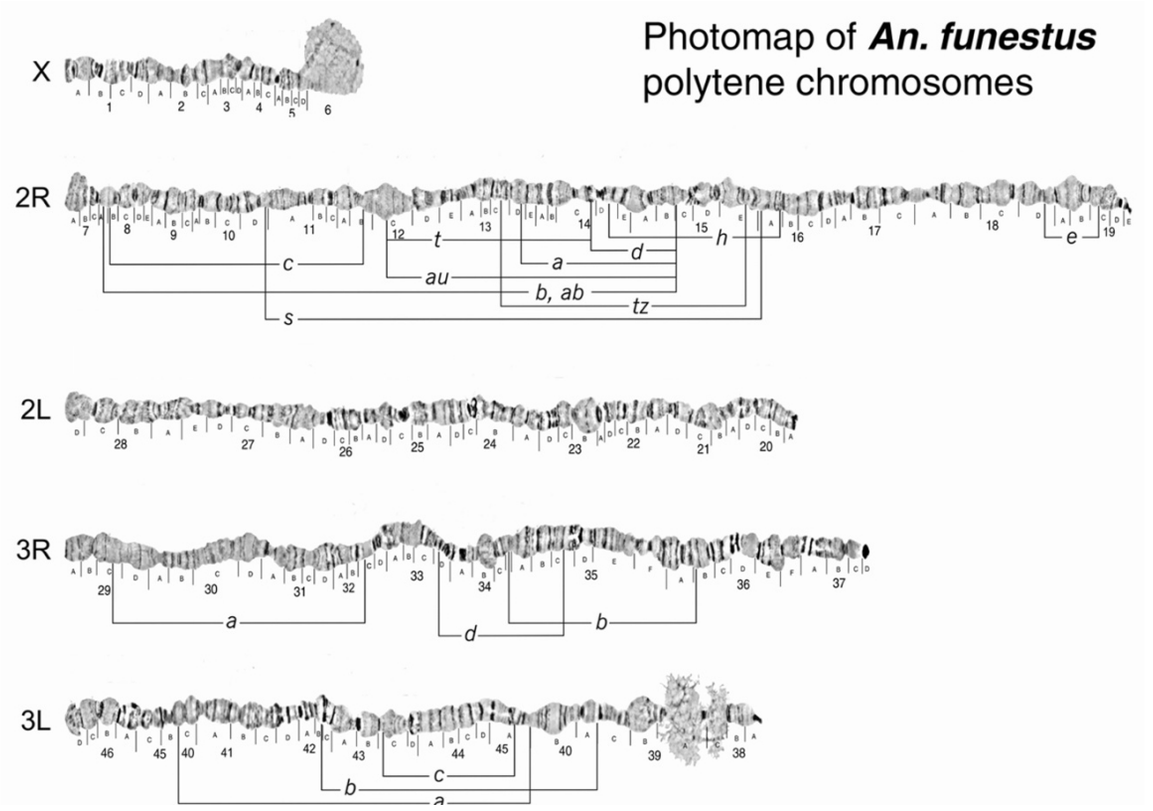

Figure 3. Chromosome map of *An. funestus*

For its predominant role as malaria vector and its wide geographical distribution across sub-Saharan Africa, *An. funestus* has been the most studied species of the group, although greatly exceeded by the studies in *An. gambiae* [82, 104, 105]. Seventeen chromosomal inversions have been recognized, with specific distribution through the African continent [6]; [52]; [84, 106-108]; (D. Ayala pers. comm.). Among them, four inversions are found all across the continent (2Ra, 3Ra, 3Rb, 3La), while others have a regional distribution (i.e. 2Rt in West Africa or 2Rh in South and Central Africa), or a very localized distribution (2Rd in the southern forested areas of Cameroon). These distributional patterns could be due to environmental selection, demographic effects or historical events [109].

Chromosomal inversions have been widely implicated in the process of speciation and local adaptation in a wide range of animals and plants [110, 111]. In recent years, studies on the chromosome composition of the populations of *An. funestus* were conducted in several African countries. These results showed a great complexity with different trends. In Burkina Faso, a deficit of heterozygotes and linkage disequilibrium among some rearrangements, led Costantini et al. [42] to identify two chromosomal forms: Kiribina and Folonzo, with a certain parallelism with the chromosomal forms of *An. gambiae* from Mali [104, 112]. These

two forms are also differentiated at the ecological level. While Kiribina appears better adapted to arid conditions, Folonzo inhabits more humid habitats [84, 113]. The presence of these two chromosomal forms was not observed in other countries such as Angola, Madagascar or Kenya [108, 114] (LeGoff, pers. comm.). Nevertheless, deficits of heterozygotes were also detected, particularly in inversions of the 3R and 3L arm, in some areas of Cameroon and Senegal [52, 83, 86, 115]. These studies did not show a clear division between the "chromosomal forms from Burkina Faso", rather a non-random distribution of chromosomal inversions and their frequencies through different habitats and environments. This fact suggests that most inversions frequencies in *An. funestus* do not follow a neutral pattern. Ayala et al. [86] observed a sharp contrast between population structure measured at neutral microsatellite markers and at chromosomal inversions. Microsatellite data detected only a weak signal of population structure due to distance among geographical zones in Cameroon, as previously described by Cohuet et al. [83]. By contrast, strong differentiation among habitats was revealed by chromosomal inversions, strongly suggesting a role of environmental selection in shaping their distribution. Moreover, in the same study, there was no apparent difference between microsatellite loci (F_{ST} estimates) lying within and outside polymorphic chromosomal inversions [86].

4.3. Molecular markers

The first assays to characterize wild populations of this mosquito were based on mitochondrial (Internal Transcribed Spacer 2, ITS2) and ribosomal DNA (cytochrome b gene, cyt-b) [116]. The results did not show any differentiation between chromosomal forms previously described by Costantini [42], rather one panmictic population. At the beginning of this century, new microsatellite markers were developed, which allowed more precise studies [89-92]. At the country scale, the results have evidenced a general trend to only one population, with a slight but significant isolation by distance. In Kenya, Braginets et al. [94] did not find any population genetic structure throughout the country, however, an important sub-division due to Rift Valley was found. A similar pattern was already observed in *An. gambiae* [117]. In Madagascar, Ayala et al. [95] did not find a population structure at the island level, rather a correlation between genetic and geographic distance across vector populations. In Senegal, Cohuet et al. [40] also showed genetic differentiation due to distance, without a clear relationship between "Burkina Faso chromosomal forms" and genetic data.

Similar results were obtained in Cameroon, where for the first time, a latitudinal cline across different environments was analyzed [83, 86]. As in previous studies, genetic differentiation among populations might be explained by isolation by distance. On the other hand, in Burkina Faso, Michel et al. [85] showed a genetic divergence between chromosomal forms on the basis of five microsatellite markers and sequence of a mitochondrial gene (ND-5). These results validated in some extend those precluded by Costantini et al. [42] and Guelbeogo et al. [84]. Unfortunately, they still remain restricted to Burkina Faso, similarly to chromosomal forms of *An. gambiae* in West Africa [118]. In recent years, several population genetic studies have been conducted at the sub-region and/or continental scale. Temu et al. [119], showed a similar pattern to the other studies at the country level for five countries in Eastern and Southern

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Africa: the genetic distance limited the gene flow among populations and promoted genetic differentiation among populations. A comprehensive study using samples across the continent provided important findings [93]. *Anopheles funestus* was subdivided into three large blocks: West Africa, East Africa and Central Africa [120, 121]. This subdivision was roughly similar than that observed in *An. gambiae* across Africa [122]. Despite these results and the unquestionable accuracy of the analysis, the question about the incipient speciation of *An. funestus*, still remains to be elucidated.

The very rapid pace of development of genetic and molecular tools will allow characterizing *An. funestus* populations in a very detailed fashion. New molecular tools, such as SNP chip, RAD-tag or DNA microarrays, will certainly contribute to a better understanding of the biology of this mosquito. The expected *An. funestus* genome sequencing will undoubtedly boost new advances in order to elucidate a variety of biological processes involved in local adaptation, speciation, parasite transmission or the immunity system among others. It will also enable comparative studies with other anopheline species, particularly, *An. gambiae*.

5. Conclusion

During the last decade, we have seen how new molecular advances have elevated *An. gambiae* to the level of model species with regard to the number of data and tools available. *Anopheles funestus* is still far from this point. Undoubtedly, it is one of the major and more deadly malaria vectors worldwide. Its capacity to adapt to a wide range of ecological settings coupled with the appearance of insecticide resistance highlight the importance for studying this mosquito. However, the extreme difficulty to establish colonies in insectary conditions has hindered its study. Now, its upcoming genome sequencing and the availability of new molecular tools preclude a promising future for the study of this malaria mosquito.

The *An. funestus* geographical distribution mirrors *An. gambiae*'s across the whole African continent, with presumably similar environmental pressures. This mosquito exhibits a large number of chromosomal and genetic polymorphisms. Furthermore, it belongs to a group of morphologically undistinguishable species. This malaria mosquito is suspected to be at the heart of an ongoing speciation process, as its congener *An. gambiae*. Once the new techniques and vector control strategies have achieved their goals in *An. gambiae*, *An. funestus* will become the new target for succeeding malaria control programs. Moreover, the parallel study between both species will help to elucidate the ecological and genetics mechanisms involved in many biological processes from immunity system to local adaptation or speciation.

In this chapter, we revisited the *state-of-the-art* of this malaria mosquito as well as the other species of the Funestus Group. Detailed descriptions were provided on their biology, role in malaria transmission and insecticide resistance status. We examined the new genomic advances and how they can be useful for improving vector control strategies. To sum up, we strongly believe that a general knowledge about this mosquito is essential for the success of its control and the ultimate aim to reduce the malaria burden in Africa.

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