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The *kdr* mutation occurs in the Mopti form of *Anopheles gambiae s.s.* through introgression

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Abstract

***Anopheles gambiae s.s.* is a complex of sibling taxa characterized by various paracentric inversions. In west and central Africa, where several taxa are sympatric, a *kdr* mutation responsible for pyrethroid resistance has been described in only one (the S taxon), suggesting an absence of gene flow between them. Following a thorough sampling, we have found a *kdr* mutation in another taxon (M). To establish whether this mutation is the same event or not, the large intron upstream of the *kdr* mutation was sequenced to find polymorphic sites in susceptible/resistant and M/S mosquitoes. The low genetic diversity found in this DNA region indicates that a local genetic sweep has recently occurred. However, some polymorphic sites were found, and it is therefore concluded that the *kdr* mutation in the M taxon is not an independent mutation event, and is best explained by an introgression from the S taxon. These results are discussed within the context of possible gene flow between members of *An. gambiae s.s.* taxa, and with the possible spread of the *kdr* mutation in other closely related malaria vectors of the *An. gambiae* complex.**

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Introduction

Resistance to pyrethroid insecticides has been recently identified (Elissa *et al.*, 1993) in the main malaria vector in Africa (*Anopheles gambiae s.s.* Giles), potentially representing a major threat to the implementation of malaria prevention programmes based on the use of insecticide-impregnated bednets. This resistance results from a single point mutation (leucine TTA phenylalanine TTT) in a gene that encodes a sodium channel (Martinez-Torres *et al.*, 1998), and gives the characteristic 'knockdown resistance' or *kdr* phenotype. *Kdr* resistance has been reported so far in the Ivory Coast and Burkina Faso (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999) (Fig. 1), although *An. gambiae s.s.* can be found in the forest and savannah areas throughout Africa. *Anopheles gambiae s.s.* is usually split into five taxonomic units, characterized by the presence or absence of paracentric inversions on the second chromosome (Coluzzi *et al.*, 1985; Touré *et al.*, 1998). The exact nature of these taxa (designated by a non-Linnean nomenclature: Forest, Savanna, Bissau, Mopti and Bamako) is unclear, nor is it clear whether gene flow is possible between them (Cianchi *et al.*, 1983; Favia *et al.*, 1997; Lanzaro *et al.*, 1998). There is also a convenient molecular classification, M and S, corresponding in the Northern range of *An. gambiae* distribution to Mopti (M) and other taxa (S) (Favia *et al.*, 1997). The *kdr* mutation has only been reported so far in the S forms, and not in the sympatric M taxon (Chandre *et al.*, 1999), although both are subjected to heavy pyrethroid pressure, suggesting a substantial prezygotic reproductive isolation between them.

An extensive survey of the *kdr* mutation has been performed in *An. gambiae s.s.* from West and Central Africa, and the exact taxon involved has been identified. The *kdr* has been found in the M form, and DNA sequence of the intron upstream of the *kdr* mutation has been determined in several resistant/susceptible and M/S mosquitoes to establish if the presence of the *kdr* mutation in M was the result or not of a *de novo* mutation.

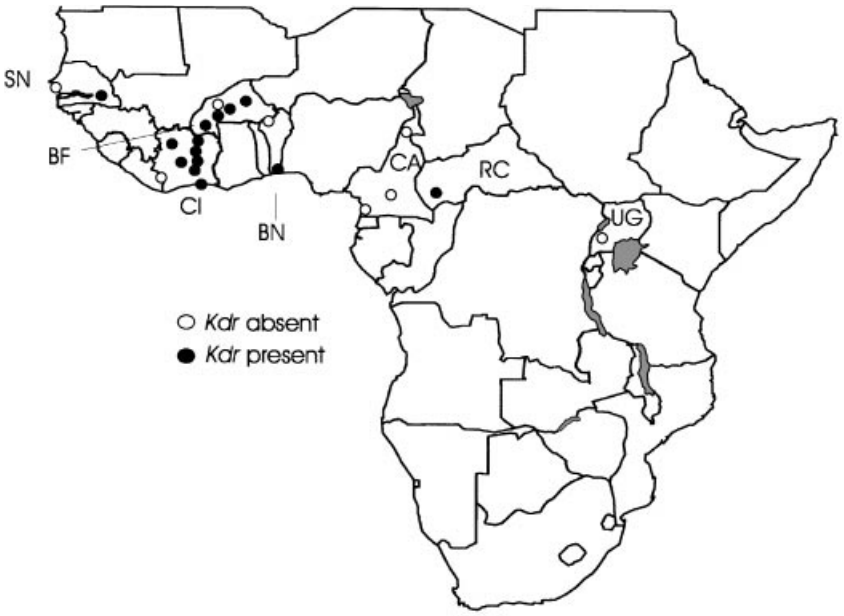

Figure 1. Geographic distribution of the *kdr* resistance mutation (●) in the main African malaria vector, *Anopheles gambiae s.s.* Absence of *kdr* resistance is represented by ○. Countries are referred to as a two-letter code: Senegal (SN), Cameroon (CA), Benin (BN), Central Africa (RC), Ivory Coast (CI), Burkina Faso (BF) and Uganda (UG).

Results and discussion

Through a large and extensive sampling (Senegal, $n = 44$; Cameroon, $n = 100$; Benin, $n = 274$; Central Africa, $n = 28$; Ivory Coast, $n = 572$; Burkina Faso, $n = 433$; and Uganda, $n = 36$), the *kdr* mutation was found in the M taxon from Benin (frequency = 0.69) and not in M mosquitoes from other countries (e.g. Ivory Coast, Bukina Faso, Cameroon, $n > 150$) (Chandre *et al.*, 1999; Brengues *et al.* unpublished). All other *kdr* mutations were found in the S taxon (Senegal, Ivory Coast, Burkina Faso and Central Africa). To understand better the recent history of this resistance allele in *An. gambiae s.s.* and to assess the number of times that it has independently arisen, we have searched for polymorphic sites in introns of the sodium channel gene near the *kdr* mutation. The first intron upstream (designated here as intron 1) and downstream (intron 2) of the *kdr* mutation were sequenced in fourteen mosquitoes from the Ivory Coast and Burkina Faso (including as an outgroup 4 *An. arabiensis*). No polymorphism was found for intron 2. For intron 1, four positions were differentiating the two sibling species, and two positions (702 and 896, Fig. 2) were polymorphic within *An. gambiae s.s.* No variation was apparent in the *An. arabiensis* sample. Subsequently, 540 bp of this intron encompassing these two polymorphic sites were sequenced from ninety mosquitoes of the M or S taxa, and no additional informative polymorphic sites were found. We have compared resistant and susceptible mosquitoes of both M and S taxa in various geographical samples (Table 1). Among susceptible mosquitoes, the M taxa displayed mostly the C-A combination (at the 702 and 896 polymorphic positions, respectively), while S were mostly T-C, independently of their geographical origin. This indicates that these positions are characteristic of the M and S taxa, although not exclusively. In contrast, all resistant mosquitoes (homozygous for the *kdr* mutation) were T-C, whether or not they were S or M. The number of occurrences of the *kdr* mutation in the S taxon cannot be determined, as resistant mosquitoes bear the T-C combination which is prevalent in susceptible individuals. For the M taxon, *kdr* mutation probably arose only once, as the T-C combination found in resistant individuals has not been found in susceptible individuals. A *kdr* mutation could occur by chance on a rare allele. However, a more likely explanation is that the resistance gene, along with the intron displaying the T-C combination, came from the S taxon through introgression. Such an event must be uncommon and recent, as no M mosquitoes displaying the *kdr* mutation were found outside Benin. These results indicate that hybridization among members of the *An. gambiae s.s.* complex does exist, although it is probably uncommon. This is in support of the incipient speciation hypothesis occurring within *An. gambiae s.s.* (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985), based on adaptation to distinct ecological environments associate to human settlements (Coluzzi, 1994; Coluzzi, 1999).

The relatively low polymorphism found in introns of the sodium channel gene deserves some comments, as introns of the same gene in another mosquito species are highly polymorphic (Martinez-Torres *et al.*, 1999). This low polymorphism indicates either a recent population bottleneck or a local genetic sweep. The former requires a low polymorphism throughout the genome of *An. gambiae s.s.*, which is not the case, either when analysed with allozymes

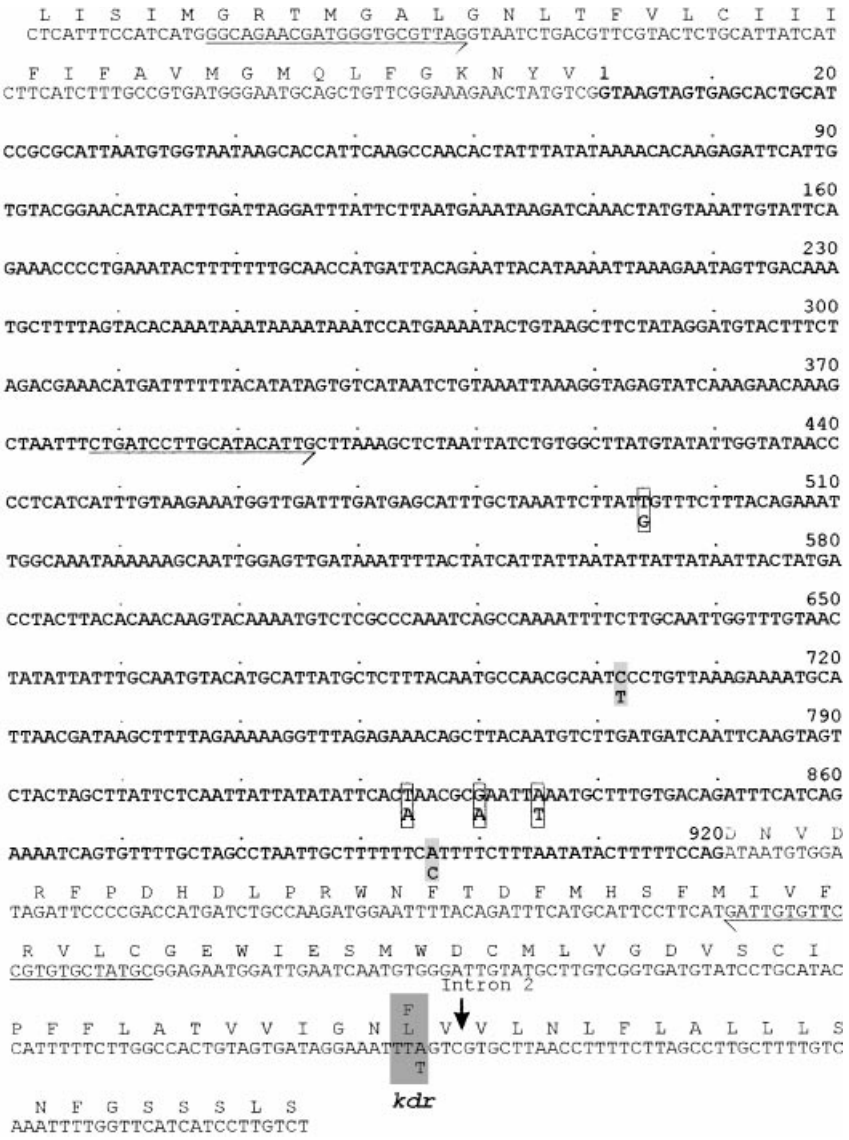

Figure 2. Partial sequence of the sodium channel gene: the 920 bp of intron 1 (bold characters) and the position (arrow) of the 57 bp intron 2; the darker shaded box corresponds to the *kdr* mutation. Four positions were differentiating the two sibling species (empty boxes), and two positions (702 and 896, lighter shaded boxes) of the larger intron were polymorphic within *An. gambiae s.s.* See text for details.

Table 1. Intron polymorphism of susceptible and resistant mosquitoes in the two taxa (M and S) of *Anopheles gambiae s.s.* The first polymorphic site (position 702) is C or T, and the second polymorphic site (position 896) is A or C, or ● for non-identified. The number of mosquitoes with each combination is indicated. The prevalent combination for *kdr* resistant and susceptible insects of each taxon is indicated in bold characters. The association between position 702 and resistance was tested, whenever possible, using a 2×2 Fisher exact test (P).

	Susceptible					Resistant				
	C-A	C-●	C-C	T-C	T-●	C-A	C-C	T-C	T-●	*P*
M taxon										
Benin	**8**	**0**	0	0	0	0	0	**7**	**2**	$< 10^{-4}$
Ivory Coast	**5**	**1**	1	0	0	–	–	–	–	–
Burkina Faso	**4**	**3**	1	0	0	–	–	–	–	–
All	**17**	**4**	2	0	0	0	0	**7**	**2**	$< 10^{-5}$
S taxon										
Ivory Coast	1	0	0	**0**	**4**	0	0	**19**	**1**	0.20
Burkina Faso	0	0	0	**2**	**0**	0	0	**5**	**2**	1.0
Cameroon	0	0	0	**7**	**0**	–	–	–	–	–
Central Africa	0	0	0	**11**	**0**	–	–	–	–	–
Uganda	0	0	0	**6**	**0**	–	–	–	–	–
All	1	0	0	**26**	**4**	0	0	**24**	**3**	1.0

or microsatellites (Cianchi *et al.*, 1983; Lehmann *et al.*, 1996; Lanzaro *et al.*, 1998). The latter is therefore more likely, although it remains to be established how large the DNA region affected by the sweep is, and which gene is responsible for it. It remains to be established by additional sampling if the same region is also depauperated of genetic diversity in *An. arabiensis* in order to know if this genetic sweep took place before the divergence of *An. arabiensis* and *An. gambiae s.s.*

Adaptive genes such as those producing insecticide resistance allow the detection of rare hybridization events among closely related taxa. Introgression with an already resistant sister taxon could be more frequent than mutation, so that the former phenomenon might predominate during the evolution of adaptive genes within a group of related taxa. This raises the risk that this resistance gene will spread rapidly throughout the five taxa of *An. gambiae s.s.*, and eventually invade other malaria vectors still susceptible to pyrethroids, such as the sibling species *An. arabiensis* or *An. bwambae*, known to potentially hybridize with *An. gambiae s.s.* (e.g. Coluzzi *et al.*, 1979; della Torre *et al.*, 1997; Thelwell *et al.*, 2000).

Experimental procedures

Mosquito populations

Samples of *An. gambiae s.s.* were collected in several African countries: Benin (Cotonou, peri-urban), Cameroon (Mfou, forest area), Central Africa (Bangui, urban), Senegal (Wassadou, rural) and Uganda (Fort portal, rural). Samples from Burkina Faso (Boromo, peri-urban and Bobo Dioulasso, urban) and the Ivory Coast (Khorogo, Nombolo and Mbé, rural) were from Chandre *et al.* (1999). Two *An. arabiensis* from Mauricius island and two from Sudan (Kartoom) were also included. Mosquitoes were identified morphologically (*An. gambiae s.l.*), then by polymerase chain reaction (PCR) (Scott *et al.*, 1993) (*An. gambiae s.s.* or *An. arabiensis*).

DNA diagnostic test for kdr *alleles in single mosquitoes*

Genomic DNA was extracted from single mosquitoes according to Martinez-Torres *et al.* (1998). 10–50 ng of genomic DNA were combined in a 25 µl total volume with the four primers Agd1, Agd2, Agd3 and Agd4 (Martinez-Torres *et al.*, 1998). The PCR conditions were 30 s at 94 °C, 30 s at 48 °C and 30 s at 72 °C for forty-five cycles. Amplified fragments were analysed by electrophoresis on a 1.5% agarose gel.

M/S taxon determination

Ten to fifty nanograms of genomic DNA were PCR amplified according to Favia *et al.* (1997), using primers A0 and Ag1.3. The PCR conditions were 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C for thirty-five cycles with a final extension step at 72° for 5 min. PCR products were then digested with restriction enzymes *Tru*9I and *Hha*I during 3 h at 65 °C and 37 °C, respectively, and analysed on 2% agarose gel. The restriction profiles were analysed as described in Favia *et al.* (1997) to determine the M or S taxon.

Intron sequence determination

The genomic region containing intron 1 was PCR amplified using primers I1dir (5′-GGCAGAACGATGGGTGCGTTAG-3′) and I1rev (5′-GCATAGCACACGGAACACAATC-3′). The genomic region containing intron 2 was PCR amplified using primers Agd1 and Agd2. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN). Automated sequencing was performed on a ABI prism 377 (Perkin Elmer) using the same primers and primer I1revseq (5′-CTGATCCTTGCATACATTG-3′) for the large intron 1.

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