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The spread of the Leu-Phe *kdr* mutation through *Anopheles gambiae* complex in Burkina Faso: genetic introgression and *de novo* phenomena

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Summary During extensive sampling in Burkina Faso and other African countries, the Leu-Phe mutation producing the *kdr* pyrethroid resistance phenotype was reported in both *Anopheles gambiae ss* and *A. arabiensis*. This mutation was widely distributed at high frequency in the molecular S form of *A. gambiae* while it has been observed at a very low frequency in both the molecular M form and *A. arabiensis* in Burkina Faso. While the mutation in the M form is inherited through an introgression from the S form, its occurrence is a new and independent mutation event in *A. arabiensis*. Three nucleotides in the upstream intron of the *kdr* mutation differentiated *A. arabiensis* from *A. gambiae ss* and these specific nucleotides were associated with *kdr* mutation in *A. arabiensis*. Ecological divergences which facilitated the spread of the *kdr* mutation within the complex of *A. gambiae ss* in West Africa, are discussed.

keywords pyrethroids, *kdr* mutation, introgression, *Anopheles gambiae ss*, *Anopheles arabiensis*, molecular forms, Burkina Faso, Africa

Introduction

Pyrethroids are a large group of highly insecticidal compounds. They have been widely used in controlling many insect pests since the 1970s. However, their important use in the last 20 years has led to the development of resistance in many insect species (Dong 1996). One main resistance mechanism is reduced target-site sensitivity to these compounds in the insect nervous system, known as knockdown resistance (*kdr*). The *kdr* has been first reported against dichlorodiphenyltrichloroethane (DDT) in the early 1950s in houseflies (Busvine 1951; Milani 1954), then lately in various insects, such as *Musca domestica* (Williamson *et al.* 1993), *Blattella germanica* (Dong & Scott 1994), *Heliothis virescens* (Taylor *et al.* 1993; McCaffery *et al.* 1995). This phenotype results from a single point mutation in a gene that encodes the sodium channel (Williamson *et al.* 1996).

Pyrethroid resistance was first reported in *Anopheles gambiae ss* in Côte d'Ivoire (Elissa *et al.* 1993). It was probably selected by the intensive use of DDT and, later pyrethroids for cotton crop protection (Chandre *et al.* 1999a; Diabaté *et al.* 2002a). As in several other insect species, a single nucleotide substitution [leucine (TTA) to phenylalanine (TTT)] in the *p*-sodium channel gene is the mutation responsible for pyrethroid resistance in *A. gambiae ss* from West Africa (Martinez-Torres *et al.* 1998). A second *kdr* mutation on the same amino acid [leucine (TTA) to serine (TCA)], produces pyrethroid resistance in *A. gambiae ss* from East Africa (Ranson *et al.* 2000) and recently pyrethroid resistance because of a mono-oxygenase-based mechanism was observed in both *A. funestus* and *A. gambiae ss* (Hargreaves *et al.* 2000; Etang *et al.* 2003). Following the availability of a rapid polymerase chain reaction (PCR)-based diagnostic test (Martinez-Torres *et al.* 1998), several studies were conducted to estimate the prevalence and assess the current distribution of the Leu to Phe mutation in natural *A. gambiae* populations (Chandre *et al.* 1999b; Weill *et al.* 2000; Della Torre *et al.* 2001). This mutation was observed, sometimes reaching high frequencies, in the

S molecular form of *A. gambiae ss* only, while it was not observed in sympatric and synchronous M form mosquitoes, or in *A. arabiensis* (Brooke *et al.* 1999; Chandre *et al.* 1999a). This strengthened earlier evidences for genetic heterogeneity within *A. gambiae ss*, formerly split into five chromosomal forms (Coluzzi *et al.* 1985; Touré *et al.* 1998). The issue of reproductive isolation of the M and S forms of *A. gambiae ss* (and, more broadly, of incipient speciation within this mosquito species) is a moot point and it is still unclear whether these forms can actually be considered as 'true' species (Gentile *et al.* 2001; Della Torre *et al.* 2001; Taylor *et al.* 2001; Tripet *et al.* 2001; Wondji *et al.* 2002; Diabaté *et al.* 2003a). A few years after the *kdr* mutation was described in the S molecular form of *A. gambiae ss*, it was reported in the M form in the forest belt of the littoral of Benin (Fanello *et al.* 2000). Subsequent molecular analysis of the DNA sequence of a large upstream intron suggested that this mutation arose in the M form through genetic introgression from the S form (Weill *et al.* 2000). Despite an extensive survey of this phenomenon in *A. gambiae* M form, the mutation was observed at high frequency only in the littoral forest belt of West Africa, while it was rare inland (Fanello *et al.* 2000; Weill *et al.* 2000; Della Torre *et al.* 2001; Diabaté *et al.* 2002b, 2003b; F. Chandre, unpublished data). The introgression event and subsequent spread could be a recent and ongoing process in this mosquito population.

We aimed at reporting in this paper, the detection of the Leu-Phe mutation in *A. arabiensis* and its distribution within the *A. gambiae* complex in Burkina Faso. We investigated whether this mutation has arisen in *A. arabiensis* through genetic introgression or through a *de novo* mutation by looking at the polymorphism of the intron upstream of the mutation.

Materials and methods

Mosquito populations

Larvae of *A. gambiae sl* were collected in Burkina Faso from 26 sites throughout the country. Because larvae samples can be biased with respect to *kdr* (there may be high levels of consanguinity among larvae from the same pool), special effort was made to collect large samples from different breeding sites and pooling them. The larvae were kept in the laboratory until adults emerged before proceeding to PCR analysis. *Anopheles arabiensis* was sampled from several African countries: Burkina Faso, Benin, Mali, Mauritania, Cameroon, Sudan, Chad, Kenya, Mozambique, Mauritius Island, Reunion Island, Madagascar Island, Djibouti. Mosquitoes were identified morphologically before PCR analysis.

DNA diagnostic test for *kdr* alleles in single mosquito

Genomic DNA was extracted from single mosquitoes according to Collins *et al.* (1987). Overall 10–50 ng of genomic DNA were combined in a 25 µl total volume with four primers Agd1, Agd2, Agd3 and Agd4 according to 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 45 cycles. Amplified fragments were analysed by electrophoresis on 1.5% agarose gel.

PCR identification of the *A. gambiae* complex

Each single mosquito was PCR identified for *A. gambiae* complex determination according to Scott *et al.* (1993). The genomic DNA was mixed with the four primers AA (specific for *A. arabiensis* species), AG (specific for *A. gambiae ss* species), AM (specific for *A. melas* and *A. merus*) and UN (common for all the species) in a total volume of 25 µl. The PCR was carried out with a programme of 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s. Ten microlitres of amplified product were run onto an 1.5% agarose gel and visualized by ethidium bromide staining under UV light.

M/S taxon determination

About 10–50 ng of genomic *gambiae s.s.* DNA were PCR amplified according to Favia *et al.* (2001) using primers R3, R5, Mopint and B/Sint. The PCR conditions were 30 s at 94 °C, 30 s at 63 °C and 30 s at 72 °C for 25 cycles with a final extension step at 72 °C for 7 min. Amplification products were run in a 1.4% agarose gel. The results were analysed as described in Favia *et al.* (2001) to determine M or S taxon.

Intron sequence determination

The *kdr* and knockdown-susceptible (*kds*) alleles were separately amplified and sequenced. Resistant allele was amplified using the primers I1dir (5′-AATTTGCAT TACTTACGACA-3′, Weill *et al.* 2000) and Agd3 (5′-AATTTGCATTACTTACGACA-3′), according to Martinez-Torres *et al.* (1998). For the susceptible allele, we determined a new reversed primer, AgdS (5′-AATTTG CATTACTTACGACT-3′), located at the same place than Agd3, from the position 312 of the sequence published by Martinez-Torres *et al.* (1998), but different from one base, at the 3′-extremity (on the *kdr* point mutation). The end of this primer, as Agd3, is located into the intron 2, which is situated only 4 bp apart downstream of the *kdr* mutation.

About 10–50 ng of genomic DNA were combined with I1dir (Weill *et al.* 2000) and Agd3 (Martinez-Torres *et al.* 1998) for the *kdr* allele and, separately I1dir and AgdS for the susceptible one. The PCR conditions were 30 s at 94 °C, 30 s at 63 °C and 30 s at 72 °C for 35 cycles with a final extension at 72 °C during 10 min. PCR fragments were gel purified using the QIAquick Gel Extraction Kit (Qiagen) then automated sequencing was performed using the same primers.

Results

Distribution of the *kdr* mutation in *Anopheles gambiae* complex in Burkina Faso

Sampling was conducted throughout Burkina Faso (26 localities) to assess geographical distribution of the *kdr* mutation in both *A. gambiae ss* and *A. arabiensis*. Overall, 546 *A. gambiae* S form specimens, 795 *A. gambiae* M forms and 232 *A. arabiensis* specimens were analysed for the *kdr* Leu-Phe mutation with a minimum of 50 specimens per village (Figure 1). A total of 571 additional *A. arabiensis* specimens were collected from 11 different countries (Benin, $n = 15$; Mali, $n = 10$; Mauritania, $n = 40$; Cameroon, $n = 50$; Sudan, $n = 30$; Chad, $n = 30$; Kenya, $n = 30$; Mozambique, $n = 136$; Mauritius Island, $n = 60$; Reunion Island, $n = 100$; Madagascar Island, $n = 30$; Djibouti, $n = 70$) and analysed for the same mutation. In a total of 26 localities sampled in Burkina Faso, the molecular S form was detected in 22 sites, the M form in 20 sites and *A. arabiensis* in 18 sites. While the molecular M form is widely distributed throughout the country, the S form and *A. arabiensis* are observed preferentially in humid and dry areas, respectively. The *kdr* mutation was found in the molecular S form wherever present and its frequency ranged from 0.17 to 0.96 (Figure 1). In the M form, the mutation was observed in just one site namely VK7 at a frequency of 0.02 (four

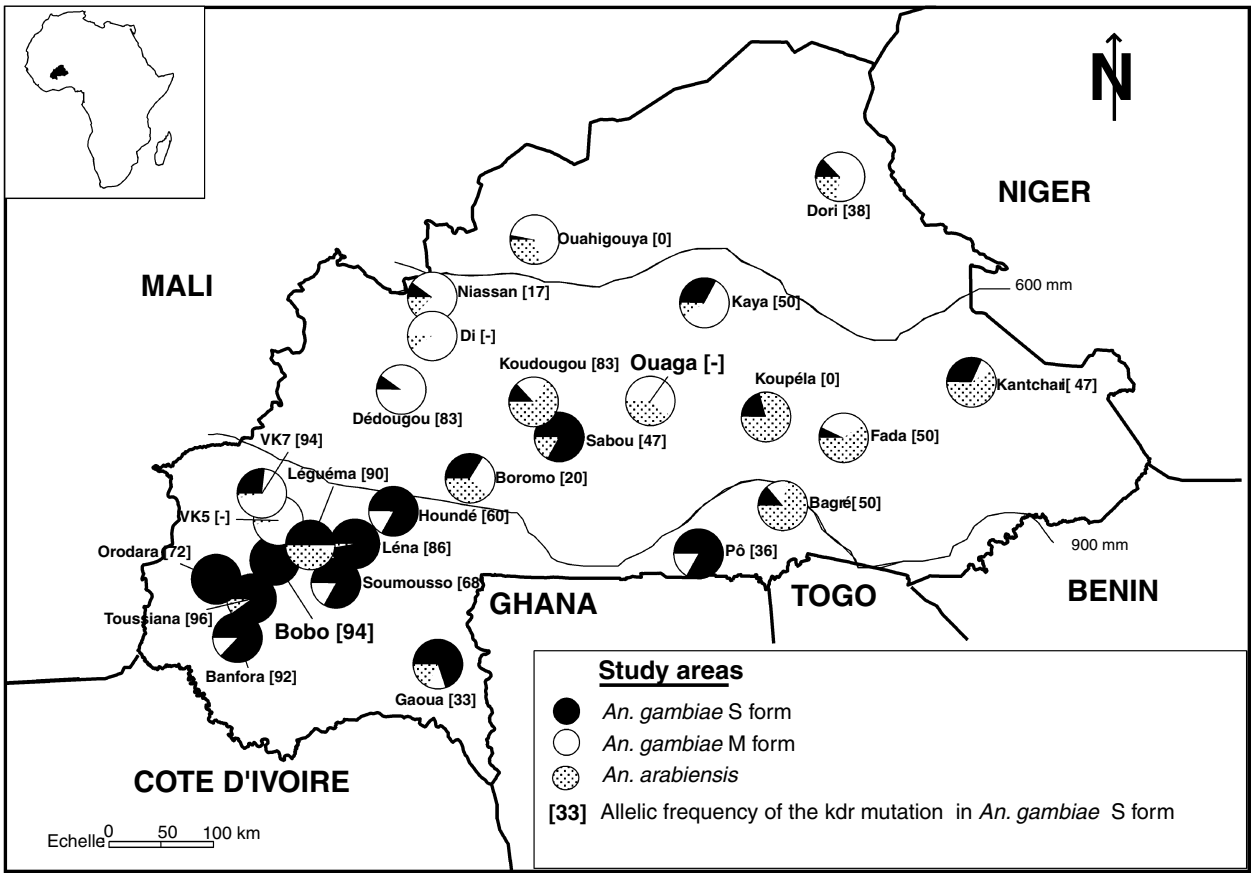

Figure 1 Geographic distribution and resistance profile of *Anopheles gambiae sl* to pyrethroids and dichlorodiphenyltrichloroethane (DDT) in Burkina Faso.

heterozygous of 173 specimens analysed for the *kdr* mutation). Despite large and extensive sampling, the *kdr* mutation was observed in a single *A. arabiensis* specimen from Burkina Faso.

Intron polymorphism of susceptible and resistant *A. gambiae ss* and *A. arabiensis*

To better understand the history of the *kdr* allele in both *A. gambiae* M form and *A. arabiensis*, we sequenced 540 bp of the intron upstream the sodium channel gene near the *kdr* mutation. Both alleles were sequenced in 69 mosquitoes (*A. gambiae* S form, $n = 15$; *A. gambiae* M form, $n = 20$ and *A. arabiensis*, $n = 34$). *Anopheles gambiae ss* specimens were collected in the villages of Lena and VK7 in Burkina Faso (Figure 1). *Anopheles arabiensis* specimens were from Burkina Faso, Soudan, Cameroon and Mauritius Island. The Leu-Phe mutation (TTA-TTC) was observed in both *A. gambiae ss* and *A. arabiensis* in all resistant alleles ($n = 30$). A leucine residue was found in susceptible alleles at amino acid 1014 regardless of the species ($n = 108$). No Leu-Ser (TTA-TCA) substitution was detected, as described in East Africa resistant *A. gambiae ss* (Ranson *et al.* 2000). Two polymorphic sites (positions 702 and 896) differentiated the two molecular forms as previously reported in Weill *et al.* (2000). However, both M and S forms displayed the T-C combination at positions 702–896, associated with the *kdr* allele (Table 1). The same pattern was observed with the susceptible allele in *A. gambiae* S form, while the susceptible *A. gambiae* M form consistently displayed C-C or C-A combinations. Three positions (824–830–835) were found to consistently differentiate *A. arabiensis* from *A. gambiae ss* (Table 2). Nucleotide at position 494 was fixed in *A. gambiae ss* and polymorphic in *A. arabiensis*. Because the sequence of nucleotides at positions 824–830–835 was specific to *A. arabiensis* in both resistant and susceptible alleles, the *kdr* mutation in *A. arabiensis* is likely to be a *de novo* event rather than the result of introgression.

Table 1 Discriminating nucleotides (702–896) in the upstream intron of the knockdown-resistant (*kdr*) mutation within M and S molecular forms of *Anopheles gambiae ss* in Burkina Faso

	Susceptible (Leu)			Resistant (Phe)		
	C-A	C-C	T-C	C-A	C-C	T-C
M taxon						
VK7	4	27	0	0	0	5
Léna	0	4	0	–	–	–
Total	4	31	0	0	0	5
S taxon						
VK7	–	–	–	0	0	16
Léna	0	0	6	0	0	8
Total	0	0	6	0	0	24

Polymorphism observed in position 702 and 896. A T-C combination is observed in both molecular S and resistant molecular M form while the susceptible M form consistently displayed C-A or C-C combination.

Table 2 Discriminating nucleotides (494–824–830–835) between *Anopheles gambiae ss* and *A. arabiensis* in the upstream intron of the *kdr* mutation

				Nucleotide position			
Forms/species	Country	Allele	*N*	494	824	830	835
A. gambiae S form	Burkina Faso	*kds*	6	T	T	G	A
		kdr	24	T	T	G	A
A. gambiae M form	Burkina Faso	*kds*	35	T	T	G	A
		kdr	5	T	T	G	A
A. arabiensis	Burkina Faso	*kds*	12	T	A	A	T
		kds	17	G	A	A	T
		kdr	1	G	A	A	T
	Soudan	*kds*	8	T	A	A	T
		kds	10	G	A	A	T
	Cameroon	*kds*	6	T	A	A	T
		kds	6	G	A	A	T
	Mauritius	*kds*	8	G	A	A	T

kds, knockdown-susceptible (Leu); *kdr*, knockdown-resistant allele (Phe); *N*, number of allele sequenced.

Discussion

After extensive sampling, the Leu-Phe *kdr* mutation was detected in both *A. gambiae ss* and *A. arabiensis* in Burkina Faso. This mutation was widely distributed at high frequency in the molecular S form, but occurred at a very low frequency in both *A. arabiensis* and the molecular M form of *A. gambiae ss*.

The unequal distribution of the resistant phenotype in *A. arabiensis* and the molecular M and S forms of *A. gambiae ss* is probably the result of differential insecticide pressure selection. The distribution and temporal dynamics of the molecular S form should expose it to higher insecticide selection pressure (Diabaté *et al.* 2002a). As the *kdr* mutation confers cross-resistance to both DDT and pyrethroids, it is likely that the present pattern of this resistance allele distribution in *A. gambiae ss* is a consequence of the important use of DDT in cotton crops in the 1960–1970s, replaced by pyrethroids in the 1980s. The *kdr* mutation has probably been selected some time ago in West Africa, as DDT-resistant, *A. gambiae ss* were reported there in the 1960s (Hamon *et al.* 1968). This, coupled with subsequent pyrethroid exposure, may explain why the *kdr* mutation is observed at such a high frequency in West Africa.

A substitution of the same amino acid in various species of insects is rather a rare event. However, the *kdr* mutation has been reported in many species of insects (Dong 1996; Williamson *et al.* 1996; Jamroz *et al.* 1998; Martinez-Torres *et al.* 1998; Ranson *et al.* 2000; Enayati *et al.* 2003). The leucine replacement by an other amino acid is the most common and that suggests that the leucine residue is very important in the recognition and/or binding of pyrethroids and DDT (Ranson *et al.* 2000). The occurrence *de novo* of this mutation in *A. arabiensis* in a sympatric area with highly resistant *A. gambiae ss*, suggests that the hybridization rate between these two sibling species is very low (Touré *et al.* 1998; Taylor *et al.* 2001). Interestingly, the same mutation found in both M and S forms is resulting from an introgression from one form to the other (Weill *et al.* 2000). We believe that the *kdr* mutation distribution in *A. gambiae* complex provides some indication on the level of gene exchange between and within these species and thus is an important genetic marker to assess the reproductive isolation within this complex of species. Of course, a larger sample size of *kdr-arabiensis* would have strengthened our results, but it is worth noting that overall 803 *arabiensis* specimens were analysed for the *kdr* mutation. Furthermore, *kdr-gambiae* resistance is already observed at very high frequencies in Benin and Burkina Faso where both *A. gambiae ss* and *A. arabiensis* are sympatric. That suggests that the *kdr* mutation in *A. arabiensis* is certainly a recent and ongoing process. The patchy distribution of the *kdr* mutation in the molecular M form of *A. gambiae ss* in West Africa is unclear and raises the question of the origin and frequency of resistance in natural M populations. It may be a recent and unique event, which arose in this form through introgression from the S form in the forest belt and subsequently spread inland. However, the current pattern of distribution of the resistant M populations of *A. gambiae ss* do not support this hypothesis. Highly resistant M populations are observed alongside the littoral while low resistance levels are recorded in only a few and discrete places inland. According to Black and Lanzaro (2001) gene flow with partial reproductive isolation among molecular forms occurs only in certain geographical locations or during certain seasons. If that is true, then the current patchy distribution of the *kdr* mutation in the M form of *A. gambiae ss* is probably resulting from different events of introgression within *A. gambiae ss*. Introgression has probably occurred when *A. gambiae* M and S forms are found in sympatry at high densities, but where one form is predominant (Diabaté *et al.* 2003b). The *kdr* mutation distribution in relation with the dynamic of both molecular and chromosomal forms of *A. gambiae ss* in a wide scale including the ecological description has not been thoroughly investigated. Such a study may be helpful to understand the ongoing process of the *kdr* mutation in *A. gambiae ss*.

The report of *kdr* in *A. arabiensis*, another major malaria vector in Africa, is of great significance at both fundamental and applied levels. Its potential impact on the efficacy of malaria vector control interventions will have to be evaluated and results taken into consideration by malaria control programmes. The very low frequency of this allele in both *A. arabiensis* and in the M form of *A. gambiae ss* suggests that these were recent phenomenon, but it may spread quickly in these mosquitoes in areas of intensive insecticide use. Further, characterization throughout the range distribution of the *A. gambiae* complex will be very informative to understand the history and the contrasting distribution of this new allele in mosquito field populations.

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