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Species abundance and insecticide resistance of *Anopheles gambiae* in selected areas of Ghana and Burkina Faso

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Abstract. The Ghanaian National Malaria Control Programme has prioritized insecticide-treated materials as a key strategy for malaria control. We report on a survey of the distribution of the molecular forms of *Anopheles gambiae* Giles (Diptera: Culicidae) and insecticide resistance (the *kdr* mutation), carried out by sampling mosquitoes from 11 locations in Ghana and one additional site in Burkina Faso. The molecular M and S forms of *An. gambiae* were found to occur in sympatry in southern Ghana. The S form predominated throughout its distribution in the coastal savannah, except at one location in the strand and mangrove zone where rice was cultivated. The M form was the only form collected in northern Ghana and was the predominant form (97.5%) in Burkina Faso. No M/S hybrids were detected. The *kdr* mutation was observed at very high frequencies (98–100%) within the S form but reached a maximum of only 3.38% in the M form in one population at an irrigation scheme in the Ghanaian coastal savannah zone.

Key words. *Anopheles gambiae*, introgression, *kdr* mutation, malaria, pyrethroids, selection, sympatry, Ghana.

Introduction

The Ghanaian National Malaria Control Programme has prioritized insecticide-treated nets or other materials as a key strategy for malaria control. The implementation of large-scale pyrethroid-impregnated bed net strategies will require knowledge of vector distribution and biology, and particularly insecticide resistance frequencies in the *Anopheles* sp. (Diptera: Culicidae) involved in transmission. In previous studies in Ghana, *Anopheles gambiae* Giles *sensu stricto* and *An. funestus* Giles were found to be the most abundant and widespread vectors in the coastal savannah zones of Ghana (Appawu *et al*., 1994). *Anopheles gambiae sensu lato* and *An. funestus* were found to be the major human-biting species in Dodowa in the coastal savannah zone, whereas *An. gambiae s.l.* and *An. pharaoensis* were the most common biting mosquitoes in Prampram in the strand and mangrove zone (Appawu *et al*., 2001). *Anopheles gambiae*, *An. funestus* and *An. rufipes* are the most common anophelines in the north, but no published data exist on the vector species in the middle rainforest belt. *Anopheles gambiae s.l.* and *An. funestus* are the most widespread malaria vectors in Ghana (Appawu *et al*., 2001).

Polytene chromosome analysis of the *An. gambiae s.s.* (Appawu *et al*., 1994) has indicated the existence of three main populations characterized by different inversion frequencies: the Forest chromosomal form, typical of the moist semi-deciduous forest, characterized by inversion systems 2Rb, 2Rd and 2La; the Savannah form typical of the more arid zones of the coastal and interior savannas, characterized by inversion arrangements 2Rbc and 2Rbcd; the Mopti chromosomal form characterized by inversion 2Rbc/2Ru, and sympatric with the Savannah form in the drier areas of both coastal and interior savannas (Appawu *et al*., 1994). Characterization of the chromosomal forms within *An. gambiae s.s.* (Bryan *et al*., 1982; Coluzzi *et al*.,

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1985) using nucleotide sequences from the intergenic spacer of rDNA region of the X-chromosome (Favia *et al*., 1997; della Torre *et al*., 2001) has revealed two forms termed S and M. In Mali and Burkina Faso, M always corresponded to chromosomal Mopti whereas S corresponded to sympatric populations of Savannah and a cytotype termed Bamako. However, outside Mali and Burkina Faso this linkage broke down, and both molecular genotypes are found in the Savannah and Forest chromosomal forms (della Torre *et al*., 2001). A recent study showed very strong positive assortative mating, with 98.8% within-form mating (Tripet *et al*., 2001). The levels of reproductive isolation and incipient speciation within the forms have become the focus of much research on the *An. gambiae* complex and the issue has generated much debate (della Torre *et al*., 2001; Taylor *et al*., 2001; Tripet *et al*., 2001; Besansky *et al*., 2003; Donnelly *et al*., 2004). Recent microsatellite, mitochondrial and rDNA data from the Navrongo area of northern Ghana demonstrated that the M form exists in the north of the country (Lehmann *et al*., 2003; Donnelly *et al*., 2004) but the rest of the country remains unexplored.

The increased development of mosquito resistance to pyrethroids is of particular concern for many integrated malaria control programmes that utilize insecticides for vector control. Pyrethroid knockdown resistance (*kdr*) allele frequencies in *An. gambiae* have been reported at over 90% (Guillet *et al*., 2001). But despite the fact that Ghana is a country where pyrethroid-impregnated materials form a major component of the National Malaria Control Programme, studies on the uses of insecticides and the development of resistance in *An. gambiae* have been very limited (see Kristan *et al*., 2003). Pyrethroid knockdown resistance (*kdr*), resulting from a single point mutation in the gene that encodes the sodium channel (Martinez-Torres *et al*., 1998), was previously confined to the S form (Chandre *et al*., 1999), but has now been observed at low frequencies in the M form in countries bordering Ghana (Diabaté *et al*., 2003).

Although the implications of the spread of resistance for the future of pyrethroid-impregnated materials in malaria control remain to be understood, the need for monitoring that spread is clear. In this study, we present the distribution of the two molecular forms of *Anopheles gambiae s.s*. in Ghana, including the far north and into Burkina Faso, and describe the distribution of the *kdr* mutation.

Materials and methods

Collection of material

In southern Ghana, adult female *An. gambiae* were collected by hand using aspirators or pyrethrum spray catches inside human dwellings between June and September 2002, and in northern Ghana and Burkina Faso between April and May 2003 (Fig. 1). Larvae, some of which were reared to adults, were collected between June and July 2003 in the middle rain forest belt (Sample i, Kumasi). Collected mosquitoes were preserved dry over silica gel in 1.5 mL Eppendorf tubes until transported to the laboratory.

In southern Ghana, three villages in the strand and mangrove zone were sampled: Mampong (a) (05°24.736′ N, 000°36.948′ W) and Okyereko (b) (05°24.874′ N, 000°36.253′ W), which are adjacent to a rice irrigation scheme, and Abia (c) (05°42.926′ N, 000°07.691′ W), a fishing village. In this zone the highest mean monthly temperature of 30°C occurs between March and April and the lowest of 26°C in August. Relative humidity is high (65–75%) throughout the year and vegetation consists mainly of grass with isolated patches of shrub and sparse trees. The major rainfall season is in June–July and is followed by a long dry season. Five villages were sampled in the coastal savannah zone: Dodowa (d) (05°52.673′ N, 000°06.365′ W), Osurogba (e) (05°52.752′ N, 000°06.577′ W), Odumasy (f) (05°53.864′ N, 000°04.725′ W), Ayenya (g) (05°56.593′ N, 000°01.892′ W), and Ayikuma (h) (05°55.227′ N, 000°03.197′ W). This zone is humid with persistent rainfall. The main crops in all five villages were cassava and mangoes with some small-scale vegetable production. The city of Kumasi (i) (6°42′ N, 1°37′ W) is located in the middle rainforest belt. Korania (j) (10°53.16′ N, 001°05.40′ W) and Bonia (k) (10°52.05 N, 001°07.25′ W) are situated in the northern savannah and form part of the Tono irrigation scheme. The most northerly site, Koubri (l) (12°11.56′ N, 001°23.46′ W) is situated in southern Burkina Faso in the Sahel Region.

Laboratory investigations

Anopheles spp. mosquitoes were identified morphologically using the keys of Gillies & Coetzee (1987). DNA was extracted using the phenol–chloroform–isoamyl alcohol method of Ballinger-Crabtree *et al*. (1992). *Anopheles gambiae* complex member species were identified by the polymerase chain reaction (PCR) method of Scott *et al*. (1993). Molecular identification of forms within *An. gambiae* s.s. was based on the method of Fanello *et al*. (2003). Detection of the standard Leu–Phe '*kdr*' mutation was performed following Martinez-Torres *et al*. (1998), with slight modifications. Genomic DNA, extracted as described, was diluted at 1 : 10 with double-distilled H_2O and 1 μL was combined in a 25 μL total reaction volume with the four primers Agd1, Agd2, Agd3 and Agd4. The PCR conditions were: 15 min at 95°C, 30 s at 94°C, 1 min at 55°C and 1 min at 72°C for 44 cycles; 30 s at 94°C, 1 min at 55°C and a final extension step at 72°C for 10 min. Amplified fragments were analysed by electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining under ultraviolet light.

Results and discussion

The numbers and morphological identifications of mosquitoes collected are shown in Table 1. A total of 935

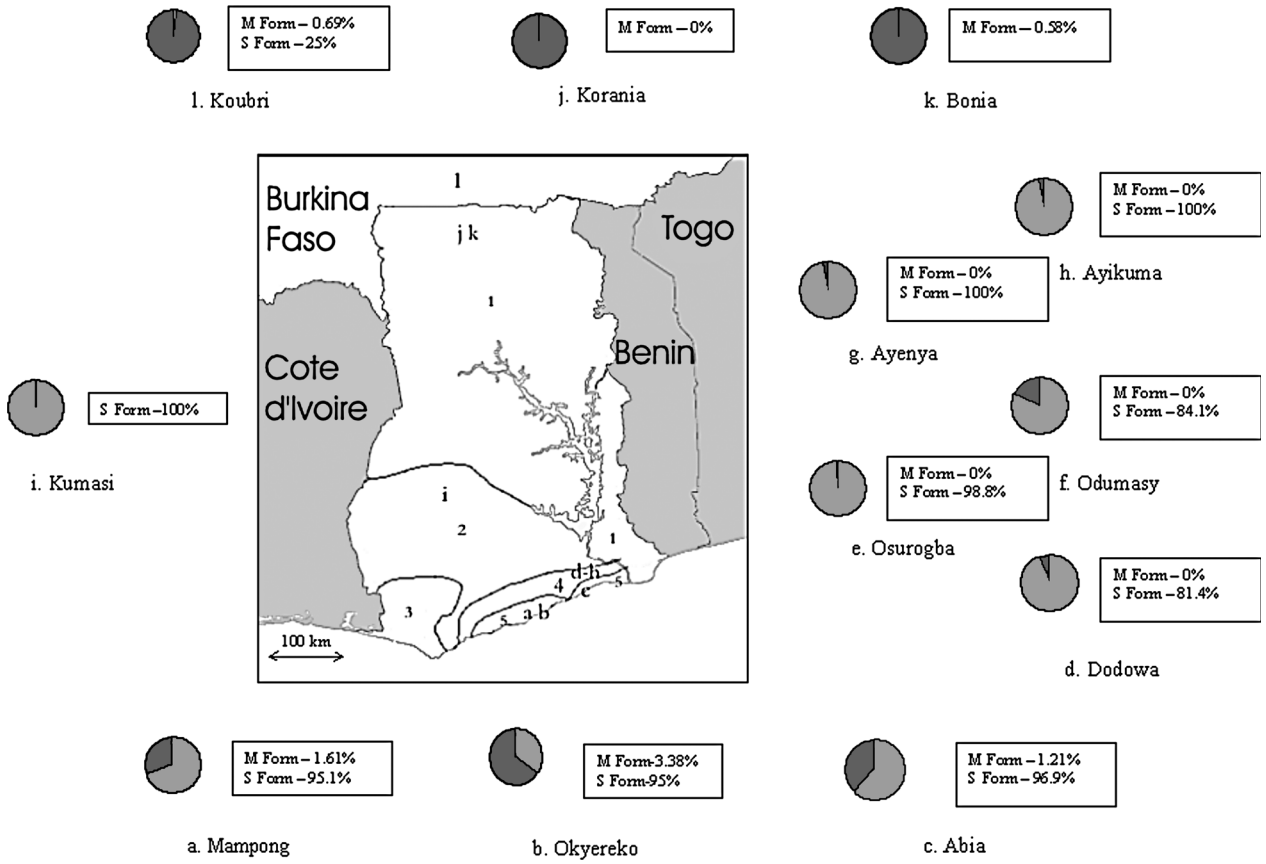

Fig. 1. Distribution of the molecular forms of *Anopheles gambiae* in Ghana and Burkina Faso. The pie charts display the relative frequency of the two forms in each collection area: M form in grey and S form in black. The boxes adjacent to each pie chart show the frequency of the *kdr* mutation in the population sample. Numbers 1–5 are the eco-climatic zones mentioned in the text. (1) interior wooded savannah, (2) semi-deciduous forest, (3) rainforest, (4) coastal savannah, (5) strand and mangrove zone. The map is orientated due north.

Table 1. Mosquito collection dates, numbers and morphological identifications in the in the different localities.

Location	Date	No of PKDs¶	*Anopheles gambiae s.l.*	*Anopheles funestus*	*Anopheles rufipes*	*Anopheles arabiensis*	*Anopheles melas*
Dodowa	10/06/02–06/06/03	27	510	163	–	–	–
Osurogba	13/06/02–21/08/02	10	283	58	–	–	–
Odumasy	07/06/02–03/07/02	15	321	260	–	–	–
Ayenya*	13/08/02	3	119	33	–	–	13
Ayikuma	20/08/02	3	52	13	–	–	–
Okyereko	15/06/02–27/06/03	8	618	138	–	–	–
Mampong	29/08/02–31/08/02	5	441	45	–	–	–
Abia†	21/06/02–11/06/03	6	133	–	–	–	1
Aburi	10/08/02	1	2	3	–	–	–
Kumasi‡	05/05/02–01/08/02		52	–	–	–	–
Bonia	25/04/03–24/04/03	8	572	72	66	–	–
Korania	23/04/03–26/04/03	6	244	603	46	–	–
Koubri (BF)§	08/05/03–14/05/03	8	103	52	–	2	–

*Thirteen of 96 individuals identified to specific status were *An. melas*.
†1One of 108 individuals identified to specific status was *An. melas*.
‡Sampling involved larval collection.
§Two of 103 individuals identified to specific status were *An. arabiensis*.
¶PKD, collections by the pyrethrum knockdown method.

mosquitoes were identified to species level and to molecular form. Of these, the *kdr* genotype was scored for 921 individuals (14 mosquitoes consistently failed to amplify).

Anopheles gambiae s.l. was always collected in sympatry with *An. funestus*. One individual (total catch, $n = 108$) from Abia (site c, Fig. 1) on the coast and 13 ($n = 96$) individuals from Ayenya (g), which is 19 km inland from Abia, were identified as *An. melas*. *Anopheles rufipes* was found sympatrically with *An. gambiae* and *An. funestus* in the savannah region of the north of Ghana. Two *An. arabiensis* ($n = 103$) were collected in Koubri (l) in southern Burkina Faso.

The two molecular forms of *An. gambiae* were sympatric in all locations, except in Kumasi (i, central Ghana), and both Korania and Bonia (j, k, northern Ghana), where only S form and M forms, respectively, were identified (Table 2). In southern Ghana, the S form predominated mainly in locations in the coastal savannah zone (sites d–h, at frequencies of 81–99%), in the strand and mangrove zone at Mampong (a, 69% S form), but not at the rice irrigation scheme of Okyereko (b, 65% M form). In the single location sampled in Burkina Faso, only two S form individuals were found (2.5% of the total number analysed from this region).

The M form may have predominated in Okyereko (b), Abia (c) and Mampong (a) and in the two sites in northern Ghana (j, k) due to the proximity of permanent breeding conditions provided by rice fields. The M form of *An. gambiae* is known to be associated with flooded/irrigated sites, typified by extensive rice cultivation, whereas the S form is characteristically found in rain-dependent breeding sites (Touré *et al.*, 1998; Diabaté *et al.*, 2003). Our rainy season collections, where S form predominated over M form (81–100% S form in sites d–i; Table 3) in all but the irrigated sites in the coastal savannah, provide circumstantial support for this hypothesis. No hybrid M/S form individuals were detected in any of the collection sites, although low levels of hybridization between M and S molecular forms have been reported in the field at

Table 2. Frequencies of the molecular form of *Anopheles gambiae s.s.* in samples from Southern Ghana to Southern Burkina Faso.

Locality	Ecological zone type	*n*	Percentage of S molecular form collected
Ghana			
Dodowa	Coastal savannah	70	94
Osurogba	Coastal savannah	82	99
Odumasy	Coastal savannah	54	81
Ayikuma	Coastal savannah	37	97
Ayenya	Coastal savannah	77	97
Okyereko	Mangrove	114	35
Mampong	Mangrove	106	69
Abia	Mangrove	108	61
Kumasi	Central forest	52	100
Korania	Northern savannah	95	0
Bonia	Northern savannah	86	0
Burkina Faso			
Koubri	Northern savannah	80	2.5

No M/S hybrids were observed.

Table 3. Frequency distribution of the *kdr* (insecticide resistance) mutation along transect from southern Ghana to Burkina Faso.

	kdr frequency			
	M form		S form	
Abia	42	1.21%	66	96.9%
Dodowa	4	0%	66	81.4%
Osurogba	1	0%	81	98.8%
Odumasy	10	0%	44	84.1%
Okyereko	74	3.38%	40	95%
Ayikuma	1	0%	36	100%
Ayenya	1	0%	76	100%
Mampong	33	1.61%	73	95.1%
Kumasi	–	0%	52	100%
Korania	95	0%	–	0%
Bonia	86	0.58%	–	0%
Koubri	78	0.69%	2	25%

frequencies ranging from 0.26% (della Torre *et al.*, 2001) and 0.3% (Tripet *et al.* 2001) to 0.71% (Taylor *et al.*, 2001).

The *kdr* mutation occurred in both M and S forms from southern Ghana but at very different frequencies across the two ecological zones (Table 3). The mutation was more common in the S molecular form, with frequencies of 81.1–100% in the coastal savannah and mangrove zones (Table 3). It was absent in the M form except in the mangrove and strand zone, Mampong (a), Okyereko (b) and Abia (c), and in the northern savannah at Bonia (k) and Koubri (l). The maximum frequency of the *kdr* allele in the M form was at Okyereko (3.38%; Table 3).

The high frequency of the *kdr* mutation in the S form is consistent with other reports, where selection pressure resulting from agricultural insecticide usage has been postulated as the cause (Guillet *et al.*, 2001). The frequencies in M form populations are low (<5%) and similar to those found in Burkina Faso (Diabaté *et al.*, 2003), and are more alike to Mali (Fanello *et al.*, 2003), Cote d′ Ivoire (della Torre *et al.*, 2001) and Nigeria (Awolola *et al.*, 2003) where the mutation has not been documented, than in neighbouring Benin where Corbel *et al.* (2004) recorded *kdr* frequencies in the M form of 78%. What causes this extreme spatial variation in *kdr* frequencies in the M from is unknown. It has been postulated that the *kdr* mutation reached the M form through introgression from the S form (Weill *et al.*, 2000), although further work will be necessary to determine if this has occurred in our study populations. However, even though the *kdr* mutation is now widely dispersed in M form populations, frequencies, Benin populations aside, are much lower than in sympatric S form populations (della Torre *et al.*, 2001). This is particularly curious given that the M form is thought to be associated with flooded/irrigated sites, typified by extensive rice cultivation, which in our study area are sites of intensive insecticide usage and presumably selection pressure. A brief investigation of practices at the rice and vegetable farms in Okyereko and Mampong revealed heavy usage of three pyrethroids throughout the growing season [Chemotrim 100 EC (100 g/μL permethrin), Dursban B 18/150 EC (18 g/L

alphacypermethrin + 150 g/L chlorpyriphos ethyl) and Polytrin C 180 EC (30 g/L cypermethrin + 150 g/L profenofos)] to control a wide range of phytophagous pest insects, particularly rice stem borers (Lepidoptera: Pyralidae).

Further work on the fitness costs of the *kdr* mutation are required to determine whether this is the cause of the much lower frequency in the M form. To date only limited work has been performed on the evolution of the *kdr* mutation and has mainly investigated the correlation between resistance profile and *kdr* locus genotype (Chandre *et al*., 2000; Corbel *et al*., 2004). Moreover, these studies used colonized specimens from vastly different geographical areas (Chandre *et al*., 2000) or even different molecular forms (Corbel *et al*., 2004). As Bourguet *et al*. (2004) noted, such strains can have different life histories or may have become adapted to colonization. Unless studies are performed on strains with a uniform genetic background it is impossible to attribute any observed effects solely to the resistance alleles.

This is the first report on the distribution of the *kdr* mutation and of the molecular forms in the different ecological zones in Ghana. These data contrast with those of Kristan *et al*. (2003), who performed insecticide bioassays on *An. gambiae* from south-western Ghana and concluded that there was no evidence of *kdr*-type resistance despite the widespread use of pyrethroids and DDT for pest control on crops in the region. However, careful examination of their Table 2 shows that KDT_{90} and KDT_{50} values were significantly higher in field-collected mosquitoes from Tarkwa (Ghana) and the coastal region than in controls (KWA strain), which is suggestive of a knockdown type resistance mechanism. The operational significance of the occurrence of the *kdr* resistance mechanism in *An. gambiae* populations is far from clear, although it is interesting to note that the *kdr* mutation was found at very low frequencies in the two samples obtained from the north of Ghana where Binka *et al*. (1996) conducted an extensive insecticide treated net trial. Furthermore, preliminary evidence using entomological proxies for malaria transmission was thought to indicate that the presence of the mutation did not reduce the efficacy of the insecticide-treated bednets (Chandre *et al*., 2000). The data presented here represent a first stage in documenting the current state of insecticide resistance in the two forms of the main malaria vector in Ghana and we are presently investigating the less tractable, although possibly more important, mechanisms of metabolic resistance in *An. gambiae* in this area.

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