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Stable Chromosomal Inversion Polymorphisms and Insecticide Resistance in the Malaria Vector Mosquito *Anopheles gambiae* (Diptera: Culicidae)

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ABSTRACT *Anopheles gambiae* Giles has been implicated as a major vector of malaria in Africa. A number of paracentric chromosomal inversions have been observed as polymorphisms in wild and laboratory populations of this species. These polymorphisms have been used to demonstrate the existence of five reproductive units in West African populations that are currently described as incipient species. They have also been correlated with various behavioral characteristics such as adaptation to aridity and feeding preference and have been associated with insecticide resistance. Two paracentric inversions namely 2La and 2Rb are highly ubiquitous in the wild and laboratory populations sampled. Both inversions are easily conserved during laboratory colonization of wild material and one shows significant positive heterosis with respect to Hardy-Weinberg proportions. Inversion 2La has previously been associated with dieldrin resistance and inversion 2Rb shows an association with DDT resistance based on this study. The stability and maintenance of these inversions as polymorphisms provides an explanation for the transmission and continued presence of DDT and dieldrin resistance in a laboratory strain of *An. gambiae* in the absence of insecticide selection pressure. This effect may also be operational in wild populations. Stable inversion polymorphism also provides a possible mechanism for the continual inheritance of suitable genetic factors that otherwise compromise the fitness of genetically modified malaria vector mosquitoes.

KEY WORDS *Anopheles gambiae*, chromosome inversions, heterosis, insecticide resistance, Mali, Cote d'Ivoire

THE AFRICAN MALARIA vector mosquito *Anopheles gambiae* Giles is one of six fully described members of the *An. gambiae* species complex. A species complex is best defined by the absence of unambiguous interspecific morphological differences between members. The other five members include the malaria vectors *An. arabiensis* Patton, *An. merus* Donitz, *An. melas* Theobald, *An. bwambae* White, and the nonvector *An. quadriannulatus* Theobald. These taxa were originally assigned species status on the basis of cytogenetic, isoenzyme, and cross-mating studies correlated with behavioral variation. A review of the history of the differentiation of the *An. gambiae* complex into specific taxa is given by Davidson et al. (1967), White (1974) and Hunt and Coetzee (1995). Recently, a seventh member currently designated as *An. quadriannulatus* species B was described by Hunt et al. (1998) from Ethiopia.

The nominal member of the complex, *An. gambiae* sensu strictu, has been further subdivided into five reproductive units in the West African region based on cytogenetic studies. Coluzzi et al. (1977; 1979, 1985) studied the chromosomal inversion polymorphisms found in West African *An. gambiae* populations and demonstrated clear evidence of positive assortative mating based on heterokaryotype deficiencies. Overlapping sets of karyotypes referring to five paracentric inversions found on arm 2R have been assigned to each of the five reproductive units. Although the taxonomic status of these reproductive units remains a contentious issue, each unit has been designated as a particular chromosomal form. These five forms are known as the Forest, Savannah, Mopti, Bamako, and Bissau forms. Two or more chromosomal forms are often found in sympatry (Toure et al. 1998) and a complete description of them can be found in Powell et al. (1999).

Recent investigations involving molecular techniques have led to significant modifications of hypotheses regarding the proposed reproductive isolation of these forms. All possible crosses between the various chromosomal forms have been performed under laboratory conditions. There is no evidence of reproductive isolation across all permutations (Coluzzi et al.

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1985; Toure et al. 1983, 1998). Since then, the role of the 2R chromosomal inversion systems in mate recognition has come under review. Restriction fragment-length polymorphism (RFLP) analysis of a segment of ribosomal DNA including a portion of the 28S coding region as well as a portion of the adjacent intergenic spacer region (IGS) has identified two molecular forms namely M and S (Favia et al. 1997). Two of the restriction enzymes used in the RFLP analyses differentiated Mopti (M) from Savannah and Bamako (S) forms. Subsequent analysis (Della Torre et al. 2001) using the RFLP system on samples from several west African countries showed the existence of two molecular forms identified as M and S but without the original correlation of M with Mopti and S with Savannah and Bamako forms. Della Torre et al. (2001) suggest that chromosome 2 inversions seem to be involved in ecotypic adaptation rather than mate recognition.

The assortment of polymorphic paracentric inversions on the second chromosome of wild *An. gambiae* populations has been correlated with adaptation to levels of aridity. Coluzzi et al. (1979, 1985) showed a cline in the frequencies of certain chromosome 2 inversions with increasing levels of aridity. These inversions include three on the right arm (inversions 2Rb, 2Rc, and 2Rd) and one on the left arm (inversion 2La). The general trend shows that the wild-type arrangements for these inversions are associated with a wetter climate while the inverted arrangements are associated with drier climates. Inversion 2La has also been linked to resting and feeding behavior (Coluzzi et al. 1979) and *Plasmodium* infection rates (Petrarca and Beier 1992) in *An. gambiae.*

This study identifies potentially stable inversion polymorphisms in wild populations and laboratory strains of *An. gambiae* and highlights their association with adaptive characteristics such as insecticide resistance, feeding preference and ecological adaptation. It is postulated that stable inversion polymorphism may exert a direct effect on the phenotypic expression of genes linked to these inversions.

Materials and Methods

Ovarian polytene chromosomes were prepared from the ovarian nurse cells of half-gravid females according to the method of Green and Hunt (1980). Polytene chromosomes were prepared from wild-caught female *An. gambiae* complex samples from three localities in Cote d'Ivoire (Bouake, Yaokoffikro, and Danane), two villages of close proximity in southern Mali (Sanso and Morila) and three villages in the Bagamoyo district, Tanzania (Kongo, Matimbwa and Yombo). The sample from Danane included *An. gambiae* females caught in villages where insecticide treated bed-nets have been used extensively as well as females caught in villages where there were no mosquito control measures in place. Inversion frequencies were compared between these samples.

Chromosomes were also prepared from samples of the *An. gambiae* laboratory strains Ian P20, CIG and NAG currently housed at the National Health Laboratory Service (NHLS), Johannesburg. The Ian P20 strain originated from northern Nigeria and was established more than 20 yr ago at the London School of Hygiene and Tropical Medicine (Hemingway 1981). It has been kept in colony at the NHLS since 1978. The CIG strain originates from Cote d'Ivoire and was established ≈3 yr ago at the NHLS. The NAG strain also originates from Nigeria and was established ≈1 yr ago at the NHLS. Chromosome preparations were scored for all known inversion systems. Species identifications were based on X chromosome banding sequences and corroborated by the polymerase chain reaction method (Scott et al. 1993) where necessary.

Selections for DDT resistance using newly emerged adults from the *An. gambiae* Ian P20 laboratory strain were conducted using 250 ml glass bottles coated with 400 μg DDT. This method was based on the CDC bottle bioassay method described by Brogdon and McAllister (1998). Survivors from samples following either 0.5- or 1-h exposures to DDT were transferred to cages and allowed a 2-d resting period after which the females were offered a blood meal. Ovarial tissue was dissected from these females at the half-gravid stage and polytene chromosomes were prepared and scored for polymorphic inversions. Inversion frequencies from the unselected, 0.5- and 1-h selected samples were compared.

Results

Table 1 gives the frequencies of the inverted arrangements of listed inversion systems in each sample.

Table 1. Frequencies of the inverted arrangements for inversion systems 2Rb, 2Rc, 2Ru, 2Rj, 2Rd, 2La, and 2Ln in *Anopheles gambiae* from wild-caught samples from Cote d'Ivoire, Mali, and Tanzania as well as colony samples

Country/Colony	Locality	*n*	2Rb	2Rc	2Ru	2Rj	2Rd	2La	2Ln
Cote d'Ivoire	Bouake	97	0.23	0	0	0	0	0.51	0.02
	Yaokoffikro	59	0.31	0	0	0	0	0.32	0.01
	Danane	52	0.38	0.01	0.12	0	0.03	0.07	0
Mali	Sanso	89	0.77	0.11	0.11	0.03	0.006	0.85	0.02
	Morila	23	0.81	0.1	0.1	0.07	0	0.74	0.05
Tanzania	Kongo	52	0.106	0	0	0	0	0.63	0
	Matimbwa	77	0.078	0	0	0	0	0.65	0
	Yombo	34	0.044	0	0	0	0	0.57	0
Colony	Ian P20	126	0.08	0	0	0	0	0.58	0
	CIG	58	0.33	0.12	0	0	0	0.5	0
	NAG	30	0.33	0.27	0	0	0	1	0

n refers to sample size, (0) and (1) refer to samples fixed for wild-type or inverted arrangements respectively.

Table 2. History of the assortment of inversions 2Rb, 2Rc, 2Rj, and 2La in the *Anopheles gambiae* Ian P20 laboratory strain

Year		2Rb			2Rc			2Rj			2La		
		b/b	b/+	+/+	c/c	c/+	+/+	j/j	j/+	+/+	a/a	a/+	+/+
1982	Obs.	1	50	47	0	50	48	0	46	52	55	43	0
	Exp.	7	38	53	6	37	55	6	35	57	59	34	5
	Stat.		$\chi^2 = 9.61$; $P = 0.008$			$\chi^2 = 11.46$; $P = 0.003$			$\chi^2 = 9.89$; $P = 0.007$			$\chi^2 = 7.65$; $P = 0.02$	
1999	Obs.	1	19	106	0	0	126	0	0	126	31	84	12
	Exp.	1	19	106							42	62	23
	Stat.		NA			NA			NA			$\chi^2 = 19.8$; $P < 0.001$	

NA refers to non-applicability of analysis.

The wild-caught samples from Cote d'Ivoire (Bouake, Yaokoffikro, and Danane) showed karyotypes suggesting a mixture of *An. gambiae* Savannah and Forest forms. The pooled samples from southern Mali revealed *An. gambiae* composed predominantly of the Savannah form with small proportions of Bamako and Mopti forms. The Tanzanian samples were also *An. gambiae* most likely of the Savannah form. Inversion 2Rb was polymorphic in all samples and inversion 2La was polymorphic in all but the NAG sample.

Chromosomal data from an Ian P20 sample recorded in 1982 was compared with the latest sample from this same colony recorded in 1999. Table 2 shows inversion polymorphism assortment for these two samples. Inversions 2Rb, 2Rc, 2Rj, and 2La all showed a significant excess of heterokaryotypes based on chi-square analysis in the 1982 sample. Only inversions 2Rb and 2La were recorded as polymorphisms in the 1999 sample of which only 2La showed a significant excess of heterokaryotypes.

Bottle bioassay results for the 0.5- and 1-h DDT resistance selections on the unselected Ian P20 strain are detailed in Table 3. A two-sample *t*-test by means showed a significant difference in percentage mortality between the 0.5- and 1-h samples ($P < 0.0001$).

Allele frequencies for inversions 2La and 2Rb in the unselected and DDT resistance selected stains are shown in Table 4. Regression analysis and analysis of variance showed a significant increase in the frequency of 2Rb with increasing length of exposure to DDT. The frequency of inversion 2La remained unchanged between unselected and selected samples.

Table 5 shows the frequencies of the inverted arrangements for inversions 2Rb, 2Rc, 2Ru, 2Rd, and 2La in *An. gambiae* in the respective samples from Danane. All inversion systems were assorting according to Hardy-Weinberg expectations across both samples. Chi-square analysis showed that only the karyotype frequencies of inversion 2La were significantly different between the two samples ($\chi^2 = 9.72$, $P = 0.002$).

Discussion

Associations between inversion polymorphism and insecticide resistance phenotypes in the *An. gambiae* complex have been documented previously. Nigatu et al. (1995) found an association between DDT resistance and inversion 2Rb in *An. arabiensis* samples from southern Ethiopia. Hunt (1987) mapped dieldrin resistance in *An. gambiae* to linkage group II on chromosome 2, where it is located close to microsatellite marker AG2H772, which probably falls within inversion 2La (Zheng et al. 1996). Haridi (1974) showed that DDT and dieldrin resistance in *An. gambiae* were both linked to the diamond marker associated with linkage group II. Benedict et al. (1999) induced and isolated a pericentric inversion, In(2)2, in a strain of *An. gambiae* using cobalt irradiation. They showed that this inversion, which covers two-thirds of chromosome 2 and partially overlaps the region covered by inversion 2La, was marked with a dominant allele for dieldrin resistance. By inbreeding inversion In(2)2 heterozygotes, a stock was isolated in which the inversion was maintained as a stable polymorphism by an unidentified recessive lethal. In an earlier study we documented a direct association between dieldrin resistance and inversion 2La in two *An. gambiae* labo-

Table 3. Bottle bioassay results of unselected Ian P20 *Anopheles gambiae* samples following 0.5- and 1-h exposures to DDT (400 μg/bottle)

Ian P20 exposure to DDT			Mean % mortality (95% CI)
Exposure time, h	*n*	No. replicates	
½	526	4	62.5 (59.1–65.9)
1	1,478	8	83.9 (80–87.8)

Table 4. Allele frequencies for inversions 2La (p_a) and 2Rb (p_b) in unselected and DDT resistance selected *Anopheles gambiae* Ian P20 strains

Sample	*n*	Inversion 2La p_a	Inversion 2Rb p_b
Unselected	127	0.58	0.08
0.5 h selected	173	0.54	0.16
1 h selected	65	0.56	0.22
R^2		$R^2 = 0.16$	$R^2 = 0.99$
ANOVA		$P = 0.74$	$P = 0.016$

Reciprocal allele frequencies are given by 1-p.

Table 5. Frequencies of the inverted arrangements for inversion systems 2Rb, 2Rc, 2Ru, 2Rd, and 2La in *Anopheles gambiae* wild-caught samples from Danane, Cote d'Ivoire

	n	2Rb	2Rc	2Ru	2Rd	2La
Base-line	52	0.038	0.01	0.12	0.03	0.07
Treated nets	32	0.047	0	0.094	0.03	0.13

The base-line sample was collected from villages where no control measures are in place. The treated nets sample refers to samples collected simultaneously from villages where insecticide treated bed-nets are used.

ratory strains (Brooke et al. 2000). Inversion 2La assortment in both of these strains showed significant positive heterosis and it was postulated that dieldrin resistance in these strains would be maintained at a high level in the absence of selection pressure as long as inversion 2La was maintained as a stable polymorphism. This effect was explained on the basis of genes linked to inversions. Loci distributed within or near to one of the breakpoints of polymorphic inversion systems show strong linkage disequilibrium with that inversion. Alleles of specific loci linked to inversion systems in this way assort in tandem with their respective inversion arrangements as a result of crossover suppression associated with inversion polymorphism. The *kdr* point mutations associated with pyrethroid and DDT resistance in west (Martinez-Torres et al. 1998, Chandre et al. 1999) and east (Ranson et al. 2000a) African *An. gambiae* do not show any association with inversion polymorphism in this species. Ranson et al. (2000a) show that the region of the voltage-gated sodium channel gene carrying these mutations maps to division 20C on the left arm of chromosome 2. This region was not associated with any inversion polymorphism recorded in *An. gambiae.*

Inversion stability and the maintenance of polymorphism is associated with heterozygote advantage. Dobzhansky (1947) asserted that when a population has been found to be polymorphic for an inversion, it has often been shown that heterozygotes for this inversion are fitter than either homozygote. Haldane (1957) stated that this was always so when a polymorphism was stable although certain parameters concerning inversion polymorphism stability should be considered. These are as follows: (1) the two mutually inverted segments could differ at several loci, (2) structural heterozygosity at the chromosomal level would not necessarily confer an advantage by itself, and (3) structurally indistinguishable inversions in different populations may carry different genes. Heterozygote advantage (possibly leading to positive heterosis at the population level) refers to the phenotypic effect of those loci in which the heterozygous genotype carries the greatest fitness. The reason for heterozygote advantage at the cytological level may then be based on the multiple advantage conferred by heterozygosity at several such loci linked to an inversion system. At least half of the zygotes formed within such a system with respect to these loci would conform to one of the possible homozygous states leading to multiple disadvantages. It follows that structural heterozygosity at the cytological level may carry the greatest level of fitness by default through cross-over suppression and the multiple advantage of the fitter heterozygote genotype at several loci linked to that inversion.

Inversions 2Rb and 2La are highly ubiquitous as polymorphisms in *An. gambiae* wild populations and laboratory strains. The significant increase in the frequency of inversion 2La in villages where insecticide treated nets are used compared with those where no control measures are in place suggests an association between this inversion system and insecticide avoidance in *An. gambiae.* The use of treated nets may inadvertently lead to selection for *An. gambiae* 2La karyotypes that preferentially feed outdoors. These associations may be mechanistic in the maintenance of these inversion systems as polymorphisms.

The association between inversion 2Rb and DDT resistance in the Ian P20 strain showed a significant trend with increasing DDT resistance although the frequency of the inverted arrangement remained comparatively low even in the 1-h selected sample. Most documented cases of DDT resistance involve elevated glutathione-S-transferase (GST) based detoxification, with the *kdr* mutations in west and east African *An. gambiae* as exceptions. Biochemical analysis of the Ian P20 strain suggests that in this case it is the operational mechanism (B.D.B., unpublished data). Ranson et al. (1997) implicate class I GSTs in DDT resistance in *An. gambiae* and show that clones of class I GST genes hybridize to chromosome 2R, division 18B. This region was not associated with inversion 2Rb. Two genetic factors proposed to be responsible for the up-regulation of GSTs, mixed function oxidases and other detoxifying enzymes have been linked to microsatellite markers assorting on chromosome arms 3R and 2L (Ranson et al. 2000b). The region associated with the latter slightly overlaps with inversion 2La and includes inversion 2Ln. The former is not associated with any inversion polymorphism described in *An. gambiae.* It seems likely that the major factor associated with GST-based DDT resistance was not associated with inversion 2Rb although a co-factor may be linked to this inversion. Candidate genes for this cofactor include genes associated with a physical characteristic such as a thicker cuticle or genes associated with avoidance behavior.

Colonization of wild mosquito material usually involves the transfer of inversion polymorphisms from field to laboratory strain. After colonization, the general trend is for inversion polymorphism to be lost as would be expected from inevitable bottleneck effects and genetic drift. Despite these effects, inversions 2La and 2Rb in *An. gambiae* tend to remain polymorphic

suggesting that both are closely associated with adaptive characteristics that favor inversion heterozygosity under laboratory conditions. Inversion stability by heterozygote advantage provides a mechanism whereby particular phenotypes associated with an inversion heterokaryotype will be maintained as long as the inversion is maintained as a polymorphism and cross-over suppression is applied. Continual inheritance of dieldrin (Brooke et al. 2000) and DDT resistance in the *An. gambiae* Ian P20 strain without insecticide selection provide candidate phenotypes for this effect which may also be operational in wild populations. Stable inversion polymorphisms also provide a useful mechanism for the continual inheritance of suitable genetic factors that otherwise compromise the fitness of genetically modified mosquitoes.

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