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Chromosomal evidence of incipient speciation in the Afrotropical malaria mosquito *Anopheles funestus*

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Abstract. The analysis of chromosomal polymorphism of paracentric inversions in anopheline mosquitoes has often been instrumental to the discovery of sibling species complexes and intraspecific genetic heterogeneities associated with incipient speciation processes. To investigate the population structure of *Anopheles funestus* Giles (Diptera: Culicidae), one of the three most important vectors of human malaria in sub-Saharan Africa, a three-year survey of chromosomal polymorphism was carried out on 4638 karyotyped females collected indoors and outdoors from two villages of central Burkina Faso. Large and temporally stable departures from Hardy–Weinberg equilibrium due to significant deficits of heterokaryotypes were found irrespective of the place of capture, and of the spatial and temporal units chosen for the analysis. Significant linkage disequilibrium was observed among inversion systems on independently assorting chromosomal arms, indicating the existence of assortative mating phenomena. Results were consistent with the existence of two chromosomal forms characterized by contrasting degrees of inversion polymorphism maintained by limitations to gene flow. This hypothesis was supported by the reestablishment of Hardy–Weinberg and linkage equilibria when individual specimens were assigned to each chromosomal form according to two different algorithms. This pattern of chromosomal variability is suggestive of an incipient speciation process in *An. funestus* populations from Burkina Faso.

Key words. *Anopheles funestus*, cytogenetics, chromosomal inversion, speciation, Burkina Faso.

Introduction

Cytogenetic studies have been instrumental to the investigation of taxonomic status and vectorial capacity in relation to population structure of insect disease vectors. A typical example is represented by the *Anopheles gambiae sensu lato* (*s.l.*) species complex. This is composed of seven isomorphic sibling species, most of which are distinguishable based on fixed paracentric inversions observable on the banding pattern of polytene chromosomes (Coluzzi *et al.*, 1979). Because of diverse bionomics, this species complex includes the world's most efficient vectors of malaria, *An. gambiae sensu stricto* (*s.s.*) Giles and *An. arabiensis* Patton, and also species of negligible medical importance as *An. quadriannulatus* species A and B (White, 1974; Hunt *et al.*, 1998). Historically, cytogenetic recognition of the members of the complex offered a simple means for routine identification of field and laboratory specimens, before novel techniques were developed for identification by molecular protocols (Scott *et al.*, 1993; Favia *et al.*,

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1997). Moreover, the observation of non-random distribution of chromosomal inversions within the nominal taxon *An. gambiae s.s.* led to the discovery of several chromosomal forms showing intrinsic limitations to gene flow, and characterized by different biogeographies and ecological traits (Coluzzi *et al.*, 1985). Such studies spurred the analysis of the genetic structure of this mosquito using molecular markers, leading to the recognition within *An. gambiae s.s.* of significant barriers to gene flow interpreted as the manifestation of an ongoing speciation process (Black & Lanzaro, 2001; della Torre *et al.*, 2002; Gentile *et al.*, 2002; Tripet *et al.*, 2004; Stump *et al.*, 2005a, b).

Like *An. gambiae s.s.* and *An. arabiensis*, *An. funestus* is an important malaria vector in Africa, sometimes rivaling or exceeding the role of other vectors in malaria transmission. In Burkina Faso, at the end of the rainy season, *An. funestus* follows in peak abundance the vectors of the *An. gambiae* complex, thereby extending malaria transmission into the dry period. In this area, infectious rates in *An. funestus* frequently average around 5–10% (Costantini *et al.*, 1999), so that any malaria vector control campaign cannot afford to ignore this species. However, efforts to address its genetic structure by means of cytogenetic studies have been lacking until recently (Lochouarn *et al.*, 1998; Dia *et al.*, 2000a,b; Sharakhov *et al.*, 2001; Kamau *et al.*, 2002). Several reasons can be evoked, but perhaps the most significant is that, relative to *An. gambiae s.l.*, the polytene chromosomes of *An. funestus* are difficult to work with.

Early cytogenetic studies reported the occurrence in *An. funestus* of 10 polymorphic paracentric inversions (Green, 1982). More recent studies described new inversion systems (Boccolini *et al.*, 1992, 1998; Sharakhov *et al.*, 2004). By the analysis of chromosomal inversion polymorphism in populations from Burkina Faso, large departures from Hardy–Weinberg (HW) and linkage equilibrium were reported (Costantini *et al.*, 1999), suggesting the existence of two strictly sympatric chromosomal forms showing contrasting degrees of chromosomal polymorphism. In this study we present results of a longitudinal survey of the chromosomal inversion polymorphism of *An. funestus* conducted over three consecutive breeding seasons in a different area of Burkina Faso 5 years subsequent to the original study. This independent and extensive data set offers the opportunity to validate the algorithm proposed by these authors, which attempts to classify individual specimens into alternative chromosomal forms based on their karyotype, as well as to test the hypothesis of the existence of sympatric chromosomal forms within *An. funestus*.

Materials and methods

Mosquito collections were carried out in two rural villages *c.* 35 km south of Ouagadougou, the capital of Burkina Faso. Koubri (12°11′54 N; 1°23′43 W) and Kuiti (12°11′36 N; 1°23′11 W) lie in the arid Sudan savanna vegetation belt of West Africa, and are located about 1 km apart on the opposite margins of a permanent swamp (Fig. 1). Family units live in individual compounds (dots in Fig. 1) composed of 1–13 closely spaced huts delimited by a mud-brick fence. This area was chosen because of the abundance of *An. funestus* and the diversity of anopheline larval habitats. Resting anophelines were collected between September 1999 and March 2002 inside human dwellings and in artificial outdoor shelters (Service, 1993). Indoor-resting mosquitoes were collected in the afternoon by spray-sheet catches or manual collections with electric aspirators. Outdoor-resting mosquitoes were aspirated from 'Muirhead-Thomson'-type pit-shelters. After collection, adult mosquitoes were morphologically identified under a dissecting microscope using standard identification keys (Gillies & Coetzee, 1987). Half-gravid females of *An. funestus* were immediately dissected and their cropped ovaries preserved in individual microcentrifuge tubes containing Carnoy's fixative (one part of glacial acetic acid in three parts of absolute ethanol). Tubes were subsequently stored at −20°C until chromosomal squash preparations were made. Polytene chromosomes were squashed and stained according to standard cytogenetic techniques (Hunt, 1973). The slides were examined under a phase-contrast microscope, and chromosomal arrangements were scored according to the chromosomal map and nomenclature reviewed by Sharakhov *et al.*, 2004).

For the purposes of analysis, inverted and standard arrangements were treated as alternative alleles at the same locus. Because inversions 2R*a* and 2R*s* overlap, these and the corresponding standard arrangement were treated as three alleles of the same 2R*as* locus. Conformance to Hardy–Weinberg (HW) equilibrium and linkage disequilibrium were assessed using exact probability tests performed with GENEPOP version 3.3 (Raymond & Rousset, 1995). Deviations from HW equilibrium were assessed using Weir and Cockerham's estimate of the F_{IS} statistic. Positive values of F_{IS} denote a deficit of heterozygotes with respect to frequencies expected under panmictic conditions. Conversely, negative values indicate an excess of heterozygotes. Whenever multiple samples were tested, probability values were corrected using the Bonferroni procedure (Weir, 1996). Assignment tests were performed using the software GENECLASS version 1.0.02 (Cornuet *et al.*, 1999).

Results

Our analysis relied on 2129 specimens from Koubri and 2509 from Kuiti, which were successfully scored for at least one chromosomal inversion system. Two polymorphic paracentric inversions were observed on arm 2R (2R*a*, 2R*s*), two on arm 3R (3R*a*, 3R*b*), and one on arm 3L (3L*a*). Inversion 3R*a* was the most frequent (0.22) followed by inversions 3R*b* (0.12), 2R*a* (0.11), 2R*s* (0.05) and 3L*a* (0.02).

Hardy–Weinberg and linkage disequilibrium

Samples were stratified by village and analysed by month of collection. Significant departures from HW equilibrium

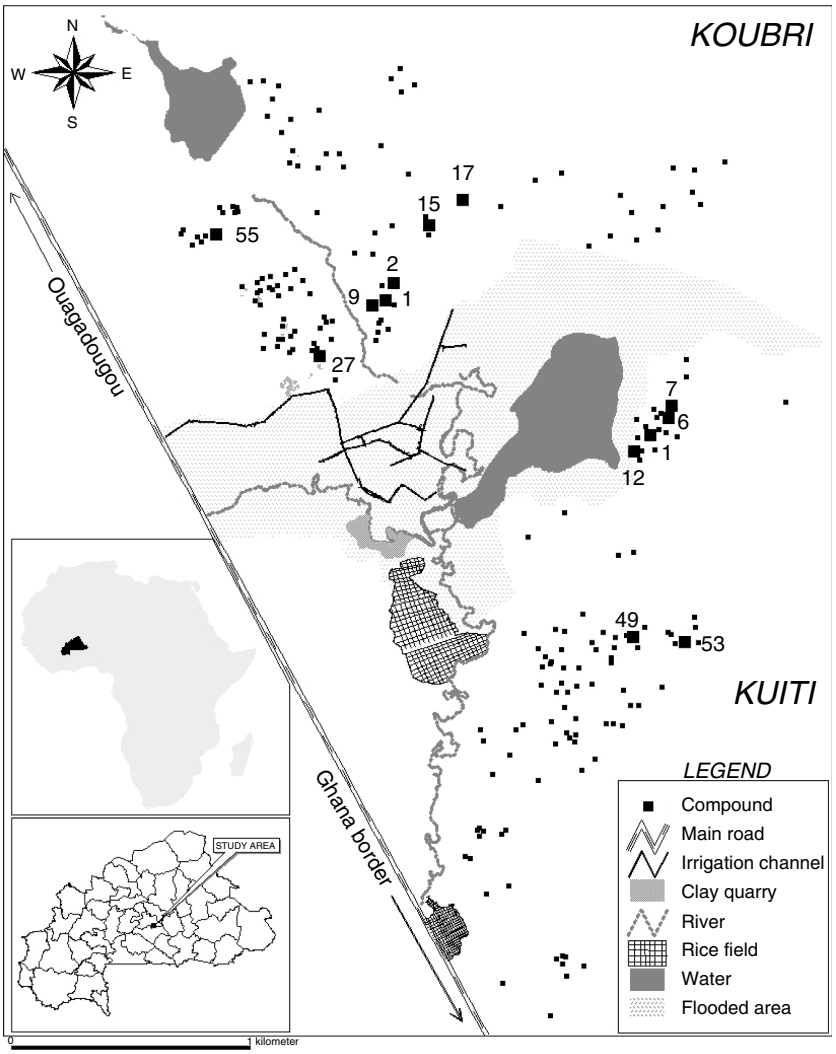

Fig. 1. Map of the study area showing the two villages where mosquito collections were performed. Sampled compounds (dots in the map) are identified by serial numbers corresponding to those presented in Table 3.

for individual loci were observed in 51% (68/134) of the samples; these were always due to a deficit of heterokaryotypes. Higher values of the F_{IS} statistics were observed for arrangement 3R*a* (mean $F_{IS} \pm$ SD: 0.70 $\pm$ 0.13; Table 1b), followed by arrangements 3R*b* (0.37 $\pm$ 0.22; Table 1c), 2R*as* (0.29 $\pm$ 0.14; Table 1a) and 3L*a* (0.09 $\pm$ 0.20; Table 1d). When tested across arrangements by Fisher's global test, all 39 comparisons yielded highly significant ($P < 0.001$) departures from equilibrium. Linkage disequilibrium analysis for each pair of loci for which an exact probability value could be computed yielded 0–89% of significant tests out of 25–39 comparisons after the Bonferroni correction. The pair of arrangements lying on the same chromosomal arm, i.e. 3R*a* vs. 3R*b*, showed the highest degree of significant linkage disequilibrium (89% of significant tests out of 38 comparisons), followed by inversion systems 2R*as* vs. 3R*a* (69%), and 2R*as* vs. 3R*b* (45%), despite the fact that these arrangements are on independently assorting chromosomes. The lowest frequency of statistically significant comparisons applied to inversion 3L*a* vs. all other arrangements, for which the proportion of significant tests ranged 0–4%.

Outdoor-resting samples

A deficit of heterokaryotypes in indoor-resting samples might result from the tendency of carriers of heterokaryotype arrangements to leave houses after having taken a bloodmeal, and/or to feed preferentially on hosts other than humans and hence rest mainly outdoors. To test this hypothesis, we stratified the outdoor samples by village and month of collection. In this case, sample sizes were substantially lower, hence we restricted our analysis only to samples with $N > 10$. Similar to what was found indoors, most samples exhibited a deficit of heterokaryotypes for inversion systems 3R*a*, 3R*b* and 2R*as*. A statistically significant deficit was observed in 23% (6/26) of the samples tested (Table 2). Fisher's global test across loci yielded four highly significant departures from HW equilibrium out of

nine samples tested. Following the Bonferroni correction, significant linkage disequilibrium was observed only between arrangements on the 2R vs. 3R arms in 17–29% of tests out of four to nine comparisons. Thus, the hypothesis of a differential degree of exophily for heterokaryotypes could not account for their deficit in indoor-resting samples.

Wahlund Effect

Our samples showed significant departures from HW and linkage equilibria independently of the time or place of collection; however, pooling of subsamples having different allele frequencies could complicate interpretation of results because of the Wahlund effect. Thus, we reanalysed samples at the minimum possible temporal and spatial scale. In September 1999 and December 2001, extensive spray-sheet catches in both villages were performed during 1 week, yielding 39–134 karyotyped females from each of three to four compounds per village (Table 3 and Fig. 1). Again, we observed strong departures from HW equilibrium due to a deficit of heterokaryotypes in most samples (Table 3). The global test across loci was highly significant in all but one of 14 tests. Significant linkage disequilibrium between arrangements on the 2R vs. 3R arms was observed in 60–80% of 15 possible tests after the Bonferroni correction. Only one significant test out of seven (involving inversion 3R*a*) was found between 3L*a* and the other arrangements.

Chromosomal forms analysis

The pattern of chromosomal variation observed is not compatible with the existence of a panmictic population. It is consistent with the hypothesis of the existence of two chromosomal forms sharing a common set of inversions, while exhibiting contrasting degrees of polymorphism. Costantini and colleagues (Costantini *et al.*, 1999) proposed an algorithm to assign karyotyped specimens to alternative chromosomal forms named with a non-Linnean nomenclature Folonzo and Kiribina. A pseudocode version of their algorithm can be written as follows:

Rule I. IF 2R*s*> 0 THEN "Kiribina" ELSE GOTO Rule II;
Rule II. IF (3R*a* OR 3R*b* OR 2R*a*)=2 THEN "Folonzo" ELSE GOTO Rule III;
Rule III. IF (3R*a* OR 3R*b*)> 0 AND 2R*a*> 0 THEN "Folonzo" ELSE GOTO Rule IV;
Rule IV. IF (3R*a* AND 3R*b*)=1 THEN "Folonzo" ELSE "Kiribina".

Under this notation chromosomal arrangements are recognized by integers, with zero identifying the homokaryotype standard, one the heterokaryotype, and two the homokaryotype inverted. The symbols AND, and OR represent Boolean operators, and IF, THEN, ELSE logical operators.

Our data provide an opportunity to test this algorithm with an independent set of karyotypes. We stratified our samples by month and village of collection and assigned individuals to each chromosomal form following the algorithm. Hardy–Weinberg equilibrium was restored within each chromosomal form in all but two samples (Tables 1a–d). Linkage equilibrium was restored within each form for all pairs of loci (range: 21–37 tests for the Folonzo form, 18–55 tests for the Kiribina form).

If we calculate inversion frequencies within each chromosomal form, as expected these show contrasting values between the two forms. In Folonzo, the inverted arrangements appear at high frequency (0.87 for 3R*a*, 0.50 for 3R*b*, 0.48 for 2R*a*, and 0.04 for 3L*a*), whereas in Kiribina most individuals are monomorphic standard, hence this form is characterized by much lower frequencies of the inverted arrangements (0.02 for 3R*a*, 0.01 for 3R*b*, 0.01 for 2R*a*, 0.01 for 3L*a,* and 0.07 for 2R*s*). Under this scenario, inversion 2R*s* is uniquely associated to the Kiribina form.

The proposed algorithm is deterministic, in the sense that it assigns individual specimens to each chromosomal form at the expense of some degree of misclassification, due to the unilateral assignment of multiple heterokaryotypes and inverted homokaryotypes to the Folonzo form. An alternative way of assigning individual karyotypes to each chromosomal form with a certain level of probability is the assignment test of Waser & Strobeck (1998). The principle of the test is to assign an individual to the population in which the individual's genotype is most likely to occur by comparing a likelihood or distance measure of the individual's genotype to the mean genotypic composition of each population. Thus, karyotypes were *a priori* assigned to one chromosomal form based on the algorithm, and then each individual karyotype was removed in turn from the population it was classified in and its genotype compared to the mean composition of the two chromosomal forms based on the product of expected genotype frequencies at each locus calculated without the removed test karyotype. There are several versions of this test depending on the discrimination criterion (likelihood vs. genetic distance), and the deterministic or probabilistic outcome of the test. Here, we have used the likelihood distance as a measure of genetic differentiation between individuals and populations, and the Bayesian approach in the calculation of population allelic frequencies based on the simulation of a population of 10 000 individuals (Cornuet *et al.*, 1999). For the purposes of this analysis, we employed only monthly samples with $\geq$50 fully karyotyped specimens collected over a period spanning 6 months in Kuiti and 10 months in Koubri. Of 2728 specimens that could be tested, only 0.7% were assigned to a chromosomal form different from that determined from the algorithm (Fig. 2 and Table 4). Some of these discrepancies were anticipated, due to sharing of arrangements such as 2R*a* between forms. Assignment to the Folonzo form of 12 individuals carrying the arrangement 2R*s* deserves special mention. According to the algorithm, inversion 2R*s* is unique to Kiribina. Does this indicate that 2R*s* is actually a shared ancestral arrangement in both forms, or that 2R*s* is present in Folonzo due to gene flow between the two chromosomal forms? The two

hypotheses are not mutually exclusive, and it seems likely on the basis of this more extensive data set that both processes are at work. Accordingly, to take into account the evidence that the presence of inversion 2R*s* cannot discriminate Kiribina from Folonzo, the algorithm should be modified as follows:

Table 1. Intra-population *F*-statistic and number of observed heterokaryotypes (H_O) out of *N* karyotyped specimens in *Anopheles funestus* indoor samples before (pooled samples) and after assignment to alternative chromosomal forms (Folonzo, Kiribina) by the algorithm. (a) Inversion system 2R*as*, (b) inversion system 3R*a*, (c) inversion system 3R*b*, (d) inversion system 3L*a*. Probability values are calculated after the Bonferroni correction. The sum of totals for the two chromosomal forms do not always coincide to the total of observed heterokaryotypes due to individuals that could not be classified because of non-readable loci. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; –, not computable.

Table 1(a). Inversion system 2R*as*

	Pooled samples			Folonzo			Kiribina		
	N	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}
Koubri									
September 1999	248	43	0.09	32	17	−0.10	216	26	−0.06
October 1999	59	16	0.41	37	14	0.26	21	2	0.32
November 1999	28	12	0.16	13	7	−0.06	15	5	−0.13
December 1999	44	10	0.27	15	7	0.08	27	3	−0.04
January 2000	91	17	0.37*	30	12	0.18	61	5	0.25
February 2000	30	7	0.37	10	3	0.44	20	4	−0.09
September 2000	39	7	0.39	4	2	0.14	34	5	0.37
October 2000	90	16	0.47***	22	8	0.24	66	8	−0.05
November 2000	80	28	0.07	18	9	0.00	62	19	−0.07
December 2000	84	17	0.11	10	4	0.22	74	13	−0.09
January 2001	83	6	0.48*	10	3	0.39	73	3	0.39
February 2001	24	5	−0.08	0	0	–	23	4	−0.07
September 2001	79	9	0.43**	12	3	0.53	67	6	−0.04
October 2001	77	13	0.31	26	7	0.36	51	6	−0.04
November 2001	46	10	0.45*	17	4	0.55	29	6	−0.10
December 2001	516	105	0.32***	102	41	0.20	410	65	0.06
January 2002	111	22	0.01	18	12	−0.38	92	10	−0.05
February 2002	57	5	0.42	4	2	−0.20	53	3	0.55
Total	1786	348		380	155		1394	193	
Kuiti									
September 1999	252	48	0.36***	44	17	0.19	208	31	−0.08
October 1999	74	17	0.40*	30	15	0.15	47	10	0.05
November 1999	38	9	0.53	32	8	0.33	16	2	−0.03
December 1999	71	14	0.43*	35	8	0.42	43	6	−0.04
January 2000	80	20	0.20	39	15	0.08	47	7	−0.04
February 2000	29	7	0.41	10	5	−0.05	19	2	−0.03
March 2000	28	6	0.32	8	3	0.30	20	3	−0.06
September 2000	36	10	0.26	4	2	−0.20	32	8	0.08
October 2000	92	21	0.27***	15	4	0.47	77	17	−0.12
November 2000	109	18	0.36**	27	11	0.20	80	7	−0.04
December 2000	105	15	0.33	17	10	−0.19	88	5	−0.02
January 2001	100	22	0.10	15	9	−0.19	85	13	0.01
February 2001	102	11	0.11	10	2	0.42	92	9	−0.05
March 2001	82	9	0.27	5	2	0.27	77	7	−0.04
September 2001	114	14	0.37***	15	2	0.72	98	12	0.09
October 2001	93	20	0.40*	42	15	0.29	51	5	−0.03
November 2001	82	20	0.41***	28	10	0.30	54	10	0.08
December 2001	449	82	0.39***	95	41	0.13	350	43	0.10
January 2002	107	21	0.01	18	9	−0.15	89	12	−0.07
February 2002	98	19	0.26	18	11	−0.20	81	8	0.16
March 2002	72	7	0.19	2	0	1.00	69	7	−0.05
Total	2213	410		494	199		1777	224	

Table 1(b). Inversion system 3R*a*

	Pooled samples			Folonzo			Kiribina		
	N	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}
Koubri									
September 1999	253	17	0.70***	34	10	−0.16	216	7	−0.01
October 1999	67	11	0.67***	40	7	−0.08	21	3	−0.05
November 1999	37	9	0.52	20	6	−0.15	15	2	−0.04
December 1999	52	9	0.59***	16	5	−0.15	27	4	−0.06
January 2000	105	14	0.70***	37	9	−0.13	61	4	−0.03
February 2000	37	3	0.81***	11	2	−0.05	20	1	–
September 2000	45	7	0.45	6	2	−0.11	34	5	−0.07
October 2000	96	13	0.63***	24	8	−0.18	66	3	−0.02
November 2000	91	7	0.78***	20	3	−0.06	62	4	−0.03
December 2000	92	9	0.61***	14	5	−0.18	74	4	−0.02
January 2001	84	6	0.66***	10	3	−0.13	73	3	−0.01
February 2001	24	1	0.66	0	0	–	23	1	–
September 2001	85	3	0.86***	14	1	0.77	67	1	–
October 2001	97	7	0.85***	41	4	−0.04	51	3	−0.02
November 2001	53	6	0.77***	22	4	−0.08	29	2	−0.02
December 2001	572	33	0.82***	126	24	0.23	409	8	0.19
January 2002	114	6	0.80***	19	3	0.33	92	3	−0.01
February 2002	58	4	0.57	5	2	−0.14	53	2	−0.01
Total	1962	165		459	98		1393	60	
Kuiti									
September 1999	264	34	0.51***	47	23	−0.24	207	8	0.18
October 1999	83	4	0.89***	30	4	0.46	47	7	−0.04
November 1999	51	12	0.53*	32	9	−0.14	16	1	–
December 1999	84	6	0.85***	35	2	0.77*	43	4	−0.04
January 2000	99	12	0.74***	39	9	−0.11	47	3	−0.02
February 2000	36	6	0.59	11	4	−0.18	19	2	−0.03
March 2000	37	4	0.66*	11	4	0.26	20	0	–
September 2000	37	1	0.88***	5	1	–	32	0	–
October 2000	100	11	0.64***	19	5	−0.13	77	6	−0.03
November 2000	118	11	0.77***	32	5	−0.07	80	5	−0.03
December 2000	108	8	0.75***	18	2	0.46	88	6	−0.03
January 2001	104	8	0.73***	17	3	−0.07	85	5	−0.02
February 2001	106	5	0.71***	10	3	−0.13	92	1	–
March 2001	86	5	0.42	6	4	−0.43	77	1	–
September 2001	121	4	0.89***	20	1	–	98	2	−0.01
October 2001	114	19	0.66***	55	16	−0.05	51	2	−0.01
November 2001	94	11	0.74***	35	9	−0.13	54	2	−0.01
December 2001	466	32	0.79***	101	21	0.11	348	11	−0.02
January 2002	110	7	0.79***	20	3	−0.06	89	4	−0.02
February 2002	105	12	0.65***	23	8	−0.19	81	4	−0.02
March 2002	78	1	0.85***	4	0	1.00	69	1	–
Total	2401	213		585	136		1774	75	

Rule I. IF (3R*a* OR 3R*b* OR 2R*a*)=2 THEN "Folonzo" ELSE GOTO Rule II;
Rule II. IF (3R*a* OR 3R*b*)> 0 AND 2R*a*> 0 THEN "Folonzo" ELSE GOTO Rule III;
Rule III. IF (3R*a* AND 3R*b*)=1 THEN "Folonzo" ELSE "Kiribina".

Discussion

Across three consecutive breeding seasons in two villages near Ouagadougou, samples of indoor-resting *An. funestus* showed a marked, significant, and temporally stable deficit of heterokaryotypes, as well as significant linkage between paracentric inversions located on independently assorting chromosomes. The possibility of a confounding Wahlund effect was discarded on the basis of a set of subsamples collected over the course of 1 week from the smallest feasible spatial scale, a family compound, which consists of several huts separated by no more than 20 m. The same observations were repeated in outdoor-resting samples, and confirmed the observed pattern of deficit of heterokaryotypes and inversion linkage found indoors.

Table 1(c). Inversion system 3R*b*

	Pooled samples			Folonzo			Kiribina		
	N	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}
Koubri									
September 1999	251	16	0.59***	33	15	0.05	216	1	–
October 1999	62	15	0.46*	36	15	0.16	21	1	–
November 1999	35	13	0.17	19	13	−0.41	15	0	–
December 1999	44	7	0.45	12	6	0.02	27	1	–
January 2000	104	17	0.42*	37	16	0.14	61	1	–
February 2000	32	3	0.72*	9	2	0.54	20	1	–
September 2000	44	3	−0.02	6	2	−0.11	34	1	–
October 2000	93	14	0.22	23	14	−0.22	66	0	–
November 2000	87	7	0.62***	18	4	0.58	62	3	−0.02
December 2000	90	11	0.36	13	9	−0.50	74	2	−0.01
January 2001	83	7	0.18	9	7	−0.51	73	0	–
February 2001	23	0	–	0	0	–	23	0	–
September 2001	83	6	0.22	12	6	−0.08	67	0	–
October 2001	94	21	0.33	38	21	−0.09	51	0	–
November 2001	51	10	0.27	20	9	0.07	29	1	–
December 2001	553	71	0.35***	121	65	−0.07	409	6	−0.01
January 2002	113	10	0.50***	19	10	−0.05	92	0	–
February 2002	58	3	0.38	5	3	−0.09	53	0	–
Total	1900	234		430	217		1393	18	
Kuiti									
September 1999	257	29	0.30**	46	26	−0.13	207	3	−0.01
October 1999	80	6	0.71***	29	10	0.49	47	2	−0.01
November 1999	44	16	0.14	27	17	−0.20	16	0	–
December 1999	76	13	0.56***	29	12	0.16	43	1	–
January 2000	94	19	0.23	38	21	−0.08	47	0	–
February 2000	35	5	0.38	11	4	0.26	19	0	–
March 2000	35	4	0.61	10	4	0.22	20	0	–
September 2000	37	5	−0.06	5	5	−1.00	32	0	–
October 2000	96	13	0.25	17	11	−0.27	76	2	−0.01
November 2000	116	20	0.32	31	19	−0.22	80	0	–
December 2000	107	7	0.60***	17	7	0.19	88	0	–
January 2001	102	10	0.40**	16	8	0.03	85	2	−0.01
February 2001	105	7	0.19	10	7	−0.37	92	0	–
March 2001	86	4	0.32	6	3	0.06	77	1	–
September 2001	117	9	0.57***	17	7	0.16	98	1	–
October 2001	111	31	0.11	52	31	−0.22	51	0	–
November 2001	92	14	0.48**	33	14	0.17	54	0	–
December 2001	455	54	0.39***	97	52	−0.07	346	2	0.00
January 2002	109	10	0.45*	19	8	0.18	89	2	−0.01
February 2002	105	12	0.44*	23	11	0.07	81	1	–
March 2002	77	0	1.00**	3	2	1.00	69	1	–
Total	2336	288		551	279		1770	18	

These observations are not compatible with the existence of a single panmictic population of *An. funestus* in this study area. The simplest explanation is coherent with the previously reported working hypothesis that populations of *An. funestus* in Burkina Faso consist of two strictly sympatric chromosomal forms characterized by contrasting degrees of chromosomal polymorphism. In analogy to the chromosomal forms of *An. gambiae s.s.*, the designation of 'forms' is intentionally ambiguous to denote units of as yet uncertain taxonomic status. These forms show restrictions to gene flow suggestive of an ongoing incipient speciation process perhaps promoted by positive assortative mating, although post–mating reproductive barriers cannot be excluded at this stage. The equally parsimonious hypothesis of the demise of two species by hybridization would imply the existence of two previously unrecognized taxa within *An. funestus* that did not diverge enough to establish reproductive barriers.

On the basis of the evidence gathered so far, alternative explanations to the speciation hypothesis appear less likely. One possibility is that our samples consist of admixtures of allopatric and/or allochronic subpopulations exhibiting different levels of chromosomal polymorphism, caused by

Table 1(d). Inversion system 3L*a*

	Pooled samples			Folonzo			Kiribina		
	N	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}
Koubri									
September 1999	61	0	–	7	0	–	54	0	–
October 1999	57	7	0.16	34	6	0.17	19	0	–
November 1999	30	2	−0.02	15	2	−0.04	15	0	–
December 1999	40	0	–	12	0	–	25	0	–
January 2000	100	3	−0.01	38	2	−0.01	59	1	–
February 2000	31	1	–	10	0	–	19	1	–
September 2000	40	0	–	5	0	–	33	0	–
October 2000	92	2	−0.01	22	2	−0.02	66	0	–
November 2000	88	9	0.13	19	5	−0.13	62	4	0.31
December 2000	88	5	−0.02	13	3	−0.09	71	2	−0.01
January 2001	81	2	0.49	9	2	−0.07	71	0	1.00
February 2001	24	0	–	0	0	–	23	0	–
September 2001	82	1	–	13	0	–	67	0	–
October 2001	89	1	–	36	1	–	51	0	–
November 2001	51	2	−0.01	20	1	–	29	1	–
December 2001	489	12	0.39***	109	8	0.17	394	4	0.60***
January 2002	105	2	−0.01	19	1	–	90	1	–
February 2002	58	0	–	5	0	–	53	0	–
Total	1606	49			386	33	1201	14	
Kuiti									
September 1999	25	2	−0.02	5	0	–	22	2	−0.02
October 1999	79	4	−0.02	29	6	−0.07	47	0	–
November 1999	41	8	−0.10	26	7	−0.13	15	1	–
December 1999	66	2	0.49	25	1	0.66	38	1	–
January 2000	89	2	−0.01	36	1	–	46	1	–
February 2000	33	0	–	11	0	–	18	1	–
March 2000	27	2	−0.02	10	2	−0.06	17	0	–
September 2000	36	1	–	5	1	–	31	0	–
October 2000	93	0	–	15	0	–	76	0	–
November 2000	114	5	−0.02	31	2	−0.02	80	3	−0.01
December 2000	107	0	–	18	0	–	87	0	–
January 2001	101	2	−0.01	16	1	–	84	1	–
February 2001	103	0	–	10	0	–	92	0	–
March 2001	82	0	–	5	0	–	76	0	–
September 2001	117	0	–	18	0	–	98	0	–
October 2001	98	2	−0.01	49	2	−0.01	49	0	–
November 2001	87	0	–	32	0	–	54	0	–
December 2001	430	10	0.28**	95	9	0.14	331	1	0.67
January 2002	106	1	−0.01	19	0	–	88	1	–
February 2002	101	2	−0.01	23	1	–	79	1	–
March 2002	70	0	–	3	0	–	66	0	–
Total	2005	43			494	33	1548	13	

migrants from contiguous areas subjected to differential selection pressures. Evidence for the association between chromosomal inversions and environmental heterogeneities in anophelines is well documented (Coluzzi *et al*., 1979; Coluzzi, 1992; Touré *et al*., 1994, 1998; Powell *et al*., 1999). If this explanation is correct, then the pattern of genetic variation observed would be unstable. Instead, our longitudinal survey indicated that the patterns of HW and linkage disequilibria were not transitory phenomena, but were stable across 3 years of the survey. A second possibility is that the two villages of this study represent an area of hybridization between intergrading allopatric populations, an explanation that is consistent with the pattern of chromosomal polymorphism observed in populations of *An. funestus* from Senegal and Cameroon (Lochouarn *et al*., 1998; Dia *et al*., 2000b; Cohuet *et al*., 2004a,b). This explanation, however, is not consistent with previous studies in Burkina Faso that showed a country-wide deficit of heterokaryotypes in individual locales (Costantini *et al*., 1999), unless the area of hybridization consists of a wide belt spanning several hundreds of kilometers. Moreover, the distribution of chromosomal diversity within Burkina

Table 2. Intra-population *F*-statistic and number of observed heterokaryotypes (H_O) out of *N* karyotyped specimens in *Anopheles funestus* outdoor samples stratified by village and month of collection. The last three columns show results of a global test across loci constructed using Fisher's method. Other symbols and statistics as in Table 1

	Inversion System 2R*as*			Inversion System 3R*a*			Inversion System 3R*b*			Inversion System 3L*a*			
Sample	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	w^2
Koubri													
September 1999	19	4	0.27	20	4	0.40	17	1	0.65	17	1	–	14.6
October 1999	47	6	0.51*	48	3	0.81***	48	5	0.40	45	1	–	47.9***
January 2000	15	0	–	15	3	0.31	15	2	−0.04	13	0	–	2.2
November 2000	16	2	0.47	18	1	0.83	18	1	0.83	15	1	–	26.6**
October 2001	27	3	0.00	31	3	0.76**	28	0	1.00*	26	0	–	33.3***
Total	124	15		132	14		126	9		116	3		
Kuiti													
October 1999	50	8	0.28	50	7	0.63***	48	6	0.44	49	2	−0.01	36.8***
January 2000	13	2	−0.04	16	2	0.61	16	2	−0.03	14	0	–	6.0
October 2000	16	2	0.47	20	1	0.66	20	1	0.66	15	0	–	15.7
October 2001	13	0	–	13	0	1.00*	12	2	−0.05	8	0	–	13.4
Total	92	12		99	10		96	11		86	2		

Faso appears associated to local ecological conditions. Because of the likely adaptive role of chromosomal inversions in *An. funestus*, the existence of nonequilibrium strong selection pressures against carriers of certain arrangements cannot be *a priori* excluded as an alternative explanation. In this case, the manifestly stable linkage disequilibrium observed among inversions on independently assorting chromosomal arms calls for genome-wide selection of coadapted gene patterns captured by the inversions. Evidence from temporal changes in inversion frequencies, however, is not in good agreement with this explanation (W. M. Guelbeogo, O. Ghrushko, D. Boccolini, P. A. Ouédraogo, N. J. Besansky, N'F. Sagnon, C. Costantini, unpublished data). Moreover, its corollary is that the high genetic load entrained by such selection phenomena should be temporally stable and cover a large geographical area.

In further support of the speciation theory are results from an investigation of these alternative hypotheses using selectively neutral markers, namely a set of microsatellite loci dispersed over all chromosomal arms, and

Table 3. Intra-population *F*-statistic and number of observed heterokaryotypes (H_O) out of *N* karyotyped specimens in *Anopheles funestus* indoor samples collected during the course of 1 week in individual compounds (see Fig. 1 for their location within the villages). Other symbols and statistics as in Table 1

	Inversion System 2R*as*			Inversion System 3R*a*			Inversion System 3R*b*			Inversion System 3L*a*			
Compound	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	*N*	H_O	F_{IS}	w^2
Koubri													
September 1999													
#01	39	7	0.30	41	2	0.73*	41	4	0.29	–	–	–	30.9***
#02	40	10	−0.09	40	2	0.81***	40	2	0.65	–	–	–	22.8
#09	42	5	0.25	43	3	0.64	43	1	0.79	–	–	–	29.0**
December 2001													
#15	108	25	0.26**	122	10	0.78***	117	14	0.47***	107	1	–	110.4***
#17	120	29	0.33***	134	5	0.89***	129	18	0.36*	114	5	0.43	161.8***
#27	70	14	0.42**	82	5	0.81***	76	9	0.34	73	4	−0.02	77.0***
#55	62	9	0.25	65	0	1.00***	62	2	−0.01	59	0	–	30.8***
Kuiti													
September 1999													
#01	119	21	0.36***	126	12	0.55***	123	12	0.35	–	–	–	66.9***
#07	52	11	0.27	53	8	0.42	52	8	−0.07	–	–	–	22.2*
#12	45	10	0.40	50	5	0.70***	47	4	0.62*	–	–	–	49.9***
December 2001													
#06	102	21	0.39***	109	9	0.80***	108	20	0.31	104	3	0.56	115.8***
#07	79	19	0.28	81	7	0.76***	78	13	0.23	74	2	−0.01	61.5***
#49	80	16	0.49***	81	5	0.81***	79	7	0.59***	73	1	–	104.7***
#53	97	14	0.36*	100	4	0.76***	97	4	0.65***	93	3	0.01	70.1***

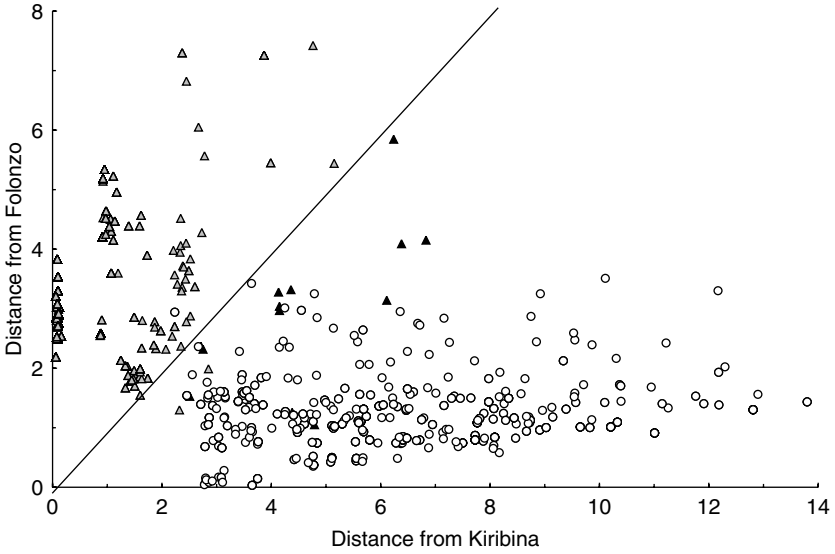

Fig. 2. Classification by the Assignment Test of individual *Anopheles funestus* previously assigned to the Folonzo (open circles) or Kiribina (shaded triangles) chromosomal forms according to the algorithm. The line identifies points with equal coordinates defining the limit of the areas with a higher probability of belongingness to Folonzo (below the line) or Kiribina (above the line). Closed triangles represent Kiribina individuals 'misclassified' by the algorithm because carriers of the 2R*s* arrangement (Rule I), though linked to arrangements on arm 3R that would put them under Folonzo by the other three rules of the algorithm (see also Table 4). Note that some points in the diagram corresponding to different individuals overlap.

located outside the inversions characterizing the two chromosomal forms (Sharakhov *et al*., 2004), and the mitochondrial *ND5* gene. Slight but significant differentiation at these markers was found between chromosomal forms, which is consistent with the hypothesis of incipient speciation between Kiribina and Folonzo, and suggest a role for selection and perhaps drift in their differentiation (Michel *et al*., 2005). Neutral markers will contribute to the understanding of the population structure of *An. funestus* from the microspatial to the continental scale, and they should help us recognize the exact nature of the chromosomal forms. However, it is uncertain whether it will be possible to discern by genetic studies alone the amount of residual gene flow vs. ancestral polymorphism, given that such speciation process is likely to be recent and/or incomplete.

By applying the algorithm proposed by Costantini and colleagues to assign individual females to one of two alternative chromosomal forms, HW and linkage equilibria were restored for all loci within each form. One form has been named with a provisional non-Linnean nomenclature

Table 4. Karyotypes of specimens showing a discrepancy between the two methods employed to discriminate between the two chromosomal forms, i.e. the algorithm or the Assignment Test performed with GeneClass. Chromosomal arrangements are identified by integers as follows: 0 = homokaryotype standard; 1 = heterokaryotype; 2 = homokaryotype inverted

			Chromosomal arrangement				
Specimen ID	Algorithm	Assignment test	2R*a*	2R*s*	3R*a*	3R*b*	3L*a*
99-00353	Kiribina	Folonzo	1	0	0	0	0
99-00476	Kiribina	Folonzo	0	1	2	1	0
99-00780	Kiribina	Folonzo	0	1	1	1	0
01-00458	Kiribina	Folonzo	0	1	1	1	0
01-01138	Kiribina	Folonzo	0	1	1	1	0
01-01583	Kiribina	Folonzo	0	0	1	0	0
01-03114	Kiribina	Folonzo	0	0	1	0	0
01-03805	Kiribina	Folonzo	0	0	1	0	0
01-05248	Kiribina	Folonzo	0	1	1	1	1
01-05421	Kiribina	Folonzo	0	1	2	0	0
01-05455	Kiribina	Folonzo	1	1	1	0	0
01-05474	Kiribina	Folonzo	0	1	0	1	0
01-05651	Kiribina	Folonzo	0	2	1	1	0
01-06511	Kiribina	Folonzo	0	1	1	0	2
01-06632	Kiribina	Folonzo	0	0	0	0	2
01-07262	Kiribina	Folonzo	0	1	1	1	0
02-01771	Kiribina	Folonzo	0	1	1	1	1
00-04029	Folonzo	Kiribina	2	0	0	0	0
01-05286	Folonzo	Kiribina	2	0	0	0	0
01-06170	Folonzo	Kiribina	2	0	0	0	0

'Kiribina', and is characterized mainly by the standard arrangement, and the other, named 'Folonzo', is characterized by a higher degree of polymorphism with inversions 3R*a*, 3R*b*, and 2R*a*, floating at high frequency, and the arrangement 3L*a* floating at lower frequencies. Our data set also suggests that the arrangement 2R*s,* previously presumed to be exclusive of Kiribina, may float at very low frequencies within Folonzo too, possibly due to occasional hybridization with Kiribina.

It is tempting to draw analogies with what is known of the pattern of chromosomal variability within *An. gambiae s.s.* in this same region, where several chromosomal forms characterized by restrictions to gene flow due to pre-mating mechanisms have been identified (Coluzzi *et al.*, 1985; Touré *et al.*, 1998). Substantial reproductive isolation between these forms has been confirmed by molecular markers (della Torre *et al.*, 2001; Tripet *et al.*, 2001; Gentile *et al.*, 2002). It appears that this region in Africa is characterized by profound anthropogenic modifications of the natural environment. These environmental modifications create opportunities for breeding in an otherwise inhospitable arid habitat at the margin of the distribution of these species, and promote speciation processes by flush and crash mechanisms (Coluzzi, 1982).

Contrary to the situation in West Africa, cytogenetic studies have shown that populations of *An. funestus* in Central (Dia *et al.*, 2000a; Boccolini *et al.*, 2005), Eastern (Sharakhov *et al.*, 2001; Kamau *et al.*, 2002), and Southern Africa (Green & Hunt, 1980; Boccolini *et al.*, 1992) are in panmixia. Also, the degree of chromosomal polymorphism in West African savanna populations appears lower than the rest of the continent (Lochouarn *et al.*, 1998; Costantini *et al.*, 1999; Dia *et al.*, 2000b). This might result from the exclusive presence of the Kiribina chromosomal form in the drier areas of this region. In conclusion, it will be relevant to define precisely the spatial and temporal distribution of the two forms, their relationship with other populations of *An. funestus* across Africa, as well as their specific behaviour, ecology, and exact role in malaria transmission.

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