Am. J. Trop. Med. Hyg., 73(6), 2005, pp. 1071–1076

CHROMOSOMAL DIFFERENTIATION OF *ANOPHELES FUNESTUS* FROM LUANDA AND HUAMBO PROVINCES, WESTERN AND CENTRAL ANGOLA

DANIELA BOCCOLINI, GIAN CARLO CARRARA, IBRAHIMA DIA, FILOMENO FORTES, PEDRO JORGE CANI, AND CARLO COSTANTINI*

Department of Infectious, Parasitic, and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy; Parasitology Unit, Department of Public Health, University of Rome "la Sapienza," Rome, Italy; Pasteur Institute, Dakar, Senegal; Ministry of Health – National Program of Malaria Control, Luanda, Angola

Abstract. The chromosomal polymorphism of *Anopheles funestus* sensu stricto from Angola was analyzed from indoor-resting samples collected in 11 peri-urban and rural sites of the Luanda and Huambo Provinces, which are > 450 km apart and have distinct eco-climatic conditions. Five polymorphic paracentric inversions were observed (scored chromatids range = 202 to 248): 2R*a*, 2R*h*, 3R*a*, 3R*b*, and 3L*a*. Inversions 3R*b* and 3L*a* were highly polymorphic; the 2R*a* and 3R*a* arrangements were absent in Luanda. No significant departures from Hardy-Weinberg and linkage equilibria were found at the locality, commune, or province level (sites ≤ 50 km from each other), indicating panmixia in each locale. Pooling the Luanda and Huambo samples produced a Wahlund effect, with significant levels of genetic differentiation suggestive of restrictions to gene flow due to geographic distance. The observation that differentiation was limited to inversions 2R*a* and 3R*a* can also be interpreted as divergent selection acting on these chromosomal regions between populations from the two provinces.

INTRODUCTION

Anopheles funestus Giles sensu stricto (s.s.) is one of the most important and widespread malaria vectors in sub-Saharan Africa, second only to *Anopheles gambiae* Giles s.s. with respect to the overall contribution to transmission across the continent. Previous studies indicated that in Angola, *An. funestus* can play a significant role in malaria transmission, with sporozoite rates ranging from 0% to 13% in the central regions.[1] Vector control can benefit from a detailed knowledge of the bionomics and genetic structure of *An. funestus*, and such knowledge is, to date, still insufficient for Angolan populations of this species.

In the face of significant advances in the molecular scrutiny of *An. funestus* and its relatives,[2] cytogenetic analysis remains a reliable and useful tool for the identification of the species constituting the Funestus group, and, perhaps more importantly, in ecological genetic studies, analogous to investigations with other anophelines, most notably the sister species of the *An. gambiae* sensu lato (s.l.) complex.[3] Moreover, in Malian populations of the nominal species of the *An. gambiae* complex, chromosomal markers were instrumental in the detection of assortative mating phenomena that led to the definition of three chromosomal forms assumed to represent the diverging taxonomic units of an incipient speciation process.[4,5] This view has been largely confirmed, at least in its general outline, by several classes of molecular markers, although the original simple relationship established in Mali between chromosomal forms and operational taxonomic units appears nowadays more complex on the basis of further cytogenetic and molecular evidence on a regional and continental scale.[6]

Analogously, previous cytogenetic studies of *An. funestus* populations from West Africa have shown the presence of marked chromosomal heterogeneities associated with behavioral and vectorial differences among carriers of alternative arrangements.[7,8] In Burkina Faso, spatially and temporally stable departures from Hardy-Weinberg and linkage equilibria at three common inversions (2R*a*, 3R*a*, and 3R*b*) were observed in strictly sympatric populations, suggesting the existence of two chromosomal forms with limitations to gene flow. These forms were named with a non-Linnean nomenclature "Kiribina", which is mainly characterized by the standard arrangement over all the polytenic complement, and "Folonzo", which is characterized by a high degree of polymorphism especially for inversions 3R*a*, 3R*b*, and 2R*a*.[7] Similar findings have been confirmed with an independent and extensive data set from a different region of Burkina Faso (Guelbeogo and others, in press). Molecular analyses of the same data set using simple tandem repeats (microsatellites) and the mitochondrial DNA (mtDNA) ND5 gene support the view of an incipient speciation process and suggest a role for selection in the differentiation of the two chromosomal forms Michel and others.[8]

However, in analogy with what has been observed for the vector species of the *An. gambiae* complex, a composite picture has emerged from the molecular and chromosomal inversion analysis of other *An. funestus* populations across the continent, in West Africa,[9–11] as well as in central,[12,13] eastern,[14–16] and southern Africa[17] and in Madagascar.[18] With a few exceptions in Senegal and Cameroon, the common feature of these studies is the panmixia of *An. funestus* in all locales. Transects along eco-climatic clines associated with humidity, however, have revealed intergrading chromosomal inversion frequencies[10,13]; significant genetic differentiation has been found with both chromosomal[14,15] and molecular[16] markers between populations separated by geographic barriers, such as the Rift Valley in Kenya. Genetic differentiation in allopatry associated with no evidence for reproductive isolation in sympatry, as observed in *An. funestus* from countries other than Burkina Faso, is in agreement with a population structure model of diverging populations isolated by distance or other geographic features. Divergence could be the outcome of any combination of interacting evolutionary forces such as genetic drift, founder effects, or selective pressures on carriers of alternative karyotypes. These observations suggest that population genetic structuring of *An. funestus* between West and East Africa could be different. Despite these dif-

* Address correspondence to Carlo Costantini, Institut de Recherche pour le Développement, 01 BP 182, Ouagadougou, Burkina Faso. E-mail: carlo-costantin@ird.bf

ferences, however, it is clear that such structuring may well affect the spread of insecticide resistance genes, such as those conferring resistance to pyrethroids reported from South African populations of this species.[2]

Thus, in this paper we report the results of a preliminary study on the population structure of *An. funestus* from Angola using chromosomal inversion markers. We have collected samples from several sites of the Luanda and Huambo Provinces with the aim to test whether Angolan populations are in panmixia, as well as to assess the geographic and environmental effects on the chromosomal polymorphism of this mosquito.

MATERIALS AND METHODS

Study area. The study was carried out in peri-urban and rural sites of the Luanda and Huambo provinces in Angola (Figure 1). These regions lie in quite distinct eco-climatic zones. The Luanda Province is located on the northwestern coastal lowland, an area of arid savanna, characterized by 300–500 mm annual rainfall. The rainy season lasts approximately from November to the beginning of May. In the cool dry season, from mid-May to September, average temperatures drop to 22°C. Here, adult mosquitoes were collected in two peri-urban and in six rural sites (Figure 1). The peri-urban sites were represented by two *bairros* located on the outskirts of Cacuaco town along the Atlantic coast (Saõ Francisco da Praia and Nazarè-Vidrul: 08°42′S, 13°23′E) and another four rural *bairros* close to the small town of Funda (Kilunda: 08°51′S, 13°36′E; Mulundu, 08°49′S, 13°29′E; Pinto, 08°47′S, 13°28′E; and Saõ Miguel, 08°50′S, 13°31′E). The other rural sites were two villages of the Viana Commune: Calumbo Pembele (09°09′S, 13°24′E) and Bita Tanke (09°09′S, 13°20′E). (Note: The term *bairros* usually indicates the town's residential quarters, but in this case it denotes temporary settlements of refugees from the rural populations that, due to insecurity procured by the Angolan civil war, were forced to move to towns or their environs. Such settlements have evolved during the past three decades into permanent shanty towns characterized by an extremely high density of houses and the absence of basic urban facilities).

The Huambo Province lies on the high plateau of central Angola. The area is a humid savanna (miombo woodland) interspersed with tropical montane forest. Annual rainfall ranges from 1,500 to 2,000 mm, with a single rainy season lasting from October to April; during this period, the mean temperature can fall to 20°C. Here, mosquito collections were carried out in one peri-urban site and in two rural villages (Figure 1): Camussamba, Commune of Cacilhas, which is a *bairro* around the extensive peripheral outskirts of the large Huambo town (12°46′S; 15°44′E; 1,721 m a.s.l) and in the villages of Cossango and Tchilonga, Commune of Chipipa (12°33′S; 15°44′E; 1,608 m a.s.l.).

Mosquito collection and processing. Mosquito sampling was carried out in June–July 2001, in April 2002 (during the dry season, and at the peak of the rainy season, respectively) in the Luanda Province, and in December 2003–January 2004 (in the middle of the rainy season) in the Huambo Province. Resting anophelines were collected manually in the morning (0700–0800) with mouth-operated aspirators inside human dwellings. Mosquitoes were kept alive in moistened cool-boxes until they reached the proper gonotrophic stage for polytene chromosome analysis (Christophers Stage III of ovarian development, also known as half-gravid stage by the external appearance of the abdomen). At that time, either whole female mosquitoes or their cropped ovaries were dropped in Carnoy's fixative solution (1 part of glacial acetic acid in 3 parts of absolute ethanol). Specimens were stored at

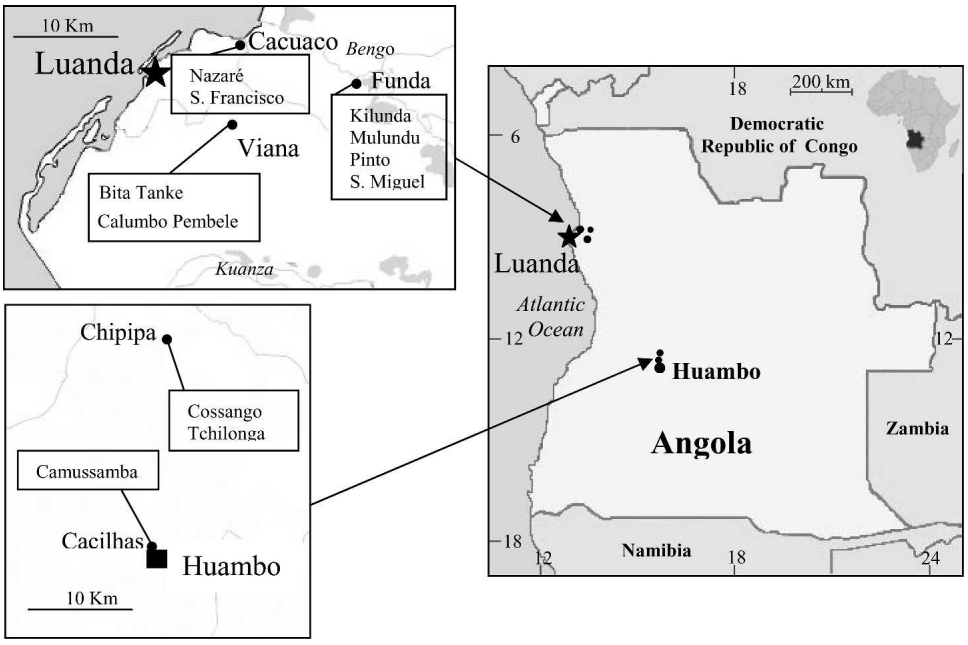

FIGURE 1. Maps showing the location of the sites near Luanda and Huambo where the *Anopheles funestus* samples of this study were collected. For analytical purposes, samples were regrouped at commune level: S. Francisco da Praia and Nazaré under Cacuaco; Kilunda, Mulundu, Pinto, and S. Miguel under Funda; Bita-Tanke and Calumbo Pembele under Viana; Cossango and Chilonga under Chipipa; and Camussamba under Cacilhas.

–20°C until processing. Preparations of polytene chromosomes were obtained by squashing the ovarian nurse cells stained with orcein according to the protocol of Green.[19] Chromosomes were examined under a phase-contrast microscope (160×; 400×), and inversions were identified and scored according to the map and nomenclature of Sharakhov and colleagues.[20]

Data analysis. Statistical analysis was performed with the software FSTAT v. 2.9.3.2[21] and GENEPOP v. 3.4.[22] Statistical inference in FSTAT is based on randomization tests, whereas GENEPOP implements several test algorithms. For analytical purposes, the standard and inverted arrangements of each chromosomal inversion system were considered as alternative alleles at a locus. Because the two inversions 2R*a* and 2R*h* overlap, they were considered as multiple alleles of the same 2R*ah* inversion system. Due to the limited number of chromosomal scorings from each site, to compare levels of population differentiation, we pooled samples from ecologically comparable sites whose distance is < 15 km. Thus, five samples (i.e., areas), which are identified by the name of the corresponding commune, were distinguished: the peri-urban *bairros* of Cacuaco and Cacilhas, the rural *bairros* of Funda, and the villages of Viana and Chipipa (Figure 1). Pooling was warranted by the absence of statistically significant differences in the distribution of genotypes across neighboring sites, as inferred by log-likelihood exact tests in GENEPOP. Inbreeding coefficients and genetic differentiation between geographical populations was examined by F-statistics calculated as in Weir and Cockerham.[23] Conformance to Hardy-Weinberg equilibrium was tested in GENEPOP with Fisher's exact test or its Markov chain equivalent[24] whenever sample size was too large to allow for the construction of all the contingency tables needed by the exact test. By pooling samples in a nested spatial fashion, Hardy-Weinberg equilibrium was assessed at different hierarchical level of geographic structure, namely countrywide, between the two provinces of Luanda and Huambo, and at commune level. Significance of F_{ST} values of pairwise population comparisons was tested using the G-based exact test of genotypic differentiation[25] using the Bonferroni correction as implemented in FSTAT.

RESULTS

A total of 196 half-gravid females suitable for chromosomal analysis were collected; of these, 123 (63%) were successfully scored for at least one inversion. Five polymorphic paracentric inversions were observed: two on arm 2R (2R*a*, 2R*h*), two on arm 3R (3R*a*, 3R*b*), and one on arm 3L (3L*a*). A diagrammatic representation of their location over the polytenic complement is reported in Figure 2. Inversion 2R*h* was observed only in heterozygotes. In accordance with previous studies, no inversions were found on the autosomal arm 2L and the X heterosome.

Inversions 3L*a* and 3R*b* were observed in populations from all communes. Inversion 2R*h* was found only in Funda and Viana from the Luanda Province. Inversions 2R*a* and 3R*a* were observed only in populations of the Huambo Province. Overall, inversions 3L*a* and 3R*b* were the most frequent, followed by inversions 3R*a*, 2R*a*, and 2R*h*, in decreasing order of frequency (Table 1).

In view of the heterogeneous distribution of inversion frequencies, a significant Wahlund effect emerged from the nested spatial analysis when samples were pooled across increasingly larger geographical areas: samples from individual localities (i.e., communes) were in Hardy-Weinberg equilibrium at all loci, even when assessed by Fisher's exact test across loci (Table 1). The same was found when samples were pooled at the province level (Table 1). When analyzing all our Angolan samples pooled together, however, highly significant departures from Hardy-Weinberg equilibrium due to a deficit of heterokaryotypes were found for inversions 2R*a* and 3R*a* (Table 1). The global test across loci was highly significant. Population structure was also investigated by calculating pairwise F_{ST} values among populations across loci and for individual loci across populations. Geographical populations separated by less than 50 km showed lower and statistically non-significant F_{ST} values; conversely, large and significant F_{ST} values were observed for populations separated by more than 450 km (Table 2). Large F_{ST} values were observed only for inversions 2R*a* ($F_{ST} = 0.877$) and 3R*a* ($F_{ST} = 0.920$), the remaining three inversions 2R*h*, 3R*b*, and 3L*a* having F_{ST} values ≤ 0.063, demonstrating that significant differentiation between populations did not involve the whole polytenic complement. No linkage disequilibrium was detected for any locus (i.e., inversions) pair across samples (i.e., geographical populations at commune level) by Fisher's method.

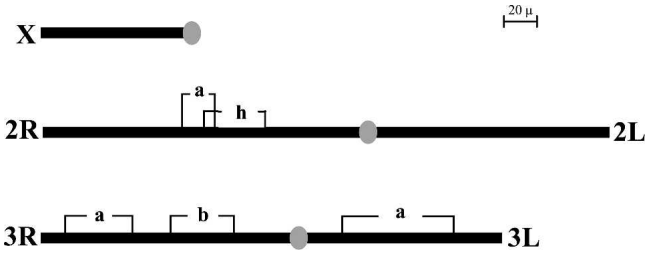

FIGURE 2. Diagrammatic representation of the polytene chromosomes of *Anopheles funestus*, showing the location of the paracentric inversions observed in Angolan samples of this species.

DISCUSSION

The chromosomal analysis of *An. funestus* sensu stricto populations from several localities of the Luanda and Huambo provinces in Angola showed the presence of five paracentric inversions (2R*a*, 2R*h*, 3R*a*, 3R*b*, 3L*a*) originally described by Green and Hunt,[17] and—with the exception of inversion 2R*a*—previously reported from other central African populations of this species.[12] High levels of chromosomal polymorphism were found in the samples collected from both provinces. Departures from panmictic conditions were not revealed in any population. A different distribution of inversion frequencies, however, was detected between the two provinces despite the availability of only a limited number of samples.

Inversion 2R*h* has been described from populations of central and eastern Africa. In three villages of southern Cameroon, it was the most frequently observed on the 2R arm.[12] In Madagascar and in Kenya, it was rarely found in heterokaryotypes.[18,26] Inversion 2R*a*, which is commonly observed in many continental populations as well as in Madagascar, was found in our samples only in villages of the Huambo

TABLE 1

Frequencies of the chromosomal inversions of *Anopheles funestus* observed in samples from the Communes of Luanda and Huambo Provinces, Western and Central Angola

Geographic level	2*N* [range]	Inversion system 2R*ah*					Inversion system 3R*a*			Inversion system 3R*b*			Inversion system 3L*a*					
		2R*a*	F_{IS}	2R*h*	F_{IS}	*P*	3R*a*	F_{IS}	*P*	3R*b*	F_{IS}	*P*	3L*a*	F_{IS}	*P*	χ^2	d.f.	*P*
Country																		
Angola	202–248	0.05	0.79	0.14	–0.16	<0.0001	0.05	0.76	<0.0001	0.50	0.00	1.00	0.71	0.06	0.64	56.2	8	<0.0001
Province																		
Luanda	188–232	0.00	–	0.15	–0.17	0.21	0.00	–	–	0.48	–0.02	1.00	0.69	0.04	0.81	3.5	6	0.74
Huambo	14–18	0.71	0.37	0.00	–	0.44	0.81	–0.17	1.00	0.69	0.19	1.00	0.94	0.00	–	1.6	6	0.95
Commune																		
Cacuaco	12–16	0.00	–	0.00	–	–	0.00	–	–	0.50	–0.25	1.00	0.50	–0.36	0.51	1.3	4	0.85
Funda	134–172	0.00	–	0.18	–0.21	0.11	0.00	–	–	0.53	–0.05	0.81	0.70	–0.01	1.00	4.9	6	0.56
Viana	42–44	0.00	–	0.10	–0.08	1.00	0.00	–	–	0.31	0.11	1.00	0.71	0.32	0.27	2.6	6	0.86
Chipipa	6–8	1.00	–	0.00	–	–	0.63	–0.50	1.00	0.50	0.14	1.00	1.00	–	–	0.0	4	1.00
Cacilhas	8–10	0.50	0.14	0.00	–	1.00	1.00	–	–	0.88	0.00	–	0.90	0.00	–	–	–	–
Unweighted frequencies		0.30		0.06			0.33			0.54			0.76					

2*N* = number of scored chromatids; F_{IS} = inbreeding coefficient (negative values indicate an excess of heterozygotes, while positive values denote heterozygote deficiency). *P* = probability of conformance to Hardy-Weinberg equilibrium (null hypothesis: $F_{IS} = 0$); χ^2, d.f., and *P* values of Fisher's exact test across inversions are reported in the last three columns of the table.

Province. On chromosomal arm 3R, arrangement 3R*a* was found at high frequency only in the two villages of the Huambo Province, whereas inversion 3R*b* floated at intermediate frequencies in all populations, without any evidence of linkage disequilibrium between this pair of arrangements. Thus, the high levels of linkage between 3R*a* and 3R*b* observed in some populations of Senegal,[27] Mali,[11] and Cameroon,[12] as well as in some villages of Burkina Faso[7] did not apply to our Angolan samples. In the Huambo populations, the 3L*a* inverted arrangement was found almost fixed, as previously observed in Cameroon[12] and in Madagascar.[18]

According to the algorithm proposed by Costantini and colleagues,[7] the specimens from the Huambo Province fall within the definition of the Folonzo chromosomal form by virtue of the high degree of polymorphism for inversions 3R*a*, 3R*b*, and 2R*a*. However, in the populations of the Luanda Province, inversions 3R*a* and 2R*a*—which are characteristic of the Folonzo form—were absent. The suitability of the algorithm constructed to define the chromosomal forms of *An. funestus* detected in Burkina Faso to other populations of this species across Africa is at the moment unclear, and most probably its application should not be attempted until further studies with other genetic markers clarify the relationships between the chromosomal forms in Burkina Faso and other continental populations of this species.

No significant departures from Hardy-Weinberg and linkage equilibria were observed when chromosomal data were analyzed at the locality, commune, or province levels. These results suggest the presence of interbreeding populations in each locale, where geographical populations are separated by no more than 50 km. This is in accordance with findings from western and coastal Kenya, where significant differentiation began to appear beyond 50–80 km,[16] as well as in populations from Senegal and Cameroon where an isolation by distance model estimated differentiation to appear at 20–50 km.[10,13] Also, as the Luanda samples were collected on consecutive years and different seasons, this would suggest that in this area, the degree of polymorphism and inversion frequencies remained fairly consistent across time.

Conversely, departures from Hardy-Weinberg equilibrium emerged when samples from the Luanda and Huambo provinces (more than 450 km apart) were pooled and analyzed as a single population, thereby denoting a marked Wahlund effect; such genetic differentiation is suggestive of restrictions to gene flow between the populations of these two regions due to geographic distance. The Luanda and Huambo provinces lie in quite distinct geographic zones characterized by different rainfall values, average temperatures, altitude above sea level, and vegetation. There is extensive evidence in other anophelines, particularly species of the afrotropical *An. gambiae* complex, for significant associations between some of these eco-climatic parameters and chromosomal inversions,[3,5,28,29] thereby supporting the notion that chromosomal inversions can play a significant role in ecotypic adaptation. The observation that marked differentiation was limited only to inversions 2R*a* and 3R*a*, therefore, can also be interpreted as a consequence of divergent selection acting on these chromosomal regions between populations from the two geographic areas.

At present, the role of different evolutionary forces promoting differentiation between the Luanda and Huambo populations cannot be untangled. Selectively neutral markers such as, for example, microsatellites, mtDNA genes, or single nucleotide polymorphisms, can contribute to uncover the population structure of this species. Indeed, larger samples, collected at other sites and times are needed for a more comprehensive understanding of the spatial and temporal variability of the chromosomal inversion polymorphism of *An.*

TABLE 2

Matrix of pairwise F_{ST} values for *Anopheles funestus* populations from the Communes of the Luanda and Huambo Provinces (shown below the main diagonal)

	Cacuaco	Funda	Viana	Chipipa	Cacilhas
Cacuaco	–	23	50	498	519
Funda	0.020	–	43	477	498
Viana	0.033	0.031	–	460	480
Chipipa	**0.598**	**0.509****	**0.558****	–	25
Cacilhas	**0.586***	**0.515****	**0.592****	0.261	–

Statistically significant values ($P < 0.05$) are formatted in bold.

Asterisks denote statistical significance after the Bonferroni correction: *$P < 0.05$; **$P < 0.01$.

Distances in km between localities are shown above the main diagonal.

funestus in Angola and of its relationship with other conspecific populations across Africa.

Despite these limitations, it is obvious from this study that genetically differentiated populations of *An. funestus* exist in the coastal lowlands and central highlands of Angola. On the basis of inversion frequencies, the populations from the coastal lowlands appears related somewhat more closely to those of central Africa (e.g., forest villages of southern Cameroon, except for the absence of the 3R*a* arrangement), whereas populations of the central plateau show the pattern of chromosomal polymorphism observed in some southern African populations (e.g., Okavango river in Namibia[16]). The chromosomal inversions responsible of such differentiation have been sometimes associated with vectorial and behavioral heterogeneities.[7,8] In this context, it is interesting to note that in the Luanda Province, the human blood index of indoor-resting *An. funestus* was reported to be 68%, a fairly low figure for such kind of samples of this species elsewhere in Africa, denoting a more zoophilic host selection pattern.[30] Thus, it will be worthwhile to investigate whether the observed genetic differences can affect the malaria transmission potential of this species in the two regions.

Received September 24, 2004. Accepted for publication February 10, 2005.

Acknowledgments: The authors thank Andrè Francisco Sebastião, Mpova Zambote, Alberto Bunga, and Manuel Alfredo Paulo (Instituto Nacional da Saúde Publica, Luanda) for their technical assistance during the field collections. We are especially grateful to Dr. Stefano Ferroni, Project Manager of the "Programma di Cooperazio ne Socio-Sanitaria AID 5810," and the Trappist Nuns in Huambo for their hospitality. We would like to acknowledge Nora Besansky, Mario Coluzzi, and Roberto Romi for their support and for helpful suggestions that improved an earlier draft of this paper.

Financial support: The Health and Social Program for Cooperation to Development "AID 5810" of the Italian Ministry of Foreign Affairs and UNICEF-Angola supported field work. The COFIN program of the Italian Ministry of Education, University, and Research supported C.C.; D.B. and C.C. benefited by financial assistance from the Pal+ project of the French Ministry of Research.

Authors' addresses: Daniela Boccolini, Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Roma, Italy, Telephone: +39 06 49903108, Fax: +39 06 49387065. Gian Carlo Carrara, Sezione di Parassitologia, Dipartimento di Scienze di Sanità Pubblica, Università degli Studi di Roma "la Sapienza," P.le Aldo Moro 5, 00185, Roma, Italy, Telephone: +39 06 4455780, Fax: +39 06 49914653. Ibrahima Dia, Laboratoire d'Entomologie Médicale, Institut Pasteur de Dakar, BP 220, Dakar, Sénégal, Telephone: +221 8399228, Fax: +221 8399210. Filomeno Fortes and Pedro Jorge Cani, Ministério da Saúde – Programa Nacional de Controle da Malária, Luanda, Angola. Carlo Costantini (formerly at the Department of Public Health, University of Rome "la Sapienza," Italy), Institut de Recherche pour le Développement, 01 BP 182, Ouagadougou, Burkina Faso, Telephone: +226 50306737, Fax: +226 50310385.

Reprint requests: Dr. Daniela Boccolini, Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Roma, Italy.

REFERENCES

1. Ribeiro H, Ramos HC, 1975. Research on the mosquitoes of Angola. *Garcia de Orta, Sér Zool 4:* 1–40.
2. Coetzee M, Fontenille D, 2004. Advances in the study of *Anopheles funestus*, a major vector of malaria in Africa. *Insect Biochem Mol Biol 34:* 599–605.
3. Powell JR, Petrarca V, della Torre A, Caccone A, Coluzzi M, 1999. Population structure, speciation, and introgression in the *Anopheles gambiae* complex. *Parassitologia 41:* 101–113.
4. Coluzzi M, Petrarca V, Di Deco MA, 1985. Chromosomal inversion intergradation and incipient speciation in *Anopheles gambiae. Boll Zool 52:* 45–63.
5. Toure YT, Petrarca V, Traore SF, Coulibaly A, Maiga HM, Sankare O, Sow M, Di Deco MA, Coluzzi M, 1998. The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. *Parassitologia 40:* 477–511.
6. della Torre A, Costantini C, Besansky NJ, Caccone A, Petrarca V, Powell JR, Coluzzi M, 2002. Speciation within *Anopheles gambiae*–the glass is half full. *Science 298:* 115–117.
7. Costantini C, Sagnon N, Ilboudo-Sanogo E, Coluzzi M, Boccolini D, 1999. Chromosomal and bionomic heterogeneities suggest incipient speciation in *Anopheles funestus* from Burkina Faso. *Parassitologia 41:* 595–611.
8. Michel AP, Guelbeogo WM, Grushko O, Schemerhorn BJ, Kern M, Willard MB, Sagnon NF, Costantini C, Besansky NJ, 2005. Molecular differentiation between chromosomally defined incipient species of *Anopheles funestus. Insect Mol Biol 14:* 375–387.
9. Lochouarn L, Dia I, Boccolini D, Coluzzi M, Fontenille D, 1998. Bionomical and cytogenetic heterogeneities of *Anopheles funestus* in Senegal. *Trans R Soc Trop Med Hyg 92:* 607–612.
10. Cohuet A, Dia I, Simard F, Raymond M, Fontenille D, 2004. Population structure of the malaria vector *Anopheles funestus* in Senegal based on microsatellite and cytogenetic data. *Insect Mol Biol 13:* 251–258.
11. Boccolini D, Sagnon NF, Touré YT, 1998. Chromosomal polymorphism in *Anopheles funestus* and description of new inversions in Burkina Faso and Mali. *Parassitologia 40:* 14.
12. Dia I, Boccolini D, Antonio-Nkondjio C, Costantini C, Fontenille D, 2000. Chromosomal inversion polymorphism of *Anopheles funestus* from forest villages of South Cameroon. *Parassitologia 42:* 227–229.
13. Cohuet A, Dia I, Simard F, Raymond M, Rousset F, Antonio-Nkondjio C, Awono-Ambene PH, Wondji CS, Fontenille D, 2004. Gene flow between chromosomal forms of the malaria vector *Anopheles funestus* in Cameroon, Central Africa, and its relevance in malaria fighting. *Genetics 169:* 301–311.
14. Kamau L, Hunt R, Coetzee M, 2002. Analysis of the population structure of *Anopheles funestus* (Diptera: Culicidae) from western and coastal Kenya using paracentric chromosomal inversion frequencies. *J Med Entomol 39:* 78–83.
15. Kamau L, Munyekenye GO, Koekemoer LL, Hunt RH, Coetzee M, 2003. A survey of the *Anopheles funestus* (Diptera: Culicidae) group of mosquitoes from 10 sites in Kenya with special emphasis on population genetic structure based on chromosomal inversion karyotypes. *J Med Entomol 40:* 664–671.
16. Braginets OP, Minakawa N, Mbogo CM, Yan G, 2003. Population genetic structure of the African malaria mosquito *Anopheles funestus* in Kenya. *Am J Trop Med Hyg 69:* 303–308.
17. Green CA, Hunt RH, 1980. Interpretation of variation in ovarian polytene chromosomes of *Anopheles funestus* Giles, *A. parensis* Gillies, and *A. aruni? Genetica 51:* 187–195.
18. Boccolini D, Rakotoson R, Ralisoa O, Sabatini A, Randrianarisoa E, Coluzzi M, 1992. Polimorfismo cromosomico di *Anopheles funestus* in Madagascar. *Parassitologia 34:* 14–15.
19. Green CA, 1972. Cytological maps for the practical identification of females of the three freshwater species of the *Anopheles gambiae* complex. *Ann Trop Med Parasitol 66:* 143–147.
20. Sharakhov I, Braginets O, Grushko O, Cohuet A, Guelbeogo WM, Boccolini D, Weill M, Costantini C, Sagnon N, Fontenille D, Yan G, Besansky NJ, 2004. A microsatellite map of the African human malaria vector *Anopheles funestus. J Hered 95:* 29–34.
21. Goudet J, 1995. FSTAT (vers. 1.2): a computer program to calculate F-statistics. *J Hered 86:* 485–486.
22. Raymond M, Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered 86:* 248–249.
23. Weir BS, Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure. *Evolution 38:* 1358–1370.
24. Guo SW, Thompson EA, 1992. Performing the exact test of

Hardy-Weinberg proportions for multiple alleles. *Biometrics 48:* 361–372.
25. Goudet J, Raymond M, de Meeus T, Rousset F, 1996. Testing differentiation in diploid populations. *Genetics 144:* 1933–1940.
26. Sharakhov IV, Sharakhova MV, Mbogo CM, Koekemoer LL, Yan G, 2001. Linear and spatial organization of polytene chromosomes of the African malaria mosquito *Anopheles funestus. Genetics 159:* 211–218.
27. Dia I, Lochouarn L, Boccolini D, Costantini C, Fontenille D, 2000. Spatial and temporal variations of the chromosomal inversion polymorphism of *Anopheles funestus* in Senegal. *Parasite 7:* 179–184.
28. Coluzzi M, Sabatini A, Petrarca V, Di Deco MA, 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. *Trans R Soc Trop Med Hyg 73:* 483–497.
29. Touré YT, Petrarca V, Traore SF, Coulibaly A, Maiga HM, Sankare O, Sow M, Di Deco MA, Coluzzi M, 1994. Ecological studies in the chromosomal form Mopti of *Anopheles gambiae* s.str. in Mali, West Africa. *Genetica 94:* 213–223.
30. Carrara GC, Santolamazza F, Fanello C, Cani PJ, della Torre A, Petrarca V, 2002. Preliminary data on the Anopheline malaria vectors at two sites of Western Angola. *Parassitologia 44:* 43.