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MULTILOCUS ENZYME ELECTROPHORESIS SUPPORTS SPECIATION WITHIN THE *ANOPHELES NILI* GROUP OF MALARIA VECTORS IN CAMEROON

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Abstract. Multilocus enzyme analysis of the genetic variability and population structure was conducted among three malaria vector species of the *Anopheles nili* group in Cameroon: *An. nili*, *An. carnevalei,* and the recently described *An. ovengensis*. We detected species-specific alleles and large differences in shared allele frequencies at six of nine loci (e.g., PGM, GOT_1, IDH_1, IDH_2, PGI, and α-GPD). This non-random distribution of alleles leads to high and significant values of differentiation indexes ($0.569 < Fst < 0.874$, $P < 10^{-4}$). These results fully agree with standard morphologic descriptions, and therefore provide further support for recent taxonomic classification within the *An. nili* group.

The bionomics of *Anopheles nili* mosquitoes was recently updated after the description of a new malaria vector species from central African regions, namely *Anopheles ovengensis*.[1] The *An. nili* group includes 4 recognized species that can be identified through slight morphologic diagnostic characters observable at the larval and/or adult stages: *An. nili*, *An. somalicus*, *An. carnevalei,* and *An. ovengensis*. Mosquitoes of this group are recognized as major human malaria vectors in tropical Africa, especially along streams and rivers that represent typical larval development sites.[2,3] However, in spite of its epidemiologic importance for malaria transmission, very few studies have yet targeted this species group. Recent analysis of DNA sequence variation in the ribosomal DNA region revealed 4 different clusters corresponding each to one species.[4] This genetic polymorphism provided the basis for the development of a PCR-based molecular identification assay. However, no study was undertaken to explore the level of intra-specific genetic variation or genetic relationship between these taxonomic units. Here, we used multilocus enzyme electrophoresis as a preliminary tool for assessing genetic variability and differentiation within the *An. nili* group of malaria vectors.

Adult females *An. nili* were captured from March to September 2001 in 4 villages within the equatorial forest domain, south of Cameroon (Figure 1): Mbébé (4°10'N; 11°04'E), Afan Essokié (2°23'N; 10°00'E), Oveng (2°24'N; 10°21'E), and Simbock (3°51'N; 11°30'E). Geographic distance between locations ranged from 50 km (Afan Essokié-Oveng) to 250 km (Mbébé-Afan Essokié). Species within the *An. nili* group were determined morphologically according to dichotomous keys and recent descriptions.[1,5] Each individual mosquito was stored in liquid nitrogen until processed in the laboratory for isoenzyme analysis. Seven enzyme systems were studied: mannose-6-phosphate-isomerase (MPI, E.C 5.3.1.8.); phosphoglucomutase (PGM, E.C 2.7.5.1); glutamate-oxaloacetate transaminase (GOT, E.C 2.6.1.1); hexokinase (HK, E.C 2.7.1.1); isocitrate dehydrogenase (IDH, E.C 1.1.1.42); phosphogluco-isomérase (PGI, E.C 5.3.1.9); alpha glycerophosphate dehydrogenase (α-GPD, E.C 1.1.1.8). Migration patterns were analyzed at 9 interpretable neutral loci, namely MPI, PGM, GOT_1, HK_1, HK_2, IDH_1, IDH_2, PGI, and α-GPD, using horizontal starch gel electrophoresis.[6] For each locus, conformity with Hardy-Weinberg (HW) expectations was estimated for each species and overall. Statistical significance and linkage disequilibrium between pairs of loci were assessed using the exact probability tests available in GENEPOP 3.2.[7] Genetic differentiation between cryptic species of the *An. nili* group was examined by Wright's *F* statistics.[8,9] Significance of *Fst* was assessed using the G-based exact test for genotypic differentiation.[10]

We analyzed 127 specimens including 66 *An. nili* (from Mbébé and Simbock), 45 *An. ovengensis* (from Oveng), and 16 *An. carnevalei* (from Afan-Essokié). Samples from Mbébé and Simbock were merged together because all specimens

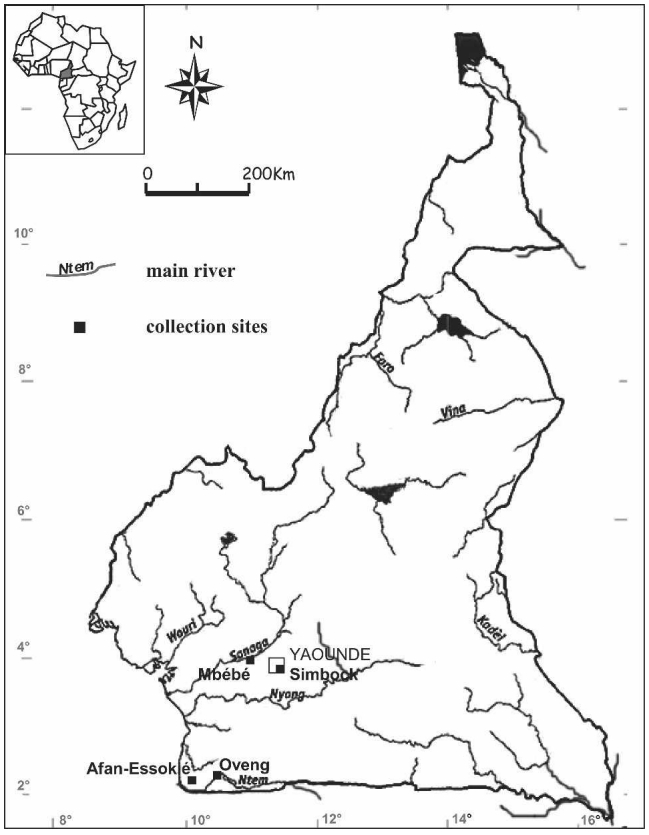

FIGURE 1. Map of Cameroon showing the main hydrographic network, the capital city, and the 4 mosquito collection sites.

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were morphologically identified as *An. nili*. Indeed, as shown in Table 1, no deficit in heterozygote could be indicative of a mixing of different gene pools (Whalund effect). The percentage of polymorphic loci (with more than 1 allele) was 55.6% for *An. nili* and 66.7% for both *An. ovengensis* and *An. carnevalei*. The average number of alleles (± standard error) per locus ranged from 1.78 (± 0.63) to 2.00 (± 0.82), and the observed heterozygosity from 0.030 (± 0.015) in *An. nili* to 0.437 (± 0.022) in *An. carnevalei*. These estimates fall within the range of values reported for other anophelines species.[11–13] When we considered all samples as a single gene pool, significant heterozygote deficits inducing a deviation from HW equilibrium was observed at PGM, GOT_1, IDH_1, IDH_2, and α-GPD loci ($P < 0.05$ after correction for multiple tests). In agreement with this genetic heterogeneity, significant linkage disequilibrium was detected for 18 of 36 pairs of loci ($P < 0.05$; single test level). In contrast, HW expectations were respected at all loci when we analyzed each species as a

TABLE 1

Genetic variability and goodness of fit to Hardy-Weinberg equilibrium in *An. nili, An. ovengensis,* and *An. carnevalei* populations from Cameroon

		Allele	*An. nili* s.s.	*An. ovengensis*	*An. carnevalei*	All samples
MPI	N		66	44	16	126
		1	0	0.102	0.063	0.044
		2	1.000	0.898	0.937	0.956
	(Ho)		–	(0.114)	(0.125)	(0.056)
	Fis		–	**0.391**	–0.034	0.338
PGM	N		66	45	16	127
		1	0.022	0	0	0.012
		2	0.970	0	0	0.504
		3	0.008	0	0	0.004
		4	0	0.022	0.125	0.024
		5	0	0.011	0	0.004
		6	0	0.967	0.875	0.452
	(Ho)		(0.061)	(0.067)	(0.250)	(0.087)
	Fis		–0.018	–0.015	–0.111	**0.841**
GOT_1	N		63	45	16	124
		1	0	0.011	0	0.004
		2	0.016	0.900	0	0.334
		3	0.008	0.089	0	0.036
		4	0.960	0	0.937	0.609
		5	0.016	0	0.063	0.016
	(Ho)		(0.079)	(0.200)	(0.125)	(0.129)
	Fis		–0.020	–0.088	–0.034	**0.751**
HK_1	N		66	45	16	127
		1	0.015	0	0	0.008
		2	0.985	1.000	1.000	0.992
	(Ho)		(0.030)	–	–	(0.016)
	Fis		–0.008	–	–	–0.004
HK_2	N		66	45	16	127
		1	0.015	0	0	0.008
		2	0.985	1.000	1.000	0.992
	(Ho)		(0.030)	–	–	(0.016)
	Fis		–0.008	–	–	–0.004
IDH_1	N		66	45	15	126
		1	0.288	0.944	0.533	0.552
		2	0.712	0	0.467	0.428
		3	0	0.045	0	0.016
		4	0	0.011	0	0.004
	(Ho)		(0.333)	(0.111)	(0.400)	(0.262)
	Fis		0.194	–0.038	0.229	**0.491**
IDH_2	N		66	45	16	127
		1	0	1.000	1.000	0.480
		2	1.000	0	0	0.520
	(Ho)		–	–	–	(0.000)
	Fis		–	–	–	**1.000**
PGI	N		66	45	16	127
		2	0	0	0.063	0.008
		3	1.000	0.989	0.781	0.968
		4	0	0.011	0.156	0.024
	(Ho)		–	(0.022)	(0.437)	(0.063)
	Fis		–	–	–0.180	–0.022
α-GPD	N		66	45	16	127
		1	1.000	0.022	0.031	0.532
		2	0	0.978	0.969	0.468
	(Ho)		–	(0.044)	(0.062)	(0.024)
	Fis		–	–0.011	–	**0.953**

N, sample size; (Ho), observed heterozygosity.
Fis was calculated according to Weir & Cockerham, 1984.[9]
Bolded characters: $P < 0.05$ after taking into account multiple tests.
–: Irrelevant because only one genotype was detected.

TABLE 2
Loci showing species diagnostic alleles (*) or significant differences in shared allele frequencies among the *An. nili* group of malaria vectors

	An. ovengensis	*An. carnevalei*
An. nili	PGM,* GOT, IDH_1, IDH_2,* α-GPD	PGM,* IDH_2,* PGI, α-GPD
An. ovengensis	–	GOT,* IDH_1, PGI

different taxonomic unit, with the exception of locus MPI that showed marginally significant heterozygote deficit in *An. ovengensis*. High values of genetic differentiation indexes (*Fst*) across all loci were observed between *An. nili* and *An. ovengensis* (Fst = 0.874, $P < 10^{-4}$), between *An. nili* and *An. carnevalei* (Fst = 0.791, $P < 10^{-4}$), and between *An. ovengensis* and *An. carnevalei* (Fst = 0.569, $P < 10^{-4}$). These findings are consistent with the occurrence of non-overlapping species-diagnostic alleles and large differences in shared allele frequencies at PGM, GOT_1, IDH_1, PGI, and α-GPD loci. *An. nili* displayed a diagnostic private allele at IDH_2, differing from the allele shared by both *An. ovengensis* and *An. carnevalei* at the same locus. No heterozygote was observed at this locus. Moreover, *An. carnevalei* and *An. ovengensis* revealed different fixed alleles for the locus GOT. As shown in Table 2, the combination of such species-diagnostic loci can be used for species identification within the *An. nili* group.

The amount of fixed and/or large differences in allele profiles and differentiation indexes across all loci we observed in this study further supports speciation between *An. nili*, *An. carnevalei,* and *An. ovengensis*, and is in agreement with current classification based on specific morphologic traits and segregating sequences in the ribosomal DNA.[1,4] As shown in this study, the combination of multiple polymorphic enzyme loci can be useful to explore the extent and distribution of genetic polymorphism within the *An. nili* group, as was formerly demonstrated in other anopheline[11–16] and culicine groups.[17] Multiple enzyme electrophoresis provided preliminary data on the population genetic structure of members of the *An. nili* group in Cameroon. Recent availability of microsatellite DNA markers should allow more refined assessment of the genetic diversity and population structure of these neglected mosquito vector groups.[11,18]

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