

## 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<sub>1</sub>, IDH<sub>1</sub>, IDH<sub>2</sub>, PGI, and  $\alpha$ -GPD). This non-random distribution of alleles leads to high and significant values of differentiation indexes ( $0.569 < F_{st} < 0.874$ ,  $P < 10^{-4}$ ). 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*.<sup>1</sup> 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.<sup>2,3</sup> 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.<sup>4</sup> 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.<sup>1,5</sup> 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.); phosphoglucosmutase (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 ( $\alpha$ -GPD, E.C 1.1.1.8). Migration patterns were analyzed at 9 interpretable

neutral loci, namely MPI, PGM, GOT<sub>1</sub>, HK<sub>1</sub>, HK<sub>2</sub>, IDH<sub>1</sub>, IDH<sub>2</sub>, PGI, and  $\alpha$ -GPD, using horizontal starch gel electrophoresis.<sup>6</sup> 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 GENETPOP 3.2.<sup>7</sup> Genetic differentiation between cryptic species of the *An. nili* group was examined by Wright's *F* statistics.<sup>8,9</sup> Significance of *F*<sub>st</sub> was assessed using the G-based exact test for genotypic differentiation.<sup>10</sup>

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

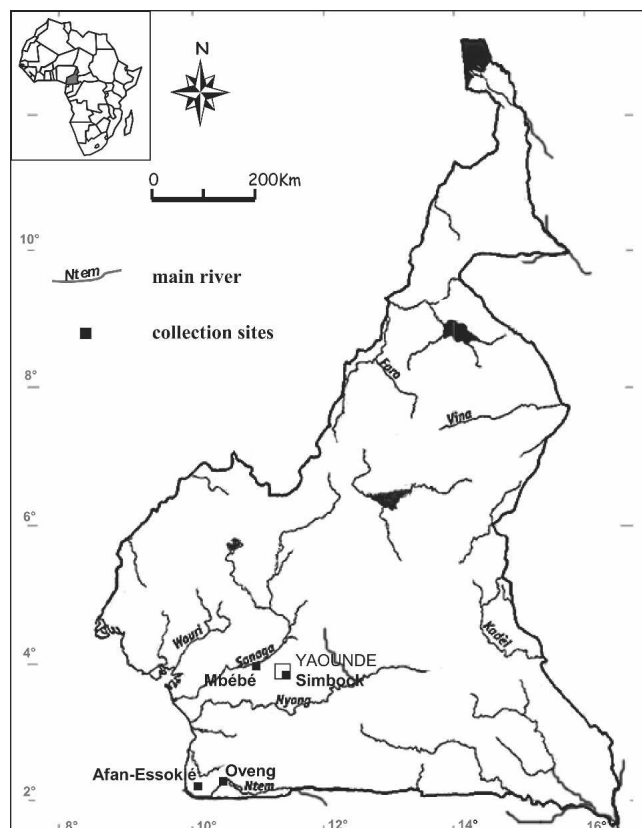


FIGURE 1. Map of Cameroon showing the main hydrographic network, the capital city, and the 4 mosquito collection sites.

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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 (Wahlund 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 ( $\pm$  standard error) per locus ranged from 1.78 ( $\pm$  0.63) to 2.00 ( $\pm$  0.82), and the observed heterozygosity from 0.030 ( $\pm$  0.015) in *An. nili* to 0.437 ( $\pm$  0.022) in *An. carnevalei*. These estimates fall within

the range of values reported for other anophelines species.<sup>11-13</sup> When we considered all samples as a single gene pool, significant heterozygote deficits inducing a deviation from HW equilibrium was observed at PGM, GOT<sub>1</sub>, IDH<sub>1</sub>, IDH<sub>2</sub>, and  $\alpha$ -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	<i>An. nili</i> s.s.	<i>An. ovengensis</i>	<i>An. carnevalei</i>	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)
		<i>Fis</i>	-	-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)
	<i>Fis</i>	-0.018	-0.015	-0.111	<b>0.841</b>	
GOT <sub>1</sub>	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)	
	<i>Fis</i>	-0.020	-0.088	-0.034	<b>0.751</b>	
HK <sub>1</sub>	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)
	<i>Fis</i>	-0.008	-	-	-0.004	
HK <sub>2</sub>	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)
	<i>Fis</i>	-0.008	-	-	-0.004	
IDH <sub>1</sub>	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)
	<i>Fis</i>	0.194	-0.038	0.229	<b>0.491</b>	
IDH <sub>2</sub>	N		66	45	16	127
		1	0	1.000	1.000	0.480
		2	1.000	0	0	0.520
		(Ho)	-	-	-	(0.000)
	<i>Fis</i>	-	-	-	<b>1.000</b>	
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)	
	<i>Fis</i>	-	-	-0.180	-0.022	
$\alpha$ -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)
	<i>Fis</i>	-	-0.011	-	<b>0.953</b>	

N, sample size; (Ho), observed heterozygosity.  
*Fis* was calculated according to Weir & Cockerham, 1984.<sup>9</sup>  
 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

	<i>An. ovengensis</i>	<i>An. carnevalei</i>
<i>An. nili</i>	PGM <sub>1</sub> *, GOT, IDH <sub>1</sub> , IDH <sub>2</sub> *, α-GPD	PGM <sub>1</sub> *, IDH <sub>2</sub> *, PGI, α-GPD
<i>An. ovengensis</i>	–	GOT*, IDH <sub>1</sub> , 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<sub>1</sub>, IDH<sub>1</sub>, PGI, and α-GPD loci. *An. nili* displayed a diagnostic private allele at IDH<sub>2</sub>, 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.<sup>1,4</sup> 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<sup>11–16</sup> and culicine groups.<sup>17</sup> 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.<sup>11,18</sup>

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