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# Morphological variability in the malaria vector, *Anopheles moucheti*, is not indicative of speciation: evidences from sympatric south Cameroon populations

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**Abstract**

*Anopheles moucheti* is a major human malaria vector in the vicinity of slow moving rivers in the tropical forests of Central Africa. Morphological variations in natural populations of *A. moucheti* led to the designation of three morphological forms named *A. moucheti moucheti*, *A. moucheti nigeriensis* and *A. moucheti bervoetsi*. Using allozyme markers, we investigated to which extent morphological and/or geographical populations of *A. moucheti* were genetically differentiated. Mosquitoes were collected from four villages 20–200 km distant apart in south Cameroon, where specimens from each morphological form were found in sympatry. All populations appeared highly homogenous across both morphological type and geographic location. Significant genetic differentiation was only observed between two locations 150 km apart ($F_{st} = 0.029$; $P = 0.006$), while no pairwise $F_{st}$ estimate between morphological forms reached statistical significance. Further evidence against any taxonomic value of this morphological classification was provided by direct observation of morphological variation within the progeny of field-collected females from all three types. Single female offspring always belonged to at least two morphologically recognised types and most often, a mixture of all three forms was observed. Our results therefore demonstrate that morphological variability within *A. moucheti* natural populations is not indicative of speciation. With this respect, restricted migration of individuals across river systems may be a more important factor in shaping population genetic structure of *A. moucheti*.


*Keywords:* Malaria; *Anopheles moucheti*; Isozyme; Morphology; Cameroon

*Anopheles moucheti* is a major human malaria vector in Africa's equatorial region (Languillon et al., 1956; Njan Nloga et al., 1993; Manga et al., 1995; Antonio-Nkondjio et al., 2002). This vector is found in villages or towns situated along slow moving streams and large rivers. *A. moucheti* is described in the literature as a group of three morphological forms: the type form *A. moucheti moucheti* Evans 1925, *A. moucheti nigeriensis* Evans 1931 and *A. moucheti bervoetsi* D'Haenens 1961. At the adult stage *A. moucheti nigeriensis* differs from the type form by the presence of a well-marked pale band at the apex of the fourth segment of the fore tarsus and by a pale spot present opposite sixth vein on the wing fringe. In addition to these characters, the pale spots of the accessory sector and sector of vein 1 of the wing are merged in *A. moucheti bervoetsi* (Gillies and De Meillon, 1968; Brunhes et al., 1998). Although, initially called "subspecies" based on allopatric distributions, the taxonomic status of these forms was subsequently questioned as they were reported to occur in sympatry (Brunhes et al., 1998).

As part of a large scale ongoing investigation of the bionomics and population genetics of *A. moucheti s.l.* in Africa, we studied isozyme variability and genetic differentiation between geographical and/or morphological populations of *A. moucheti* from southern Cameroon. Mosquitoes were collected in the villages of Simbock (3°51′N; 11°30′E), Olama (3°24′N; 11°18′E), Mbalmayo (3°30′N; 11°30′E) and Nyabessan (2°80′N; 10°25′E) from March to July 2001. Geographic distance between locations ranged from 20 (between Olama and Mbalmayo) to 200 km (between Simbock and Nyabessan). All these villages are located within the Guineo-congolese bioclimatic domain in an equatorial forest environment, with annual rainfall around

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1500 mm spread all over the year. Adult mosquitoes were collected after landing on human volunteers, before feeding. They were identified to species using the morphological keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1987), and stored in liquid nitrogen until processed in the laboratory. A total of 198 *A. moucheti s.l.* specimens were used for genetic analyses. Isozymes were separated by horizontal starch gel electrophoresis as described in Pasteur et al. (1987).

Only 10 specimens were available from Mbalmayo but in all other locations, sample sizes were above 50 (Table 1). All three morphological types were present in each locality and no significant difference in their distribution was revealed among sites (Fisher's exact test; $P = 0.51$). *A. moucheti moucheti* was the most frequent in all sites. Technical and operational issues in the field, however, prevented confident identification of 29 specimens down to their morphological type. Thus, only 169 out of 198 specimens were included in the isozyme analysis when mosquitoes were clustered according to morphological classification (Table 1). Nine allozyme systems were tested among which three were discarded because of low allelic resolution, and one showed identical migration patterns in all samples. Five systems including six putative loci, revealed polymorphic, showing two–four alleles/locus and overall heterozygosity ranging from 0.02 for locus α-GPD to 0.34 for locus IDH1 (Table 1). These values are similar to what has been reported in other anopheline species (Lanzaro et al., 1995; Manguin et al., 1999).

Goodness of fit to Hardy–Weinberg expectations was assessed for each locus in each population using the exact probability test available in Genepop 3.2 (Raymond and Rousset, 1995). Although, several single locus deviations were observed, most of the loci conformed to Hardy–Weinberg expectations whether grouping specimens according to their geographical origin, or morphological form (after adjusting significance thresholds to account for simultaneous testing at the population level, see Table 1). However, significant deficit in heterozygote persisted at the PGI locus in Olama ($F_{is} = +0.593$; $P < 0.05$) as

Table 1
Genetic variability at six isozyme loci and goodness of fit to Hardy–Weinberg equilibrium in natural populations of *A. moucheti* from south Cameroon

| OTU | Population | | Locus[a] | | | | | | Mean across all loci |
|---|---|---|---|---|---|---|---|---|---|
| | | | PGM | GOT | IDH1 | IDH2 | PGI | α-GPD | |
| Geographic location | Simbock ($n = 53$) | $N_{all}$ | 4 | 4 | 3 | 1 | 2 | 1 | 2.1 |
| | | $H$ | 0.192 | 0.280 | 0.360 | –[b] | 0.091 | – | 0.116 |
| | | $F_{is}$ | +0.119 | +0.124 | +0.284 | – | −0.040 | – | NC |
| | Olama ($n = 59$) | $N_{all}$ | 3 | 4 | 3 | 2 | 4 | 1 | 2.4 |
| | | $H$ | 0.204 | 0.476 | 0.395 | 0.050 | 0.083 | – | 0.151 |
| | | $F_{is}$ | −0.099 | +0.005 | *+0.229* | *+0.663* | **+0.593** | – | NC |
| | Nyabessan ($n = 76$) | $N_{all}$ | 4 | 3 | 3 | 2 | 2 | 2 | 2.3 |
| | | $H$ | 0.290 | 0.214 | 0.305 | 0.026 | 0.088 | 0.052 | 0.122 |
| | | $F_{is}$ | +0.047 | +0.156 | +0.051 | −0.007 | +0.257 | −0.020 | NC |
| | Mbalmayo ($n = 10$) | $N_{all}$ | 2 | 4 | 2 | 1 | 2 | 1 | 1.8 |
| | | $H$ | 0.268 | 0.553 | 0.189 | – | 0.100 | – | 0.139 |
| | | $F_{is}$ | −0.125 | +0.287 | −0.059 | – | – | – | NC |
| Pooled populations ($n = 198$) | | $N_{all}$ | 4 | 4 | 3 | 2 | 4 | 2 | 2.6 |
| | | $H$ | 0.238 | 0.337 | 0.342 | 0.025 | 0.087 | 0.020 | 0.131 |
| | | $F_{is}$ | +0.018 | *+0.119* | *+0.176* | *+0.395* | **+0.249** | −0.008 | NC |
| Morphological form | *A. moucheti nigeriensis* ($n = 39$) | $N_{all}$ | 3 | 4 | 3 | 1 | 3 | 2 | 2.3 |
| | | $H$ | 0.102 | 0.254 | 0.379 | – | 0.076 | 0.026 | 0.105 |
| | | $F_{is}$ | −0.031 | +0.195 | +0.259 | – | *+0.324* | – | NC |
| | *A. moucheti moucheti* ($n = 121$) | $N_{all}$ | 4 | 4 | 3 | 2 | 3 | 2 | 2.5 |
| | | $H$ | 0.237 | 0.375 | 0.317 | 0.250 | 0.080 | 0.017 | 0.136 |
| | | $F_{is}$ | 0.000 | +0.066 | **+0.225** | *+0.665* | *+0.380* | −0.004 | NC |
| | *A. moucheti bervoetsi* ($n = 9$) | $N_{all}$ | 2 | 3 | 2 | 2 | 2 | 1 | 1.8 |
| | | $H$ | 0.471 | 0.521 | 0.503 | 0.111 | 0.209 | – | 0.227 |
| | | $F_{is}$ | +0.059 | +0.158 | +0.351 | – | −0.067 | – | NC |

OTU: operational taxonomic unit; $N_{all}$: number of alleles; $H$: expected heterozygosity under Hardy–Weinberg equilibrium (Nei, 1978). $F_{is}$ was calculated according to Weir and Cockerham (1984) and goodness of fit to Hardy–Weinberg equilibrium was estimated by the exact test available in Genepop 3.2 (Raymond and Rousset, 1995).

[a] α-GPD: alpha glycerophosphate dehydrogenase (E.C 1.1.1.8); PGM: phosphoglucomutase (E.C 2.7.5.1); PGI: phosphogluco-isomérase (E.C 5.3.1.9); HK: hexokinase (E.C 2.7.1.1); IDH: isocitrate dehydrogenase (E.C 1.1.1 42); GOT: glutamate-oxaloacetate transaminase ( E.C 2.6.1.1).

[b] –: Irrelevant because only one allele was detected in more than one copy; NC: mean $F_{is}$ across loci was not computed; italic values: $P < 0.05$, single test level; bolded values: $P < 0.05$ after taking into account multiple tests using a Bonferroni procedure (Holm, 1979).

well as when all samples were pooled together ($F_{is} = F_{it} = +0.249$; $P < 0.05$). Hardy–Weinberg equilibrium was restored at this locus when grouping according to morphological type while locus IDH1 showed significant heterozygote deficit within the *A. moucheti moucheti* form ($F_{is} = +0.225$; $P < 0.05$). Although, deviation at locus IDH1 may result from increased statistical power to detect geographical heterogeneity within *A. moucheti moucheti* due to the combination of high sample size in this population ($n = 121$) and high polymorphism at this locus ($N_{all} = 4$; overall $H = 0.34$; see Table 1), this unique deviation is not strengthened by other loci in the dataset. Thus, it is unlikely to reflect population substructure. On the other hand, the pattern shown by locus PGI may reflect genetic heterogeneity between morphological forms. However, if this was the case, significant deviation from Hardy–Weinberg equilibrium should be observed at other loci as well, as expected when mixing different gene pools (Whalund, 1928). Consistent with these evidences of panmixia both within and across populations, no significant linkage disequilibrium was observed at any level of the analysis ($P > 0.15$).

To further investigate to which extent may genetic variation be partitioned within *A. moucheti s.l.* natural populations, analysis of molecular variance (AMOVA) was performed using the Arlequin 1.2 software (Schneider et al., 1997). Independently of the putative genetic structure tested, more than 95% of the total variance in genotypic frequencies was explained by intrapopulation variation while less than 1.2% was generated by a priori grouping specimens by morphological form or geographic location. Accordingly, levels of genetic differentiation estimated by $F_{st}$ (Weir and Cockerham, 1984) between geographical and/or morphological populations were low and in most cases, insignificant (not shown). Statistically significant differentiation ($F_{st} = 0.029$; $P = 0.006$, single test level) was observed only between Olama and Nyabessan populations, while no pairwise $F_{st}$ estimates between morphological forms reached statistical significance. Thus, *A. moucheti s.l.* populations from south Cameroon appeared highly homogenous across both morphological forms and geographic locations.

Additional observations of morphological variations within the offspring of single field-collected *A. moucheti s.l.* females, lends credence to the erroneous taxonomic value of current morphological classification within the *A. moucheti* group. Indeed, as shown in Table 2, the progeny of wild (inseminated) females, morphologically identified

Table 2
Morphological variations in the adult progeny of single field-collected *A. moucheti* females

| Field-collected inseminated females | Progeny | | | |
|---|---|---|---|---|
| | *A. moucheti moucheti* | *A. moucheti nigeriensis* | *A. moucheti bervoetsi* | All |
| *A. moucheti moucheti* | | | | |
| 1 | 5 | 8 | 4 | 17 |
| 2 | 7 | 1 | 3 | 11 |
| 3 | 3 | 11 | 5 | 19 |
| 4 | 9 | 6 | 4 | 19 |
| 5 | 11 | 4 | 6 | 21 |
| 6 | 25 | 12 | 6 | 43 |
| 7 | 11 | 1 | 1 | 13 |
| 8 | 16 | 4 | 1 | 21 |
| 9 | 4 | 2 | 5 | 11 |
| All | 91 | 49 | 35 | 175 |
| *A. moucheti nigeriensis* | | | | |
| 1 | 7 | 5 | 1 | 13 |
| 2 | 2 | 3 | 0 | 5 |
| 3 | 12 | 22 | 2 | 36 |
| 4 | 7 | 4 | 0 | 11 |
| 5 | 7 | 20 | 0 | 27 |
| All | 35 | 54 | 3 | 92 |
| *A. moucheti bervoetsi* | | | | |
| 1 | 20 | 6 | 3 | 29 |
| 2 | 10 | 1 | 5 | 16 |
| 3 | 11 | 1 | 2 | 14 |
| 4 | 3 | 6 | 6 | 15 |
| 5 | 1 | 0 | 5 | 6 |
| 6 | 10 | 4 | 3 | 17 |
| All | 55 | 18 | 24 | 97 |
| Overall | 181 | 121 | 62 | 364 |

Gravid field-collected *A. moucheti* females were placed individually in paper cups and allowed to lay eggs. Each egg batch was collected on humid filter paper and placed on breeding sites water for hatching. Mosquitoes development was monitored up to emergence of adults. Mothers and their offspring that reached the adult stage were identified morphologically and classified according to Gillies and De Meillon (1968) and Brunhes et al. (1998).

as *A. moucheti moucheti* ($n = 9$), *A. moucheti nigeriensis* ($n = 5$) or *A. moucheti bervoetsi* ($n = 6$) consistently appeared as a mixture of at least two morphological types. Most of the time (16 out of 20 progeny), all three morphological forms were represented. Moreover, only in 11 out of 20 cases was the morphological type of the female predominant in its own progeny, specimens identified as *A. moucheti moucheti* often being the most represented. This direct evidence definitively argues against any use of currently indexed morphological variations within *A. moucheti* natural populations as an indicator of genetic structuring. Similar observations were already reported by Ramsdale (in Gillies and De Meillon, 1968), based on the progeny of a single *A. moucheti nigeriensis* female.

Our results therefore, do not support splitting *A. moucheti* into three species and suggest high gene flow between locale populations at the geographical scale studied (20–200 km). Our findings of genetic similarity across distances as wide as 200 km may reflect limited power of isozymes to detect intraspecific genetic differentiation (Estoup et al., 1998). However, weak but statistically significant genetic differentiation was revealed between populations from the villages of Olama and Nyabessan (150 km apart), which may reflect geographic differentiation among *A. moucheti* natural populations. High population densities are often recorded on the edges of rivers where *A. moucheti* breeds (Mouchet and Gariou, 1966; Njan Nloga et al., 1993), but adult mosquitoes hardly penetrate surrounding forested environments (Languillon et al., 1956). Thus, migration of individuals across river systems may be highly restricted, while active or wind-assisted dispersal along rivers may still be permitted. Passive dispersal of larvae carried by water current may also contribute to unidirectional population admixture on a wide geographical scale (Wanson et al., 1947). We are currently developing microsatellite markers to explore in greater details this hypothesis. In addition, our results need to be validated by more extensive studies including populations sampled from throughout the species range and characterised at their ecological, behavioural and genetic levels. In particular, valuable information would be obtained from specimens collected in areas of allopatry, namely the type localities of *A. moucheti nigeriensis* (Lagos, Nigeria) and *A. moucheti bervoetsi* (Tsakalakuku, Democratic Republic of Congo).

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