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Impact of insecticide-treated bed nets implementation on the genetic structure of *Anopheles arabiensis* in an area of irrigated rice fields in the Sahelian region of Cameroon

C. WONDJI,*† F. SIMARD,*† T. LEHMANN,‡ E. FONDJO,§ A. SAMÈ-EKOBO¶ and D. FONTENILLE**
*Laboratoire de Recherche sur le Paludisme (LRP), Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC), PO Box 288, Yaoundé, Cameroon, †Institut de Recherche pour le Développement (IRD), UR016, PO Box 1857, Yaoundé, Cameroon, ‡Entomology Branch, Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Bufford Hwy, Chamblee, GA 30041, USA, §Programme National de Lutte contre le Paludisme, Ministère de la Santé Publique, PO Box 14306 Yaoundé, Cameroon, ¶Faculté de Médecine et des Sciences Biomédicales, Université de Yaoundé I, PO Box 8046, Yaoundé, Cameroon, **Laboratoire de Lutte contre les Insectes Nuisibles (LIN-UR016), Institut de Recherche pour le Développement (IRD), 911, avenue Agropolis, PO Box 64501, 34394 Montpellier cedex 5, France*

Abstract

Variation at 12 microsatellite loci was investigated to assess the impact of the implementation of insecticide-treated bed nets (ITNs) on the genetic structure of *Anopheles arabiensis* in Simatou, a village surrounded by irrigated rice fields in the Sahelian area of Cameroon. The *An. arabiensis* population of Simatou was sampled twice before ITN implementation, and twice after. Effective population size estimates (N_e) were similar across each time point, except for the period closely following ITN introduction where a nonsignificant reduction was recorded. Hence, we believe that ITN implementation resulted in a temporary bottleneck, rapidly followed by a demographic expansion. The genetic diversity of the population was not significantly affected since different genetic parameters (allele number, observed and expected heterozygosities) remained stable. Low estimates of genetic differentiation between the populations from Simatou and Lagdo, separated by 300 km, suggested extensive gene flow among populations of *An. arabiensis* in the Sahelian region of Cameroon. A decrease in the susceptibility to deltamethrin was observed following ITN introduction, but no kdr mutation was detected and a metabolic resistance mechanism is probably involved. The temporary effect of ITNs on the genetic structure of *An. arabiensis* population suggests that, to optimize the success of any control programme of this species based on ITNs, the control area should be very large and the programme should be implemented for a long period of time.

Keywords: *Anopheles arabiensis*, Cameroon, effective population size, ITNs, malaria, microsatellite

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Introduction

The Roll Back Malaria initiative intends to halve the burden caused by this disease by 2010 (Nabarro 1999). Amongst the control tools available for this initiative, insecticide-treated nets (ITN) are one of the most widely used. In many African countries such as Cameroon, there is an increase in the use of ITNs by inhabitants mainly among pregnant women and children (Samè Ekobo, unpublished).

Exposure to ITNs could have several impacts on mosquito populations. Until now, studies on the modifications induced in malaria vector populations by ITNs have investigated behaviour (Hossain & Curtis 1989), vectorial capacity (Darriet *et al*. 1984; Karch *et al*. 1993) and susceptibility to insecticide (Vulule *et al*. 1994). However, little is known about the impact of ITNs on the genetic parameters of vector populations, a knowledge that would be of strategic importance to control programmes.

The use of ITNs may influence vector population size by inducing a decrease in vector abundance, which, in turn,

Correspondence: Charles Wondji, Present address: Vector Research Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. Tel: +44 (0)151 705 3107; Fax: +44 (0)151 705 3369; E-mail: c.s.wondji@liverpool.ac.uk

could alter the genetic composition of the population. Furthermore, the use of ITNs can also reduce the susceptibility of malaria vectors to insecticide (Vulule *et al*. 1994), because ITNs could operate selective pressure on vector populations leading to the selection of insecticide resistance genes within the population.

The changes induced by ITN implementation on population size can be monitored by estimating the effective population size (N_e) of this population at different time periods. This indirect approach relies on the relationship between temporal variation in allele frequencies (genetic drift) and population size, such that large fluctuations are expected in small populations, while minor changes would occur in large populations (Waples 1991; Taylor *et al*. 1993). N_e is defined as a size of an ideal population (i.e. a hypothetical panmictic population with nonoverlapping generations, in which the sex ratio is 1 and each individual has the same reproductive potential) that experiences drift at the same rate as the natural population under consideration (Wright 1931, 1938). When population size varies over generations, N_e approximates the harmonic mean of the effective population sizes in all individual generations, and hence is dominated by the smallest value (Nei & Tajima 1981; Pollak 1983). This means that N_e is sensitive to bottlenecks (Hartl & Clark 1989).

To assess the impact of ITN implementation on the genetic structure of the malaria vector *Anopheles arabiensis*, we monitored allelic variation at 12 microsatellite loci in the population of Simatou, a village surrounded by irrigated rice fields in northern Cameroon. The *An. arabiensis* population of Simatou was sampled twice before ITN implementation, and twice after. Estimates of N_e were computed across each time point and genetic diversity parameters were compared between time points. Susceptibility of this population to deltamethrin, the insecticide used on bed nets, was also monitored before and after ITN implementation. A sample from the locality of Lagdo, 300 km from Simatou and also surrounded by rice fields, was concomitantly genotyped in order to estimate the level of gene flow between *An. arabiensis* populations in the Sahelian area of North Cameroon.

Materials and methods

Study sites

The village of Simatou (10°34′N, 14°30′E), with 800 inhabitants, is located in the Sahelian area of Cameroon, in the Maga subdivision, Far North Province (Fig. 1). The vegetation of this area is made of steppe and belongs to the Sudanese–Sahelian domain. The climate exhibits two contrasting seasons: one short rainy season from July to October and a long dry season from November to June. The presence of a large artificial lake serving as a water

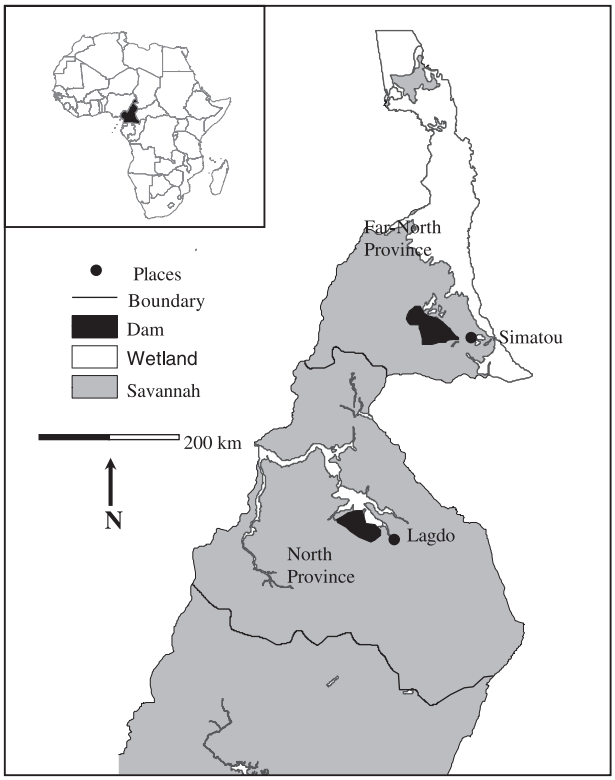

Fig. 1 Geographical location of the study sites in Cameroon, the villages of Simatou and Lagdo.

reservoir allows the inhabitants to practice an irrigated rice culture (two crops per year during rainy and dry seasons) on over 5000 ha. This activity generates many breeding sites for mosquito larvae. People live in mud huts with thatched roofs which offer resting refuges for endophilic mosquitoes. The main malaria vectors in this village are *Anopheles arabiensis* and *Anopheles funestus* (Fondjo 1996). Because of the nuisance-biting rate of mosquitoes, 65% of inhabitants were using nonimpregnated bed nets before our study (Cameroon Malaria Control Program, unpublished).

To evaluate the level of gene flow between geographical populations of *An. arabiensis* in the northern region of Cameroon, we collected mosquitoes in the village of Lagdo (9°05′N, 13°40′E), 300 km southwest of Simatou. This village is located in the tropical basin of river Benoue. Mean annual rainfall is 900–1000 mm with a 6-month dry season, and mean annual temperature is 26 °C. Like Simatou, Lagdo is surrounded by irrigated rice fields, but there is no control programme based on ITNs in this village.

Bed nets implementation and mosquito sampling

Bed nets were impregnated in Simatou in March 2001 during a community-based operation led by the National

Malaria Control Program. The insecticide used was deltamethrin (currently used by the Malaria Control Program across the country) at a final concentration of 25 mg/m², with an emulsifying concentrate at 25 g/L. It was the first time that this insecticide was used at such a large scale in this area. This pyrethroid presents a high efficacy on malaria vectors across Cameroon. Initial coverage was 70% but in November 2001, another operation achieved 100% of inhabitants using ITNs, and the implementation of ITNs was extended to neighbouring villages.

In Simatou, mosquito collections were conducted twice before ITN implementation, in November 1999 and in March 2001, and twice after, in August 2001 and in August 2002. In Lagdo, mosquitoes were collected in December 2001.

Female anophelines were collected inside human dwellings, either by aspiration or after pyrethrum spraying. Anophelines were identified using the morphological identification keys of Gillies & de Meillon (1968) and Gillies & Coetzee (1987). Field specimens were stored individually in tubes with desiccant (silica gel) and kept at –20 °C until processed in the OCEAC laboratory in Yaoundé.

Insecticide susceptibility assays

Standardized WHO test kits were used in the field to assess susceptibility to DDT deltamethrin and permethrin in *An. arabiensis* before (November 1999) and to deltamethrin after (August 2002) implementation of ITNs. The test was run according to WHO guidelines (WHO 1980), using 2- to 3-day-old unfed females obtained from larval collections in several breeding sites in Simatou.

Microsatellite genotype scoring

DNA from the legs or wings of individual specimens was extracted using the technique described by Collins *et al.* (1987). After species identification using species-specific polymerase chain reaction (PCR) following the technique of Scott *et al.* (1993), only *An. arabiensis* specimens were included in subsequent analysis.

Twelve microsatellite loci distributed across all three chromosomes were selected according to Zheng *et al.* (1996). Two (53 and 7) were on the X chromosome, five on chromosome 2 (417, 46, 803, 26 and 141) and five on chromosome 3 (93, 555, 170, 750 and 45C). Microsatellite alleles were PCR amplified as previously described (Simard *et al.* 1999; Wondji *et al.* 2002). PCR products were loaded on 15% (w/v) nondenaturing polyacrylamide gels and the rapid silver staining method from Sanguinetti *et al.* (1994) was used to visualize the allelic bands.

We attempted to genotype more than 100 chromosomes per locus in each sample from Simatou while only 90 chromosomes/locus were genotyped from Lagdo, due to low *An. arabiensis* density in December in this locality (*An. funestus* is the most abundant species at that period).

Data analysis

Genetic variability parameters (mean number of alleles per locus, allelic and genotypic frequencies, observed and expected heterozygosities under Hardy–Weinberg equilibrium) were assessed for each locus in each population using GENEPOP version 3.3 (Raymond & Rousset 1995). Tests for deviation from Hardy–Weinberg expectations and for linkage disequilibrium between loci were computed using exact tests available in GENEPOP 3.3. The significance of each test was adjusted to take into account the number of tests using the sequential Bonferroni procedure (Holm 1979).

Waples (1989a) pointed out that F_{ST} estimates are biased when temporally spaced samples of the same population are compared. For this reason an unbiased estimate of the P value of a log-likelihood (G) based exact test was performed (Goudet *et al.* 1996) to estimate the genotypic differentiation between the four samples of Simatou. Genetic differentiation between samples from Simatou and Lagdo was examined using the *F*-statistics (Wright 1978) calculated according to Weir & Cockerham (1984).

Estimation of the effective population size

We estimated N_e of the *An. arabiensis* population from Simatou based on temporal variation in allelic frequencies. The assumptions underlying this procedure are generations are assumed to be discrete; selection, mutation and migration are negligible; and sampling of a homogeneous gene pool is representative of the population (Waples 1989b, 1991). Because specimens were sampled without replacement from a very large population and many generations passed between samples, we followed the methods pertaining to sampling before reproduction [sampling plan II (Nei & Tajima 1981; Waples 1989b)]. Thus estimates of N_e were obtained using equation 11 in Waples (1989b):

$$N_e = \frac{t}{2\left(F - \frac{1}{2S_0} - \frac{1}{2S_t}\right)}$$

where S_0 and S_t represent respectively sample sizes (number of individuals) at generation 0 and t while F estimates the change in allele frequency from one time period to another. Several ways of computing F have been proposed as described by Waples (1989b). These methods generally lead to very similar results (Waples 1989b; Taylor *et al.* 1993; Lehmann *et al.* 1998; Simard *et al.* 2000). We also verified this accordance and therefore, only results based on F_k (Pollak 1983) will be presented. The following

equation has been considered during this study to estimate the change in allele frequency from one time period to another at one locus:

$$F_k = \frac{1}{K-1}\sum_{i=1}^{K}\frac{(x_i - y_i)^2}{(x_i + y_i)/2}$$ Pollak (1983).

Where K is the number of alleles, x_i and y_i represent the frequency of allele i at generation 0 and t, respectively.

Extreme allele frequencies could introduce a bias in the estimation of F leading to an overestimation of N_e (Waples 1989b). Therefore, alleles with frequencies lower than 2% at both time points of the considered interval were pooled into one class (Lehmann *et al*. 1998). For estimation of F over all loci, we computed weighted means of single-locus values as:

$$F = \frac{\sum(K_j - 1)F_j}{\sum(K_j - 1)}$$

where j stands for the different loci. This weighted mean was then used to estimate N_e based on the combination of information from all loci. Calculation of the 95% confidence intervals (CI) followed equation 16 of Waples (1989b).

We assumed, like in previous studies (Taylor *et al*. 1993; Simard *et al*. 2000), that 12 generations occurred during a year for *An. arabiensis* in Simatou. N_e values for X-linked loci were adjusted assuming that these N_e are equivalent to three-quarters of the values of autosomes.

Results

Genotypes of 54 females were scored at 12 microsatellite loci in each sample from Simatou while 45 were scored from Lagdo. All loci were highly polymorphic with the number of distinct alleles per locus ranging from 4 (locus 417) to 14 (loci 26 and 170) (Table 1). When combining information from all loci, mean number of alleles per population ranged from 7.5 to 8.

Hardy–Weinberg test

Significant departures from Hardy–Weinberg equilibrium (HWE) within the four samples from Simatou and that from Lagdo were found in 19 out of a total of 60 tests after the application of the Bonferonni test. Among these 19 significant values of F_{IS} ($P < 0.05$), 16 were found in four loci (93, 170, 141 and 46). This clustering of significant F_{IS} in only a few loci suggests that these departures from HWE are a locus-specific effect rather than a genome-wide effect. Because ITNs could generate departures from HWE in loci linked to genes under selection, we investigated whether there was a difference in the number of loci showing departure from HWE between samples collected before ITNs and those collected after. We did not observe any difference between the two groups of samples; those loci with departure from HWE were identified in samples collected both before and after ITNs (46, 141, 170) (ANOVA $F = 1.207$, d.f. = 3, $P = 0.318$). All significant departures from HWE were associated with positive F_{IS} indicating a heterozygote deficit. Heterozygote deficits across the genome suggest that samples represent several subpopulations pooled together (Wahlund effect), or inbreeding, or null alleles (Callen *et al*. 1993). Wahlund effect and inbreeding that affects the entire genome can be ruled out as causes of the observed deviations, because deficits were clustered in only 4 of 12 loci (93, 141, 46, 170). Furthermore, several specimens repeatedly failed to generate a PCR product for some loci (141, 46, 7) suggesting that they represented homozygotes for null alleles. Similar deviations were reported in previous microsatellite studies on *Anopheles arabiensis* and *Anopheles gambiae* (Kamau *et al*. 1998; Lanzaro *et al*. 1998; Lehmann *et al*. 1999; Simard *et al*. 1999; Wondji *et al*. 2002).

Linkage disequilibrium analysis within each sample from Simatou provided further support to exclude the Wahlund effect and/or inbreeding as a cause of heterozygote deficits. Wahlund effect and/or inbreeding should cause linkage disequilibrium within a population because members of the different subpopulations would have different probabilities of carrying certain combinations of alleles. However, if null alleles caused the heterozygote deficits, linkage equilibrium is expected since all individuals are equally likely to carry a null allele, and the distribution between alleles from different loci is not disturbed. Exact test for linkage disequilibrium within each sample after Bonferroni correction resulted after in 12 significant values out of 264 comparisons (13.2 significant values are expected at the 5% level) with values between 0.00 and 0.034. Only one pair of loci was in disequilibrium in the sample from Lagdo. No pair of loci appeared linked in more than one sample.

Altogether, our results suggest that heterozygote deficits observed are most likely due to null alleles, rather than pooling of subdivided populations, inbreeding or selection following ITN implementation.

Changes in population diversity

Genetic variability indicators were first compared between both samples collected in Simatou before ITN implementation, to assess temporal stability of the *An. arabiensis* population in the absence of control measures (Fig. 2). By using the ANOVA test, no significant variation was observed ($P > 0.05$) between the expected heterozygosities (H_E) of the two samples collected before ITN (0.71 and 0.67 for the first and second samples, respectively). The observed heterozygosity (H_O) is also

Table 1 Genetic variability and significance level with respect to Hardy–Weinberg equilibrium within *Anopheles arabiensis* populations from Simatou and Lagdo

Locus		Populations						
		Before ITNs		After ITNs				
		Simatou November 99 (2n = 108)	Simatou March 01 (2n = 108)	Simatou August 01 (2n = 108)	Simatou August 02 (2n = 108)	Simatou pooled (2n = 424)	Lagdo December 01 (2n = 90)	All (2n = 514)
Chromosome X								
53	N_{all}	6	8	7	6	8	9	10
	H_O	0.58	0.58	0.59	0.33	0.52	0.59	0.53
	H_E	0.62	0.59	0.61	0.51	0.58	0.62	0.59
	F_{IS}	+0.063	+0.022	+0.042	**+0.345**	**+0.118**	+0.053	**+0.105**
7	N_{all}	7	7	7	9	9	8	9
	H_O	0.59	0.55	0.47	0.52	0.53	0.81	0.58
	H_E	0.65	0.64	0.61	0.67	0.64	0.73	0.66
	F_{IS}	+0.081	+0.129	+0.237	**+0.217**	+0.166	−0.109	+0.111
Chromosome 2								
417	N_{all}	3	3	2	4	4	2	4
	H_O	0.52	0.56	0.38	0.45	0.48	0.60	0.50
	H_E	0.43	0.45	0.47	0.48	0.46	0.51	0.47
	F_{IS}	−0.212	−0.258	+0.189	+0.060	−0.055	−0.189	−0.048
46	N_{all}	10	10	8	9	10	6	11
	H_O	0.55	0.70	0.31	0.42	0.49	0.35	0.46
	H_E	0.87	0.86	0.76	0.82	0.83	0.81	0.82
	F_{IS}	**+0.367**	+0.185	**+0.615**	**+0.499**	**+0.416**	**+0.574**	**+0.448**
803	N_{all}	6	7	7	7	8	7	8
	H_O	0.63	0.65	0.62	0.64	0.63	0.58	0.62
	H_E	0.74	0.74	0.77	0.76	0.75	0.74	0.75
	F_{IS}	+0.150	+0.122	+0.196	+0.170	**+0.159**	**+0.213**	**+0.170**
26	N_{all}	14	14	13	13	14	12	14
	H_O	0.76	0.76	0.73	0.72	0.74	0.83	0.76
	H_E	0.87	0.87	0.83	0.82	0.85	0.89	0.86
	F_{IS}	+0.125	**+0.134**	+0.124	+0.132	**+0.129**	+0.068	**+0.117**
141	N_{all}	7	7	8	10	10	8	10
	H_O	0.57	0.56	0.47	0.62	0.55	0.47	0.53
	H_E	0.75	0.65	0.80	0.79	0.75	0.67	0.73
	F_{IS}	**+0.247**	+0.137	**+0.415**	**+0.229**	**+0.257**	**+0.299**	**+0.265**
Chromosome 3								
93	N_{all}	9	12	7	8	13	9	13
	H_O	0.61	0.57	0.41	0.36	0.49	0.54	0.50
	H_E	0.84	0.70	0.67	0.64	0.71	0.65	0.70
	F_{IS}	**+0.283**	+0.187	**+0.393**	**+0.437**	**+0.325**	+0.161	**+0.292**
555	N_{all}	9	7	7	7	9	7	9
	H_O	0.87	0.81	0.60	0.67	0.74	0.64	0.72
	H_E	0.77	0.69	0.54	0.58	0.64	0.58	0.63
	F_{IS}	−0.138	−0.163	−0.118	−0.160	−0.145	−0.090	−0.134
170	N_{all}	7	6	13	10	14	10	14
	H_O	0.39	0.36	0.53	0.51	0.45	0.44	0.45
	H_E	0.67	0.57	0.73	0.63	0.65	0.61	0.64
	F_{IS}	**+0.425**	+0.387	**+0.278**	**+0.195**	**+0.321**	**+0.284**	**+0.314**
750	N_{all}	5	6	7	6	7	6	7
	H_O	0.56	0.73	0.52	0.61	0.60	0.59	0.60
	H_E	0.65	0.72	0.67	0.67	0.68	0.63	0.67
	F_{IS}	+0.122	−0.018	+0.222	+0.097	**+0.106**	+0.053	+0.095
45C	N_{all}	7	5	7	7	7	7	7
	H_O	0.48	0.50	0.69	0.71	0.60	0.51	0.58
	H_E	0.68	0.56	0.68	0.75	0.67	0.62	0.66
	F_{IS}	+0.295	+0.108	−0.011	+0.064	**+0.114**	+0.177	**+0.127**
Mean over loci								
	N_{all}	7.5	7.8	7.75	8	9.4	7.6	9.7
	H_O	0.59	0.61	0.53	0.54	0.57	0.58	0.57
	H_E	0.71	0.67	0.67	0.67	0.68	0.67	0.68
	F_{IS}	+0.151	+0.081	**+0.215**	+0.190	**+0.159**	+0.124	**+0.155**

All refers to populations pooled; N_{all}, number of alleles per locus; 2n, number of chromosomes scored; H_O, observed heterozygosity; H_E, expected heterozygosity (Nei 1978); F_{IS} is calculated according to Weir & Cockerham (1984). When populations are pooled, $F_{IS} = F_{IT}$. Significance levels were corrected according to Bonferroni procedure (Holm 1979) to take into account multiple tests (12 tests per sample). For F_{IS}, bold values indicate a level of significance at $P < 0.05$ whereas bold and underlined values are for $P < 0.01$.

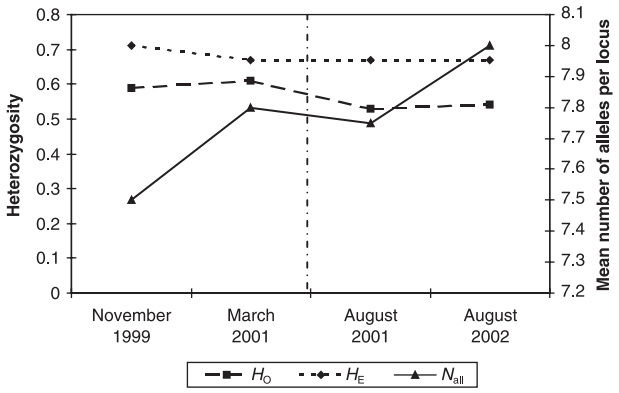

Fig. 2 Temporal evolution of genetic parameters of *Anopheles arabiensis* population of Simatou before and after ITNs. H_O, mean observed heterozygosity; H_E, mean expected heterozygosity; N_{all}, mean number of alleles per locus; the vertical dotted line indicates the time of the ITN implementation.

similar for both samples. The same result was obtained for the average number of alleles per locus (N_{all}) with no significant difference ($P > 0.05$) between the two samples (7.5 and 7.8 for the first and second samples, respectively). These results therefore demonstrated stability of the genetic structure of *An. arabiensis* in Simatou before implementation of the ITNs.

To assess the impact of ITNs on the genetic diversity of the population of *An. arabiensis*, we compared the average expected heterozygosity (H_E), observed heterozygosity (H_O) and allele number (N_{all}) before and after ITNs. Samples collected after ITNs showed smaller H_O (0.53 and 0.54) than samples collected before ITNs (0.59 and 0.61). However, the ANOVA test showed that this difference is not statistically significant ($F = 1.21$, d.f. = 3, $P > 0.05$). H_E estimates remained stable (0.67) for samples collected after ITNs implementation. No significant difference ($F = 1.206$, d.f. = 3, $P > 0.05$) was observed between the mean number of allele per locus in samples collected before (7.5 and 7.8) and after (7.75 and 8) ITN implementation. However, we noted the appearance of novel rare alleles in the last sample collected in August 2002, mainly in loci 170, 141, 417 and 7.

Temporal genotypic differentiation between samples from Simatou

In total, 27 significant *P* values were observed over 72 cases (Table 2) with at least four significant *P* values for each pairwise comparison after the Bonferroni test. The highest number of significant *P* values (6) was recorded between the first sample collected before ITN and the last sample collected after, probably an influence of time elapsed between the two collections. Eleven of the 27 significant *P* values appear to be associated to a specific sample as that on locus 7, 803, 750 and 45C linked respectively to the last, the first and the second of the four samples. One locus, 417, showed significant *P* values only when samples collected before ITN were compared to the sample collected just after (August 2001).

Genetic differentiation between populations of Simatou and Lagdo

A similar level of genetic differentiation is observed when single samples of Simatou are compared to the population of Lagdo. F_{ST} estimates are 0.0135, 0.0058, 0.0116 and 0.0130 (with $P < 0.01$) respectively obtained when the first, the second, the third and the fourth samples of

Table 2 Temporal genotypic differentiation (*P* values) between samples of Simatou

Locus	Simatou November 1999/ March 2001	Simatou November 1999/ August 2001	Simatou November 1999/ August 2002	Simatou March 2001/ August 2001	Simatou March 2001/ August 2002	Simatou August 2001/ August 2002
53	0.2016	0.1909	0.1470	0.7103	0.1375	0.1326
7	0.9996	0.9809	**0.0000**	0.9918	**0.0000**	**0.0000**
417	0.8552	**0.0016**	0.7156	**0.0039**	0.9637	0.7156
46	0.9197	0.3929	0.3015	0.2314	0.2018	0.9915
803	**0.0000**	**0.0000**	**0.0000**	0.3975	0.2014	0.0074
26	0.9990	0.2753	**0.0000**	**0.0000**	**0.0000**	**0.0000**
141	0.3691	0.4697	**0.0001**	**0.0059**	**0.0000**	**0.0000**
93	0.0343	0.0336	0.0245	0.3781	0.2831	0.9972
555	**0.0000**	**0.0000**	**0.0000**	0.0151	**0.0004**	**0.0028**
170	0.9937	0.3724	0.5773	0.5634	0.7566	0.0073
750	**0.0000**	**0.0000**	**0.0000**	**0.0019**	0.0279	0.1472
45C	**0.0000**	0.3484	0.0377	**0.0039**	0.0472	0.5826

Bold values, significant after Bonferroni test at $P < 0.05$; bold underlined values, significant after Bonferroni at $P < 0.01$.

Table 3 Spatial differentiation (F_{ST}) between Simatou and Lagdo

Locus	Simatou November 1999/ Lagdo	Simatou March 2001/ Lagdo	Simatou August 2001/ Lagdo	Simatou August 2002/ Lagdo	Simatou pre-ITN/ Lagdo	Simatou post-ITN/ Lagdo	All Simatou/ Lagdo
53	–0.0035	–0.0048	0.0089	0.0045	–0.0030	0.0044	0.0009
7	–0.0031	–0.0005	0.0020	0.1652	0.0011	0.0110	–0.0063
Chromosome X	–0.0033	–0.0026	0.0054	0.0848	–0.0009	0.0077	–0.0027
417	0.0252	0.0124	0.0601	–0.0060	0.0191	–0.0225	–0.0024
46	0.0077	0.0006	0.0053	–0.0039	0.0067	0.0083	0.0026
803	0.0005	–0.0049	–0.0032	–0.0104	0.0287	–0.0067	0.0061
26	0.0137	0.0088	0.0220	0.0336	0.0136	0.0144	0.0071
141	–0.0034	–0.0070	0.0368	**0.0968**	–0.0064	0.0223	0.0041
Chromosome 2	0.0094	0.0043	0.0248	0.0265	0.0123	0.0031	0.0035
93	**0.0539**	–0.0084	–0.0090	–0.0085	0.0141	–0.0020	0.0060
555	0.0310	0.0253	0.0000	0.0058	**0.0752**	0.0011	0.0172
170	0.0000	0.0000	0.0101	0.0000	0.0933	**0.0935**	**0.0978**
750	0.0000	0.0131	0.0117	–0.0035	0.0536	0.0036	0.0086
45C	**0.0385**	–0.0094	0.0158	0.0126	–0.0041	0.0080	0.0010
Chromosome 3	0.0246	0.0281	0.0075	0.0037	0.0464	0.0224	0.0261
All loci	**0.0135**	**0.0058**	**0.0116**	**0.0130**	**0.0238**	**0.0129**	**0.0112**

Bold values $P < 0.05$; bold underlined values, $P < 0.01$.

Simatou are compared to the population of Lagdo (Table 3). The F_{ST} estimates are 0.0238 and 0.0129 (with $P < 0.01$), respectively, when mixed samples collected before ITN and mixed samples collected after are compared to Lagdo. When the four samples of Simatou are grouped as a single sample and compared to Lagdo, the F_{ST} estimate is 0.0112 similar to the estimates obtained between individual samples of Simatou and Lagdo.

Effective population size in Simatou

The effective population size of *An. arabiensis* in Simatou was estimated for each time period by using the temporal method. Results are shown in Table 4.

As commonly observed in large populations, the upper 95% confidence intervals (CI) of the N_e estimates was infinity for almost all single-locus estimates (35 of 48 cases), but combining the information across the 12 loci yielded N_e estimates with defined 95% CI.

The N_e estimates we obtained for the time period following ITN (5 months after ITN implementation) is substantially lower than the N_e estimates for the time period before ITN implementation. However, there is a slight overlapping of the respective 95% CIs. These N_e estimates are 649 (301–2437) and 135 (70–334) respectively before and just after ITNs. The N_e estimates for all other time intervals are not statistically different, as evidenced through substantial overlapping of their respective 95% CIs.

Over the entire timeframe of the study, the N_e was estimated at 484 (290–845), a value lower than what was observed in other studies. For example, Simard *et al*. (2000) found $N_e = 1046$ (490–2768) in a Sahelian area of Senegal, with similar geography and climate to our study area in Simatou.

The pattern of N_e estimates presented in this study is similar to what was obtained while using other methods also based on the temporal changes in allele frequencies. Using the N_e estimator program, we effectively found similar patterns of N_e estimates with the Bayesian-based approach called TM3 (Berthier *et al*. 2002) and with the maximum-likelihood-based approach called MCLEEPS (Anderson *et al*. 2000).

Like *Anopheles gambiae*, several polymorphic inversions are found on chromosome 2 in *An. arabiensis*. Chromosomal arrangements in *An. gambiae* have been found to be under strong selective pressure due to environmental conditions (Coluzzi *et al*. 1979). Two loci of the 12 studied here are located within inversions. Locus 26 is located within the polymorphic inversion 2Rb, whereas locus 141 may be linked to inversion 2Rd (Zheng *et al*. 1997) although its exact cytological location is unknown. Previous cytological studies on *An. arabiensis* in a similar geographical area did not detect any departure from panmixia (Petrarca *et al*. 1987). The reduction in N_e after the implementation of ITNs is more important and reaches statistical significance when loci within polymorphic inversions are considered [58 (27–107) after ITNs vs. 862 (331–2130) before] than when loci outside inversions are considered [114 (59–256) after ITNs vs. 349 (187–720) before]. A similar pattern is observed when comparing N_e estimates for loci on chromosome 2 [71 (40–124) before vs. 540 (273–1108) after] to estimates obtained from loci on chromosome X and 3 [102 (55–196) before vs. 280 (150–529) after].

Table 4 Effective population size estimates based on F_k (Pollak 1983) computations

Locus	Before ITNs November 1999–March 2001	Just before and just after ITNs March 2001–August 2001	After ITNs August 2001–August 2002	Total time period November 1999–August 2002
X				
53	1388 (85–∞)	∞ (83–∞)	467 (51–∞)	1229 (105–∞)
7	∞ (∞–∞)	∞ (360–∞)	2227 (100–∞)	3479 (269–∞)
Chromosome 2				
417	∞ (270–∞)	14 (0–193)	36 (1–698)	∞ (45–∞)
46	∞ (525–∞)	111 (15–∞)	∞ (605–∞)	912 (125–∞)
803	107 (21–417)	558 (27–∞)	300 (40–∞)	2462 (126–∞)
26Φ	∞ (∞–∞)	193 (38–∞)	∞ (478–∞)	2220 (239–∞)
141Φ	992 (62–∞)	50 (9–231)	163 (35–876)	297 (68–1218)
Chromosome 3				
93	318 (73–∞)	3578 (33–∞)	67 (11–∞)	126 (35–∞)
555	188 (38–944)	80 (11–1258)	169 (22–∞)	268 (55–1219)
170	∞ (127–∞)	∞ (52–∞)	469 (80–∞)	∞ (182–∞)
750	886 (51–∞)	46 (7–226)	403 (41–∞)	∞ (293–∞)
45C	90 (15–361)	50 (7–441)	1914 (47–∞)	340 (48–4634)
All loci	649 (301–2437)	135 (70–334)	355 (175–1050)	484 (290–845)
Outside inversion	349 (187–720)	114 (59–256)	239 (126–524)	400 (245–659)
Within inversionΦ	862 (331–2130)	58 (27–106)	213 (98–394)	350 (174–607)
Chromosome 2	540 (273–1108)	71 (40–124)	312 (157–664)	605 (338–1053)
Chromosome X +3	280 (150–529)	102 (55–196)	168 (95–296)	292 (172–475)

Twelve generations per year were assumed. 95% confidence intervals are shown in parenthesis.
ΦLoci within polymorphic paracentric inversions.

Susceptibility of Anopheles arabiensis *population to insecticide before and after ITNs*

Before ITN implementation in Simatou, the susceptibility of *An. arabiensis* populations to DDT and pyrethroids (permethrin and deltamethrin) was evaluated in November 1999. This population was completely susceptible to these different insecticides. In August 2002, 17 months after implementation of ITNs, susceptibility of the population was evaluated only for deltamethrin since this insecticide was used to treat the nets. Of 80 specimens tested, seven survived after the 24-h observation period, indicating a decrease in susceptibility to deltamethrin. No resistant kdr (mutation in the sodium channel gene) allele was detected among survivors.

Discussion

We investigated the genetic variation at 12 microsatellite loci in the *Anopheles arabiensis* population from Simatou, in an attempt to assess the impact of ITN implementation on the genetic structure of this population.

No significant variation was recorded among genetic parameters such as mean number of alleles per locus, observed and expected heterozygosities before and after ITN implementation, even if a slight decrease was noted for observed heterozygosity. One of the explanations of the absence of diversity loss after ITNs is in the size of the remnant population and the time elapsed since so far (Queney *et al*. 2000). Effectively, even if ITNs induced a high mortality rate in the *An. arabiensis* population, the size of the remnant population was still large enough to retain almost all the genetic diversity.

The pattern of the genotypic differentiation between the four samples of the population of Simatou was apparently more influenced by locus-specific effect or the number of generations elapsed between samples than by ITNs. Indeed, a similar number of significant *P* value was observed between all the pairwise comparisons independently of the ITNs.

The pattern of genetic differentiation observed between populations of Simatou and Lagdo is not influenced by the ITNs because similar F_{ST} estimates were obtained when samples collected before or after ITNs were compared to Lagdo. F_{ST} estimates obtained between samples from Simatou and the population of Lagdo were always low suggesting a low level of genetic differentiation between these two populations despite being separated by a geographical distance of 300 km. This result suggests extensive gene flow among populations of *An. arabiensis* in the Sahelian region of Cameroon. It also supports the fact already noted by other authors that *An. arabiensis* is genetically less structured compared to a species such as *Anopheles gambiae* (Besansky *et al*. 1997; Petrarca *et al*. 2000; Simard *et al*. 2000), even if some evidence of extensive

differentiation among its population has been shown in eastern Africa (Donnelly & Townson 2000).

A transient reduction in the effective population size of the *An. arabiensis* population of Simatou was observed just after the ITN implementation although the 95% CIs of the N_e estimates slightly overlapped. In previous studies of temporal variation of *An. arabiensis* population structure, Simard *et al*. (2000) did not find any significant difference between the different N_e values obtained for each time point over a period of 4 years. Even in an area with a severe dry season, such as the Sahelian region of Senegal, significant reduction of N_e has not been noted, nor was any significant difference of the allelic and genotypic distribution between samples from different time period over 4 years seen. A reduction in the *An. arabiensis* population in Simatou is unlikely to be due to environmental factors since many breeding sites are available throughout the year (rice fields and several irrigation channels), while in Senegal the dry season did not allow the presence for breeding sites to *An. arabiensis*. If despite the favourable conditions for a continuous abundance of *An. arabiensis* throughout the year in Simatou, a decrease in its effective population size was observed just after the implementation of ITNs, we conclude that this reduction is probably a consequence of ITN implementation.

Analysis of our result suggests that inversions or chromosomal location of loci may have influenced our N_e estimates since a more significant reduction in N_e just after ITN implementation has been observed for loci on chromosome 2 or within inversions than for loci on chromosomes X and 3 or outside inversions. Similar differences in N_e estimates due to chromosomal location of loci have been detected by Simard *et al*. (2000) in an *An. arabiensis* population in Senegal. Selection on some genes within the inversion may influence variation in microsatellite loci within and adjacent to polymorphic inversions through a reduced recombination, a process called 'hitch-hiking' (Slatkin 1995; Lanzaro *et al*. 1998). Linkage disequilibrium between those loci is expected if such an effect encompasses the whole chromosome arm, as reported in *An. gambiae* by Lanzaro *et al*. (1998). This linkage disequilibrium pattern was not observed between loci on chromosome 2 even when we pooled the four samples from Simatou, indicating that the presence of a potential selection on inversion regions does not encompass this whole chromosome arm. Rather than confounding our data, the difference in response of these loci supports the central proposition of a decrease in N_e just after ITNs. However, even if loci on chromosome 2 are excluded, our conclusions do not change significantly.

A probable expansion of the *An. arabiensis* population of Simatou after the reduction in effective population size gives an indication of its genetic structure. This pattern contradicts theoretical expectations, in which N_e remains stable or declines with increasing time between sampling, because it approximates the smallest N_e in a series of generations and accordingly, it hardly changes after a large N_e, but declines quickly if a small N_e was included between the time points. To justify this phenomenon, we used the concept called 'permeable demes' from T. Lehmann (unpublished). This concept takes into consideration the fact that a deme is surrounded by many other demes. Because this deme is permeable to migrants, it can receive many alleles from the neighbouring demes in such a way that changes in allelic frequency can be stabilized in a few generations. The fraction of migrants that the deme can receive is estimated to be 2–5% per generation. After one to three generations, migrant alleles are low in frequency while after more generations (e.g. > 15), these migrant alleles constitute most of the population. Since the 'ocean' of neighbouring demes is large and changes in allele frequency in these demes cancel out each other, the ocean acts as a buffer that constrains the variance in allele frequency over time. As Cornuet & Luikart (1996) proposed, the appearance of novel rare alleles in the second samples after ITNs may be a result of migration from neighbouring demes and not a consequence of mutation, since the number of generations are limited between the different collections.

The temporary reduction in effective population size after ITNs followed by a new expansion of the population and the stability of the genetic parameters of this population suggest that implementation of ITNs in Simatou did not maintain a constant selection pressure in the *An. arabiensis* population. Migration has probably played a major role in this situation. In fact, Simatou is located in a large area of rice field with abundant breeding sites for *An. arabiensis* such that a local action on a population is probably quickly attenuated by the high gene flow between the different populations. Therefore, an important and significant impact can only be obtained in control programmes against this principal malaria vector if the area concerned is large. In our case, this can be achieved by implementing ITNs in the entire administrative subdivision of Maga and not just at the level of the village of Simatou.

ITNs have probably induced a decrease of the susceptibility to deltamethrin within the *An. arabiensis* populations of Simatou even if this needs to be confirmed by further bioassays tested on field samples. Nevertheless, the probable resistance is at a low frequency perhaps because the time since the implementation of ITNs was short. The fact that the most likely mutation in the sodium channel gene was not found may be an indication that other mechanisms of resistance are acting within this population (Etang *et al*. 2003). According to Chandre *et al*. (1999), resistance developed by mosquito populations under selective pressure due to ITNs is driven mainly by metabolic resistance mechanism rather than by target site alterations such as kdr. The over-expression of detoxifying proteins such as esterases or cytochrome P450 monoxygenases is the most likely mechanism (Nikou *et al*. 2003).

Conclusion

ITNs implementation in Simatou resulted in either no or minor changes in genetic diversity of *Anophelesarabiensis* population, with broadly stable genetic parameters. Nevertheless, this implementation resulted in a measurable nonsignificant reduction in N_e. The effect measured was weak, suggesting that high coverage over a large area is required before the genetic parameters of this mosquito's population can be significantly modified. A longer time period will better assess the real impact of ITNs on the genetic structure of such a population provided selective pressure (i.e. insecticide efficacy/impregnation quality control) is maintained. The implementation of an *An. arabiensis* control programme should take into consideration the genetic homogeneity amongst geographical populations of this vector as indicated by the low genetic differentiation between populations from Simatou and Lagdo. In fact, this relative homogeneity could be a disadvantage because the high level of gene flow between populations would facilitate the spread of any emerging resistance. On the other hand, the genetic homogeneity of the *An. arabiensis* population could provide an advantage to malaria control programmes based on the replacement of wild populations by genetically modified mosquitoes incapable of transmitting *Plasmodium* spp. to human, since the gene responsible for this trait could spread quickly within and between populations.

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C. Wondji is a medical entomologist investigating population genetics and molecular genetics in *Anopheles* species. F. Simard is a medical entomologist working on population genetics and biology of mosquitoes. T. Lehmann is an entomologist working on evolution of insect vectors. E. Fondjo is an entomologist working in malaria vector control programme. A. Same-Ekobo is a parasitologist and a medical doctor involved in malaria control program. D. Fontenille is a medical entomologist working on taxonomy, systematic, biology and evolution of mosquitoes.
