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Ace-1 duplication in Anopheles gambiae: a challenge for malaria control

Luc Djogbénou†1,2, Pierrick Labbé*,†3, Fabrice Chandre1, Nicole Pasteur4 and Mylène Weill4

Address: 1Institut de Recherche pour le Développement, UR 016, 01 BP 4414 RP Cotonou, Benin, 2Centre de Recherche Entomologique de Cotonou (CREC), 06 BP 2604 Cotonou, Benin, 3Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK and 4Université Montpellier 2 – CNRS, Institut des Sciences de l’Evolution, Equipe Génétique de l’Adaptation, C.C. 065, Place Eugène Bataillon, 34095 Montpellier, France

E-mail: Luc Djogbénou - Luc.Djogbenou@ird.fr; Pierrick Labbé - Pierrick.Labbe@ed.ac.uk; Fabrice Chandre - Fabrice.Chandre@ird.fr; Nicole Pasteur - nicole.pasteur@univ-montp2.fr; Mylène Weill - mylene.weill@univ-montp2.fr

*Corresponding author †Equal contributors

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Abstract

Background: Insecticide resistance is a rapid and recent evolutionary phenomenon with serious economic and public health implications. In the mosquito Anopheles gambiae s.s., main vector of malaria, resistance to organophosphates and carbamates is mainly due to a single amino-acid substitution in acetylcholinesterase 1 (AChE1). This mutation entails a large fitness cost. However, a resistant duplicated allele of the gene encoding AChE1 (ace-1), potentially associated to a lower fitness cost, recently appeared in An. gambiae.

Methods: Using molecular phenotype data collected from natural populations from West Africa, the frequency of this duplicated allele was investigated by statistical inference. This method is based on the departure from Hardy-Weinberg phenotypic frequency equilibrium caused by the presence of this new allele.

Results: The duplicated allele, Ag-ace-1D, reaches a frequency up to 0.65 in Ivory Coast and Burkina Faso, and is potentially present in Benin. A previous study showed that Ag-ace-1D, present in both M and S molecular forms in different West Africa countries, was generated by a single genetic event. This single origin and its present distribution suggest that this new allele is currently spreading.

Conclusion: The spread of this less costly resistance allele could represent a major threat to public health, as it may impede An. gambiae control strategies, and thus increases the risk of malaria outbreaks.

Background

Since early 1950s, humans have controlled the populations of many agricultural or medical arthropod pests, mostly with chemical insecticides. After years of success, evolutionary adaptations to these new conditions began...
economic and public health implications. In the arms race between arthropods and humans, the mosquito *Anopheles gambiae*, the main vector of malaria, seems to have just moved up a gear with the emergence of a resistant duplicated allele of the gene encoding acetylcholinesterase 1 (AChE1).

AChE1 is a critical enzyme in nerve transmission and the target of two of the most commonly used types of insecticides (organophosphates, OPs, and carbamates, CXs). Like several other mosquito species (including *Culex pipiens*, the well-studied vector of West Nile virus), *An. gambiae* displays resistance due to a single amino-acid substitution, from a glycine to a serine at the position 119, in the AChE1 catalytic site (G119S)[2]. In *C. pipiens*, there is direct and indirect evidence that the resistance allele (*ace-1R*) entails a large fitness cost, probably due to the mutated AChE1 having a much lower level of activity. Homozygous *ace-1R* mosquitoes survive in the presence of insecticide, but are rapidly outcompeted in the absence of insecticide (see review in [3]). Heterozygotes are subject to smaller costs than resistant homozygotes in the absence of insecticide. In treated areas, they survive better than susceptible homozygotes, but are less resistant than *ace-1R* homozygotes. Due to the patchy nature of mosquito control, the generalist heterozygote is advantaged across treated and non-treated areas, although the more specialist resistant and susceptible homozygotes are locally selected in treated and non-treated environments respectively. Moreover, heterozygotes cannot invade due to the segregation burden leading to the loss of the advantage in half of their progeny.

Several duplicated alleles (*ace-1D*) have recently appeared, which link a susceptible and a resistant copy of the *ace-1* gene on the same chromosome [4]. Duplication thus creates a "permanent heterozygote" allele. The first case of *ace-1* gene duplication was recently discovered in *An. gambiae* [5]. Molecular analysis showed this duplicated allele (*Ag-ace-1D*) to be present at several sites and to have probably spread among the two molecular forms S and M of *An. gambiae* s.s., by introgression.

Unfortunately, it is not possible to design a simple test for studying the frequency of *Ag-ace-1D* due to the lack of features specific to this duplication, as with available genotyping methods carriers of this duplicated allele cannot be distinguished from classical heterozygotes. Thus an indirect method previously developed for *C. pipiens* was used to estimate *Ag-ace-1D* frequency in the field [6]. The results of this analysis and the potential consequences for *An. gambiae* population management and on malaria control are discussed.

### Methods

**Data collection**

The study area is shown in Figure 1. Both published data [5,7] and data from new samples were used. The date and location of the sampling sites are shown in Table 1. For each locality, several ponds where sampled in an area a few hundred meter-squares to insure a representative sample of the local population.

**Molecular analysis**

All samples were collected at the larval stage and reared to adulthood in the laboratory. Genomic DNA was extracted from each field mosquito. The protocol used is a simplified version of Collins et al. [8]: a single mosquito is homogenized in a 1.5 ml Eppendorf tube containing 200 μl of CTAB buffer (100 mM Tris HCL, pH 8.0, 10 mM EDTA, 1.4 M NaCl, 2% CTAB) and incubated at 65°C for 5 min; then 200 μl of chloroform are added. After centrifugation (room temperature, 5 min, 12000 g), the supernatant is moved to a fresh tube, 200 μl of isopropyl alcohol are added, and the mix is centrifuged again (12000 g, 15 min). After discarding supernatant, the pellet is washed with 70% ethanol, dried and resuspended in DNAse Free water. The molecular form of each individual was determined by a PCR-based test,

### Figure 1

*Ag-ace-1D* frequency in Western Africa. The frequency of *Ag-ace-1D* is given for each *An. gambiae* molecular form: M (red) and S blue). Samples are described in Table 1. Samples in which *Ag-ace-1D* was detected by molecular analysis are bolded and underlined (Table 2). Significant presence of the duplicated allele (before Bonferroni correction, see Methods) is given with * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. 
The frequency of the recently discovered duplicated allele of the ace-1 gene in An. gambiae, Ag-ace1D, was investigated in natural populations from West Africa by considering the departure from Hardy-Weinberg proportions caused by its presence [6].
Figure 1 shows the predicted spatial distribution of Ag-ace-1D in the S and M forms of An. gambiae, as shown by previous molecular investigations and analyses of heterozygote excess. The probability of Ag-ace-1D being present was significant in nine samples (five after Bonferroni correction) from Ivory Coast (four samples) and Burkina Faso (five samples), in both M (two samples) and S (seven samples) molecular forms of An. gambiae (Figure 1 and Table 2). In these samples, the frequency of Ag-ace-1D was up to 0.65, with the lowest significant frequency being 0.24, consistent with the expected highly conservative output of the method used. Indeed, this method will detect low frequencies only in large samples; for example, Ag-ace-1D was not detected with this method in one of the analysed populations (Boromo, population #26, Table 2), whereas molecular methods proved this duplication to be present [5]. The frequency and the geographic distribution of this duplication are therefore probably underestimated. For example, the analysis of each Benin population independently did not provide any indication supporting the presence of the duplication in this country (Figure 1 and Table 2). Nevertheless, the pooled analysis yields a P-value of 0.052, which points toward the potential presence of Ag-ace-1D as this method underestimate the excess of heterozygotes and thus its frequency. However, more data are required to confirm the presence of the duplicated allele in Benin (Table 2). The complete lack of variation of the Ag-ace-1D sequence over several countries [5] indicates that this allele was generated by

Table 2: Ag-ace-1D frequency in West Africa

| #  | Locality   | M form | S form |
|----|------------|--------|--------|
|    |            | R      | S      | D      | P-value | R      | S      | D      | P-value |
| 1  | Abomey     | 3      | -      | I      | -      | 68     | 0      | 0.87   | 0.13   | 0.144NS |
| 2  | Bohicon    | 2      | -      | I      | -      | -      | 3      | 0.82   | 0.18   | 0.65NS  |
| 3  | Houebo     | 9      | -      | I      | -      | -      | 62     | 0.97   | 0.03   | 0.715NS |
| 4  | Niaouli    | 50     | -      | -      | 1      | -      | 12     | 0.96   | 0.04   | 0.835NS |
| 5  | Paougnan   | 0      | -      | -      | -      | -      | 41     | 0.9    | 0.1    | 0.352NS |
| 6  | Zogbodomey | 13     | -      | I      | -      | -      | 9      | 0.94   | 0.06   | 0.808NS |
| 7  | Cana       | 38     | -      | I      | -      | -      | 26     | 0.83   | 0.17   | 0.227NS |
| 8  | Bembrereke | 0      | -      | -      | -      | -      | 62     | 0.96   | 0.04   | 0.647NS |
| 9  | Parakou    | 0      | -      | -      | -      | -      | 20     | 0.97   | 0.03   | 0.873NS |
| 10 | Bassila    | 0      | -      | -      | -      | -      | 76     | 0.97   | 0.03   | 0.68NS  |
| 11 | Tangueta   | 0      | -      | -      | -      | -      | 47     | 0.96   | 0.04   | 0.673NS |
| 12 | Nattingou  | 0      | -      | -      | -      | -      | 48     | 0.99   | 0.01   | 0.918NS |
| 13 | Djougou    | 0      | -      | -      | -      | -      | 46     | 0.97   | 0.03   | 0.750NS |
| 14 | Dassa      | 0      | -      | -      | -      | -      | 64     | 0.96   | 0.04   | 0.652NS |
| 15 | Savalou    | 0      | -      | -      | -      | -      | 29     | 0.96   | 0.04   | 0.789NS |
| 16 | Total Bénin| 115    | -      | I      | -      | -      | 221    | 0      | 0.91   | 0.09   | 0.052NS |
| 17 | Darsalamy  | 7      | -      | I      | -      | -      | 2      | 0      | 0      | 1      | 0.096NS |
| 18 | Dioulassoba| 1      | -      | I      | -      | -      | 23     | 0.21   | 0.55   | 0.24   | 0.044*  |
| 19 | Kuinima    | 0      | -      | -      | -      | -      | 27     | 0      | 0.58   | 0.42   | 0.007** |
| 20 | Mombamba   | 8      | -      | I      | -      | -      | 7      | 0.85   | 0.15   | 0.563NS |
| 21 | Sabou      | 0      | -      | I      | -      | -      | 14     | 0      | 0.76   | 0.24   | 0.198NS |
| 22 | Samandeni  | 0      | -      | -      | -      | -      | 25     | 0      | 0.57   | 0.43   | 0.002** |
| 23 | Ségouéré   | 10     | -      | I      | -      | -      | 8      | 0      | 0.5    | 0.5    | 0.049*  |
| 24 | Soumoussé  | 32     | -      | I      | -      | -      | 12     | 0      | 0.71   | 0.29   | 0.153NS |
| 25 | Vallée du Kou| 86    | 0      | 0.96   | 0.04  | 0.641NS| 10     | 0.16   | 0.34   | 0.51   | 0.000***|
| 26 | Yeugresso  | 8      | 0      | 0.87   | 0.13  | 0.592NS| 10     | 0      | 0.71   | 0.29   | 0.560NS |
| 27 | Boromo     | 38     | 0.05   | 0.95   | 0      | 1.000NS| 3     | 0      | 0      | 1      | 1.000NS |
| 28 | Total Burkina Faso| 212    | 0.03   | 0.97   | 0      | 1.000NS| 202    | 0.12   | 0.53   | 0.35   | 0.000***|
| 29 | Tounouri   | 18     | 0      | 0.41   | 0.59  | 0.001***| 0      | -      | -      | -      | -      |
| 30 | Niamoue    | 24     | 0.35   | 0      | 0.65  | 0.000***| 0      | -      | -      | -      | -      |
| 31 | Toubakoro  | 19     | 0.23   | 0.61   | 0.16  | 0.195NS| 5      | 0      | -      | 0      | 1      |
| 32 | Yaokoikiko| 0      | -      | -      | -      | -      | 19     | 0      | 0.32   | 0.32   | 0.009** |
| 33 | Total Ivory Coast| 61     | 0.26   | 0.4    | 0.34  | 0.000***| 24     | 0.29   | 0.29   | 0.42   | 0.001***|
| 34 | Lomé (Togo)| 73     | 0      | 0.93   | 0.07  | 0.39NS | 13     | 0      | 0.88   | 0.12   | 0.531NS |

For each population sampled, the number of mosquitoes of each of the S and M molecular forms of An. gambiae is given, together with the estimated frequency of the various alleles: R, S and D for ace-1R, ace-1S and ace-1D, respectively. A global estimation is also presented for each country sampled. The populations in italics are those in which Ag-ace-1D has been identified by molecular analysis [5]. Finally, the p-value for the test for the presence of ace-1D is also given for each population (see Methods), with NS for P ≥ 0.05, * for P < 0.05, ** for P < 0.01 and *** for P < 0.001 (except when no estimate was possible, i.e. when all the mosquitoes of a sample were susceptible). The P-values that remained significant after Bonferroni correction (see methods) are presented in bold; P-values no longer significant after Bonferroni correction are shown in italics.
a single genetic event and its current distribution suggests that it is probably spreading.

Unfortunately, the spread of this new resistance allele poses a potential major threat to public health, as *An. gambiae* is the main vector of malaria. Indeed, several studies of a similar allele in *C. pipiens* have indicated that the duplication entails a lower fitness cost than the single-copy resistance gene, ace-1R [4,6] (but see [16]). This is probably also the case for *An. gambiae*, as the mutated AChE1 gene is also associated with a strong decrease in enzyme activity [17]. The presence and spread of the Ag-ace-1D allele may greatly impede *An. gambiae* control strategies designed to maintain resistance alleles at low frequencies through the use of different insecticides with no cross-resistance in a mosaic or rotation strategy. It has been clearly demonstrated [18,19] that the efficiency of such strategies increases with the fitness cost of resistance.

**Conclusion**

Insecticides for controlling malaria vectors are a major weapon in the battle between humans and malaria. Unfortunately, these insecticides exert strong selection pressure on vector populations, causing the spread of resistance genes, such as the resistance allele observed at the ace-1 locus in *An. gambiae*. The long-term use of an insecticide promotes the selection of new resistant variants, with a high risk of selecting a low (or null)-cost variant. The ace-1 duplicated allele recently appeared in *An. gambiae* is probably an example of such a low-cost variant. It is shown here that the presence of this duplicated allele, known from the molecular analysis of a few mosquitoes in some samples from Burkina Faso and Ivory Coast [5] is largely distributed in several countries of Western Africa, sometimes at high frequencies, and that it is probably spreading.

To prevent such spreads of resistance genes, it is crucial to develop the largest possible number of complementary means of control (e.g. larval insecticides, mosquito nets, biological agents, etc.) and to use them wisely. However, the emergence of ace-1 duplication in natural populations of *An. gambiae*, has just given mosquitoes the edge in this particular battle, seriously undermining our efforts to control vector populations and increasing the risk of malaria outbreaks.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

LD designed the study, acquired the data and wrote the manuscript. PL analysed the data and wrote the manuscript. NP, FG and MW contributed to the design of the study and for draft and revision of the manuscript. All authors read and approved the final manuscript.

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