A molecular test to identify resistance alleles at the amplified esterase locus in the mosquito *Culex pipiens*

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Abstract: A new PCR/RFLP method is presented to identify all described alleles involved in resistance at the *Ester* locus in the mosquito *Culex pipiens*. The efficiency of this method as well as its advantages compared with the traditional identification technique (starch gel electrophoresis) have been tested in four natural samples from France, Tunisia and California. This simple and fast molecular test is a very convenient tool for studies in field populations and laboratory strains.

Keywords: esterase; insecticide resistance gene; molecular test; mosquito; *Culex pipiens*

1 INTRODUCTION

The super-locus *Ester* is one of the two genome areas in the mosquito *Culex pipiens* L. involved in organophosphorus (OP) insecticide resistance.[1] This super-locus is composed of two loci on chromosome II, *Est-3* and *Est-2*, separated by an intergenic DNA fragment of 2–6 kb,[2–4] and both loci encode for detoxifying esterase. The resistance mechanism at *Ester* corresponds to an esterase over-production at one or both loci.[5] This over-production is the result of two non-exclusive mechanisms: gene amplification of one (*Est-2*) or both loci,[4,6–9] or change in gene regulation.[3] Eight *Ester* alleles involved in resistance have been described so far (Table 1): four correspond to the co-amplification of both *Est-2* and *Est-3* loci ($Ester^2$, $Ester^4$, $Ester^5$ and $Ester^8$, encoding esterases A2-B2, A4-B4, A5-B5 and A8-B8, respectively), one corresponds to the exclusive amplification of *Est-2* ($Ester^{B1}$, encoding esterase B1), one corresponds to an up-regulation of *Est-3* ($Ester^1$, encoding esterase A1). The exact nature of $Ester^6$ and $Ester^7$, encoding esterases B6 and B7, respectively, is unclear,[10] and may correspond to an exclusive amplification of *Est-2*. Other putative amplified esterase genes have been proposed, although further evidence is required.[8] *Est-3* and *Est-2* loci have always been found in maximal linkage disequilibrium for alleles involved in resistance in field studies (see reviews in References 5 and 11, which justify the concept of *Ester* super-locus).

The traditional technique used to identify the various alleles involved in resistance at *Ester* is through the determination of the over-produced esterases by

Table 1. Presentation of the eight previously described alleles involved in resistance at the *Ester* super-locus

Strain[a]	*Allele*	*Overproduced esterases*	*Origin*	*Reference*
BARRIOL*	$Ester^1$	A1	Arles, France	13
VIM*	$Ester^4$	A4-B4	Montpellier, France	9
SELAX*	$Ester^2$	A2-B2	California, USA	24
EDIT*	$Ester^{B1}$	B1	California, USA	16
BICHON*	$Ester^5$	A5-B5	Cyprus	9
MAO*	$Ester^8$	A8-B8	Guangzhou, China	25
No strain available	$Ester^6$	B6	Foshan, China	10
No strain available	$Ester^7$	B7	Chengdu, China	10

[a] Stars indicate the *Ester* homozygous strains used to set up the present PCR/RFLP method.

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either starch or acrylamide gel electrophoresis.[12] However, this method does not allow discrimination between resistant homozygotes and heterozygotes with a susceptible allele. In addition, this method cannot easily discriminate between A4-B4 and A5-B5 esterases, due to their similar electrophoretic migrations.[9] More generally, this method will miss the identification of additional alleles involved in resistance which display electrophoretic migration similar to that of previously described alleles.

We have developed a molecular test to circumvent those difficulties, based on a specific PCR amplification of both parts of *Ester* (*Est-2* and *Est-3*), followed by digestion with a restriction enzyme. This test was set up using laboratory strains homozygous for resistance alleles, and its usefulness was assessed on field samples.

2 EXPERIMENTAL METHODS

2.1 Insects

Six mosquito strains were used, each of them being homozygous for one of the six alleles involved in resistance available at both *Est-3* and *Est-2* loci (Table 1): $Ester^1$, $Ester^4$, $Ester^2$, $Ester^5$, $Ester^{B1}$ and $Ester^8$. In addition, four field populations were used to test the method: two populations from southern France collected at Viols-le-Fort (corresponding to sample K5 in Reference 13) and at Martigues in June 1991 and July 1996, respectively, pooled for convenience in sample P1; P2, collected in the San Diego Zoo (California) in September 1992; P3, collected in Tunis, Tunisia, in June 1996 (corresponding to sample 4 in Reference 14).

2.2 DNA extraction and PCR

DNA extraction was performed according to Roger and Bendich[15] on single adults. Whole mosquitoes were used, except for the individuals from field populations, for which half of the abdomen was kept for starch gel electrophoresis.[12] The extraction products were incubated for 1 h with 0.02 mg of RNAse. Both B (*Est-2*) and A (*Est-3*) esterase genes were amplified with the same protocol. The primers were: 5′ GCAACGGGGGTCGATTACTAC 3′ and 5′ ACTTCATTCGTTCCTGCTCCG 3′ for *Est-3* (in exon 1 and exon 7, respectively), 5′ TGCTGGGACAGGAGTACTTCA 3′ and 5′ CAGCTTCGGGTCGATCATCAT 3′ for *Est-2* (in exon 1 and exon 4, respectively).[3,16] The expected size of the amplified fragments was 2.1 kb for *Est-2* (*c* 85% of the total gene sequence) and 1.8 kb for *Est-3* (*c* 85% of the total gene sequence).[3,16] The PCR mix was made of 20 ng of genomic DNA, 500 pmol of each primer, 100 μM of each dNTP, 2.5 units of *Taq* polymerase (Goldstar Polymerase, Eurogentec, Louvain-la-Neuve, Belgium) in a 1 × reaction buffer HCl Tris-HCl (pH 9.0; 75 mM), $(NH_4)_2SO_4$ (20 mM), Tween 20 (0.1 g litre^{-1})], and $MgCl_2$ (1.25 mM) to give a final volume of 50 μl. The PCR was run on a PTC100 thermocycler (MJ Research, Inc) with a denaturing step at 93 °C for 4 min, followed by 30 cycles of 93 °C for 30 s, 60 °C for 30 s, and 2 min at 72 °C, and a final step of 10 min at 72 °C.

2.3 RFLP determination

A total of 10 restriction enzymes was assessed: *Bam*H1, *Eco*R1, *Eco*RV, *Hae*III, *Hin*dIII, *Kpn*1, *Mun*1, *Sac*1, *Sau*3A and *Xho*1, with the same protocol.

Twenty μl of the PCR product were digested by 20 units of the restriction enzyme (Eurogentec, Louvain-la-Neuve, Belgium) in its 1 × reaction buffer (according to the distributor's requirements) at 37 °C for 1 h for a final volume of 30 μl. After a brief centrifugation, the digestion products were separated on a 1.5% agarose gel stained with ethidium bromide and viewed under UV light.

3 RESULTS AND DISCUSSION

For all strains, the amplification products were of the expected size, ie 1.8 kb and 2.1 kb for each part of the *Ester* super locus (*Est-3* and *Est-2*, respectively). The only exception concerned the *Est-2* part of the $Ester^8$ allele, which displayed a band at around 3.1 kb, ie corresponding to an insertion of *c* 1.1 kb. Thus, individuals homozygous or heterozygous for the $Ester^8$ allele can easily be identified by migration of the amplification products (Fig 1).

All 10 enzymes were first used to digest both *Est-3* and *Est-2* PCR products of $Ester^1$, $Ester^2$ and $Ester^4$ alleles (detail not shown). Three restriction enzymes (*Hae*III, *Eco*RV and *Bam*H1) generated clear and different profiles according to the three alleles involved in resistance, and were subsequently used on the PCR products of all other alleles. Among these three restriction enzymes, *Hae*III generated distinct and repeatable profiles for both parts (*Est-2* and *Est-3*) of each allele (Fig 2). This enzyme was subsequently used to identify the alleles involved in resistance in the field samples.

For the P1 field samples ($n = 33$ mosquitoes), starch gel electrophoresis disclosed 18 mosquitoes without over-produced esterase, 10 with only esterases A4-B4 and four with esterases A1. Independent analysis of the same individual mosquitoes with the present molecular test disclosed the following classes: 18 mosquitoes without any known alleles involved in resistance, 11 mosquitoes displaying $Ester^4$ (six of which were apparently homozygous and five heterozygous with a susceptible allele), four mosquitoes displaying $Ester^1$ (three of which were apparently homozygous and one heterozygous with a susceptible allele). Results from both methods were consistent, with the exception of those for two mosquitoes: one insect was identified as displaying A4-B4 by starch electrophoresis and showing a susceptible pattern with the present test; the converse was the situation for the second. This situation may result from the low

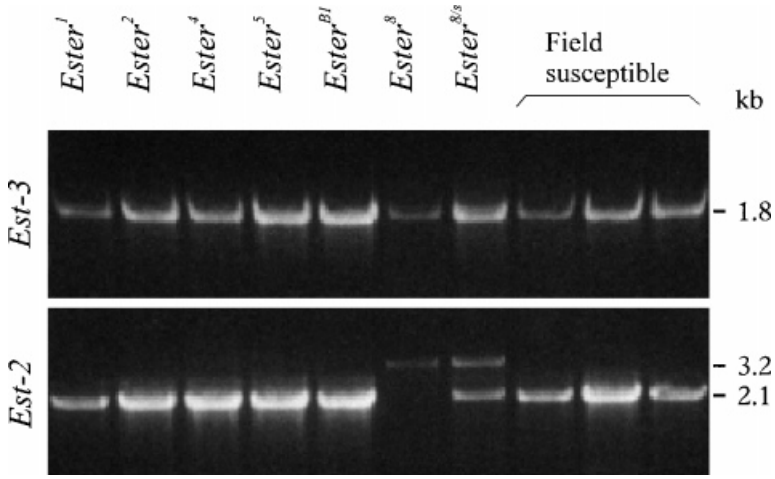

Figure 1. PCR products for both parts (*Est-2* and *Est-3*) of all the resistant *Ester* alleles studied, one heterozygous individual for *Ester*^{8} and a susceptible allele, and for three field susceptible mosquitoes.

overproduction of A4-B4, which is difficult to identify correctly in small individuals (and particularly in half an abdomen, as in this case). If this explanation is correct, then the molecular test provides the correct answer. Another explanation for the second mosquito may result from the high variability displayed by susceptible alleles in field populations (Fig 2), so that some susceptible alleles could present a similar restriction pattern to that of a resistant allele.

For the P2 field sample ($n = 30$ mosquitoes), starch gel electrophoresis disclosed one mosquito without over-produced esterase, 10 with only esterase B1, one with esterases A2-B2 and 18 with esterases A2-B2 and B1. Independent analysis of the same individual mosquitoes with the present molecular test disclosed the following classes: one mosquito without known alleles involved in resistance, 10 mosquitoes displaying *Ester*^{B1} (nine of which are apparently homozygous and one heterozygous with a susceptible allele), three mosquitoes homozygous for *Ester*^{2} and 16 displaying both *Ester*^{2} and *Ester*^{B1}. Results from both methods were consistent, with the exception of two mosquitoes: these were seen with both A2-B2 and B1 in starch electrophoresis and were seen to be homozygous for *Ester*^{2} with the molecular test. One of these mosquitoes displayed a low quantity of PCR product, so that the *Ester*^{2} bands were faint. It is therefore probable that the usually fainter *Ester*^{B1} bands could not be seen. This was not the case for the second mosquito.

For the P3 field sample ($n = 26$ mosquitoes), starch gel electrophoresis disclosed one mosquito without over-produced esterase, three with esterase A2-B2, and 22 with esterases A4-B4 or A5-B5 (no mosquito displayed the additional esterase incorrectly named B8 in Reference 14). These results are not different (2×3 contingency table, $P > 0.5$) from the data of the same sample.[14] Independent analysis of the same individual mosquitoes with the present molecular test disclosed the following classes: one mosquito without known resistant allele, three mosquitoes with *Ester*^{2} (two homozygous, one heterozygous with a susceptible allele), five displaying *Ester*^{4} (three homozygous, two heterozygous with a susceptible allele), 15 displaying *Ester*^{5} (eight homozygous, seven heterozygous with a susceptible allele), and two mosquitoes heterozygous *Ester*^{4}/*Ester*^{5}. Results from both methods were con-

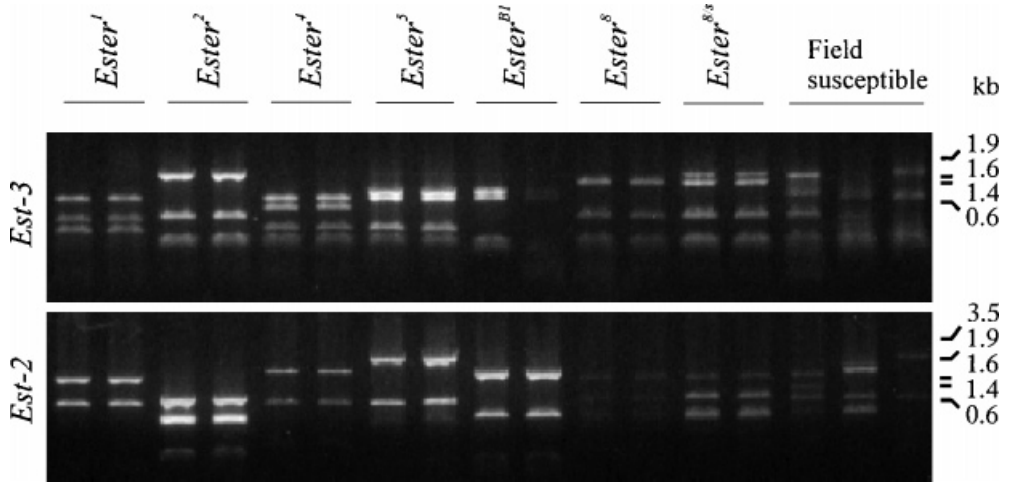

Figure 2. *Hae*III restriction profiles of PCR products for both parts (*Est-2* and *Est-3*) of each resistant *Ester* allele studied (two mosquitoes for each), two heterozygous individual for *Ester*^{8} and a susceptible allele and for three field susceptible mosquitoes.

sistent for all mosquitoes. It should be noted that the esterases A4-B4 and A5-B5 could not be discriminated in the previous study,[14] because both have the same mobility in starch gel electrophoresis.[9] The molecular test has unambiguously identified the presence of *Ester*^{5} in Tunisia, which is the first record of this allele in Africa. The present known distribution of the esterase A5-B5 is therefore Cyprus,[9] Italy[17] and Tunisia (this study).

The geographical distribution of the various alleles involved in resistance at *Ester* is irregular. Some alleles have an extended distribution, and are present in several continents. This is the case in particular for *Ester*^{2} (encoding esterase A2-B2), which is present in South and North America, Eurasia and Africa, plus several archipelagos (eg Polynesia), as a result of migration and passive transportation.[18,19] The same situation is found for the other alleles, with, however, a less dramatic geographical extension.

There is co-occurrence of more than one resistant allele in some geographical areas. In the Mediterranean, for example, four *Ester* alleles could be found (*Ester*^{1}, *Ester*^{2}, *Ester*^{4} and *Ester*^{5}), and three of them could occur simultaneously in the same population, as is the case in southern France (*Ester*^{1}, *Ester*^{2}, *Ester*^{4}), northern Italy (*Ester*^{1}, *Ester*^{4}, *Ester*^{5}) and Tunisia (*Ester*^{2}, *Ester*^{4}, *Ester*^{5}). All these alleles involved in resistance are prone to extensive migration, either through active or passive dispersal.[18,20,21] The case of *Ester*^{4} and *Ester*^{5} is particularly puzzling: *Ester*^{5} was initially localized in the eastern Mediterranean, and has subsequently invaded northern Italy[9,17,22] and is now present in Tunisia (this study). Considering that the frequency of *Ester*^{4} in most western Mediterranean populations is rather high, the only possibility of easily detecting the invasion and spread of the esterase A5-B5 in this geographical area is through the present molecular test.

In conclusion, this new PCR/RFLP method to identify alleles involved in resistance at the *Ester* superlocus presents some advantages. It is a useful and fast technique to identify homozygote strains for particular resistance alleles at *Ester* as opposed to starch gel electrophoresis for which these alleles are dominant towards susceptible alleles. In field studies, this molecular method is a very convenient tool to differentiate susceptible/heterozygous individuals from homozygote resistants, which cannot be identified by conventional gel electrophoresis; it is also useful when different resistance alleles have a similar electrophoretic migration, and are segregating in the same populations (such as *Ester*^{4} and *Ester*^{5} in northern Italy and Tunisia). It is noteworthy that the exclusive use of this PCR/RFLP technique to identify genotypes in field populations may miss some resistant heterozygous (as one mosquito misinterpreted in the P2 sample) and may lead to over-estimation of the frequency of resistance genes (except for *Ester*^{8}, due to distinct size of the PCR product), particularly in areas where susceptible alleles display a high genetic variability.[23] These problems could be partially circumvented by the joint analysis of *Est-3* and *Est-2* on the same mosquitoes, as proposed in the present molecular test. It is therefore recommended that the present PCR/RFLP method is used in complement with other techniques such as conventional gel electrophoresis.

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REFERENCES

1 Lenormand T, Guillemaud T, Bourguet D and Raymond M, Evaluating gene flow using selected markers: a case study. *Genetics* **149**:1383–1392 (1998).
2 Heyse D, Catalan J, Nancé E, Britton-Davidian J and Pasteur N, Unconventionnal organization of esterase B amplified gene in insecticide-resistant mosquitoes of the *Culex pipiens* complex. *J Am Mosq Cont Assoc* **12**:199–205 (1996).
3 Rooker S, Guillemaud T, Bergé J, Pasteur N and Raymond M, Coamplification of esterase A and B genes as a single unit in *Culex pipiens* mosquitoes. *Heredity* **77**:555–561 (1996).
4 Guillemaud T, Makate N, Raymond M, Hirst B and Callaghan A, Esterase gene amplification in *Culex pipiens*. *Insect Mol Biol* **6**:319–327 (1997).
5 Raymond M, Chevillon C, Guillemaud T, Lenormand T and Pasteur N, An overview of the evolution of overproduced esterases in the mosquito *Culex pipiens*. *Phil Trans Royal Society, London B* **353**:1–5 (1998).
6 Mouchès C, Pasteur N, Bergé JB, Hyrien O, Raymond M, Robert de Saint Vincent B, De Silvestri M and Georghiou GP, Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito. *Science (Washington)* **233**:778–780 (1986).
7 Raymond M, Beyssat-Arnaouty V, Sivasubramanian N, Mouchès C, Georghiou GP and Pasteur N, Amplification of various esterase B's responsible for organophosphate resistance in Culex mosquitoes. *Biochem Genet* **27**:417–423 (1989).
8 Vaughan A, Hawkes N and Hemingway J, Co-amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide-resistant *Culex quinquefasciatus*. *Biochem J* **325**:359–365 (1997).
9 Poirié M, Raymond M and Pasteur M, Identification of two distinct amplifications of the esterase B locus in *Culex pipiens* (L) mosquitoes from Mediterranean countries. *Biochem Genet* **30**:13–26 (1992).
10 Xu J, Qu F and Liu W, Diversity of amplified esterase B genes responsible for organophosphate resistance in *Culex quinquefasciatus* from China. *J Med Coll PLA* **9**:20–23 (1994).
11 Chevillon C, Raymond M, Guillemaud T, Lenormand T and Pasteur N, Population genetics of insecticide resistance in the mosquito *Culex pipiens*. *Biol J Linn Soc* **68**:147–157 (1999).
12 Pasteur N, Pasteur G, Bonhomme F and Britton-Davidian J, *Practical isozyme genetics*, Ellis Horwood Ltd, Chichester, UK (1988).
13 Chevillon C, Addis G, Raymond M and Marchi A, Population structure in Mediterranean islands and risk of genetic invasion in *Culex pipiens* L (Diptera: Culicidae). *Biol J Linn Soc* **55**:329–343 (1995).
14 Ben Cheikh H, Ben Ali-Haouas Z, Marquine M and Pasteur N,

Resistance to organophosphorus and pyrethroid insecticides in *Culex pipiens* (Diptera: Culicidae) from Tunisia. *J Med Entomol* **35**:251–260 (1998).

15 Roger SO and Bendich AJ, Extraction of DNA from plant tissues, in *Plant Molecular Biology Manual*, ed by Gelvin SB and Schilperoort RA, Kluwer Academic Publishers, Boston, pp 1–10 (1988).

16 Guillemaud T, Raymond M, Tsagkarakou A, Bernard C, Rochard P and Pasteur N, Quantitative variations and selection of esterase gene amplification in *Culex pipiens*. *Heredity* **83**:87–99 (1999).

17 Severini C, Romi R, Marinucci M, Guillemaud T and Raymond M, Esterases A5-B5 in organophosphate-resistant *Culex pipiens* from Italy. *Med Vet Entomol* **11**:123–126 (1997).

18 Raymond M, Callaghan A, Fort P and Pasteur N, Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature (London)* **350**:151–153 (1991).

19 Guillemaud T, Rooker S, Pasteur N and Raymond M, Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data. *Heredity* **77**:535–543 (1996).

20 Pasteur N, Marquine M, Rousset F, Failloux A-B, Chevillon C and Raymond M, The role of passive migration in the dispersal of resistance genes in *Culex pipiens quinquefasciatus* within French polynesia. *Genet Res* **66**:139–146 (1995).

21 Curtis CF and White GB, *Plasmodium falciparum* transmission in England: entomological data relative to cases in 1983. *J Trop Med Hyg* **87**:101–194 (1984).

22 Silvestrini F, Severini C, Di Pardo V, Romi R, De Matthaeis E and Raymond M, Population structure and dynamics of insecticide resistance genes in *Culex pipiens* populations from Italy. *Heredity* **81**:342–348 (1998).

23 Raymond M, Qiao CL and Callaghan A, Esterase polymorphism in insecticide susceptible populations of the mosquito *Culex pipiens*. *Genet Res* **67**:19–26 (1996).

24 Raymond M, Pasteur N, Georghiou GP, Mellon RB, Wirth MC and Hawley MK, Detoxification esterases new to California, USA, in organophosphate-resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* **24**:24–27 (1987).

25 Qiao C-L, Marquine M, Pasteur N and Raymond M, A new esterase amplification involved in OP resistance in *Culex pipiens* mosquitoes from China. *Biochem Genet* **36**:417–426 (1998).