mate gynes were closely related and all wasps usually belonged to a single extended matriline presumably originating from the original founding queen. The overall structure of perennial *V. germanica* colonies was broadly consistent with expectations based on kin selection theory within polygyne colonies. Multiple queens successfully reproduced, and new queens were usually recruited into their natal nests so that workers raised relatives and received high inclusive fitness benefits.

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Recombination Between Two Amplified Esterase Alleles in *Culex pipiens*

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Esterase gene amplification at the *Ester* superlocus provides organophosphate resistance in the mosquito *Culex pipiens* (L.). In this study we explored the possibility of recombination between two amplified esterase alleles, thus generating a composite amplified allele. To do that, females heterozygous for two distinct amplified alleles (*Ester*^{2} and *Ester*^{4}) were crossed with males homozygous for a third resistance allele (*Ester*^{8}). Among analyzed offspring, one recombinant composite allele (*Ester*$^{2–4}$) was detected, providing a rate of recombination of approximately 0.2%. This is the first report of a recombination between two distinct amplified esterase alleles. This phenomenon renders the predictability of allele evolution considerably more complex than was previously thought.

General models of population genetics that try to infer the outcome of adaptive genes are often based on simple approximations, like the existence of one or two loci with two alleles. Concerning resistance to organophosphorous (OP) insecticides in *Culex pipiens* mosquitoes, the adaptive system is more complex. The *Ester* superlocus, which is one of the main genome areas involved in this resistance (Lenormand et al. 1998), presents multiple resistance alleles. This superlocus is in fact composed of two loci on chromosome II, *Est-3* and *Est-2,* separated by an intergenic DNA fragment of 2–6 kb (Guillemaud et al. 1997; Heyse et al. 1996; Rooker et al. 1996) and both loci encode for detoxifying esterases A and B, respectively. The resistance conferred by *Ester* is due to an esterase overproduction that is the result of two nonexclusive mechanisms: gene amplification of one (*Est-2*) or both loci, or change in gene regulation (for a review, see Raymond et al. 1998). Six *Ester* alleles involved in resistance have been described: four correspond to the coamplification 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), and one corresponds to an upregulation of *Est*-3 (*Ester*^{1}, encoding esterase A1). Two other amplified esterase genes have been reported, but direct comparison with the above alleles is needed to confirm that they are distinct alleles (Vaughan et al. 1997; Xu et al. 1994). *Est-3* and *Est-2* loci have always been found in maximal linkage disequilibrium for alleles involved in resistance in field studies (see review in Chevillon et al. 1999; Raymond et al. 1998), which justifies the concept of *Ester* superlocus.

Recently molecular data have confirmed that the amplification level of amplified alleles is variable in natural populations (Callaghan et al. 1998; Weill et al. 2000). Moreover, level of resistance, esterase ac-

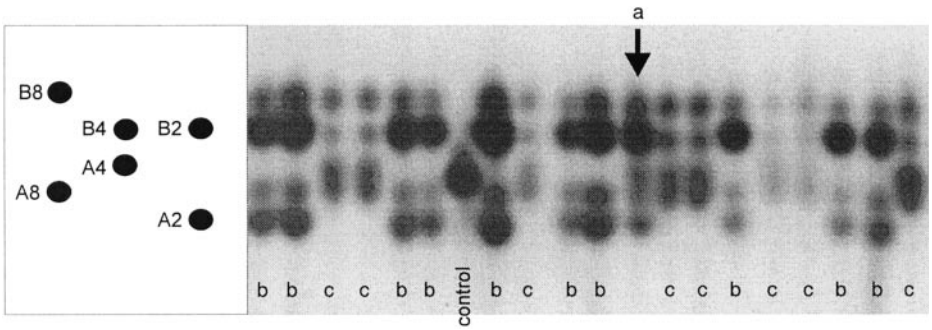

Figure 1. Identification by starch-gel electrophoresis of esterase phenotype of the offspring from the cross between F_1 {female} displaying A2-B2 and A4-B4 with MAO {male} displaying only A8-B8. a: individual displaying the recombinant phenotype A2-B2/A4-B4/A8-B8; b and c: individuals displaying A2-B2/A8-B8 and A4-B4/A8-B8 phenotype, respectively; control: control mosquito displaying esterase B1.

tivity, and amplification covary so that the evolution of the amplification level can take place under selection (Guillemaud et al. 1999). The mechanism generating the amplification or the variability of copy number is currently unknown. We report here a laboratory experiment which shows that recombination between distinct amplified alleles is possible. To do that we crossed mosquitoes between three strains homozygous for different amplified alleles. We searched for recombinants characterized by the coexistence of the three overproduced esterases.

Materials and Methods

Insects

Three laboratory mosquito strains were used: MAO from China (Qiao et al. 1998), SELAX from California (Raymond et al. 1987), and VIM from France (Poirié et al. 1992). They are homozygous for *Ester*^{8}, *Ester*^{2}, and *Ester*^{4} alleles, respectively. These strains display the overproduced esterases A8-B8, A2-B2, and A4-B4, respectively.

Crossing

Mass crossings between VIM females and SELAX males were performed in order to obtain F_1 heterozygous mosquitoes *Ester*^{4}/*Ester*^{2}. The F_1 females were then crossed with males of the MAO strain (*Ester*^{8}/*Ester*^{8}). The offspring of this cross were then reared to adult and deep frozen for further analyses.

Identification of Esterase Phenotypes

Esterase phenotypes were established by starch-gel electrophoresis in TME 7.4 buffer systems as described by Pasteur et al. 1988. Esterases A2-B2, A4-B4, and A8-B8 present clearly distinct electrophoregrams, that is, each of them has at least one of its two spots (esterase A and B) which has different electrophoretic mobility from the others. This allows us to easily detect individuals possessing all three.

Results and Discussion

Of the 548 mosquitoes analyzed from the cross {female}(VIM*SELAX) * {male}MAO, 547 displayed an heterozygote phenotype, corresponding to *Ester*^{4}/*Ester*^{8} and *Ester*^{2}/*Ester*^{8} genotypes (in frequency, 0.46 and 0.54, respectively, not different from a 1:1 ratio, binomial exact test, $P > .05$). One mosquito displayed a phenotype with A2-B2 (*Ester*^{2}), A4-B4 (*Ester*^{4}), and A8-B8 (*Ester*^{8}), as shown in Figure 1. It must be noted that this phenotype does not result from a mixture between the two flanking samples because the A8 and B8 bands are really less intense than would be expected from a mixture scenario. Thus this situation is easily interpreted by a recombination between *Ester*^{4} and *Ester*^{2} in the F_1 females, generating a composite allele with both types of amplified alleles, designated *Ester*$^{2-4}$ (Figure 2). This is the first evidence that there is recombination between two different amplified esterase alleles.

The rate of recombination between amplified alleles observed here ($1/548 \approx 0.2\%$; 95% confidence interval 0.0007–0.004) is probably an underestimation. This is because if the recombination occurs at a distal part of one of the amplified alleles involved, then the composite recombinant allele might produce an insufficient amount of one esterase type for detection by starch-gel electrophoresis. Thus with this technique the phenotype of individuals resulting from such distal recombination could be misinterpreted.

Composite alleles, that is, different amplified esterase alleles within one amplification, have never been observed in field populations. This is probably not because intra-amplification recombination is rare, as the present estimate is relatively large. This absence of detection is best explained by the paucity of geographic areas where at least three different resistance alleles at *Ester* could be simultaneously detected in a single mosquito. Formation of a composite recombinant allele must occur in gametes of a heterozygote for two distinct amplified alleles. Its detection is then possible by starch-gel electrophoresis analysis of the offspring of this individual, provided that they carry a third distinct resistance allele, since susceptible esterases are not detectable in the presence of overproduced ones. Finally, the probability of detection of a composite allele would be maximized when the formation of both types of individuals described above (heterozygotes for two distinct amplified alleles and their offspring with a third distinct resistant one) is maximized, that is, for equifrequency of the three resistance alleles. A very low frequency of

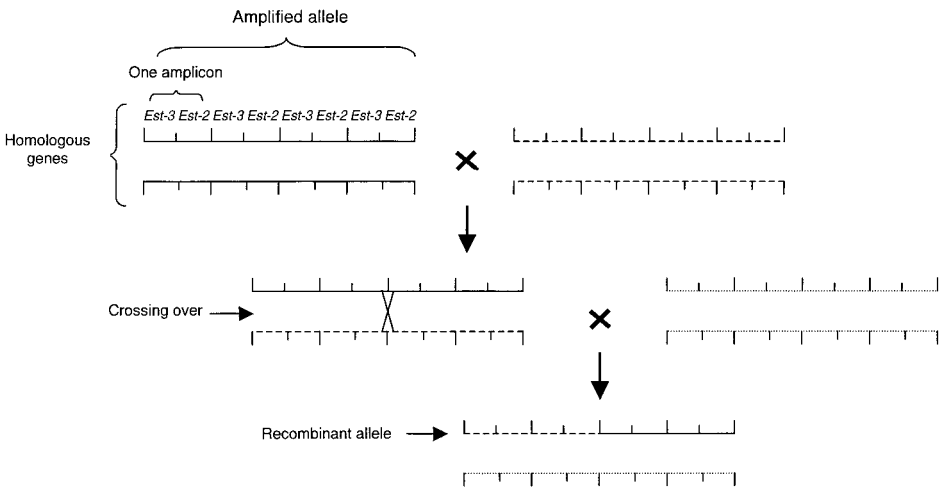

Figure 2. Schematic representation of the formation of the composite recombinant allele observed. Full, dashed, and dotted lines correspond to *Ester*^{2}, *Ester*^{4}, and *Ester*^{8} amplified alleles, respectively.

one of the three alleles could then compromise the detection of one recombinant.

Consequently no composite amplified allele could be detected in the Americas and Polynesia, where only *Ester*^{2} and *Ester*^{B1} were reported, in Africa, and in most parts of Asia where only *Ester*^{2} was reported (Georghiou 1992; Qiao and Raymond 1995; Raymond et al. 1991, 1998; Raymond and Pasteur 1996; Yébakima et al. 1995). Mediterranean countries and China are the only known areas where at least three resistance *Ester* alleles coexist (Chevillon et al. 1999; Raymond et al. 1998). In China, despite this favorable situation (Qiao et al. 1998; Xu et al. 1994), the rarity of population studies did not give a chance to detect any putative composite allele. In northern Italy there are three amplified alleles (*Ester*^{2}, *Ester*^{4}, and *Ester*^{5}) plus the nonamplified resistant one (*Ester*^{1}) (Silvestrini et al. 1998). If we assume that recombination occurs through unequal recombination between amplicons, then recombination involving *Ester*^{1} will be extremely rare because this allele is composed of a single unit, that is, one amplicon (the esterase overproduction of this allele is the result of a regulatory mutation; Rooker et al. 1996). Moreover, esterases encoded by *Ester*^{4} and *Ester*^{5} have a similar electrophoretic mobility (Poirié et al. 1992), such that a putative composite allele *Ester*$^{4-5}$ could not be identified by starch-gel electrophoresis. Thus composite alleles that might be detected in Italy would be either *Ester*$^{2-4}$ or *Ester*$^{2-5}$ (indistinguishable from one another), provided that the other allele in the mosquito is *Ester*^{1}. However, studies by Silvestrini et al. (1998) have shown that these three alleles coexisted only in two samples and that they were far from equifrequency with a very low frequency for *Ester*^{2}. This explains why no *Ester*$^{2-4}$/*Ester*^{1} (or *Ester*$^{2-5}$/*Ester*^{1}) heterozygote was detected. *Ester*^{1}, *Ester*^{2}, and *Ester*^{4} coexist in Mediterranean France too, especially around Montpellier, where field population studies have been intensively performed for 25 years. Among about 14,000 individuals characterized so far, the putative composite allele *Ester*$^{2-4}$ (which might be detected here provided that the other allele in the mosquito is *Ester*^{1}) has never been reported (Guillemaud et al. 1998; Lenormand et al. 1998; Lenormand and Raymond 2000). All three resistant alleles are now concentrated within the same costal populations, but *Ester*^{2} only recently appeared (1991) so the composite *Ester*$^{2-4}$ allele could not be formed before the 1990s (Chevillon et al. 1995; Guillemaud et al. 1998; Lenormand et al. 1998). Since then, *Ester*^{4} frequency is greater than 0.4, and *Ester*^{2} and *Ester*^{1} maximal frequencies are less than 0.02 and 0.10, respectively, such that probability for detecting *Ester*$^{2-4}$/*Ester*^{1} mosquitoes remains very low, as long as *Ester*$^{2-4}$ remains at the frequency of formation.

The relatively low number of resistant alleles at *Ester,* recorded on a world scale for a pest species with large population sizes, indicates that advantageous mutations (e.g., amplification) at this locus could be limiting (Raymond et al. 1998). However, this paucity of resistance gene is compensated for by a variation of gene copy number for a given amplified allele (Guillemaud et al. 1999; Weill et al. 2000), and by the possible formation of composite alleles (this study). These two phenomena, which constantly generate new types of resistance alleles, cannot be ignored in field studies and could render the predictability of allele frequency evolution considerably more complex than was previously thought.

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