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On the worldwide spread of an insecticide resistance gene: a role for local selection

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Abstract

Adaptation occurs by gene replacement (or transient balanced polymorphism). Replacement may be caused by selection (local or global) and/or genetic drift among alleles. In addition, historical events may blur the respective effects of selection and drift during the course of replacement. We address the relative importance of these processes in the evolution of insecticide resistance genes in the mosquito *Culex pipiens*. The resistance allele, *Ester*^{2}, has a broad geographic distribution compared to the other resistance alleles. To distinguish between the different processes explaining this distribution, we reviewed the literature and analysed updated data from the Montpellier area of southern France. Overall, our data indicate that *Ester*^{2} prevails over other *Ester* resistance alleles in moderately treated areas. Such conditions are common and favour the hypothesis of selection acting at a local level. This places an emphasis on the importance of ecological conditions during the evolution of resistance. Finally, we highlight that historical events have contributed to its spread in some areas.

Introduction

Adaptation occurs by gene replacement at one or several loci. In a large or structured population, several adaptive alleles may segregate simultaneously at a single locus. In the long run different outcomes are possible: (i) one of these alleles may prevail, i.e. the one that most likely confers the best adaptation (i.e. global selection); (ii) several adaptive alleles may coexist if they confer different phenotypes, which are favoured in different environments (i.e. local selection), or (iii) the different alleles may be adaptively equivalent and may coexist in a neutral way (i.e. genetic drift). Discriminating between these scenarios is relatively easy in the long run, when equilibrium has been reached. However, before equilibrium has been reached, due to historical events (i.e. initial conditions and contingency events), it is not easy to distinguish how these three scenarios influence the course of allele replacement (Fig. 1). Moreover, these processes are not mutually exclusive and can combine. Balanced polymorphism is also a possibility, although it will eventually be fixed (for example through duplication, Haldane, 1932). Sometimes it is possible to distinguish, at least partly, between selection (local or global), genetic drift, and history. For example, the *G6PD* A^- deficient allele, conferring malarial resistance in humans (i.e. it gives a selective advantage), is at higher frequencies than other deficient alleles of the *G6PD* gene. However, the frequency of another deficient allele, *Med*, providing less resistance than A^-, is as high as 70% in some populations, where apparently A^- has not spread yet. In this example, historical events are the most likely explanation for the prevalence of the *Med* allele (Tishkoff *et al.*, 2001). A second example is the *Adh* gene in *Drosophila melanogaster*. Three different alleles are found: a wild one (named *Standard* or *Low* allele), an inverted allele (*In(2L)t*), most common in Africa, and a *Fast* allele, found essentially in Europe. In this example too, historical events and global selection are the most likely explanation for the distribution of alleles with the independent appearance of *In(2L)t* in Africa and *Fast* in Europe, and the subsequent spread of the *Fast* allele in Africa (Veuille *et al.*, 1998). This spread is thought to

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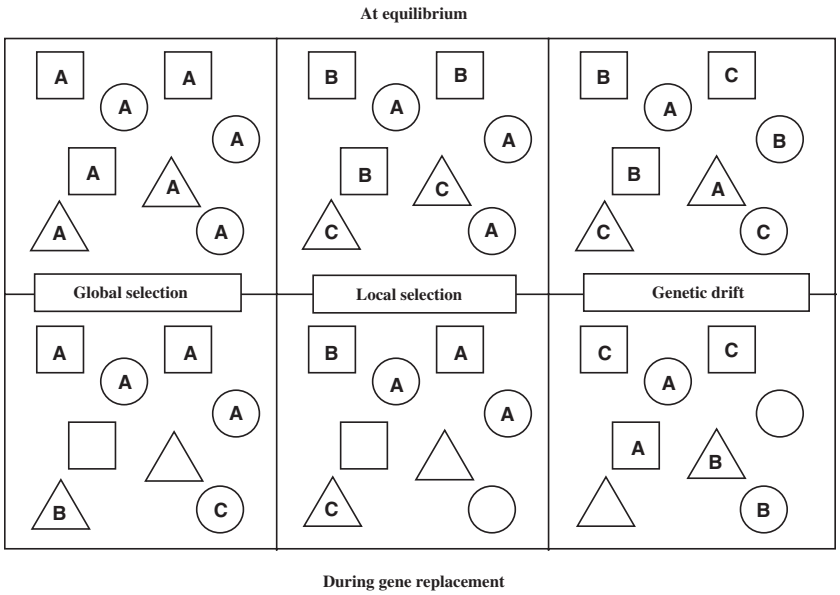

Fig. 1 Expected patterns under the different evolutionary hypotheses. The different shapes represent different environmental conditions. A, B, C are different adaptive alleles. Global selection: A is the fittest in each condition. Local selection: each allele is selected in a different environment. Genetic drift: the different adaptive alleles are neutral with respect to each other. To take account of historical contingency, expected patterns at equilibrium are indicated above and those occurring during gene replacement, and thus submitted to historical events, at the bottom. An absence of letters indicates the absence of an adaptive gene.

occur because of a selective global advantage of the *Fast* allele. In these examples, the importance of historical events was inferred, although their roles remain uncertain due to the incomplete knowledge of the selective process at work.

The wide use of pesticides to control pests of agricultural and public-health importance has been a powerful and recent agent of selection in natural populations of many insect species. Here we took advantage of the knowledge on insecticide resistance genes in the mosquito *Culex pipiens* L. to investigate the relative importance of historical events, genetic drift between resistance alleles, and selection. This mosquito, common in temperate and tropical countries, is subjected to insecticide control in many places, particularly with organophosphate insecticides (OP), because it is a nuisance and a vector of human diseases (West Niles encephalitis, filariasis). These insecticides inhibit acetylcholinesterase (or AChE) in the central nervous system, causing death. This mosquito has rapidly developed various adaptations to this new and toxic component of its environment. This example of microevolution has been thoroughly investigated as an opportunity to characterize precisely both the new adapted phenotypes and the associated genetic changes. In addition, OP resistance in this mosquito was studied from its first appearance in one geographic area, and has been followed up in the context of a long-term project. In this species, only three loci have developed major OP resistance alleles: *Est-2*, *Est-3* and *ace-1*. The first two loci, *Est-2* and *Est-3*, code for detoxifying carboxylester hydrolases (or esterases). Resistance alleles correspond to an over-production of esterase (which binds or metabolizes the insecticide) relative to susceptible alleles. Several resistance alleles (each corresponding to a distinct over-produced allozyme) have been described at both loci (see for review Raymond *et al.*, 2001). For most resistance alleles, the over-production of esterase is the result of gene amplification (i.e. several copies of the same gene are found in the same genome), affecting one or both *Est* loci. The latter situation, the co-amplification of two esterase loci that are adjacent in the genome, explains the tight statistical association of some electromorphs, e.g. A2–B2 (Guillemaud *et al.*, 1996; Rooker *et al.*, 1996). Although, strictly speaking, A2 is coded by an allele of the *Est-3* locus, and B2 by an allele at the *Est-2* locus, A2–B2 behave as if coded by an allele (named *Ester*^{2}) of a single super locus (named *Ester*) due to the complete linkage in amplification of *Est-2* and *Est-3*. Gene regulation is also present (i.e. esterase over-production is higher than expected by the amplification level), and is the major mechanism of over-production of A1 (Rooker *et al.*, 1996). A third locus, *ace-1*, codes the insecticide target (acetylcholinesterase). The wild type and susceptible forms of this enzyme are inhibited by OP insecticides. Several resistance alleles at this locus have been described with reduced sensitivity towards OP, associated with their modified catalytic properties (Bourguet *et al.*, 1997; Lenormand *et al.*, 1998a; Weill *et al.*, 2003,2004).

A particular OP resistance allele, *Ester*^{2}, has a worldwide distribution. It has been shown by various molecular studies that this situation is the result of extensive migrations, and not due to independent origins (Raymond *et al.*, 1991; Guillemaud *et al.*, 1996; Callaghan *et al.*, 1998). These migrations have been driven by human activity, as there is direct evidence of passive transportation of mosquitoes, including *C. pipiens*, by ships and planes (Asahina, 1970; Highton & van Someren, 1970; Curtis & White, 1984). The worldwide

distribution of *Ester*^{2} has four possible and non-exclusive explanations. First *Ester*^{2} could be the first OP resistance gene to have occurred and spread in treated areas worldwide; this is the *historical contingency hypothesis*. Second and third, *Ester*^{2} could present some fitness advantage over other resistance genes, for example a lower cost or a better resistance level, its actual distribution thus being the result of a competitive advantage. This advantage may be local (i.e. only in some areas) or global (i.e. in all environments encountered); these are the *local* and *global selection hypotheses*. Fourthly, *Ester*^{2} may be as fit as the other resistance alleles at the *Ester* locus, with its higher prevalence being explained only by chance; this is the *drift hypothesis*. In practice, different patterns of gene replacement are expected under these hypotheses (Fig. 1): local selection would cause *Ester*^{2} to be prevalent only in some type of environments, other resistance alleles being more frequent in others, whereas in the case of global selection, an intrinsic advantage would make *Ester*^{2} prevalent in all environmental conditions. Historical events would cause a less fit allele to be prevalent in environments where *Ester*^{2} would eventually prevail once introduced. With genetic drift among resistance alleles, no predictable pattern would emerge, different alleles prevailing independently of the type of selection or environmental variables.

In order to distinguish between these four hypotheses, we first reviewed all the locations where *Ester*^{2} has been reported, to establish if it is the only *Ester* resistance allele present and whether it occurred first or appeared later. Secondly, we present detailed studies from one particular area (Montpellier, southern France) where *Ester*^{2} was not the first *Ester* resistance allele to occur, in order to document its possible evolution at the population level.

Material and methods

Recorded data

To find all data available on *Ester*^{2}, we performed literature searches in several databases for recently published studies, and then used cited literature to find older studies. As the nomenclature concerning resistance alleles at this locus has not been stable and consensual, all known designations were considered (*Ester*^{2}, *Est3*$^{A'}$, A2–B2, *est*α2^1 and *est*β2^1). Unpublished data, i.e. samples not formally and fully presented in a publication but available from the laboratory, were also used.

Mosquito sampling

Pupae and larvae were collected along a 50 km northwest-southeast transect crossing the treated-untreated boundary near the Montpellier area and studied previously (Guillemaud *et al.*, 1998; Lenormand *et al.*, 1999). This cline was sampled in July 1999 ($n = 411$), July 2001 ($n = 736$) and August 2002 ($n = 521$). Locations of samples are described in Fig. 2. Pupae and larvae were reared in the laboratory until emergence, and then adults were frozen and stored at −80 °C for further analyses. Data from the same transect, sampled in summer 1986 and 1991 ($n = 354$ and 217, respectively, Guillemaud *et al.*, 1998), in summer 1995 ($n = 1203$, Lenormand *et al.*, 1998b) and in summer 1996 ($n = 512$, Lenormand & Raymond, 2000), were used for an overall comparison.

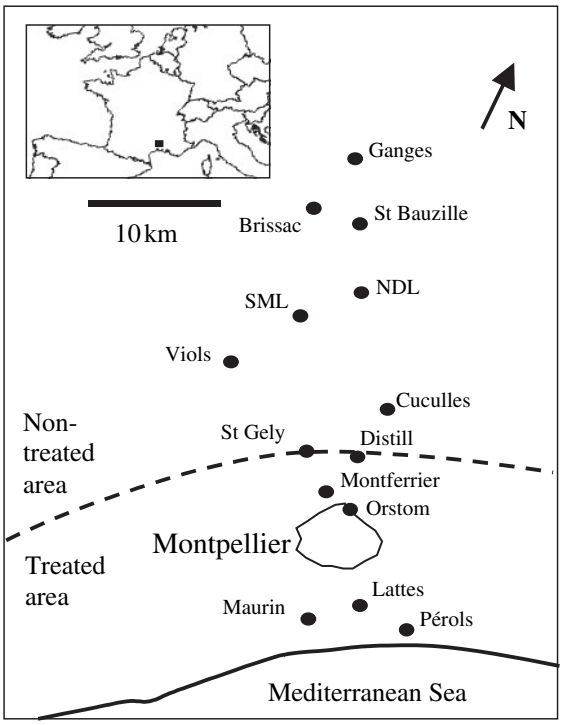

Fig. 2 Sample site locations in the northwest southeast transect in the Montpellier area. Samples are indicated with black circle. The dashed line represents approximately the border between treated and untreated areas (modified from Lenormand *et al.*, 1999). *C. pipiens* is present in the whole area.

Identification of overproduced esterases

For each mosquito, the thorax and abdomen were used to detect overproduced esterases using starch-gel electrophoresis (Tris–Malate–EDTA 7.4 buffer, Pasteur *et al.*, 1988) and to identify resistance alleles at the *Ester* locus. Heads were used to establish the phenotype at the *ace-1* locus; these data will be reported elsewhere. Overproduced esterases are dominant over non-overproduced esterases under our electrophoretic conditions. Thus, this method does not allow complete genotype identification. See Lenormand *et al.* (1998b)) for the correspondence between each genotype and its corresponding phenotype detected by starch gel electrophoresis.

Descriptive statistics

To test for the existence of frequency clines for each resistance allele along the transect and in order to compare frequency patterns among years, phenotypic data were fitted to a descriptive cline function. Frequency clines for each resistance allele i ($i = 1$, 2 or 3, respectively for *Ester*^{1}, *Ester*^{2}, and *Ester*^{4}) at time j ($j =$ 1986, 1991, 1995, 1996, 1999, 2001 and 2002) were simultaneously fitted to a scaled negative exponential

$$p_{ij} = h_{ij} \exp[-(a_{ij}x^2 + b_{ij}x)],$$

where x is the distance from the coast and h_{ij}, b_{ij} and a_{ij} are the estimated parameters (Lenormand & Raymond, 2000). b_{ij} and a_{ij} describe rates of decline in allele i frequency at time j with distance and with the square of distance from the coast, respectively. h_{ij} is the frequency of resistance allele i at time j and at $x = 0$ (i.e. at the coast). In order to test for trends in allelic replacements over the period 1986–2002, we supposed that h_{ij} values followed a logistic function of time

$$h_{ij} = \frac{\exp(\alpha_i t_{1j} + \beta_i t_{2j} + \gamma_i)}{1 + \exp(\alpha_i t_{1j} + \beta_i t_{2j} + \gamma_i)},$$

where t_{1j} is the number of years after 1986 when the year of sampling is before 1986 + t^* and is t^* otherwise. t_{2j} is the number of years after 1986 + t^*. α_i, β_i, γ_i and t^* are estimated parameters. Overall change in frequency over the 1986–2002 period is measured for each resistance allele i by α_i and β_i, which measure the rate of frequency change between 1986 and 1986 + t^* and between 1986 + t^* and 2002, respectively. We introduced t^* to allow for changes in the rate of allele replacement due to the appearance of *Ester*^{2} allele. Parameter γ_i is related to the initial frequency h_{i0} of each allele i as: $h_{i0} = \exp(\gamma_i)/[1 + \exp(\gamma_i)]$. The occurrence of allele replacement was tested by comparing models where α_i and β_i are estimated or set to zero. Changes in rates of allele replacement due to the presence of *Ester*^{2} allele were tested by comparing models where α_i and β_i are estimated independently or constrained such that $\alpha_I = \beta_i$.

Expected phenotypic distributions were computed using allelic distributions and assuming each locus to be at Hardy–Weinberg equilibrium, at each location. The phenotype was considered to be a seven-state random variable. The log-likelihood of a sample was computed from the phenotypic multinomial distribution. Let n_{ij} and f_{ij} be the observed number and expected frequency of individuals having phenotype i in population j, respectively. The log likelihood L of observing all the data is proportional to

$$L = \sum_i \sum_j n_{ij} \ln(f_{ij}).$$

It was maximized for parameters joint estimation, using the Metropolis algorithm (see Lenormand *et al.*, 1999,1998b; Lenormand & Raymond, 2000). Model simplification was performed using likelihood ratio tests corrected for over-dispersion (Lebreton *et al.*, 1992; Anderson *et al.*, 1994). Over-dispersion was computed from the full model as the ratio of residual deviance over residual d.f. We computed the percentage of total deviance explained by a model (%TD) as

$$\mathrm{TD}(\%) = (D_{\max} - D_{\mathrm{model}})/(D_{\max} - D_{\min}),$$

where the maximal deviance ($D_{\max}$) is obtained by estimating the allele's average frequency among populations and the minimal deviance ($D_{\min}$) by estimating the allele's frequency independently in each population.

Departure from Hardy–Weinberg proportions F at the *Ester* locus was tested (and Bonferroni corrected) in each population (comparing models where F is either estimated or set to zero) and overall (comparing models where F is estimated for each population, averaged over populations or set to zero for all populations) by likelihood ratio tests. Testing for departure from Hardy–Weinberg proportion is possible at the *Ester* locus because seven phenotypes can be observed on electrophoresis gels for four alleles (all alleles are co-dominant markers except the susceptible allele, which is recessive, as for O in ABO blood groups).

Results

Literature data

All data available on the distribution of *Ester*^{2} are summarized in Table 1. The first confirmed report of this gene dates from Liberia and Nigeria in 1977 (Villani *et al.*, 1983; Urbanelli *et al.*, 1985), although the earliest report could possibly be in 1970 in Japan (Yasutomi, 1970). Its present distribution encompasses Africa, Asia, Europe, North and South America, and French Polynesia (Fig. 3).

In some areas, *Ester*^{2} is the only resistance allele reported at the *Ester* locus, such as the sub-Saharan Africa. In other places, one or several resistant alleles are present, in addition to *Ester*^{2}, e.g. China or in southern France. In the latter situations, different *Ester* alleles are competing, and allelic replacement among resistance alleles is possible. In Asia, North America, South America, Caribbean's and French Polynesia, it is most often found with *Ester*^{B1}. In Mediterranean countries, *Ester*^{2} is present with different alleles: *Ester*^{4} mainly in occidental parts, *Ester*^{5} mainly in oriental ones, and *Ester*^{1} in all the Mediterranean Basin. Countries with the most diverse esterase-mediated insecticide resistance are in Asia, with five resistance alleles described in addition to *Ester*^{2}: *Ester*^{B1}, *Ester*^{8}, *Ester*^{9}, *Ester*^{B6} and *Ester*^{B7}. There is several other resistance alleles not yet well characterized which are found together with *Ester*^{2}: one in Tunisia (named A9 in Ben Cheikh *et al.*, 1998) and one in Brazil and Venezuela (described as A6–B6 by Yébakima *et al.*, 1995b; Gonzalez *et al.*, 1999).

Thus, on a world scale, *Ester*^{2} is in competition with all the reported alleles of the *Ester* locus. In the majority of

Table 1 Geographic distribution of *Ester*[2] within each geographic area.

Geographic area	Country	Year of first observation	*Ester* alleles	References
Europe				
Western	Italy, Greece, Spain, Portugal and France	1984	*Ester*[4] *Ester*[1] *Ester*[5]	Bonning *et al.* (1991), Chevillon *et al.* (1995a), Chevillon *et al.* (1995b), Chevillon *et al.* (1995c), Eritja & Chevillon (1999), Gazave *et al.* (2001), Georghiou *et al.* (1988), Guillemaud *et al.* (1998), Rivet *et al.* (1993), Severini *et al.* (1994), Silvestrini *et al.* (1998), Villani & Hemingway (1987), and Unpublished data
Eastern	Cyprus	1987	*Ester*[1] *Ester*[5]	Georghiou *et al.* (1987), Wirth & Georghiou (1996)
Asia				
Southern–west	Sri Lanka, Pakistan and India	1978		Georghiou (1992), Georghiou *et al.* (1987), Peiris & Hemingway (1993), Villani *et al.* (1983) and Unpublished data
Southern–east	Thailand, South Korea, China and Vietnam	1980? 1981	*Ester*^{B1} *Ester*[8] *Ester*[9] *Ester*^{B6} *Ester*^{B7}	Callaghan *et al.* (1998), Georghiou *et al.* (1987), (1988), Jinfu (1999), Liu *et al.* (2000), Maruyama *et al.* (1984), Pasteur *et al.* (2001), Villani *et al.* (1983), Weill *et al.* (2001), and Xu *et al.* (1994)
Japan	–	1970? 1980		Georghiou (1992), Georghiou *et al.* (1987), Maruyama *et al.* (1984), Yasutomi (1970) and Yasutomi (1983)
Middle East	Saudi Arabia and Israel	1983	*Ester*[1]	Georghiou *et al.* (1987), Hemingway *et al.* (1990), (2000), Vaughan *et al.* (1997) and Villani *et al.* (1986)
Africa				
Western	Liberia, Nigeria, Burkina–Faso, Cape Verde, Niger, Senegal, Ivory Coast, Mali, Mauritania and Congo	1977		Beyssat-Arnaouty *et al.* (1989), Callaghan *et al.* (1998), Chandre *et al.* (1997), Magnin (1986), Magnin *et al.* (1988), Majori *et al.* (1986), Urbanelli *et al.* (1985), Villani *et al.* (1983) and Unpublished data
Southern	Tanzania, Kenya, Zimbabwe and South Africa	1979		Callaghan *et al.* (1998), Curtis & Pasteur (1981), Guillemaud *et al.* (1996), Vaughan *et al.* (1997), Villani *et al.* (1983) and Unpublished data
Northern	Egypt, Tunisia and Algeria	1983	*Ester*[4] *Ester*^{A9} *Ester*[5]	Ben Cheikh *et al.* (1998), Ben Cheikh & Pasteur (1993), Villani *et al.* (1986) and Unpublished data
America				
North America	USA (Louisiana, New–Mexico, Texas, California, New Jersey and Tennessee)	1984	*Ester*^{B1}	Georghiou *et al.*, 1987, 1988; Pasteur & Georghiou, 1989; Pietrantonio *et al.* (2000), and Raymond *et al.* (1987)
South America	Brazil, Venezuela and French Guyana	1991	*Ester*[6] *Ester*^{B1}	Gonzalez *et al.* (1999), Qiao & Raymond (1995) and Yébakima *et al.* (1995b))
Caribbean islands	Most countries (Martinique)	1988	*Ester*^{B1}	Small *et al.* (1998), Yébakima *et al.* (1995a)) and Yébakima *et al.* (1995b))
Oceania				
Polynesia	French Polynesia (Society Is., Tuamotu Is., Australs Is.)	1990	*Ester*^{B1}	Pasteur *et al.* (1995)

The country of first observation is underlined. Putative years of first observation are indicated with a question mark (see text). The presence of other resistance alleles of *Ester* locus is also indicated.

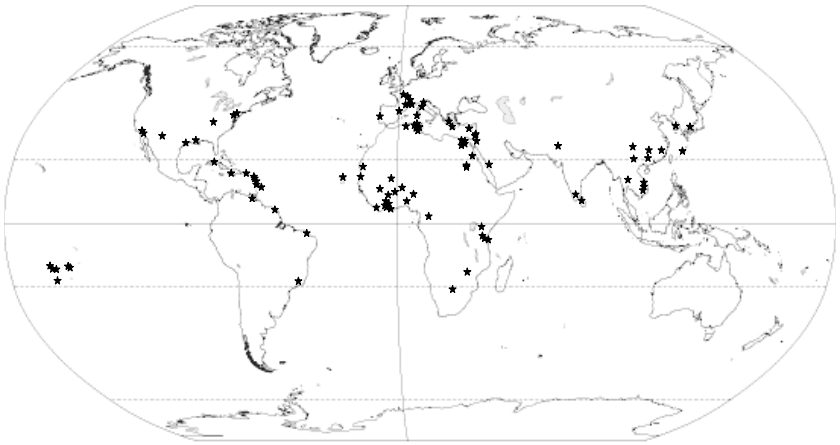

Fig. 3 Global distribution of *Ester*^{2}. Stars (*) show the different places where this resistance gene has been reported (see references in Table 1).

cases, *Ester*^{2} is found at relatively high frequencies (>0.2), and often being the most prevalent resistance gene. There is one exception in the western Mediterranean, where it is found at low frequencies (Severini *et al.*, 1993; Guillemaud *et al.*, 1998; Eritja & Chevillon, 1999).

Information on the process explaining the distribution of *Ester*^{2} and its evolutionary dynamics are limited. Indeed the new occurrence of *Ester*^{2} in a particular region is seldom documented except in France, where its occurrence is closely monitored. Rivet *et al.* (1993) showed that *Ester*^{2} was first detected in southern France in 1986, near the international airport and seaport of Marseille. Then it spread in southern France, and was first detected in the Montpellier area in 1990, where it remained at a low frequency (0.03 in 1995, <0.05 in 1997, Guillemaud *et al.*, 1998).

Experimental data

In order to extend the monitoring (data from 1986 to 1996), the Montpellier area was sampled in summers 1999, 2001 and 2002 (Table 2). Cline models were fitted for resistance alleles frequencies. We first checked if there was no departure from Hardy–Weinberg proportions before using this assumption for estimating allelic clines.

Hardy–Weinberg equilibrium

Hardy–Weinberg equilibrium was not rejected in any population. Only one population displayed a significant deficit in heterozygote before Bonferroni correction (Pérols population, 2001 transect, $F = 0.707$, $\chi^2_1 = 6.12$, $P < 0.05$). Allelic frequencies for each

Table 2 Sampling results for *Ester* phenotypes.

		1999								2001								2002							
Site	Kilometers from the sea	*n*	[0]	[1]	[2]	[4]	[12]	[14]	[24]	*n*	[0]	[1]	[2]	[4]	[12]	[14]	[24]	*n*	[0]	[1]	[2]	[4]	[12]	[14]	[24]
Pérols	2.2	–	–	–	–	–	–	–	–	56	3	5	6	36	0	4	2	–	–	–	–	–	–	–	–
Maurin	5	58	4	5	3	36	2	6	2	78	4	3	6	43	1	8	13	58	3	2	8	34	1	3	7
Lattes	5.2	–	–	–	–	–	–	–	–	58	2	3	6	31	1	6	9	58	4	3	3	32	3	6	7
Orstom	14	58	25	3	2	26	0	2	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Montferrier	17	12	5	2	0	5	0	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Distill	18	–	–	–	–	–	–	–	–	58	16	4	0	34	0	3	1	58	15	2	2	32	1	4	2
StGely	21	–	–	–	–	–	–	–	–	60	19	2	1	33	1	2	2	–	–	–	–	–	–	–	–
Cuculles	26	–	–	–	–	–	–	–	–	58	16	5	3	29	0	3	2	58	21	1	1	35	0	0	0
Viols	30	58	29	1	1	27	0	0	0	58	37	1	0	17	0	2	1	58	32	5	2	19	0	0	0
SML	34	58	37	0	1	19	0	1	0	69	29	2	0	35	0	3	0	58	35	3	1	17	0	1	1
NDL	35	58	41	3	0	12	0	2	0	58	28	0	3	23	0	2	1	58	27	1	0	29	0	0	1
Brissac	43	–	–	–	–	–	–	–	–	58	43	1	2	11	0	0	1	–	–	–	–	–	–	–	–
StBauzille	44	51	28	2	0	19	0	2	0	58	36	2	0	19	0	1	0	57	25	1	0	30	0	1	0
Ganges	49	58	35	2	1	19	0	0	1	68	49	4	1	14	0	0	0	58	27	3	2	23	1	2	0
Total		411	204	18	8	163	2	13	3	737	282	32	28	325	3	34	32	521	189	21	19	251	6	17	18

For each year the numbers of individuals presenting each phenotype are indicated: for nomenclature, see Lenormand *et al.* (1998b)). Briefly, phenotype [i] corresponds to genotypes *Ester*^{i}/*Ester*^{0} or *Ester*^{i}/*Ester*^{i}, and phenotype [ij] correspond to genotype *Ester*^{i}/*Ester*^{j}. *n* represents the total number of mosquitoes analysed for each year at each sampling site. (–) Indicates that the site was not sampled in the corresponding year. See Fig. 2 for details of the sampling location.

population and each year are presented in Table 3. Each year, overall Hardy–Weinberg proportions were not rejected either (for each cline the best model is an average F equal to zero, F-test, $P > 0.05$ in each case).

Descriptive models

A decrease in allele frequency from the coast inland was observed for all resistance alleles (i.e. slope parameters a and/or b different from zero in the best model after model simplification, F-test, $P < 0.05$ in each case) and each year, except for the *Ester*^{2} allele, which presents a clinal pattern only since 1999. The majority of cline models were best fitted by an $\exp(-x)$ shape, with the exception of *Ester*^{4} cline of year 2001 and *Ester*^{1} cline of year 1995, which were best fitted by an $\exp(-x^2)$ shape. The estimated parameters for allelic clines are consistent with those obtained in previous studies (see Table 4 and Guillemaud *et al.*, 1998; Lenormand *et al.*, 1998b; Lenormand & Raymond, 2000). Over-dispersion is low around fitted models, between 0.99 and 1.57. All fitted models explain more than 50% of the total observed deviance (see Table 4).

Time variations of allelic frequencies

After model simplification, the best model describing frequency variations over the 1985–2002 period explains 62.05% of the total deviance with low over-dispersion (= 1.29). This model indicates that (i) *Ester*^{1} changes in frequency at a constant (α_1 not different from β_1, $F_{1,327} = 3.52$, $P = 0.062$) and negative ($\alpha_1 = -0.1452$, $F_{1,327} = 61.38$, $P < 0.0001$) rate, (ii) *Ester*^{2} changes in frequency at a constant (α_2 not different from β_2, $F_{1,327} = 1.35$, $P =$ n.s.) and positive ($\alpha_2 = 0.3835$, $F_{1,327} = 42.26$, $P < 0.0001$) rate, (iii) *Ester*^{4} frequency changes at different rates before and after ~1996 ($t^* = 9.84$, $t_{\min} = 9.43$, $t_{\max} = 10.31$, α_4 different from β_4, $F_{1,327} = 19.11$, $P < 0.0001$): it first increases ($\alpha_4 = 0.3016$, $F_{1,327} = 47.15$, $P < 0.0001$) and then remains constant (β_4 not different from zero, $F_{1,327} = 1.02$, $P =$ n.s.). The fitted frequency variations are illustrated on Fig. 4.

This overall pattern is consistent with previous studies of the decade 1985–1995, during which *Ester*^{2} was almost absent from Montpellier area, and has been detailed by Guillemaud *et al.* (1998). It corresponds to the replacement of *Ester*^{1} by *Ester*^{4} allele. However, in the most recent period (i.e. after ~1996), *Ester*^{4} has not increased further in frequency, whereas *Ester*^{2} frequency increased rapidly. *Ester*^{2} was first reported in 1990 and remained at low frequency ($h < 0.01$) without a clear clinal pattern until 1999. Over the period 1999–2002, however, *Ester*^{2} increased sharply in frequency ($\alpha_2 = 0.3835$) and started to exhibit a clinal pattern (Table 4 and Fig. 5), almost reaching the frequency of 0.2 at the coast in 2002.

Discussion

Global distribution

Ester^2 is widely distributed over the planet, as it has been reported in most places investigated. The actual geographic distribution probably corresponds to areas where OP insecticides are regularly applied. This is indicated by the disappearance of *Ester*^{2} whenever OP insecticides are no longer used. In Spain, OP insecticides were replaced by *Bacillus sphaericus* toxin the year after the first report of *Ester*^{2} (1993), and this allele was not found in later samples (Eritja & Chevillon, 1999). In Lucca, Italy, insecticide treatments were stopped in 1989, and *Ester*^{2}, which was detected in 1988, no longer found in 1992. Across Italy, *Ester*^{2} was reported from places where OPs were continuously used, and was not detected in places where treatments have been stopped (Silvestrini *et al.*, 1998). This suggests that a fitness cost is associated with *Ester*^{2}, thus precluding the presence of *Ester*^{2} at high frequencies in non-treated areas.

Dynamics of *Ester*^{2} invasion

The current geographical distribution of *Ester*^{2} could be explained by four non-exclusive hypotheses. First, *Ester*^{2} could have been the first resistance gene to occur at the *Ester* locus, thus having an opportunity to spread widely, before other resistance alleles appeared (*historical contingency hypothesis*). Alternatively, *Ester*^{2} may possess a fitness advantage over the other resistant alleles, either locally (i.e. only in some environmental conditions) or globally (i.e. in all areas), and its present distribution would be the result of its competitive advantage (*local* or *global selection hypotheses*). Third, it may be neutral with respect to other resistance genes, and chance alone explains this distribution (*drift hypothesis*). It is probably impossible to reject the *historical contingency hypothesis*, due to the lack of data during the 1960s and early 1970s, i.e. at a time where the first *Ester* resistance gene occurred. The pattern of replacement in resistance alleles and their global distribution do not suggest that *genetic drift* between resistance alleles (i.e. neutrality among resistance alleles) plays a predominant role: drift would generally create random fluctuations, which are not observed at a local scale (for example in the Montpellier area). Moreover, due to the large population sizes observed for this species, the time needed for a replacement between two resistance alleles (e.g. 7 years, Guillemaud *et al.*, 1998) would be much longer than that observed if the process was driven by genetic drift only. Longitudinal studies are required to evaluate the *selection* hypotheses. To our knowledge, only eight cases are documented in the literature (Table 5), and five of them document the interaction of *Ester*^{2} with another resistance allele. In California, only *Ester*^{B1} was reported before 1979 (Pasteur & Georghiou, 1980). Then, *Ester*^{2}

Table 3 Estimated frequencies for each population.

	1995				1996				1999				2001				2002			
Site	*Ester*^{1}	*Ester*^{2}	*Ester*^{4}	F_{is}	*Ester*^{1}	*Ester*^{2}	*Ester*^{4}	F_{is}	*Ester*^{1}	*Ester*^{2}	*Ester*^{4}	F_{is}	*Ester*^{1}	*Ester*^{2}	*Ester*^{4}	F_{is}	*Ester*^{1}	*Ester*^{2}	*Ester*^{4}	F_{is}
Brissac	–	–	–	–	–	–	–	–	–	–	–	–	0.009	0.026	0.109	0.000	–	–	–	–
Cournonteral	0.150	0.016	0.372	0.000	–	–	–	0.000	–	–	–	–	–	–	–	–	–	–	–	–
Cuculles	–	–	–	–	–	–	–	–	–	–	–	–	0.072	0.044	0.356	0.297	0.009	0.009	0.372	0.000
Distill	–	–	–	–	–	–	–	–	–	–	–	–	0.062	0.009	0.411	0.000	0.061	0.044	0.409	0.000
Ganges	0.011	0.000	0.139	0.000	0.024	0.006	0.138	0.000	0.017	0.017	0.190	0.025	0.030	0.007	0.109	0.000	0.053	0.026	0.246	0.000
Lattes	–	–	–	–	0.140	0.000	0.481	–	–	–	–	–	0.091	0.152	0.548	0.295	0.108	0.117	0.532	0.000
Maurin	0.109	0.003	0.476	0.000	–	–	–	0.000	0.119	0.063	0.527	0.438	0.080	0.137	0.567	0.000	0.053	0.153	0.524	0.472
Montferrier	–	–	–	–	–	–	–	–	0.088	0.000	0.240	0.000	–	–	–	–	–	–	–	–
NDL	0.017	0.006	0.156	0.003	0.016	0.000	0.116	0.000	0.044	0.000	0.128	0.000	0.018	0.036	0.260	0.000	0.009	0.009	0.304	0.000
Orstom	–	–	–	–	–	–	–	–	0.044	0.017	0.281	0.348	–	–	–	–	–	–	–	–
Perols	0.141	0.029	0.427	0.000	–	–	–	0.000	–	–	–	–	0.128	0.114	0.520	**0.707**	–	–	–	–
SML	0.029	0.003	0.168	0.000	0.008	0.008	0.197	0.856	0.009	0.009	0.190	0.000	0.037	0.000	0.327	0.000	0.035	0.017	0.179	0.000
St Bauzille	0.039	0.004	0.156	0.051	0.024	0.000	0.162	0.000	0.040	0.000	0.231	0.000	0.026	0.000	0.190	0.000	0.018	0.000	0.324	0.001
St Gely	0.079	0.005	0.246	0.621	0.087	0.000	0.293	–	–	–	–	–	0.042	0.034	0.379	0.000	–	–	–	–
Triadou	0.066	0.000	0.389	0.000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Viols	0.043	0.008	0.203	0.000	–	–	–	–	0.009	0.009	0.270	0.000	0.026	0.009	0.188	0.000	0.044	0.017	0.181	0.000
Sumène	–	–	–	–	0.023	0.000	0.145	–	–	–	–	–	–	–	–	–	–	–	–	–

For each year and each population the frequency of each *Ester* allele, and the estimated F_{is} is indicated. Bold numbers identify significant F_{is} (likelihood ratio tests, but see text). (–) Indicates that the site was not sampled in the corresponding year. See Fig. 2 for details of sampling location.

Table 4 Parameters of the best-fitted clines.

	Allele *Ester*^{1}			Allele *Ester*^{2}			Allele *Ester*^{4}			
Year	h	$a \times 10^{-4}$	$b \times 10^{-2}$	h	$b \times 10^{-4}$	$b \times 10^{-2}$	h	$a \times 10^{-4}$	$b \times 10^{-2}$	TD (%)
1995	0.129	10.23	–	0.008	–	–	0.515	–	2.915	70.7
1996	0.191	–	4.813	0.002	–	–	0.486	–	2.591	59.9
1999	0.127	–	4.504	0.07	–	6.339	0.501	–	2.451	50.6
2001	0.1	–	3.301	0.152	–	6.573	0.551	6.702	–	73.4
2002	0.087	–	2.856	0.195	–	7.61	0.583	–	2.072	60.9

The estimated value of parameters h_{ij}, a_{ij} and b_{ij} of the best model after likelihood ratio tests simplification procedure are given for each allele and each year. TD (%) is the part of total deviance explicated by the model (see text). (–) Means that this parameter is not significantly different from zero in the best model for the year considered, see text.

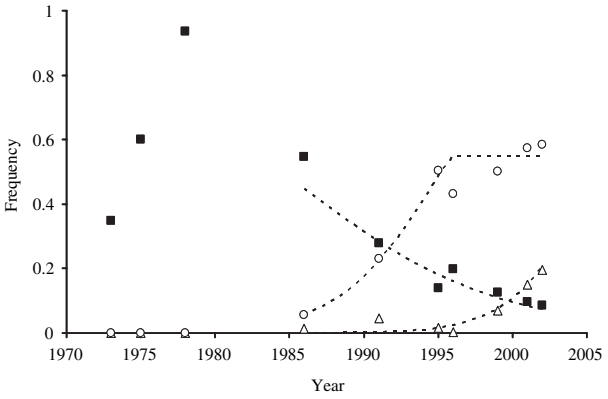

Fig. 4 Evolution of the insecticides resistant alleles in the Montpellier area since the beginning of the organophosphate treatments. The frequencies presented are the maximum frequencies, i.e. at the coast. The first allele that appeared was *Ester*^{1} (black squares), then replaced by *Ester*^{4} (white rounds) (Guillemaud *et al.*, 1998). Recently, a third allele, *Ester*^{2}, invaded the Montpellier area (white triangles). Dotted lines represent the fitted values according to the best logistic model (see material and methods).

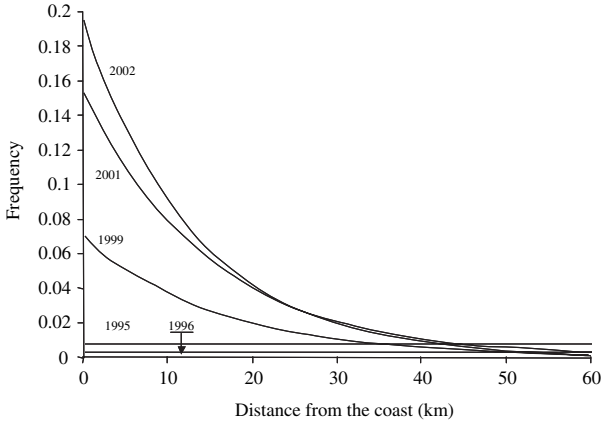

Fig. 5 Recent evolution of *Ester*^{2}-frequency clines in the Montpellier area. Clines of 5 years, over a period extending from 1995 to 2002, are presented: 1995, 1996, 1999, 2001 and 2002. See text for explanations.

was also detected in the same populations in 1984, sometimes at a relatively high frequency (>0.5 at Long Beach in 1985, Raymond *et al.*, 1987), suggesting that the replacement of *Ester*^{B1} by *Ester*^{2} was going on. Unfortunately no additional published data are available to document the evolution of this situation. In Cuba, the same process has apparently taken place: *Ester*^{B1} was first present, then *Ester*^{2} was detected, and its frequency rose and it became the most frequent resistance gene (Small, 1996). In Houston (Texas), *Ester*^{2} is also replacing *Ester*^{B1}. In 1994, 20% of analysed mosquitoes were found carrying *Ester*^{B1}, 40% *Ester*^{B1} and *Ester*^{2}, and 40% only *Ester*^{2} ($n = 104$), whereas in 1998, the frequencies were respectively ($n = 84$) 11.5%, 11.9% and 68.7%, with 7.1% being susceptible mosquitoes (Pietrantonio *et al.*, 2000). The authors explain the replacement by a decrease, since 1993, in malathion (an OP) use: both the number of areas treated and the number of applications decreased, to very low levels (even zero in some areas). In Martinique, the opposite situation is found: in 1990, *Ester*^{2} was at a high frequency (>37% in average, >66% in one population), while *Ester*^{B1} was very infrequent (<3%) and not found in three of seven populations sampled. Nine years later, *Ester*^{B1} had spread to all the sampled populations, with an overall frequency >30%, whereas the susceptible allele has decreased from >60 to <15%. In the same time, *Ester*^{2} decreased in some populations (e.g. from >60 to <30% in Carbet population), while increasing in few others (e.g. from <40 to >90% in Fort de France, alleles frequencies were computed using maximum likelihood from phenotype data provided in Yébakima *et al.*, 2004). At the locations where *Ester*^{B1} has replaced *Ester*^{2}, the authors explain this situation by a change of insecticide usage: during the increase of *Ester*^{B1} frequency, the intensity of insecticide treatment also increased (~10-fold) and the authors show that *Ester*^{B1} confers a higher resistance to insecticides used in Martinique (Temephos) than *Ester*^{2}. In Lucca (Italy), there is a possible replacement of *Ester*^{1} by *Ester*^{2} around 1985–1988 (Bonning *et al.*, 1991; Villani & Hemingway, 1987) although published data are too scarce to firmly document it. However, *Ester*^{2} was present in 1985 in Lucca (Bonning *et al.*, 1991), but it disap-

Table 5 Literature data on allele replacement.

Area	Allele replacement	Observations
California	*Ester*^{2} taking over *Ester*^{B1}	No change in insecticide
Cuba	*Ester*^{2} taking over *Ester*^{B1}	No change in insecticide
Texas	*Ester*^{2} taking over *Ester*^{B1}	OP quantities decreased
Martinique	*Ester*^{B1} taking over *Ester*^{2}	OP quantities increased
Italy	*Ester*^{0} taking over *Ester*^{2}	OP insecticides removed
Barcelona	*Ester*^{0} taking over *Ester*^{2}	OP insecticides removed
Montpellier	*Ester*^{1} taking over *Ester*^{0}	OP introduced
Montpellier	*Ester*^{4} taking over *Ester*^{1}	Temephos partially replacing chlorpyrifos OP quantities decreased
Montpellier	*Ester*^{2} taking over *Ester*^{1} and *Ester*^{4}	No change in insecticide

For Montpellier different replacements occurred through time and are all indicated separately, see text for explanation and references.

peared in 1992, probably because of the stoppage of insecticide treatments (Severini *et al.*, 1994). Overall, this fragmentary dataset suggests that there is no overall trend, and that *Ester*^{2} could either replace or be replaced by another *Ester* allele. Thus, this dataset contradicts the *global selection hypothesis*, and is more consistent with the *local selection hypothesis*. Environmental conditions are probably involved in this interaction, such as the type or quantity of insecticide used. In Martinique, *Ester*^{2} decreased in frequency when treatments were increased, whereas in Houston its frequency increased when treatments were decreased. Both situations suggest that *Ester*^{2} is at a disadvantage when insecticide treatments are intensive. Its fitness advantage in California and Cuba, in conditions where insecticide doses are reduced, could be explained by lower fitness costs rather than better resistance. However, in most places, little information is available on pesticide application practices, precluding any firm conclusions. Nevertheless, this indicates that environmental conditions play a role during the course of adaptation at the local scale. There is an additional situation where a detailed longitudinal study has taken place, allowing a clearer understanding of the selection hypotheses.

Montpellier area

The Montpellier area is the subject of a long-term longitudinal study for the evolution of the resistance genes since 1972 (Pasteur & Sinègre, 1975; Chevillon *et al.*, 1995c; Guillemaud *et al.*, 1998; Lenormand *et al.*, 1999). Since the 1960s, OP treatments have been used on an area extending from the coast to approximately 20 km. Resistance to these insecticides is conferred in this area by different *Ester* alleles. The first to appear was *Ester*^{1}, in 1972, followed by *Ester*^{4} in 1984 and then, *Ester*^{2}, in 1990 (Guillemaud *et al.*, 1998). The *Ester*^{1} and *Ester*^{4} alleles are known to harbour deleterious effects in the absence of OP insecticides (the so-called 'cost' of resistance), because they decline in frequency along transects from treated to non-treated areas (Guillemaud *et al.*, 1998; Lenormand *et al.*, 1999) and they alter fitness-related life history traits (e.g. Berticat *et al.*, 2002a,2004; Agnew *et al.*, 2004). In this study, we show that *Ester*^{2} also is costly in the absence of insecticides, as this allele declines in frequency from the treated coastal area to untreated inland areas (Fig. 5). During the nineties, *Ester*^{4} has replaced *Ester*^{1} (Fig. 4, Guillemaud *et al.*, 1998). This increase in the frequency of *Ester*^{4} also corresponds to an environmental change, as the quantities of OP decrease dramatically from about 2700 L applied per year in the Languedoc–Roussillon region, to about 300 L/year from 1987 to 1992, and to a change from chlorpyrifos to temephos (EID, 1992). As *Ester*^{4} is known to confer a slightly lower OP resistance level, its advantage over *Ester*^{1} could possibly be due to lower costs (Guillemaud *et al.*, 1998).

Although the appearance of *Ester*^{2} in southern France was documented in 1986, it was detected in the Montpellier area for the first time in 1990, at a low frequency (Rivet *et al.*, 1993). It remained at a low frequency (<0.1) during the following years (Guillemaud *et al.*, 1998; Gazave *et al.*, 2001). We report here that it increased in frequency since 1996, reaching a frequency of 0.2 in treated areas. Since the increase in frequency of *Ester*^{2}, the frequency of *Ester*^{1} has continued to decrease at the same rate. During the same period, the frequency of *Ester*^{4} stabilized, after a sharp increase during the period 1986–1996. Although the data seems to indicate a slow increase, we did not detect a significant variation over the 1996–2002 period and strongly rejected the idea of a slower but continuous rise of *Ester*^{4}. Moreover, the higher frequency in 1995 is not an artifact as more than 1200 mosquitoes were analysed for that particular year. Thus, overall, the replacement of *Ester*^{1} by *Ester*^{4} started in 1986 and stopped in 1996, with *Ester*^{2} now replacing both *Ester*^{1} and *Ester*^{4}. This pattern of replacement clearly indicates that *Ester*^{2} enjoys a competitive advantage, at least locally, over previously prevalent resistance alleles. This change in the course of allele replacement is, however, not correlated with any noticeable change in insecticide treatment practices since 1996. This result

confirms that selection plays a major role on the evolution of *Ester* resistance alleles and strengthens the *local selection hypothesis*. It also emphasizes the role of treatment practices in this evolutionary dynamic, at least in the replacement of *Ester*^{1} by *Ester*^{4}.

Four advantages could exist for *Ester*^{2}. First, it could provide better protection against the insecticide used, as the levels of resistance to OP insecticides used in the Montpellier area (temephos and chlorpyrifos) are higher for *Ester*^{2} than for *Ester*^{1} or *Ester*^{4} (Raymond *et al*., 1986; Wirth *et al*., 1990; Poirié *et al*., 1992). Second, *Ester*^{2} may confer lower fitness costs than other *Ester* resistance alleles. Comparisons of life history traits have revealed that mosquitoes bearing *Ester*^{2} fare better than those with other resistance alleles, in particular for larval mortality (Duron *et al*. unpublished data), and endure a lower density of endosymbiotic bacteria (*Wolbachia*, Berticat *et al*., 2002b). These trends are confirmed by population cage experiments in the absence of insecticide, showing that the fitness costs associated with *Ester*^{2} are lower than that of *Ester*^{1}, *Ester*^{4} or *Ester*^{B1} (Berticat, 2001; Berticat *et al*., 2004). The nature of the cost displayed by the various resistance alleles is currently unknown, thus the reduced costs associated with *Ester*^{2} are also enigmatic. Third, as proposed by Guillemaud (1997) and Berticat (2001) for *Ester*^{4} when replacing *Ester*^{1}, *Ester*^{2}, having a greater gene copy number, may recombine less than the other alleles with the locus *ace-1* (same linkage group), whose resistance allele (*ace-1*^{R}) confers a much higher level of insecticide resistance. Thus, *Ester*^{2} would be favoured by hitchhiking with *ace-1*^{R} resistance gene. This hypothesis is currently under consideration. Lastly, the advantage of *Ester*^{2} could be indirect, and due to another gene located in its amplicon, coding for an aldehyde-oxydase (Hemingway *et al*., 2000). According to these authors, this gene is thought to provide resistance against a carbamate insecticide (aldicarb), although this effect has not yet been shown. The effect of a co-amplified gene remains unclear for any advantage it may confer, although it may enhance fitness costs, through biochemical perturbation due to over-expression. Further comparative data between the various *Ester* resistance alleles could settle this issue.

Conclusions

The allele *Ester*^{2} is found globally and its current distribution probably reflects the areas where OP insecticides are regularly used. There are four possible explanations for this prevalence: the *historical contingency*, the *drift* and the *global* and *local selection* hypotheses. The data available in the literature, although not refuting the *historical contingency* hypothesis, show that *selection* is also at work, as in five cases *Ester*^{2} has replaced one or several previous *Ester* resistance alleles. *Drift* between resistance alleles does not appear to be a major mechanism in this process. A long-term longitudinal study the Montpellier area has found evidence that *Ester*^{2} is taking over previously prevalent resistance alleles (*Ester*^{1} and *Ester*^{4}). So, at least in southern France, this allele confers a sufficient fitness advantage to be selected for in conditions where other resistant alleles were already present at high frequencies, offering a unique opportunity to distinguish between selection, drift and historical events at the gene level. Different types of advantages could explain the selection of *Ester*^{2}, although the data are currently inconclusive, and it is not excluded that several coexist. However, in Martinique another allele is currently replacing *Ester*^{2}, showing that the selective advantages of *Ester*^{2} do not appear to be constitutive and are probably dependent on environmental conditions, weakening the *global selection hypothesis*. The data suggest that *Ester*^{2} may be the best allele so far, but only in moderately treated areas. This conclusion is consistent with the *local selection hypothesis*. The global distribution of this allele would then simply reflect the fact that most treated areas are now moderately treated. The traditional way of using insecticides is a high dose strategy, which favours alleles conferring strong resistance despite a high cost (Denholm & Rowland, 1992). Modern practices are more refined, thus decreasing insecticide quantities, with the consequences that an alternative resistance allele becomes fitter. This must be taken into account in insecticide treatment strategies. The importance of environmental conditions should also be considered when studying the evolution of such adaptations to anticipate and predict the long-term outcomes. In the case of insecticide resistance, an increased knowledge of pesticide applications across the planet is required to improve our understanding of the process of allele replacement at local and global scales.

Allele replacement is not an instantaneous process: it took approximately 7 years for *Ester*^{4} to become prevalent over *Ester*^{1} (Fig. 4) and *Ester*^{1} is still present at a low frequency 14 years later (Guillemaud *et al*., 1998; this study). So, in countries where *Ester*^{2} was the only allele described during the 1980s (e.g. Sub-Saharan Africa and southern-western Asia, see Table 1), allele replacement is probably not yet complete. In these regions, *Ester*^{2} was probably the first allele to appear and spread and, there, this historical event contributed to its present prevalence.

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