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POPULATION STRUCTURE AND DYNAMICS OF SELECTED GENES IN THE MOSQUITO *CULEX PIPIENS*

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Abstract.—To determine the outcome of the combined effects of gene flow, genetic drift, and selection on the evolution of insecticide-resistance genes in the mosquito *Culex pipiens*, samples were collected along three transects crossing treated and nontreated areas in northern Spain and southern France. Electrophoretic polymorphisms of five presumably neutral genes disclosed that differentiation among samples was low, and that both Wright *F*-statistics and Slatkin private-alleles methods provided a high estimate for *Nm*. In contrast, there was a strong differentiation in the distribution of resistance genes closely associated with insecticide treatments. These divergent situations are explained in relation to both the very recent origin of some resistance genes that are still localized geographically (A2-B2 and C1), and the high fitness cost of the older and ubiquitous ones in nontreated areas (A1, A4-B4, and Ace^R).

Key Words.—Fitness cost, gene flow, insecticide resistance genes, mosquito, selection.

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The wide use of organic insecticides to control agricultural and public-health pests has been a powerful agent of selection in natural populations of many insect species that have developed various degrees of resistance (Georghiou and Mellon 1983; Georghiou and Lagunes-Tejeda 1991). These species provide a unique opportunity to study genetic adaptation to new environmental conditions in nature. As with industrial melanism in insects (e.g., Bishop and Cook 1981), resistance to insecticides has developed extremely rapidly, that is, over the last 50 years. The physiological basis of insecticide resistance is well understood in many cases (e.g., Mullin and Scott 1992), and, in a few species, such as the mosquito *Culex pipiens*, identification of each resistance gene in single individuals is possible. Thus, unlike industrial melanism, adaptation to the new environment can be analyzed in relation to changes in gene frequencies, rather than through their global phenotypic expression.

In *Culex pipiens*, resistance to OP (organophosphorous) insecticides is due to two main mechanisms: (1) modification of the target acetylcholinesterase that becomes less susceptible to inhibition by OPs (Raymond et al. 1986; Bisset et al. 1991), probably because of point mutation (Fournier et al. 1993), and (2) increased detoxification through an increased production of esterases (Fournier et al. 1987) that degrade or sequester OPs before they reach their target (Cuany et al. 1993). Recent studies (Raymond et al. 1991) pointed out that oversea migration (probably associated with human transports) was an important factor in explaining the present worldwide distribution of certain resistance genes in the species. Here we have investigated the outcome of gene flow, genetic drift, and selection on the distribution of resistance genes in adjacent populations. This study was conducted in the Languedoc-Roussillon Province (southern France), where the evolution of OP resistance genes has been documented since the early seventies when they were first detected (Pasteur and Sinègre 1975; Sinègre et al. 1977), as well as in regions south (Spain), east (French Riviera), and north (Rhône-Alpes valley) of this Province.

The *Culex pipiens* control program (with an organization treating up to 20,000 larval breeding sites each year), involving only the OP chlorpyrifos, was initiated along the Languedoc-Roussillon Mediterranean coast in 1969. The first resistance gene (the detoxifying esterase A1) occurred in 1972, and was initially restricted to a single village. Esterase A1 spread rapidly and was present throughout the treated area in 1978 (Pasteur et al. 1981a). In 1978, chlorpyrifos resistance increased sharply, an event that was subsequently attributed to the appearance of a second resistance gene, Ace^R, which codes an altered acetylcholinesterase target (Raymond et al. 1985b, 1986). Like esterase A1, the Ace^R gene rapidly increased in frequency and geographical range, impairing the efficacy of chlorpyrifos to the extent that other insecticides were needed to regain adequate control. Consequently, temephos and fenitrothion (two OP insecticides) were introduced into the control program from 1981 onward. Later, two other resistance factors occurred, the associated esterases A4 and B4, and the associated esterases A2 and B2 (hereafter termed A4-B4 and A2-B2). A4-B4 and A2-B2 were first detected in Languedoc-Roussillon in 1984 (Poirié et al. 1992) and 1986 (Rivet et al. 1993), respectively.

Control programs against *Culex pipiens* were initiated in other regions of France, Italy, and northern Spain during the seventies and early eighties (Villani et al. 1982; Consell comarcal del baix Llabregat 1989; Sinègre et al. 1976; Rivet et al. 1994), all using OP insecticides. Whenever genetic surveys were conducted on these more recently treated areas, the resistance genes detected were the same as those of southern France (Villani et al. 1982; Severini et al. 1993, 1994; Rivet et la. 1994; Raymond and Marquine 1994; Raymond and Pasteur unpubl. data). The only exception was the esterase named C1, which has only been found in Corsica and the central part of eastern France (Raymond and Marquine 1994; Rivet et al. 1994).

Although these results suggest that migration plays an important role in the evolution of resistance in these populations, they give no information on gene flow or the impor-

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TABLE 1. Characteristics of the populations sampled along the three transects I, J, and K. Location refers to the nearest town or village. Treatments are qualified by a letter: A indicates no treatment; B indicates treated with temephos since 1990; C indicates treated with temephos since 1983; D indicates treated with chlorpyrifos since 1969 plus temephos since 1981; E indicates sporadical treatment; F indicates treated with chlorpyrifos and temephos since 1975; G indicates treated with temephos since 1980; H indicates treated with temephos since 1973.

Code	Location	Collection date (mo–d)	Type of site	Treatment
I transect				
I1	Alcanar	6–04	Ditch	A
I2	Deltebre	6–04	Ditch	B
I2b	Camarles	6–04	Sewage station	B
I3	Montbrio del Campo	6–03	Water tank	A
I4	Gava (02.04)	6–06	Ditch	G
I4b	Gava (26.03)	6–06	Ditch	G
I5	Montseny	6–07	Ditch	A
I6	El Pont de la Selva	6–03	Sewage station	A
I7	Villamalla	6–07	Sewage station	C
I8	Canohès	6–11	Sewage station	D
I9	Peyriac	6–11	Sewage station	D
I10	Vendres	6–21	Sewage station	D
I11	Saint Brés	7–14	Sewage station	D
I12	Saujean	7–31	Ditch	D
I13	Rognac	6–14	Sewage station	E
I14	Signes	6–26	Sewage station	E
I14b	Cannet-des-Maures	6–26	Sewage station	E
I15	Puget s/ Argens	6–26	Ditch	E
I15b	Bagnols-en-Forêt	6–26	Sewage station	E
I16	Rouret	6–26	Sewage station	E
I16b	Chateauneuf-les-Grasses	6–16	Sewage station	E
I17	Galéria (Pirio)	7–01	Polluted puddle	H
J transect				
J1	Rognonas	6–26	Sewage station	E
J2	Grillon	6–26	Sewage station	E
J3	Suze-la-Rousse	6–26	Sewage station	E
J4	Chonas	6–05	Ditch	A
J5	Jonage	8–26	Ditch	F
J5b	Neyronde	6–06	Sewage station	A
J5c	Crey	7–05	Sewage station	F
J6	Macon	6–12	Sewage station	F
J7	Branges	7–02	Ditch	A
K transect				
K1	Montpellier (St Pierre)	6–24	Sewer	D
K2	Montpellier (Univ.)	5–25	Polluted puddle	D
K3	Prades-le-Lez (Distil.)	6–18	Sewage station	D
K4	Triadou	6–20	Sewage station	D
K5	Viols le Fort	6–13	Sewage station	A
K6	St Martin de Londres	6–13	Sewage station	A
K7	Brissac	6–13	Sewer	A
K8	St Bauzille de Putois	6–13	Sewage station	A
K9	Ganges	6–13	Sewage station	A

tance of recurrent migration. Occurrence of a resistance gene in discrete controlled areas can be due to an anecdotal migration of a single resistant individual, the offspring of which has been selected because of insecticide treatments. Conversely, its absence in an area close to a controlled zone may result from either a limited migration or a high pleiotropic cost associated with the resistance gene.

Here we address the following points: (1) Is the balance between genetic drift and gene flow sufficient to explain the rapid spread of resistance genes in France and Spain? (2) Is the variability of electrophoretic variants (presumably neutral) and resistance genes differently distributed geographically? (3) Are resistance genes neutral in untreated areas?

MATERIALS AND METHODS

Mosquito Samples.—Forty epigeous breeding sites of *Culex pipiens* were sampled as egg rafts, larvae, or pupae from June 3 to July 31 1991, following three transects I, J, and K (table 1, fig. 1). To obtain a regular sampling distribution, each transect was divided in segments of equal length, and one (sometimes two) breeding site was sampled in each segment. Segment lengths measured 50 km for transects I and J, and 6 km for transect K. Transect I covers 850 km and follows the Mediterranean coast from Valencia (Spain) to Nice (French Riviera); it contains 16 segments (21 samples) and also includes an outgroup (I17) collected in Corsica. Transect J length measures 360 km and goes upstream through the

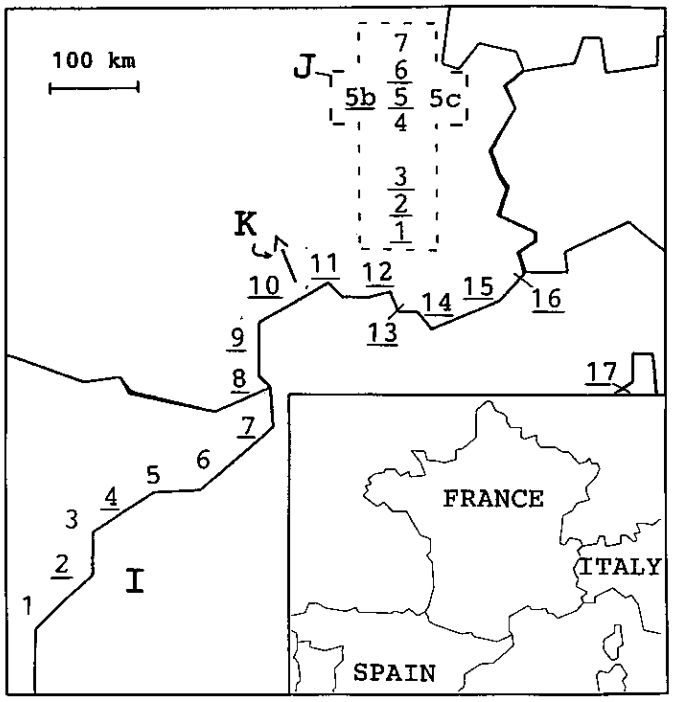

FIG. 1. Geographical location of sampled breeding sites in the three transects. Samples separated by less than 15 km are not indicated. The K transect is represented by a line because of its small range. Underlines figures indicate the presence of insecticide control (for further information on treatments, see table 1).

Rhône valley from the Mediterranean coast; it contains 8 segments (7 samples) and two additional samples 50 km west (J51) and east (J52) of the J5 segment. Transect K length measures 50 km; it is perpendicular to the coastline from Montpellier to Ganges, and contains 9 segments (9 samples). Preimaginal stages were reared until the adult stage under standard laboratory conditions (Raymond et al. 1987).

Insecticide Control against Culex pipiens.—Mosquito control is patchily distributed along the three transects (table 1). In Spain, only three small areas are treated with temephos (I2, I4, and I7), separated by untreated places. In France, the coast from I8 to I12 is treated with OP insecticides (mainly chlorpyrifos, temephos, and fenitrothion); from I13 to the Italian border, treatments are sparse and occasional. On the J transect, treatments are occasional in the south (J1-J3) and intensive and recent around Lyon (J5b-J6), where temephos is mainly used (Rivet et al. 1994). Transect K runs across the coastal areas with intensively treated OP (K1-K4) and goes into the untreated inland (K5-K9).

Identification of Known Resistance Genes.—The acetylcholinesterase genotypes were determined on single mosquitoes by the microplate test of Raymond and Marquine (1994). S-LAB (Georghiou et al. 1966), strains were used as susceptible controls and MSE (Raymond et al. 1986) strains were used as (Ace^{SS}) and resistant (Ace^{RR}) controls.

Presence or absence of highly active esterases was determined on single mosquito homogenates by starch-gel electrophoresis in TME 7.4 buffer systems (Pasteur et al. 1988). Mosquitoes with known overproduced esterases were run in each gel as controls: esterase A1 (strain BARRIOL which replaces the S54 strain of Pasteur et al. 1981b), esterases A4-B4 (strain VIM, Poirié et al. 1992), and esterases A2-B2 (strain SELAX, Wirth et al. 1990). Because of the fast staining procedure needed to score highly active overproduced esterases, esterases B with normal activity, such as the $Est\text{-}2^{0.64}$ allele, were only noted when there was no possible ambiguity ($Est\text{-}2^{0.64}$ occurs in the referenced BARRIOL strain).

Overproduced esterases A4-B4 and A5-B5 that have the same electrophoretic mobilities under our experimental conditions were discriminated by the RFLP (restriction fragment length polymorphism) pattern of the amplified region encompassing the esterase-B structural gene (Poirié et al. 1992). This was conducted on single mosquitoes from seven samples (K2 and K4 to K9). DNA was obtained, as described in Raymond and Marquine (1994), digested with *Eco*RV restriction enzyme, run on agarose gel, transferred onto nylon membrane, and hybridized with the 1.3 kb cDNA B1 probe of Mouchès et al. (1990), as described in Raymond et al. (1989a).

Genotype Determination of Neutral Genes.—The electrophoretic polymorphism at five enzymatic loci was studied by starch-gel electrophoresis (TME 7.4 buffer systems) as described in Pasteur et al. (1988): *Aat-1* and *Aat-2* (two aspartate amino transferases, EC 2.6.1.1.1), *Pgm* (phosphoglucomutase, EC 2.7.5.1.), *Gpi* (glucose phosphate isomerase, EC 5.3.1.9), and *Hk* (hexokinase, EC 2.7.1.1.). Strains used for mobility references were the same as above.

Statistics.—Hardy-Weinberg equilibrium was tested by the Fisher exact test (Fisher 1935 in Weir 1991) for loci with four or less alleles, using either the BIOSYS package (release 1.7) when two alleles were present, or a Quick-Basic version of the Louis and Dempster (1987) program that was translated from a Fortran listing provided by E. J. Louis (Institute of Molecular Medicine, Oxford) and tested with the table 2 data of Louis and Dempster. For loci with 5 or more alleles, the exact P-value was estimated by resampling without replacement, as described by Guo and Thompson (1992). The Quick-Basic program built for these computations uses the pseudorandom number generator of Marsaglia et al. (1990) and was thoroughly tested by comparing results with those published by Louis and Dempster (1987) and Guo and Thompson. As suggested by Guo and Thompson, 17,000 resamplings were performed for each test.

Possible slight but systematic excesses or deficits in heterozygotes for each locus were analyzed with the program provided by Rousset and Raymond (1995) that combines to obtain overall statistics and the independent exact probabilities of excess or deficit in heterozygotes computed within each sample.

F-statistics were computed using the BIOSYS package (release 1.7) or Weir's (1990) program provided by J. Goudet (University of Bangor, Wales). The significance of the mean F_{is} for each locus and sample was considered to be the same as that of the above for the Hardy-Weinberg equilibrium test. Mean and variance of each locus F_{st} estimate were established using a jackknife procedure over populations, and the 95% confidence limits of multiloci estimates were established using a bootstrap procedure over loci (Weir 1990). The homogeneity of the allelic composition between samples was

TABLE 2. Frequencies (expressed in percentages) of mosquitoes with overproduced esterases and of the Ace^R allele. *Tot* refers to the frequency of mosquitoes with at least one overproduced esterase. For the *Ace* locus, the frequency of the Ace^R gene and the F_{is} parameter are indicated, and *P* refers to the Hardy-Weinberg exact test. Significant ($P < 0.05$) nonzero values are indicated by a star and are in bold characters when they are still significant, taklng into account multiple testing. Sample sizes are in parentheses.

	Esterases						*Ace*			
Sample	A1	A4-B4	A2-B2	C1	*Tot*	(*N*)	Ace^R	*P*	F_{is}	(*N*)
I1	—	—	—	—	0	(28)	63	0.003	−0.60*	(28)
I2	—	—	—	—	0	(31)	64	0.398	−0.24	(26)
I2b	—	3	—	—	3	(28)	64	0.004	−0.60*	(29)
I3	—	—	—	—	0	(31)	50	0.003	−0.59	(29)
I4	32	67	—	—	100	(30)	56	0.001	−0.65*	(27)
I4b	36	32	—	—	45	(31)	62	0.021	−0.46*	(29)
I5	—	—	—	—	0	(25)	0	/	/	(29)
I6	5	—	—	—	5	(30)	2	1	−0.02	(29)
I7	3	9	—	—	13	(32)	7	1	−0.07	(29)
I8	47	9	—	—	56	(32)	76	0.154	−0.32	(29)
I9	—	12	—	—	12	(32)	66	0.096	−0.31	(29)
I10	28	22	—	—	47	(32)	64	0.045	−0.42*	(29)
I11	31	50	—	—	66	(32)	43	0.131	−0.34	(29)
I12	24	18	—	—	58	(29)				
I13	25	6	19	—	43	(32)	64	0.045	−0.42	(29)
I14	—	6	10	—	16	(31)	3	1	−0.04	(29)
I14b	3	3	6	—	12	(32)	9	1	−0.10	(29)
I15	10	0	—	—	10	(31)	0	/	/	(29)
I15b	—	0	—	—	7	(28)	2	1	−0.02	(29)
I16	3	13	3	—	19	(31)	5	1	−0.05	(29)
I16b	3	—	—	—	3	(31)	2	1	−0.02	(29)
I17	18	25	—	7	46	(28)	9	1	−0.09	(29)
J1	31	34	—	—	55	(32)	12	1	−0.14	(29)
J2	6	9	—	—	12	(32)	10	1	−0.11	(29)
J3	22	16	—	—	31	(32)	23	0.287	−0.29	(29)
J4	3	—	—	—	3	(32)	3	1	−0.04	(29)
J5	15	—	—	—	15	(32)	31	0.077	0.36	(29)
J5b							0	/	/	(29)
J5c							5	1	−0.05	(29)
J6	15	—	—	—	15	(32)	31	0.077	0.36	(29)
J7	—	—	—	—	0	(32)	0	/	/	(29)
K1	9	16	—	—	25	(32)	71	0.765	−0.02	(53)
K2	26	13	—	—	34	(30)	40	0.435	−0.22	(29)
K3	34	17	6	—	51	(29)	65	0.578	0.15	(13)
K4	47	37	—	—	66	(32)	62	0.158	−0.41	(21)
K5	37	16	—	—	50	(32)	21	0.296	−0.27	(28)
K6	47	34	—	—	61	(31)	53	1	−0.04	(29)
K7	19	—	—	—	21	(32)	23	1	−0.10	(28)
K8	27	9	—	—	35	(31)	26	0.636	−0.17	(29)
K9	19	9	—	—	30	(30)	36	0.239	−0.27	(29)

also tested with an unbiased approximation of the Fisher exact test on R × C contingency tables (Raymond and Rousset 1994).

Gametic associations were tested in computing the unbiased composite-linkage disequilibrium, or Δ_{ij} (Weir 1991), for all possible pairs of alleles at distinct loci in each population. Either genetic drift or systematic selection pressures acting on pairs of loci can create a linkage disequilibrium between two alleles *i* and *j*. To discriminate between the two situations, Ohta (1982) proposed to decompose the observed gametic associations on the whole data set (D_{it}) in four indices that estimate the parts created within populations (D_{is} and D'_{is}) and between populations (D_{st} and D'_{st}). The discrimation is based on the comparisons of D_{is} and D_{st} values, on one hand, and of D'_{is} and D'_{st} values, on the other hand. These indices were computed using the Linkdis program (Black and Krasfur 1985) provided by J. Cuguen (University of Lille I, France).

The number of effective migrants (*Nm*) was estimated by two methods: first, from the *F*-statistics of each locus according to the equation $Nm = (1/F_{st} - 1)/4$ (Wright 1969) (this formula assumes the neutrality of the polymorphic genes and an island model of migration [e.g., Hartl and Clarke 1989]); second, by the method of private alleles described by Slatkin (1985). Isolation by distance was tested as described by Slatkin (1993) on data from the I transect. As this transect follows the seacoast, the geographical distances between samples were measured along the coast, assuming that the sea was a barrier to migration.

Independence among loci was investigated among samples, using the nonparametric Spearman test of correlation, and within samples, using a χ^2 contingency table.

The significance level for each test was adjusted to take into account the other tests using the sequential Bonferroni method as described by Rice (1989). Overall significance of several independent tests was estimated by Fisher's method (Fisher 1970 in Manly 1985).

TABLE 3. Comparisons of resistance-genes frequencies between treated and untreated areas in each transect and in the entire data set. χ^2 is calculated on 2 × 2 contingency tables (df = 1), P refers to the probability of a bilateral test.

	I		J		K		IJK	
Transect	χ^2	P	χ^2	P	χ^2	P	χ^2	P
A1	16	$5{,}10^{-5}$	10	10^{-3}	0.06	0.8	1.9	0.17
A4–B4	21	7.10^{-6}	8	4.10^{-3}	2.6	0.1	18	2.10^{-5}
Ace^R	3.5	0.06*	31	2.10^{-8}	15	8.10^{-5}	31	2.10^{-7}

* When the Spanish part of this transect was removed, this test became highly significant (Fisher exact test, $P < 10^{-5}$).

RESULTS

Insecticide Resistance Genes

Acetylcholinesterase (AChE).—An *Ace*^{R} allele (coding an insensitive AChE) was observed in the three transects with a mean frequency of 0.31. The null hypothesis of homogeneity of *Ace*^{R} frequency was strongly rejected (Fisher exact test, $P < 10^{-5}$). The presence of the *Ace*^{R} gene was significantly associated with insecticide control in transect J (table 3), and its frequency decreased significantly (Spearman rank correlation: $R = -0.65$; $P < 0.05$) along transect K running from a heavily controlled coastal area to an untreated zone. In transect I, the association of *Ace*^{R} with treated areas was less significant ($P < 0.03$, unilateral test, table 3). This was due to the Spanish part of this transect, where *Ace*^{R} frequencies seem independent of treatments (fig. 2). Hardy-Weinberg equilibrium was not rejected (sequential Bonferroni procedure, $P > 0.05$ for 35 samples), except in the I4 sample, which was collected after an insecticide treatment and displayed an excess in heterozygotes. However, overall excess of *Ace*^{RS} heterozygotes was observed (32 of 35 samples displayed a negative F_{is}) and confirmed by the overall statistic computed following Rousset and Raymond's (1995) method ($\chi^2 = 117.4$, df = 66, $P = 0.0001$).

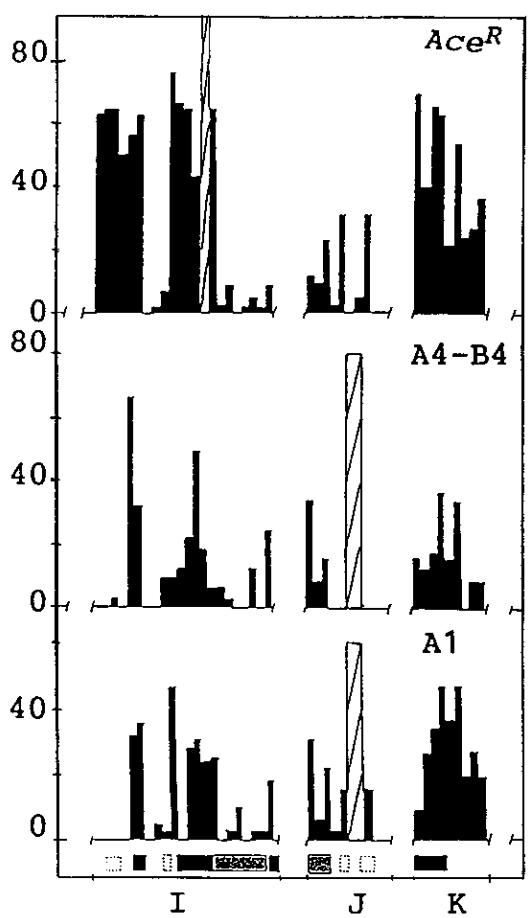

FIG. 2. Frequency distribution (in percentages) of resistance genes *Ace*^{R}, A4–B4, and A1 along the transects I, J, and K. Striped bands indicate missing data. Intensity of insecticide treatments (as coded in table 1) are represented below—histograms by a black bar for D, G, or H types, a gray bar for E type, and a white bar for B, C, or F types.

Overproduced Esterases.—Starch-gel electrophoresis revealed overproduced esterase A1, A2 and B2, A4 and B4, and C1. Esterase A1, observed in all transects with a mean frequency of 0.16, was significantly associated with treated areas in transects I and J, but not in transect K (table 3). Esterases A2 and B2 were always found together, as in every previous survey (Rivet et al. 1993, 1994). They were detected in four samples from the I and K transects, with a mean frequency in these samples of 11%. Esterase C1 was only observed in Corsica (I17), with a frequency of 7%.

Overproduced esterases A4 and B4, or A5 and B5 (see below), were present in the three transects, at a mean frequency of 0.14. They were strongly and significantly associated throughout the entire dataset ($\chi^2 = 814$, df = 1, $P < 0.0001$) and within each population ($\chi^2 > 7.5$, df = 1, $P < 0.006$). Because A4-B4 and A5-B5 esterases cannot be discriminated by electrophoresis, the RFLP pattern of the amplified esterase-B region was studied on individual mosquitoes from seven transect K populations. No mosquito exhibited the B5 pattern. Because of the strength of the association between A and B esterases (see above), the absence of A5 is probable. The B4 pattern was identified in 49 of 143 mosquitoes, which is significantly ($P < 0.001$) higher than the proportion (38/180) of B4 detected by electrophoresis (this discrepancy is expected, because mosquitoes with a very low level of amplification are always detected with DNA techniques but may produce an amount of B4 protein insufficient for electrophoretic detection). Esterases A4-B4 were significantly ($P < 0.001$) associated with treated areas in transects I and J, but not in transect K (table 3).

Linkage Disequilibrium between Esterase A1 and the Est-2$^{0.64}$ allele.—The presence or absence of the *Est-2*$^{0.64}$ allele was unambiguously determined in 640 individuals from 19 populations. In this restricted data set, 9 of 129 individuals with A1 lacked the *Est-2*$^{0.64}$ allele, and one individual with *Est-2*$^{0.64}$ lacked A1. This association is highly significant for

TABLE 4. Allelic frequencies observed at five electrophoretic genes for the 34 populations. Alleles are coded by a letter, and *N* is the sample size.

	Samples																
Locus	I1	I2	I2b	I3	I4	I4b	I5	I6	I7	I8	I9	I10	I11	I12	I13	I14	I14b
Aat-1																	
(*N*)	28	32	61	26	30	63	36	55	30	28	30	30	32	24	32	31	32
A	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0	0	0	0
C	0.02	0.02	0.03	0	0	0.04	0.07	0.11	0.10	0.02	0	0.03	0.03	0	0	0	0
D	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0
E	0.80	0.83	0.76	0.89	0.92	0.83	0.63	0.73	0.65	0.80	0.87	0.67	0.66	0.73	0.83	0.84	0.91
F	0	0	0	0.10	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0.18	0.16	0.21	0	0.08	0.13	0.30	0.16	0.23	0.18	0.13	0.28	0.31	0.27	0.16	0.16	0.09
I	0	0	0	0	0	0	0.01	0	0	0	0	0.02	0	0	0.02	0	0
Aat-2																	
(*N*)	29	32	62	28	28	62	28	62	32	29	30	32	32	28	32	31	32
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0.02	0.03	0	0	0.04	0.02	0.02	0.03	0	0.05	0.05	0.11	0.02	0.03	0.08	0.03
E	0.88	0.78	0.82	0.93	0.88	0.87	0.84	0.87	0.78	0.88	0.83	0.78	0.75	0.89	0.81	0.86	0.88
F	0.12	0.20	0.15	0.07	0.13	0.08	0.14	0.10	0.17	0.12	0.18	0.11	0.09	0.09	0.16	0.07	0.09
G	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
I	0	0	0	0	0	0	0	0.01	0.02	0	0	0.06	0.05	0	0	0	0
Pgm																	
(*N*)	28	32	32	0	0	31	35	32	32	28	32	31	32	0	31	30	32
A	0	0	0			0	0	0	0	0	0	0	0		0	0	0
B	0	0	0			0	0	0.01	0	0	0	0	0		0	0	0
C	0.11	0.05	0.06			0.05	0.13	0.13	0.16	0.11	0.25	0.08	0.13		0.02	0.07	0.09
D	0	0	0			0	0	0	0	0	0	0	0		0	0	0
E	0.88	0.92	0.88			0.92	0.84	0.78	0.73	0.88	0.66	0.84	0.83		0.97	0.88	0.83
F	0	0	0			0	0	0	0	0	0	0	0		0	0	0
G	0.01	0.03	0.06			0.03	0.03	0.08	0.11	0.02	0.10	0.08	0.05		0.02	0.05	0.08
I	0	0	0			0	0	0	0	0	0	0	0		0	0	0
Pgi																	
(*N*)	29	31	31	24	31	57	33	32	0	32	0	30	32	0	23	31	26
D	0	0.02	0	0	0	0	0	0		0		0	0		0	0	0.04
E	0.43	0.35	0.40	0.52	0.53	0.49	0.61	0.47		0.45		0.70	0.41		0.87	0.57	0.52
F	0.50	0.63	0.55	0.46	0.45	0.47	0.38	0.52		0.52		0.28	0.45		0.13	0.32	0.33
G	0	0	0	0.02	0	0.03	0	0.02		0.03		0.02	0.14		0	0.11	0.12
H	0.07	0.01	0.05	0	0.02	0.01	0.02	0		0		0	0		0	0	0
Hk																	
(*N*)	28	32	28	29	31	30	34	30	0	0	0	27	32	28	32	31	32
D	0.45	0.45	0.57	0.43	0.63	0.53	0.57	0.58				0.56	0.50	0.45	0.61	0.44	0.58
E	0.55	0.52	0.32	0.57	0.37	0.47	0.43	0.42				0.44	0.50	0.55	0.39	0.56	0.42
F	0	0.03	0.11	0	0	0	0	0				0	0	0	0	0	0

the whole data set and within each population (Fisher exact tests, $P < 0.0001$).

Independence between Variables.—Independence between resistance genes was rejected for all combinations when the entire data set was considered (Spearman rank correlation, $P < 0.05$). This statistical dependence may be the result of either (1) a linkage disequilibrium, or (2) an allelic relationship of the considered characters, or else (3) a covariation in the frequency of resistance genes. The first explanation is probably true for esterases A4 and B4, A2 and B2, and A1 and *Est-2*$^{0.64}$, because all populations concerned displayed a significant positive association (see above), and esterases A and B are known to be coded by two distinct loci at less than 1 centimorgan (de Stordeur 1976; Pasteur et al. 1981a; Wirth et al. 1990; Poirié et al. 1992). The second explanation is probable for the A1/A4 couple; five of the seven samples in which the expected number of mosquitoes with both enzymes was at least two displayed a repulsion (highly significant in three samples; Fisher exact test, $P < 0.003$). The third explanation seems likely for the presence of overproduced esterases and *Ace*^{R}, but could not be tested, because these characters were not determined on the same mosquitoes.

Electrophoretic Genes

The electrophoretic polymorphisms of 5 loci (*Aat-1, Aat-2, Pgm, Gpi,* and *Hk*) were studied on the same mosquitoes in 25 of 34 samples, on different mosquitoes in 3 samples (I2b, I4b, and I6). In 6 samples (I3, I4, I7, I8, I9, and I12), the genotypes at one or two loci were missing (table 4). Because of these differences, the following analyses were performed on different data sets.

Hardy-Weinberg equilibrium was rejected at the 5% significance level in 17 of 161 tests. Taking into account the number of independent tests (sequential Bonferroni test; Rice 1989), only one test remained significant (*Gpi* in population

TABLE 4. Extended.

	Samples																
Locus	I15	I15b	I16	I16b	I17	J1	J2	J3	K1	K2	K3	K4	K5	K6	K7	K8	K9
Aat-1																	
(*N*)	27	27	31	31	28	31	25	31	31	23	28	32	31	31	32	32	29
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
C	0.04	0.06	0.02	0.02	0	0.03	0.02	0	0	0	0.11	0.02	0	0	0.08	0.06	0.05
D	0	0	0	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0
E	0.78	0.85	0.77	0.74	0.66	0.66	0.68	0.71	0.73	0.74	0.64	0.70	0.50	0.66	0.64	0.69	0.73
F	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0.18	0.09	0.18	0.24	0.34	0.30	0.28	0.29	0.27	0.26	0.25	0.28	0.50	0.34	0.26	0.25	0.22
I	0	0	0.02	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0
Aat-2																	
(*N*)	30	27	31	31	28	29	28	29	30	25	29	32	31	31	31	30	30
C	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0	0
D	0.02	0.02	0.02	0.02	0.04	0	0.05	0	0	0.02	0.02	0.03	0.03	0.05	0.03	0.03	0.02
E	0.85	0.91	0.87	0.79	0.91	0.91	0.93	0.90	0.80	0.96	0.86	0.85	0.79	0.74	0.87	0.80	0.95
F	0.12	0.07	0.11	0.18	0.04	0.09	0.02	0.10	0.18	0.02	0.05	0.09	0.15	0.21	0.10	0.17	0.03
G	0.01	0	0	0	0	0	0	0	0.01	0	0.03	0.03	0	0	0	0	0
H	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0
I	0	0	0	0.01	0	0	0	0	0	0	0.01	0	0.03	0	0	0	0
Pgm																	
(*N*)	31	29	32	31	28	32	32	32	32	31	29	32	32	29	32	32	32
A	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0.11	0.14	0.08	0.11	0.27	0.11	0.14	0.27	0.02	0.21	0.07	0.05	0.14	0.12	0.11	0.06	0.03
D	0	0	0	0	0	0	0	0	0.06	0	0	0	0	0	0	0	0
E	0.84	0.79	0.84	0.86	0.64	0.83	0.86	0.68	0.92	0.74	0.90	0.89	0.78	0.85	0.83	0.81	0.89
F	0	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	0.05	0.03	0.08	0.03	0.09	0.06	0	0.05	0	0.05	0.02	0.06	0.06	0.03	0.06	0.13	0.08
I	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0
Pgi																	
(*N*)	31	29	30	31	27	32	31	32	27	30	26	31	32	31	32	30	29
D	0	0.05	0	0.02	0.06	0	0	0.05	0.03	0	0.40	0	0	0	0	0	0
E	0.55	0.50	0.47	0.58	0.54	0.64	0.52	0.59	0.57	0.60	0.46	0.65	0.56	0.58	0.64	0.70	0.67
F	0.42	0.45	0.50	0.40	0.37	0.33	0.48	0.36	0.39	0.30	0.14	0.34	0.42	0.37	0.36	0.30	0.33
G	0.03	0	0.03	0	0.03	0.03	0	0	0	0.10	0	0	0.02	0.05	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0	0	0
Hk																	
(*N*)	31	29	29	31	27	26	29	32	32	31	28	31	32	30	32	32	32
D	0.46	0.37	0.59	0.55	0.77	0.48	0.48	0.50	0.47	0.47	0.17	0.55	0.56	0.58	0.36	0.50	0.55
E	0.54	0.62	0.41	0.45	0.22	0.52	0.52	0.50	0.53	0.53	0.55	0.45	0.44	0.42	0.64	0.50	0.45
F	0	0	0	0	0	0	0	0	0	0	0.27	0	0	0	0	0	0

I14b). A slight and systematic deficit in heterozygotes was observed using the program provided by Rousset and Raymond (1995) at the three following locus: *Aat-2* ($\chi^2 = 98.76$; df = 68; $P = 0.0088$), *Pgm* ($\chi^2 = 87.98$; df = 62; $P = 0.017$), and *Pgi* ($\chi^2 = 93.07$; df = 62; $P = 0.0065$).

Gametic associations were significant for 21 out of 217 Δ_{ij} tests, taking into account multiple testing (sequential Bonferroni test, Rice 1989). Among these significant Δ_{ij}, 7 concerned pairs of alleles observed in less than 8 populations, and only 2 were still significant when the 25 populations were pooled (table 5). For all pairs of loci, D_{is} was lower than D_{st} and D'_{is} greater than D'_{st} (table 6). Thus, the observed situation can be mainly explained by the action of genetic drift, even if a selection pressure on some *i,j* allele pairs in a few populations cannot be rejected (table 6).

Although F_{st} values were low in all transects, they were all significantly different from zero ($P < 0.05$, table 7), indicating a slight and significant differentiation at all geographic scales of the study. The lower bound of the confidence limits of *Nm* computed according to Wright (1969) from F_{st} values was higher than 4 for all loci and transects, with the exception of *Gpi* and *Hk* in transect K (1 and 1.3, respectively). These values were consistent with the overall *Nm* estimate (3.88) obtained with the Slatkin's private-allele method. The graph of *Nm* values against geographical distances for pairs of samples from transect I (fig. 3) suggested an absence of isolation by distance between 10 km and 850 km, but could not be tested statistically (Slatkin 1993).

DISCUSSION

1 Resistance and Electrophoretic Genes: Small Isolated Populations or a Single Large One?

When insecticide-resistance genes are considered, a strong differentiation is found between samples with sometimes sharp transitions over short distances, as along the K transect.

TABLE 5. Analysis of linkage disequilibrium between electrophoretic loci within samples. Only pairs of alleles with a significant disequilibrium after a sequential Bonferroni test are indicated. A/B indicates that allele A (of the locus indicated in the column) is associated with allele B (in the locus indicated in the row. (*S,N*) indicates that the combinations occurred in *N* samples and is significant in the *S* sample. Associations significant on the entire data set are underlined. Private alleles are indicated with a star.

	Aat-1	*Aat-2*	*Pgm*	*Pgi*
Aat-2	C/F (I16b,18)			
Pgm	C/E (I11,17)	E/F* (I15b,1)		
	C/C (K4,17)	I/C (K2,4)		
		D/C (K5,22)		
		D/E (K5,22)		
Pgi	E/E (K2,25)	D/H (I2,3)	C/F (I2,25)	
	G/E (K2,25)	E/F (I5,25)	G/D (J3,8)	
	E/E (I17,25)	D/G (K5,10)	C/G (K5,12)	
	G/E (I17,25)	E/G (K5,12)		
Hk			C/D (J3,8)	D/D (K3,8)
				F/D (K3,25)
				F/F* (K3,1)

The distribution pattern of each resistance gene is different (fig. 2, table 2); it is restricted to small geographic areas for esterases A2-B2 and C1 ("pocket distributions") and covers the entire sampled area for A1, A4-B4, and Ace^R, which present a great heterogeneity in frequencies between samples (mosaic distributions).

When genes with a polymorphism independent of insecticide selection pressure (*Aat-1* and *-2, Pgm, Gpi* and *Hk*) are considered, the estimated gene flow between populations is high and independent of distances over the whole range of our study (850 km), suggesting that migration is counteracting the effect of drift. This conclusion is based upon: (1) the statistical independence of the five loci as described by the $\Delta_{i,j}$ parameters, (2) the concordant local differentiation and migrant estimates obtained for the five loci, (3) the absence of a strong viability selection, suggested by the absence of deviations from Hardy-Weinberg equilibrium, and (4) the unlikeliness of a systematic selection on pairs of alleles at distinct loci, as indicated by *D*-statistics.

However, a possible balancing selection acting simultaneously on the five loci cannot be excluded from this data set. But isolation by distance could be detected for this species if a wider geographical scale were considered (e.g., reanalysis of *Culex pipiens* data in Cheng et al. 1982, for *Pgi, Hk,* and *Pgm*), which is best explained by a limitation of migration only at a very large scale. This characteristic seems to be very common in other widespread mosquito species associated with human activities, like *Aedes aegytpi* or *A. albopictus* (data of Powell and Tabachnick 1980; W. J. Tabachnik 1982; Black et al. 1988, reanalyzed with the methods described here), and the possibility of regular migration over several hundred kilometers is documented for at least one *Culex* mosquito species (Ming et al. 1993).

Similarly, the possibility exists that gene flow was high in the past, but reduced now. Separated populations could keep traces of ancient migrations for a long time, because of the reduced drift in huge mosquito populations. However, in Mediterranean *C. pipiens* populations, present and recurrent migration is indicated by the occurrence of A1 in several untreated areas soon after its first and localized occurrence (e.g., in 1975, mosquitoes with A1 reached a frequency of 49% in a location 20 km away from the treated area, Pasteur and Sinègre 1978). A similar observation was made with A4-B4: less than 2 yr after it had been first detected in the Languedoc-Roussillon treated coast, it reached a frequency of around 10% in two breeding sites 20 km from the treated area (Magnin 1986).

In conclusion, present migration is extensive between *C.*

TABLE 6. Ohta's indices computed on the 25 samples where genotypes at the five loci were identified. For all pairs of loci $D_{is} < D_{st}$, and $D'_{is} > D'_{st}$ (for explanations, see the text).

Compared loci	D_{is}	D'_{is}	D_{st}	D'_{st}	D_{it}
Aat-1/Aat-2	0.005	0.07	0.018	0.00007	0.07
Aat-1/Pgm	0.003	0.08	0.018	0.00005	0.08
Aat-1/Pgi	0.007	0.12	0.028	0.00007	0.12
Aat-1/Hk	0.006	0.09	0.022	0.00008	0.09
Aat-2/Pgm	0.003	0.05	0.012	0.00049	0.05
Aat-2/Pgi	0.006	0.11	0.027	0.00014	0.11
Aat-2/Hk	0.006	0.09	0.023	0.00028	0.09
Pgm/Pgi	0.006	0.13	0.030	0.00035	0.13
Pgm/Hk	0.006	0.09	0.021	0.00054	0.09
Pgi/Hk	0.010	0.11	0.026	0.00104	0.11

TABLE 7. Analysis of differentiation at two different geographical scales, local (K transect) and regional (I and J transects). For both scales, the F_{st} estimate of Weir and Cockerham's (1984) θ is given with its standard deviation (in parentheses) for each locus and with its 95% confidence limits for the multilocus estimate. *P* refers to the exact probability of the Fisher exact test on the $R \times C$ contingency table.

	K		IJ	
Locus	θ	*P*	θ	*P*
Aat-1	0.014 (0.002)	<0.05	0.020 (0.007)	<0.00001
Aat-2	0.020 (0.004)	<0.00001	0.004 (0.0003)	<0.002
Pgm	0.011 (0.001)	<0.03	0.013 (0.003)	<0.001
Pgi	0.040 (0.016)	<0.00001	0.034 (0.021)	<0.00001
Hk	0.036 (0.013)	<0.001	0.012 (0.003)	<0.00001
All	0.015–0.036		0.010–0.027	

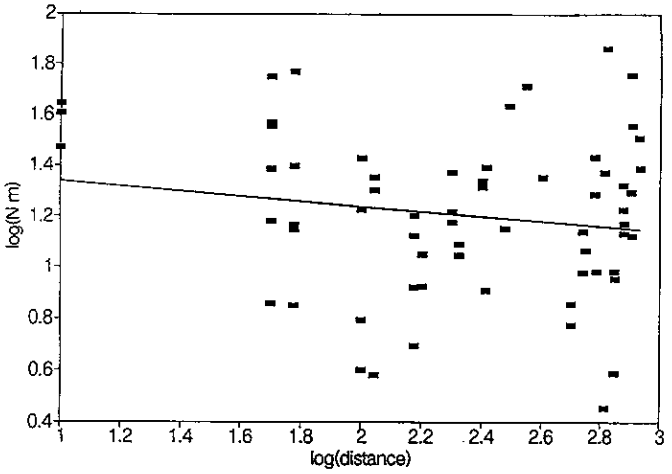

FIG. 3. Isolation by distance (d in Km) along the I transect using Slatkin's (1993) method. Pairwise estimates of Nm were calculated using the G_{st} parameter of Nei (1973), because the F_{st} estimate of Weir and Cockerham (1984) was sometimes negative. The equation of the regression line is Log (Nm) = 1.434 − 0.097 * Log(d).

pipiens populations, even at a scale of several hundred kilometers.

2 Why are distinct resistance genes differently distributed?

The focused distribution of esterases A2-B2 and C1 ("pocket distribution") is best explained by their recent occurrence in the studied area. A2-B2 was first detected in 1986 in a single breeding site near Marseille International Airport (i.e., close to the I13 sample), and its progression east, west, and north of this first location was documented until 1990 (Rivet et al. 1993). This 1991 survey is consistent with the previous ones in showing that A2-B2 has an extremely low frequency, except near Marseille (I13-I16). Esterase C1 was observed in the first study of Corsica in 1988 (Raymond and Marquine 1994) and was found in 1990 in the central part of eastern France (Rivet et al. 1994). In the 1991 survey, its presence was confirmed in Corsica, but not in continental France. Its spread is probably slow, because of the low resistance level it provides to OP insecticides (Raymond and Marquine 1994).

A1 and A4-B4 resistance genes that occurred in the south of France in 1972 (A1) and between 1978 and 1984 (A4-B4), respectively (Pasteur et al. 1981b; Raymond et al. 1992; Poirié et al. 1992), displayed a mosaic distribution over the entire geographic range studied.

Here, as in all previous investigations (Pasteur and Sinègre 1975; Pasteur et al. 1981b; Severini et al. 1993; Raymond and Marquine 1994; Rivet et al. 1994), A1 was strongly associated with *Est-2*$^{0.64}$, as were the esterases A4 and B4. These linkage disequilibria suggest strongly that A1, and A4-B4, have each occurred once in the region studied and increased their geographic range through the combined effect of migration and selection (see also Raymond et al. 1992).

There is no experimental proof that *Ace*^{R} is the same allele over the entire sampled area. However, since the biochemical test used to detect variants relies on the high propoxur insensitivity of the allele described in Languedoc-Roussillon (Raymond et al. 1986), it is clear that all alleles that may have been pooled under the *Ace*^{R} name have to share this property. Then, it is possible that, in addition to extensive migration of the *Ace*^{R}, which first occurred in southern France about 15 years ago, recurrent mutations have caused the observed co-occurrence of *Ace*^{R} in distant geographic areas.

The structured distributions of *Ace*^{R} (especially along the 50 km-long K transect), A1, and A4-B4 (on the entire sampled area) cannot be explained by a limitation of migration, as they have spread in all treated areas in a few years. As expected, their frequencies are well correlated on a large scale with mosquito control in Spain and southern France (I transect), and along the Rhône valley (J transect). Resistance genes are selected in treated areas, and their frequencies increase. As migration is high, resistance genes are constantly imported in nontreated areas, and susceptible genes are constantly exported into treated areas. This should result in the overall decrease of susceptible genes unless resistance genes are costly, that is, unless they have a reduced fitness in an environment lacking insecticide. In laboratory conditions, such fitness costs have been demonstrated in other resistant insects (e.g., *Lucilia cuprina,* Clarke and Mckenzie 1987) as well as in *C. pipiens* for esterases A1, B1, and *Ace*^{R} (Raymond et al. 1985a, 1985b, 1992). In natural populations, they were often suspected of causing the observed decline of resistance when insecticide treatments were stopped, and tentative estimates suggested that they were high (see Bishop and Cook 1981).

When A1 or A4-B4 are considered, a fitness cost is apparent throughout the sampled area, as their frequency is always higher in treated areas than in the nearby nontreated ones. The only exception is the K transect, where neither A1 nor A4-B4 seems to decrease in frequency in the nontreated part (fig. 2, table 3). This may be explained by the intense gene flow within this restricted geographic area (50 km), which overcomes the effect of fitness cost.

The fitness cost of *Ace*^{R} is also indirectly demonstrated by its sharp variation in frequencies between treated and nontreated areas, even on the small scale of transect K (fig. 2, table 3). The only exception is the Spanish part of transect I, in which the high *Ace*^{R} frequency seems independent of OP treatments (fig. 2). The existence of a distinct *Ace*^{R} allele in Spain with a reduced fitness cost cannot be excluded. Alternatively, if the *Ace*^{R} gene is the same in the entire sampled area, its cost could be reduced if modifier genes are present in Spain. Such modifier genes, which reduce the fitness cost of resistance genes, have been found in few other situations (Clarke and Mckenzie 1987; Bishop and Cook 1981). Molecular data are required to clarify this situation.

CONCLUSION

The distributions of A2-B2 and C1 in localized areas are probably transitory, as the extensive gene flow at this geographical scale will bring them in all treated areas rapidly. Their equilibrium frequencies in untreated areas will depend on the value of their fitness cost, as shown by A1 and A4-B4.

Theoretical work, considering situations where a high symmetrical gene flow occurs between treated and untreated ar-

eas, shows that conditions under which the present distribution of A1, A4-B4, and Ace^R could be a stable equilibrium, is conditioned by the relation between gene flow and selection pressures (e.g., Slatkin 1973), as well as by the ratio between fitness cost and selective advantage in a treated area (Nagylaki 1974). This last condition is difficult to test. First, because interaction between the different resistance genes present in southern France should be taken into account. Resistance provided by an insensitive target (Ace^R) and an overproduced esterase combines additively (Raymond et al. 1989b), but how fitness costs of distinct resistance genes combine remains to be determined; second, because of the situation of the Ace^R allele in Spain. As we have seen, the absence of difference in Ace^R frequency between treated and not treated areas suggests that an Ace^R modifier gene or another Ace^R allele with a reduced cost is occurring in Spain. If this is the case, we expect that the normal susceptible Ace^S allele will greatly decrease, even in untreated areas. Under such a scenario and if OP insecticides are continuously used, the Ace^R will increase until fixation at equilibrium, even in untreated areas. It is worth noting that if the hypothesis of an Ace^R modifier gene is confirmed, this situation will provide a good model to study the process of peak shifting (sensu Wright) in the wild.

It is unlikely that the present situation of strong and opposite selection pressures in adjacent areas could lead to a speciation event, because of the high gene flow encountered between these populations. This is perhaps a general conclusion for pest resistance, because most, if not all, concerned species display a high migration propensity likely to maintain a high gene-flow level and have extremely large population sizes (unless they are not pests). This may explain why the only report of an incipient speciation directly associated with xenobiotics resistance concerns a nonweed plant with relatively limited migration capability that was not deliberately treated (MacNair et al. 1989).

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