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Commensalism, adaptation and gene flow: mosquitoes of the *Culex pipiens* complex in different habitats

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Summary

Two ecotypes have been described for *Culex pipiens* mosquitoes of the temperate zone: a human commensal type and a feral type, but their degree of evolutionary differentiation and taxonomic status are still unclear. The commensal form is characterized by life-history traits probably adaptive to underground man-made environments. This situation has sometimes been considered as an example of recent speciation although the existence of intermediate forms indicates that the balance between gene flow and disruptive selection should first be assessed. The present study was concerned with (1) the determination of biological traits involved in adaptation to commensalism, and (2) the pattern of gene flow within and between ecotypes in a restricted area. It was found that (1) significant differences in biological traits exist between mosquitoes from different habitats, (2) characteristics of the commensal type are not universal in mosquitoes from underground man-made habitats, (3) allozyme markers do not clearly differentiate ecotypes and (4) insecticide resistance genes, which reveal recent migration, occur in each ecotype. These results are discussed in the context of possible speciation due to commensalism.

1. Introduction

Several opportunist animal and plant species use human habitats to feed or reproduce, and they are generally designated as commensal. For a given species, a commensal and a non-commensal form generally coexist (e.g. the house mouse *Mus musculus domesticus*, Auffray *et al.* 1988; 1990*a*, *b*). Such a situation allows the study of the evolution of adaptation to a new habitat (e.g. Ganem, 1991; Said & Britton-Davidian, 1991).

For the *Culex pipiens* mosquitoes of the temperate zone, two ecotypes are traditionally described (Roubaud, 1929, 1933; Barr, 1981; Mattingly, 1951, 1967). First, a commensal form, breeding in underground (or hypogeous) human habitats such as flooded cellars and basements, cesspits, pit latrines, etc. Four particular life history traits are associated with this form, and are viewed as specific adaptations to these new habitats: the ability to mate in a restricted volume (stenogamy), the ability to lay a first batch of eggs without a blood meal (autogeny), a host preference for mammals (mammophily), and the ability to reproduce throughout the year (homodynamy). Secondly, a non-commensal form, breeding in open (or epigeous) habitats such as pools, ponds, ditches, canals, etc. This form lacks adaptations to commensalism and requires a large space for mating in a swarm (eurygamy), it cannot produce any eggs without a blood meal (anautogeny), it feeds mainly on birds (ornithophily) and adult females hibernate (heterodynamy). The commensal form is usually urban while the non-commensal is usually rural.

The taxonomic status of these two forms is unclear. They are sometimes considered as valid species (the commensal form being called *C. molestus*; e.g. Harbach, 1986; Miles & Paterson, 1979) or as subspecies (*C. p. molestus* for the commensal form and *C. p. pipiens* for the rural one, e.g. Urbanelli *et al.* 1985). The existence of intermediate forms (e.g. Roubaud & Ghelelovich, 1956; Rioux & Pech, 1961; Dobrotworsky, 1967; Ishii, 1983; Pasteur *et al.* 1977; Urbanelli *et al.* 1985) suggests the possible existence of

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a continuum, and indicates that ecotypic variation is involved (Barr, 1981; Mattingly *et al.* 1951).

In order to clarify this situation, a population genetic study was undertaken in an area where both open air and underground habitats are common. In addition, an area under insecticide mosquito control was selected, because the detection of known recent resistance genes could indicate recent migration events. This is particularly true for some resistance genes which have been shown to have a unique origin, so that their presence in a new treated place indicates necessarily at least one migration event (Raymond *et al.* 1991; Raymond & Marquine, 1994; Qiao & Raymond, 1995).

The present study was conducted in the area of Barcelona (Catalonia, Iberian peninsula) which has been subject to insecticidal control since 1982. Only temephos (an organophosphate (OP) insecticide) was used until 1992 when it was replaced by *Bacillus* toxins. The following points were addressed. Are the various life history traits clustered in two distinct forms? Are mosquitoes from underground and open air sites genetically differentiated? What is the pattern of gene flow between and within the two habitats and does it preclude any speciation event?

2. Material and methods

(i) *Mosquito sampling and biological traits*

Forty five larval breeding sites were sampled from March 8th to August 27th, 1993 (Table 1). Thirty were open air habitats and fifteen were underground habitats. Among the underground sites, five could be considered as having no obvious opening to the surface (designated fully closed) and 10 had some obvious opening (designated intermediate).

Table 1. *Habitat types of* Culex pipiens *sampled in Barcelona area in 1993*

Habitat type	Sample number
Fully closed	
Flooded cellar	4
Underground sewer	1
Intermediate	
Underground water tank	5
Underground drain	2
Drain	2
Cesspit	1
Open air:	
Pit	2
Ditch	10
Flooded meadow	2
Abandoned bath tub	1
Clean ditch	2
Open drain from a latrine	1
Open drain from a farmyard	1
Reservoir	1
Concrete lined reservoir	2
Flower pot	1
Sewage lagoon	1
Fountain	1
Stagnant river	2
Agricultural pit	2
Pool	1

From each breeding site, 500 larvae were collected and reared under standard conditions (e.g. Raymond *et al.* 1985). Forty 4th-instar larvae were randomly isolated for genetical analyses as well as morphological measurements which will be presented elsewhere, and the remaining adults were allowed to emerge in a $24 \times 25 \times 35$ cm cage (maximum density of adults/cage was 160) in order to assess autogeny and stenogamy. Adults were only supplied with a sugar solution for feeding, no water cup was supplied for egg laying. Stenogamy was determined as the proportion of mated females after 20 d, after dissecting spermathecae and examination for presence of spermatozoa. Autogeny was assessed on the follicular development of the same females. When ovaries were found after 20 d developed beyond stage IIa (Christophers, 1911), the female was considered autogenous.

(ii) *Electrophoresis*

The polymorphism at four 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), *Pgm* (phosphoglucomutase EC 2.7.5.1), and *Pgi* (phosphate glucose isomerase EC 5.3.1.9). Strains used for mobility references are those described in Chevillon *et al.* (1995).

(iii) *Identification of known resistance genes*

The acetylcholinesterase genotypes were determined on single mosquitoes by the microplate test described by Raymond & Marquine (1994). Presence or absence of highly active esterases was determined on single mosquito homogenates by starch gel electrophoresis in TME 7·4 buffer systems (see above). Mosquitoes with known highly active esterases were run in each gel as controls (see Chevillon *et al.* 1995 for details).

(iv) *Hardy–Weinberg equilibrium*

Hardy–Weinberg proportions were tested by the exact test proposed by Haldane (1954), using the algorithm of Louis & Dempster (1987) for up to four alleles. For five alleles or more, an unbiased estimate of the exact *P*-value was computed using the Markov chain method of Guo & Thompson (1992). The Markov chain was set to at least 100000 steps, and 2000 steps of dememorization (see Guo & Thompson, 1992 for details) in order to obtain standard error estimates below 0·005. The GENEPOP software (version 1·2) was used for these computations (Raymond & Rousset, 1995*b*). The overall significance of multiple tests for each sample was estimated by Fisher's combined

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probability test (Fisher, 1970). This method assumes that P values for each of the several independent tests follow a uniform distribution between 0 and 1, and it is therefore slightly inaccurate (Yates, 1955). However, when sample size or allele numbers are high, the continuous approximation can be made because the number of distinct genotypic tables (hence of probability values) considered becomes large. Whenever this number was below 10, the corresponding probability was not included in overall testing.

F_{is} values were computed according to Weir & Cockerham (1984). Multisample heterozygote deficits were tested using an exact test procedure described by Rousset & Raymond (1995*a*).

(v) *Differentiation among populations*

The F_{st} parameter was computed according to Weir & Cockerham (1984). Due to significant departure from Hardy–Weinberg equilibrium (see above), the exact test of population differentiation of Raymond & Rousset (1995*a*) could not be used. The type-I error probability of the null hypothesis (Ho: $F_{st} = 0$, no differentiation) was computed using an F_{st}-based exact test (Raymond & Rousset, unpublished). The principle of this test is to define a rejection region as the sum of all tables (populations × genotypes) with the same marginal and having a F_{st} equal or larger than the observed one. An unbiased estimate of this probability was obtained with a Markov chain method as explained by Raymond & Rousset (1995*a*). For all tests, the Markov chain was set to at least 100000 steps, and 2000 steps of dememorization (see Raymond & Rousset, 1995*a*). The overall significance of multiple tests for each locus was estimated by Fisher's combined probability test (Fisher, 1970).

(vi) *Linkage disequilibrium and D statistics*

For each population, the global disequilibrium between pairs of loci was estimated using the common correlation coefficient (Weir, 1990, pp. 111–113), and tested using Fisher's test on $R \times C$ contingency table (see above). For each pair of loci, a global measure was obtained by averaging the correlation coefficients across populations, and a global test was obtained by combining (Fisher's method) the probability for each population.

Either genetic drift or directional 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) suggested rearrangement of the gametic associations observed in the whole data set (D_{it}) into four indices which estimate the parts created within (D_{is} and D'_{is}) and between populations (D_{st} and D'_{st}). The discrimination is based on the comparisons of D_{is} and D_{st} values, on the one hand, and of D'_{is} and D'_{st} values, on the other. These indices were computed using the LINKDOS program (Garnier-Gere & Dillman, 1992).

(viii) *Effective migrants*

The number of effective migrants (Nm) was estimated by two methods. First, it was estimated from the F statistics of each locus according to the equation $Nm = (1/F_{st} - 1)/4$ (Wright, 1969). This formula assumes neutral polymorphism and an island model of migration (see e.g. Hartl & Clark, 1989). Secondly, Nm was estimated by the method of 'private alleles' described by Slatkin (1985)

(viii) *Multiple tests*

The significance level for each test was adjusted to take into account the other tests using the sequential Bonferroni method as described by Holm (1979).

3. Results

(i) *Life history traits*

Stenogamous and eurygamous insects were not randomly distributed among the habitats (Table 2).

Stenogamous females were significantly (Fisher exact test, $P < 10^{-5}$) more numerous in fully closed habitats than in more open habitats but there was no significant difference between intermediate and fully open air sites (Fisher exact test, $P > 0{\cdot}19$). From the different fully closed sites, the frequency of stenogamous females varied between 64 and 97%, but it varied between 0 and 63% in the different open air sites (details not shown).

Autogenous females were found in the three habitats, but were significantly (Fisher exact test, $P < 10^{-5}$) more numerous in fully closed sites. No difference in frequency of autogenous females was found between intermediate and open air habitats (Fisher exact test, $P > 0{\cdot}7$). The frequency of autogenous females varied between 45 and 100 in the different fully closed sites, and between 0 and 87% in different open air sites (details not shown). The frequency of stenogamy and of autogeny were significantly correlated (Spearman rank correlation, $rs = 0{\cdot}60$, $N = 41$, $P < 0{\cdot}001$).

(ii) *Population genetic analysis*

Independence between loci. Genotypic association between each pair of loci was measured by the common correlation coefficient and was tested with an exact test (details not shown). Among all the pairs of loci, non-independence was only rejected for the pair Sex-*Pgi* (Common correlation: 0·23, $P = 0{\cdot}004$). This non-independence does not remain significant at the 0·05 level when multiple testing was taken into account (Holm, 1979). The analysis of Ohta's indices does not reject the hypothesis that the observed gametic associations were only due to the action of drift, as for all samples the ranking of Ohta's indices were: $D_{is} < D_{st}$ and $D'_{is} > D'_{st}$ (details not shown).

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Table 2. *Female life history traits in the three habitat types (percentage in parentheses). Habitat types which share the same letter do not differ significantly ($P > 0{\cdot}1$) in proportion of mosquitoes with each trait*

	Mating		Egg laying	
Habitat types	Stenogamy	Eurygamy	Autogeny	Anautogeny
Fully closed	126 (86)a	20 (14)	112 (77)a	34 (23)
Intermediate	71 (20)b	291 (80)	14 (4)b	348 (96)
Open air	180 (17)b	889 (83)	37 (3)b	1042 (97)

Hardy–Weinberg equilibrium. Hardy–Weinberg equilibria were rejected ($P < 0{\cdot}05$) in 40 out of 158 tests (Table 3), which is higher than expected by chance under the null hypothesis. When testing across loci within each sample, Hardy–Weinberg equilibria were rejected ($P < 0{\cdot}05$) for 19 out of 45 samples. Four of them remained significant when multiple tests were taken into account, heterozygotes being significantly in deficit (details not shown). For each habitat type, the mean F_{is} computed according to Weir & Cockerham (1984) was positive for all loci, indicating extensive heterozygote deficit (Table 4), and these deficits were significant ($P_d < 0{\cdot}05$, Table 4) in 10 out of 12 cases. When all loci were considered, significant ($P_d < 10^{-4}$) heterozygote deficit was found in all habitat types (Table 4).

Population differentiation. The overall differentiation among populations was highly significant ($P < 10^{-5}$) and corresponded to a F_{st} value of 0·067 (Table 5). In order to investigate the differentiation hierarchically, several groups were considered. First, the inter-habitat differentiation, tested by the genetic composition of fully closed, intermediate and open air populations, was significant ($P < 10^{-5}$) with a corresponding F_{st} value of 0·055 (Table 5). Secondly, the comparison of fully closed and intermediate *v.* open air habitats indicated a significant differentiation ($F_{st} = 0{\cdot}013$, $P < 10^{-5}$). Thirdly, pairwise comparisons of habitats showed that only open air and intermediate habitats were not significantly differentiated ($F_{st} = 0{\cdot}0028$, $P > 0{\cdot}05$, Table 5). Fourthly, differentiation was estimated within each habitat, and all three were found significantly differentiated ($F_{st} > 0{\cdot}032$, $P \leqslant 10^{-4}$).

Number of migrants. Wright's F_{st} or Slatkin's private allele methods of estimating the number of migrants, provided similar values (Table 5), except for the open air *v.* intermediate and the underground *v.* open air comparisons. The number of migrants between populations or groups of populations was around four for all the considered subdivisions (Table 5), except for the open air *v.* fully closed habitats, where it was just above 2.

(iii) *Insecticide resistance genes*

Both highly active esterases and insensitive acetylcholinesterase target (*Ace*) were found in the three types of habitat (Table 6). The frequency of the various highly active esterases differed between the types of habitat, indicating differences in selection pressures. Esterases A2–B2 were found in only four mosquitoes from three intermediate sites. The identity of A1 esterase was confirmed by the presence of the $Est\text{-}2^{0{\cdot}64}\text{-}A1$ linkage disequilibrium found in all previously sampled populations (Pasteur *et al.* 1981; Raymond & Marquine, 1994; Chevillon *et al.* 1995; details not shown).

There was no difference in the frequency of Ace^R among habitat types (Table 6), indicating that this gene experiences similar selection in all the populations sampled.

4. Discussion

(i) *Are the various life history traits clustered in two distinct forms?*

Both stenogamy and autogeny were found in the three habitat types, so that they do not represent diagnostic characters. Similar situations have been observed in other places (e.g. southern France, Pasteur *et al.* 1977; Italy, Urbanelli *et al.* 1985; Japan, Ishii, 1983; Russia, Vinogradova, 1992). However, fully closed habitats displayed a significantly higher proportion of autogenous and stenogamous females than do the other habitats. There was no difference in these life history traits between open air and intermediate habitats.

Models studying the evolution of autogeny/anautogeny (Tsuji, 1989; Tsuji *et al.* 1990) in relation to various life history traits have shown that searching time to locate a blood meal (T), the relative size of the autogenous and initial anautogeneous egg batches (P), and the probability of surviving accidental death during blood feeding (S) have the strongest influences. Thus, autogeny appeared more advantageous when T and P are high and S low; larval conditions that lengthen larval development or induce high larval mortality reduce the advantage of autogeny, whereas high adult mortality reduces the advantage of anautogeny. In our study, fully closed habitats are certainly more favourable for autogeny than other habitats on

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Table 3. *Allelic frequencies observed at four loci (N = sample size)*

Locus	C1	C2	C3	C4	C5	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10
Aat-1															
(N)	(27)	(22)	(28)	(16)	(25)	(21)	(5)	(4)	(8)	(21)	(13)	(16)	(20)	(26)	(22)
1	0·83	0·52	0·77	0·78	0·90	1	0·90	0·87	0·56	0·81	0·96	0·66	0·77	0·61	0·91
2	0·17	0·48	0·23	0·19	0·08	—	0·10	—	0·13	0·07	—	0·19	0·13	0·25	0·04
3	—	—	—	0·03	0·02	—	—	0·13	0·31	0·12	0·04	0·16	0·03	0·14	0·04
4	—	—	—	—	—	—	—	—	—	—	—	—	0·07	—	—
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F_{is}	0·061	0·20	0.12	−0·21	−0·071	—	—	—	0·40	0·29	—	0·17	−0·17	0·24	0·22
P	**0·0091**	0·41	0·60	1	1	—	—	—	*0.16*	**0.023**	—	0·10	1	0·093	0·14
Aat-2															
(N)	(25)	(28)	(34)	(12)	(26)	(10)	(14)	(12)	(12)	(30)	(18)	(28)	(25)	(19)	(18)
1	1	0·96	0·97	1	1	1	1	0·79	0·87	0·88	0·69	0·96	0·94	0·79	1
2	—	0·04	0·03	—	—	—	—	0·08	0·13	0·12	0·25	0·04	0·04	0·13	—
3	—	—	—	—	—	—	—	—	—	—	—	—	0·02	0·08	—
4	—	—	—	—	—	—	—	0·13	—	—	0·06	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F_{is}	—	1	−0.15	—	—	—	—	−0·15	0·64	0·84	0·65	1	−0·029	0·71	—
P	—	***0·018***	*1*	—	—	—	—	*1*	*0.13*	**0.006**	**0·0023**	**0·18**	1	**0·0121**	—
Pgm															
(N)	(33)	(29)	(37)	(31)	(25)	(17)	(19)	(18)	(34)	(34)	(20)	(31)	(16)	(19)	(24)
1	0·86	0·79	0·63	0·77	0·84	0·97	0·95	0·86	0·85	0·84	0·90	0·85	0·88	0·87	0·63
2	0·14	0·21	0·37	0·23	0·14	—	0·05	0·14	0·09	0·15	0·02	0·10	0·12	0·05	0·37
3	—	—	—	—	—	0·03	—	—	0·03	0·01	0·05	0·05	—	0·08	—
4	—	—	—	—	0·02	—	—	—	0·03	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	0·02	—	—	—	—
F_{is}	0·12	−0·033	0.26	0·28	0·15	—	−0·029	−0·13	0·34	−0·16	−0·48	0·39	0·45	0·36	0·48
P	0·47	1	0·16	0·15	0·48	—	*1*	1	**0.0011**	1	*1*	**0·031**	0·19	0·11	**0·029**
Pgi															
(N)	(40)	(32)	(38)	(31)	(27)	(19)	(19)	(19)	(33)	(33)	(23)	(30)	(34)	(25)	(29)
1	0·11	0·16	0·09	0·11	0·19	0·66	0·47	0·50	0·48	0·58	0·65	0·42	0·53	0·56	0·48
2	0·89	0·83	0·88	0·84	0·65	0·32	0·42	0·47	0·45	0·36	0·35	0·58	0·40	0·30	0·43
3	—	0·02	0·03	—	0·17	—	0·03	—	0·02	0·02	—	—	0·07	0·08	0·09
4	—	—	—	—	—	—	—	0·03	0·03	—	—	—	—	—	—
5	—	—	—	—	—	0·03	—	—	0·02	—	—	—	—	—	—
6	—	—	—	0·05	—	—	0·08	—	—	0·04	—	—	—	0·06	—
F_{is}	0·14	0·68	−0.095	0·10	0·51	0·30	−0·13	−0·076	0·20	0·28	0·25	0·26	0·22	0·40	0·47
P	0·40	**0·0004**	*1*	0·11	**0·0003**	0·36	**0·041**	1	0.24	**0.26**	0·36	**0·25**	0·16	**0·0066**	**0·0081**
All															
F_{is}	0·31	0·30	0·12	0·048	0·30	0·27	−0·11	−0·126	0·34	0·28	0·34	0·29	0·12	0·41	0·44
P	**0·047**	**0·0078**	0·32	0·22	**0·0063**	0·36	**0·041**	1	**0.0022**	**0·0002**	**0·0064**	**0·0046**	0·54	**0·0001**	**0·0021**

[*continued overleaf*

Table 3. (*Cont.*)

Locus	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15
Aat-1															
(N)	(16)	(4)	(16)	(13)	(13)	(17)	(9)	(14)	(8)	(13)	(14)	(8)	(12)	(22)	(21)
1	0·78	0·75	0·72	0·61	0·73	0·65	0·89	0·57	0·94	0·65	0·93	0·63	0·88	0·52	0·67
2	0·16	0·25	0·16	0·31	0·23	0·23	0·05	0·32	—	0·31	—	0·31	0·08	0·39	0·33
3	0·06	—	0·12	0·08	—	0·12	0·05	0·11	0·06	0·04	0·07	0·06	0·04	0·04	—
4	—	—	—	—	—	—	—	—	—	—	—	—	—	0·04	—
5	—	—	—	—	0·04	—	—	—	—	—	—	—	—	—	—
F_{is}	0·51	1	0.32	0·006	−0·083	0·22	−0·032	0·27	—	−0·42	−0·04	0·56	−0·065	−0·007	−0·26
P	***0·014***	*0·14*	0·06	1	0·30	*0·054*	*1*	0·27	—	0·30	*1*	*0·077*	*1*	0·62	0·34
Aat-2															
(N)	(18)	(11)	(27)	(17)	(17)	(21)	(14)	(14)	(13)	(19)	(17)	(10)	(23)	(24)	(17)
1	0·75	1	0·90	0·88	0·94	0·90	0·93	0·93	0·92	0·90	0·91	0·85	0·83	0·83	0·88
2	0·22	—	—	0·09	0·06	0·10	0·07	0·07	0·08	0·05	0·09	0·15	0·06	0·06	0·09
3	—	—	0·02	—	—	—	—	—	—	—	—	—	—	0·06	—
4	—	—	0·06	0·03	—	—	—	—	—	0·05	—	—	0·11	0·04	—
5	0·02	—	0·02	—	—	—	—	—	—	—	—	—	—	—	0·03
F_{is}	0·45	—	0.37	−0·076	−0·032	0·47	1	−0·040	−0·043	−0·059	0·65	0·64	0·16	0·45	0·20
P	***0·033***	—	0·18	*1*	*1*	0·14	***0·037***	*1*	*1*	*1*	*0·091*	*0·16*	0·14	***0·0070***	*0·18*
Pgm															
(N)	(23)	(12)	(24)	(22)	(22)	(19)	(16)	(23)	(15)	(25)	(20)	(15)	(22)	(16)	(23)
1	0·96	1	0·85	0·86	0·75	0·74	0·84	0·87	0·87	0·82	0·88	0·87	0·80	0·94	0·85
2	0·04	—	0·04	0·09	0·16	0·26	0·16	0·09	0·10	0·16	0·05	0·10	0·16	0·03	0·11
3	—	—	0·06	0·05	0·09	—	—	0·04	0·03	0·02	—	0·03	0·02	0·03	—
4	—	—	0·04	—	—	—	—	—	—	—	—	—	—	—	0·04
5	—	—	—	—	—	—	—	—	—	—	0·07	—	0·02	—	—
F_{is}	−0·023	—	−0·088	0·091	0·34	0·21	0·32	−0·091	−0·087	0·092	−0·080	−0·087	0·35	−0·017	0·21
P	*1*	—	*1*	*0·32*	0·055	0·55	*0·31*	*1*	*1*	0·58	*1*	1	0·19	*1*	***0·040***
Pgi															
(N)	(23)	(7)	(27)	(19)	(25)	(24)	(18)	(22)	(20)	(25)	(24)	(15)	(24)	(28)	(24)
1	0·67	0·22	0·57	0·37	0·50	0·67	0·52	0·48	0·47	0·50	0·58	0·60	0·52	0·38	0·29
2	0·33	0·64	0·43	0·53	0·48	0·33	0·36	0·48	0·43	0·36	0·42	0·33	0·46	0·55	0·63
3	—	0·14	—	0·10	0·02	—	—	—	—	0·08	—	—	—	0·07	0·08
4	—	—	—	—	—	—	—	0·04	—	0·02	—	—	0·02	—	—
5	—	—	—	—	—	—	0·06	—	0·10	—	—	0·03	—	—	—
6	—	—	—	—	—	—	0·06	—	—	0·04	—	0·03	—	—	—
F_{is}	0·71	0·25	−0.27	0·11	0·019	−0·10	0·36	−0·066	−0·002	0·17	0·16	0·27	0·14	0·30	0·37
P	**0·0011**	*0·10*	0·24	0·20	1	1	**0·0048**	0·59	0.68	**0.025**	0·67	0·22	0·68	**0·019**	**0·012**
All															
F_{is}	0·52	0·57	0.036	0·044	0·071	0·15	0·35	0·054	−0·034	−0·055	0·16	0·36	0·16	0·19	0·12
P	**0·0011**	—	0·064	0·25	0·22	0·29	**0·0048**	0·45	0.68	0.095	0·67	0·35	0·24	0·064	**0·027**

Locus	O16	O17	O18	O19	O20	O21	O22	O23	O24	O25	O26	O27	O28	O29	O30
Aat-1															
(N)	(14)	(14)	(12)	(13)	(3)	(14)	(13)	(5)	(20)	(12)	(13)	(15)	(17)	(14)	(13)
1	0·86	0·61	0·92	0·92	0·50	0·86	0·73	0·80	0·75	0·54	0·58	0·53	0·59	0·93	0·46
2	0·14	0·39	—	—	—	—	0·08	0·10	0·17	0·20	0·15	0·10	0·23	0·03	0·39
3	—	—	0·04	0·08	0·50	0·14	0·19	0·10	0·08	0·17	0·19	0·33	0·18	0·03	0·15
4	—	—	—	—	—	—	—	—	—	—	0·08	0·03	—	—	—
5	—	—	0·04	—	—	—	—	—	—	0·08	—	—	—	—	—
F_{is}	−0·13	−0·31	−0.023	−0·043	−1	0·45	0·49	0·50	0·029	−0·017	−0·24	−0·089	0·20	−0·020	0·53
P	*1*	*0·32*	1	*1*	*0·40*	0·22	**0·024**	*0·11*	0.12	0.28	1	0·82	**0·020**	*1*	***0·021***
Aat-2															
(N)	(19)	(17)	(15)	(18)	(4)	(10)	(20)	(5)	(27)	(15)	(16)	(18)	(20)	(20)	(12)
1	0·92	0·97	0·90	0·94	0·37	0·80	0·77	1	0·87	0·80	0·94	0·97	0·97	0·93	1
2	—	0·03	0·10	0·06	—	0·05	0·17	—	0·07	0·03	—	0·03	—	0·07	—
3	—	—	—	—	—	0·10	—	—	0·02	0·10	—	—	0·03	—	—
4	—	—	—	—	0·63	0·05	0·03	—	0·02	0·07	0·03	—	—	—	—
5	0·08	—	—	—	—	—	0·03	—	0·02	—	0·03	—	—	—	—
F_{is}	−0·059	—	−0.077	−0·030	0·57	0·46	0·21	—	0·23	0·26	−0·017	—	—	0·65	—
P	*1*	—	*1*	1	*0·43*	**0·0093**	0·23	—	0.36	0.083	*1*	—	—	*0·077*	—
Pgm															
(N)	(19)	(13)	(17)	(19)	(19)	(10)	(21)	(14)	(34)	(6)	(5)	(8)	(16)	(18)	(25)
1	0·90	0·81	0·94	0·92	0·82	0·70	0·76	0·89	0·94	0·92	0·90	1	0·93	0·94	0·86
2	0·10	0·19	0·06	0·08	0·18	0·15	0·19	0·07	0·03	0·08	0·10	—	0·06	0·06	0·02
3	—	—	—	—	—	0·10	—	0·04	0·03	—	—	—	—	—	0·08
4	—	—	—	—	—	—	0·05	—	—	—	—	—	—	—	0·04
5	—	—	—	—	—	0·05	—	—	—	—	—	—	—	—	—
F_{is}	−0·091	−0·20	−0.032	0·65	−0·20	0·00	−0·52	0·66	0·49	—	—	—	−0·034	1	0·54
P	*1*	*1*	*1*	*0·81*	1	*0·65*	**0·0052**	***0·037***	***0.030***	—	—	—	*1*	***0·028***	**0·0063**
Pgi															
(N)	(20)	(24)	(25)	(17)	(19)	(22)	(22)	(14)	(35)	(17)	(23)	(25)	(20)	(20)	(24)
1	0·47	0·60	0·58	0·47	0·18	0·45	0·64	0·71	0·46	0·50	0·41	0·30	0·42	0·30	0·54
2	0·33	0·40	0·42	0·50	0·47	0·45	0·36	0·29	0·50	0·44	0·52	0·58	0·52	0·70	0·46
3	—	—	—	—	0·03	0·07	—	—	0·04	0·06	0·04	0·12	0·05	—	—
4	—	—	—	—	0·11	—	—	—	—	—	—	—	—	—	—
5	0·20	—	—	0·03	0·21	0·02	—	—	—	—	0·02	—	—	—	—
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
F_{is}	−0·17	−0·46	0.12	−0·20	−0·28	−0·15	0·24	−0·37	0·17	−0·14	−0·15	0·23	0·74	−0·17	0·014
P	0·73	**0·036**	0·69	0·47	**0·019**	0·66	0·36	*0·50*	0.13	0·46	0·18	0·10	**0·002**	0·62	1
All															
F_{is}	−0·14	−0·34	0.031	−0·018	−0·26	0·097	0·36	0·14	0·16	−0·011	−0·18	0·057	0·40	0·16	0·34
P	0·73	**0·036**	0·94	0·82	**0·021**	**0·040**	**0·0034**	—	0.11	0·17	0·49	0·30	**< 0·0001**	0·62	**0·0022**

F_{is} is calculated according to Weir & Cockerham (1984). *P* refers to type-I error probability of the Hardy–Weinberg exact probability test, or its unbiased estimate with a standard deviation of less than 0·005. 'All' refers to multilocus estimates of F_{is} and to overall testing across loci (Fisher's combined probability tests). Italics indicate cases not taken into account for overall testing (see Material and methods for details). Bold characters indicate significant ($P < 0·05$) deviations from Hardy–Weinberg expectations, and bold underlined characters indicate significant ($P < 0·05$) values when multiple testing is taken into account. Samples are numbered consecutively C1–C5 for fully closed, I1–I10 for intermediates and O1–O15 for open air habitats).

Table 4. *Mean F_{is} values (computed according to Weir and Cockerham, 1984) for each locus in each habitat type. P_d refers to the multisample test of departure from Hardy–Weinberg equilibrium due to heterozygote deficit. Significant values ($P < 0.05$) are in bold characters*

	Habitat type							
	Closed		Intermediate		Open		All	
Locus	F_{is}	P_d	F_{is}	P_d	F_{is}	P_d	F_{is}	P_d
Aat-1	0·17	0·05	0·18	0·057	0·079	**0·002**	0·11	**0·0006**
Aat-2	0·50	**0·015**	0·56	**$< 10^{-4}$**	0·27	**$< 10^{-4}$**	0·35	**$< 10^{-4}$**
Pgm	0·17	**0·026**	0·22	**$< 10^{-4}$**	0·18	**$< 10^{-4}$**	0·19	**$< 10^{-4}$**
Pgi	0·029	**0·0002**	0·24	**$< 10^{-4}$**	0·066	**$< 10^{-4}$**	0·13	**$< 10^{-4}$**
All	0·22	**$< 10^{-4}$**	0·26	**$< 10^{-4}$**	0·12	**$< 10^{-4}$**	0·16	**$< 10^{-4}$**

Table 5. *Differentiation among populations from different habitat types*

	F_{st}					Nm	
Loci	*Aat-1*	*Aat-2*	*Pgm*	*Pgi*	All	F_{st}	Priv. allele
Comparison							
All samples	0·067 (**$< 10^{-5}$**)	0·046 (**0·001**)	0·034 (**$< 10^{-5}$**)	0·090 (**$< 10^{-5}$**)	0·067 (**$< 10^{-5}$**)	3·5	—
Inter habitat							
Closed *v.* interm. *v.* open	0·010 (**0·0055**)	0·014 (**0·0030**)	0·019 (**$< 10^{-5}$**)	0·12 (**$< 10^{-5}$**)	0·055 (**$< 10^{-5}$**)	4·3	6·0
Closed + interm. *v.* open	0·0044 (0·063)	0·0037 (0·063)	0·010 (**0·0012**)	0·023 (**$< 10^{-5}$**)	0·013 (**$< 10^{-5}$**)	19	4·0
Closed *v.* interm.	0·021 (**0·012**)	0·041 (**0·00061**)	0·026 (**0·00042**)	0·27 (**$< 10^{-5}$**)	0·13 (**$< 10^{-5}$**)	1·7	5·3
Open *v.* interm.	0·0075 (**0·034**)	−0·0013 (0·57)	−0·0006 (0·49)	0·0023 (0·089)	0·0028 (0·055)	89	4·8
Closed *v.* open	0·011 (**0·017**)	0·030 (**0·0023**)	0·042 (**$< 10^{-5}$**)	0·021 (**$< 10^{-5}$**)	0·10 (**$< 10^{-5}$**)	2·2	2·1
Intra habitat							
Closed	0·091 (**0·00020**)	−0·0095 (0·67)	0·034 (**0·028**)	0·029 (**0·010**)	0·051 (**$< 10^{-4}$**)	4·7	3·7
Intermediate	0·047 (**0·024**)	0·055 (**0·0070**)	0·050 (**0·002**)	0·0046 (**0·021**)	0·032 (**0·0001**)	7·6	3·4
Open	0.063 (**$< 10^{-5}$**)	0·034 (**0·0027**)	0·012 (0·053)	0·029 (**0·0039**)	0·037 (**$< 10^{-5}$**)	6·5	—

F_{st} is computed according to Weir and Cockerham (1984). Type-I error probabilities of the F_{st} exact test are given in parentheses, with a standard error of less than 0·005. 'All' refers to the overall statistics for all loci (the combined probability, using Fisher's method, is given in parentheses). *Nm* refers to the number of migrants computed using either Wright's method (F_{st}) or the private allele method (Slatkin, 1985).

Table 6. *OP-Insecticide resistance gene distribution in relation to habitat types*

	Overproduced esterases				*Ace* locus	
Habitat types	A1	A4–B4	A2–B2	(N)	Ace^R	(N)
Closed	4[a]	0[a]	0[a]	(137)	38[a]	(81)
Intermediate	44[b]	49[b]	4[a]	(278)	224[a]	(154)
Open	79[b]	224[c]	0[a]	(676)	557[a]	(457)

N refers to sample size. Where habitat types share letters within a column these differences are not significant using the Fisher exact test on contingency table.

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two accounts: (1) time to find a host for blood feeding is certainly high due to the scarcity of vertebrates in such environments (mainly small rodents and occasional dogs, cats and humans), and (2) larval mortality is probably low due to a relative stability of environmental parameters such as temperature, and absence of predators (which are often abundant in open air habitats), and the generally high concentration of organic material ensuring a high nutritional value of the medium. Further studies are required to analyse whether the other parameters may explain the differences in frequency of autogeny observed between the different habitats. Another parameter which might be worth investigating in the future is the influence of mosquito control on the evolution of autogeny/anautogeny in natural populations. By disturbing larval and adult survival rates in both, exposure to insecticide might considerably change the relative frequencies of these characters.

The presence of eurygamous females (between 3 and 36%) in fully closed habitats is unexpected. Such a trait seems non-adaptive in such habitats, as swarm formation is inhibited in confined space. However, the evaluation of stenogamy in laboratory conditions does not exactly model the real life situation, and results may be strongly influenced by the size of the containers used for testing the character. Undoubtedly the volume (0·21 m^3) of the cages used is much smaller than any of the underground sites studied.

(ii) *Are mosquitoes from underground and open air sites genetically differentiated?*

The absence of differentiation between open air and intermediate sites indicates that the underground/open air classification of *Culex pipiens* ecotypes is probably artificial. The only classification with biological significance is one group corresponding to fully closed underground sites and a second group including both intermediate and open air sites, as suggested by both the differences or similarities of life history traits (Table 2) and genic differentiation (Table 5) between habitat types.

However, within each of these two 'groups', a significant differentiation was found. This observation is compatible with the population dynamics of this mosquito which experiences repetitive extinctions (including those resulting from insecticidal control) and rapid recolonizations, a situation which could enhance the effect of drift (Wade & McCauley, 1990; Njiokou *et al.* 1994). This hypothesis is supported by the high frequency of heterozygote deficits, an indication of population mixing of differentiated subpopulations (Wahlund, 1928), and of migration of predominantly already mated females (Subra, 1972; Smittle *et al.* 1973; Weidhaas *et al.* 1973).

However, such static data of allele frequency distribution are insufficient to precisely study the complex interaction of drift, migration and extinction/recolonization, and is also inadequate to test the complete neutrality of the genetic markers. Further work is needed to fully understand the dynamics of mosquitoes in these habitat types.

(iii) *How much gene flow exists between habitat types?*

Estimates of gene flow between habitat types were all above 1, indicating that migration is too strong to allow for the fixation of alternate alleles in different habitat types due to drift alone. The possibility exists that gene flow was high in the past but reduced now. Separated populations could retain traces of past migrations for a long time, due to the low level of drift when mosquito populations become large.

However, the presence of the same resistance genes in all habitats (except A2–B2, see below) is a clear indication that migration exists between these habitats. A1, A4–B4 and A2–B2 have been shown to each have a unique and recent origin, so that their present geographic distribution is only explained by migration (Raymond *et al.* 1991, 1992; Rivet *et al.* 1993; Raymond & Marquine, 1994; Chevillon *et al.* 1995*a*; Qiao & Raymond, 1995).

A2–B2 is currently under a world-wide expansion (Raymond *et al.* 1991; Rivet *et al.* 1993), and this is the first report of its presence in the Iberian peninsula. Its frequency is low, which is compatible with a recent introduction and further supported by its absence in a previous, but more limited, sampling of the same area in 1991 (Chevillon *et al.* 1995).

It is not known whether the Ace^R detected in Barcelona is identical to that observed in southern France. Indirect evidence indicates that two resistance alleles may be present in the three habitats (unpublished data), so that Ace^R could represent a composite allelic class. The similar frequency of this allele class in all habitats indicates a similar selection pressure for this gene in the two groups of habitats, between which migration occurred at least once.

5. Conclusion

Commensal forms have a recent origin, not older than the neolithic period for most of them. One of the best studied cases concerns house mouse commensalism which preceded agriculture and has probably been promoted by building practices (Auffray *et al.* 1990). Commensal mice display some behavioural modifications (e.g. Ganem, 1991) and sometimes show recent and major genetic modifications such as Robertsonian chromosome fusions (e.g. Britton-Davidian *et al.* 1989; Said & Britton-Davidian, 1919). However, there is apparently sufficient gene flow between commensal and feral forms to diminish the effect of drift (Auffray *et al.* 1990*a*), and it is unclear whether characters associated with commensalism will promote speciation.

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For the mosquito *Culex pipiens*, no archaeological data are available to date the origin of commensal forms, but both animal husbandry and urbanization actively contribute in producing rich larval habitats. Stenogamy, which is associated with commensal forms, is a major change in mating behaviour and thus a process of particular importance in terms of speciation. Unfortunately, the genetic determination of this character is unknown, as eurygamous females cannot be reared in standard laboratory conditions. Autogeny is also strongly associated with commensalism in *C. pipiens* from European temperate areas, but it has also been selected in non-commensal populations in areas where host availability is reduced (desert regions of Turkestan and Azerbaijan in the former USSR, Babayants & Karapetyan, 1970; Vinogradova, 1961) or where the climate is warmer (Egypt: Knight & Malek, 1951; Tunisia: Dancesco *et al.* 1975; Israel: Nudelman *et al.* 1988). Its genetic determination is not simple (at least two genes are involved according to Spielman, 1957; Aslamkhan & Laven, 1970), penetrance is variable and expressivity often incomplete and modulated by environmental factors including photoperiod, and larval nutrition (Clements, 1992). A better characterization of the genes controlling these two characters and the development of methods for identifying their allelic forms are needed to understand their evolution in natural populations and to determine how selection and migration interact.

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