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Migration/selection balance and ecotypic differentiation in the mosquito *Culex pipiens*

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Abstract

Ecotypic differentiation is well described in the mosquito *Culex pipiens*, separating populations breeding in subterranean and human-made sites (hypogeous habitat) from those in open-air sites (epigeous habitat). The pattern of population differentiation observed at the *Aat-1* locus has been suspected to be associated with such ecotypic differentiation via habitat-dependent selection, but this supposition is still the subject of debate in the literature. We analysed differentiation patterns for *Aat-1* and another four loci among populations from both habitat types in the French Alps. We showed that the *Aat-1*^{A} allele is favoured within hypogeous habitats but selected against within epigeous habitats. Comparisons of our results with other data reported in the literature indicate that the *Aat-1*^{A} allele is generally evolving under habitat-differential selection, but that the precise balance of migration and selection that determines equilibrium allele frequencies varies greatly across Europe. The nature of this habitat-dependent selection, and its resulting (geographically varying) equilibrium point, are discussed in relation to the biology of this mosquito species.

Keywords: allozymes, *Culex pipiens*, ecotypic differentiation, migration, selection

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Introduction

In *Culex pipiens*, two ecotypic forms were described by Roubaud (1933) in northern France (Strasbourg and Paris areas), based on differentiating physiological and behavioural traits. 'Epigeous' populations breed in rural, open-air habitats (brooks, rivers, swamps, ditches, or any artificial open-air collection of water). Females from epigeous habitats require a blood meal to produce their first batch of eggs (anautogeny), are unable to mate in confined spaces, such as in laboratory conditions (eurygamy), hibernate during the winter (heterodynamy), and have a propensity to feed on birds (ornithophily). 'Hypogeous' populations breed in underground urban habitats (cellars, sanitary spaces under buildings, septic tanks). Females from hypogeous habitats do not require a blood meal to produce their first batch of eggs (autogeny), are able to mate in confined spaces (stenogamy), do not hibernate (homodynamy), and have a tendency to feed on mammals (mammophily). These habitat-associated differences in the biology of the hypogeous type are viewed as recent adaptive changes to human-associated hypogeous habitats. Microclimatic conditions are more stable in confined spaces, and opportunities for blood feeding are mainly restricted to a few commensal rodents. The same strict association between physiological traits and ecology is observed in North American and Australian regions with severe winters (Marshall & Staley 1937; Spielman 1964; Miles 1976). By contrast, in Mediterranean countries with mild winters, epigeous and hypogeous populations often display variable degrees of autogeny and/or stenogamy (Roubaud 1939; Knight & Maleek 1951; Rioux *et al*. 1961; Dancesco *et al*. 1975; Pasteur *et al*. 1977; Chevillon *et al*. 1995a).

Geographic variation in the distribution of these adaptive biological traits among habitat types could reflect variation in the migration/selection balance acting on the genes involved. These adaptive traits are not convenient markers for investigating the migration/selection balance, however, as the relative importance of genetic and environmental factors on their expression is not precisely known. For instance, the ability to lay eggs without blood feeding (autogeny, a hypogeous characteristic) is genetically determined

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(Aslamkhan & Laven 1970), but its expression can be modulated by environmental factors such as quality of larval nutrition and photoperiod (Clements 1992). Previous studies indicated that an aspartate–aminotransferase (*Aat-1*) locus could be involved in the adaptation to hypogeous habitats, thus providing a useful marker for the analysis of ecotypic differentiation. Byrne (1996) recently showed that the same allele (hereafter referred as *Aat-1*^{A}) is fixed in most hypogeous populations from London, UK, and that it tends to display low frequencies in the surrounding epigeous populations. A similar situation (although less striking) has been found in southern France (Pasteur 1977). However, no habitat-dependent differentiation in *Aat-1* composition has been found in Italy (Urbanelli *et al*. 1985), Egypt or Israel (Villani *et al*. 1986), and Spain (Chevillon *et al*. 1995a). These results could then well support the conclusions of Urbanelli *et al*. (1985), i.e. that the differentiation pattern observed by Pasteur (1977) was incidental and that *Aat-1* is not involved in ecotypic differentiation. Alternatively, another hypothesis would be that hypogeous and epigeous populations are ecologically more distinct in places where winter is more severe than in Mediterranean countries (cf. Byrne & Nichols 1998).

In the present study, we investigated *Aat-1* differentiation among populations from Chambéry (French Alps) where the winters are cold, and where epigeous and hypogeous mosquitoes display distinct physiological and behavioural traits. The divergence among habitat types for the *Aat-1* locus was compared with that of four other allozyme loci. As insecticide control of *C. pipiens* populations is intense in the Chambéry area, we also compared the *Aat-1*^{A} results with the distributions of resistance genes among habitat types. Some of these resistance mutants have been shown to have a unique origin (Raymond *et al*. 1992; Guillemaud *et al*. 1996), so that only recent gene flow can explain their presence in both habitat types. This comparative framework is used to investigate the relative importance of migration (gene flow) and selection on *Aat-1* differentiation among populations in Chambéry. The analysis was completed by incorporating published data on *Aat-1*^{A} frequencies of neighbouring epigeous and hypogeous populations from several other geographical areas. Our aim is to evaluate the relative effects of geography and ecology on *Aat-1*^{A} differentiation among populations, allowing us to propose hypotheses on the nature of *Aat-1*^{A} selection, and its relevance for ecotypic differentiation.

Materials and methods

Mosquito samples

All samples of *Culex pipiens* were collected as larvae during the summers of 1989 and 1990, from an area of less than 10 km^2 in Chambéry and its suburbs. The five hypogeous sites (H1–H5) were sanitary spaces under buildings, in which larvae were observed throughout the winter of 1989–90. Each of these sites was well isolated from the exterior and was comparable to the 'closed habitats' described by Chevillon *et al*. (1995a). Nine epigeous habitats were also sampled. They included large (200 L) drums for water storage in garden areas (E1, E2 and E3), ditches (E6, E7 and E9), and waterholes (E4, E5 and E8). Distances between two hypogeous breeding sites range from 1.8 km to 4.4 km (mean 2.9 km), between two epigeous samples range from 600 m to 11 km (mean 6.6 km), and distances between hypogeous and epigeous breeding sites range from 1.1 km to 8.8 km (mean 4.8 km). Some of these samples have also been studied with regard to the evolution of insecticide resistance genes (Rivet & Pasteur 1993; Rivet *et al*. 1994). The collected larvae were reared to imagoes in the laboratory, then stored in liquid nitrogen until further processing.

Genetic markers

The polymorphism at seven loci has been analysed. The *Ace* locus, coding an acetylcholinesterase, was assayed by the method of Raymond *et al*. (1985). The polymorphisms at the other loci were analysed by starch gel electrophoresis (Pasteur *et al*. 1988). They included: *Aat-1* and *Aat-2* (aspartate–aminotransferases), *Hk* (hexokinase), *Pgm* (phosphoglucomutase), *Pgi* (phosphoglucoisomerase), *Est-2* and *Est-3* (esterases). We used the reference strains described by Rivet *et al*. (1994)

Population structure analysis

We tested for the presence of directional selection, acting on pairs of loci, using *D* statistics (Ohta 1982). The genotypic associations observed over the whole data set (D_{IT}), were decomposed into four indices, to discriminate between the portions within (D_{IS} and D'_{IS}) and among (D_{ST} and D'_{ST}) populations (Ohta 1982). These indices were computed with the LINKDOS program (Garnier-Gere & Dillmann 1992).

F statistics were estimated according to Weir & Cockerham (1984) and using the GENEPOP (version 3.1) software (Raymond & Rousset 1995). Deviations from Hardy–Weinberg equilibrium at each locus and in each population were tested by the probability test described by Guo & Thompson (1992), using a complete enumeration method (Louis & Dempster 1987) for loci with up to four alleles and a Markov chain method (Guo & Thompson 1992) for loci with more than four alleles. Under the assumption of frequent mixing of genetically differentiated populations within breeding sites, a general tendency for heterozygote deficits is expected. The multisample extension of the score test described by

Rousset & Raymond (1995) was then applied to data, using the alternative hypothesis 'H_1: heterozygote deficits'.

Genotypic differentiation among populations was tested using the G_a-based exact test described by Goudet *et al*. (1996), with a Markov–Chain method (see Guo & Thompson 1992) of 100 000 steps. When three levels (habitats, populations and individuals) were considered, we estimated the genetic differentiation between (F_{CT}) and within (F_{SC}) habitat types by computing the estimates defined by Weir & Cockerham (1984). Under habitat-dependent selection, we expect a larger differentiation between than within habitat types, and values of F_{CT} significantly greater than zero. Genetic differentiation between habitat types was tested for each locus by comparing the observed value of $\hat{F}_{CT}$ with the distribution estimated by 5000 permutations of whole populations among habitats. These permutations were performed with the AMOVA (version 1.55) program (Excoffier *et al*. 1992).

Isolation by distance was tested by Mantel tests (Smouse & Long 1992) performed between pairwise estimates of $[F_{ST}/(1 - F_{ST})]$ and of geographical distances as described by Rousset (1997).

Discrimination between two habitat-dependent effects

The tendency of hypogeous populations to evolve in greater isolation (on average) than do epigeous populations has been previously reported (Byrne 1996; Chevillon *et al*. 1995a). This habitat-dependent (effective population size) effect can be separated from the effect of habitat-dependent selection *per se*. If habitat-dependent selection is the primary evolutionary force driving this system, then each hypogeous sample should (on average) be genetically more similar to other hypogeous samples than to the epigeous counterparts. Conversely, if the primary habitat-associated effect is a difference in the balance between migration and drift, with hypogeous populations being more isolated, then each hypogeous sample should (on average) be genetically less similar to other hypogeous samples than to epigeous counterparts. These predictions can be formalized and tested, as follows. The F_{ST} estimate computed between the *i*-th and *j*-th populations is denoted by θ_{ij}. We denote the mean θ_{ij} value between the *i*-th hypogeous population and all the other hypogeous populations by $\theta_{i\text{-}hypo}$. Similarly, we denote the mean θ_{ij} value between the *i*-th hypogeous population and the collection of epigeous populations by $\theta_{i\text{-}epi}$. For those loci evolving under strict habitat selection, we expect ($\theta_{i\text{-}hypo} < \theta_{i\text{-}epi}$), and we expect the order ($\theta_{i\text{-}hypo} > \theta_{i\text{-}epi}$) if the effect of genetic drift is stronger (on average) within hypogeous than within epigeous populations. For each hypogeous sample (*i*) and each locus, the null hypothesis of identical distributions for $\theta_{i\text{-}hypo}$ and $\theta_{i\text{-}epi}$ was tested with a nonparametric Mann–Whitney test (Siegel & Castellan 1988).

Meta-analysis of Aat-1 *differentiation*

The extent of *Aat-1* differentiation among neighbouring epigeous and hypogeous populations was analysed in several different localities (Pasteur 1977; Urbanelli *et al*. 1985; Villani *et al*. 1986; Byrne & Nichols 1998; Chevillon *et al*. 1995a). Statistical analyses were restricted to published cases where information on sample sizes were available: eight hypogeous and 12 epigeous populations from London, UK (Byrne 1996), five hypogeous and 30 epigeous populations from Barcelona, Spain (Chevillon *et al*. 1995a), seven hypogeous and 19 epigeous populations from Montpellier, France (Pasteur 1977), and five hypogeous and nine epigeous populations from Chambéry (present study). Despite variation in allelic nomenclature among published research, the allele invariably found at high frequency in hypogeous populations was identical to that designated as the *Aat-1*^{A} allele in this study: all common reference strains are monomorphic for this allele.

To analyse how the ecology and the geography of sampling affect the distribution in *Aat-1*^{A} frequency among population groups, we compared alternative models describing this trait for each of the following questions. (i) Do the epigeous and hypogeous distributions in *Aat-1*^{A} frequency significantly differ from one another within localities? For each locality, a model describing a similar distribution in *Aat-1*^{A} frequency across habitat was compared to the alternative model where the expected *Aat-1*^{A} frequency differs between habitat types. (ii) Is the habitat-dependent difference in *Aat-1*^{A} frequency the same among all localities? The overall data set was then fitted by two models which allows differences in *Aat-1*^{A} frequency between localities and between habitat types, but where the habitat-dependent difference in frequency is fixed in one model and is allowed to vary among localities in the other. (iii) If habitat-dependent differences in *Aat-1*^{A} frequency vary among the localities, do such variations mainly reflect differences within habitat types between Mediterranean (Montpellier and Barcelona) and non-Mediterranean (London and Chambéry) localities? Among samples collected within each habitat type, we first investigated for possible differences in *Aat-1*^{A} frequencies between the two climatic groups, and then for residual differences within these groups.

These analyses were performed using the GLIM software (Baker & Nelder 1985) by modelling *Aat-1*^{A} frequency as a variable with binomial error, in order to correct for the heterogeneity of variances which must be larger for intermediate frequencies. Alternative models were compared using *F*-derivative tests after correction

for overdispersion, i.e. deviation from binomial variance among samples (see Crawley 1993).

Results

Distribution of polymorphism among populations in Chambéry

Aat-1. The allele *Aat-1*^{A} displayed significantly (Mann–Whitney test, $P = 0.01$) higher frequencies within hypogeous (range, 0.24–1.00, Table 1) than within epigeous populations (range, 0.18–0.60, Table 1). Significant deviations from Hardy–Weinberg expectations were observed in two samples where only homozygous individuals were observed ($P < 0.05$ in H1 and H4). *Aat-1* presented an overall tendency to display significant ($P < 0.005$) heterozygote deficits, and this tendency remained significant ($P < 0.005$) when only hypogeous populations were considered.

Independence between Aat-1, Aat-2, Hk, Pgm, *and* Pgi. Genotypic associations among all pairs of loci were analysed within each sample. The null (genetic equilibrium) hypothesis was only rejected ($P < 0.05$, details not shown) in seven of the 117 tests, i.e. in a number of cases not higher than expected under the null hypothesis. The absence of linkage disequilibrium (within samples) between *Aat-1*, *Aat-2*, *Hk*, *Pgm*, and *Pgi* indicated that these loci provide independent information on population structure.

Ohta's (1982) *D* statistics were computed for all samples and for samples collected within each habitat type in order to detect possible selection acting on pairs of loci. The null hypothesis that observed that gametic associations were due to the action of drift alone could not be rejected (details not shown; $D_{IS} < D_{ST}$ and $D'_{IS} > D'_{ST}$ for all pairs of loci, in all three sets of tests), indicating that selection acting on pairs of loci is unlikely.

Aat-2, Hk, Pgm, *and* Pgi. These four loci exhibited from two to six alleles each (Table 1). All epigeous populations were polymorphic at all loci. This was not the case among hypogeous populations; population H1 was monomorphic at both *Pgm* and *Aat-2* loci and population H5 at the *Pgm* locus. Interestingly, the allele fixed within hypogeous populations was the most frequent allele in epigeous populations (Table 1). Significant ($P < 0.05$) deviations from Hardy–Weinberg expectations were observed at the *Pgm* locus in four samples (H3, E1, E6 and E7) and at the *Hk* locus in one epigeous sample (E3). Global tests of Hardy–Weinberg expectations were performed using the alternative hypothesis 'H_1: heterozygote deficits'. Three samples displayed significant heterozygote deficits across loci ($P = 0.021$ in H3; $P = 0.011$ in E1 and $P = 0.017$ in E6). *Pgm* presented a significant ($P < 0.005$) tendency to display heterozygote deficits within populations, such a tendency remaining significant ($P < 0.001$) when only epigeous populations were considered.

Insecticide resistance genes. Three loci (*Ace*, *Est-2* and *Est-3*) are involved in resistance to organophosphate insecticides (OP); OPs were used extensively in the Chambéry area at the time of collection (Rivet & Pasteur 1993; Rivet *et al*. 1994). Two alleles were present at the *Ace* locus: *Ace*^{S}, coding for the wild-type acetylcholinesterase, and *Ace*^{R}, coding for an enzyme insensitive to inhibition by OP. The *Ace*^{R} allele was observed in four of the five hypogeous populations and in all epigeous populations. Highly active esterases A1 and A4, which are encoded by dominant alleles of the *Est-3* gene and involved in OP resistance, were observed in all samples except H5 (from which both A1 and A4 were absent) and E7 (in which A4 was not recorded). A strict association was observed between *Est-2*$^{0.64}$ and the highly active esterase A1, as well as between highly active esterases B4 and A4, encoded, respectively, by the *Est-2* and *Est-3* loci. Such high linkage disequilibria between these *Est-2* and *Est-3* alleles have been observed in all previous studies from western Mediterranean countries (e.g. Severini *et al*. 1993; Raymond & Marquine 1994; Rivet *et al*. 1994; Chevillon *et al*. 1995a,b), strongly suggesting that both A1 and A4–B4 are probably the results of unique mutational events (see Raymond *et al*. 1992). Their presence in a particular population is an indication of recent genetic exchange among populations.

Population differentiation within and between habitat types

Aat-1. Differences in habitat types explain a large and significant part of the differentiation observed for *Aat-1* ($\hat{F}_{CT} = 0.29$, $P = 0.010$, Table 2), but the mean differentiation within habitat types was as high as the divergence between them ($\hat{F}_{SC} = 0.23$, $P = 0.005$, Table 2). A stronger differentiation was observed among hypogeous than among epigeous populations ($\hat{F}_{ST\ hypo} = 0.212$, $P < 10^{-5}$ and $\hat{F}_{ST\ epi} = 0.094$, $P < 10^{-5}$). As already observed through the analysis of allelic frequencies (see above and Table 1), pairwise differentiation estimates θ_{ij} indicate that H3 is the only hypogeous sample to be similar to epigeous populations at the *Aat-1* locus (see Table 3). However, it is noteworthy that H3 is not the only sample involved in the strong differentiation observed within the hypogeous habitat: *Aat-1* composition of H2 is also significantly and strongly different from that observed within all other hypogeous sites (see θ_{ij} in Table 3).

Other loci. Two loci (*Hk* and *Pgi*) provided significant estimates of F_{CT} ($P < 0.039$), but all four loci provided estimates of F_{CT} that were much smaller ($\hat{F}_{CT} < 0.06$) than the *Aat-1* locus ($\hat{F}_{CT} = 0.29$). In addition, the extent of differentiation

seems larger within ($\hat{F}_{SC}$ = 0.042) than between ($\hat{F}_{CT}$ = 0.026) habitat types (Table 2). In order to clarify the situation, population differentiation was separately analysed within each habitat. Within the epigeous habitat, no differentiation was observed among populations (averaged over all loci, $\hat{F}_{ST}$ = – 0.0001, P = 0.07; for *Pgm* $\hat{F}_{ST}$ = 0.094, P = 0.013; but for the other three loci, $\hat{F}_{ST}$ < 0.0013, P > 0.15). The pairwise differentiation estimates θ_{ij} indicated weak divergence among all pairs of epigeous samples (Table 3). A very different pattern was observed within the hypogeous ecotype where a strong differentiation was observed at all loci (averaged over all loci, $\hat{F}_{ST}$ = 0.29, $P < 10^{-5}$; for *Aat-2* and *Hk*, $\hat{F}_{ST} \approx 0.04$ (P < 0.002), but for *Pgm* and *Pgi*, $\hat{F}_{ST}$ > 0.15, $P < 10^{-5}$). All pairwise comparisons of hypogeous populations displayed high differentiation except H2 and H3 (see θ_{ij} in Table 3), i.e. the two very hypogeous populations which had already been noticed for their particular *Aat-1* composition (see section above).

We investigated the possibility of isolation by distance. Within each habitat type, no increase of genetic differentiation was observed with increasing distance (epigeous, P = 0.39; hypogeous, P = 0.84). Among all samples, genetic differentiation tends to decrease (b = – 0.013, P = 0.062, see Fig. 1) with increasing geographical distance, rather than increase. This tendency seems to be a result of shorter geographical distances among pairs of highly differentiated hypogeous populations than among pairs of weakly differentiated epigeous populations (see Fig. 1).

Habitat-dependent effects on population differentiation

The strong genetic differentiation observed among nearby hypogeous populations deserved further investigation on the relative importance of selection and genetic drift. Each hypogeous population was compared with the other populations to determine whether it was genetically more similar to the other hypogeous ($\theta_{i\text{-}hypo} < \theta_{i\text{-}epi}$) or to epigeous populations ($\theta_{i\text{-}hypo} > \theta_{i\text{-}epi}$).

Aat-1. The null hypothesis of identity between $\theta_{i\text{-}hypo}$ and $\theta_{i\text{-}epi}$ could not be rejected (P > 0.1) for hypogeous populations H1, H2, and H4. We observed $\theta_{i\text{-}hypo} < \theta_{i\text{-}epi}$ in population H5 (P = 0.05), as expected under the selection model for *Aat-1*^{A}. By contrast, H3 was significantly (P = 0.005) more similar to epigeous ($\theta_{i\text{-}hypo} > \theta_{i\text{-}epi}$) than to other hypogeous populations for its *Aat-1* composition. H5 and H3 are the hypogeous samples with the highest (hypogeous-typical) and lowest (epigeous-typical) *Aat-1*^{A} frequencies, respectively (Table 1). Under the assumption of habitat-dependent selection acting on *Aat-1*^{A}, these results would then indicate that the efficiency of the hypogeous-positive selection (relative to migration between habitat types) greatly varies among hypogeous sites, from a strong effect in H5 to no effect in H3, and intermediate effects in the other three sites.

The other four loci. For the *Aat-2* locus and all hypogeous populations, $\theta_{i\text{-}hypo}$ and $\theta_{i\text{-}epi}$ were not significantly (P > 0.1) different. For *Pgm* and *Pgi* in populations H2 and H3 and for *Hk* in population H2, we found that $\theta_{i\text{-}hypo} > \theta_{i\text{-}epi}$ (P < 0.05). When all four loci were considered simultaneously, we found that $\theta_{i\text{-}hypo} > \theta_{i\text{-}epi}$ (P < 0.05) in populations H2 and H3, which were also the only hypogeous populations between which the absence of genetic divergence was not rejected (see Table 3). These results indicate that genetic drift (relative to migration) is highly variable among hypogeous populations, e.g. with stronger effects within H1, H4 and H5 than within H2 and H3 populations. These differences (as discussed below) are congruent with H2 and H3 having experienced large immigration waves in a more recent past than did the other three hypogeous populations.

Effects of ecology and geography on Aat-1 *differentiation patterns*

Information available from the literature on *Aat-1* composition among neighbouring epigeous and hypogeous populations is summarized in Table 4. Statistical analyses were performed on those populations from Chambéry, London, Barcelona and Montpellier for which sample sizes were available.

Effects of habitat type on the distribution of Aat-1^A *frequencies*

With the exception of Barcelona (P = 0.15), the type of habitat significantly affects the distribution of *Aat-1*^{A} frequencies (see 'Habitat' in Table 4, P < 0.05). The global trend among the four regional studies is a significant (Fisher's method, χ^2 = 62, d.f. = 8, $P = 1.5 \times 10^{-10}$) divergence of frequencies between epigeous and hypogeous populations. This result indicates higher *Aat-1*^{A} frequencies within hypogeous than within nearby epigeous populations (see Table 4). However, the habitat-dependent difference in *Aat-1*^{A} frequency significantly varies among localities (interaction HABITAT × LOCALITY: $F_{3,83}$ = 11.4, $P < 10^{-5}$). Such a variation requires further investigation into the geographical variation in *Aat-1*^{A} frequencies among populations colonizing a single habitat type.

Effects of geographical factors within each habitat

Barcelona and Montpellier are grouped together, as localities experiencing similar Mediterranean climatic conditions, whereas London and Chambéry are grouped together as localities experiencing more temperate climatic conditions. Within the hypogeous collection, significant (P < 0.01)

Table 1 Polymorphism observed in hypogeous and epigeous habitats in the region of Chambéry (France). Polymorphism at the *Est-3* locus is described by the number of mosquitoes exhibiting different phenotypes which are defined by the single or joint presence of the overproduced esterases A1 and A4 or by the absence of any overproduced esterase (phenotype Null). The polymorphism at the *Est-2* locus is not reported, due to maximal linkage disequilibrium between *Est-2* and *Est-3* dominant alleles (i.e. every mosquito displaying A1 (A4) also displays *Est-2*$^{0.64}$ (B4, respectively) and vice versa). For codominant allozyme loci, allelic frequencies and $\hat{F}_{IS}$ values are reported

	Hypogeous habitat					Epigeous habitat								
Locus	H1	H2	H3	H4	H5	E1	E2	E3	E4	E5	E6	E7	E8	E9
Est-3														
(N)	58	58	58	116	58	58	44	116	58	58	58	58	58	63
A1	4	29	10	27	0	23	6	28	30	8	2	3	22	7
A4	3	0	1	9	0	6	2	3	3	2	1	0	6	3
A1A4	0	4	0	1	0	0	2	3	0	0	0	0	0	1
Null	51	25	47	80	58	29	34	82	25	48	55	55	30	52
Ace														
(N)	58	58	58	105	58	58	44	116	58	58	58	58	58	58
R	0.121	0.491	0.155	0.510	–	0.397	0.705	0.448	0.629	0.233	0.190	0.207	0.353	0.379
S	0.879	0.509	0.845	0.490	1.000	0.603	0.295	0.552	0.371	0.767	0.810	0.793	0.647	0.621
$\hat{F}_{IS}$	+0.034	**–0.268**	–0.043	–0.043	–	**–0.361**	+0.138	+0.168	+0.159	–0.005	+0.223	+0.168	–0.237	–0.090
Aat-1														
(N)	28	20	29	31	34	22	24	–	30	32	24	33	28	28
A	0.929	0.700	0.241	0.968	1.000	0.591	0.396	–	0.183	0.188	0.188	0.258	0.339	0.554
B	–	–	–	–	–	–	–	–	–	0.016	0.021	–	–	–
C	–	–	–	–	–	–	–	–	–	–	–	–	–	0.036
D	0.071	0.225	0.741	0.032	–	0.409	0.604	–	0.800	0.781	0.792	0.727	0.589	0.393
I	–	0.075	0.017	–	–	–	–	–	0.017	0.016	–	0.015	0.054	–
K	–	–	–	–	–	–	–	–	–	–	–	–	0.018	0.018
$\hat{F}_{IS}$	**+1**	–0.299	+0.049	**+1**	–	–0.487	+0.063	–	+0.199	+0.133	+0.157	+0.116	+0.016	–0.178
Aat-2														
(N)	29	22	29	31	31	24	24	30	–	32	24	33	28	28
0	1.000	0.818	0.879	0.935	0.839	0.896	0.833	0.883	–	0.781	0.792	0.788	0.839	0.857
B	–	–	–	–	–	–	0.021	–	–	0.016	0.021	–	0.018	0.018
C	–	0.182	0.121	0.065	0.161	0.104	0.146	0.067	–	0.203	0.188	0.197	0.125	0.125
K	–	–	–	–	–	–	–	0.050	–	–	–	0.015	0.018	–
$\hat{F}_{IS}$	–	–0.200	+0.205	–0.053	–0.176	**+0.349**	–0.154	–0.080	–	–0.061	+0.034	+0.302	–0.133	+0.158
Hk														
(N)	29	23	29	30	34	30	24	35	31	30	24	27	34	29
A	0.776	0.435	0.534	0.567	0.618	0.517	0.542	0.471	0.387	0.450	0.583	0.519	0.515	0.483
B	0.224	0.565	0.448	0.433	0.382	0.483	0.458	0.529	0.613	0.550	0.417	0.481	0.471	0.517
C	–	–	0.017	–	–	–	–	–	–	–	–	–	0.015	–
$\hat{F}_{IS}$	+0.125	–0.578	–0.261	–0.206	–0.106	+0.016	+0.348	**–0.538**	–0.071	–0.264	+0.164	–0.169	+0.041	–0.226

Table 1 Continued

	Hypogeous habitat					Epigeous habitat								
Locus	H1	H2	H3	H4	H5	E1	E2	E3	E4	E5	E6	E7	E8	E9
Pgm														
(N)	29	23	29	32	34	32	24	34	32	32	24	33	34	30
A	1.000	0.652	0.672	0.953	1.000	0.688	0.875	0.721	0.859	0.703	0.750	0.773	0.809	0.783
C	–	0.065	–	–	–	–	–	–	–	0.063	0.063	–	–	–
D	–	0.239	0.155	0.047	–	0.234	0.083	0.206	0.109	0.203	0.083	0.182	0.147	0.133
E	–	0.043	0.069	–	–	0.047	0.042	0.074	0.031	0.031	0.021	0.045	0.015	0.083
F	–	–	0.103	–	–	0.031	–	–	–	–	0.083	–	0.029	–
$\hat{F}_{IS}$	–	–0.083	**+0.270**	–0.033	–	**+0.083**	–0.087	–0.140	+0.135	–0.072	**+0.225**	**+0.517**	+0.195	+0.003
Pgi														
(N)	29	23	26	32	34	32	24	32	32	29	24	33	34	30
A	0.241	0.717	0.769	0.125	0.559	0.594	0.563	0.734	0.719	0.638	0.646	0.652	0.588	0.600
B	–	–	–	–	–	0.016	0.083	0.016	0.016	0.017	–	–	–	–
C	0.759	0.283	0.231	0.875	0.441	0.391	0.333	0.250	0.266	0.345	0.354	0.333	0.368	0.400
E	–	–	–	–	–	–	0.021	–	–	–	–	0.015	0.044	–
$\hat{F}_{IS}$	+0.076	+0.270	+0.153	–0.127	+0.060	–0.122	+0.063	–0.162	+0.031	+0.071	+0.019	–0.160	–0.181	+0.183

	Locus					Multilocus	
	Aat-1	*Aat-2*	*Pgm*	*Pgi*	*Hk*	with *Aat-1*	without *Aat-1*
$\hat{F}_{ST}$	0.46	0.019	0.060	0.15	0.029	0.17	0.070
$\hat{F}_{SC}$	0.23	0.010	0.044	0.10	0.013	0.10	0.047
$\hat{F}_{CT}$	0.29	0.008	0.016	0.052	0.016	0.082	0.026

Table 2 Measures of differentiation within and between ecotypes. F_{ST}, F_{SC} and F_{CT} are the Weir & Cockerham (1984) parameters, describing the differentiation among all populations, among populations within habitats, and between habitats, respectively. Significant ($P < 0.05$) deviations are underlined

Table 3 Comparisons of θ_{ij} estimates computed with Aat-1 (above the diagonal) or another four loci (below the diagonal) for all pairs of populations. Significant differentiation ($P < 0.05$) is indicated in bold type

Other loci	*Aat-1* H1	H2	H3	H4	H5	E1	E2	E3	E4	E5	E6	E7	E8	E9
H1		**0.11**	**0.63**	−0.02	0.05	**0.27**	**0.47**	–	**0.69**	**0.43**	−0.01	**0.60**	**0.47**	**0.25**
H2	**0.27**		**0.35**	**0.19**	**0.29**	0.04	**0.19**	–	**0.43**	**0.41**	**0.42**	**0.34**	**0.19**	**0.04**
H3	**0.25**	−0.00		**0.69**	**0.75**	**0.20**	0.03	–	−0.01	−0.01	−0.01	−0.02	0.02	**0.18**
H4	**0.06**	**0.25**	**0.26**		0.00	**0.37**	**0.56**	–	**0.75**	**0.73**	**0.76**	**0.67**	**0.54**	**0.33**
H5	**0.12**	**0.08**	**0.07**	**0.15**		**0.46**	**0.64**	–	**0.81**	**0.78**	**0.81**	**0.73**	**0.61**	**0.41**
E1	**0.18**	−0.00	0.00	**0.16**	**0.02**		0.06	–	**0.28**	**0.26**	**0.26**	**0.18**	**0.07**	−0.01
E2	**0.16**	**0.04**	0.01	**0.16**	**0.03**	0.00		–	**0.08**	0.07	0.07	0.02	−0.01	**0.05**
E3	**0.26**	−0.00	−0.00	**0.25**	**0.07**	0.00	0.01		–	–	–	–	–	–
E4	**0.29**	0.01	0.02	**0.28**	**0.07**	0.02	0.01	0.01		−0.02	−0.02	−0.00	0.06	**0.25**
E5	**0.21**	0.01	0.01	**0.19**	**0.01**	0.01	0.02	0.00	0.01		−0.02	−0.01	0.05	**0.23**
E6	**0.11**	**0.03**	0.01	**0.19**	**0.03**	0.00	−0.00	0.01	**0.03**	0.02		−0.01	**0.05**	**0.24**
E7	**0.19**	0.00	−0.00	**0.19**	**0.01**	0.00	−0.01	0.00	0.01	0.00	0.01		0.01	**0.16**
E8	**0.16**	0.01	0.00	**0.16**	**0.02**	−0.00	−0.00	0.01	0.01	0.01	−0.00	0.00		0.06
E9	**0.17**	−0.00	0.00	**0.15**	**0.02**	−0.00	0.00	0.00	0.01	0.01	0.00	0.00	–	0.00

differences in distributions of *Aat-1*^{A} frequencies were found within but not between climatic groups (Table 5). Pairwise comparisons between studies indicate that the main source of heterogeneity is a significant ($P < 0.0001$) difference in the hypogeous distributions of *Aat-1*^{A} frequencies observed in Montpellier and in Barcelona.

Within the epigeous habitat, significant ($P < 0.0001$) differences in distributions of *Aat-1*^{A} frequencies were detected between temperate and Mediterranean groups of populations, but not within climatic groups (Table 5). Comparisons between all pairs of localities confirmed these results: the assumption of a common distribution of *Aat-1*^{A} frequency was not rejected among Mediterranean as well as among non-Mediterranean groups of epigeous populations ($P > 0.3$ for Montpellier–Barcelona and for London–Chambéry comparisons, see Table 5), but was rejected in all four Mediterranean/non-Mediterranean comparisons ($P < 0.0001$ for all cases, see Table 5).

Discussion

A detailed understanding of the mechanisms involved in ecotypic differentiation is difficult to achieve, especially when ecotypes are defined by phenotypic differences which can be modulated by environmental conditions. The presence of a genetic marker evolving under habitat-dependent selection is thus a particularly useful addition to any test of the relative importance of selection and population subdivision on ecotypic divergence. Previous investigators proposed that one allozyme allele, *Aat-1*^{A}, might be involved in the adaptation of *Culex pipiens* mosquitoes to human-constructed subterranean (hypogeous) breeding sites. The present study was performed to investigate the information brought by *Aat-1* differentiation to the subject of ecotypic differentiation. In Chambéry, genetic differentiation among nearby (maximal distance of about 11 km) populations was analysed for several loci, in order to test the relative importance of gene flow (a generic demographic process) and selection (acting on the *Aat-1* locus). We then investigated the effects of geography and of divergent regional ecology on *Aat-1* differentiation among populations collected over a wider geographical area.

Mechanisms involved in Aat-1 *differentiation in Chambéry*

The results are consistent with selection driving *Aat-1*^{A} allele frequencies in opposite directions in hypogeous and

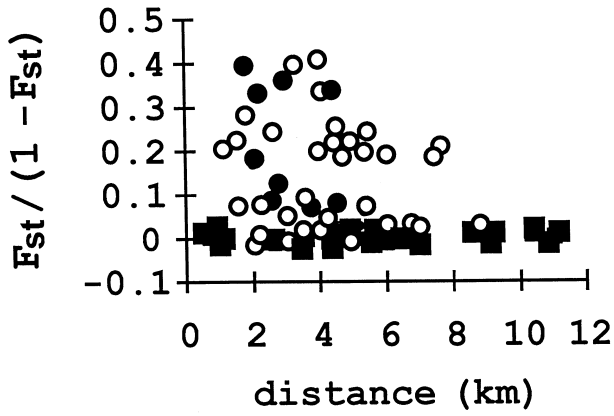

Fig. 1 Absence of isolation by distance in Chambéry. Pairwise estimates of $[F_{ST}/(1 - F_{ST})]$ are plotted against pairwise geographical distances, as described by Rousset (1997). Open circles correspond to comparisons between one hypogeous and one epigeous populations, filled circles to comparisons between two hypogeous populations, and squares indicate comparisons between two epigeous populations.

epigeous ecotypes. First, differentiation between habitat types is much larger for *Aat-1* than for all other loci. Second, this habitat-dependent effect corresponds to elevated *Aat-1*^{A} frequencies in hypogeous populations of Chambéry, compared with the surrounding epigeous populations.

The pattern of genetic differentiation displayed at the other four loci, none of them thought to be involved in adaptation to the alternate habitat regimes, suggests that hypogeous populations are relatively isolated from each other, relative to epigeous populations, and/or that the population effective sizes tend to be smaller within the hypogeous than within the epigeous habitats. This is consistent with the routine observation of monomorphic loci in hypogeous populations but not in the epigeous counterparts. We also observe high genetic differentiation among hypogeous populations, as opposed to nondifferentiation among epigeous populations, over comparable geographical distances. In addition, none of the hypogeous populations displayed a genetic divergence higher from epigeous ($\theta_{i\text{-}epi}$) than from other hypogeous ($\theta_{i\text{-}hypo}$) populations. This suggests that preferential gene flow among hypogeous populations (as compared to gene flow between habitat types) is unlikely. Finally, the high estimates of pairwise differentiation θ_{ij} computed for hypogeous populations confirm that the migration/drift balance tends to be biased toward genetic drift in the hypogeous habitat, despite variations among sites.

Isolation of hypogeous populations is not absolute, however, as indicated by the presence of OP resistance genes in four of the five hypogeous populations. Their presence in any particular population can only have resulted from migration inputs from other populations, as these alleles are each known to have been the result of a

Table 4 *Aat-1*^{A} divergence between ecotypes in several localities. For each locality, numbers of hypogeous (hypo) and epigeous (epi) populations sampled and average *Aat-1*^{A} frequency (ranges in parentheses) are indicated. When sample sizes were available, the effect of habitat type on *Aat-1*^{A} frequency distributions was tested using GLIM models (see text for explanation). *All* refers to a global test (Fisher's method) across studies

	Aat-1^A frequency within ecotype:		Habitat differences
Region	Hypogeous	Epigeous	*P* value
London (8 hypo; 12 epi)	0.91 (0.30–1.0)	0.35 (0.10–0.85)	$<2\times10^{-4}$
Chambéry (5 hypo; 9 epi)	0.78 (0.24–1.0)	0.33 (0.18–0.59)	0.012
Barcelona (5 hypo; 24 epi)	0.77 (0.52–0.90)	0.72 (0.53–0.93)	0.15
Montpellier (7 hypo; 19 epi)	0.99 (0.93–1.0)	0.66 (0.31–0.91)	$<10^{-6}$
All			$<10^{-8}$
Italy (2 hypo; 13 epi)	0.96* (0.52–0.90)	0.62 (0.52–0.93)	NT†
Egypt-Israel (3 hypo; 7 epi)	0.95* (0.93–1.0)	0.94 (0.31–0.91)	NT†

* Identity of *Aat-1*^{A} with the alleles called *Aat-1*^{100} by Villani *et al*. (1985) and Urbanelli *et al*. (1986) has not been tested, but is probable. The strain S54 used by Pasteur (1977) and in the present study, as well as all the reference strains used in Chevillon *et al*. (1995a) and in Byrne (1996), are monormorphic for this allele.
†No formal test was performed, in the absence of detailed information on sample sizes.

	Aat-1 differentiation within habitats	
	Hypogeous	Epigeous
Levels investigated		
Among the four localities	**$F_{(1,23)}$ =9.66****	**$F_{(3,59)}$ = 53*****
Between climatic groups	$F_{(1,23)}$ = 2.89	**$F_{(1,61)}$ = 52*****
Within climatic groups	**$F_{(2,21)}$ = 6.80***	$F_{(2,59)}$ = 1.05
Pairwise comparisons		
Chambéry–London	$F_{(1,11)}$ =0.8	$F_{(1,18)}$ = 0.2
Chambéry–Montpellier	$F_{(1,10)}$ = 8.2	**$F_{(1,30)}$ = 45*****
Chambéry–Barcelona	$F_{(1,8)}$ = 1.0	**$F_{(1,25)}$ = 19*****
London–Montpellier	$F_{(1,13)}$ = 3.1	**$F_{(1,29)}$ = 17*****
London–Barcelona	$F_{(1,11)}$ = 0.6	**$F_{(1,34)}$ = 36*****
Montpellier–Barcelona	**$F_{(1,10)}$ = 11.8*****	$F_{(1,41)}$ = 0.9

Table 5 Geographical variation in *Aat-1*^{A} frequencies within ecotypes. The first part of the table summarizes the effects of localities, of climatic groups, and of localities within climatic groups, on the overall distribution in *Aat-1*^{A} frequencies within each habitat type. The second part of the table summarizes the effects of locality on all pairwise comparisons. The effect of each geographical factor is indicated by the corresponding *F* value (d.f. in parentheses). Significant ($P < 0.05$) effects are indicated in bold type

*Significant at $P < 0.01$.
**Significant at $P < 0.0005$.
***Significant at $P < 0.0001$.

unique mutation event (e.g. A1 and A4–B4, see Raymond *et al*. 1992; Guillemaud *et al*. 1996). A 2-year longitudinal survey of one hypogeous population from Chambéry suggested that immigration from epigeous into hypogeous breeding sites is likely to occur whenever epigeous females seek shelter for hibernation (Rivet & Pasteur 1993).

The exact pattern of gene flow among habitat types is not known, but the possibility of asymmetric gene flow cannot be ruled out; in particular, we might have migration from epigeous to hypogeous populations more frequently than the converse. The assumption of immigrants into hypogeous populations being primarily of epigeous origin is consistent with the evidence that some hypogeous populations are genetically more similar to nearby epigeous populations than to other hypogeous populations. For these epigeous-similar hypogeous populations, *Aat-1*^{A} frequency is low, compared with other hypogeous populations, suggesting that further selection on *Aat-1*^{A} will shift the frequency upward. This scenario seems indeed to be supported by comparing the H3 and H2 populations. These populations are genetically similar at all loci except *Aat-1* (see Table 3, $\theta_{ij} = 0.35$ for *Aat-1* and is null otherwise), neither have diverged strongly from surrounding populations (as far as the other loci are concerned), and both are strongly different from other hypogeous populations. These results are consistent with an evolutive scenario of populations colonizing hypogeous sites where: (i) H3 represents an early stage of colonization (no drift, no selection); (ii) H2 represents a slightly later stage (no drift but habitat-dependent selection); and (iii) the other three samples represent later stages (after drift and selection). Although further longitudinal studies are necessary to confirm this evolution of hypogeous populations, our regional survey clearly shows that habitat-dependent selection is acting on the *Aat-1* locus. It is very unlikely that genetic drift alone can independently enhance the frequency of the very same allele (*Aat-1*^{A}) within the 25 hypogeous sites investigated, knowing that (i) there has recently been gene flow between habitat types (as attested by resistance gene distributions in Chambéry and in Barcelona (see Chevillon *et al*. 1995a)), and (ii) this allele sometimes displays very low frequencies within surrounding epigeous populations (as observed in London, UK; Byrne & Nichols 1998).

Ecological and geographical effects on Aat-1 *differentiation*

It has been suggested that *Aat-1*^{A} is involved in adaptation to a hypogeous ecology through a functional connection with autogeny, another hypogeous-adaptive trait (Pasteur 1977). Autogeny is the ability to lay a first batch of eggs without a prior blood meal. Reduction and suppression of autogeny is observed when there is a food limitation or high competition during larval development (Clements 1992). These results suggest that energy transfer from larval uptake to subsequent egg material is probably an important limiting factor for autogenous female fertility under suboptimal larval feeding conditions. Pasteur (1977) has suggested that the *Aat-1*^{A} allele could promote more effective transamination (more efficient turnover of amino acids), which could be crucial under hypogeous conditions. For anautogenous females, on the other hand, a more efficient turnover of amino acids may be less crucial. Clements (1992) reports that anautogenous female reproductive potential is established by the end of larval development, but that the subsequent exploitation

of that potential varies with multiple factors of adult nutrition, the quality and quantity of blood ingested, even the availability of nectar. Their fertility is thus affected in more complex ways than for autogenous females, and more effective transamination might be a secondary consideration.

On a larger geographical scale, analyses of *Aat-1* differentiation indicate that *Aat-1*^{A} is probably adaptive (in general) under hypogeous conditions but selected against under epigeous conditions. Nevertheless, the effect of habitat type on *Aat-1*^{A} frequency varies among regions, indicating that the balance of migration and selection is variable.

The distribution of *Aat-1*^{A} frequencies within habitat types varied geographically. Within the epigeous ecotype, *Aat-1*^{A} frequencies were higher in Mediterranean localities than in northern European localities. No geographical variation was observed within the hypogeous ecotype. These patterns indicate that (i) the efficiency of the selection against *Aat-1*^{A} in epigeous habitats decreases in the Mediterranean basin, relative to more northern areas, but that (ii) the efficiency of the selection for *Aat-1*^{A} in hypogeous habitats is much less variable.

The geographical pattern observed in the epigeous ecotype could reflect a gradual increase, from northern to southern Europe, in the level of gene flow between ecotypes, such that the homogenizing influence of substantial gene flow overwhelms the action of selection in Mediterranean areas. This model is consistent with the greater level of polymorphism of habitat-specialized biological traits (autogeny vs. anautogeny, stenogamy vs. eurigamy) within both epigeous and hypogeous breeding sites in the Mediterranean basin than in more northern areas (Roubaud 1939; Knight & Maleek 1951; Rioux *et al*. 1961; Dancesco *et al*. 1975; Pasteur *et al*. 1977; Chevillon *et al*. 1995a; Byrne & Nichols 1998). It is noteworthy, however, that if the level of gene flow between ecotypes does increase in Mediterranean areas, it is not sufficient to affect the efficiency of the selection for *Aat-1*^{A} in hypogeous habitats.

Under this scenario, the gradual decrease from northern Europe to the Mediterranean basin of the efficiency of counter-selection of *Aat-1*^{A} in epigeous populations will simply reflect the gradual increase in frequency of autogenous females observed in this habitat. The discrepancies observed between Montpellier and Barcelona areas in the distributions of *Aat-1*^{A} frequencies in hypogeous populations could also be explained by this scenario, as there are lower frequencies of autogeny in hypogeous populations from Barcelona (0.77, Chevillon *et al*. 1995a) than in hypogeous populations from Montpellier (0.88, Pasteur 1977), matching the difference in *Aat-1*^{A} frequencies. A direct relationship between autogeny and *Aat-1* genotype could not be tested, as in neither study were these characters identified from the same individuals.

Overall, the comparison of local and geographically widespread levels of genetic divergence between *Culex pipiens* ecotypes leads to several conclusions. First, we have shown that *Aat-1*^{A} is favoured in the hypogeous ecotype but (relatively) disfavoured in the epigeous ecotype. Second, the complex effects of ecological and geographical factors on *Aat-1* differentiation among populations is compatible with a plausible functional relationship between the activity of the aminotransferase enzyme encoding by this gene and other habitat-adaptive biological traits. Third, analysis of several other loci, not thought to be involved in ecotypic adaptation, confirms the conclusion that hypogeous populations have evolved in greater isolation from neighbouring populations than have epigeous populations. Such habitat-dependent demographic effects would explain why local adaptation to a hypogeous ecology is easier to achieve than local adaptation to an epigeous ecology in places where gene flow between ecotypes increases. A level of genetic swamping sufficient to impede local adaptation would not be as likely to occur in hypogeous habitats as in epigeous habitats (Dias 1996).

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This paper is part of larger studies on the evolution of insecticide resistance genes and on population differentiation in the *Culex pipiens* mosquito complex. These studies have been the main focus of interest of Yannick Rivet, Christine Chevillon, Michel Raymond and Nicole Pasteur. François Rousset is investigating the interactions between *Culex pipiens* and the endosymbiont *Wolbachia pipientis*, involved in reproductive incompatibilities of this host, and developing tools for the analysis of population differentiation. Peter Smouse is a population geneticist whose recent work has centred on statistical analysis of population differentiation. Present problems investigated by Peter Smouse and Christine Chevillon concern the use of mitochondrial markers for analyses of human colonization of the Americas.
