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Genetic differentiation of *Anopheles claviger s.s.* in France and neighbouring countries

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Abstract. An investigation of polymorphism of 11 autosomal and one sex-linked allozyme loci was made on 18 samples of *Anopheles claviger* Meigen (Diptera: Culicidae) from localities across France and neighbouring sites in Germany and Switzerland, plus one sample of *Anopheles petragnani* Del Vecchio from the French Pyrénées. Genetic differentiation between these two sibling species was confirmed (Nei genetic distance 0.33–0.44) and two genetically distinct groups of populations were identified within *An. claviger*. These two forms of *An. claviger* showed contiguous geographical distributions, Group I found across western and Central France, Group II in eastern France and nearby parts of Germany and Switzerland. The two groups were in contact in a region near the Rhone Valley where two intermediate samples were found. The taxonomic significance of this finding is discussed in the context of the recent climatic history of Europe and in relation to the vector potential of each member of the *An. claviger* complex.

Key words. *Anopheles claviger*, *An. petragnani*, isoenzymes, population genetics, sibling species, France.

Introduction

The western Palaeartic mosquito originally described as *Anopheles claviger* Meigen (Diptera: Culicidae) is now known to represent a complex of species distributed abundantly in Europe, Scandinavia and the Mediterranean Region. The former medical importance of *An. claviger s.l.* as a vector of human malaria, especially around the eastern Mediterranean (Russell *et al.*, 1963), has been superseded by its new role in transmission of myxomatosis (Service, 1971) and possibly other pathogens such as *Borrelia* and tularaemia.

Two morphological forms of *An. claviger*, designated *missiroli* and *petragnani*, were first recognized in Italy (Del Vecchio, 1939; Lupascu, 1941). Coluzzi (1962) showed that these two forms represent reproductively isolated species: *An. claviger* (Meigen 1804) *sensu stricto* (type locality Germany, corresponding to the Italian *missiroli* form) and *An. petragnani* Del Vecchio 1939, type locality Latium, Italy. These two sibling species can be distinguished by some morphological characters of the immature stages (Coluzzi *et al.*, 1965). Also, Cianchi *et al.* (1980, 1981) found diagnostic alleles at eight of 27 loci coding isoenzymes studied in *An. petragnani* (from Buonamico, Italy) compared with *An. claviger s.s.* (from Vicalvi, Italy, and Strasbourg, France). In terms of genetic distance D (Nei, 1978), both samples of *An. claviger s.s.* were similar (D = 0.02), equally distinct from the single sample of *An. petragnani* (D = 0.60).

In some parts of eastern France, *An. claviger s.s.* is sufficiently anthropophilic to be regarded as a nuisance and is therefore subjected to control activities (Schaffner, 1992). Elsewhere in France, as in Scandinavia, Central Europe and the British Isles, *An. claviger s.l.* does not seem to cause problems, although population densities seem to be similar and it is the predominant anopheline in most situations (Martini, 1930; Marshall, 1938). In Italy, Greece, Turkey, Lebanon and other countries of eastern Europe and eastern Mediterranean, the behaviour and ecology of the *An. claviger* complex are more variable and sometimes vectorial (Senevet & Andarelli, 1955; Russell *et al.*, 1963; Postiglione *et al.*, 1972). The present study was undertaken to investigate the genetic variability and population structure of *An. claviger s.s.* in France and neighbouring countries, in an attempt to interpret the behavioural and ecological contrasts between eastern and western populations. We obtained samples of *An. claviger s.s.*

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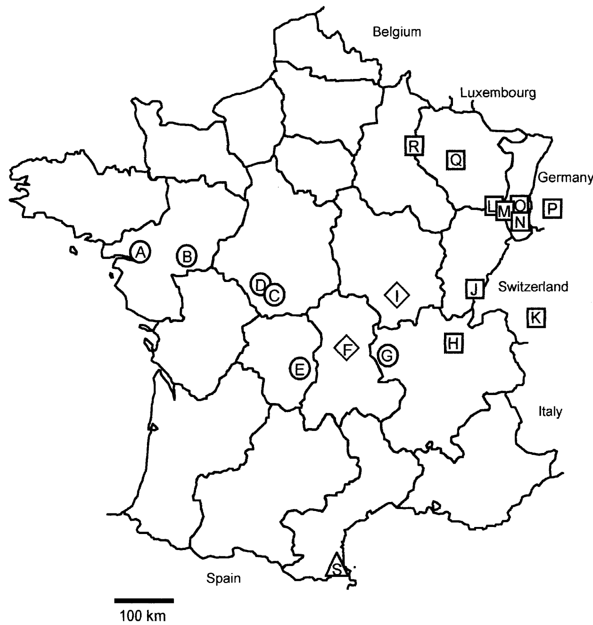

Fig. 1. Sampling localities for the *An. claviger* complex (see Table 1 for explanation of code letters). The triangle refers to the *An. petragnani* sample. Circles and squares represent samples attributed to Group I and Group II, respectively. Diamonds represent two ungrouped samples (F and I) from intermediate localities. Administrative regions are outlined.

(Fig. 1) from a wide range of representative sites across France (spanning >1000 km) from sea level up to 1000 m altitude. One sample of putative *An. petragnani* from the Pyrénées was included for comparative purposes.

Materials and methods

Mosquitoes

Larvae and pupae of *An. claviger s.l.* were collected during 1993–95 from a total of 19 localities (Table 1), representing France (17), Germany and Switzerland. After rearing to adulthood, the exuviae, head, wings, legs and male genitalia were mounted on slides in Canada balsam for morphological examination, which will be presented elsewhere. From freshly killed adults, the thorax and abdomen were stored in liquid nitrogen for isoenzyme analyses.

Electrophoresis

Electrophoretic polymorphisms of the following nine enzymes were studied by starch gel electrophoresis (TM 7.4 buffer system) on adult mosquito homogenates (Pasteur *et al.*, 1988): α-glycerophosphate dehydrogenase (EC 1.1.1.8), esterases (EC 3.1.1.1/2), glutamate-oxaloacetate transaminase (EC 2.6.1.1), hexokinase (EC 2.7.1.1), isocitrate dehydrogenase (EC 1.1.1.42), malic enzyme (EC 1.1.1.40), mannose-phosphate isomerase (EC 5.3.1.8), phosphogluco-isomerase (EC 5.3.1.9) and phosphoglucomutase (EC 2.7.5.1). The German sample P, from Schwarzwald, was used as reference standard. For each enzyme the predominant electromorph band (putatively the commonest allele at each locus) was designated '100' and other electromorphs (alleles) were numbered according to their relative electrophoretic mobility, i.e. 110, 120, etc. for faster bands; 90, 80, etc. for slower bands.

Data analysis

Conformity with Hardy–Weinberg (HW) expectations was tested for the proportions of genotypes at each locus, using the exact U-score test in presence of the alternative hypothesis of heterozygote deficiency (Rousset & Raymond, 1995). A global test across samples and/or loci was also performed (Rousset & Raymond, 1995). Genotypic associations between each pair of loci in each population were tested using the probability test described by Raymond & Rousset (1995a). For each locus pair, global tests (Fisher's method) were performed across all populations. Departure from HW was measured using the Fis estimator proposed by Weir & Cockerham (1984). Genotypic differentiation between populations was tested by computing an unbiased estimate of the *P*-value of a log-likelihood (G) based exact test (Goudet *et al.*, 1996). Population differentiation was measured using the Fst estimator (Weir & Cockerham, 1984). Isolation by distance was analysed as described by Rousset (1997), i.e. computing the relationship between pairwise estimates of $\hat{F}_{st}/1–\hat{F}_{st}$ and logarithm of geographical distance. A possible positive relationship was tested with a Mantel test, using the Spearman Rank correlation coefficient statistic. Geographical distances between samples were taken as the shortest measurement on a map. Computations were performed by Genepop version 3.1d (Raymond & Rousset, 1995b) and multiple testing used the Bonferroni method (Hochberg, 1988). Genetic distances (Nei, 1978) were estimated using BIOSYS-1 release 1.7 (Swofford & Selander, 1981).

Results

Description of polymorphism

Among the nine enzymes systems investigated, 12 of the putative loci were polymorphic: *Gpd*, *Est-2*, *Est-3*, *Got-1*, *Got-2*, *Hk-1*, *Idh-1*, *Idh-2*, *Me-1*, *Mpi*, *Pgi* and *Pgm*. We first analysed the genotypic composition of males and females of each sample to determine whether any of these loci was sex-linked. At the *Mpi* locus, no heterozygote was observed among the 277 males analysed, whereas 85 were present among the 264 females. This highly significant ($P = 10^{-9}$) difference indicates that the *Mpi* locus is carried on the X chromosome. At all other loci, heterozygotes were observed in males and females and there was no significant

Table 1. Characteristics of the *Anopheles claviger s.s.* (A–R) and *An. petragnani* (S) populations studied.

Code	Name	Latitude	Longitude	Locality (administrative area, country)	Sample date	Habitat	Altitude (m a.s.l.)
A	Erdre	47°22′ N	1°24′ W	St-Mars-du-Désert (Loire-Atlantique, France)	11/94	Pasture pool	6
B	Layon	47°14′ N	0°30′ W	Faveraye-Mâchelles (Maine-et-Loire, France)	11/94	Pasture ditch	55
C	Brenne	46°48′ N	1°10′ E	St-Michel-en-Brenne (Indre, France)	11/94	Ditch, forest	110
D	Vendœuvres	48°48′ N	1°21′ E	Vendœuvres (Indre, France)	11/94	Ditch, marsh	125
E	Millevaches	45°40′ N	2°12′ E	Sornac (Corrèze, France)	12/94	Peat pool	720
F	Allier	45°54′ N	3°14′ E	Ennezat (Puy-de-Dôme, France)	12/94	Ditch, garden	315
G	Loire	45°46′ N	4°08′ E	Sainte-Foy-Saint-Sulpice (Loire, France)	12/94	Ditch, meadow	350
H	Lavours	45°52′ N	5°43′ E	Talissieu (Ain, France)	03/94	Peat spring	235
I	Charollais	46°37′ N	4°34′ E	Joncy (Saône-et-Loire, France)	12/94	Pasture pool	238
J	Chapelle	46°36′ N	6°07′ E	Chapelle-des-Bois (Doubs, France)	11/95	Peat pool	1080
K	Poutafontana	46°15′ N	7°25′ E	Pramagnon (Valais, Switzerland)	09/94	Marsh, forest	500
L	Bussang	47°53′ N	6°51′ E	Bussang (Vosges, France)	09/94	Tank	650
M	Mollau	47°52′ N	6°58′ E	Mollau (Haut-Rhin, France)	04/94	Marsh (*Carex*)	475
N	Amélie	47°48′ N	7°14′ E	Wittelsheim (Haut-Rhin, France)	02/94	Brook (*Joncus*)	258
O	MarieLouise	47°50′ N	7°15′ E	Staffelfelden (Haut-Rhin, France)	03/94	Pool, forest	243
P	Schwarzwald	47°52′ N	8°06′ E	Lenzkirch (Bade-Wurtemberg, Germany)	11/93	Peat bog	835
Q	Boucq	48°45′ N	5°46′ E	Boucq (Meurthe-et-Moselle, France)	09/94	Spring, forest	260
R	Possesse	48°53′ N	4°48′ E	Possesse (Marne, France)	04/95	Brook, forest	172
S	Argelès	42°33′ N	3°01′ E	Argelès-sur-Mer (Pyrénées-Orientales, France)	04/94	Brook, forest	30

($P > 0.05$) difference in the proportions of heterozygotes between sexes. These 11 loci are therefore autosomal, so male and female samples were pooled for assessment of their HW equilibria.

Genotypic associations were tested at each pair of loci in each sample. Random association was rejected ($P < 0.05$) in 12 of 416 tests, although none remained significant when taking into account multiple tests. A global test across populations for each locus pair revealed two pairs with significant values (*Gpd* and *Pgm*, $P = 0.037$; *Pgi* and *Pgm*, $P = 0.008$), although none remained significant when taking into account the number of pair of loci tested (61).

Significant departure from HW equilibrium, due to heterozygote deficiency, was observed in eight of 109 cases (Table 2). They concerned the *Est-2* locus in sample G, the *Est-3* locus in samples A and G, the *Idh-1* locus in sample N, the *Mpi* locus in sample M, and the *Pgm* locus in samples A, L and P. Thus, for all loci and samples, a highly significant ($P < 10^{-3}$), although moderate ($= 0.044$), heterozygote deficiency was found.

Genetic differentiation between An. claviger s.s. *and* An. petragnani

In the small sample of *An. petragnani*, only 10 of the 12 putative loci investigated could be studied. Among these loci, three (*Got-1*, *Est-2* and *Idh-1*) possessed one or more diagnostic alleles, i.e. rare or absent in *An. claviger s.s.* Nei's genetic distances ranged from 0.33 to 0.43 between *An. petragnani* and the 18 samples of *An. claviger s.s.*, compared with only 0–0.07 among *An. claviger s.s.* samples.

Genetic differentiation among samples of An. claviger s.s.

Genotypic differentiation among *An. claviger s.s.* was highly significant (considering all loci: $= 0.06$, $P < 10^{-5}$), and this differentiation was found to be carried by six loci (*Est-2, Est-3, Me-1, Pgi, Pgm* and *Mpi*). To better understand the forces controlling genetic exchange among *An. claviger s.s.* populations, we examined variation between $\hat{F}_{st}/1–\hat{F}_{st}$ and the log of geographical distance (Rousset, 1997). This analysis considered only females at the *Mpi* locus, but combined both sexes for the other five autosomal loci. Values of $\hat{F}_{st}/1–\hat{F}_{st}$ increased significantly (one-tailed Mantel test, $P < 10^{-4}$) with geographical distance, indicating relative isolation by distance (Fig. 2). Examination of pairwise values (Table 3) disclosed that most samples of *An. claviger s.s.* segregated as two Groups (I and II) characterized by large between group values (range: 0.09–0.26) and low intragroup values (range: 0–0.06). Group I (comprising samples A, B, C, D, E and G) represented localities in the west of our sampling area; whereas Group II (comprising samples H, J, K, L, M, N, O, P, Q and R) represented localities in the east of our sampling area. Samples F and I could not be attributed to either group, as their values (when compared to samples in both the other groups) were uniformly low ($0 < \hat{F}_{st} < 0.09$). Samples F and I were collected from localities nearly intermediate between the allopatric ranges of Groups I and II (Fig. 1).

Within each group, genotypic differentiation was low but significant (Group I: $\hat{F}_{st} = 0.011$, $P = 0.04$; Group II: $\hat{F}_{st} = 0.011$,

Table 2. Allelic frequencies observed at 11 putative allozyme loci for *An.petragnani* (sample S) and *An. claviger s.s.* from 18 localities. Number of mosquitoes analysed at each locus is in parenthesis. *Idh-2* was polymorphic in a single sample (Q) for a rare allele at a frequency of 0.03. Fis estimates are in italics when there is a significant ($P < 0.05$) departure from HW, due to heterozygote deficiency; those remaining significant ($P < 0.05$) when taking into account multiple tests are in bold characters (see text for explanations).

Locus	Populations A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	All
Gpd	(13)	(22)	(17)	(11)	(24)	(17)	(37)	(52)	(12)	(6)	(54)	(20)	(17)	(55)	(45)	(98)	(31)	(15)	(9)	
100	1.00	1.00	0.97	1.00	1.00	1.00	1.00	0.97	0.96	1.00	1.00	1.00	1.00	1.00	0.98	0.98	0.98	1.00	1.00	
(a)	0	0	0.03	0	0	0	0	0.03	0.04	0	0	0	0	0	0.02	0.02	0.02	0	0	
Fis	–	–	–	–	–	–	–	−0.02	–	–	–	–	–	–	−0.01	−0.01	–	–	–	−0.01
Est-2	(10)	(18)	(15)	(9)	(22)	(14)	(29)	(47)	(10)	(7)	(41)	(19)	(22)	(54)	(48)	(94)	(23)	(15)	(8)	
120	0	0	0.03	0	0	0	0	0.03	0.05	0	0	0.03	0	0.04	0.01	0.04	0.04	0	0	
100	0.85	0.86	0.90	0.61	0.93	0.96	0.88	0.95	0.95	1.00	0.94	0.90	0.93	0.93	0.95	0.94	0.94	1.00	0	
90	0.15	0.14	0.07	0.33	0.07	0.04	0.09	0.01	0	0	0.04	0.08	0.07	0.02	0.01	0.01	0.02	0	0	
80	0	0	0	0	0	0	0.03	0.01	0	0	0	0	0	0.01	0.03	0.01	0	0	0	
(b)	0	0	0	0.06	0	0	0	0	0	0	0.02	0	0	0	0	0	0	0	1.00	
Fis	+0.64	−0.13	−0.05	+0.19	+0.66	–	***+0.54***	+0.18	–	–	−0.04	+0.47	+0.66	−0.04	−0.03	−0.04	−0.03	–	−0.11	+0.14
Est-3	(13)	(19)	(16)	(9)	(23)	(16)	(25)	(49)	(11)	(6)	(42)	(19)	(23)	(57)	(47)	(93)	(25)	(15)	(9)	
110	0.31	0.21	0.25	0.11	0.17	0.03	0.10	0.02	0.18	0	0	0.03	0.04	0.03	0.02	0.02	0.04	0	0.39	
100	0.65	0.63	0.63	0.72	0.74	0.88	0.70	0.80	0.73	0.83	0.68	0.84	0.74	0.76	0.77	0.77	0.64	0.77	0.11	
90	0	0.08	0	0.11	0.07	0.03	0.08	0.08	0.05	0.17	0.27	0.08	0.13	0.13	0.10	0.11	0.22	0.17	0.39	
80	0	0.08	0.12	0	0.02	0.06	0.08	0.09	0.05	0	0.04	0.05	0.06	0.08	0.10	0.09	0.10	0.07	0.11	
(c)	0.04	0	0	0.06	0	0	0.02	0.01	0	0	0.01	0	0	0	0.02	0.01	0	0	0	
Fis	+*0.54*	+0.06	+0.21	−0.18	+0.29	−0.06	+*0.28*	−0.04	+0.21	−0.11	+0.09	+0.28	−0.19	+0.07	−0.07	−0.04	+0.12	+0.16	−0.10	+0.06
Got-1	(10)	(16)	(14)	(8)	(19)	(10)	(22)	(7)	(8)	(3)	(32)	(16)	(9)	(10)	(27)	(52)	(17)	(4)	(8)	
100	1.00	0.97	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	0.97	1.00	1.00	0.90	1.00	1.00	1.00	1.00	0	
90	0	0	0	0	0	0	0.04	0	0	0	0	0	0	0.10	0	0	0	0	0	
(d)	0	0.03	0	0	0	0	0	0	0	0	0.03	0	0	0	0	0	0	0	1.00	
Fis	–	–	–	–	–	–	−0.02	–	–	–	−0.02	–	–	−0.06	–	–	–	–	–	−0.02
Got-2	(12)	(19)	(14)	(8)	(17)	(12)	(23)	(9)	(10)	(2)	(26)	(10)	(9)	(10)	(27)	(46)	(13)	–	–	
100	1.00	1.00	1.00	1.00	0.94	0.96	0.98	0.83	0.95	0.75	0.92	1.00	0.89	0.95	0.98	0.95	1.00	–	–	
(e)	0	0	0	0	0.06	0.04	0.02	0.17	0.05	0.25	0.08	0	0.11	0.05	0.02	0.05	0	–	–	
Fis	–	–	–	–	−0.03	–	–	+0.64	–	–	−0.06	–	+1	–	–	+0.30	–	–	–	+0.22
Hk-1	(13)	(22)	(17)	(11)	(24)	(17)	(36)	(52)	(12)	(7)	(54)	(20)	(23)	(58)	(49)	(93)	(31)	(15)	(9)	
100	1.00	1.00	0.97	1.00	0.98	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	0.97	0.99	1.00	1.00	1.00	1.00	
(f)	0	0	0.03	0	0.02	0	0	0.02	0	0	0	0	0	0.03	0.01	0	0	0	0	
Fis	–	–	–	–	–	–	–	−0.01	–	–	–	–	–	−0.02	–	–	–	–	–	−0.01
Idh-1	(12)	(14)	(15)	(8)	(19)	(14)	(26)	(39)	(12)	(7)	(30)	(15)	(19)	(39)	(40)	(89)	(22)	(15)	(9)	
110	0	0.07	0	0.13	0	0.04	0.10	0.06	0.08	0.07	0.03	0.10	0.11	0.08	0.10	0.10	0.05	0.10	0	
100	1.00	0.93	1.00	0.87	1.00	0.96	0.88	0.92	0.92	0.93	0.97	0.90	0.89	0.92	0.90	0.90	0.95	0.90	0.33	
(g)	0	0	0	0	0	0	0.02	0.01	0	0	0	0	0	0	0	0	0	0	0.67	
Fis	–	−0.04	–	+1	–	–	−0.09	+0.30	−0.05	–	−0.02	−0.08	+0.46	+*0.29*	+0.18	+0.03	−0.02	−0.08	−0.03	+0.11
Me-1	(14)	(22)	(17)	(11)	(24)	(17)	(37)	(42)	(12)	(7)	(53)	(20)	(22)	(24)	(44)	(87)	(31)	(15)	(9)	
100	0.96	1.00	1.00	1.00	0.90	1.00	0.96	1.00	0.96	1.00	0.99	1.00	1.00	1.00	1.00	0.99	0.98	1.00	1.00	
80	0	0	0	0	0.08	0	0.02	0	0	0	0.01	0	0	0	0	0	0.02	0	0	
(h)	0.04	0	0	0	0.02	0	0.02	0	0.04	0	0	0	0	0	0	0.01	0	0	0	
Fis	–	–	–	–	+0.36	–	−0.01	–	–	–	–	–	–	–	–	–	–	–	–	+0.13
Mpi	(12)	(21)	(17)	(11)	(23)	(16)	(32)	(51)	(12)	(7)	(51)	(20)	(31)	(57)	(49)	(90)	(27)	(15)	(6)	
120	0.06	0	0.03	0	0	0.14	0.12	0.19	0.29	0.36	0.09	0.37	0.16	0.17	0.20	0.17	0.19	0.26	0.11	
100	0.94	1.00	0.97	1.00	1.00	0.86	0.88	0.79	0.71	0.64	0.88	0.63	0.84	0.81	0.80	0.80	0.76	0.74	0.89	
80	0	0	0	0	0	0	0	0.02	0	0	0.03	0	0	0.02	0	0.03	0.05	0	0	
Fis	–	–	–	–	–	–	−0.14	−0.23	+0.06	−0.50	−0.11	−0.65	+*0.23*	−0.15	−0.07	−0.01	−0.03	−0.27	–	−0.12
Pgi	(14)	(22)	(17)	(11)	(24)	(17)	(36)	(50)	(12)	(7)	(53)	(19)	(23)	(55)	(49)	(87)	(31)	(15)	(9)	
110	0	0	0	0	0	0	0	0.02	0.04	0	0	0	0.02	0.01	0	0	0	0.07	0	
100	0.71	0.50	0.59	0.68	0.60	0.71	0.53	0.73	0.63	1.00	0.82	0.90	0.89	0.86	0.85	0.89	0.81	0.80	0.89	
90	0.21	0.46	0.38	0.32	0.35	0.26	0.43	0.25	0.29	0	0.18	0.10	0.07	0.13	0.14	0.11	0.18	0.13	0.11	
80	0.07	0.04	0.03	0	0.04	0.03	0.04	0	0.04	0	0	0	0.02	0	0.01	0.01	0.02	0	0	
Fis	−0.27	+0.18	−0.37	−0.43	−0.13	−0.06	+0.08	+0.07	+0.24	–	+0.18	−0.09	−0.06	+0.02	+0.15	−0.01	+0.01	−0.15	+1	+0.02
Pgm	14	20	17	11	24	15	35	52	12	7	51	19	23	58	48	95	30	15	9	
120	0	0	0	0	0	0	0	0.01	0.02	0	0	0.03	0	0	0	0	0.03	0	0	
110	0.07	0.08	0	0.05	0.04	0.03	0.03	0.05	0.08	0	0.04	0.03	0.11	0.02	0.07	0.09	0.05	0.13	0	
100	0.50	0.55	0.41	0.68	0.52	0.77	0.34	0.92	0.67	0.93	0.90	0.82	0.87	0.95	0.90	0.86	0.85	0.80	1.00	
90	0.43	0.37	0.56	0.27	0.33	0.13	0.51	0.01	0.25	0	0.06	0.10	0.2	0.03	0.03	0.02	0.05	0.07	0	
80	0	0	0.03	0	0.10	0.07	0.10	0	0	0.07	0	0.03	0	0.01	0	0.02	0.02	0	0	
Fis	+*0.52*	+0.21	+0.01	−0.14	−0.22	+0.18	+0.12	−0.05	+0.35	–	+0.15	+*0.53*	−0.11	+0.14	−0.08	+*0.02*	+0.16	−0.15	–	+0.08

(a) represents allele Gpd^{120} in samples C, H and I, alleles Gpd^{120} and Gpd^{80} in sample O (each at a frequency of 0.01), and allele Gpd^{80} in sample P.
(b) represents allele $Est\text{-}2^{110}$ in sample D, allele $Est\text{-}2^{130}$ in sample K, and three alleles, $Est\text{-}2^{140}$, $Est\text{-}2^{150}$ and $Est\text{-}2^{160}$ in sample S (at frequencies of 0.06, 0.81 and 0.13, respectively)
(c) represents allele $Est\text{-}3^{120}$ in sample D and allele $Est\text{-}3^{70}$ in other samples.
(d) represents allele $Got\text{-}1^{120}$ in samples B and K, and allele $Got\text{-}1^{80}$ in sample K.
(e) represents allele $Got\text{-}2^{80}$ in samples E, F, and K, and allele $Got\text{-}2^{120}$ in other samples.
(f) represents allele $Hk\text{-}1^{110}$ in sample C and allele $Hk\text{-}1^{80}$ in other samples.
(g) represents allele $Idh\text{-}1^{150}$ in sample H, allele $Idh\text{-}1^{80}$ in sample G and alleles $Idh\text{-}1^{80}$ (frequency = 0.61) and $Idh\text{-}1^{60}$ (frequency = 0.06) in sample S.
(h) represents allele $Me\text{-}1^{120}$ in sample G, allele $Me\text{-}1^{110}$ in sample A and allele $Me\text{-}1^{90}$ in other samples.

$P = 0.001$) and no isolation by distance was found (Mantel test, $P > 0.40$).

Nei's genetic distance was D = 0.03 between Groups I and II of *An. claviger s.s.*, and D = 0.39 and 0.35 between *An. petragnani* and Group I or Group II, respectively. Table 4 compares these values to those observed between sibling species of other *Anopheles* complexes.

Discussion

Our isoenzyme study of the *An. claviger* complex in France, plus two samples from neighbouring countries (Germany and Switzerland) confirmed the major differences between isoenzymes of *An. claviger s.s.* and *An. petragnani*. Of the eight diagnostic allozymes reported by Cianchi *et al.* (1980, 1981), we confirmed the contrast at three loci (*Got-1*, *Est-2* and *Idh-1*), but the others could not be studied (*Idh-2*, *Got-2*) or were not investigated (*Mdh*, *Adk*, *Xdh*). Nei's genetic distances found here between *An. claviger s.s.* and *An. petragnani* (0.33–0.43) are lower that the value (D = 0.60) determined by Cianchi *et al.* (1980), but both values are higher than those between most sibling species of mosquitoes: 0.1–0.3 (Bullini, 1982). *Anopheles petragnani* from the Pyrénées (this study) seems not different from the Italian sample of *An. petragnani* described by Cianchi *et al.* (1980), although this result is based on few loci and a thorough comparison is required to conclude.

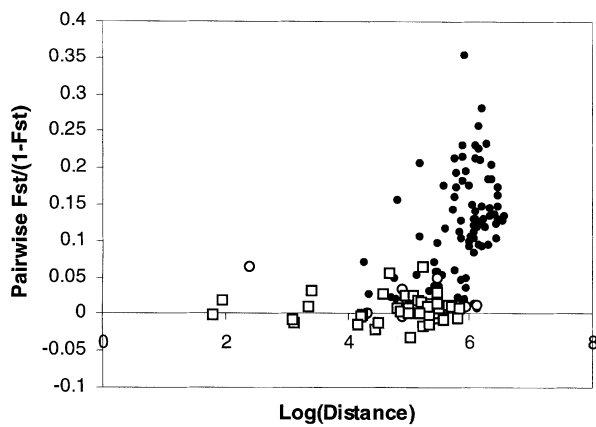

Fig. 2. Relationship between pairwise $\hat{F}_{st}/1-\hat{F}_{st}$ values and logarithm of geographical distances for *An. claviger s.s.* Open symbols refer to intra groups values (○ and □ for Group I and Group II, respectively) and ● refer to intergroups values.

Our study revealed the allopatric occurrence of two genetically divergent population groups of *An. claviger*: Group I in western and central France; Group II in eastern France (between Bourgogne and Alsace), Switzerland and Germany. Within each group, there was no trend of isolation by distance over the geographical range surveyed, i.e. each group was relatively homogeneous among samples studied from several sites. The existence of two genetically differentiated allopatric groups of *An. claviger s.s.* populations may be associated with the behavioural and ecological contrasts between *An. claviger* from these different parts of France. This raises the question of the relative taxonomic status of *An. claviger* Groups I and II. Among the 12 loci studied, none had diagnostic alleles, indicating that Groups I and II are not genetically isolated from another, or their geographical isolation may be too recent for diagnostic genetic loci to have evolved. Additional analyses are required to characterize these two groups by morphological and cytological studies, and by means of genetic markers such as maternally inherited DNA (mtDNA) or highly variable loci (e.g. microsatellites).

Complexes of sibling species are plentiful in the genus *Anopheles*: allozymes or molecular markers have been used to separate and identify members of such complexes. In the *An. maculipennis* and *An. gambiae* complexes for example, the different species could be identified by several diagnostic loci,

Table 3. Fst estimates observed between pairs of *An. claviger s.s.* samples. Groups I (upper left) and II (lower right) have been boxed with a single line. The Fst estimates corresponding to comparison of samples from different groups are boxed with a double line.

	A	B	C	D	E	G	F	I	H	J	K	L	M	N	O	P	Q
B	0.00	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C	−0.01	0.00	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D	0.01	0.01	0.06	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E	0.00	0.00	0.01	0.03	–	–	–	–	–	–	–	–	–	–	–	–	–
G	0.01	0.01	0.00	0.05	0.02	–	–	–	–	–	–	–	–	–	–	–	–
F	0.05	0.06	0.10	0.05	0.03	0.07	–	–	–	–	–	–	–	–	–	–	–
I	0.01	0.03	0.05	0.05	0.03	0.02	0.00	–	–	–	–	–	–	–	–	–	–
H	0.12	0.13	0.18	0.10	0.10	0.14	0.01	0.02	–	–	–	–	–	–	–	–	–
J	0.17	0.22	0.26	0.19	0.18	0.17	0.09	0.05	−0.02	–	–	–	–	–	–	–	–
K	0.11	0.12	0.17	0.09	0.10	0.15	0.02	0.07	0.02	0.05	–	–	–	–	–	–	–
L	0.11	0.16	0.19	0.12	0.13	0.13	0.05	0.02	0.03	0.01	0.06	–	–	–	–	–	–
M	0.09	0.13	0.18	0.08	0.10	0.14	0.01	0.04	0.00	−0.03	0.01	0.02	–	–	–	–	–
N	0.13	0.16	0.21	0.11	0.12	0.16	0.02	0.05	0.00	0.01	0.01	0.03	−0.01	–	–	–	–
O	0.12	0.14	0.18	0.11	0.11	0.15	0.01	0.04	0.01	0.02	0.02	0.01	−0.01	0.00	–	–	–
P	0.12	0.15	0.19	0.11	0.12	0.16	0.01	0.05	0.01	0.01	0.02	0.03	−0.01	0.00	0.00	–	–
Q	0.09	0.11	0.15	0.09	0.09	0.11	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	–
R	0.09	0.11	0.15	0.09	0.09	0.10	0.01	0.00	−0.01	−0.01	0.01	0.00	−0.02	0.00	−0.01	−0.01	−0.02

Table 4. Nei's genetic distance values, based on isoenzyme comparisons (or microsatellite DNA*), observed between members of *Anopheles* complexes. ? = uncertain.

Reference	Complex	Species compared	No. of populations	D value
Bullini & Coluzzi (1982)	*An. gambiae*	*An. arabiensis – An. gambiae*	?	0.133
	An. maculipennis	*An. messeae – An. subalpinus*	?	0.119
		An. melanoon – An. subalpinus	?	0.154
		An. maculipennis s.s.– An. subalpinus	?	0.162
		An. maculipennis s.s – An. melanoon	?	0.228
		An. atroparvus – An. labranchiae	?	0.250
		An. melanoon – An. sacharovi	?	0.526
	An. claviger	*An. claviger s.s. – An. petragnani*	?	0.597
Lokki *et al.* (1979)	*An. maculipennis*	*An. beklemishevi – An. messeae*	7–14	0.35
		Various populations	21	0.006–0.05
Cianchi *et al.*, 1981	*An. claviger*	*An. claviger* populations	2	0.017
	An. maculipennis	seven species	13	0.12–0.67
		An. labranchiae populations	3	0.011–0.014
		An. maculipennis populations	3	0.017–0.047
Cianchi *et al.*, 1983	*An. gambiae*	*An. arabiensis – An. gambiae – An. melas*	0.11–0.25	
(in Cianchi *et al.*, 1985)		*An. gambiae s.s.* cytotypes	0.01	
Lambert (1983)	*An. marshallii*	*An. marshallii* A, B, C and E	?	0.03–0.22
Bullini (1984)	*An. maculipennis*	Six species	?	0.12–0.42
		An. sacharovi + six species	?	0.12–0.71
	An. gambiae	Four species	?	0.11–0.41
Narang *et al.* (1990)	*An. quadrimaculatus*	*An. quadrimaculatus* species C populations	10	0.01–0.20
Narang *et al.* (1993)	*An. albitarsis*	*An. marajoara – An. deaneorum*	3–1	0.27–0.40
		An. marajoara populations	3	0.07–0.24
Lanzaro *et al.* (1995)	*An. gambiae*	*An. arabiensis – An. gambiae*	1	0.090
				0.282*
This study	*An. claviger*	*An. claviger – An. petragnani*	18–1	0.33–0.43
		An. claviger s.s. populations	18	0–0.07
		An. claviger s.s. Groups I–II	6–10	0.03

with Nei's genetic distances of 0.07–0.71 between species and 0.006–0.24 between local populations (Table 4). Ayala (1975) utilized the same approach on the *Drosophila willistoni* group of fruitflies (Diptera: Drosophilidae) and estimated the average genetic distance (D) to be about 0.03 between local populations, about 0.2 between pairs of subspecies and semispecies, and about 0.7 between pairs of sibling species.

The genetic distance (D = 0.03) between the two French groups of *An. claviger* is only a preliminary estimate. We intend to make more extensive samplings from areas where both groups are parapatric or overlap, such as in the middle Rhone Valley (Fig. 1). Some samples from this region belong to Group I (G) or II (H), whereas others remain ambiguous/intermediate (e.g. F and I) either because they contained a mixture of the two groups or because they were hybrids/intergrades.

Possibly Groups I and II of *An. claviger* represent two allopatric members of a species complex, forming a hybrid zone in mid-France. Such hybrid zones are relatively common in western Europe, e.g. in the mouse, *Mus musculus* L. (Boursot *et al.*, 1993), the crow *Corvus corone* L. (Rolando, 1993) and the oak tree *Quercus robur* L. (Ferris *et al.*, 1998). The reason for this situation is usually attributed to recent climatic fluctuation, with genetic differentiation taking place between southern refugia during cold episodes, and the hybrid zone arizing from re-colonization of Europe during a warm phase. The classical refugia are the southern peninsulas, such as Spain, Italy and Greece. Accordingly, it could be proposed that *An. claviger* Group I in western France originates from the Spanish peninsula, whereas the eastern *An. claviger* Group II originates from one of the other peninsula. *Anopheles claviger s.s.* prefers breeding sites with cold water (see p. 140 in Bates, 1949) and is now distributed in northern Europe and southern Scandinavia (Natvig, 1948; Utrio, 1975; Mehl, 1996), corresponding approximately to the distribution limit of the oak *Quercus robur* (Ferris *et al.*, 1998). It overwinters as larvae in pasture pools and marshes, so that freezing aquatic conditions limit the geographical distribution of the immature stages. From the shared current northern limit of oaks and *An. claviger* it seems likely that, during the last and coldest glaciation, the geographical distribution of *An. claviger* was probably confined to the same three peninsular refugia where oaks survived in southern Europe (Blondel, 1995; Ferris *et al.*, 1998).

This 'glaciation scenario' could be tested by comparing *An. claviger* populations from the three southern peninsulas: it is expected that the genetic variability is higher within each peninsula, with Spanish samples resembling *An. claviger* Group I, whereas samples from the Italian or Greek peninsula

would be closer to *An. claviger* Group II. If another hybrid zone exists (probably north-east Italy) it would correspond to the interface between *An. claviger* populations originating from Greek and Italian refugia. Clarification of the genetic structure of *An. claviger* populations across the Palaeartic should help to interpret variations in their vector potential and control options.

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References

Ayala, F.J. (1975) Genetic differentiation during the speciation process. *Evolutionary Biology*, **8**, 1–78.

Bates, M. (1949) *The Natural History of Mosquitoes*. The MacMillan Company, New York.

Blondel, J. (1995) *Biogéographie, Approche Écologique et Évolutive*. Masson, Paris.

Boursot, P., Auffray, J.-C., Britton-Davidian, J. & Bonhomme, F. (1993) The evolution of house mice. *Annual Review of Ecology and Systematics*, **24**, 119–152.

Bullini, L. (1982) Genetic, ecological and ethological aspects of speciation process. *Mechanisms of Speciation* (ed. by C. Barrigozzi), pp. 241–264. Alan R. Liss, Inc., New York.

Bullini, L. (1984) Genetic differentiation and speciation in European and African malaria vectors. *Memoire di Scienze Fisiche e Naturali Serie V*, **8**, 57–69.

Bullini, L. & Coluzzi, M. (1982) Evolutionary and taxonomic inferences of electrophoretic studies in mosquitoes. *Recent Developments in the Genetics of Insect Disease Vectors* (ed. by W. W. M. Steiner, W. J. Tabachnick, K. S. Rai and S. Narang), pp. 465–482. Stipes Publishing Co, Champaign, IL.

Cianchi, R., Sabatini, A., Bullini, L. & Coluzzi, M. (1981) Differenziazione morphologica e genetica nei complessi *Anopheles maculipennis* e *Anopheles claviger*. *Parassitologia*, **23**, 158–163.

Cianchi, R., Sabatini, A., Coluzzi, M. & Bullini, L. (1980) Divergenza genetica tra due specie gemelle del genere *Anopheles*: *An. claviger* e *An. petragnanii* (Diptera, Culicidae). *Atti XII Congresso Nazionale Italia Entomologica*, *Roma*, 261–263.

Cianchi, R., Urbanelli, S., Villani, F., Sabatini, A. & Bullini, L. (1985) Electrophoretic studies in mosquitoes: recent advances. *Parassitologia*, **27**, 157–167.

Coluzzi, M. (1962) Le forme di *Anopheles claviger* Meigen indicate con i nomi *missiroli* e *petragnanii* sono due specie riproduttivamento isolate. *Rendiconti della Accademia Nazionale Lincei*, **32**, 1025–1030.

Coluzzi, M., Sacca, G. & Feliciangeli, D. (1965) Il complesso *A. claviger* nella sottoregione mediterranea. *Cahiers ORSTOM, série Entomologie médicale et Parasitologie*, pp. 97–102.

Del Vecchio, G. (1939) Sulle varietà di *Anopheles claviger*. *Rivista di Parassitologia*, **3**, 27–37.

Ferris, C., King, R.A., Väinölä, R. & Hewitt, G.M. (1998) Chloroplast DNA recognizes three refugia sources of European oaks and suggests independent eastern and western immigration to Finland. *Heredity*, **80**, 584–593.

Goudet, J., Raymond, M., De Meeüs, T. & Rousset, F. (1996) Testing differentiation in diploid populations. *Genetics*, **144**, 1933–40.

Hochberg, Y. (1988) A sharper Bonferroni procedure for multiple tests of significance. *Biometrika*, **75**, 800–802.

Lambert, D.M. (1983) A population genetical study of the African mosquito *Anopheles marshallii* (Theobald). *Evolution*, **37**, 484–495.

Lanzaro, G.C., Zhaneg, L., Toure, Y.T., Traore, S.F., Kafatos, F.C. & Vernick, K.D. (1995) Microsatellite DNA and isozyme variability in a West African population of *Anopheles gambiae*. *Insect Molecular Biology*, **4**, 105–112.

Lokki, J., Saura, A., Korvenkontio, P. & Ulmanen, I. (1979) Diagnosing adults *Anopheles* mosquitoes. *Aquilo. Series Zoologica*, **20**, 5–12.

Lupascu, G. (1941) Sull'existanza di due varieta di *Anopheles claviger*. *Rivista di Parassitologia*, **5**, 25–44.

Marshall, J.F. (1938) *The British Mosquitoes.* British Museum, London.

Martini, E. (1930) *Culicidae.* E. Schweizerbant, Stuttgart.

Mehl, R. (1996) Culicidae Stikkmygg. *Limnofauna Norvegica. Katalog Over Norsk Ferskvannsfauna* (ed. by K. Aagaard and D. Dolmen), pp. 202–205. Tapir, Trondheim.

Narang, S.K., Klein, T.A., Perera, O.P., Bento Lima, J. & Toda Tang, A. (1993) Genetic evidence for the existence of cryptic species in the *Anopheles albitarsis* complex in Brazil: allozymes and mitochondrial DNA restriction fragment length polymorphisms. *Biochemical Genetics*, **31**, 97–112.

Narang, S.K., Seawright, J.A. & Kaiser, P.E. (1990) Evidence for microgeographic genetic subdivision of *Anopheles quadrimaculatus* species C. *Journal of the American Mosquito Control Association*, **6**, 179–187.

Natvig, L.R. (1948) *Contribution to the Knowledge of the Danish and Fennoscandian Mosquitoes. Culicini.* Norsk Entomologisk Tidsskrift, Oslo.

Nei, M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Pasteur, N., Pasteur, G., Catalan, J., Bonhomme, F. & Britton-Davidian, J. (1988) *Practical Isozyme Genetics*. Ellis Horwood Ltd, Chichester, U.K.

Postiglione, M., Tabanli, S. & Ramsdale, C.D. (1972) *Anopheles claviger* in Turkey. *Rivista di Parassitologia*, **33**, 220–229.

Raymond, M. & Rousset, F. (1995a) An exact test for population differentiation. *Evolution*, **49**, 1280–1283.

Raymond, M. & Rousset, F. (1995b) Genepop (Version 1.2), a population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rolando, A. (1993) A study on the hybridization between carrion and hooded crow in northwestern italy. *Ornis Scandinavica*, **24**, 80–83.

Rousset, F. (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.

Rousset, F. & Raymond, M. (1995) Testing heterozygote excess and deficiency. *Genetics*, **140**, 1413–1419.

Russell, P.F., West, L.S., Manwell, R.D. & MacDonald, G. (1963) *Practical Malariology*. Oxford University Press, London.

Schaffner, F. (1992) *Les Moustiques de Haute Alsace. I. Systématique.* Conseil Général du Haut Rhin, Strasbourg.

Senevet, G. & Andarelli, L. (1955) Races et variétés de l'*Anopheles*

claviger Meigen, 1804. *Archives de l'Institut Pasteur Algérie*, **33**, 128–137.

Service, M.W. (1971) A reappraisal of the role of mosquitoes in the transmission of myxomatosis in Britain. *Journal of Hygiene*, **69**, 105–111.

Swofford, D.L. & Selander, R.B. (1981) Biosys-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity*, **72**, 281–283.

Utrio, P. (1975) *Anopheles claviger* (Meig.), *Aedes pionips* Dyar and *A. beklemishevi* Den. (Diptera, Culicidae) found in Finland. *Notulae Entomologicae*, **55**, 63–64.

Weir, B.S. & Cockerham, C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

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