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POPULATION GENETICS OF THE MOSQUITO *CULEX PIPIENS PALLENS* REVEALS SEX-LINKED ASYMMETRIC INTROGRESSION BY *CULEX QUINQUEFASCIATUS*

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Abstract

The *Culex pipiens* complex in Asia includes a temperate subspecies, *Culex pipiens pallens*, of uncertain taxonomic status. The shape of the male genitalia suggests it is a hybrid between *Cx. pipiens* and *Cx. quinquefasciatus*. We studied populations of *Cx. p. pallens* in Japan, Korea, and China and compared them to local populations of *Cx. quinquefasciatus* and *Cx. p. pipiens*. We examined variation in a nuclear intron in the acetylcholinesterase-2 gene [ACE] and eight microsatellite loci. We found a distinct microsatellite signature for *Cx. p. pallens* indicating restricted gene flow between Eastern and Western populations of *Cx. pipiens*, supporting the existence of two subspecies. Furthermore, a multilocus genotype analysis revealed current hybridization between *Cx. p. pallens* and *Cx. quinquefasciatus* in southern Japan, Republic of Korea, and China but not in Hokkaido, in northern Japan. Surprisingly, however, we found that the sex-linked ACE locus in chromosome I has introgressed asymmetrically through the males such that all male *Cx. p. pallens* have a copy of the *Cx. quinquefasciatus* ACE locus. This result highlights some of the potential consequences of hybridization between local and introduced species to disease transmission worldwide.

Keywords

HYBRIDIZATION; GENETIC INTROGRESSION; SPECIATION; INVASIVE SPECIES; DISEASE VECTORS; ASIA; SEX-LINKED

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INTRODUCTION

Natural hybridization, defined as "Successful matings in nature between individuals from two populations, or groups of populations, that are distinguishable on the basis of one or more heritable characters"(Harrison 1990, Arnold 2004b), can provide favorable conditions for rapid evolution and even lead to speciation (Arnold 1997, 2004a, Jiggins et al. 2008). Although hybridization processes are harder to observe when they involve populations of the same species or cryptic taxa, the advent of molecular techniques has allowed their detection (Ellstrand and Schierenbeck 2000, Meixner et al. 2002, Kovarik et al. 2005, Fonseca et al. 2006). We can now further our studies of hybridization as an evolutionary force and, in particular, examine the contribution of the horizontal transfer of advantageous genes (Arnold 2004b, Currat et al. 2008). Nonetheless, the consequences of natural hybridization can be difficult to study since each is a "natural experiment" often unique or with multiple events locally correlated. Exceptionally in the *Culex pipiens* complex a group of important disease vectors, hybrid zones appear to have been created repeatedly and independently across the World, as the two main species in the complex, *Cx. pipiens* and *Cx. quinquefasciatus* were introduced into new areas with migrating people and commercial traffic (Mattingly et al. 1951).

Recurring hybridization occurs in the *Culex pipiens* complex mostly between the two widespread species in the complex, *Culex (Culex) pipiens* L. 1758 and *Cx. (Cx.) quinquefasciatus* Say 1823. Hybrids have been documented in North America, Argentina, as well as in Madagascar (Barr 1957, Urbanelli et al. 1995, Urbanelli et al. 1997, Humeres et al. 1998), and have been hypothesized in East Asia (Smith and Fonseca 2004, Cui et al. 2007). This is therefore a system where comparative analyses are potentially feasible. With that aim, we have been examining the *Cx. pipiens* complex in East Asia. A temperate sub-species of *Cx. pipiens, Cx. pipiens pallens* Couquillett 1898 is restricted to Eastern Asia where it vectors lymphatic filariasis and canine dogworm and has been well studied especially under the threat of introduction of West Nile virus (Choi et al. 2002, Jang et al. 2002, Oda et al. 2002, Yang et al. 2003). Its relationship to the remaining members of the complex has, however, remained a mystery. Because the male genitalia (phallosome) of *Cx. p. pallens* has a shape very similar to that of hybrids of *Cx. pipiens* and *Cx. quinquefasciatus*, which is intermediate between the two parent species (Barr 1957, Tanaka et al. 1979), *Cx. p. pallens* has often been described as a hybrid (Bekku 1956, Laven 1967, Cornel et al. 2003). Bekku (1956) found a north-south gradient in the morphology of the phallosome of *Cx. p. pallens* in which specimens from northern Japan were more like the temperate species, *Cx. pipiens*, and specimens from southern Japan were more like *Cx. quinquefasciatus*, the tropical species. In Japan, *Cx. quinquefasciatus* are restricted to the Ryukyu Islands (Kasai et al. 2008), and while they have not been reported in the Republic of Korea (commonly referred to as "South Korea" and from now on abbreviated ROK), this species occurs in southern China (Cui et al. 2007). Furthermore, *Culex p. pipiens*, has two epidemiologically distinct forms: form "pipiens" and form "molestus" (Harbach et al. 1984). The urban form of *Cx. p. pipiens* (form molestus) was introduced into northern East Asia likely during WWII (Mattingly et al. 1951), but the feral form (*Cx. p. pipiens* form pipiens) has not been found in Asia (Cui et al. 2007, Kasai et al. 2008).

While mitochondrial DNA has failed to show informative variation, possibly as a result of selective sweeps driven by *Wolbachia pipientis* (Guillemaud et al. 1997), members of the *Cx. pipiens* complex can be identified by rapid assays using nuclear loci (Smith and Fonseca 2004, Bahnck and Fonseca 2006, Kasai et al. 2008). The objective of this study was to test the hypothesis that populations of *Cx. p. pallens* are the result of hybridization between the western *Cx. pipiens* (*Cx. p. pipiens)* and *Cx. quinquefasciatus* as well as examine the overall population structure of this subspecies in eastern Asia (China, ROK, and Japan). To do so we used variation

in the acetylcholinesterase-2 locus [ACE] previously found to be useful in examining hybridization in the *Cx. pipiens* complex (Smith and Fonseca 2004, Kothera et al. 2009), as well as a panel of microsatellite loci developed for the *Cx. pipiens* complex.

MATERIAL AND METHODS

We examined variation at a nuclear intron (the ACE locus) and at eight microsatellite loci. The microsatellite loci were developed from other members of the *Cx. pipiens* complex but were optimized in *Culex pipiens pallens* (Smith et al. 2005). Figure 1 depicts a geographic map of the relative positions of the collection sites. The specimens used are listed in Table 1 and were obtained by us and from local entomologists. DNA was extracted using a standard phenol/chloroform method (Fonseca et al. 2000), and the presence of diagnostic ACE bands for members of the *Cx. pipiens* complex was scored (Smith and Fonseca 2004). Specimens identified as *Cx. p. pipiens* were further examined with a second rapid assay (Bahnck and Fonseca 2006) to identify the form ("pipiens" vs. "molestus"). We also compared the microsatellite signature of Asian *Cx. p. pallens* populations to those of a sample of *Cx. p. pipiens* form pipiens from Northern Europe previously published (Fonseca et al. 2004).

The ACE locus

A small part of exons 2 and 3 and the entire intron II of the *ace-2* gene (the ACE locus,(Bourguet et al. 1998) were amplified using a PCR+1 protocol to prevent polymerase errors and heteroduplex formation (Borriello and Krauter 1990). First, an asymmetric amplification was performed in 50-μL reactions containing 0.25 μM of the primer F1457 (5′-GAGGAGATGTGGAATCCCAA-3′), 2.5 μM of B1246 (5′-TGGAGCCTCCTCTTCACGGC-3′), 1X Easy-A reaction buffer, 500 μM of each dNTP, 2.5 units Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA), and approximately 6 ng of DNA template. The amplification program consisted of 95°C for 2 min, 30 cycles of 95°C for 40 sec, 54°C for 30 sec, 72°C for 1 min, followed by 72°C for 7 min. Twenty microliters of the PCR product were used in a new 50-μl reaction for one additional cycle (the PCR+1 step, times and temperatures identical to those above) with 2.5 μM of a third primer F1457MluI (5′-ACGCGTGAGGAGATGTGGAATCCCAA-3′) with an extra 5μl of 1 X Easy-A buffer, additional dNTPs (to 500μM), and 2.5 units of Easy-A. The primer F1457MluI contains a cut-site for the restriction enzyme *Mlu* I.

The ACE locus of *Cx. p. pallens* was amplified using the PCR+1 protocol and cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were amplified with the sequencing primers M13F(-20) and M13R. Reactions were carried out in 50-μL volumes containing 0.75 μM of each primer, 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 500 μM of each dNTP, and 1.5 units *Taq* polymerase (Applied Biosystems, Foster City, CA). The M13 product was cleaned using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and digested with the restriction enzyme *Mlu* I (New England Biolabs, Beverly, MA) according to manufacturer's instructions. The digestion product was electrophoresed on 1% agarose gel to detect clones that contained the cut site from the PCR+1 amplification step. Only clones with the cut site (less than 30% of the digested clones) were sequenced since it ensured they were produced during the last PCR step using the third primer and therefore did not result from priming by partially extended DNA fragments, which can lead to PCR cloning artifacts. Two clones per specimen were sequenced using standard cycle sequencing conditions (Big Dye, ABI, Foster City, CA). The resulting fragments were analyzed by electrophoresis in a slab gel (ABI 377) automated sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned in Sequencher version 4.1 (GeneCodes, Ann Arbor, MI).

Microsatellite analysis

We used eight of the twenty microsatellite loci currently optimized for the *Cx. pipiens* complex (CQ11F2R2, CQ26FR, CxqGT4F3R, CxqGT6bFR, EmmaFR, CxpGT12F2R2, CxpGT46FR, and GT9FR) since they appeared to amplify consistently in *Cx. p. pallens* (Smith et al. 2005). The loci were multiplexed and amplified in 20-μL reactions containing 0.2 μM of each primer, 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 200 μM of each dNTP, 250 μM $MgCl_2$, 150 μg/mL of bovine serum albumin, 0.5 units of *Taq* polymerase (Applied Biosystems, Foster City, CA), and approximately 6 ng of the DNA template. The amplification program consisted of one cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 52°C or 54° C (depending on primer, (Smith et al. 2005) for 30 sec, 72°C for 30 sec, and one cycle of 72° C for 5 min. A positive control with the clone used to design the microsatellite primers and a water negative control were included in each batch of samples. Microsatellite regions were sized in an ABI3100 automatic sequencer (Applied Biosystems, Foster City, CA) and analyzed with GeneMapper 3.7 (Applied Biosystems, Foster City, CA).

Statistical analyses

The ACE locus sequence data was analyzed in Arlequin (Schneider et al. 2000) to obtain measures of molecular diversity, calculate F_{ST}values, and their significance, as well as obtain exact tests of population differentiation. The microsatellite data was first examined for compliance with Hardy-Weinberg equilibrium GENEPOP 1.2. (Raymond and Rousset 1995), and then pair-wise F_{ST}values and their significance, were obtained using Arlequin (Schneider et al. 2000). Further, we assigned individuals to clusters based on their microsatellite multilocus genotypes with a maximum likelihood algorithm implemented in the program Structure 2.0 (Pritchard et al. 2000b). This method combines all the individual multilocus genotypes and separates them into distinct clusters analogous to the hierarchical branching of tree diagrams (Rosenberg et al. 2001). We used 20,000 burn-in steps and 1,000,000 runs with a model of uncorrelated allele frequencies allowing admixture (lambda = 0.64, calculated at K = 1 (Pritchard et al. 2000a). In this analysis, the origin of each specimen is not disclosed but the number of clusters (K) is decided *a priori* for each run. To assess the consistency of the analysis we performed an exhaustive comparison of 10 runs at each K scoring the similarity coefficient described in Rosenberg and others (2002).

RESULTS

We examined 115 specimens of *Culex pipiens pallens*, 24 of *Cx. quinquefasciatus*, and 23 of *Cx. pipiens pipiens* form molestus (Table 2). All specimens of *Cx. p. pipiens* from Japan and ROK examined had the genetic signature of the “molestus” form.

The ACE locus was polymorphic across and within the populations of *Cx. p. pallens* examined (gene diversity = 0.87±0.03 with 13 polymorphic sites/612 total basepairs examined, Table 2). We excluded from this analysis sequences that matched those obtained from *Cx. quinquefasciatus* (Smith and Fonseca 2004) since we had independent evidence of hybridization (see below) and wished to examine just *Cx. p. pallens* sequences for phylogeographic purposes. To increase sample sizes, we grouped sequences by country (Japan, ROK, and China) and found that on average one quarter of the ACE sequences were unique to each country (Table 2), although we did not find evidence of population differentiation based on this locus (all pair-wise F_{ST} values failed to be statistically significant, data not shown).

The rapid assay based on diagnostic bands in the ACE locus revealed complete hybridization between *Cx. p. pallens* and *Cx. quinquefasciatus* as all male specimens had the “quinques” diagnostic band as well the “pallens” band (Table 3, Figure 3). However, this assay did not reveal extensive hybridization in females as they have overwhelmingly only the “pallens” ACE

diagnostic band (Table 3). A few males and females in very southern populations had only the "quinques" diagnostic band (Table 3).

One of the eight-microsatellite loci used in the analyses (CxpGT9) amplified very poorly in all populations of *Cx. pipiens pallens* and was excluded from further analyses although it is in Hardy-Weinberg (H-W) equilibrium in all populations of *Cx. p. pipiens* examined to date (Fonseca et al. 2004). There were also departures from H-W equilibrium in CQ11 and CxqGT6b in some populations (Supplementary Information). Since a multilocus genotype analysis assumes all loci are in H-W (Pritchard et al. 2000a), we ran analyses with all seven loci, as well as excluding CQ11 (the locus with the most significant departures from H-W), and both CQ11 and CxqGT6b (Figure 2). Irrespective of the number of loci used, the results of the multilocus genetic structure analysis, which combines all the individual multilocus genotypes and separates them into most likely clusters, show hybridization between *Cx. p. pallens* and *Cx. quinquefasciatus* (Figure 2). Both males and females have a similar hybrid microsatellite signature (Figure 3). The uniformity of results across 10 replicates was high (0.9-0.99). Microsatellite alleles unique to *Cx. quinquefasciatus* (see GT4 in Supplementary Materials) are not found in populations from the northernmost island in Japan (Hokkaido) while they are particularly common in Chinese populations and even dominant in Shanghai specimens resulting in specimens indistinguishable from "pure" *Cx. quinquefasciatus* (Figure 2). Furthermore, two specimens, one in Suwon, ROK, and another in Tianjin, near Beijing, China, show strong ancestries from *Cx. pipiens pipiens* form molestus (Figure 2).

Analysis of population differentiation using the five loci that fit H-W expectations in all populations revealed significant differentiation mostly between hybrid and non-hybrid populations irrespective of geographic distance (Table 4) although the hybrid zone has a geographic component with more hybrids in the southernmost locations (Figure 2). The small sample sizes of the populations from Paju and Suwon likely decreased the power of the comparisons to other populations. Since Shijiazhuang, Beijing, Tianjin, and Taiuman are all locations on or very near Beijing, we grouped them to increase the sample size. A pair-wise comparison using the 5 microsatellite loci revealed significant differentiation between the western and eastern subspecies of *Cx. pipiens* (F_{ST} values ranged from 0.148 - 0.253, Table 5), while among northern European *Cx. p. pipiens* populations the F_{ST} values ranged from only 0.001 to 0.138 and were not different from zero, indicating considerable gene flow.

DISCUSSION

Although our analyses have uncovered extensive hybridization between *Culex pipiens pallens* and *Cx. quinquefasciatus* in Eastern Asia, they also provide strong evidence that *Cx. p. pallens* is not simply a hybrid of the European *Cx. p. pipiens* and *Cx. quinquefasciatus*. The multilocus genotype analysis based on the microsatellite loci identified two specimens with a mixed signature involving *Cx. p. pipiens* but all of *Cx. p. pipien*s tested revealed the diagnostic *Cx. p. pipiens* form molestus CQ11-band (Bahnck and Fonseca 2006), corroborating the hypothesis that only the molestus form has been introduced to Japan. Further our extensive testing and sequencing of the ACE locus across the *Cx. p. pallens* range did not recover the diagnostic pipiens-ACE sequence, only the "pallens" as well as "quinquefasciatus" bands were present (Smith and Fonseca 2004). The ACE sequences we obtained from *Cx. p. pallens* specimens are diagnostic and *Cx. p. pallens* populations have a unique microsatellite genotype signature when compared to populations from Northern Europe, both indicating lack of gene flow. We therefore conclude that *Cx. p. pallens* differs from hybrids of *Cx. p. pipiens* and *Cx. quinquefasciatus*.

However, we also found many hybrids of *Cx. p. pallens* and *Cx. quinquefasciatus*, an observation that explains morphological clines (Bekku 1956). As expected, a detailed analysis

found evidence of a north-south population differentiation most likely because of differences in the extent of hybridization with *Cx. quinquefasciatus*. Interestingly, although the ACE locus was considerably polymorphic, we did not find a significant geographic assortment of alleles. These results, as well as the low F_{ST} values among northern European populations (Table 5), agree with expectations of extensive movement in mosquitoes belonging to the *Culex pipiens* complex possibly as a result of their close association with humans (Vinagradova 2000).

The north-south microsatellite signature of *Cx. p. pallens* in Japan (Figure 2) resembles the situation in North America where *Cx. p. pipiens* and *Cx. quinquefasciatus* hybridize extensively (Kothera et al. 2009, Fonseca and others unpublished data). However, while the ACE rapid assay in North America is a good measure of the extent of the hybridization between the two species (Smith and Fonseca 2004,Kothera et al. 2009), such is not the situation in Japan (Figure 2). In Japan, all males identified as *Cx. p. pallens* have two DNA bands, one diagnostic of "pallens" the other of "quinquefasciatus" (Figure 3). A few exceptions occur in the southernmost populations where some males have just a "quinquefasciatus" band and are likely either males of *Cx. quinquefasciatus* or the result of extensive hybridization with *Cx. quinquefasciatus*. Our survey failed to detect a male with just the "pallens" band (Table 3). Females, in contrast, have overwhelmingly only the "pallens" band, although specimens with both bands and just the "quinquefasciatus" band also occur (Table 3), again mostly in the southernmost populations. Indeed, such ACE-quinques males and ACE-hybrid females are found exclusively in areas of high hybridization detected using microsatellites e.g. Saga (88% hybrids based on multilocus genotype, where a hybrid is being defined as a specimen with more than 5% probability of ancestry from two or more taxa) and China (76% hybrids). We therefore conclude there has been asymmetric introgression of *Cx. quinquefasciatus* genetic material across chromosomal regions into *Cx. p. pallens*, similar to that found in birds (Parsons et al. 1993), where traits under selection (male sexual plumage) introgressed further than neutral markers like microsatellite loci. These results expand the findings of Smith and Fonseca (2004) and Kasai and colleagues (2008).

Gender in the *Cx. pipiens* complex is thought to be determined by a male determining locus (MDL), where males are heterozygous, *Mm*, and females are homozygous, *mm* (Gilchrist and Haldane 1947). The ACE locus is within the *ace-2* gene, which is physically linked (distance calculated at ≤ 0.8 cMorgans) to the MDL (Malcolm et al. 1998). Our results suggest that the *Cx. quinquefasciatus* ACE locus and possibly its associated *M* allele have introgressed to fixation in the *Cx. p. pallens* populations examined. If so, all male *Cx. p. pallens* have a "pallens", ACE^P, and a "quinquefasciatus", ACE^Q, allele at the MDL, while females are homozygous for ACE^P. If ACE and MDL are linked, then females are homozygous for $(m/ACE)^P$, while male *Cx. p. pallens* must be $(m/ACE)^P + (M/ACE)^Q$. This suggests that the male determining locus of *Cx. quinquefasciatus* may have replaced that of *Cx. p. pallens* in all the populations we examined. The altered phallosome morphology that distinguishes *Cx. p. pallens* from *Cx. p. pipien*s may be the result.

An alternative to this scenario is that because of the way male *Cx. p. pallens* are identified (by the shape of their phallosome), we have missed the "true" pallens males in our analyses (i.e. our analysis includes only female *Cx. p. pallens* and hybrid males). However, the unique "pallens" microsatellite signature is shared between males and females in the two populations from the northern island of Hokkaido and those specimens (males and females) do not have a hybrid microsatellite signature. The specimens with the *Cx. p. pipiens* phallosome that we examined in Asia (Japan and Korea) all had a *Cx. p. pipiens* f. molestus microsatellite signature (Fonseca et al. 2004). It is apparent therefore that we are examining a representative sample of the taxon. There is the additional possibility that the current gender difference in introgression of the ACE locus may involve negative selection on the heterozygous females.

The discrepancy between microsatellite and ACE loci, and the similarity in microsatellite signature between males and females, however, do not support that hypothesis.

The function of *ace*-2 is still unknown (Malcolm et al. 1998) since in *Cx. pipiens* it has been shown that AchE insecticide insensitivity is linked to *ace*-1, a paralogous gene (Weill et al. 2002). It is therefore still unclear if the asymmetric introgression we uncovered is related to the MDL, the *ace*-2 locus, or some other gene in this region. We do not yet know what are the selective forces driving or maintaining this apparent introgression of genetic material but are currently examining Single Nucleotide Polymorphisms (SNPs) in the chromosome 1 to understand this phenomenon. Although the transfer of advantageous traits across hybrid zones has been proposed as an important consequence of hybridization (for reviews see Arnold 2004b, Currat et al. 2008), the proposed complete swap of a *Cx. p. pallens* nuclear region by that of *Cx. quinquefasciatus* may be an extreme example of the power of hybridization to affect the evolution of an organism. The need to examine this possibility is made more pressing by the medical importance of these vectors of human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic geographic map of parts of northeastern Asia depicting the relative positions of the sources of specimens of *Culex pipiens pallens* examined in this study. NK= Democratic People's Republic of Korea; ROK= Republic of Korea. Please refer to Smith and Fonseca (2004) for a world map depicting the approximate distributions of the various members of the *Culex pipiens* complex.

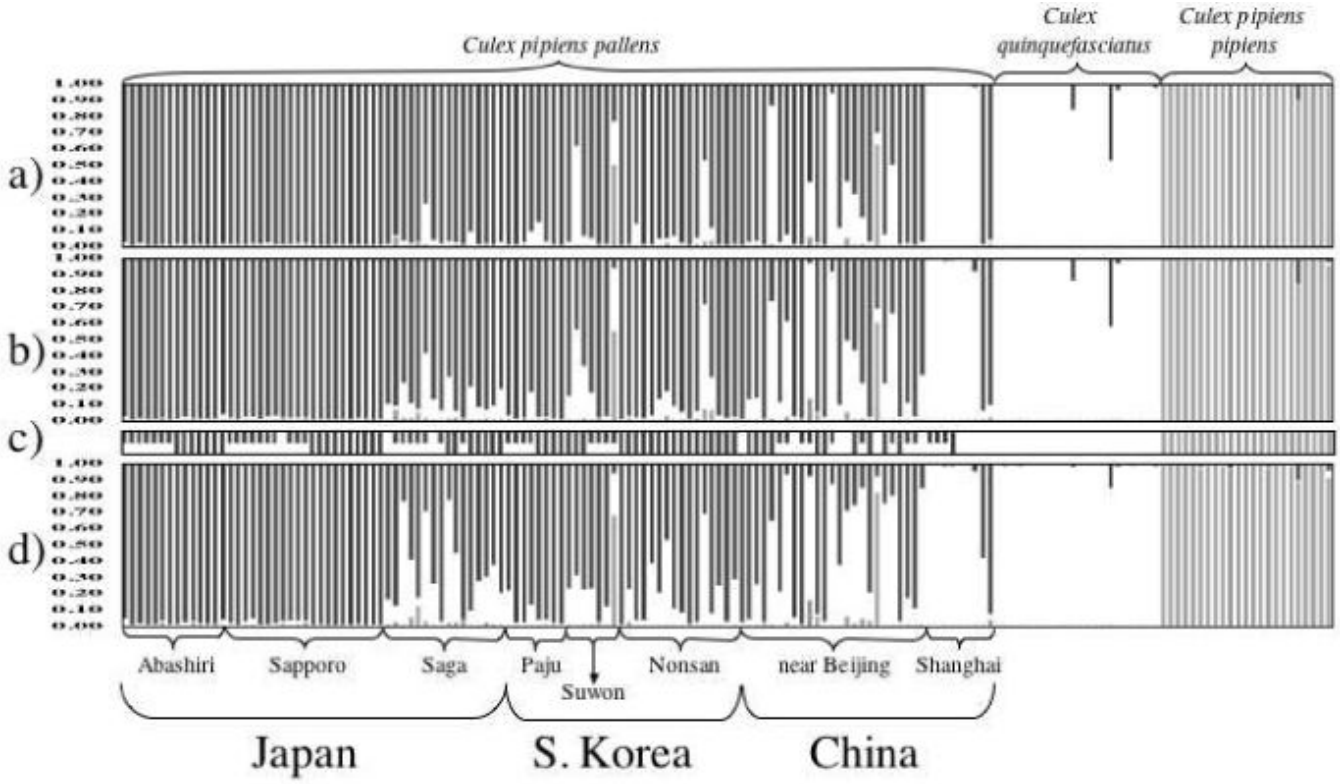

Figure 2.

Comparison of the results of multilocus genotype analyses of Japanese, ROKn, and Chinese populations of mosquitoes in the *Culex pipiens* complex using (a) 7, (b) 6, and (d) 5 microsatellite loci. Each of the individuals is represented by a column partitioned into three shaded segments that represent the individual's probability of ancestry from one of three genetic clusters (dark grey, *Culex pipiens pallens*, white, *Cx. quinquefasciatus*, light grey, *Cx. p. pipiens* form molestus). For this representation individuals were grouped by location (bracketed). Populations of *Cx. p. pallens* are ordered from North to South within each country. (c) Summary of the individual ACE rapid assays. The diagnostic bands are represented by a bar of a single shade or, in the case of hybrids, by a bar divided into two shades (the shades are the same as in the multilocus genotype analyses).

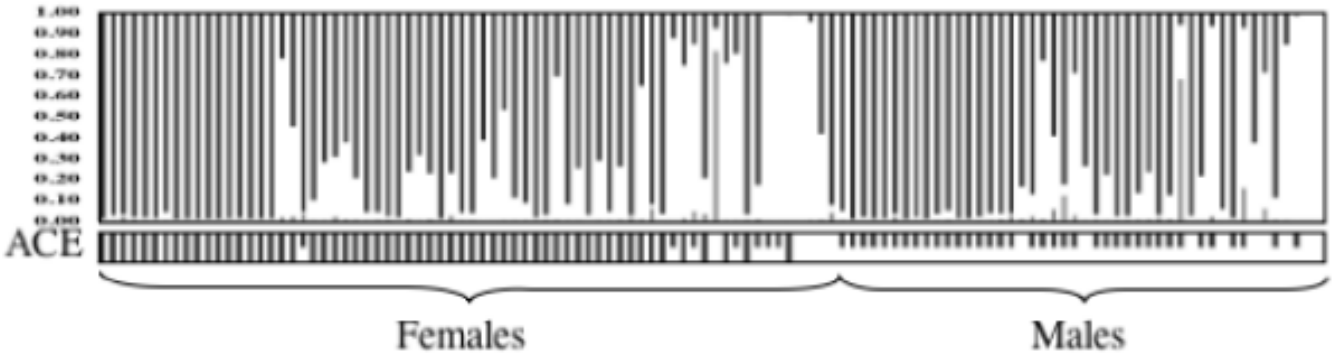

Figure 3.
Comparison of the multilocus genotype signatures of male and female *Culex pipiens pallens*. The specimens are in the same latitudinal order as in Figure 2 but sorted by sex. Only the multilocus genotype analysis using five microsatellite loci is depicted.

Table 1

Populations of *Culex pipiens* complex mosquitoes analyzed in this study. N_m and N_{ACE} are the numbers of specimens from each population examined with microsatellite markers and the ACE locus, respectively

Species	Location	Latitude (deg min)	N_m	N_{Ace}	Collection date	Source*
Culex pipiens pallens and hybrids	Abashiri, Japan	44 01 N	14	3	07/2004	M. Mogi
	Sapporo, Japan	43 05 N	22	3	06/2001	M. Mogi
	Saga, Japan	43 05 N	16	4	06/1999	M. Mogi
	Paju, ROK	37 80 N	8	2	08/2003	M. Turrell
	Suwon, ROK	37 17 N	7	2	11/2003	H-C. Kim
	Nonsan, ROK	36 12 N	16	3	06/2004	H-C. Kim
	Beijing, China	39 55 N	8	1	10/2002	T. Zhao/C. Curtis
	Tianjin, China	39 08 N	7	1	10/2002	T. Zhao/C. Curtis
	Shijiazhuang, China	38 03 N	5	2	10/2002	T. Zhao/C. Curtis
	Taiuman, China	37 55 N	5	1	10/2002	T. Zhao/C. Curtis
	Shanghai, China	31 14 N	7	0	04/2004	P. L. M. Rueda
Cx. quinquefasciatus	Okinawa, Japan	26 20 N	22		06/1999	I. Miyagi
	Shanghai, China	31 14 N	2		04/2004	P. L. M. Rueda
Cx. pipiens pipiens	Seoul, ROK	37 33 N	8		11/2003	H-C. Kim
	Busan, ROK	32 06 N	15		10/2003	H-C. Kim

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Table 2

Polymorphisms in the second intron of the ace-2 gene. Of the 612 bp sequenced only the variable positions are shown. Position 1 corresponds to position 1 in GenBank accession #AY497524.(Smith and Fonseca 2004) Deletions were not included unless the position was also polymorphic, and are indicated by a dash (-). JPN, SKO, and CHN, refer to the frequency of each allele in Japan, ROK, and China, respectively

	06 6	11 5	15 1	16 4	17 5	22 1	23 7	27 5	31 2	38 4	38 7	45 1	47 2	**JPN**	**SKO**	**CHN**
A1	C	C	C	G	-	C	T	C	G	T	T	A	C	**2**	**0**	**0**
A2	.	.	.	.	.	.	.	.	.	.	.	G	.	**1**	**0**	**0**
A3	.	.	.	A	T	.	.	T	.	.	.	G	.	**1**	**1**	**0**
A4	T	A	.	.	A	.	.	.	.	.	.	.	.	**6**	**1**	**0**
A5	.	.	.	.	T	.	.	.	T	.	C	G	.	**1**	**0**	**0**
A6	.	.	.	.	T	.	.	.	T	.	.	G	.	**3**	**3**	**3**
A7	T	A	.	.	.	.	.	.	.	.	.	.	T	**1**	**5**	**0**
A8	.	.	.	.	.	A	A	.	.	C	.	G	.	**0**	**0**	**1**
A9	.	.	T	.	.	.	.	.	.	.	.	.	.	**0**	**0**	**1**
A10	.	.	.	.	A	.	.	.	.	.	.	.	.	**0**	**2**	**0**
A11	.	.	T	.	A	.	.	.	.	.	.	.	.	**0**	**1**	**0**
A12	.	.	.	.	.	.	.	.	.	C	.	G	.	**1**	**0**	**0**

Table 3

Comparison of rapid assay (ACE) results and gender in *Culex pipiens pallens*. Names between quotes refer to the presence of a single ACE diagnostic band

	"pallens"	"quinquefasciatus"	both bands	Total
male	0	8	38	46
female	58	5	7	70

χ^2 probability of gender parity <0.001

Table 4

Pairwise F_{ST} values (upper diagonal) among populations of *Culex pipiens pallens* and respective p-values (lower diagonal) based on the allelic frequency distributions at 5 microsatellite loci

	Abashiri	Sapporo	Saga	Paju	Suwon	Nonsan	Beijing	Shanghai
Abashiri		0.038	0.115	0.060	0.062	0.052	0.062	0.270
Sapporo	0.008		0.195	0.136	0.144	0.082	0.122	0.328
Saga	0.001*	0.001*		0.091	0.019	0.041	0.026	0.189
Paju	0.003	0.001*	0.001*		0.043	0.031	0.065	0.295
Suwon	0.012	0.001*	0.135	0.057		0.001	0.019	0.158
Nonsan	0.001*	0.001*	0.004	0.058	0.420		0.020	0.169
Beijing	0.001*	0.001*	0.026	0.003	0.169	0.048		0.137
Shanghai	0.001*	0.001*	0.001*	0.001*	0.002	0.001*	0.001*	

Beijing=specimens from Beijing, Tianjin, Shijiazhuang, and Taiuman, China; Shan= Shanghai, China.

*significant p-values after Bonferroni correction

Table 5

Pairwise F_{ST} values (upper diagonal) among populations of *Culex pipiens pallens* from Abashiri and Sapporo in Hokkaido, the northernmost island where hybridization based on microsatellite analysis is not evident, and *Cx. p. pipiens* (form pipiens) from several Northern European populations. In the lower diagonal are the p-values based on the allelic frequency distributions at 5 microsatellite loci

	Abashiri	Sapporo	Mens	Camb	Wed	Lough	Alsace	Germ
Abashiri		0.038	0.164	0.193	0.167	0.148	0.163	0.180
Sapporo	0.008		0.220	0.253	0.227	0.202	0.225	0.246
Mens	0.001*	0.001*		0.138	0.008	0.009	0.026	0.024
Camb	0.001*	0.001*	0.135		0.008	0.001	0.012	0.017
Wed	0.001*	0.001*	0.189	0.180		0.001	0.027	0.017
Lough	0.001*	0.001*	0.315	0.730	0.694		0.019	0.012
Alsace	0.001*	0.001*	0.036	0.225	0.009	0.108		0.010
Germ	0.001*	0.001*	0.054	0.072	0.036	0.234	0.405	

Please refer to Fonseca and colleagues (2004) for more detailed location, sample, and source information. Mens=Menstrie, Scotland; Camb=Cambridge, England; Wed=Wedmore, England; Lough=Loughborough, England; Alsace= Staffelfelden and Cernay, France; Germ= Nonnenweier and Altrip, Germany.

*significant p-values after Bonferroni correction