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THE ACETYLCHOLINESTERASE GENE *ACE*: A DIAGNOSTIC MARKER FOR THE *PIPIENS* AND *QUINQUEFASCIATUS* FORMS OF THE *CULEX PIPIENS* COMPLEX

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ABSTRACT. The taxonomy of the *Culex pipiens* complex remains a controversial issue in mosquito systematics. Based on morphologic characters, 2 allopatric taxa are recognized, namely *Cx. pipiens* (including the form "*molestus*") in temperate areas and *Cx. quinquefasciatus* in tropical areas. Here we report on variability at the nucleotide level of an acetylcholinesterase gene in several strains and natural populations of this species complex. Few polymorphisms were found in coding regions within a subspecies but many polymorphisms were observed between subspecies in noncoding regions. We describe a method based on a restriction enzyme polymorphism in polymerase chain reaction-amplified DNA, in which the presence or absence of one restriction site discriminates *Cx. pipiens, Cx. quinquefasciatus,* and their hybrids. This technique reliably discriminates mosquitoes from more than 30 worldwide strains or populations. Polymerase chain reaction amplification of specific alleles may also be a useful tool for characterizing specific alleles of each sibling taxon.

KEY WORDS Acetylcholinesterase gene, *Culex pipiens* complex, diagnostic marker, sibling species, *Culex torrentium, Culex pipiens "molestus"*

INTRODUCTION

The mosquito *Culex pipiens* represents a species complex that is incompletely understood (see Harbach et al. [1985] for a review). Based on morphologic characters, 3 types have thus far been described: *Culex quinquefasciatus* Say (Sirivanakarn and White 1978), *Culex pipiens "molestus"* Forskål (Harbach et al. 1984), and *Culex pipiens* Linnaeus (Harbach et al. 1985). The last 2 types are sympatric and are considered by some authors to be ecotypes of the same form (Roubaud 1933; Mattingly 1951; Pasteur 1977; Barr 1981; Chevillon et al. 1995a, 1998; Vinogradova et al. 1996; Eritja, 1998), as they are mainly distinguished by ecological and physiologic characteristics. *Culex p. "molestus"* breeds in underground urban habitats (hypogeous habitats such as cellars, sanitary spaces under buildings, and septic tanks), and *Cx. pipiens* breeds in rural open-air habitats (epigeous habitats such as brooks, rivers, swamps, ditches, or any artificial open-air collection of water). 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). In contrast, 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). The same association between physiologic traits and habitat types is observed in northern Europe and in North American and Australian regions with cold winters (Roubaud 1933, Marshall and Stanley 1937, Spielman 1964, Miles 1976).

Culex pipiens (including *Cx. p. "molestus"*) is largely a temperate form, whereas *Cx. quinquefasciatus* is cosmotropical (Mattingly et al. 1951, Barr 1957). *Cx. quinquefasciatus* is homodynamous, stenogamous, and anautogenous. Extensive areas of overlap and hybridization exist in the Middle and Far East, North and South America, Australia, and Africa (Barr 1982; Urbanelli et al. 1995, 1997). The main morphologic differences between *Cx. pipiens* and *Cx. quinquefasciatus* are found in the male genitalia, and can be quantified using the DV/D ratio (Sundararaman 1949), where DV is the distance from the tip of the ventral arm of the phallosome to its intersection with the dorsal arm and D is the distance between the tips of the dorsal arms of the phallosome. Values of DV/D below 0.2 characterize *Cx. pipiens,* whereas values above 0.4 characterize *Cx. quinquefasciatus.* Although this ratio has proven to be reliable outside hybrid zones by several authors (Mattingly et al. 1951, Barr 1957), its use is restricted to adult males. More recently, biochemical and molecular techniques have been used to find diagnostic markers (Miller et al. 1996, Severini et al. 1996, Crabtree et al. 1997). Recently, part of an acetylcholinesterase gene, referred to as *Ace,* was cloned for a *Cx. pipiens* strain (Malcolm

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et al. 1998), thus offering a new opportunity to compare *Cx. pipiens* and *Cx. quinquefasciatus* at the genomic level. Here we report partial sequences of the *Ace* locus for different collections of the *Cx. pipiens* complex. Variation in this region clearly discriminates *Cx. pipiens* from *Cx. quinquefasciatus.* Based on these sequences we propose and test a restriction enzyme pattern as a diagnostic marker for the 2 subspecies.

MATERIALS AND METHODS

Mosquitoes: Origins and references of the strains and populations used in this study are given in Table 1. Mosquitoes from populations or strains close to putative hybrid zones (Mattingly et al. 1951) such as BED (South Africa), Killcare (Australia), DC3 (Washington DC, USA), and BEIJING (China) were classified as *Cx. pipiens* or *Cx. quinquefasciatus* by means of DV/D ratios of male genitalia (Barr 1957). Females of the strain S-LAB (*Cx. quinquefasciatus*) were crossed with males of 2 different *Cx. pipiens* strains from southern France to obtain hybrid individuals that were referred to as MSE-F1 and RSV, respectively (Table 1). Mosquitoes from 2 populations of *Culex torrentium* Martini (see Table 1) were also used for comparison.

Polymerase chain reaction (PCR) amplification and sequencing: For the MSE, BRUGES A, Praias, S-LAB, SUPERCAR, MRES, and BEIJING strains, genomic DNA extraction of up to 100 mosquitoes was performed as described by Raymond et al. (1989). The DNA from the DC3, Hilo, and McCandless strains was extracted from individual mosquitoes using a standard phenol–chloroform protocol (Sambrook et al. 1989). A 700-base pair (bp) fragment (which encompassed part of exon 2, intron 2, and part of exon 3, see Fig. 1 and Malcolm et al. [1998]) of the *Ace* gene was amplified using the oligonucleotide primers F 1457 (5′-GAGGAGATGTGGAATCCCAA-3′) and B 1246 (5′-TGGAGCCTCCTCTTCACGGC-3′) (Eurogentec, Seraing, Belgium). Amplifications were performed in a 50-μl volume containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.1% (w/v) Tween 20, 1.25 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate (dNTP), 100 ng of each primer, 10–100 ng of DNA, and 2.5 units of *Taq* polymerase (Eurogentec). The tubes were then quickly transferred to the thermal cycler (Thermocycler Crocodile II, Appligene, Illkirch, France). After 5 min at 93°C, reactions were cycled 35 times through the following temperature profile: 93°C for 1 min, 52°C for 1 min, and 72°C for 90 sec. The tubes were finally incubated at 72°C for 10 min. One hundred microliters of PCR products of MSE, BRUGES A, Praias, S-LAB, SUPERCAR, MRES, and BEIJING were purified (Geneclean II Kit, Bio 101 Inc., Vista, CA) and resuspended in 20 μl H_2O. The purified PCR products were then sequenced following the procedure described by Rousset et al. (1992) with the PCR primers. For the DC3, Hilo, McCandless, and Macapà populations, the PCR conditions were identical to those described above but reagents from ABI/Perkin Elmer (Norwalk, CT) and an MJ Research Peltier thermocycler (MJ Research, Inc., Waterton, MA) were used instead. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA). One microliter of clean DNA was cycle sequenced using AmpliTaq DNA FS polymerase and dye-labeled terminators (PE Biosystems, Foster City, CA), and was examined on an automated sequencer (ABI/Perkin Elmer).

Restriction fragment length polymorphism (RFLP) analysis: At least 3 mosquitoes from each strain or population were analyzed except for Fort Knox and Simpson where RFLP analyses were performed on genomic DNA of up to 100 mosquitoes. Single mosquito genomic DNAs were obtained following Qiao and Raymond (1995). The 700-bp fragment of the *Ace* gene was amplified as described above. Aliquots of 10 μl of each amplification were digested with the *Sca*I restriction enzyme and loaded onto a 1.5–2% (w/v) agarose gel with tris borate EDTA (TBE) buffer.

RESULTS AND DISCUSSION

Ace polymorphism

At least 363 nucleotide sites (44 in exon 2, 158 in intron 2, and 161 in exon 3) have been sequenced at the *Ace* locus for several strains and populations from various geographic areas (China, Hawaii, Brazil, Ivory Coast, Cuba, and California for *Cx. quinquefasciatus* and France, Belgium, Portugal, and Washington, DC, for *Cx. pipiens*). Variable nucleotides are shown in Fig. 2. Variable sites are mainly located in intron 2 and substitutions in the exons did not change the inferred amino-acid sequence. This indicates that *Ace* is probably not a pseudogene, although its exact function remains unknown (Malcolm et al. 1998). The polymorphism among strains of the same subspecies is low and *Taq* errors may not be excluded. In contrast, we found many differences (37 variable sites out of 710 sequenced) between the *Ace* sequences of *Cx. pipiens* and those of *Cx. quinquefasciatus.* The *Ace* gene of the *Cx. pipiens* complex is characterized by the presence of 10 introns (Malcolm et al. 1998). With the exception of intron 4, these introns are very large, resembling more the structure of the *Drosophila melanogaster Ace* Meigen gene (Fournier et al. 1989) than that of *Anopheles stephensi* (Malcolm and Hall 1990).

A diagnostic marker

A *Sca*I restriction site that discriminates *Cx. pipiens* from *Cx. quinquefasciatus* alleles was found in intron 2 (Fig. 3). The 700-bp amplified *Ace* frag-

Table 1. Strains and populations of *Culex pipiens* complex used in this study.

Taxa	Name	Origin	P/S[1]	Reference
Culex pipiens				
Ecotype *pipiens*	Ebre	Spain	P	Chevillon et al. 1995b
	Praias	Portugal	S	Bourguet et al. 1996
	MSE	France	S	Raymond et al. 1986
	BRUGES A	Belgium	S	Raymond et al. 1995
	Rothamsted	England	P	Unpublished
	Fort Knox	Kentucky, USA	S	Unpublished
	Gamart	Tunisia	P	Ben Cheikh et al. 1998
	Simpson	California, USA	S	Beyssat-Arnaouty et al. 1989
	DC3	Washington, DC, USA	P	Unpublished
Ecotype *molestus*	Alsace	France	P	Unpublished
	Heteren	Netherlands	S	Unpublished
	Killcare	Australia	P	Guillemaud et al. 1997
Culex quinquefasciatus	BED	South Africa	S	Raymond et al. 1991
	BSQ	South Africa	S	Unpublished
	Ouagadougou	Burkina Faso	P	Unpublished
	SUPERCAR	Ivory Coast	S	Chandre et al., unpublished
	Récife	Brazil	P	Unpublished
	Macapá	Brazil	P	Unpublished
	Reparto	Venezuela	P	Unpublished
	MRES	Cuba	S	Bisset et al. 1990
	Haïti	Haiti	P	Yébakima et al. 1995
	S-LAB	California, USA	S	Georghiou et al. 1966
	Mahape	Tahiti	P	Pasteur et al. 1995
	Hilo	Hawaii, USA	P	Unpublished
	McCandless	Hawaii, USA	P	Unpublished
	Maduraï	India	S	Unpublished
	Lahore	Pakistan	S	Raymond et al. 1991
	Thaï	Thailand	S	Unpublished
	Guang zhou	China	S	Qiao et al., unpublished
	BEIJING	China	S	Qiao and Raymond 1995
Culex pipiens–Cx. quinquefaciatus hybrid	MSE-F1	Laboratory	S	Raymond et al. 1987
	RSV	Laboratory	S	Unpublished
Culex torrentium	Alsace 366	France	P	Unpublished
	Uppsala	Sweden	P	Unpublished

[1] P, natural population; S, strain.

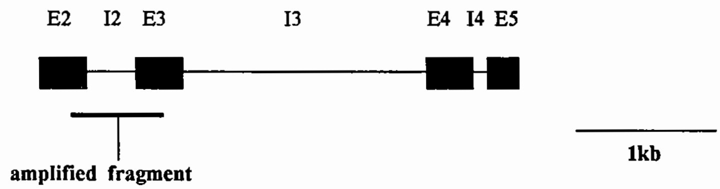

Fig. 1. Map of the *Ace* gene in the *Culex pipiens* complex, indicating the location of exons (boxes E2 to E5) and introns (lines I2 to I4) already known, and the amplified fragment used in this study (see Malcolm et al. 1998 for a detailed nucleotide sequence).

ment of *Cx. quinquefasciatus* possesses 2 *Sca*I restriction sites but only one is shared with the *Ace* sequence of *Cx. pipiens* (Fig. 3). Thus, the presence or absence of this restriction site may be used to distinguish between the 2 subspecies. This technique has been used on single mosquitoes from the worldwide populations and strains listed in Table 1. The restriction profile patterns found for *Cx. pipiens, Cx. quinquefasciatus,* and their hybrids are shown in Fig. 4. All mosquitoes possess an identical *Sca*I site, generating 2 fragments of 470 and 230 bp. The extra *Sca*I restriction site of *Cx. quinquefasciatus* alleles cuts the 470-bp fragment into 2 fragments (350 + 120 bp). *Culex pipiens* mosquitoes are characterized by the presence of 2 fragments (470 and 230 bp), whereas *Cx. quinquefasciatus* has 3 fragments (350, 230, and 120 bp). Hybrid mosquitoes display the 4 predicted bands (470, 350, 230, and 120 bp).

Culex pipiens and *Cx. p.* "*molestus*" ecotypes share a similar *Sca*I restriction profile, supporting the hypothesis that significant gene flow occurs between them (Chevillon et al. 1998). Genomic DNA from *Cx. torrentium,* a species known to be closely related to members of the *Cx. pipiens* complex (Miller et al. 1996), was used to determine whether the presence of the 2nd *Ace Sca*I site is a derived or an ancestral character. The amplified *Ace* fragment from *Cx. torrentium* had a lower molecular weight and the *Sca*I restriction profile did not resemble that of *Cx. pipiens* or that of *Cx. quinquefasciatus.*

A PCR assay for discriminating the 2 sibling taxa has also been developed by Crabtree et al. (1997). By using subtractive hybridization, they isolated a DNA fragment containing a sequence specific to *Cx. pipiens.* They used this sequence to design PCR primers that amplified a specific product from *Cx. pipiens* but not from *Cx. quinquefasciatus* genomic DNA. Although use of these primers was one of the first molecular tools for examining the *Cx. pipiens* complex at the taxonomic level, the method does not provide a perfect diagnostic marker. First, the presence or absence of PCR product amplification segregates as a dominant marker so that *Cx. pipiens* and *Cx. pipiens–Cx. quinquefasciatus* hybrids cannot be differentiated. Second, identification of *Cx. quinquefasciatus* is based on the absence of amplified product and therefore cannot distinquish the presence of *Cx. quinquefasciatus* DNA from the absence of adequate template DNA. Our assay uses a codominant marker that allows identification of each taxon and their hybrids based on distinct restriction profiles.

The technique of PCR amplification of specific alleles first described by Sommer et al. (1992) has had wide applicability for the determination of point mutations involved in insecticide resistance

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                          Exon2                    Intron2                  Exon3
                          <---><------------------------------------------><------->
                          0111111111112222222222222222222222333333333333333344444444444456666677
                          6001445678900111223345566777889001111122345599255566777788921458900
                          7014484060007029060440246259794571235601714729478907136824031902678
C.p.pip.      MSE         CACCCTTTTAGGCAGTAATACGAGTGACCGGA---GCA---ATT-TCTTTTTC-GC-CCTTGTGTTC
              DC3         -.....A........................ATTC..TTT...T........T..G....AA.-C-
              BRUGESA     C..........................
              PRAIAS      C..........................

C.p.quinq.    Hilo        -GATTAAGACA-TGT..GAGGAGAATG.TAGGATTAAATTTG.CTAA.---.-TTAGTAGCAAA-C-
              McCandless     TTAAGACA-TGT..GAGGAGAATG.TAAGATTAATTTTG.CTAAG---.-TTAGTAGCAAA-C-
              Macapá       GATTAAGACA-TGT..GAGGAGAATG.TAGGATTAAATTTGGCTAAG----CTTAGTA
              SLAB           TTAATACA-TGT..GAGGAGAATG.T
              SUPERCAR       TTAAGACA-TGT..GAGGAGAATG.T
              MRES           TTAATACA-TGT.GGAGGAGAATG.T
              BEIJING        TTAATACA-TGT-.GAGGAGAATGTT
```

Fig. 2. Variable nucleotides at the *Ace* locus in *Culex pipiens* and *Culex quinquefasciatus.* Deletions are indicated by a hyphen. The positions of variable sites in the genomic sequence are given by the number above MSE nucleotides. Position 1 corresponds to the 5′ end of exon 2 (position 1 in Fig. 2a in Malcolm et al. 1998).

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                          Exon 2              Intron 2               Exon 3

                          ------><-----------------/    /--------------><--------------
                          1                      1/    /4                            5
                          3                      6/    /8                            1
                          8                      0/    /1                            0
                                    *
C. p. pipiens             CAACGACGTATGTACTACTTCTT/...../AGGTCTTTATTGCAGTACTTCCAGGACGAT
C. p. quinquefasciatus    CAACGATGTAAGTACTACTTCTT/...../ACCTCTTTATTGCAGTACTTCCAGGACGAT
                                   ScaI                              ScaI
```

Fig. 3. *Sca*I restriction sites in the sequenced part of the *Ace* locus. The variable nucleotide at position 148 (*) disrupts the *Sca*I recognition sequence of the *Ace* locus of the 3 *Culex pipiens* strains from France, Belgium, and Portugal (MSE, BRUGES A, and Praias) and the 3 *Culex pipiens* strains from Washington, DC (DC1, DC2, and DC3).

(ffrench-Constant et al. 1994, Steichen and ffrench-Constant 1994, Martinez-Torres et al. 1998). This technique, which relies upon the specific amplification of one allele in preference to others at a given magnesium concentration within the PCR reaction, can also be used in species determination (Sommer et al. 1992). Because of the large number of differences found between *Cx. quinquefasciatus* and *Cx. pipiens Ace* sequences, a wide range of allele-specific primers could be designed.

Irrespective of the technique of choice, the *Ace* locus appears to be a useful molecular marker to discriminate the 2 subspecies *Cx. pipiens* and *Cx. quinquefasciatus* and their hybrids. This PCR-RFLP technique was also used in inheritance analysis, which revealed that the *Ace* locus is sex-linked (Malcolm et al. 1998). Clearly, further investigations of *Ace* polymorphism in mosquitoes of the *Cx. pipiens* complex may contribute to our understanding of the relationships between members of this medically important taxonomic group.

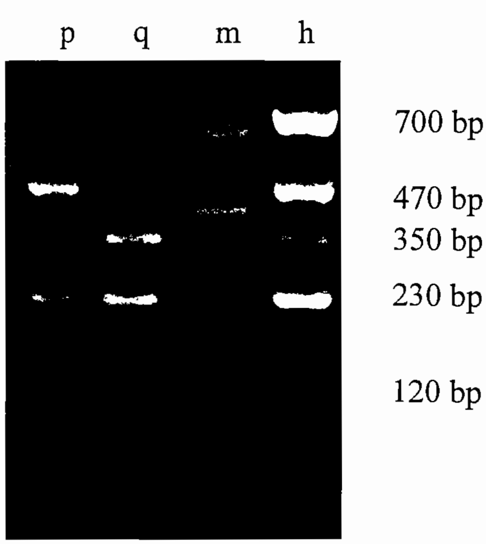

Fig. 4. *Sca*I digest of the polymerase chain reaction product derived from single mosquito extracted genomic DNA. Lanes: m, marker; p, *Culex pipiens*; q, *Culex quinquefasciatus*; h, *Cx. pipiens–Cx. quinquefasciatus* hybrid.

Culex p. "molestus" and *Cx. pipiens* apparently are not genetically differentiated, with the former probably being an ecotype of the latter. Because of the tendency for hypogeous breeding areas to be independently colonized by *Cx. pipiens* and not by mosquitoes from another hypogeous site, *Cx. p. "molestus"* is unlikely to emerge as a true species (Chevillon et al. 1998). On the other hand, *Cx. pipiens* and *Cx. quinquefasciatus* are genetically differentiated, as shown both by their different ITS2 (Severini et al. 1996) and *Ace* sequences. However, these 2 forms still exchange genes, as indicated by the spread of resistance genes across the *Cx. pipiens–Cx. quinquefasciatus* boundary (Raymond et al. 1991). Although the current taxonomic standard suggests that the 2 forms are true species, an interesting avenue of research will be to use these currently incompletely differentiated forms to study the processes that lead to speciation.

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