RAPID ASSAYS FOR IDENTIFICATION OF MEMBERS OF THE CULEX (CULEX) PIPIENS COMPLEX, THEIR HYBRIDS, AND OTHER SIBLING SPECIES
(DIPTERA: CULICIDAE)

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Abstract. Mosquitoes in the Culex (Culex) pipiens complex of species, known as vectors of periodic filariasis and deadly encephalitides, have recently emerged as important vectors of West Nile virus in the United States. Highly conserved morphology but marked differences in potential vectorial capacity require the development of polymerase chain reaction (PCR)–based tests that unambiguously distinguish among the different species. We introduce and describe a series of PCR-based assays that use polymorphisms in the second intron of the ace-2 gene to identify each taxon and may be rapidly executed even in local vector control offices.8 Our objective was to design a rapid and cost-effective PCR-based method in which each species/sub-species produces an amplification product of distinctive size.

INTRODUCTION

Mosquitoes in the Culex pipiens complex are important disease vectors with global distribution, yet remain difficult to identify in the field. The rapid and accurate identification of these mosquitoes, which vector West Nile Virus and St. Louis encephalitis in the eastern United States,2–4 periodic lymphatic filariasis (Wuchereria bancrofti),4 avian malaria,2 and other encephalitides across the world,9 is critical to control efforts. Identifying members of the Cx. pipiens complex by morphologic methods is difficult, time-consuming, and often limited to adult males.7 A rapid polymerase chain reaction (PCR) assay based on polymorphisms in ribosomal DNA was developed by Crabtree and others,8 who compared Cx. pipiens complex species from other species with similar morphology in the United States (Cx. restuans and Cx. salinarius). However, this test fails to differentiate the main taxonomic units within the Cx. pipiens complex. To date, molecular techniques used to distinguish members of the complex have included allozyme analyses,9 restriction fragment length polymorphism analysis of PCR products,10 and a PCR assay developed from subtractive hybridization that distinguishes between Cx. pipiens and Cx. quinquefasciatus by the presence of an amplification product in the former and its absence in the latter.11 These techniques only distinguish between the two major taxa of the complex: Cx. pipiens and Cx. quinquefasciatus.

Members of the Cx. pipiens complex include Cx. (Cx.) pipiens L. 1758, Cx. (Cx.) quinquefasciatus Say 1823, Cx. (Cx.) pipiens pallens Coquillett 1898, and Cx. (Cx.) australicus Dobrotworsky & Drummond 1953. While the male genitalia (phallosoma) can be used to distinguish Cx. pipiens from Cx. quinquefasciatus, their hybrids commonly have intermediate shapes,12 which are similar to the phallosomes of Cx. australicus and Cx. p. pallens. This has led some to hypothesize the latter are actually hybrid forms.13 Two closely related species, Cx. (Cx.) torrentium Martini 1925, and Cx. (Cx.) pervigilans Von Bergroth 1889, are morphologically very similar to members of the Cx. pipiens complex. As a testament to their highly cryptic morphologic differences, Cx. torrentium occurs in sympatry with Cx. pipiens throughout Europe and in some parts of Asia,13 but the species escaped notice until 1925. Indeed, it remained undetected in Great Britain until 1951, although it is now known to have been present there since at least 1900.14 Cx. pervigilans, which occurs exclusively in New Zealand and neighboring islands,15 closely resembles Cx. quinquefasciatus morphologically and some hybridization between the two has been hypothesized.15 Culex pipiens and Cx. quinquefasciatus are invasive, ubiquitous species, with geographic distributions closely overlapping those of humans, who are responsible for their introduction into many areas (Figure 1). Hybrid zones between the two species are known to occur in North America, Argentina, and Madagascar.9,16–18 The remaining species and subspecies in the complex have localized distributions (Figure 1). Our series of rapid assays is tailored to these geographic differences, so that a single assay uses fewer primers, reducing both the cost of the reaction and the chance for non-specific amplification.

Because of putative and realized differences in vectorial capacity within the complex,13 an assay that unambiguously identifies each taxon and may be rapidly executed even in local vector control offices is highly desirable.8 Our objective was to design a rapid and cost-effective PCR-based method in which each species/sub-species produces an amplification product of distinctive size.

On the heels of studies in which introgression between Cx. pipiens and Cx. quinquefasciatus was assessed using microsatellite markers, we also examined the ability of our simple diagnostic assay to provide information on the presence of hybrids. Those studies analyzed north-south transects between Massachusetts and Florida on the East Coast, and between Oregon and Jalisco, Mexico on the West Coast (Fonseca DM and others, unpublished data), mapping the extent of the hybrid zone between the two species using multilocus signatures19 unique to each species.20 Microsatellites are extremely polymorphic repeats of simple nucleotides motifs found in the genome of most organisms.21 Primers that amplify microsatellite-containing DNA fragments have been developed both for Cx. quinquefasciatus22 and Cx. pipiens,23 and there is a subset that will amplify in both species (Keyghobadi N and others, unpublished data). To evaluate the ability of the rapid assay to identify hybridization, we assessed the extent of introgression between Cx. pipiens and Cx. quinquefasciatus in 10 populations, using both a panel of eight microsatellite loci and the rapid assay. Previous studies have detected hybrids morphologically by differences in the shape of phallosoma, quantified as the DV/D ratio. This is the ratio between the extent to which the ventral arm of the phallo-
soma protrudes from the dorsal arm (DV) and the distance between the two dorsal arms (D). The use of microsatellites instead of the classic DV/D ratio to identify hybrids circumvents a potential bias due to the known effect of rearing temperature on the shape of the genitalia.

The presence of two nuclear genes that encode acetylcholinesterase (ACE) was first discovered in *Cx. pipiens* and has since been found in other mosquito species. The *ace-1* gene can confer resistance to organophosphate insecticides and is therefore subject to selection pressure. The *ace-2* gene is sex-linked, and its exact function and the selection pressures acting on it are not known. We used polymorphisms in the second intron of the *ace-2* gene to design species-specific primers for PCR-based assays.

MATERIALS AND METHODS

Mosquitoes. Origins and sources of mosquito samples are listed in Table 1. Genomic DNA was extracted from individual mosquitoes using a phenol-chloroform based protocol as previously described. Eight hybrid populations of *Cx. pipiens* and *Cx. quinquefasciatus* were first pinpointed using microsatellite allelic sizes and frequencies (Fonseca DM and others, unpublished data). The microsatellite-containing fragments (loci CQ11, CQ26, qGT4, pGT4, pGT9, pGT12, and pGT46) were amplified as previously described and sized in a ABI3100 capillary automatic sequencer (Applied Biosystems, Foster City, CA). Non-hybrid populations of *Cx. pipiens* were from Boston, Massachusetts and non-hybrid populations of *Cx. quinquefasciatus* were from Archer, Florida and New Orleans, Louisiana. The eight hybrid populations tested with our rapid assay are listed in Table 1; two additional populations were used only for sequencing because of their limited sample sizes. We analyzed a minimum of eight specimens from each population (mean ± SD = 8.7 ± 0.15).

Primer design. Sequences of sections of exons 2 and 3 and the entire intron II in the *ace-2* gene (the ACE locus) were obtained using the oligonucleotide primers F1457 (5′-GAGGAGATGTGGAATCCCAA-3′) and B1246 (5′-TGGAGCCTCCTCTTCAACGTC-3′) and the conditions described in Bourguet and others. Except for *Cx. australicus* and *Cx. pervigilans*, we had access to several populations of each species; therefore, we amplified and cloned (TOPO TA cloning kit; Invitrogen, Carlsbad, CA) the ACE locus from three specimens from several of those populations (Table 1). We sequenced two clones per specimen using standard cycle-sequencing conditions, and analyzed the resulting fragments by electrophoresis in a slab gel (ABI 377) automated sequencer (Applied Biosystems). We aligned the sequences in Sequencher version 4.1 (GeneCodes, Ann Arbor, MI) and manually added deletions/insertions. Selective forward-primers were designed for each species so that unique polymorphisms occurred within the first 2−4 basepairs (bp) of the 3′ end (Figure 2). Each of these primers was used in conjunction with the reverse primer B1246, which was modified by removing one nucleotide from the 3′ end to reduce primer dimers (Table 2). We used the online program Primer 3 to check for compatibility and self-annealing in the primers. Primers were first optimized individually, and then multiplexes (mixtures of two or more primer pairs) were created and optimized so that a single amplification reaction would distinguish any of the species present in a given geographic region (Figure 3 and Table 3).

Polymerase chain reaction assay. The PCR assays were optimized for a 20-μL volume since the product is needed only for fragment size analysis by electrophoresis on a 1.5% agarose gel. Reactions contained 1× PCR buffer, 250 μM of
amplification program consisted of one cycle at 94°C for five minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for one minute, and one cycle at 72°C for five minutes. DNA from individuals previously identified to each relevant species was included in every assay both as a positive control and as a demonstration of the expected size of each taxon-specific fragment. A negative control to which no DNA was added was also included in every run.

The diagnostic abilities of the amplification assays were tested using a minimum of six specimens from populations across the world other than from which specimens were sequenced (Table 1) in geographically appropriate mixtures (Table 3).

### RESULTS

Because ACE is a nuclear locus, the amplified PCR product had to be cloned to recover both alleles for sequencing. We cloned three individuals per population and obtained a minimum of six and a maximum of 49 (mean ± SE = 18.7 ± 7.7) sequences per species. The size of the amplified product was 634–636 bp for *C. p. pipiens* (n [number of cloned sequences] = 36), 626–634 bp for *C. quinquefasciatus* (n = 49), 636–641 bp for *C. p. pallens* (n = 6), 627–633 bp for *C. australicus* (n = 6), 691–706 bp for *C. p. pergivilans* (n = 9), and 512–513 bp for *C. torrentium* (n = 6). Intra-specifically and within the *C. p. pipiens* complex, the variation in the size of fragments is almost exclusively in single base pair microsatellites (runs of As or Ts). In contrast, relative to the *C. p. pipiens* complex, *C. pergivilans* and *C. torrentium* have large insertions (66 bp) or deletions (209 bp), respectively, in the intron region. Specimens from the *C. p. pipiens* × *C. quinquefasciatus* hybrid populations often produced clones that matched both those from *C. p. pipiens* and those from *C. quinquefasciatus*. Similarly, specimens identified as *C. p. pallens* through male genitalia analysis produced clones that matched *C. quinquefasciatus* and clones that were unique to *C. p. pallens*.

Primers were successfully designed for the identification of *C. pipiens*, *C. quinquefasciatus*, *C. p. pallens*, *C. australicus*, and *C. torrentium*. We did not design a unique primer for *C. pergivilans* since it can be amplified using the unique primer for *C. pipiens*, producing a fragment approximately 60 bp larger than in *C. pipiens*. Unfortunately, there is only a single polymorphism (A/T) that makes AC Epip unique for *C. pipiens* (Figure 2). It is therefore important to use very stringent conditions to avoid the amplification of that fragment in other species within the *C. pipiens* complex. Since the other species have their own unique fragments, this is not a major problem except during the evaluation of putative hybrids. It is important to note, however, that in hybrids the bands unique for *C. pipiens* and *C. quinquefasciatus* both amplify strongly (Figure 3), while the occasional non-specific amplification of the *C. pipiens* fragment generates a weak band (see *C. australicus* in Figure 3).

A multiplex of primers was optimized for each of five geographic regions based on which species are present there (Table 3 and Figure 1). The first regional multiplex, designed for use in Africa and the Americas, will produce a 610-bp fragment in *C. pipiens*, a 274-bp fragment in *C. quinque-
and may produce one or both fragments in hybrids of the two species (Figure 3). These are approximate sizes based on single individuals since, as mentioned, a small number of insertions and/or deletions occur intra-specifically. These fragment sizes are diagnostic for *Cx. pipiens* and *Cx. quinquefasciatus* regardless of which multiplex is used. Diagnostic primers for these two species are included in all multiplexes except Eurasia where only *Cx. pipiens* occurs. Besides the diagnostic fragments for *Cx. pipiens* and *Cx. quinquefasciatus*, 1) the Australian multiplex amplifies a 437-bp fragment in *Cx. australicus*; 2) although as mentioned earlier we did not design a primer specific for *Cx. pervigilans*, the New Zealand multiplex produces a 668-bp fragment in *Cx. pervigilans* specimens in contrast to the 610-bp fragment produced by *Cx. pipiens*; 3) the East Asian multiplex produces a 478-bp fragment in *Cx. p. pallens* and very commonly also the 274-bp fragment characteristic of *Cx. quinquefasciatus* in specimens with the male genitalia of *Cx. p. pallens*; and 4) the Eurasian multiplex produces a unique 416-bp fragment in *Cx. torrentium*.

Our rapid assay was able to detect hybrids between *Cx. pipiens* and *Cx. quinquefasciatus* in North America. We found that there is a strong correlation ($r^2 = 0.92$) between the population frequency of the ACE allele unique to one of the species (in this case we used *Cx. pipiens*) and the average probability of ancestry from *Cx. pipiens* in that population.

### Table 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>ACEaüs</td>
<td>5'-CTTGTGGTGTAGTTAGTGTTCGG-3'</td>
</tr>
<tr>
<td>ACEquin</td>
<td>5'-CTCTTCTAGAAGCTGGCGCA-3'</td>
</tr>
<tr>
<td>ACEpall</td>
<td>5'-ATGGTGGAGACGGCATGACG-3'</td>
</tr>
<tr>
<td>ACEpip</td>
<td>5'-GGAAAACAGCAGGTATGTACT-3'</td>
</tr>
<tr>
<td>ACEtorr</td>
<td>5'-TGCGTGTGTACAGTGTGT-3'</td>
</tr>
<tr>
<td>B1246s</td>
<td>5'-TGGAGGCTCTCTTCCACGG-3'</td>
</tr>
</tbody>
</table>

*The first five primers are all forward primers and need to be run with B1246s as the reverse primer.*

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*Figures and diagrams are not provided in the text.*
DISCUSSION

We found a high degree of intra-specific polymorphism in the intron region (Figure 2) of the ACE locus, especially if one considers the low levels of mitochondrial polymorphism commonly found across some of the species in the complex.5,29 The fixed polymorphisms at this nuclear intron between species in the *Cx. pipiens* complex and its sibling species might reveal more of the true phylogeny of the group than the mitochondrial DNA that has probably been highly modified by selective sweeps linked to *Wolbachia pipientis*.30 Although the primers that identify *Cx. australicus*, *Cx. torrentium*, and *Cx. p. pallens* generate fragments of very similar sizes, their distributions are not known to coincide. Therefore, we decided it was more important to separate each from their respective local species than from other species with which they do not co-exist. From Figure 2, it is apparent that other primers can be designed specifically to separate them if needed. It must be noted, however, that adding more primers to a reaction may require extensive optimization.

The finding that specimens identified as *Cx. p. pallens* through genitalia analysis will generate fragments unique to *Cx. quinquefasciatus* adds to the debate over the taxonomic status of *Cx. p. pallens*.13 In these specimens, we cloned the unique *Cx. p. pallens* allele as well as alleles characteristic of *Cx. quinquefasciatus*, leading us to hypothesize that extensive hybridization may be occurring between the two forms. However, examination of the ACE locus sequences in a phylogenetic context (Fonseca DM and others, unpublished data) does not support the hypothesis that *Cx. p. pallens* represents simply an extensive hybridization between *Cx. pipiens* and *Cx. quinquefasciatus*, as has been proposed.13,16 We are currently performing additional analyses which include populations of *Cx. p. pallens* from China and northernmost Japan. In contrast, we found that *Cx. australicus* is genetically well differentiated from both *Cx. pipiens* and *Cx. quinquefasciatus*, a result that is supported by further analyses of mitochondrial and microsatellite loci (Fonseca DM and others, unpublished data). Also, we did not find evidence of introgression between *Cx. quinquefasciatus* and *Cx. pervigilans*. This last observation is based on the analysis of a single population, so further study will be necessary. The assay we developed will allow a more extensive examination of this hypothesis.

The strong agreement on the degree of introgression between *Cx. pipiens* and *Cx. quinquefasciatus* across populations, obtained through an extensive microsatellite analysis based on a multilocus genotype analysis using the panel of eight microsatellite loci (Figure 4).

**TABLE 3**

Summary of the main geographic species groups and the appropriate primer multiplexes*

<table>
<thead>
<tr>
<th>Region</th>
<th>Species present and approximate size of diagnostic fragments</th>
<th>Primers to use</th>
<th>Primer concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa, Americas</td>
<td><em>Culex (Culex) pipiens</em> (610 bp)</td>
<td>ACEpip, ACEquin, B1246s</td>
<td>0.2, 0.4, 0.4</td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) quinquefasciatus</em> (274 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hybrids (610-bp and 274 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eurasia</td>
<td><em>Cx. (Cx.) pipiens</em> (610 bp)</td>
<td>ACEpip, ACEquin, B1246s</td>
<td>0.1, 0.4, 0.4</td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) torrentium</em> (416 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Asia</td>
<td><em>Cx. (Cx.) pipiens</em> (610 bp)</td>
<td>ACEpip, ACEquin, ACEpall, B1246s</td>
<td>0.1, 0.4, 0.3</td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) quinquefasciatus</em> (274 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) p. pallens</em> (478 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Putative hybrids (478 bp and 274 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td><em>Cx. (Cx.) pipiens</em> (610 bp)</td>
<td>ACEpip, ACEquin, ACEaus, B1246s</td>
<td>0.2, 0.2, 0.6</td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) quinquefasciatus</em> (274 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) australicus</em> (437 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td><em>Cx. (Cx.) quinquefasciatus</em> (274 bp)</td>
<td>ACEpip, ACEquin, B1246s</td>
<td>0.1, 0.1, 0.2</td>
</tr>
<tr>
<td></td>
<td><em>Cx. (Cx.) pervigilans</em> (668 bp)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*bp = basepairs.

**FIGURE 4.** Correlation between the frequency of *Culex pipiens* acetylcholinesterase (ACE) alleles and the average frequency of the “pipiens” signature (probability of ancestry from *Cx. pipiens*) based on a panel of eight microsatellite loci across 10 populations from the United States.
and the simple PCR assay we describe, was encouraging. While the power to detect low levels of introgression increases with the number of independent loci being assessed, areas of moderate hybridization can be easily pinpointed using just the quickly implemented ACE PCR assay. The much higher cost of a full microsatellite analysis can therefore be kept for questions that require a high level of precision or the need to know the likely ancestry of individual specimens.

This rapid assay does have limitations when used with pooled samples in areas of hybridization, such as the United States. It will not be possible to determine if the pooled sample contains a mixture of pure Cx. pipiens and pure Cx. quinquefasciatus, and/or their hybrids. Also, in the United States, Cx. restuans, Cx. salinarius, and Cx. pipiens complex specimens are commonly found in pooled samples of potential West Nile virus vectors. In those instances, we recommend that the assay in Crabtree and others be used in conjunction with our assay.

In conclusion, we have developed assays that identify the members of the Cx. pipiens complex and other sibling species across several geographic regions worldwide, and that also detect introgression between Cx. pipiens and Cx. quinquefasciatus. The assays involve a single PCR per specimen, and our extensive population level examination for most of the species shows they consistently generate unique fragments easily resolved by electrophoresis on agarose gels. This methodology will be helpful to researchers and will aid vector control programs by facilitating the rapid and reliable identification of local mosquitoes.

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