

DEVELOPMENT OF A SPECIES-DIAGNOSTIC POLYMERASE CHAIN REACTION ASSAY FOR THE IDENTIFICATION OF *CULEX* VECTORS OF ST. LOUIS ENCEPHALITIS VIRUS BASED ON INTERSPECIES SEQUENCE VARIATION IN RIBOSOMAL DNA SPACERS

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Abstract. *Culex pipiens* complex mosquitoes (*Cx. p. pipiens* and *Cx. p. quinquefasciatus*) are among the principal vectors of St. Louis encephalitis (SLE) virus in the eastern United States; *Cx. restuans* and *Cx. salinarius* play secondary roles in the transmission and maintenance of the virus cycle. Accurate identification of these three species in field collections is required for epidemiologic studies of SLE virus transmission. We have developed a polymerase chain reaction (PCR) assay for this purpose. Species-specific PCR primers were designed based on interspecies nucleic acid sequence variation in the first and second internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA gene array; however, insufficient variation was detected to differentiate between subspecies of the *Cx. pipiens* complex. The primers were used together in a single amplification reaction to correctly identify specimens to species using genomic DNA extracted from whole individual mosquitoes, DNA from triturated mosquito pools, or crude DNA from mosquito heads or legs.

Accurate identification of field-collected *Culex* mosquitoes is essential for epidemiologic studies and control efforts related to the transmission of St. Louis encephalitis (SLE) virus. Members of the *Cx. pipiens* complex (*Cx. pipiens pipiens* and *Cx. pipiens quinquefasciatus*) are the primary vectors of SLE in the eastern and southcentral United States. *Culex restuans* and *Cx. salinarius* are secondary vectors that contribute to enzootic virus transmission in the midwest and southeastern United States; *Cx. salinarius* may also be involved in transmission to humans.^{1,2} The geographic ranges of these mosquito species overlap; therefore, a reliable method of species identification is needed. Although males can be distinguished as larvae and adults, adult females of these species are differentiated morphologically only by minor differences in scale patterns that are often rubbed off, due to trap collection or age, making identification impossible. In addition to morphologic methods, isoenzyme analysis has been used for identification of these *Culex* species; however, the technique can be time-consuming and expensive because multiple enzyme staining may be required for definitive results.^{3,4}

Recent studies have shown that interspecies nucleotide sequence variation in noncoding regions of highly repeated segments of genomic DNA, such as the nuclear ribosomal DNA (rDNA) gene group, can be useful in developing taxonomic tools to identify related species.⁵⁻⁷ Researchers have used interspecies sequence variation in the rDNA intergenic spacer (IGS) and second internal transcribed spacer (ITS2) to develop species-diagnostic polymerase chain reaction (PCR) assays for differentiating sibling species of *Anopheles* mosquitoes.^{8,9}

We have developed a species-specific PCR assay for the identification of the aforementioned *Culex* vectors of SLE virus. A region of the rDNA cistron, including the ITS1 and ITS2, from individuals belonging to several populations each of *Cx. p. pipiens*, *Cx. p. quinquefasciatus*, *Cx. restuans*, and *Cx. salinarius* was sequenced, and the sequences were com-

pared. Primers for the PCR were designed, based on species-specific differences, and were used in a diagnostic assay to identify individual mosquitoes or the contents of mosquito pools.

MATERIALS AND METHODS

Mosquitoes. Table 1 lists the taxa, place of collection, and source of the *Culex* mosquito populations used in this study. Most of the mosquitoes were field-collected specimens (Table 1). To ensure correct species identification, only male mosquitoes were used for PCR amplification, nucleic acid sequencing, and development of the diagnostic assay. Male terminalia were clipped, cleared, and individually mounted on a slide in a drop of cobol/phenol solution without a coverslip. Terminalia were identified to species using a standard reference.¹⁰ Individuals of the *Cx. pipiens* complex were identified to subspecies by means of DV/D ratios (DV is the extension of the ventral arm of the phallosome laterally of its intersection with the dorsal arm and D is the distance between the two intersections of the dorsal and ventral arms) and other characteristics.¹¹

Mosquito genomic DNA preparation. Mosquito genomic DNA was extracted as previously reported.¹² We used a modification of the procedure reported by Porter and Collins⁹ to prepare genomic DNA from mosquito heads and legs. The head or two to four legs were removed from each individual mosquito to be tested. Parts were ground in 20 μ l of ice-cold TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with microfuge pellet pestle grinders (Kontes, Vineland, NJ), incubated at 95°C for 10 min, microfuged for 2 min to clarify the suspension, and then held on ice until used.

Pooled mosquitoes were ground in BA-1 medium (1 \times M199-H, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g/l of sodium bicarbonate, 100 units/ml of penicillin, 100 μ g/ml of streptomycin, 1 μ g/ml of fungizone) with a mortar and pestle and clarified by microcentrifugation for 5

TABLE 1
Culex mosquito species used in this study

Species	Population origin	Specimen description*	Source†
<i>Cx. p. pipiens</i>	Colorado	Field	H. Savage and B. Apostol
	Illinois	Field	B. Steiny
	Massachusetts‡	Colony	A. Spielman
	Virginia	Field	J. Kertesz
	Sweden	Field	C. Dahl
<i>Cx. p. quinquefasciatus</i>	Arkansas	Field	H. Savage and G. Smith
	Florida	Colony	J. Seawright
	Louisiana	Field	J. Stennett
	Puerto Rico	Field	B. Apostol and P. Reiter
	Texas	Field	D. Sprenger
<i>Cx. restuans</i>	Belize	Field	H. Harlan
	Illinois	Field	B. Steiny
	Louisiana	Field	R. Nasci
<i>Cx. salinarius</i>	Maryland	Field	J. Dorothy
	Arkansas	Field	H. Savage and G. Smith
	Louisiana	Field	R. Nasci and C. Moore
	Ohio	Field	R. Nasci

* Field-collected or colonized (continuous) specimens; only adult-stage mosquitoes were used in this study.

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‡ *Culex p. pipiens* form *moletus*, autogenous population.

min at 4°C. Using the GeneClean II kit (Bio101, La Jolla, CA) with a single wash step, DNA was extracted from 100 µl of this suspension and eluted in 100 µl of double-distilled water.

Cloning and sequencing. A region of the rDNA gene group, including the 3' 181 nucleotides of the 18S gene, the ITS1, the 5.8S gene, the ITS2, and the 5' 45 nucleotides of the 28S gene, was amplified by PCR from mosquito genomic DNA with the 18SFHIN and CP16 primers listed in Table 2 (Figure 1). These primers contain sequences complementary to conserved 18S rDNA and 28S rDNA regions, respectively (Porter CH, unpublished data).

Each 25-µl amplification reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 0.1 mM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Indianapolis, IN), 13 ng each of the CP16 and CP17 primers, 0.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 1 pmole of mosquito genomic DNA. Reactions were overlaid with 50 µl of mineral oil and amplified in a Techne PHC 2 Dri-Block Cycler (Techne, Princeton, NJ) programmed for one cycle at 96°C for 4 min, 35 cycles at 96°C for 15 sec, 55°C for 30 sec, 72°C for 90 sec, and one cycle at 72°C for 4 min.

The single amplification product was isolated by agarose gel electrophoresis, reamplified to obtain sufficient quantities

of DNA, and prepared for blunt-end cloning with the Double GeneClean (Bio 101) method.¹³ The DNA was cloned into the plasmid pBluescript II (Stratagene, La Jolla, CA), and plasmids were transformed into *Escherichia coli* strain XL-1 Blue (Stratagene). Recombinant colonies were picked and grown overnight and plasmid DNA was extracted from 2-ml aliquots of the bacterial suspension using the Qiagen Plasmid Mini Kit (Qiagen Inc., Chatsworth, CA). Insert sizes were verified by restriction endonuclease digestion and agarose gel electrophoresis.

Two clones per individual mosquito were sequenced by the dideoxy method using the primers listed in Table 2 and the Sequenase version 2.0 sequencing kit (Amersham Corp., Arlington Heights, IL). Each clone was sequenced in both directions, and compressions were resolved with dITP.

Species diagnostic PCR. The nucleotide sequences of the rDNA PCR fragments were aligned using the Pileup multiple sequence alignment program in the Genetics Computer Group (GCG) analysis package, Version 7.3.¹⁴ Sequence differences between the species were then used as the basis for designing species-specific PCR primers. These primers were designed as forward primers to be paired with the 28S-complementary CP16 reverse primer. The primers were first tested alone on each of the species and then were mixed together and used in a single amplification reaction.

TABLE 2
 Polymerase chain reaction primers used in this study

Primer	Sequence (5' to 3')	Description
18SFHIN	GTAAGCTTCCTTTGTACACACCCGCCG	Complementary to 18S rDNA gene
CP16	GCGGGTACCATGCTTAAATTTAGGGGTA	Complementary to 28S rDNA gene
PQ10	CCTATGTCGGCTATACTA	<i>Culex pipiens</i> complex-specific
R6	CCAAACACCCGTACCCAA	<i>Cx. restuans</i> -specific
S20	TGAGAATACATACCCTGCT	<i>Cx. salinarius</i> -specific

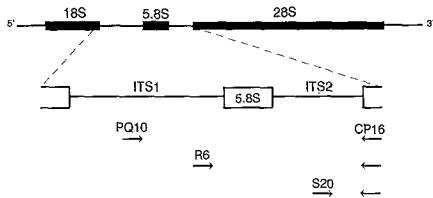


FIGURE 1. Diagram illustrating the rDNA gene group, the region of rDNA amplified and sequenced in this study, and the location and direction of primers used in the species identification assay. ITS = internal transcribed spacer.

The reaction mixture for the species identification PCR assay using purified, individual mosquito, genomic DNA was as described above. When crude DNA from mosquito heads or legs was amplified, 1 μ l of a 1:10 dilution of the clarified leg suspension was used in each reaction. When DNA from triturated mosquito pools was amplified, 5 μ l of the extracted DNA suspension was used per reaction.

The amplification program consisted of one cycle at 96°C for 4 min, 40 cycles at 96°C for 30 sec, 51°C for 30 sec, 72°C for 90 sec, and one cycle at 72°C for 4 min. Amplification reactions were electrophoresed on a 1.8% agarose gel in the presence of ethidium bromide. Amplification reactions using extracted genomic DNA from identified individuals of each species were included in every assay both as a positive control for the amplification system and to demonstrate the expected species-specific fragments. A reaction containing no template DNA was also included in each run as a negative control.

The diagnostic capabilities of the amplification assay were tested. Thirty male *Culex* mosquitoes were identified; several legs were removed from each. The legs were coded blind and were then tested in the diagnostic assay. Additionally, mosquito pools containing different numbers of mosquitoes and various mixtures of species were tested to define the limit of sensitivity of the assay when used on DNA from pools.

RESULTS

Ribosomal DNA from two individuals from each of the populations listed in Table 1 was amplified, cloned, and sequenced (Genbank accession numbers U22111–U22144). Nucleotide sequence differences were observed between clones from individual mosquitoes; therefore, two clones from each individual were sequenced. Additionally, intraspecies variation was observed. None of these differences were associated with specific populations, but rather were observed between individuals within a population as well as between clones from a single individual. The size of the amplified rDNA fragment ranged from 1,240 to 1,256 basepairs (bp) for the *Cx. p. pipiens* subspecies, 1,232–1,274 bp for *Cx. p. quinquefasciatus*, 1,125–1,143 bp for *Cx. restuans*, and 1,022–1,063 bp for *Cx. salinarius*.

The *Culex* species sequences were aligned to determine variability between the species. Few interspecies differences

were observed in the 18S, 5.8S, and 28S gene regions. Diversity in the spacers varied, with conserved regions interspersed with areas showing a high degree of heterology. Although sequence differences were observed between *Cx. p. pipiens* and *Cx. p. quinquefasciatus*, all of these sites varied between individuals within the subspecies and, therefore, none of them could be used as the basis for designing *Cx. p. pipiens*-specific or *Cx. p. quinquefasciatus*-specific amplification primers.

Amplification primers were designed for each species based on sequence differences among the species, conservation of sequence within the species, and the size of the amplified fragment. Primers were successfully constructed for identification of *Cx. restuans*, *Cx. salinarius*, and mosquitoes in the *Cx. pipiens* complex. These primers are listed in Table 2. The three forward primers, PQ10, R6, and S20, were designed to amplify a single DNA fragment each when paired with the CP16 reverse primer (Figure 1). The fragments amplified by these primers varied in size so that when used together in a single amplification reaction (four-primer reaction) the fragments could be easily separated and identified on an agarose gel. The four-primer reaction amplifies a 698-bp fragment from specimens in the *Cx. pipiens* complex, a 506-bp fragment from *Cx. restuans*, and a 175-bp fragment from *Cx. salinarius* (Figure 2). Occasionally, a double band was amplified from an individual. This was observed primarily with *Cx. salinarius* individuals and is probably the result of polymorphisms among copies of the rDNA array within an individual. Other minor bands that stained with low intensity may also occasionally be observed. These bands are most likely due to a mismatch pairing of primer and template, and they do not interfere with the accurate interpretation of species-specific bands. When DNA from other *Culex* species (*Cx. nigripalpus*, *Cx. territans*, and *Cx. erraticus*) was tested, no amplification product was observed.

We were able to use crudely prepared DNA from mosquito heads and legs in the assay to identify individual specimens (Figure 3). To test the accuracy of the assay, 30 mosquito specimens from the species listed in Table 1 were coded blind, and several legs were removed, processed, and identified to species using the four-primer reaction and agarose gel electrophoresis. One hundred percent of the samples were correctly identified.

Figure 3 also shows the results of the assay when used on DNA from mosquito pools. A pool size of 25 mosquitoes or less was optimal for detection of a single individual of a different species; however, the limit of detection was a single specimen of a different species in a pool of 40.

DISCUSSION

Polymerase chain reaction amplification techniques have been used to identify a variety of organisms and pathogens, and applying the PCR to the identification of field-collected mosquitoes can improve the accuracy and efficiency of vector surveillance studies. Often, when trap-caught adult mosquitoes are sorted for surveillance related to SLE virus transmission, a large group of specimens remain that can only be identified as *Culex* (*Culex*) species. However, due to the different roles played by *Culex* vectors in the transmission cy-



FIGURE 2. Fragments amplified from extracted genomic DNA using the four-primer species identification assay. Lane A, 123-basepair (bp) ladder size marker; lanes B–D, *Culex p. pipiens* individuals from Colorado, Virginia, and Illinois populations; lanes E–H, *Cx. p. quinquefasciatus* individuals from Texas, Florida, Louisiana, and Arkansas populations; lanes I–K, *Cx. restuans* individuals from Illinois, Maryland, and Louisiana populations; lanes L–N, *Cx. salinarius* individuals from Arkansas, Louisiana, and Ohio populations. Arrows indicate the 698-bp *Cx. pipiens* complex fragment, the 506-bp *Cx. restuans* fragment, and the 175-bp *Cx. salinarius* fragment.

cle of SLE virus, species identification of specimens is critical to an evaluation of surveillance data.

We have developed a PCR assay that allows morphologically similar SLE virus vector mosquito species to be identified. This assay is based on observed nucleotide sequence variation in the rDNA ITS1 and ITS2 regions of mosquito genomic DNA. The rDNA gene family consists of regions of highly conserved gene sequences that are flanked by transcribed and nontranscribed spacer regions that may contain variation that can be useful in differentiating between related species. Many copies of the rDNA gene array occur in the genome of an individual, making it an excellent target for PCR amplification from a small amount of genomic material.

Species-specific primers were designed, based on interspecies sequence variation in the ITS1 and ITS2. These

primers were used together in a four-primer amplification assay to identify *Cx. restuans*, *Cx. salinarius*, and *Cx. pipiens* complex mosquitoes. The assay can be performed on genomic DNA from pools of unidentifiable individuals or from small amounts of tissue, such as a head or legs, from an individual mosquito, allowing researchers to use the remainder of the insect for additional tests such as virus isolation.

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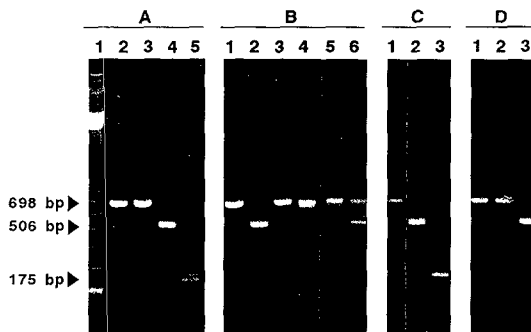


FIGURE 3. Fragments amplified using the four-primer species identification assay on A, extracted genomic DNA controls (lane 1, 123-basepair [bp] ladder; 2, *Culex p. pipiens*; 3, *Cx. p. quinquefasciatus*; 4, *Cx. restuans*; 5, *Cx. salinarius*); B, mosquito pool DNA (lane 1, 25 *Cx. p. pipiens*; 2, 25 *Cx. restuans*; 3, 25 *Cx. p. pipiens* and 1 *Cx. restuans*; 4, 25 *Cx. p. pipiens* and 1 *Cx. salinarius*; 5, 23 *Cx. p. quinquefasciatus* and 1 each of *Cx. restuans* and *Cx. salinarius*; 6, 10 each of *Cx. p. pipiens*, *Cx. p. quinquefasciatus*, and *Cx. restuans*); C, mosquito head DNA (lane 1, *Cx. p. pipiens*; 2, *Cx. restuans*; 3, *Cx. salinarius*); and D, mosquito leg DNA (lane 1, *Cx. p. pipiens*; 2, *Cx. p. quinquefasciatus*; 3, *Cx. restuans*). Arrows indicate the 698-bp *Cx. pipiens* complex fragment, the 506-bp *Cx. restuans* fragment, and the 175-bp *Cx. salinarius* fragment.

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