POLYMERASE CHAIN REACTION ASSAY IDENTIFIES CULEX NIGRIPALPUS: PART OF AN ASSAY FOR MOLECULAR IDENTIFICATION OF THE COMMON CULEX (CULEX) MOSQUITOES OF THE EASTERN UNITED STATES

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ABSTRACT. Nucleotide sequence information on internal transcribed spacer (ITS) 1 and ITS 2 regions of the nuclear ribosomal DNA multigene family was used to develop a polymerase chain reaction assay that identifies Culex nigripalpus Theobald. The assay uses species-specific forward and reverse primers for Cx. nigripalpus and can be used along with previously described primers to distinguish among 4 common taxa of Culex (Culex) of the eastern USA with a single thermal cycler program. The assay distinguishes among the 4 taxa Cx. nigripalpus, Cx. restuans Theobald, Cx. salinarius Coquillett, and members of the Cx. pipiens Linnaeus complex. This assay may be used to verify the morphological identification of individual specimens of Culex or to confirm the species composition of mosquito pools.

KEY WORDS Culex (Culex), Culex nigripalpus, internal transcribed spacers, ribosomal DNA, molecular species identification

INTRODUCTION

In North America, mosquitoes of the subgenus Culex (Culex) are the primary enzootic vectors of West Nile virus (Nasci et al. 2001b) and the primary enzootic and epidemic vectors of St. Louis encephalitis virus (Mitchell et al. 1980). Adult females of these mosquitoes are frequently collected in gravid traps (Reiter 1983), CO₂-baited Centers for Disease Control light traps, or with aspirators as part of arbovirus surveillance programs (Nasci et al. 2001a), blood host studies (Apperson et al. 2002), or other mosquito investigations. Morphological characters are used to identify specimens and sort field-collected mosquitoes into species pools. Mosquito pools can be tested for virus as part of surveillance programs and to assess the vector status of particular species. However, adult females of some Culex common in the eastern USA (Cx. pipiens Linnaeus, Cx. quinquefasciatus Say, Cx. nigripalpus Theobald, Cx. restuans Theobald, and Cx. salinarius Coquillett) are difficult to distinguish morphologically if specimens are worn or damaged during the trapping process. The correct identification of mosquito species is essential in determining the roles of vector species and for the development of effective arbovirus control and prevention strategies.

Polymerase chain reaction (PCR)–based assays that use species-specific primers based on differences in ribosomal DNA (rDNA) nucleotide sequence are useful in distinguishing among closely related anopheline (Paskewitz and Collins 1990, Porter and Collins 1991, Scott et al. 1993, Townsend and Onapa 1994, Cornel et al. 1996) and culicine (Crabtree et al. 1995, Toma et al. 2000) mosquito taxa. Ribosomal DNA makes an ideal template for species-diagnostic PCR for at least 2 important reasons. First, the high copy number of rDNA arrays within the adult mosquito genome (in Aedes aegyp-ti, see Park and Fallon [1990]) means that a small fraction of extracted DNA supplies a sufficient template for PCR. Second, rDNA contains highly conserved coding regions, ideal as annealing sites for sequencing primers, separated by less conserved spacers, which are the source of interspecific sequence variation.

Crabtree et al. (1995) describe a PCR assay for the identification of Cx. restuans, Cx. salinarius, and members of the Cx. pipiens complex. This assay makes use of interspecific nucleotide sequence differences in the noncoding internal transcribed spacers (ITS) 1 and ITS 2 of rDNA. Different PCR primers anneal to taxon-specific ITS sequences, producing a uniquely sized amplicon in the presence of the appropriate template DNA. Subtractive hybridization was used to design primers that would further distinguish among the sibling taxa Cx. pipiens and Cx. quinquefasciatus (Crabtree et al. 1997); however, only Cx. pipiens–specific primers could be designed. Recently, a PCR assay that identifies Cx. pipiens, Cx. quinquefasciatus, and their F₁ hybrids based on nucleotide sequence differences in the acetylcholinesterase gene Ace.2 has been developed (Aspen and Savage, personal communication).

The appearance of West Nile virus (WN) in Florida and the isolation of WN from Cx. nigripalpus during the summer of 2001 (CDC 2002) imposed the need to add Cx. nigripalpus to the list of taxa that can be distinguished by the assay of Crabtree et al. (1995). Within the eastern USA, Cx. nigripalpus is found in the southernmost latitudes, where it may be collected along with Cx. restuans, Cx. quinquefasciatus, and the morphologically similar species Cx. salinarius. For this study, the ITS regions of Cx. nigripalpus were sequenced and compared with the ITS sequences from 7 other taxa.
of *Culex* (*Culex*). Based upon the results of this comparison, species-specific PCR primers were designed for the identification of *Cx. nigripalpus*.

**MATERIALS AND METHODS**

*Mosquitoes:* The geographic origin and source of each mosquito population used for this study are given in Table 1.

*Mosquito genomic DNA preparation:* Mosquito genomic DNA was extracted by using the Qiagen DNeasy Tissue Kit (Qiagen Inc., St. Clarita, CA) with the following modifications. Individual or pooled mosquitoes were ground in BA-1 medium (1X M199-H, 0.05 M Tris, pH 7.6, 1% bovine serum albumin, 0.35 g/liter of sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 μg/ml of fungizone). The DNA was eluted twice in 200 μl of warm (40°C) nuclease-free water (Amresco, Solon, OH) for a total eluate volume of 400 μl. The DNA concentration was determined by using the ultraviolet absorbance at 260-nm wavelength on a spectrophotometer.

*Cloning and sequencing:* A region of *Cx. nigripalpus* rDNA (including part of the 18S gene, the ITS 1, the 5.8S gene, the ITS 2, and part of the 28S gene) was amplified as described by Crabtree et al. (1995). The PCR product was purified by using the Qiaquick PCR Purification Kit (Qiagen) and ligated into the plasmid pGEM-T Easy (Stratagene, La Jolla, CA) before transforming the *Escherichia coli* strain XL-1 Blue (Stratagene) by heat shock. Recombinant clones were selected on agar plates with tetracycline and ampicillin. Clones were sequenced with 3 different primers in each direction (the 2 PCR primers, 2 plasmid primers, and 2 primers complementary to the 5.8S gene) in an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA), by using the BigDye Terminator version 3 reaction mix (Applied Biosystems).

*Species-specific PCR primer design:* The rDNA nucleotide sequences of *Cx. nigripalpus* from the Jefferson County, FL, and Vero Beach, FL, specimens were aligned with rDNA sequences from 49 clones from 7 other taxa of *Culex* by using PILEUP in the Wisconsin Package, ver. 70.2 (Devereux et al. 1984). Other taxa included in the alignment were *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. pipiens-Cx. quinquefasciatus* hybrids, *Cx. restuans*, *Cx. salinarius*, *Cx. tarsalis* Coq. and *Cx. erythrothorax* Dyar (Genbank acquisition numbers U22114-U22144, U33018, U33022-U33024, U33030-U33032). A pair of PCR primers complementary to *Cx. nigripalpus*—specific sequences in ITS 1 (primer N901) and ITS 2 (primer NR1080) was designed by using the PrimerSelect module of Lasergene version 5.03 (DNAStar, Inc., Madison, WI). The nucleotide sequences of these primers are included in Table 2.

*Species-diagnostic PCR:* The species-diagnostic PCR described by Crabtree et al. (1995) was modified as follows. Separate reaction mixtures, containing 1 bp, base pairs.
containing different species-specific primers (with sequences listed in Table 2), were prepared for each taxon to be identified. Each 25-μl reaction mixture contained 1X GeneAmp PCR Buffer I (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% weight:volume gelatin, Applied Biosystems, Foster City, CA), 0.2 mM of each deoxynucleotide triphosphate (Roche Diagnostics Corp., Indianapolis, IN), 80 nM of each appropriate primer (see below), 1.25 ng of template DNA, and 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems). The reaction mix for identifying *C. nigripalpus* contained the forward-sense primer N901 and the reverse-sense primer NR1080. Reaction mixes for identifying *Cx. restuans*, *Cx. salinarius*, or members of the *Cx. pipiens* complex contained the reverse-sense consensus primer CP16 and the respective forward-sense species-specific primer (see Table 2). The reactions were placed in a PTC-100 thermal cycler (MJ Research, Inc., Incline Village, NV) programmed for 1 cycle at 96°C for 4 min, followed by 40 cycles of 96°C for 30 sec, 51°C for 30 sec, and 72°C for 90 sec, and completed by 1 cycle at 72°C for 4 min. The PCR products were visualized with ethidium bromide on a 1.8% agarose gel.

**RESULTS**

A region of *Cx. nigripalpus* ribosomal DNA was amplified, cloned, and sequenced. The sequences were deposited into Genbank. Three clones were sequenced from a single adult female specimen from Jefferson County, FL (Genbank accession numbers AF520974, AF521662, and AF521663), and 1 clone was sequenced from a single adult female specimen from Vero Beach, FL (Genbank accession number AF521664).

*Culex nigripalpus*-specific PCR primers N901 and NR1080 were tested on a range of DNA templates from *Culex* mosquitoes. When DNA from any of the 3 *Cx. nigripalpus* populations indicated in Table 1 was used as the template, a 404-base-pair (bp) amplicon resulted (Fig. 1). When DNA from each of the non-*Cx. nigripalpus* populations in Table 1 was used as the template, no amplifica-
tion resulted (Fig. 1). When DNA from mixed-species pools containing a fraction of *Cx. nigripalpus* specimens was used as the template, the *Cx. nigripalpus* DNA was detected, as indicated by the presence of a 404-bp amplicon (Fig. 2). The DNA from a single *Cx. nigripalpus* specimen in a pool of 40 mosquitoes was detectable (Fig. 2).

When primers N901 and CP16 were used together, a 505-bp amplicon resulted from both *Cx. nigripalpus* and *Cx. tarsalis* (results not shown). Although these 2 species are morphologically distinct, alignment of their ITS 1 nucleotide sequences revealed sufficient sequence similarity to explain the cross reaction. Primer NR1080 is based on a *Cx. nigripalpus* ITS 2 sequence that is not found in *Cx. tarsalis*. Use of the primer pair N901 and NR1080 eliminates the cross-reaction with *Cx. tarsalis* DNA (Fig. 1).

The *Cx. nigripalpus*-specific primers are compatible with a modification of the protocol of Crabtree et al. (1995), thus allowing the identification of *Cx. nigripalpus*, *Cx. restuans*, *Cx. salinarius*, and members of the *Cx. pipiens* complex with a single thermal cycler program. A 404-bp amplicon resulting from a reaction containing N901 and NR1080 is diagnostic of *Cx. nigripalpus*, a 506-bp amplicon resulting from a reaction containing R6 and CP16 is diagnostic of *Cx. restuans*, a 175-bp amplicon resulting from a reaction containing S20 and CP16 is diagnostic of *Cx. salinarius*, and a
698-bp amplicon resulting from a reaction containing PQ10 and CP16 is diagnostic of members of the Cx. pipiens complex. When DNA from a mosquito pool containing all 5 taxa was tested (Fig. 2), each of the diagnostic amplicons was observed. When DNA from each of the Cx. nigripalpus populations was tested with each of the non-Cx. nigripalpus-specific primers, no amplicon was observed (not shown), indicating that no cross-reactions occur that could lead to false-positive identification.

**DISCUSSION**

Accurate identification of field-collected mosquitoes is an important step in arbovirus surveillance, vector incrimination, blood host preference investigations and other mosquito studies. However, many species of *Culex* (*Culex*) are difficult to distinguish from one another by using morphological characters. Difficulty with the identification process can lead to some mosquito pools being mislabeled or some pools containing multiple species and labeled as *Culex* spp. The species composition of morphologically identified mosquito pools or individuals can be verified by using a PCR assay with species-specific primers. We have designed a PCR primer pair that accurately identifies Cx. nigripalpus. These primers distinguish Cx. nigripalpus from all specimens of *Culex* tested, including the morphologically similar species Cx. salinarius, and the morphologically distinct species Cx. tarsalis, a species which shares much ITS nucleotide sequence homology with Cx. nigripalpus. Species identification can be determined by the PCR assay even after specimens have been damaged or ground. A single specimen of Cx. nigripalpus in a pool of 40 (containing 39 other specimens of *Culex*) mosquitoes can be identified. This sensitivity allows arbovirus investigators to sort *Culex* mosquitoes into pools as large as 40 specimens that can be ground and tested for virus before molecular identification of the mosquito species is undertaken.

The primers for Cx. nigripalpus, in conjunction with a modified protocol based on the work of Crabtree et al. (1995) allow the identification of Cx. nigripalpus, Cx. restuans, Cx. salinarius, and members of the Cx. pipiens complex with a single thermal cycler program. This provides a means of verifying the morphological identification of *Culex* commonly collected in the eastern USA, and the presence of *Culex* species in virus-positive mosquito pools. The modified protocol described here separates each primer pair into a different reaction tube and avoids a loss of sensitivity caused by primer competition that can occur in a mixed primer or cocktail assay. In the future, it would be beneficial to develop a quantitative PCR assay that could determine the number of specimens of each species in a pool.

This assay does not distinguish among the members of the Cx. pipiens complex: Cx. pipiens, Cx. quinquefasciatus, and their hybrids. Nucleotide sequence comparison among members of the Cx. pipiens complex reveals insufficient fixed differences in the ITS 1 and ITS 2 regions to allow PCR primers to be designed that will distinguish among these mosquitoes. Recently, a PCR assay that identifies Cx. pipiens, Cx. quinquefasciatus, and their F, hybrids based on nucleotide sequence differences in the acetylcholinesterase gene *Ace.2* has been developed (Aspen and Savage, personal communication). Just as this research was spawned by the discovery of WN in Florida, the continuing spread of WN into different ecosystems may impose the need for primers that identify additional species. Polymerase chain reaction primers specific to other mosquito species likely can be designed by using techniques described here.

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