*Journal of the American Mosquito Control Association,* 19(2):115–120, 2003


# 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

STEPHEN ASPEN, MARY B. CRABTREE AND HARRY M. SAVAGE

*Division of Vector-Borne Infectious Disease, Centers for Disease Control and Prevention, Fort Collins, CO 80522*

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), $CO_2$-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, Townson 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 aegypti,* 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 $F_1$ 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

Table 1. Populations of *Culex* used in this study.

| Taxa | Geographic origin | Source |
|---|---|---|
| *Culex pipiens* complex | Alleghany County, NC | B. Harrison |
| | Los Angeles County, CA | M. Madden |
| *Cx. pipiens* | Ft. Collins, CO | H. Savage |
| | Queens, NY | C. Apperson |
| *Cx. quinquefasciatus* | Monroe, LA | H. Savage |
| | Jefferson County, FL | M. Godsey |
| *Cx. nigripalpus* | Jefferson County, FL | M. Godsey |
| | Vero Beach, FL | R. Rutledge |
| | Gainesville, FL | J. Reinert |
| *Cx. restuans* | Burlington, VT | H. Savage |
| | Alleghany County, NC | B. Harrison |
| *Cx. salinarius* | Monroe, LA | H. Savage |
| | Jefferson County, FL | M. Godsey |
| *Cx. tarsalis* | Los Angeles County, CA | M. Madden |

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. 10.2 (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, con-

Table 2. Polymerase chain reaction primers, complementary to *Culex* rDNA, used for molecular identification.

| Primer | Sequence (5′ to 3′) | Specificity | Sense | Pairs with | Amplicon size (bp[1]) |
|---|---|---|---|---|---|
| CP16 | GCGGGTACCATGCTTAAATTTAGGGGGTA | Consensus | Reverse | See below | |
| PQ10 | CCTATGTCCGCGTATACTA | *Culex pipiens* complex | Forward | CP16 | 698 |
| R6 | CCAAACACCGGTACCCAA | *Cx. restuans* | Forward | CP16 | 506 |
| S20 | TGAGAATACATACCACTGCT | *Cx. salinarius* | Forward | CP16 | 175 |
| N901 | ATACCCATGCGAAAGCATAC | *Cx. nigripalpus* | Forward | NR1080 | 404 |
| NR1080 | GTACCGCGACCACACGACTT | *Cx. nigripalpus* | Reverse | N901 | 404 |

[1] bp, base pairs.

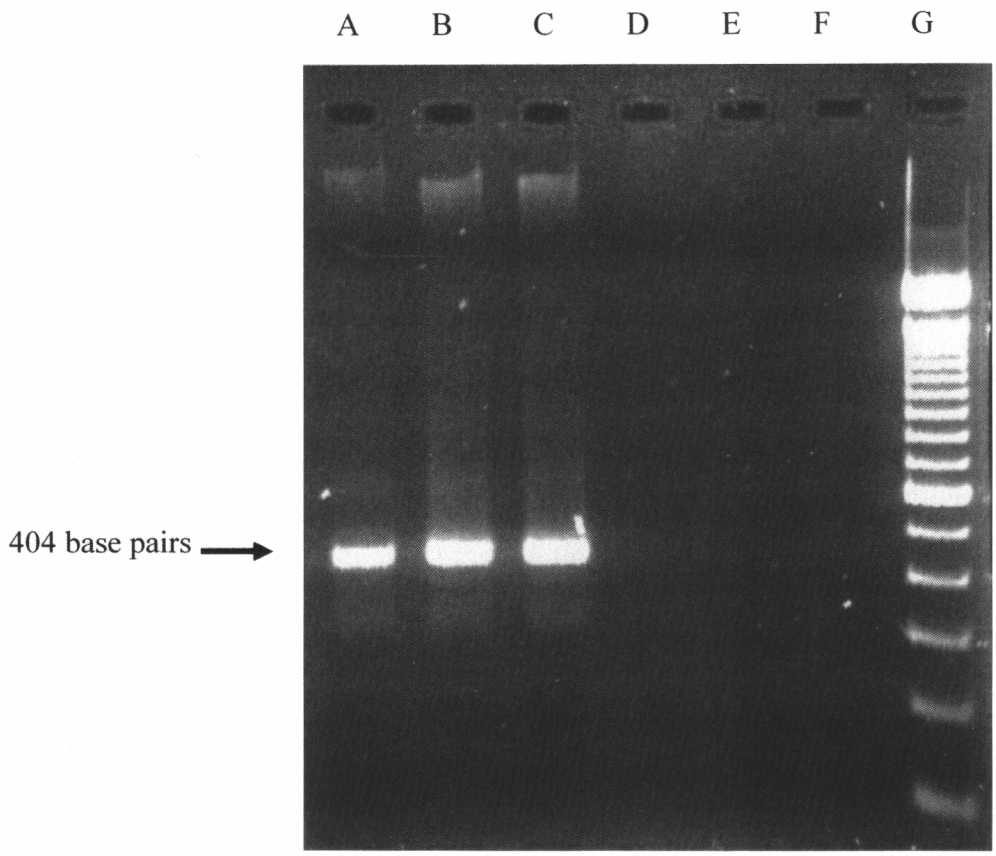


Fig. 1. Amplicons from polymerase chain reaction with primer pair N901 and NR1080. Reactions (A)–(F) contained template DNA from a single specimen of the following taxa and geographic origin: (A) *Cx. nigripalpus* (Jefferson County, FL); (B) *Cx. nigripalpus* (Vero Beach, FL); (C) *Cx. nigripalpus* (Gainesville, FL); (D) *Cx. tarsalis* (Los Angeles County, CA); (E) *Cx. salinarius* (Monroe, LA); (F) *Cx. salinarius* (Jefferson County, FL). Lane (G) contains a 100–base pair DNA marker.

taining 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 $MgCl_2$, 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 *Cx. 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 acquisition numbers AF520974, AF521662, and AF521663), and 1 clone was sequenced from a single adult female specimen from Vero Beach, FL (Genbank acquisition 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-

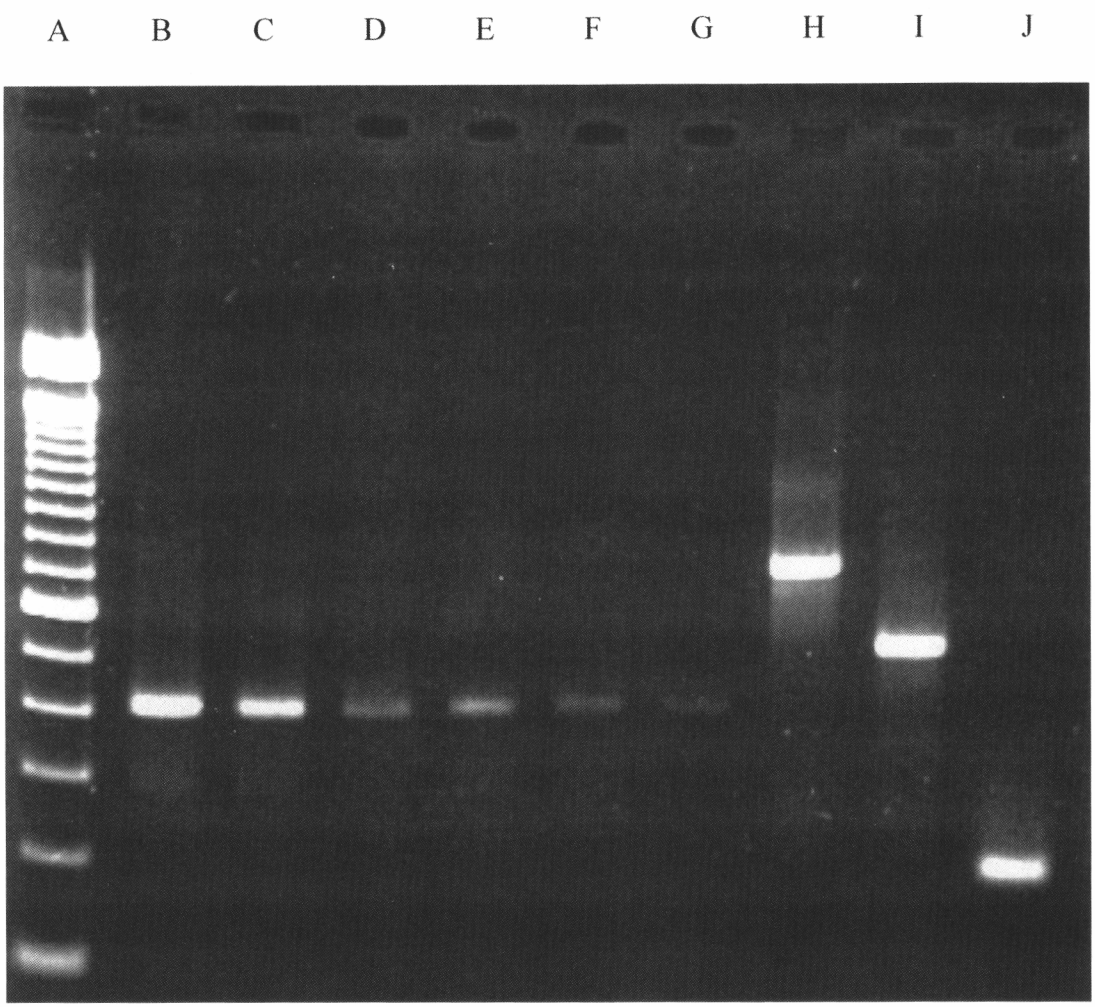


Fig. 2. Amplicons from polymerase chain reaction with DNA from mixed-species pools as the template. (A) DNA 100–base pair marker. Reactions (B)–(G) contained the *Culex nigripalpus*–specific primers and DNA from a pool with the following species composition: (B) 1 *Cx. nigripalpus*; (C) 1 *Cx. nigripalpus* and 1 *Cx. restuans*; (D) 1 *Cx. nigripalpus* and 3 *Cx. salinarius*; (E) 1 *Cx. nigripalpus,* 1 *Cx. pipiens,* 2 *Cx. quinquefasciatus,* 2 *Cx. restuans,* and 2 *Cx. salinarius*; (F) 1 *Cx. nigripalpus* and 19 *Cx. quinquefasciatus*; (G) 1 *Cx. nigripalpus* and 39 *Cx. quinquefasciatus.* Reactions (H)–(J) contained the same template DNA used in reaction (E), with the following primers: (H) *Cx. pipiens* complex–specific; (I) *Cx. restuans*–specific; (J) *Cx. salinarius*–specific.

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 $F_1$ 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.

## ACKNOWLEDGMENTS

We thank C. Apperson, North Carolina State University, Raleigh, NC; M. Godsey, Centers for Disease Control and Prevention, Ft. Collins, CO; B. Harrison, North Carolina Department of Environment and Natural Resources, Winston–Salem, NC; M. Madden, Greater Los Angeles County Vector Control District, Sante Fe Springs, CA; J. Reinert, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, FL; and R. Rutledge, Florida Medical Entomology Lab, Vero Beach, FL for providing mosquito specimens.

## REFERENCES CITED

Apperson CS, Harrison BA, Unnasch TR, Hassan HK, Irby WS, Savage HM, Aspen SE, Watson DW, Rueda LM, Engber BR, Nasci RS. 2002. Host-feeding habits of *Culex* and other mosquitoes (Diptera: Culicidae) in the borough of Queens in New York City, with characters and techniques for identification of *Culex* mosquitoes. *J Med Entomol* 39:777–785.

CDC [Centers for Disease Control]. 2002. West Nile virus activity—United States, 2001. *Morb Mortal Wkly Rep* 51(23):497–501.

Cornel AJ, Porter CH, Collins FH. 1996. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *J Med Entomol* 33:109–116.

Crabtree MB, Savage HM, Miller BR. 1995. 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. *Am J Trop Med Hyg* 53: 105–109.

Crabtree MB, Savage HM, Miller BR. 1997. Development of a polymerase chain reaction assay for differentiation between *Culex pipiens pipiens* and *Cx. pipiens quinquefasciatus* (Diptera: Culicidae) in North America based on genomic differences identified by subtractive hybridization. *J Med Entomol* 34:532–537.

Devereux J, Haeberli P, Smithies O. 1984. A comprehen-

sive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12:387–395.

Mitchell CJ, Francy DB, Monath TP. 1980. Arthropod vectors. In: Monath TP, ed. *St. Louis encephalitis* Washington, DC: American Public Health Association. p 313–379.

Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, Kerst AJ, Bennett P, Gottfried K, Lanciotti RS. 2001a. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. *Emerg Infect Dis* 7: 742–744.

Nasci RS, White DJ, Stirling H, Oliver JA, Daniels TJ, Falco RC, Campbell S, Crans WJ, Savage HM, Lanciotti RS, Moore CG, Godsey MS, Gottfried KL, Mitchell CJ. 2001b. West Nile virus isolates from mosquitoes in New York and New Jersey, 1999. *Emerg Infect Dis* 7:626–630.

Park YJ, Fallon AM. 1990. Mosquito ribosomal RNA genes: characterization of gene structure and evidence for changes in copy number during development. *Insect Biochem* 20:1–11.

Paskewitz SM, Collins FH. 1990. Use of the polymerase chain reaction to identify mosquito species of the *Anopheles gambiae* complex. *Med Vet Entomol* 4:367–373.

Porter CH, Collins FH. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *Am J Trop Med Hyg* 45:271–279.

Reiter P. 1983. A portable, battery-powered trap for collecting gravid *Culex* mosquitoes. *Mosq News* 43:496–498.

Scott JA, Brogdon WG, Collins FH. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg* 49:520–529.

Toma T, Miyagi I, Crabtree MB, Miller BR. 2000. Identification of *Culex vishnui* subgroup (Diptera: Culicidae) mosquitoes from the Ryukyu Archipelago, Japan: development of a species-diagnostic polymerase chain reaction assay based on sequence variation in ribosomal DNA spacers. *J Med Entomol* 37:554–558.

Townson H, Onapa AW. 1994. Identification by rDNA-PCR of *Anopheles bwambae*, a geothermal spring species of the *An. gambiae* complex. *Insect Mol Biol* 3: 279–282.