Development of a Multiplexed Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Assay to Identify Common Members of the Subgenera *Culex* (*Culex*) and *Culex* (*Phenacomyia*) in Guatemala

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Abstract. Morphological differentiation of mosquitoes in the subgenera *Culex* (*Culex*) and *Culex* (*Phenacomyia*) in Guatemala is difficult, with reliable identification ensured only through examination of larval skins from individually reared specimens and associated male genitalia. We developed a multiplexed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to identify common *Cx.* (*Cux.*) and *Cx.* (*Phc.*) *Culex* (*Cux.*) *chidesteri*, *Cx.* (*Cux.*) *coronator*, *Cx.* (*Cux.*) *interrogator*, *Cx.* (*Cux.*) *quinquefasciatus*, *Cx.* (*Cux.*) *nigripalpus*/*Cx.* (*Cux.*) *thriambus*, and *Cx.* (*Phc.*) *lactator* were identified directly with a multiplexed primer cocktail comprising a conserved forward primer and specific reverse primers targeting ribosomal DNA (rDNA). *Culex nigripalpus* and *Cx. thriambus* were differentiated by restriction digest of homologous amplicons. The assay was developed and optimized using well-characterized specimens from Guatemala and the United States and field tested with unknown material from Guatemala. This assay will be a valuable tool for mosquito identification in entomological and arbovirus ecology studies in Guatemala.

INTRODUCTION

Several arboviruses of public health importance circulate in Guatemala, including Eastern equine encephalitis virus, Venezuelan equine encephalitis virus (VEE), Ilheus virus, St. Louis encephalitis virus (SLE), West Nile virus (WNV), and Dengue virus.^{1–5} Of these viruses, SLE and VEE have been isolated from *Cx.* (*Culex*) *nigripalpus* Theobald,^{4,6} and WNV has been isolated from *Cx.* (*Cux.*) *quinquefasciatus* Say and *Cx.* (*Cux.*) *chidesteri* Dyar in Guatemala (Morales-Betoulle and others, unpublished data) and detected in *Cx.* (*Cux.*) *nigripalpus* and *Cx.* (*Cux.*) *interrogator* Dyar and Knab in Chiapas, Mexico.⁷ Additional WNV isolates in Guatemala were obtained from *Cx.* (*Cux.*) mosquito pools of uncertain species identification (Morales-Betoulle and others, unpublished data). Patois virus (Bunyaviridae) has also been isolated from *Cx.* (*Cux.*) *thriambus* Dyar in Mexico.⁸ Identification of actual and potential arbovirus vectors in this subgenus is a major challenge to arbovirus ecology studies in this tropical ecosystem.

Currently, 16 species in the subgenus *Culex* (*Culex*) and two species in the subgenus *Culex* (*Phenacomyia*) are known to occur in Guatemala.⁹ Accurate morphological differentiation of these species is extremely difficult, requiring careful examination of larval skins from reared specimens and associated male genitalia. Identification of adult females is further complicated by overlapping morphological variation and by the lack of a current and comprehensive morphological key. *Culex* (*Cux.*) *pseudostigmatosoma* Strickman, *Cx.* (*Cux.*) *res-tuans* Theobald, and *Cx.* (*Phc.*) *lactator* Dyar and Knab were reported from Guatemala by Strickman and others^{11–13} after the development of the morphological key by Clark-Gil and Darsie.¹⁰ Additionally, confusion has arisen regarding the identification and nomenclature of some species. Strickman¹⁴ discovered that the holotype specimen of *Cx. peus* Speiser was conspecific with *Cx.* (*Cux.*) *thriambus*. He correctly placed

Cx. thriambus as a junior synonym of *Cx. peus*, and restored *Cx.* (*Cux.*) *stigmatosoma* Dyar as the valid name for the species previously known as *Cx. peus*. However, because these changes had considerable potential for confusion among mosquito workers, the name *Cx. peus* was subsequently suppressed and *Cx. thriambus* was retained in its traditional usage.¹⁵ *Culex lactator* was elevated from synonymy with *Cx. corniger* Theobald and recognized as a distinct species.¹² Harbach and Peyton¹⁶ noted the unusual morphological characters of these two species and transferred them to the new subgenus *Phenacomyia*. Finally, no reliable morphological characters have yet been identified to differentiate between adult female specimens of *Cx.* (*Cux.*) *mollis* Dyar and Knab and *Cx.* (*Cux.*) *declarator* Dyar and Knab, or among adult female specimens of the *Cx.* (*Cux.*) *coronator* complex: *Cx.* (*Cux.*) *coronator* Dyar and Knab, *Cx.* (*Cux.*) *ousqua* Dyar, and *Cx.* (*Cux.*) *usquatus* Dyar.¹⁰ A molecular diagnostic that could be used to confirm the identity of morphologically similar species and provide the identity of unknown and damaged specimens would be a great asset to overcoming these difficulties with morphological identification.

Molecular diagnostics designed from ribosomal DNA (rDNA) gene arrays have long been used for the reliable separation of closely-related and/or morphologically indistinguishable mosquito species.^{17–21} The rDNA gene array contains three coding regions, 18S, 5.8S, and 28S, separated by two polymorphic internal transcribed spacer regions, ITS1 and ITS2.²² The organization of these conserved coding regions juxtaposed with ITS regions characteristically high in inter-specific sequence variation lends itself well to the design of multiplexed polymerase chain reaction (PCR) assays, as a conserved primer can be anchored in coding regions (18S, 5.8S, and 28S), and species-specific primers can be placed in either polymorphic region, ITS1 or ITS2. Tandem repeats of the rDNA gene array also permit the robust amplification of these multi-copy regions of DNA from minimal starting material, such as mosquito legs.²² Therefore, we designed multiplexed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays from novel rDNA sequences generated from common *Culex* mosquitoes in Guatemala. This diagnostic will be a valuable tool for confirming *Culex* (*Cux.*) and *Culex* (*Phc.*) mosquito species identification for

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entomological studies and arbovirus detection and surveillance efforts in Guatemala.

MATERIALS AND METHODS

Mosquito collection and handling. Larval mosquitoes were collected at various sites in Guatemala during June 2004 and May 2006. At each site an ecological description form was completed and Global Positioning System (GPS) coordinates recorded. Larvae were individually reared to the adult stage and associated life stages assigned a unique number. Larvae and pupae skins were held in 70% ethanol until slide mounted at the Centers for Disease Control and Prevention (CDC). Adult specimens were either pinned for morphological study, or placed in cryotubes and held on dry ice or at -70°C , or placed in 95–100% ethanol for future molecular analyses. Genitalia from selected male adult specimens were slide mounted in copal-phenol. The remaining carcass or legs from adult voucher specimens were available for DNA extraction and sequencing, assay optimization, or for use as controls (Table 1). Specimens were identified morphologically using both larval skins and male genitalia.^{10,23–25} Voucher slides are deposited in the CDC collections, Fort Collins, CO.

Additional larval specimens from the United States were collected, reared, and associated stages processed as described previously. In Chicago, IL egg rafts were collected, individually tubed, and shipped to CDC in Fort Collins, where larvae from individual rafts were reared separately to the fourth instar. Fourth instars were identified to species²⁶ and mass reared to the adult stage for use as controls.

To field test the assay, adult female mosquitoes were collected in Puerto Barrios ($\text{N}15^{\circ}43'$, $\text{W}88^{\circ}35'$), Guatemala in July and December 2007. Mosquitoes were aspirated from plastic artificial resting containers and ground vegetation using a Modified CDC Backpack Aspirator model 1412 (John W. Hock Company, Gainesville, FL) and stored in tubes with silica gel desiccant until molecular processing.²⁷ Preliminary morphological identifications were performed using the key of Clark-Gil and Darsie.¹⁰

DNA preparation. Adult mosquito bodies from voucher specimens were triturated individually in 180–200 μL of phosphate-buffered saline (PBS). The full homogenate was used for DNA extraction using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) following the protocol designed for extraction of DNA from insects. Extracted DNA was eluted from the membrane twice in 200 μL of nuclease-free water (Amresco, Solon, OH) and combined for a total eluate volume of 400 μL . The DNA was extracted from legs of some male voucher specimens by ultrasonication for 5 minutes in 40 μL 1X TE buffer. Unknown field samples were extracted by hand using a standard salt extraction.²⁸

Internal transcribed spacer region PCR. An approximately 1-kb region of rDNA gene array including both internal transcribed spacer regions was amplified from voucher specimens for cloning and sequencing.^{18,29} A 100 μL reaction was prepared with 1X of Gene Amp PCR Buffer I (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl_2 , 0.01% wt:vol gelatin) (Applied Biosystems, Foster City, CA), 1 mM of dNTP mix (Roche Diagnostics, Indianapolis, IN), 120 nM of each primer, 2.5 U of Amplitaq DNA polymerase (Applied

TABLE 1
Culex specimens used to develop and optimize the molecular assay

Species	Specimen no.	Gen Bank no.	Collection site	Collectors
I. Specimens sequenced to develop assay				
<i>Cx. (Cux.) chidesteri</i>	GU 2006-39-25	GU477612	GU: Peten Dept.	S,W,S,C,S*
<i>Cx. (Cux.) chidesteri</i>	GU 2006-39-26	GU562344	GU: Petern Dept.	S,W,S,C,S
<i>Cx. (Cux.) coronator</i>	MS 2004-01-01	GU562346	MS: Copiah Co.	W. Vamado, J. Goddard
<i>Cx. (Cux.) coronator</i>	MS 2004-01-03	GU562347	MS: Copiah Co.	W. Vamado, J. Goddard
<i>Cx. (Cux.) interrogator</i>	GU 2004-72-05	GU562345	GU: Izabal Dept.	S,B,A,G†
<i>Cx. (Cux.) interrogator</i>	GU 2006-23-05	GU562870	GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) nigripalpus</i>	FL 2005-06-28	GU562871	FL: Miami-Dade Co.	M. Williams
<i>Cx. (Cux.) quinquefasciatus</i>	CA 2004-02-24	GU562872	CA: San Diego Co.	S. Deus
<i>Cx. (Cux.) thriambus</i>	TX 2003-03-208	GU562873-GU562874	TX: Brewster Co.	H. Savage, K. Bennett
<i>Cx. (Phc.) lactator</i>	GU 2006-30-01	GU562875	GU: Peten Dept.	S,W,S,C,S
II. Specimens used to optimize assay				
<i>Cx. (Cux.) chidesteri</i>	GU 2004-80-31		GU: Izabal Dept.	S,B,A,G
<i>Cx. (Cux.) coronator</i>	GU 2004-33-12		GU: Suchitepequez Dept.	S,B,A,G
<i>Cx. (Cux.) declarator</i>	GU 2006-18-01		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) declarator</i>	GU 2004-69-25		GU: Izabal Dept.	S,B,A,G
<i>Cx. (Cux.) declarator</i>	GU 2006-47-06		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) declarator</i>	GU 2004-70-24		GU: Izabal Dept.	S,B,A,G
<i>Cx. (Cux.) interrogator</i>	GU 2006-01-04		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) mollis</i>	GU 2006-18-MR		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) mollis</i>	GU 2006-19-MR		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) mollis</i>	GU 2006-38-raft-202		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) nigripalpus</i>	GU 2006-02-02		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) pinarocampa</i>	GU 2004-06-09		GU: Sacatepequez Dept.	S,B,A,G
<i>Cx. (Cux.) pipiens</i>	IL 2006-Doty		IL: Cook Co.	J.-P. Mutebi
<i>Cx. (Cux.) quinquefasciatus</i>	GU 2006-01-14		GU: Peten Dept.	S,W,S,C,S
<i>Cx. (Cux.) restuans</i>	IL 2006-Doty		IL: Cook Co.	J.-P. Mutebi
<i>Cx. (Cux.) stigmatosoma</i>	TX 2003-3-69		TX: Brewster Co.	H. Savage, K. Bennett
<i>Cx. (Cux.) thriambus</i>	TX 2003-3-57		TX: Brewster Co.	H. Savage, K. Bennett
<i>Cx. (Cux.) thriambus</i>	TX 2003-3-60		TX: Brewster Co.	H. Savage, K. Bennett
<i>Cx. (Phc.) lactator</i>	GU 2006-01-39		GU: Peten Dept.	S,W,S,C,S

* S,W,S,C,S = H. Savage, M. Williams, S. Sousa, A. Catalan, A. Salam.

† S,B,A,G = H. Savage, K. Bennett, S. Aspen, J. Garcia.

Biosystems), and 60 ng of template DNA. Amplification was conducted under the following thermal cycler conditions: one cycle at 97°C for 4 minutes, followed by 30 cycles of 96°C for 30 seconds, 48°C for 30 seconds and 72°C for 2 minutes, and ending with a 5 minute extension step at 72°C.

Cloning and sequencing. Each molecule was cloned with the TOPO-TA ligation vector pCR4TOPO (Invitrogen, Carlsbad, CA) used to transform One Shot Chemically Competent *Escherichia coli* (Invitrogen) by heat shock per the manufacturer's instructions. Colonies of the transformed bacteria were visible on tetracycline-treated LB agar plates (Invitrogen) after 12 hours of incubation at 37°C in a CO₂ incubator. Colonies were picked and incubated in tetracycline-treated LB broth (Invitrogen) for 16 hours at 37°C in a rotating incubator before treatment with the Qiagen Qiaprep Spin Miniprep Kit (Qiagen) and eluting in 50 µL of nuclease-free water. This product was dried in a vacuum centrifuge and reconstituted in 400 µL of nuclease-free water.

The ITS inserts were recovered from individual colonies as described previously,^{18,29} and M13 Forward (Invitrogen), M13 Reverse (Invitrogen), T3 (Invitrogen). The PCR products were purified using a Qiagen PCR Purification Kit (Qiagen). The ITS amplicon was sequenced three times in the forward direction and five times in the reverse direction using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Each 20 µL sequencing reaction included 10–50 ng purified PCR product, 8.0 µL Sequencing Mix, and 3.2 pmol sequencing primer. Cycle sequencing was performed on a DNA Engine PTC-200 thermal cycler (GMI Inc., Ramsey, MN). Dyes were removed from sequenced PCR products either through ethanol purification or with the BigDye Xterminator kit (Applied Biosystems). Purified samples were analyzed on an ABI 3130 genetic analyzer. Sequences were assembled and annotated using SeqMan II, Lasergene 7.0 (DNASTAR Inc., Madison, WI). Novel sequences were submitted to GenBank (Table 1).

Multiplexed primer design and PCR. One conserved forward primer and species-specific reverse primers were manually selected from a multiple alignment of novel rDNA sequences generated from *Cx. chidesteri* from Guatemala, *Cx. coronator* from Mississippi, *Cx. interrogator* from Guatemala, *Cx. nigripalpus* from Florida, *Cx. quinquefasciatus* from California, *Cx. thriambus* from Texas, and *Cx. lactator* from Guatemala (Table 1). For the multiplexed assay, specific reverse primers designed for *Cx. quinquefasciatus* (QUIN2), *Cx. interrogator* (INTE2), *Cx. coronator* (CORO3), *Cx. chidesteri* (CHID), *Cx. nigripalpus/Cx. thriambus* (NIGR4), and *Cx. lactator* (LACT) were used in combination with the conserved forward primer (CXFOR) located in 18S (Tables 2 and 3). All species-specific reverse primers were placed in

polymorphic regions of ITS1 where the target sequence had at least five nucleotide differences from homologous regions in the other mosquito sequences and the resulting amplicons could be differentiated by agarose gel electrophoresis (Table 3). Primer sequences were checked for Tm compatibility and self-complementarity with Primer3 software.³⁰

The multiplexed primer set was optimized using individually reared, voucher specimens from Guatemala and the United States, including both target and non-target species to ensure primer specificity. Non-target species tested included *Cx. declarator*, *Cx. mollis*, *Cx. (Cux.) pinarocampa* Dyar and Knab, *Cx. (Cux.) pipiens* L., *Cx. restuans*, and *Cx. stigmatosoma* (Table 1). *Culex (Cux.) inflicus* Theobald DNA was obtained from a 2006 CDC light trap mosquito pool from Puerto Barrios, Guatemala because no voucher specimen was available.

Amplification cycles for the multiplexed assay consisted of an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of 95°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds. The final 72°C extension was 7 minutes. Each 25-µL PCR reaction consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin; 1.0 mM dNTPs; and 1.0 U of *Taq* polymerase. Each reaction also contained 25 pmol each of CXFOR, CHID, LACT, INTE2, NIGR4, CORO3, and 15 pmol QUIN2. One microliter of DNA from each extracted specimen was used as template in each reaction. All DNA amplifications were completed on a DNA Engine PTC-200 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) and visualized on ethidium bromide-stained 1.8–2% agarose gels buffered with 0.5X TBE.

Restriction fragment length polymorphism. Primers CXFOR and NIGR4 resulted in amplification of homologous ITS fragments from *Cx. nigripalpus* and *Cx. thriambus*. Novel ITS sequences were obtained from *Cx. thriambus* from Texas using the methods described previously, and sequences from these two species were analyzed for unique restriction sites using RestrictionMapper version 3 online software (www.restrictionmapper.org). The ITS1 clones from *Culex thriambus* each contained a single restriction site for *NcoI* (c/catgg) at position 108 of this amplicon, whereas *Cx. nigripalpus* lacked this unique sequence. Each 20 µL digest consisted of 18 µL PCR product, 2 µL 10x NE Buffer 3, and 2 U of *NcoI* enzyme (New England BioLabs, Ipswich, MA). For each specimen, paired control reactions were set up that contained only PCR product and 10x buffer. Reactions were incubated at 37°C for 2 hours, followed by a heat inactivation step of 65°C for 20 minutes. Digested and undigested PCR products were analyzed side by side by agarose gel electrophoresis as described previously.

Field validation. To test this assay on field specimens, 112 adult female field specimens were randomly selected from collections performed in Puerto Barrios, Guatemala in July and December 2007. Of these specimens, preliminary morphological identifications were *Cx. chidesteri* (*N* = 1), *Cx. coronator* (*N* = 18), *Cx. interrogator* (*N* = 12), *Cx. lactator* (*N* = 1), *Cx. nigripalpus* (*N* = 24), *Cx. quinquefasciatus* (*N* = 40), and *Cx. spp.* (*N* = 16).¹⁰ Two additional field specimens of *Cx. thriambus* from Texas were also tested. To test the sensitivity of this assay for detecting mixed DNA templates in mosquito pools, experiments were conducted on disproportionate ratios of mixed, known templates. Mosquito pools containing multiple species were simulated by mixing paired templates from voucher specimens (*Cx. quinquefasciatus/Cx. interrogator* and *Cx. interrogator/Cx. lactator*) such that one template

TABLE 2

Primers used to identify members of the subgenera *Culex* (*Culex*) and *Culex* (*Phenacomia*) in Guatemala based on interspecific sequence variation in the rDNA gene array

Primer name	5' to 3' Sequence	Tm	Amplicon size
CXFOR	ATCGCTGAAGTTGACCGAAC	60.26	—
CHID	TACACGGTGCGCTAAGAGATACAC	62.68	180
NIGR4	TCATGGTAGGCGATGACG	59.14	207
CORO3	AGTACGCGCATTCCGACAG	62.35	244
INTE2	GATAGGAACACAGGAGGAGTGC	60.14	354
LACT	GAAGGGTGCTGGATGGTG	60.05	446
QUIN2	ACTGTTTTATCGGTGGGTCGT	60.66	580

TABLE 3
Nucleotide substitutions present among priming site sequences*

CXFOR (-89)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	A	T	C	G	C	T	G	A	A	G	T	T	G	A	C	C	G	A	A	C
<i>Cx. interrogator</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Cx. nigripalpus</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Cx. lactator</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Cx. coronator</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Cx. quinquefasciatus</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>Cx. thriambus</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CORO3(+155)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	.	.	.	.	.	.	.	.	-	-	.	-	-	.	.	.	T	.	-	-
<i>Cx. interrogator</i>	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	T	.	-	-
<i>Cx. nigripalpus</i>	.	.	C	G	.	.	.	.	G	.	A	-	-	.	.	.	T	.	-	-
<i>Cx. lactator</i>	C	.	-	G	T	.	T	.	.	.	C	A	.	.	C	G	A	G	A	A
<i>Cx. coronator</i>	A	G	T	A	C	G	C	G	C	A	T	T	C	C	G	A	C	A	G	A
<i>Cx. quinquefasciatus</i>	T	.	.	G	T	.	T	.	A	T	C	A	.	.	.	G	G	G	A	A
<i>Cx. thriambus</i>	.	.	C	G	.	.	.	.	G	.	.	-	-	.	.	T	.	-	-	-
INTE2(+265)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	.	.	.	C	.	-	-	-	.	.	-	-	-	-	-	-	.	T	C	C
<i>Cx. interrogator</i>	G	A	T	A	G	G	A	A	C	A	C	A	G	G	A	G	G	A	G	T
<i>Cx. nigripalpus</i>	.	.	.	C	.	T	T	C	-	-	T	C	.	-	-	-	-	-	-	-
<i>Cx. lactator</i>	.	G	.	C	.	T	T	C	-	-	T	C	.	C	T	C	-	C	A	C
<i>Cx. coronator</i>	.	.	.	C	T	A	T	C	T	-	-	-	-	-	-	-	-	G	T	C
<i>Cx. quinquefasciatus</i>	.	T	.	C	.	T	T	C	G	G	T	C	.	.	T	C	.	G	T	C
<i>Cx. thriambus</i>	.	.	.	C	.	-	-	-	.	T	.	-	-	-	-	-	.	.	T	C
QUIN2(+491)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	.	.	G	.	.	.	.	.	C	.	A	.	.	.	.	.	T	G	C	A
<i>Cx. interrogator</i>	.	.	G	.	.	.	.	.	C	A	A	.	.	.	.	.	T	G	C	A
<i>Cx. nigripalpus</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	C	A
<i>Cx. lactator</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	C	A
<i>Cx. coronator</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	C	A
<i>Cx. quinquefasciatus</i>	A	C	T	G	T	T	T	T	A	-	-	T	C	G	G	T	G	-	-	T
<i>Cx. thriambus</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	C	A
CHID (+91)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	T	A	C	A	C	G	G	T	G	C	G	C	T	A	-	-	-	A	G	A
<i>Cx. interrogator</i>	C	G	.	T	A	A	.	A	.	A	.	.	G	.	G	A	T	.	T	.
<i>Cx. nigripalpus</i>	.	G	G	G	T	A	C	.	.	.	.	.	G	.	.	.	.	-	-	-
<i>Cx. lactator</i>	G	.	G	A	A	C	G	.	A	.	A	G	G	A	C	G	G	.	C	G
<i>Cx. coronator</i>	G	G	G	T	A	C	.	.	.	T	A	G	C	.	.	.	-	A	.	C
<i>Cx. quinquefasciatus</i>	.	G	.	G	A	C	A	.	T	T	.	A	A	T	G	C	G	A	G	A
<i>Cx. thriambus</i>	G	G	G	T	A	C	.	.	.	.	.	G	.	.	.	.	.	T	C	G
NIGR4 (+118)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	.	.	G	.	A	G	G	T	.	.	.	.	T	A	.	G	C	.	.	G
<i>Cx. interrogator</i>	.	.	C	A	C	C	T	G	A	C	.	.	.	.	A	G	A	.	.	.
<i>Cx. nigripalpus</i>	T	C	A	T	G	-	-	-	-	-	G	T	A	G	G	C	G	A	T	G
<i>Cx. lactator</i>	.	G	.	C	-	.	A	C	T	.	G	.	T	A	.	C	G	A	.	T
<i>Cx. coronator</i>	G	G	G	C	T	G	G	G	T	T	.	.	C	T	.	A	C	.	.	G
<i>Cx. quinquefasciatus</i>	G	G	T	C	C	G	G	A	C	T	.	G	T	.	A	C	T	G	.	T
<i>Cx. thriambus</i>	.	.	.	C	.	G	G	T	A	G	.	.	.	.	.	.	C	.	.	.
LACT (+357)																				
Mosquito species	Primer sequence 5' to 3'																			
<i>Cx. chidesteri</i>	.	.	-	-	A	.	A	.	.	G	C	.	T	A	-	T	A	T	A	G
<i>Cx. interrogator</i>	A	.	G	C	-	.	A	.	.	G	C	.	T	A	-	T	A	T	A	G
<i>Cx. nigripalpus</i>	.	.	-	-	C	.	A	.	.	G	C	.	T	A	-	T	A	T	A	G
<i>Cx. lactator</i>	G	A	A	G	G	G	T	G	C	T	G	G	A	T	G	G	-	-	-	T
<i>Cx. coronator</i>	.	G	.	-	.	A	A	.	.	G	T	G	T	.	-	T	G	T	A	G
<i>Cx. quinquefasciatus</i>	.	C	T	.	C	.	.	.	.	A	T	.	T	A	.	T	A	T	A	C
<i>Cx. thriambus</i>	.	.	.	C	A	.	A	.	.	G	C	.	T	A	-	T	A	T	A	G

*Dots indicate nucleotides shared with the reference sequence (in bold), whereas dashes represent gaps. Numbers indicate the 5' position of each primer relative to the beginning of ITSI.

concentration was fixed and the other serially diluted to a final relative concentration of 1:160. Equal volumes of fixed and diluted templates (0.75 μ L each) were combined such that the final template proportions in PCR reactions were 1:1, 1:4, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Each series of template mixtures was analyzed as described previously with the full complement of primers.

RESULTS

Multiplexed PCR and RFLP. Using the novel, multiplexed primer set, rDNA fragments of the predicted sizes were amplified from *Cx. chidesteri* (180 bp), *Cx. coronator* (244 bp), *Cx. interrogator* (354 bp), *Cx. nigripalpus* (207 bp), *Cx. quinquefasciatus* (580 bp), and *Cx. lactator* (446 bp) voucher specimens (Figure 1). Additionally, there was no amplification from *Cx. declarator*, *Cx. inflicus*, *Cx. mollis*, *Cx. restuans*, *Cx. pinarocampa*, or *Cx. stigmatosoma* (data not shown). The *Cx. quinquefasciatus* primer (QUIN2) did amplify a homologous ITS fragment from *Cx. pipiens*, however *Cx. pipiens* is not reported from Guatemala and reliable molecular diagnostics already exist to separate these two species and their hybrids in areas of sympatry.³¹⁻³⁴ Homologous fragments were amplified from *Cx. thriambus* and *Cx. nigripalpus* using primers CXFOR and NIGR4. *Culex nigripalpus* and *Cx. thriambus* were further differentiated by RFLP, exploiting a unique *NcoI* restriction site in *Cx. thriambus* (Figure 2).

Field validation. Of the 98 specimens for which a species name was preliminarily assigned, the molecular assay confirmed the identification of 88 (90%), and corrected the misidentification of 9/10 of the remaining identified mosquitoes. The most common correction was *Cx. interrogator* misidentified as *Cx. quinquefasciatus*. Of the mosquitoes classified as *Cx. spp.*, identifications were obtained for 13/16. These identifications included one *Cx. interrogator*, nine *Cx. nigripalpus*, and three *Cx. quinquefasciatus*. No product was obtained from the three remaining *Cx. spp.* or one mosquito classified as *Cx. nigripalpus*, indicating that they each represented one or more species not included in the assay. Both target templates were detected in mixed template solutions at dilutions out

FIGURE 1. Ethidium bromide-stained agarose gel depicting differentiation of six species of *Culex* in Guatemala. Lane 1: *Cx. (Cux.) quinquefasciatus* (580 bp), Lane 2: *Cx. (Phc.) lactator* (446 bp), Lane 3: *Cx. (Cux.) interrogator* (354 bp), Lane 4: *Cx. (Cux.) coronator* (244 bp), Lane 5: *Cx. (Cux.) nigripalpus* (207 bp), Lane 6: *Cx. (Cux.) chidesteri* (180 bp), Lane 7: Negative control.

FIGURE 2. Ethidium bromide-stained agarose gel showing differentiation of *Culex nigripalpus* from *Cx. thriambus* by restriction fragment length polymorphism. *Culex thriambus* contains a *NcoI* restriction site in the 207-bp amplicon, whereas *Cx. nigripalpus* lacks this site. Lane 1: *Cx. nigripalpus* (digested), Lane 2: *Cx. nigripalpus* (undigested), Lane 3: *Cx. thriambus* (digested), Lane 4: *Cx. thriambus* (undigested), Lane 5: negative control.

to 1:20, corresponding to a detection limit of 0.052 ng of contaminating template (Figure 3).

DISCUSSION

The misidentification of morphologically similar mosquito species could lead to the false incrimination of a vector and/or the misappropriation of scarce resources for vector control. With numerous species of *Culex* (*Cux.*) sharing a similar geographical distribution and an unreliable morphological key, Guatemala is one such location where a molecular assay for the identification of *Culex* species would contribute greatly to entomological and arbovirus ecology studies. We have developed a multiplex PCR-RFLP assay based on the internal transcribed spacer regions of the rDNA gene array to identify common *Culex* (*Cux.*) and *Culex* (*Phc.*) mosquitoes in Guatemala. Of the 16 *Culex* (*Cux.*) and two *Culex* (*Phc.*) species present in Guatemala, our assay consistently and reliably identified seven of these species, including species from which arboviruses have been isolated or detected in Latin America: *Cx. chidesteri*, *Cx. interrogator*, *Cx. nigripalpus*,

FIGURE 3. Ethidium bromide-stained agarose gel showing disproportionate ratios of *Culex quinquefasciatus* and *Cx. interrogator* mixed templates, simulating contaminated pools. A fixed volume of *Cx. quinquefasciatus* template was mixed with serial dilutions of *Cx. interrogator* template. Lane 1: *Cx. quinquefasciatus*, Lane 2: *Cx. interrogator*, Lane 3: equal proportions of both templates 1:1, Lane 4: 1:4, Lane 5: 1:10, Lane 6: 1:20, Lane 7: 1:40, Lane 8: 1:80, Lane 9: 1:160, Lane 10: 1:320, Lane 11: negative control.

Cx. quinquefasciatus, and *Cx. thriambus*. Morphologically similar species and/or species that key to the same couplet in Clark-Gil and Darsie,¹⁰ such as *Cx. quinquefasciatus* and *Cx. interrogator*, *Cx. lactator* and *Cx. coronator*, and *Cx. chidesterei* and *Cx. nigripalpus*, can be confirmed by this molecular assay. Consequently, this multiplex PCR-RFLP assay makes significant headway towards the correct identification of *Culex* species in Guatemala.

In addition to the correct identification of target species, no amplification was obtained from at least six additional non-target species of *Culex* (*Cux.*) indigenous to Guatemala: *Cx. declarator*, *Cx. inflicus*, *Cx. mollis*, *Cx. pinarocampa*, *Cx. restuans*, and *Cx. stigmatosoma*. A confirmatory restriction digest was designed to differentiate *Cx. nigripalpus* from *Cx. thriambus*.

Additional field specimens from outside Guatemala were not tested; however, primers for *Cx. coronator* (Mississippi), *Cx. nigripalpus* (Florida), and *Cx. quinquefasciatus* (California) were designed from sequences of specimens collected in the continental United States. In comparing our sequences with those available in GenBank, priming site sequences for CXFOR and NIGR4 were identical to *Cx. nigripalpus* ITS sequences from a previous study (AF521664 and AF521662 and AF520974),³⁵ and intraspecific ITS sequence variability was minor, with only one or two nucleotide differences at the priming site evident in some other ITS clones (e.g., U33023 and U33024 from the same specimen in Florida). Successful priming and amplification from these specimens is expected. Each of these *Cx. nigripalpus* sequences also lacked the *NcoI* restriction site at position 108 of the CXFOR-NIGR4 amplicon, confirming the use of the RFLP to separate these *Cx. nigripalpus* from *Cx. thriambus*. Furthermore, ITS amplicons from the two additional *Cx. thriambus* field specimens were each digested by *NcoI* as expected, whereas ITS amplicons from *Cx. nigripalpus* field specimens were not digested by *NcoI*. Similarly for *Cx. quinquefasciatus*, the priming site sequences for CXFOR and QUIN2 were identical between *Cx. quinquefasciatus* from California (this study), and *Cx. quinquefasciatus* from South Africa (DQ341108). Consistent amplification of ITS from Guatemalan *Cx. quinquefasciatus* field specimens in this study shows that these primers are broadly applicable across the geographic range of these species.

It is unknown whether our *Cx. lactator* primers distinguish between the two *Cx.* (*Phc.*) species known to occur in Guatemala, *Cx. corniger* and *Cx. lactator*. Similarly, we do not know if our *Cx. coronator* primers are species or complex specific. The *Cx. coronator* complex includes *Cx. coronator*, *Cx. usquatus*, and *Cx. ousqua*. Adding additional species would require development of additional multiplex assays. Therefore, future work should include the design of additional molecular diagnostics covering similar or related species such as members of the *Cx. coronator* complex and *Cx. mollis* versus *Cx. declarator*.

The sensitivity of this assay resulted in amplification of less than one nanogram of DNA template from each species in mixed pools. Therefore, this assay can be used to confirm the identification of individual field specimens and to verify species composition in virus-positive mosquito pools. Assay validation also used field specimens stored dry on silica desiccant for 2 to 12 months before DNA extraction and voucher specimens stored in 95% ethanol. Successful and consistent PCR amplification was obtained from DNA extracted by standard salt extraction, the Qiagen blood and tissue kit, and from spec-

imen legs sonicated in TE buffer. These results show the use of this assay across a variety of specimen collection, storage, and DNA preparation methods.

In conclusion, we have developed a novel multiplexed PCR-RFLP assay to identify seven species of *Culex* (*Cux.*) and *Culex* (*Phc.*) in Guatemala. It is our expectation that this diagnostic will serve as a valuable tool for *Culex* mosquito identification in Guatemala and neighboring countries in providing the ability to confirm the identity of morphologically similar species, and obtain the identity of otherwise badly damaged and unidentifiable specimens. With the inclusion of confirmed and potential arbovirus vectors, this assay will serve as an instrumental role in future ecological studies of *Culex* mosquitoes and arbovirus surveillance and control efforts in Guatemala.

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