Published in final edited form as:
J Am Mosq Control Assoc. 2010 June ; 26(2): 214–218.

UNIVERSAL PRIMERS FOR THE AMPLIFICATION AND SEQUENCE ANALYSIS OF ACTIN-1 FROM DIVERSE MOSQUITO SPECIES

MOLLY STALEY[1], **KARIN S. DORMAN**[2], **LYRIC C. BARTHOLOMAY**[3], **ILDEFONSO FERNÁNDEZ-SALAS**[4], **JOSE A. FARFAN-ALE**[5], **MARIA A. LOROÑO-PINO**[5], **JULIAN E. GARCIA-REJON**[5], **LUIS IBARRA-JUAREZ**[4], and **BRADLEY J. BLITVICH**[1]

[1] Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011

[2] Department of Genetics, Development and Cell Biology, College of Agriculture and Life Sciences, Iowa State University, Ames, IA 50011

[3] Department of Entomology, College of Agriculture and Life Sciences, Iowa State University, Ames, IA 50011

[4] Department of Entomology, Faculty of Biological Sciences, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México 66451

[5] Laboratorio de Arbovirologia, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, The Universidad Autonoma de Yucatan, Merida, Yucatan, Mexico 97000

Abstract

We report the development of universal primers for the reverse-transcription polymerase chain reaction (RT-PCR) amplification and nucleotide sequence analysis of actin cDNAs from taxonomically diverse mosquito species. Primers specific to conserved regions of the invertebrate actin-1 gene were designed after actin cDNA sequences of *Anopheles gambiae*, *Bombyx mori*, *Drosophila melanogaster*, and *Caenorhabditis elegans*. The efficacy of these primers was determined by RT-PCR with the use of total RNA from mosquitoes belonging to 30 species and 8 genera (*Aedes*, *Anopheles*, *Culex*, *Deinocerites*, *Mansonia*, *Psorophora*, *Toxorhynchites*, and *Wyeomyia*). The RT-PCR products were sequenced, and sequence data were used to design additional primers. One primer pair, denoted as Act-2F (5′-ATGGTCGGYATGGGNCAGAAGGACTC-3′) and Act-8R (5′-GATTCCATACCCAGGAAG-GADGG-3′), successfully amplified an RT-PCR product of the expected size (683-nt) in all mosquito spp. tested. We propose that this primer pair can be used as an internal control to test the quality of RNA from mosquitoes collected in vector surveillance studies. These primers can also be used in molecular experiments in which the detection, amplification or silencing of a ubiquitously expressed mosquito housekeeping gene is necessary. Sequence and phylogenetic data are also presented in this report.

Keywords

Mosquito; actin; universal primers; reverse transcription-polymerase chain reaction; sequence

Correspondence to: BRADLEY J. BLITVICH.

All mosquitoes are members of the family *Culicidae* (Knight and Stone 1977). This family contains of over 3,500 species and is divided into 3 subfamilies: Anophelinae, Culicinae, and Toxorhynchitinae. The subfamily Anophelinae is further divided into 3 genera: *Anopheles*, *Bironella* and *Chagasia* (Gwadz and Collins 1996). The genus *Anopheles* is of particular relevance to human health because all human malarial parasites are transmitted by *Anopheles* spp. Malaria is an enormous public health problem with an estimated 300–500 million cases and 1–3 million deaths occurring worldwide each year (Gwadz and Collins 1996, Snow et al. 2005). Anopheline mosquitoes are also important vectors of human filarial parasites and O'nyongnyong virus (Johnson 1988, Bartholomay and Christensen 2002).

The subfamily Culicinae contains 29 genera (Nasci and Miller 1996), 6 of which are represented in this study: *Aedes*, *Culex*, *Deinocerites*, *Mansonia*, *Psorophora*, and *Wyeomyia*. Mosquitoes in the genera *Aedes*, *Culex*, and *Mansonia* are important vectors of human filarial parasites (Bartholomay and Christensen 2002). *Aedes* spp. are also important vectors of many medically significant arboviruses; for example: dengue, yellow fever, chikungunya, Rift Valley fever, and La Crosse viruses (Weaver 2005, Gubler et al. 2007, Schmaljohn and Nichol 2007). Dengue virus has a particularly devastating impact on human health with an estimated 50 million–100 million cases of dengue fever and 500,000 cases of life-threatening dengue hemorrhagic fever occurring annually in tropical regions of the world (Gubler 2006). Many viruses that cause morbidity and mortality in humans are also transmitted by *Culex* spp.; for example: Japanese encephalitis, West Nile, St. Louis encephalitis, Western equine encephalitis, and Venezuelan equine encephalitis viruses (Weaver 2005, Gubler et al. 2007). Human pathogens, such as WNV, have been isolated from *Deinocerites* and *Psorophora* spp. mosquitoes (Granwehr et al. 2004).

The subfamily Toxorhynchitinae contains a single genus: *Toxorhynchites* (Nasci and Miller 1996). *Toxorhynchites* spp. are among the few mosquito species that do not take blood meals; adults feed primarily on plant nectar. Thus, *Toxorhynchites* spp. do not transmit infectious agents to humans.

Because of the significant burden that Anophelinae and Culicinae mosquitoes have on human health, numerous mosquito surveillance and control programs have been established throughout the world. Mosquito surveillance often involves the use of reverse-transcription polymerase chain reaction (RT-PCR) to test field-caught mosquitoes for specific pathogens. Often, however, only a small proportion of mosquitoes are positive. Because of the often small proportion of positive specimens, it is useful to amplify a reference or housekeeping gene to be sure that the RNA isolation and cDNA generation were successful. This is especially true when mosquitoes are collected in remote study sites and cannot be transported to the laboratory in a timely manner. Thus, we have developed generic RT-PCR primers for the detection of actin-1 in mosquitoes from all 3 Culicidae subfamilies.

To facilitate the design of universal actin-specific primers, the actin-1 cDNA sequences of *Anopheles gambiae* (Giles), *Bombyx mori* (Linnaeus), *Drosophila melanogaster* (Meigen), and *Caenorhabditis elegans* were downloaded from the Genbank database (accession numbers U02933. 1, NM_001126255.1, NM_079643.1, and NM_073418.4, respectively) and aligned. Highly conserved regions were identified and used to design 10 actin-specific primers, including Act-2F (5′-ATGGTCGGYATGGGNCAGAAG-GACTC-3′), Act-2R (5′-TCGCACTTCAT-GATSGAGTTGTA-3′), and Act-3R (5′-CCNGGGTACATGGTGGTACCNCCGGA-3′). Act-2F is a forward primer that aligns to nucleotides 269–294 of the actin cDNA sequence of *An. gambiae* (Ge nB a nk ac ce ss i on number U02933.1). Act-2R and Actin-3R are reverse primers that align to nucleotides 974–996 and 1037–1062, respectively of the aforementioned sequence.

The efficacy of various primer combinations was evaluated by RT-PCR with the use of 30 species of mosquitoes from 8 genera and 3 subfamilies (Table 1). Twenty-three mosquito species were collected in northern Mexico or the Yucatan Peninsula of Mexico as part of our ongoing arbovirus surveillance studies. Seven species were obtained from the insectaries at Iowa State University. Detailed protocols for the collection, identification, and homogenization of mosquitoes have been provided elsewhere (Far-fan-Ale et al. 2009). Total RNA was extracted from mosquito homogenates with the use of the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA). Complementary DNAs were generated with the use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCRs were performed with the use of *Taq* polymerase (Invitrogen).

Act-2F and Act-3R was the most consistent and efficient primer combination; RT-PCR products were detected in 29 of the 30 species tested (Table 1), although a nonspecific band of approximately 400 nt was detected with several samples. Primers Act-2F and Act-2R recognized actin-1 from 28 species, and no nonspecific bands were detected. The longest RT-PCR product of the expected size from each species was purified using the Purelink Gel Extraction Kit (Invitrogen) and sequenced using a 3730×1 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting sequences were aligned and used to design additional primers including Act-8R (5′-GATTCCATACCCAGGAAGGADGG-3′). Act-8R is a reverse primer that aligns to nucleotides 929–951 of the actin cDNA sequence of *An. gambiae* (GenBank accession number U02933.1). When primers Act-2F and Act-8R were used in combination, RT-PCR products of the expected size (683 nt) were detected in all mosquito species. Nonspecific bands were not observed in any samples. All RT-PCR products generated with the use of Act-2F and Act-8R were sequenced with the use of these same primers in the sequencing reactions. Resulting sequences were aligned and in most instances the forward and reverse sequences did not overlap at the 5′ and 3′ ends. Thus, additional primers were designed and used in subsequent sequencing reactions. The resulting sequences were once again aligned and approximately 20 nts were trimmed from the 5′ and 3′ ends to confidently yield sequences of 641 nt (GenBank accession numbers GQ981439 to GQ981468).

Alignment of the 641-nt region of actin-1 from all the mosquito spp. examined in this study using the CLUSTAL W algorithm (version 2) (Higgins and Sharp 1988, Larkin et al. 2007) revealed that this region is highly conserved. Highest (99.2%) nucleotide identity occurs between the actin cDNAs of *Ae. aegypti* (Linnaeus) and *Ae. albopictus* (Skuse), and identity was lowest (85.3%) between the actin cDNAs of *De. cancer* (Theobald) and *Mn. titillans* (Walker). A total of 465 (72.5%) nucleotide positions are strictly conserved among all mosquito species. This region also has striking identity to the homologous regions of actin cDNAs of other invertebrate species. For instance, alignment of the 641-nt regions of actin-1 from *C. elegans* and all mosquito species revealed that there are 441 (68.8%) strictly conserved nucleotide positions.

The predicted amino acid sequences of actin-1 from all the mosquito species evaluated in this study were aligned, and amino acid identities and similarities were calculated as described by Altschul et al. 2005. This analysis revealed that the sequences are 94.4–100% identical and 96.2–100% similar. A total of 186 (87.3%) amino acid residues are strictly conserved between all species. This region also has striking identity and similarity to the homologous region of actin-1 of other invertebrate species. A total of 181 (85.0%) amino acid residues are strictly conserved in actin-1 of *C. elegans* and all the mosquito spp.

Because the nucleotide and amino acid sequence alignments revealed that there is limited sequence diversity between actin-1 from different mosquito species, the universal primers reported here can not be used for RT-PCR sequencing to determine the identity of field mosquitoes that could not be classified based on morphological characteristics. In contrast, the

D2 region of 28S ribosomal DNA provides a suitable target in multiplex PCR assays for the differentiation of different members of the *An. culicifacies* complex (Raghavendra et al. 2009). The ribosomal internal transcribed spacer 1 region provides a reliable target to differentiate *Ae. aegypti*, *Ae. albopictus*, and *Ae. scutellaris* (Walker) in real-time RT-PCR assays (Hill et al. 2008).

A phylogenetic tree was constructed with the use of the MrBayes GTR+I+G model applied to the 641-nt actin region of 30 mosquito species (Fig. 1). Phylogenetic trees were also generated with the use of neighbor-joining (NJ) and maximum-likelihood (ML) methods (data not shown). To our surprise, the mosquitoes did not separate according to subfamily; no tree has the 3 expected clades corresponding to Anophelinae, Culicinae, or Toxorhynchitinae species mosquitoes. *Toxorhynchites amboinensis* (Doleschall), *Wyeomyia*, and *Mn. titillans* are positioned with the greatest uncertainty and disparity among the phylogenetic trees. In the Bayesian and NJ trees, *Wyeomyia* and *Mn. titillans* are basal to all other mosquito sequences, but cluster with the *Aedes* sequences in the ML tree. *Toxorhynchites amboinensis* is the most basal sequence in the ML tree, but clusters with some *Psorophora* spp. in the Bayesian and NJ trees. The *Psorophora* spp. do not appear as a monophyletic clade in any tree, but instead appear basal to *Culex* and *Anopheles* sequences. In particular, *Ps. albipes* (Theobald) is significantly clustered in the Bayesian tree with *Culex* and *Anopheles* spp. rather than other *Psorophora* spp. Because of the considerable amount of disagreement in the topological arrangements of the mosquito sequences in the Bayesian, NJ, and ML trees and because the mosquitoes did not separate according to subfamily, these data indicate that actin sequences are not suitable for the study of mosquito phylogeny.

In summary, we report the development of primers that detected by RT-PCR a 683-nt region of actin-1 in all mosquito species tested. Because these studies were performed with the use of a taxonomically diverse group of mosquitoes, with representatives from all 3 subfamilies, it is likely that these primers can amplify actin RNA in all mosquito species. Thus, we propose that these primers can be used to monitor the integrity of mosquito samples collected in the field. These primers, in addition to the sequence data presented here, can also be used to facilitate molecular experiments in which the detection, expression, or silencing of a mosquito housekeeping gene is required as a positive or negative control, for example, in Northern blots and RNA interference experiments. The mosquito actin sequences could also be used to design smaller primer sets for quantitative RT-PCR assays if the abundance of a target gene needs to be normalized against a ubiquitously expressed housekeeping gene.

Acknowledgments

This study was supported by Grant 5R21AI0-67281-02 from the National Institutes of Health.

REFERENCES CITED

Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, Yu Y. Protein database searches using compositionally adjusted substitution matrices. FEBS J 2005;272:5101–5109. [PubMed: 16218944]

Bartholomay, LC.; Christensen, BM. Vector–parasite interactions in mosquito-borne filariasis. In: Klei, TR.; Rajan, TV., editors. The Filaria. Dordrecht, Netherlands: Kluwer Academic; 2002. p. 9-20.

Farfan-Ale JA, Lorono-Pino MA, Garcia-Rejon JE, Hovav E, Powers AM, Lin M, Dorman KS, Platt KB, Bartholomay LC, Soto V, Beaty BJ, Lanciotti RS, Blitvich BJ. Detection of RNA from a novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 2009;80:85–95. [PubMed: 19141845]

Granwehr BP, Lillibridge KM, Higgs S, Mason PW, Aronson JF, Campbell GA, Barrett AD. West Nile virus: where are we now? Lancet Infect Dis 2004;4:547–556. [PubMed: 15336221]

Gubler DJ. Dengue/dengue haemorrhagic fever: history and current status. Novartis Found Symp 2006;277:3–16. discussion 16–22, 71–73, 251–253. [PubMed: 17319151]

Gubler, DJ.; Kuno, G.; Markoff, L. Flaviviruses. In: Knipe, DM.; Howley, PM., editors. Fields virology. Philadelphia, PA: Lippincott Williams and Wilkins; 2007. p. 1153-1252.

Gwadz, R.; Collins, FH. *Anopheline* mosquitoes and the agents they transmit. In: Beaty, BJ.; Marquardt, WC., editors. The biology of disease vectors. Niwot, CO: University Press of Colorado; 1996. p. 73-84.

Higgins DG, Sharp PM. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 1988;73:237–244. [PubMed: 3243435]

Hill LA, Davis JB, Hapgood G, Whelan PI, Smith GA, Ritchie SA, Cooper RD, van den Hurk AF. Rapid identification of *Aedes albopictus*, *Aedes scutellaris*, and *Aedes aegypti* life stages using real-time polymerase chain reaction assays. Am J Trop Med Hyg 2008;79:866–875. [PubMed: 19052295]

Johnson, BK. O'nyongnyong virus disease. In: Monath, TP., editor. The arboviruses: epidemiology and ecology. Boca Raton, FL: CRC Press; 1988. p. 217-223.

Knight, KL.; Stone, A. A catalogue of the mosquitoes of the world (Diptera: Culicidae). College Park, MD: Thomas Say Foundation and Entomological Society of America; 1977.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics 2007;23:2947–2948. [PubMed: 17846036]

Nasci, RS.; Miller, BR. *Culicine* mosquitoes and the agents they transmit. In: Beaty, BJ.; Marquardt, WC., editors. The biology of disease vectors. Niwot, CO: University Press of Colorado; 1996. p. 85-97.

Raghavendra K, Cornel AJ, Reddy BP, Collins FH, Nanda N, Chandra D, Verma V, Dash AP, Subbarao SK. Multiplex PCR assay and phylogenetic analysis of sequences derived from D2 domain of 28S rDNA distinguished members of the *Anopheles culicifacies* complex into two groups, A/D and B/C/E. Infect Genet Evol 2009;9:271–277. [PubMed: 19138765]

Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003;19:1572–1574. [PubMed: 12912839]

Schmaljohn, CS.; Nichol, ST. Bunyaviridae. In: Knipe, DM.; Howley, PM., editors. Fields virology. Philadelphia, PA: Lippincott Williams and Wilkins; 2007. p. 1741-1789.

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434:214–217. [PubMed: 15759000]

Weaver SC. Host range, amplification and arboviral disease emergence. Arch Virol Suppl 2005;19:33–44. [PubMed: 16358422]

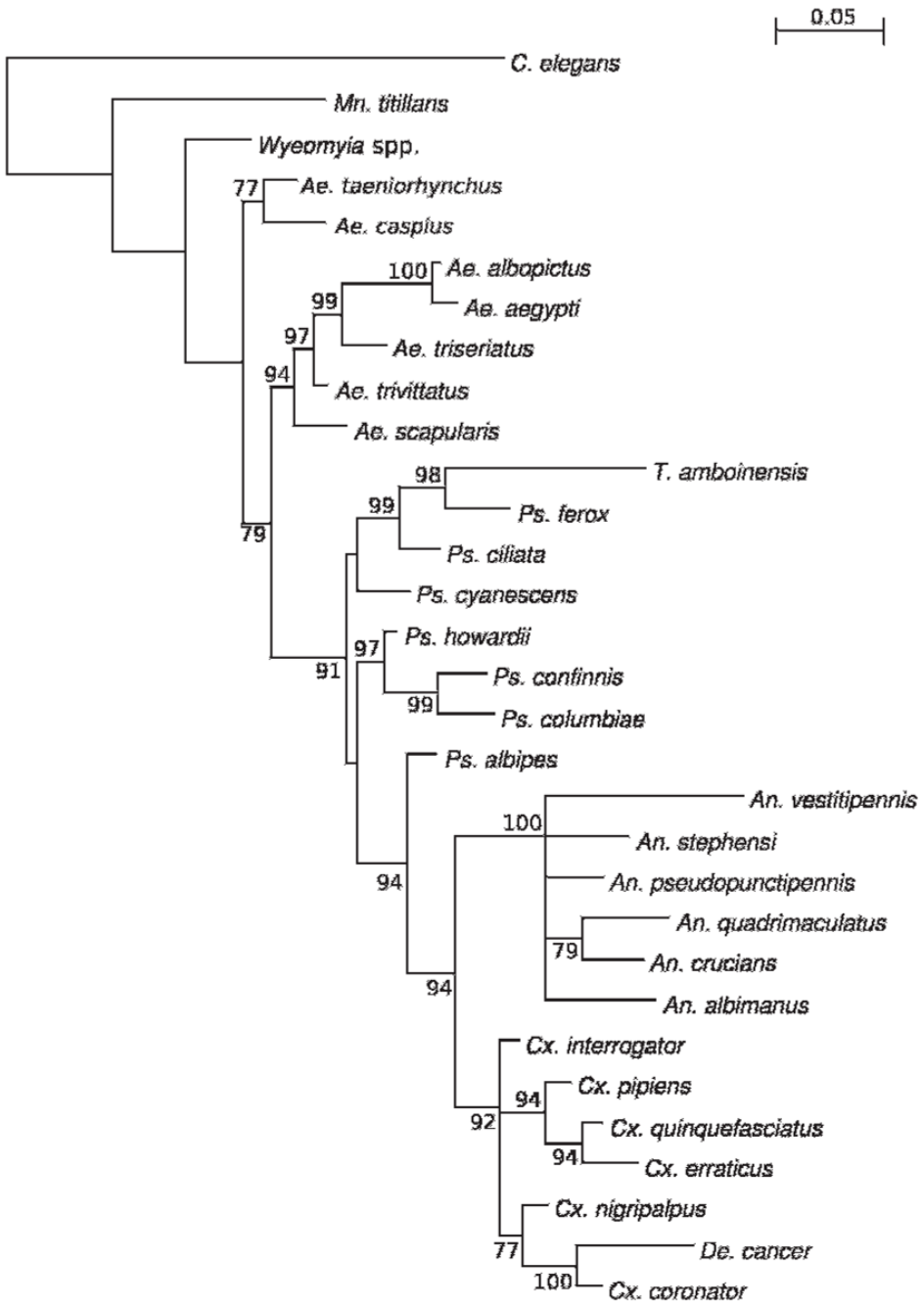

Fig. 1.

Phylogenetic analysis of a 641 nucleotide region of the actin-1 cDNA sequence of 30 species of mosquitoes. The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003). Posterior support (out of 100) for selected branches is indicated. All trees were midpoint rooted; the midpoint was on the branch to the homologous region of actin-1 of *C. elegans* (GenBank accession number NM_079643.1).

Table 1

Mosquito species used in this study, reactivities of actin-specific primers by RT-PCR, and GenBank accession numbers.

Species	Subfamily	Source of mosquitoes	Primer pair[1]			GenBank accession no.
			Act-2F + 2R	Act-2F + 3R	Act-2F + 8R	
Aedes aegypti	Culicinae	YP[2]	+[3]	+	+	GQ981439
Ae. albopictus	Culicinae	ISU[4]	+	+	+	GQ981440
Ae. caspius	Culicinae	ISU	+	+	+	GQ981456
Ae. scapularis	Culicinae	NM[5]	+	+	+	GQ981441
Ae. taeniorhynchus	Culicinae	NM	+	+	+	GQ981457
Ae. triseriatus	Culicinae	ISU	+	+	+	GQ981442
Ae. trivittatus	Culicinae	ISU	+	+	+	GQ981443
Anopheles albimanus	Anophelinae	YP	+	+	+	GQ981444
An. crucians	Anophelinae	NM	+	+	+	GQ981467
An. pseudopunctipennis	Anophelinae	NM	+	+	+	GQ981445
An. quadrimaculatus	Anophelinae	NM	+	+	+	GQ981446
An. stephensi	Anophelinae	ISU	+	+	+	GQ981447
An. vestitipennis	Anophelinae	YP	+	+	+	GQ981448
Culex coronator	Culicinae	NM	+	+	+	GQ981449
Cx. erraticus	Culicinae	NM	+	+	+	GQ981450
Cx. interrogator	Culicinae	YP	+	+	+	GQ981451
Cx. nigripalpus	Culicinae	YP	+	+	+	GQ981452
Cx. pipiens	Culicinae	ISU	+	+	+	GQ981453
Cx. quinquefasciatus	Culicinae	NM	+	+	+	GQ981468
Deinocerites cancer	Culicinae	YP	+	+	+	GQ981454
Mansonia titillans	Culicinae	YP	+	+	+	GQ981455
Psorophora albipes	Culicinae	YP	+	−	+	GQ981458
Ps. cyanescens	Culicinae	YP	+	+	+	GQ981459
Ps. ciliata	Culicinae	YP	+	+	+	GQ981460
Ps. columbiae	Culicinae	NM	+	+	+	GQ981461
Ps. confinnis	Culicinae	YP	–[6]	+	+	GQ981462
Ps. ferox	Culicinae	YP	+	+	+	GQ981463

Species	Subfamily	Source of mosquitoes	Primer pair[1]			GenBank accession no.
			Act-2F + 2R	Act-2F + 3R	Act-2F + 8R	
Ps. howardii	Culicinae	YP	+	+	+	GQ981464
Toxorhynchites amboinensis	Toxorhynchitinae	ISU	+	+	+	GQ981465
Wyeomyia spp.[7]	Culicinae	YP	−	+	+	GQ981466

[1] Sizes of RT-PCR products generated by primer pairs Act-2F + 2R, Act-2F + 3R and Act-2F + 8R are 728, 794, and 683 nt, respectively.

[2] Yucatan Peninsula of Mexico.

[3] Positive.

[4] Insectaries at Iowa State University.

[5] Northern Mexico.

[6] Negative.

[7] Mosquitoes were identified to the genus but not species level.

J Am Mosq Control Assoc. Author manuscript; available in PMC 2010 September 6.