Sequence, Secondary Structure, and Phylogenetic Analyses of the Ribosomal Internal Transcribed Spacer 2 (ITS2) in Members of the North American Signifera Group of *Orthopodomyia* (Diptera: Culicidae)

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ABSTRACT Mosquitoes of the genus *Orthopodomyia* (Diptera: Culicidae) are little known and of uncertain epidemiological importance. In the United States, there are three *Orthopodomyia species* (i.e., *Or. signifera* (Coquillett), *Or. alba* Baker, and *Or. kummi* Edwards); they are all members of the Signifera Group based on the current morphological taxonomy. In the course of identifying recently collected specimens, a problem was found with the current key morphological characters for separating the fourth instar larvae of *Or. signifera* and *Or. kummi*. Internal transcribed spacer two sequences of the rDNA were obtained to resolve the identities. The *Orthopodomyia* internal transcribed spacer two ranged in size from 193 (*Or. kummi*) to 244 bp (*Or. signifera*) (mean = 218 bp) and were slightly Adenine/Thymine enriched (44.7% Guanine/Cytosine on average). Putative secondary structures reveal structural homologies (four domains) consistent between species that also feature conserved sequences specific to mosquitoes (e.g., a conserved motif on the 3′ aspect of the longest helix: GARTACATCC). Sequence analyses suggest that in certain areas of southwestern North America, hybridization may occur between *Or. kummi* and *Or. signifera*. Furthermore, our analyses confirm that *Or. californica* (a junior synonym of *Or. signifera*) is indeed *Or. signifera*. To our knowledge, this is the first sequence-based phylogenetic and molecular analysis of the *Orthopodomyia*.

KEY WORDS ITS2 secondary structure, ribosomal DNA, *Orthopodomyia*, hybridization

There are five members of the Signifera Group of *Orthopodomyia* (Diptera: Culicidae) and only three (*Or. signifera* (Coquillett), *Or. alba* Baker, and *Or. kummi* Edwards) of these mosquito species occur in the United States (Zavortink 1968, Darsie and Ward 2005). *Orthopodomyia waverleyi* (Grabham) apparently is only endemic in Jamaica and *Or. pulcripalpis* (Rondani) is a Palearctic species that has been found in northwestern Europe, Asia Minor, and northern Morocco (Zavortink 1968, Ramsdale and Snow 2001). Previously, *Or. californica* Bohart was reduced to synonymy with *Or. signifera* (Zavortink 1968).

In North America, mosquitoes of the genus *Orthopodomyia* are little known and of uncertain epidemiological importance. The *Orthopodomyia* are generally tree hole dwelling during their immature life stages. However, they are also commonly found in artificial containers such as discarded tires (Hanson et al. 1995, Woodward et al. 1998, Qualls and Mullen 2006). Although the feeding behavior of these species is poorly understood, they are considered ornithophilic and nonanthropophagic (Carpenter and LaCasse 1955). *Or. signifera* has been shown to be a competent vector for Eastern Equine Encephalitis (EEE) and Western Equine Encephalitis viruses (Chamberlain et al. 1954). West Nile and EEE viruses, respectively, have been detected in naturally occurring *Or. signifera* from the continental United States (CDC 2012) and Mexico (Vargas 1960). Although they are not significant human pests, the role of these species in the enzootic transmission of arboviruses remains unclear.

Recently, during the course of identifying *Orthopodomyia* specimens collected from the southwestern United States, problems were encountered using morphological keys for identification (Byrd et al. 2009). Specifically, we were unable to consistently morphologically identify the fourth instar larvae of *Or. signifera* and *Or. kummi* found in sympatric areas of the southwestern United States. Our observations and problems using the current key characters (Darsie and Ward 2005) were also confounded by published descriptions of *Or. californica* (a junior synonym of *Or. signifera*) fourth instar larvae (Bohart 1950, Yamaguti and LaCasse 1951). Although *Or. californica* was synonymized with *Or. signifera* by Zavortink (1968) based predominantly on adult characters, the larvae of *Or. californica* would be identified as *Or. kummi* using

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Table 1. *Orthopodomyia* specimens included in phylogenetic analyses

Organism	Collection location	No. samples	Collector	ITS2 amplicon size[a]
Or. alba	Orleans Parish, LA	8	Authors	248
Or. alba	Concho County, TX	4	Authors	250
Or. signifera	Transylvania County, NC	11	Authors	276
Or. signifera	Lake County, CA	3	D. Woodward	284
Or. signifera	Indian River County, FL	4	Authors	266
Or. signifera	Orleans Parish, LA	6	Authors	266
Or. signifera	Santa Cruz County, AZ	5	Authors	286
Or. signifera	Sierra County, NM	4	Authors	291
Or. kummi	Brewster County, TX	9	Authors	246
Or. kummi	Santa Cruz County, AZ	7	Authors	240
Or. pulcripalpis	London, United Kingdom	4	Unknown	229

Representative sequences are deposited in GenBank (FJ867643-FJ867653).
[a] Note, the amplicon sizes reported here include 25 nts of the 5.8S and 22 nts of the 28S rDNA regions.

the current keys to North American mosquitoes by Darsie and Ward (2005). These observations are further confounded by the overlapping distributions of *Or. signifera* and *Or. kummi* and previous morphological studies suggesting that hybridization and introgression may occur between these two species (Zavortink 1968).

To address these issues, we sought to characterize and classify the North American members of the Signifera Group of *Orthopodomyia* using ribosomal DNA internal transcribed spacer two (ITS2) sequences. The nuclear rDNA array is composed of ribosomal RNA genes and spacer regions that are tandemly repeated up to ≈1,000 times in the mosquito genome (Kumar and Rai 1993). Each of the repeated units include regions for the three mature rRNA species (18S [SSU: small subunit], 5.8S and 28S [LSU: large subunit]), an external transcribed spacer (ETS) and two internal transcribed spacers (ITS1 and ITS2). Certain higher order (e.g., secondary and tertiary) RNA folding structures within the rRNA are necessary for pre-rRNA processing, maturation and subsequent ribosome synthesis and function. Although the spacers (i.e., ETS and ITS) are noncoding regions, they are essential for the synthesis of the mature rRNA species and are the site of concerted endo- and exonucleolytic posttranscriptional modifications before maturation of the active rRNA species (Venema and Tollervey 1999, Fromont–Racine et al. 2003).

The utility of the internal transcribed spacers to differentiate closely related and cryptic species is well established (Collins and Paskewitz 1996, Toma et al. 2002, Li and Wilkerson 2005). Similarly, the use of ITS sequences for phylogenetic studies is increasing (Koekemoer et al. 2009, Paredes–Esquivel et al. 2009) and recent studies suggest that inferences from ITS2 sequences and secondary structures strongly correlate with taxonomic classification (Coleman 2009). Here we describe the complete rDNA ITS2 sequences of the North American *Orthopodomyia* for classification and genetic characterization.

Materials and Methods

DNA Sources and Preparation. Specimens of *Orthopodomyia alba*, *Or. kummi*, and *Or. signifera* were collected from different localities in North America (Table 1). Specimens of *Or. pulcripalpis* were obtained from the British Natural History Museum. Genomic DNA was extracted from the samples with either the QIAmp DNeasy Tissue Kit-Insect Protocol (Qiagen, Valencia, CA) or the DNAzol reagent (Molecular Research Center, Inc., Cincinnati, OH) as recommended by the manufacturers. In all instances, genomic DNA was isolated from larvae, unmated individually reared adults, or tissue from other than the abdomen to prevent contamination by exogenous DNA.

Polymerase Chain Reaction (PCR) Amplification. Each 50 μl polymerase chain reaction (PCR) reaction mixture contained 3 μl of DNA template (2–25 ng/μl), 10.0 pmoles of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, and 22 U recombinant *Taq*DNA Polymerase (Invitrogen, Carlsbad, CA). The CP-P1A and CP-P1B primers (Wesson et al. 1992) were used to amplify the complete ITS2 from the 3′ end of the 5.8S gene to the beginning of the 28S gene. The ITS2 was amplified in 50 μl reactions with PCR conditions including an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 60 s, 58°C for 30 s, and 72°C for 60 s. The PCR was completed with a final extension of 72°C for 10 min. A negative control (H_2O en lieu of gDNA template) was included in each run. PCR amplified products were analyzed by electrophoresis on a 1.5% agarose gel and identified by ethidium bromide staining and ultraviolet (UV) illumination.

Sequencing. To obtain full length sequences of the ITS2, the PCR products were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen) and subcloned into TOPO-TA vectors (Invitrogen) according to the manufacturer's protocol. Plasmid mini-preps were purified using the Promega Wizard Plasmid Purification kit (Promega, Madison, WI) and incorporation of the PCR amplicon was confirmed by EcoR1 restriction digest of the plasmid and analysis by gel electrophoresis. In instances where the initial PCR amplicon appeared to have size polymorphisms, as evidenced by gel electrophoresis or direct sequencing heterogeneity, 8–11 clones were sequenced. In the absence of size polymorphisms or direct sequencing

heterogeneity, a minimum of two clones for each sample were sequenced. The plasmids were sequenced using the M13 sequencing primers by the Davis Sequencing Facility, University of California (Davis, CA) or the DNA Sequencing Core at the Center for Gene Therapy, Tulane University Health Sciences Center using the ABI BigDye Terminator v3.1 cycle sequencing chemistry. Electropherograms were analyzed and screened for vector contamination using the CodonCode Aligner (CodonCode Corporation, Dedham, MA) software. The sequences reported in this study were verified as ITS2 after evaluating the results of a BLAST query, secondary structure analysis, and the identification of specific sequence motifs known to exist on the ITS2 of all mosquitoes (Coleman 2007, Benson et al. 2009). Representative sequences for the major localities have been deposited in GenBank (Accession Numbers: FJ867643–FJ867653).

Sequence Analysis. DNA sequences were analyzed with the Accelrys Gene version 2.0 program (Accelrys, San Diego, CA) software. The flanking 5.8S and 28S sequences were identified using the Diptera model through the ITS2 annotation tool (Keller et al. 2009). Multiple sequence alignments were performed using the Clustal X software (Thompson et al. 2002). Secondary structure predictions were calculated using mfold version 3.2 with the default parameters (Zuker 2003). Conserved motifs were identified using multiple sequence alignments and secondary structure comparisons. Statistical analyses and distance measurements (divergence) were performed using the Molecular Evolutionary Genetics Analysis (MEGA) version four program (Tamura et al. 2007).

Phylogenetic Analysis. Phylogenetic analyses were performed with the neighbor-joining and maximum parsimony algorithms available in the MEGA four software program (Tamura et al. 2007). Gaps and missing data were considered complete deletions. Bootstrap sampling (1,000 replicates) was performed to test inferred phylogenies. Maximum likelihood analyses were performed incorporating the HKY + I + G model of nucleotide substitution, the base composition, the gamma (G) distribution of among-site rate variation, and the proportion of invariant sites (I) all estimated from the data. To explore the robustness of particular phylogenetic groupings, a bootstrap resampling analysis was undertaken. All maximum likelihood analyses were performed with the PAUP* package (Wilgenbusch and Swofford 2003). Bayesian analysis was performed using MrBayes (Ronquist and Huelsenbeck 2003) with the following settings. The maximum likelihood model employed six substitution types ("nst = 6"), with rate variation across sites modeled using a gamma distribution (rates = "gamma"). The Markov chain Monte Carlo search was run with four chains for 1,000,000 generations, with trees being sampled every 100 generations (the first 100 trees were discarded as "burnin").

Results

Nucleotide Compositions and Length. The studied taxa of North American *Orthopodomyia* (Table 1) showed an average ITS2 length of 218 bp and varied from 193 bp (*Or. kummi*) to 244 bp (*Or. signifera*). The ITS2 length of *Or. pulcripalpis* was 182 bp. The average Adenine/Thymine (AT) content was 55.3% (A = 24.8%, T(U) = 30.5%, G = 20.5%, and C = 24.2%). Intraspecies length differences (<1%) were mostly because of dinucleotide (CT) repeats that typically occur toward the 3′ end of the ITS2 before the 28S sequences. In instances where minor intraspecific variations were noted at the same locality the most common genotype was used for the phylogenetic analysis. Interspecies differences may be found throughout the ITS2 and correspond to structural differences based on putative secondary structures.

Intraindividual Variation. In most instances the intragenomic polymorphisms of the studied taxa were low or undetectable. However, in some instances clones selected from individual specimens of *Or. signifera* or *Or. kummi* collected in southeastern Arizona contained ITS2 sequences of both species. To further investigate this observation, a species specific internal primer was designed to detect *Or. signifera* ITS2 sequences. When used with the CP-P1A forward primer (Wesson et al. 1992) the *Or. signifera* primer (5′-CAAGCAGGGGTAAGTACACAAG-3′) produces a species specific amplicon product of ≈250 bp in length. This primer does not bind to the *Or. kummi* ITS2 sequences and therefore does not produce an amplicon. This primer was used to confirm the presence of *Or. signifera* ITS2 loci in specimens that contained PCR or clone dominant *Or. kummi* loci (Fig. 1). *Or. signifera* sequences were not detectable in *Or. kummi* collected in west Texas (lanes 1 and 2). These specimens were collected from the Chisos Mountains and are geographically isolated. However, *Or. signifera* ITS2 sequences were detectable in specimens (4 of 7) previously identified as *Or. kummi* in Arizona (lanes 3–6) in an area where the two species are known to occur sympatrically. The PCR dominant ITS2 sequence from these specimens were *Or. kummi* and matched with their morphological identifications. However, some level of *Or. signifera* ITS2 sequence is present in the genome of these individuals. When compared with the PCR amplicon band intensities of the *Or. signifera* specimens (Lanes 7–12), it suggests that the *Or. signifera* loci in the putative hybrid specimens are present at a much reduced number.

ITS2 Secondary Structures. The putative ITS2 secondary structures of all the study taxa were generated to aid in the construction of the multiple sequence alignment. In particular, the identification of homologous helices provided support for positional sequence homologies in the alignment. The ITS2 secondary structures of the *Orthopodomyia* are compatible with the eukaryote-universal ITS2 secondary structure (Coleman 2007). The putative ITS2 secondary structure of *Or. alba* (Fig. 2) serves as a model for all the taxa in this study. There are four structurally

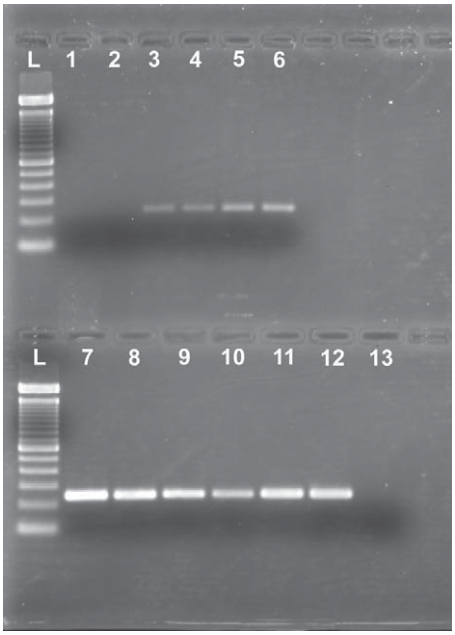

Fig. 1. *Orthopodomyia signifera* specific primer. The DNA standard ladder (L) is the Invitrogen 100 bp ladder. The 600 bp band appears brighter for internal orientation. The Signifera specific PCR products are 250 bp. Lanes 1–2, *Or. kummi* (TX); Lanes 3–4, *Or. kummi* (AZ); Lanes 5–6, *Or. kummi* (AZ); Lanes 7–8, *Or. signifera* (MS); Lanes 9–10, *Or. signifera* (NC); Lanes 11–12, *Or. signifera* (CA); Lane 13, Negative control.

homologous helices present in all of the studied taxa. However, the helices are designated with Roman Numerals I, IIa, IIb, and III to conform to the current universal paradigm. Consistent with previous studies, the hallmark structures supporting the putative ITS2 model for the *Orthopodomyia* are helices II (IIa in this study) and III (Coleman 2007). Helix IIa is highly conserved throughout the genus and is recognizable because it is unbranched and possesses a characteristic pyrimidine-pyrimidine mismatch. Helix III is longer than helix II and is branched in all of the study taxa.

All secondary structure foldings of the *Orthopodomyia* ITS2 possessed these universal structural homologies and a well conserved intermediate helix (IIb) between helices IIa and III. Furthermore, a conserved sequence motif (GARTACATCC) that can be found throughout the Culicidae is present on the 5′ side of helix III (Coleman 2007). In whole, the putative secondary structures aided in the generation of the multiple sequence alignment for the study taxa (Fig. 3). In addition, sequences in nonhelical regions between helix I and IIa (i.e., GCGCAATG) or between IIa and IIb (i.e., TCAA) were perfectly conserved in all species. The most conserved secondary structural elements were Helix IIa and IIb.

Phylogenetic Analyses and Estimates of Evolutionary Divergence. Signifera group phylograms were constructed based on the provided data matrix (Fig. 3) and rooted to *Or. pulcripalpis* as an outgroup species. Although *Or. pulcripalpis* is a member of the *Orthopodomyia* Signifera Group, it is a Palearctic species and is not found in the New World. Furthermore, phylograms with two outgroup species (e.g., *Culex pilosus* (Dyar & Knab): GenBank ID U33028 and *Anopheles maculipennis* (Meigen): GenBank ID FJ210892) from different genera yielded similar topologies for the *Orthopodomyia.* The phylogenetic data support a monophyletic relationship between *Or. signifera* and *Or. alba* that was well resolved statistically (boot-strap value: 92% and Bayesian probability of 100%; Fig. 4). Biogeographic relationships were not completely resolved in the *Or. signifera* clade. However, it is notable that the ITS2 from the California collections grouped within the *Or. signifera* clade. Phylogenetic analyses using neighbor-joining, parsimony, maximum likelihood and Bayesian inference generated trees with similar topologies. Pairwise estimates of evolutionary divergence between the ITS2 sequences are shown in Table 2.

Nucleotide Substitution Pattern. The nucleotide substitution pattern was examined using the maximum composite likelihood estimate (Tamura et al. 2004). The proportion of each type of nucleotide change was estimated using the total number of positions (Table 3). The overall transition/transversion bias is $R = 1.841$, where $R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]$. Interestingly, the observed substitution pattern has a slightly higher probability of mutation to G or to C (54.28%). This is in contrast to the overall nucleotide frequencies, where the sequences are slightly AT enriched (55.3%). In addition, we repeated the analysis on the data set after the removal of hypervariable sequences starting after the 3′ end of Helix III until the beginning of the 28S sequences. The overall substitution pattern did not change significantly and retained a higher probability of mutation to G or to C (53.54%). However, the overall transition/transversion bias decreased from 1.841 to 1.061.

Discussion

The ITS2 in the *Orthopodomyia* are slightly AT enriched, differing from the Guanine/Cytosine (GC) rich composition of many other arthropods, including other Culicinae (Wesson et al. 1992, Beebe et al. 1999). Despite this observation, the overall nucleotide substitution pattern revealed a higher probability of GC mutation. Limited intraspecific nucleotide polymorphisms in *Or. signifera* collected from throughout the United States suggests homogenization of the species specific array by concerted evolution (Williams et al. 1998, Ganley and Kobayashi 2007).

However, the observation of *Or. signifera* and *Or. kummi* ITS2 sequences within the same individual in specimens collected from areas in the southwestern United States where the two species live in sympatry supports previous statements by Zavortink (1968) suggesting that hybridization occurs between these species. A species specific internal primer was designed to detect the presence of *Or. signifera* ITS2 sequences in

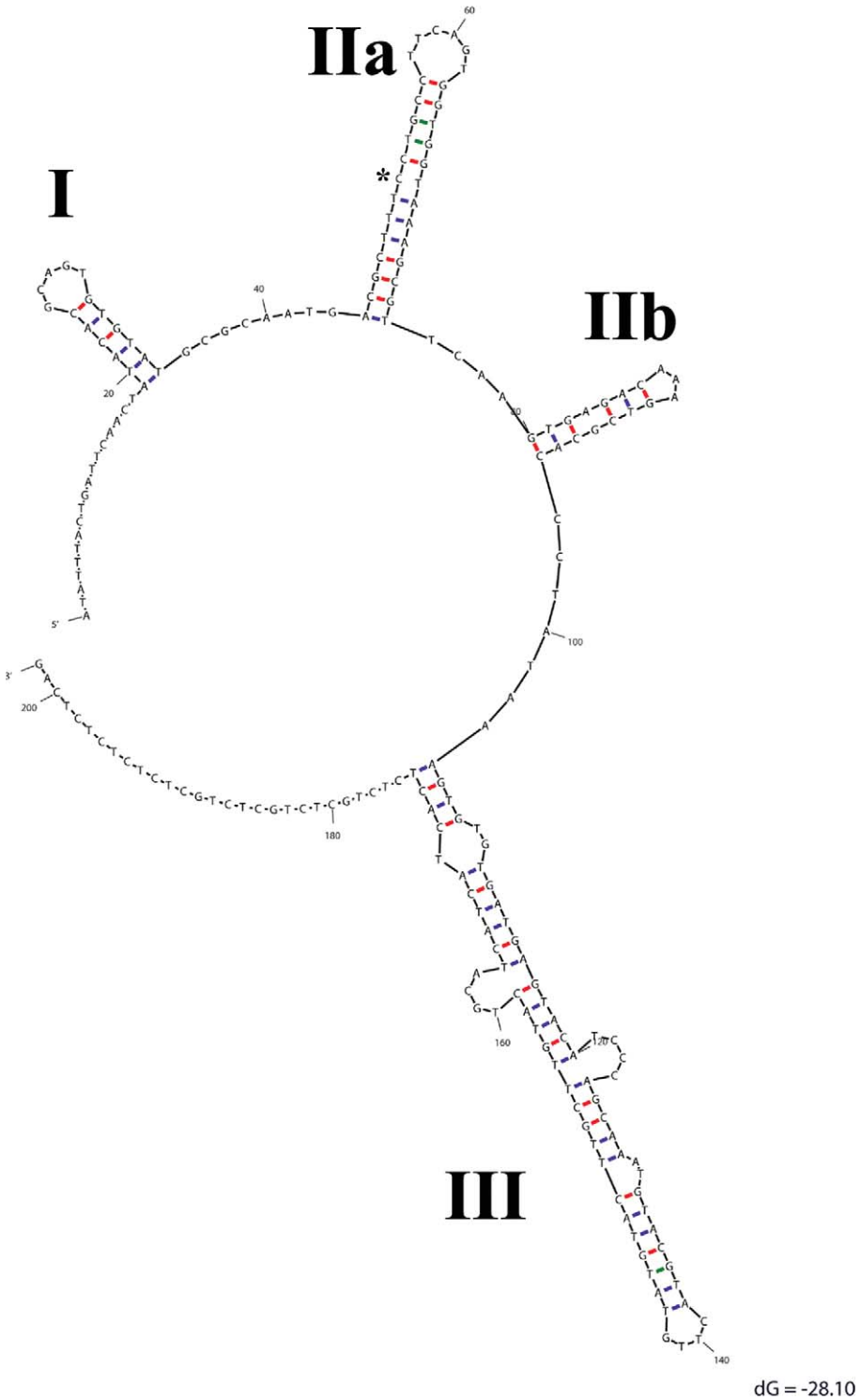

Fig. 2. Secondary structure diagram of *Orthopodomyia alba* ITS2. There are four consistent helices present in all of the studied taxa. However, the helices are designated with roman numerals I-III to conform to the current universal paradigm. The 5.8S and 28S (large subunit) sequences have been removed from the respective 5′ and 3′ ends of the ITS2. The asterisk designates the characteristic pyrimidine-pyrimidine (C or T[U]) mismatch of helix II that is consistent with the universal model. The mosquito (Culicidae) conserved motif (i.e., GARTACATCC) is found on the 5′ aspect of helix III. Sequences in nonhelical regions between helix I and IIa or between IIa and IIb were perfectly conserved in all species. (Online figure in color.)

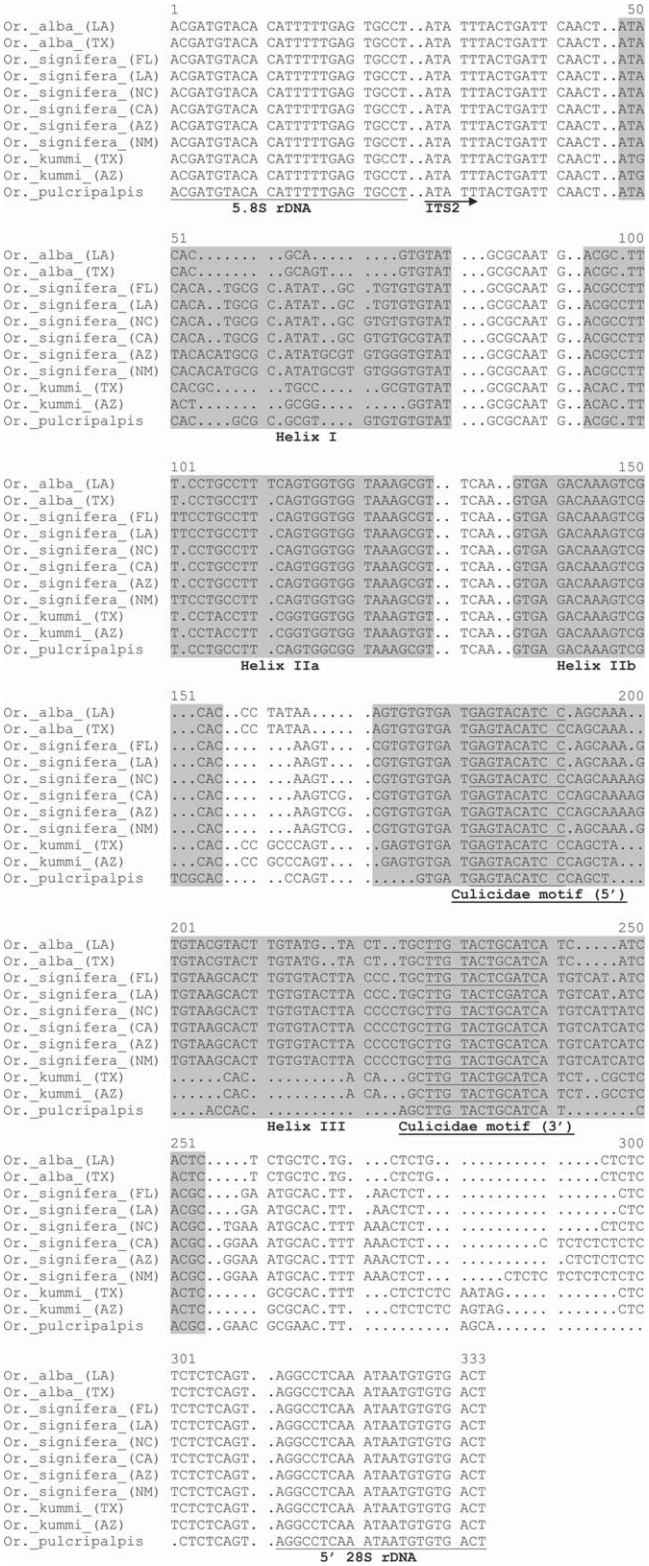

Fig. 3. Multiple sequence alignment.

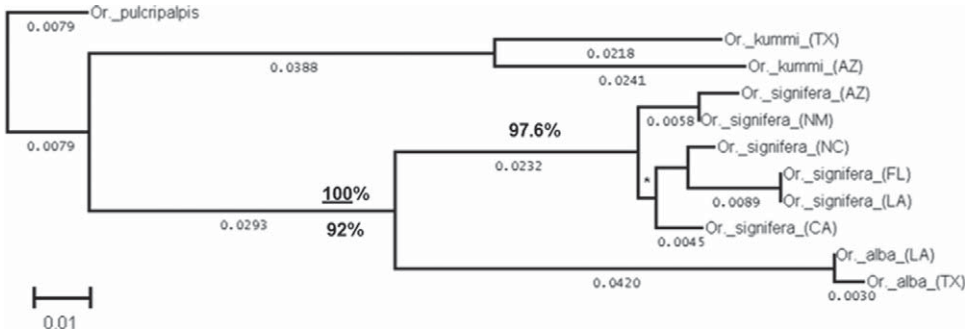

Fig. 4. Neighbor-joining (NJ) analysis (Kimura 2 Parameter); the tree is rooted to *Or. pulcripalpis.* Notable boot-strap values (1,000 replicates) are presented. All boot-strap values were >80% with the exception of one low level internal value marked with an asterisk (53% support). Bayesian probabilities are underlined.

Orthopodomyia specimens. Using this primer, we detected *Or. signifera* ITS2 sequences in specimens that had previously been both morphologically and molecularly identified by ITS2 sequencing as *Or. kummi.*

Comprehensive morphological studies led Zavortink (1968) to conclude that, with the exception of *Or. alba,* the larvae of North American members of the Signifera Group "cannot be adequately separated." In that regard, we recently encountered difficulties using the standard morphological characters (Darsie and Ward 2005) to differentiate *Or. signifera* and *Or. kummi* larvae in southwestern U.S. populations (Byrd et al. 2009). In light of these findings, it is likely that a series of concerted morphological and molecular studies will be required to solve these issues and validate any novel morphological characters.

Phylogenetic analyses were performed using neighbor-joining, maximum parsimony, maximum likelihood, and Bayesian algorithms. The small number of species in our study taxa limits our phylogenetic inference. However, the data suggest a monophyletic relationship between *Or. signifera* and *Or. alba.* This relationship is strongly supported analytically (boot strap values of 92% and a Bayesian probability of 100%) and is in concordance with previous work by Zavortink (1968) in which he divided the *Or. signifera, Or. alba,* and *Or. waverleyi* into a monotypic subgroup based on adult morphological characteristics. Unsuccessful attempts were made to amplify the ITS2 of *Or. waverleyi* as part of this study; limited specimen materials were available and they were not well preserved.

Previously, *Or. californica* Bohart, a species limited in distribution within California, was reduced to synonymy with *Or. signifera* (Zavortink 1968) based on adult morphology. Because the *Or. signifera* populations found in California are allopatric, specimens collected from Lake County, CA, were included in this study. Our sequence and phylogenetic analyses support the morphological evidence because *Or. signifera* (CA) clades within the signifera group based on the ITS2 sequences. In addition, inspection of the primary sequence homology coupled with the putative secondary structures did not reveal the presence of compensatory base changes (CBC). This supports the synonymy of *Or. californica* with *Or. signfera* because the difference of even one CBC in highly conserved portions of the ITS2 predicts the inability to cross, and therefore implies separate biological species (Coleman 2009).

The discovery of multiple ITS2 types within individual *Orthopodomyia* mosquitoes, indicating hybridization, is similar to that observed by Miller et al. (1996) between *Culex pipiens* L. and *Cx. quinquefasciatus* Say. These observations should be a reminder to investigators that the ITS2 is a product of biparental

Table 2. Estimates of Evolutionary Divergence Between Sequences

		1	2	3	4	5	6	7	8	9	10	11
1	*Or. alba* LA											
2	*Or. alba* TX	0.000										
3	*Or. signifera* NC	0.035	0.035									
4	*Or. signifera* LA	0.045	0.045	0.010								
5	*Or. signifera* CA	0.040	0.040	0.005	0.015							
6	*Or. signifera* FL	0.045	0.045	0.010	0.000	0.015						
7	*Or. signifera* AZ	0.040	0.040	0.005	0.015	0.010	0.015					
8	*Or. signifera* NM	0.035	0.035	0.000	0.01	0.005	0.010	0.005				
9	*Or. kummi* TX	0.088	0.088	0.078	0.088	0.083	0.088	0.083	0.078			
10	*Or. kummi* AZ	0.094	0.094	0.094	0.106	0.094	0.106	0.094	0.094	0.03		
11	*Or. pulcripalpis*	0.077	0.077	0.077	0.088	0.083	0.088	0.083	0.077	0.078	0.078	

The no. of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 11 sequences. Analyses were conducted using the Kimura 2-parameter method. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 204 positions in the final dataset.

Table 3. Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution in the Signifera Group

	A	T	C	G
A	—	*4.78*	*3.7*	**12.63**
T	*4.42*	—	**16.77**	*3.44*
C	*4.42*	**21.69**	—	*3.44*
G	**16.25**	*4.78*	*3.7*	—

Each entry shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The transition/transversion rate ratios are $k_1 = 3.677$ (purines) and $k_2 = 4.535$ (pyrimidines). The overall transition/transversion bias is $R = 1.841$. All positions containing gaps and missing data were eliminated from the dataset.

inheritance. Furthermore, the maternally inherited mitochondrial gene sequences, commonly used for "barcoding" initiatives, will not detect hybridization. Recent hybridization events generally produce offspring that initially possess both of the parental ITS2 genotypes. The recognition of such events may be missed if care is not taken to adequately isolate individual DNA sequences by cloning and subsequent sequencing. One mechanism to address this issue is to directly sequence the combined amplified products of multiple PCR runs. If polymorphic results appear during gel electrophoresis or from the direct sequencing of the combined products, a more thorough approach to cloning may be warranted and additional clones should be sequenced. In addition, it is easy to forget about life history traits and ecological phenomena when a field specimen is being processed into an aliquot of genomic DNA. Extreme care should be taken when choosing specimens for molecular studies. For taxonomic studies, we recommend that specimens should be individually reared and that the resulting exuviae be prepared or photodocumented for inclusion in a permanent collection. In addition, individually reared specimens should not have the opportunity to mate and therefore exogenous DNA from insemination will not be present. Similarly, if adult field specimens are used for molecular studies, DNA extraction from the abdomen should be avoided from female specimens.

To our knowledge, the work described here represents the first sequence-based phylogenetic and molecular analysis of the *Orthopodomyia.* Although the studied taxa are not significant human pests, reports of naturally acquired infections of EEE and WNV by *Or. signifera* correlate with previous studies suggesting ornithophilic feeding behavior (Chamberlain et al. 1954, Carpenter and LaCasse 1955, CDC 2012). Because these species are nonanthropophagic, they are not considered epidemiologically important from a human health perspective. However, they may play a role in the enzootic transmission of arboviruses among the avian populations and therefore may have an indirect impact on human health. Therefore, the species' role in the transmission of arboviruses remains unclear and should be studied further.

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