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Use of DNA barcoding to distinguish the malaria vector *Anopheles neivai* in Colombia

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Abstract

A reference 535 bp barcode sequence from a fragment of the mitochondrial gene cytochrome oxidase I (*COI)*, acquired from specimens of *An. neivai* Howard, Dyar & Knab, 1913 from its type locality in Panama, was used as a tool for distinguishing this species from others in the subgenus *Kerteszia*. Comparisons with corresponding regions of *COI* between *An. neivai* and other species in the subgenus (*An. bellator* Dyar & Knab 1906, *An. homunculus* Komp 1937*, An cruzii* Dyar & Knab, 1908 and *An. laneanus* Corrêa & Cerqueira, 1944) produced K2P genetic distances of 8.3–12.6%, values well above those associated with intraspecific variation. In contrast, genetic distances among 55 specimens from five municipalities in the Colombian Pacific coastal state of Chocó were all within the range of 0–2.5%, with an optimized barcode threshold of 1.3%, the limit for unambiguous differentiation of *An. neivai*. Among specimens from the Chocó region, 18 haplotypes were detected, two of which were widely distributed over the municipalities sampled. The barcode sequence permits discrimination of *An. neivai* from sympatric species and indicates genetic variability within the species; aspects key to malaria surveillance and control as well as defining geographic distribution and dispersion patterns.

Keywords

DNA Barcode; Malaria; *Kerteszia*; *COI*

Introduction

In the Neotropical region, Colombia is second only to Brazil in number of malaria cases each year (Chaparro & Padilla 2012). Among malaria vectors in Colombia, *Anopheles* (*Kerteszia*) *neivai* Howard, Dyar & Knab, 1913 is often considered to be a secondary vector due to localized distribution, apparent natural infection rates and its larval habitat (Gutiérrez *et al.* 2008; Sinka *et al.* 2010). However, in the Pacific Coast of Colombia it may be

considered a primary vector of malaria, especially in isolated municipalities where medical access is still limited such as Litoral de San Juan (Charambirá) and Santa Bárbara-Iscuandé (Astaiza *et al.* 1988; Murillo *et al.* 1988). Those municipalities are located within mangrove environments, which are ideally suited to *An. neivai*, a species that undergoes larval development in bromeliads. Within this region, in Buenaventura, Valle del Cauca, 4.76% of this species has been found to be naturally infected with *Plasmodium falciparum* (Gutiérrez *et al.* 2008). In the mangrove environment of this region, the relative abundance of *An. neivai* surpasses other known malaria vectors, such as *An. albimanus* Wiedemann, 1820, and its biting activity has been shown to peak at dawn and dusk, times of increased fishing activity by locals (Escovar *et al.* 2013).

From a phylogenetic perspective, *An. neivai* occurs within the subgenus *Kerteszia* Theobald, which includes several species associated with malaria transmission and whose larvae undergo development primarily in bromeliads located in forests (Zavortink 1973). While one of the earliest recognized species of the subgenus *Kerteszia*, it has an extensive geographic distribution extending from Chiapas, Mexico to Peru and Bolivia (Stone *et al.* 1959; Zavortink 1973). Distinguishing *An. neivai* often has been difficult; questionable records of its distribution exist, especially from topographic areas of relatively high elevation (González & Carrejo 2009).

Evidence from molecular variability in mitochondrial DNA fragments and variation in morphological characters suggests that the current concept of *An. neivai* may represent a complex of closely related species (Linton 2009; Montoya-Lerma *et al.* 1987). Several Neotropical species of *Anopheles* have been shown to consist of one or more sibling (cryptic) species (Rosa-Freitas *et al.* 1998). Even though closely related, such species may exhibit ecological and behavioral differences that may affect their malaria transmission potential or susceptibility to insecticides (Collins & Paskewitz 1996). Molecular markers are now being used to elucidate the presence of cryptic species within long established malaria vectors such as *An. cruzii*, *An. albitarsis* Lynch Arribálzaga, 1878, and *An. triannulatus (Neiva & Pinto, 1922)* (Gómez *et al.* 2013; Gutiérrez *et al.* 2010; Lehr *et al.* 2005; Rona *et al.* 2009, 2012; Rosero *et al.* 2012; Silva-do-Nascimento *et al.* 2011).

In the current study we evaluated a fragment of mitochondrial Cytochrome Oxidase I (*COI*), which represents the barcode region as described by Hebert *et al.* (2003, 2004). Standardized mitochondrial fragments from *COI* (DNA barcodes) have been used to identify mosquitoes (Cywinska *et al.* 2006; Harrison *et al.* 2012; Kumar *et al.* 2007; Ruiz-Lopez *et al.* 2012), including species from the Colombian Andes (Rozo-Lopez & Mengual 2015). Also, in Colombia, DNA barcodes were used to distinguish *An. calderoni* Wilkerson, 1991, *An. punctimacula* Dyar & Knab, 1906 and *An*, *malefactor* Dyar & Knab, 1906; part of a closely related species group with high morphological similarities and of medical importance, especially with regard to *An. calderoni* (González *et al.* 2010). DNA barcodes also have been used to evaluate intraspecific variability, especially in some Neotropical *Anopheles* (Gutierrez *et al.* 2010; Jaramillo *et al.* 2011; Mirabello & Conn 2006). Thus, when haplotype diversity in the malaria vector *An. darlingi* Root, 1926 was assessed among several municipalities in two western states of Colombia, many shared haplotypes were encountered, inferring a high level of gene flow (Gutierrez *et al.* 2010). Similarly, in the

same region of Colombia, low genetic differentiation was noted among populations of the malaria vector *An. nuneztovari* Gabaldon, 1940, where previous evidence suggests the existence of a species complex (Jaramillo *et al.* 2011).

The present study was designed to obtain a reference barcode sequence from specimens of *An. neivai* from its type locality in Panama and to evaluate the efficacy of this barcode region to differentiate *An. neivai* from other species in the subgenus *Kerteszia*. Also, haplotype diversity in *An. neivai* was evaluated over a similar size area as that described for *An. darlingi* and *An. nuneztovari* (Gutierrez *et al.* 2010; Jaramillo *et al.* 2011) but for the distinctive Pacific coastal region of Colombia. Haplotype structure in *An. neivai* was further contrasted between the Colombian municipalities and the species' type locality in Panama.

Materials and methods

We collected specimens from the type locality at Portobelo, Colon Panama; as well as five municipalities within the Colombian department of Chocó: Acandí, Jurubidá, Nuquí, Bahía Solano, and Litoral de San Juan (Fig. 1, Table 1). The Colombian localities were chosen on the basis of epidemiological indications of malaria transmission (SEM 1957, Astaiza *et al.* 1988, INS 2015). While a few adult specimens were obtained using aspirators, most were collected as larvae and pupae in tank bromeliads and reared to adults under laboratory conditions similar to those described by Pecor & Gaffigan (1997). Fourth-stage larval and pupal exuviae, as well as male genitalia, associated with some of the reared specimens were mounted in Euparal following procedures presented by Pecor & Gaffigan (1997). Identification of many specimens (n = 34) was based on morphological characters as presented in Zavortink (1973), González & Carrejo (2009) and Harrison *et al.* (2012). Specimens were deposited at Museo Entomológico Francisco Luis Gallego, at Universidad Nacional de Colombia sede Medellin (MELFG). Additionally, thirty-one specimens were later identified on the basis of the *COI*-barcode sequences obtained, and added to the genetic pool for analysis.

Total DNA was extracted from each sample using the macerate method of Collins *et al.* (1987), as adapted by Uribe *et al.* (1998) and (Uribe *et al.* 2001) in a final elution volume of 50 μL. A 650–700 bp fragment of the *COI*-barcode region was amplified with the primers described in Kumar *et al.* (2007) because the primers provided in Folmer (1994) failed to yield consistent positive amplicons. PCR amplification was carried out in a final volume of 30 μL, including 6 μL of GoTaq® 5X buffer (Promega), 1.2 μL of dNTP (2.5 mM), 0.48 μL of $MgCl_2$ (25 mM), 0.6 μL of each primer, 0.18 μL of GoTaq® DNA polymerase and 2 μL of purified DNA. All PCR amplifications were verified using electrophoresis on a 1% agarose gel with Gelstar® (Lonza), only positive PCR products were purified for cycle sequencing using a MultiScreen®HTS Vacuum Manifold (EMD Millipore). DNA sequencing was performed on both strands for each positive PCR product using BigDye 3.1® sequencing kit (ThermoFisher Scientific), in a 10 μL reaction including 2 μL ABI 5X dilution buffer, 0.5 μL Big Dye, 1 μL of primer and 2 μL of PCR product. Both strands were sequenced. Each sequencing reaction were purified using BigDye XTerminator® purification kit (ThermoFisher Scientific) using 45 μL of SAM solution, 10 μL XTerminator

and 2 μL cycle sequencing for each sample. Finally, all samples were analyzed in an ABI 3500XL® (Applied Biosystems) automated capillary sequencer.

Sequence quality was assessed with Sequence Scanner 1.0® (Applied Biosystems 2011) based on the quality estimators CRL and QV20. Consensus sequences were obtained with Bioedit 7.25 (Hall 1999) and Geneious ® 6 (Kearse *et al.* 2012). A multiple alignment was constructed using Muscle (Edgar 2004). The final aligned data matrix was comprised of 535 base pairs. The presence of nuclear copies of mitochondrial origin (NUMTs) was evaluated as in Hlaing *et al.* (2009). To verify the identity of the amplified region, the sequences were aligned with the whole mitochondrial genome of *An. albitarsis* (NCBI accession number NC020662). Sequences from the type locality were compared to deposited sequences of species in the subgenus *Kerteszia* in GenBank using the Blast algorithm implemented by Altschul *et al.* (1990) and, also, by using the Barcoding of Life (BOLD) Identification System (IDS) described by Ratnasingham & Hebert (2007). The sequences included were: *An. cruzii* (KU551285), *An. homunculus* (KU551283); *An. laneanus* (KU551288) as part of recent *Kerteszia* mitochondrial genome sequences from Oliveira *et al.* (2016), as well as those of *An. bellator* (KU551287), *An. lepidotus* Zavortink, 1973 (JQ041286) and *An. pholidotus* Zavortink, 1973 (JQ041288-87). In addition, sequence from a collected specimen of *An. marajoara* Galvao & Damasceno, 1942 and a *COI* sequence of *An. albitarsis* (NC020662) were included, since the subgenus *Nyssorhynchus* Blanchard is closely related to *Kerteszia* (Harbach 2013). No barcode sequences of *An. neivai* from the type locality were recovered from GenBank. From the BOLD database, 51 records of sequences described as *An. neivai* were retrieved, 33 of which originated from Colombia. However, none of the sequences have been published nor are they available from the BOLD database.

Polymorphism and haplotype diversity (Hd) in the *COI* barcoding region of *An. neivai* was evaluated using DNASP 5 (Librado & Rozas 2009). Intra- and inter-species variation (barcoding gap) was estimated from genetic distances based on the K2P model that distinguishes between transitions and transversions (Kimura 1980), available in MEGA 6 (Tamura *et al.* 2013). A threshold for barcode gap at the species level was calculated for detecting the possible presence of cryptic species using the SPIDER package (Brown *et al.* 2012), available in R 3.2 (R Developement Core Team 2015). To further explore the existence of cryptic species associated to *An. neivai*, a statistical parsimony network was obtained using TCS 1 (Clement *et al.* 2000), based on previously identified haplotypes. Comparisons with other species in the subgenus *Kerteszia and An. albitarsis* were derived from K2P distances using criteria presented by Hebert *et al.* (2003) and Kumar *et al.* (2007). A K2P based dendrogram with bootstrap support was estimated using the APE package (Paradis 2012), also available in R.

Results

Genetic variability within the 535 base pair barcode region of *COI* was examined for 65 individuals of *An. neivai*. Within the type locality (n=10), three haplotypes were encountered, which were divergent by four substitutions (Tables 1 and 2). These haplotypes were compared with sequences available in GenBank for other species of the subgenus *Kerteszia*. The pairwise genetic similarity between *An. neivai* and other species of *Kerteszia*

ranged between 88–92%: *An. pholidotus* (90–91%), *An. lepidotus* (88–90%), *An. cruzii* (90%), and *An. homunculus* (92%). Differences between the sequences of *An. neivai*, other species of *Kerteszia* and *An. albitarsis* are presented in terms of genetic distance (K2P distances) in Fig. 2. In this figure the first dotted box (A) of the three distinct ranges presented represents intraspecies distances for *An. neivai*, which varied between 032.5%. The second range (B) represents the differences between *An. neivai* and other species in *Kerteszia* (*An. pholidotus*, *An. lepidotus*, *An. cruzii* and *An. homunculus*), which varied between 8.3 to 12.6 %. The last range (C) represents differences between specimens of *Kerteszia* and *An. albitarsis,* which varied from 13 to 15.2%. For *An. neivai*, a barcode threshold of 1.3% (Fig. 3) was calculated, indicating the limit at which accumulative errors are minimized and no concurrence occurs with the distance rank of specimens assigned to another taxonomic identity (species) nor are nonspecific DNA sequences present (false and positive negatives) (Brown *et al.* 2012). The neighbor joining dendrogram in Fig. 4 presents a summary of K2P distances and infers that all *An. neivai* sequences conform to a single cluster with 100% bootstrap support. Considered together, these results indicate a clear separation of *An. neivai* from other species in the subgenus *Kerteszia*.

Among the 55 specimens of *An. neivai* from five municipalities in the Chocó region of Colombia, 18 *COI* haplotypes were detected in association with 26 polymorphic sites. All variation among these sites were transitions with the exception of a single transversion. The Colombian haplotypes varied from the Panamanian ones by 6312 nucleotide differences (Table 2). A majority of the Colombian specimens were from the municipalities of Bahía Solano (21) and Litoral de San Juan (19). While 11 haplotypes were detected from Bahía Solano only 5 were observed from Litoral de San Juan. Two Colombian haplotypes (1 and 8), differing by a single transition, were the most frequently encountered with similar distributions spanning approximately 500 km. In addition, they exhibited a close relationship to most of the other Colombian haplotypes (Table 1, Fig. 5). Haplotype diversity (Hd), Nei (1987) ranged from 0 at Nuquí (3 specimens) to 0.99 at Bahía Solano (21 specimens), while in Litoral de San Juan (19 specimens) and Portobelo (10 specimens) Hd values were similar (0.67–0.69 respectively).

Discussion

With an extensive geographic distribution and indications of both morphologic and molecular variability, emphasis was placed on developing a molecular marker for *An. neivai* based on specimens originating from its type locality in Panama (Linton 2009; Montoya-Lerma *et al.* 1987; Zavortink 1973). Despite of its usefulness for most metazoans, the barcode universal primers (Folmer 1994) do not always produce consistent PCR products for specimens preserved under differing conditions. Furthermore, presence of mutations at nucleotide positions where primers anneal require alternative primers sets in order to produce *COI* DNA barcodes (Hajibabaei *et al.* 2006; Kumar *et al.* 2007; Park *et al.* 2010). The 535 bp *COI* barcode region utilized in this study appears to clearly distinguish *An. neivai* from other species in *Kerteszia* (*An. bellator, An. homunculus, An. cruzii, and An. laneanus*) as revealed by K2P genetic distance divergences of 8.3312.6%. This range is similar to the interspecific divergence (mean 8.2%, range 7.638.7%) derived from *COI* sequences by Harrison *et al.* (2012) for comparisons between *An. pholidotus* and *An.*

lepidotus. Also, using the *COI* barcoding region, Linton (2009) observed interspecific differences averaging 7.2% among four species of *Kerteszia*. Furthermore, comparisons between Panamanian specimens of *An. neivai* and those from the Pacific coast of Colombia revealed only small differences in K2P distances, all less than 2.5%, and, thus, below the barcode limit of 4% (0–3.9 %) for defining a species [single taxonomic unit] (Cywinska *et al.* 2006; Escovar *et al.* 2012; Foster *et al.* 2013; Kumar *et al.* 2007; Ruiz-Lopez *et al.* 2010). In addition, Arregui *et al.* (2015) report differences from 3–7% between species complexes, such as *An. albitarsis* and *An. oswaldoi, Peryassú, 1922*. Considered together, these results indicate that the 535 bp *COI* barcoding region appears to be an effective tool for differentiating *An. neivai*.

With the relative ease in which *COI* barcode sequences are now produced and their unique specificity, the *COI* barcode region provides a means to evaluate the extensive geographic distribution of *An. neivai* and may be helpful in evaluating the existence of sibling or cryptic species from different environments and geographic regions (Hebert *et al.* 2003). Indications of morphological variation in *An. neivai* have been noted by Montoya-Lerma *et al.* (1987), and differences exceeding 3% in the *COI* barcode region have been reported by Linton (2009) among specimens from Colombia, Ecuador and Venezuela. This level of variability could indicate the presence of cryptic species, especially when compared to local distributions in Colombia and the species type locality. When comparing mutation rates of the same sequence region in *An. fluviatilis* James 1902, differences reached only 0.8 % including those between forms *S* and *T* (Kumar *et al.* 2013).

The presence of two highly frequent haplotypes (1 and 8), which differed by a single transition, among specimens from Colombia (Fig. 2) suggests they may represent hypothetical ancestors from which many of the other haplotypes were derived as was suggested for *An. pseudopunctipennis* Theobald, 1901 (Dantur Juri *et al.* 2014). Both of these haplotypes were most abundant at the southernmost Colombian municipality sampled, and while also present near the Panamanian border, they were not encountered at the type locality in Panamá. The minimal number of mutations between specimens of *An. neivai* from the type locality and those from Colombia was six. Based on a larger fragment of the *COI* gene, 978 bp, Mirabello & Conn (2006) found differences of seven mutational steps between specimens of *An. darlingi* from Central America and Colombia. Comparisons of haplotype divergence among *Kerteszia* species by Lorenz *et al.* (2015) detected a magnitude of difference between *An. cruzii* and *An. homunculus* of 23 mutations.

While *An. neivai* is primarily associated with low elevation coastal regions, several records of its presence at relatively high elevations occur in the literature including municipalities in the Colombian Departments of Antioquia, Cundinamarca, and Boyacá (González & Carrejo 2009; SEM 1957), the Venezuelan Andes (Rubio-Palis 1991) and Ecuador (Arregui *et al.* 2015). The purported presence of *An. neivai* in atypical environments may be evaluated on the basis of barcode sequences. Such analyses would be appropriate for delimiting *An. neivai*'s distribution, from the perspectives of both elevation and geographic range, and also for detecting potential cryptic species. In turn, this knowledge should be beneficial for understanding *An. neivai*'s role in malaria transmission and for undertaking control measures.

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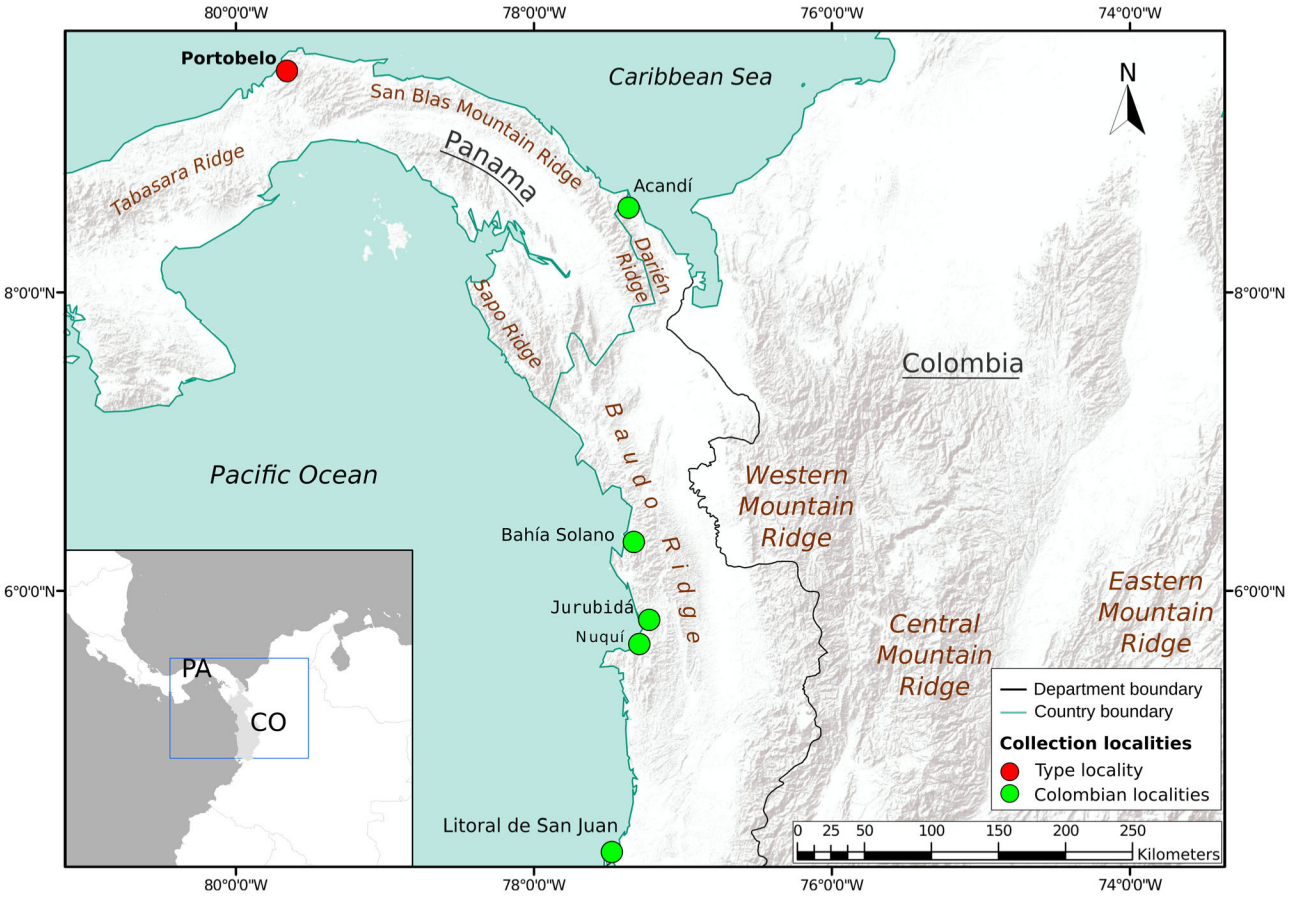

FIGURE 1.
Collection municipalities map for *Anopheles neivai* in Colombia (CO) and Panama (PA).

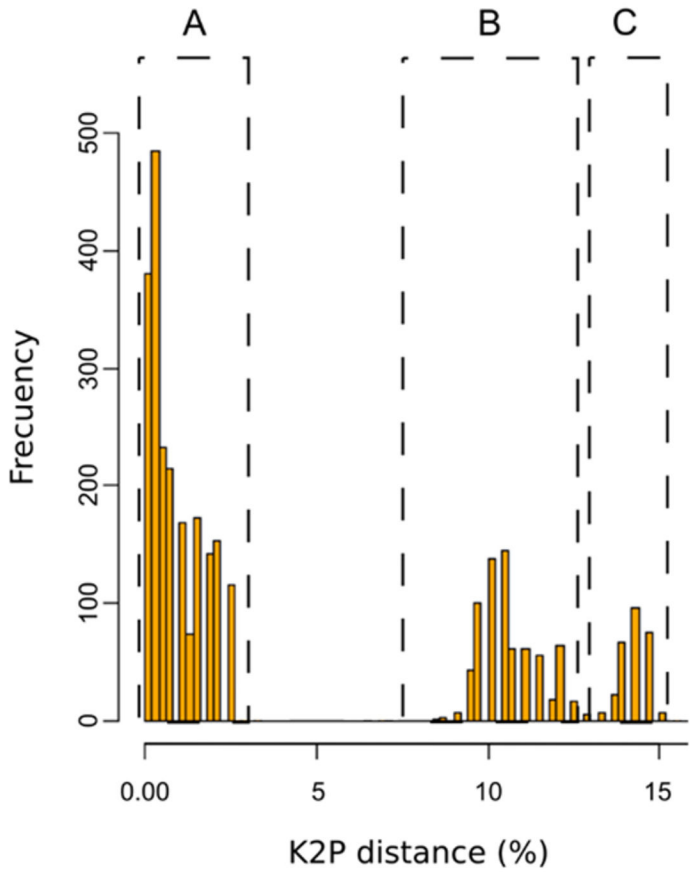

FIGURE 2.

Genetic differences (K2P) among *An. neivai* collected specimens (A) and against other species from *Kerteszia* (B) and *Nyssorhynchus* from NCBI (C).

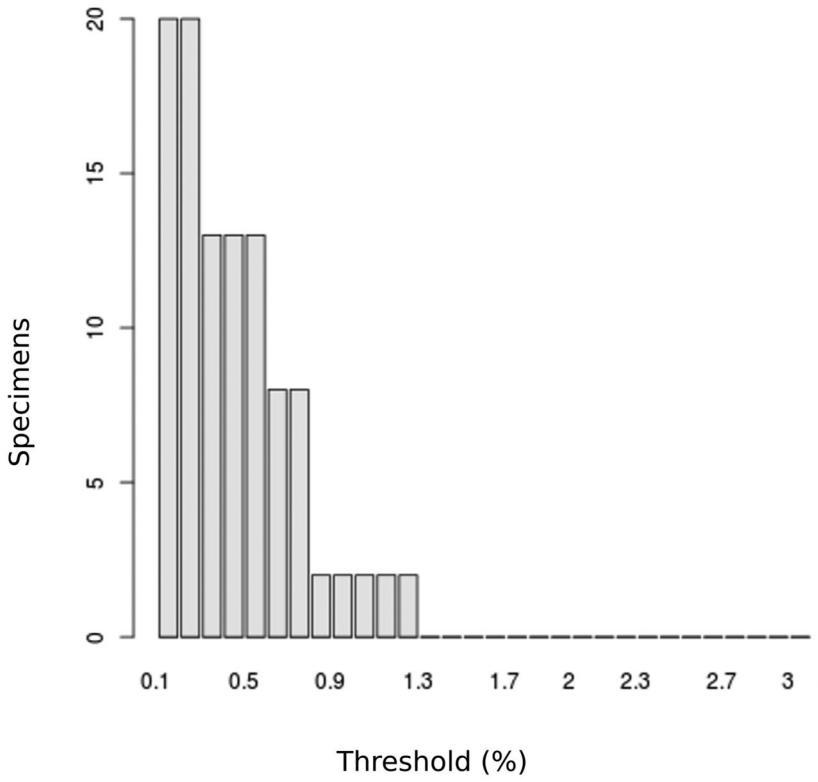

FIGURE 3.
DNA barcode threshold optimization for *An. neivai.*

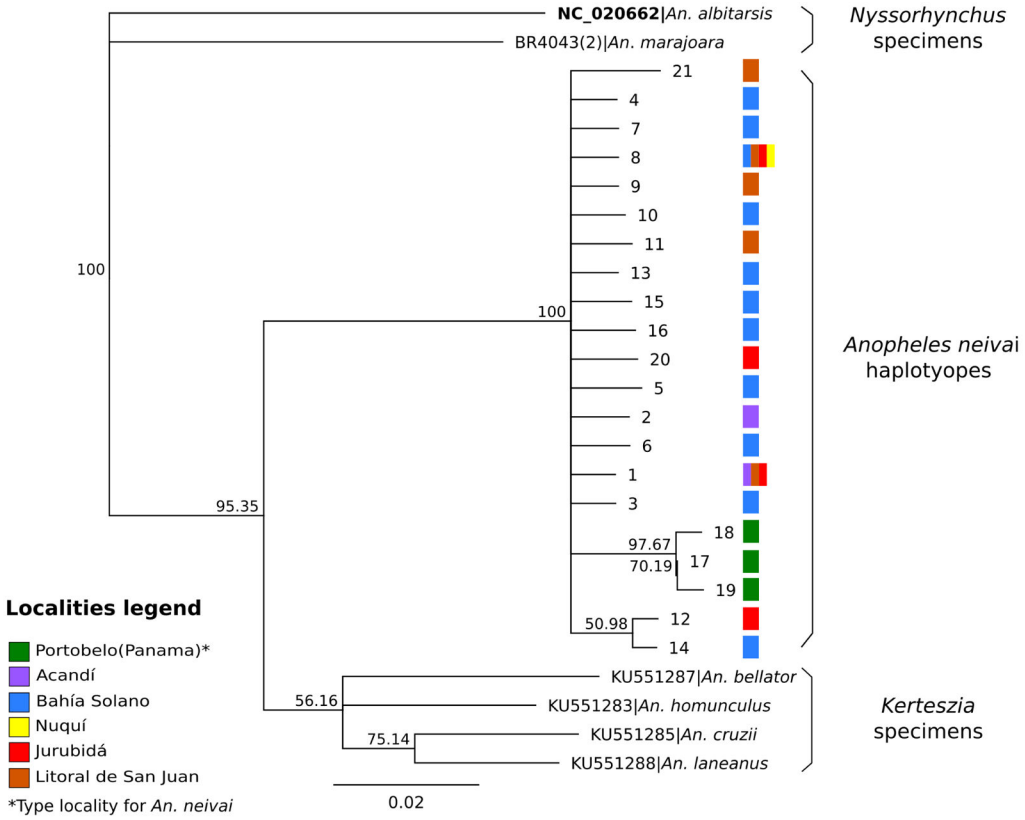

FIGURE 4.

Dendrogram (K2P) for *An. neivai* specimens from Colombia, Panama, with other *Kerteszia* and *Nyssorhynchus* species (based on a 535 bp *COI* alignment. Branch support was provided by bootstrap resampling (10000 replicates).

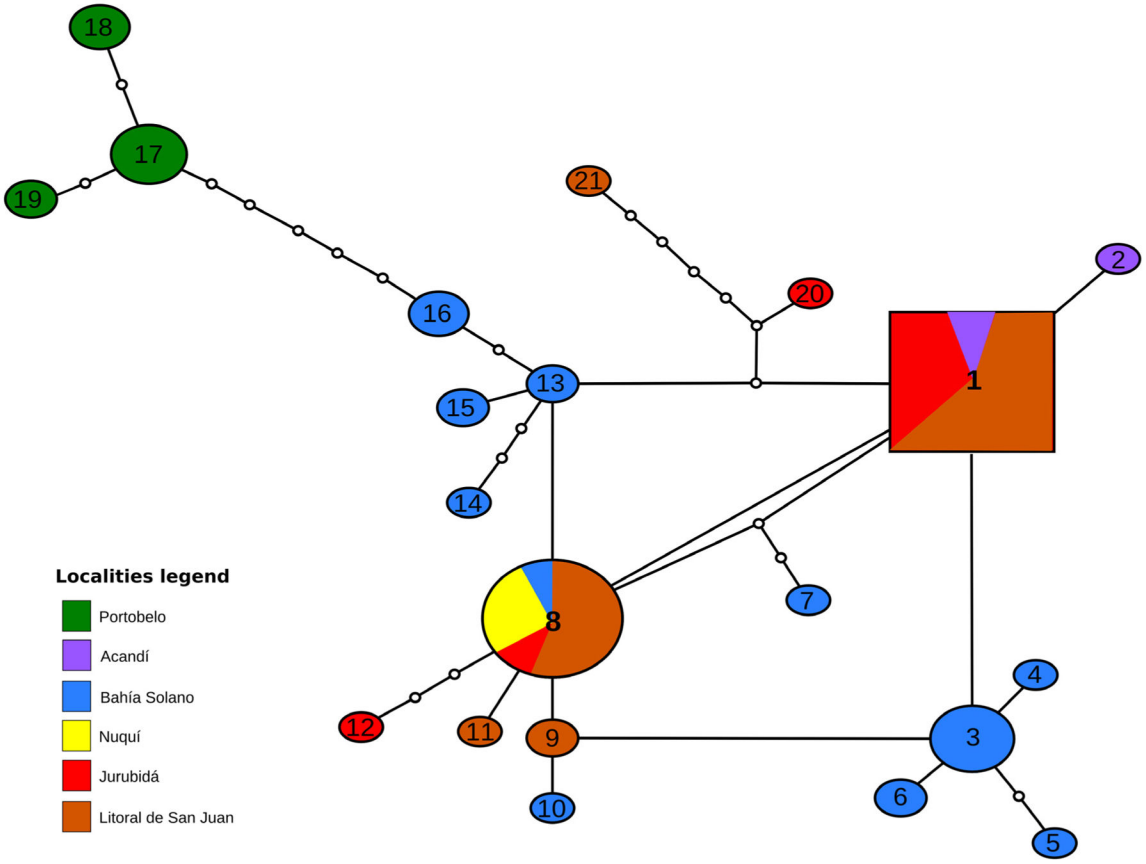

FIGURE 5.

Haplotype network for *An. neivai* collected in Colombia and Panama (based on a 535 bp *COI* alignment).

TABLE 1

Collection information for *Anopheles neivai* in Panama and Colombia and its associated haplotypes (based on a 535 bp *COI* alignment).

Country, Dept.	Municipality	n	Haplotype (n)	Capture site: [NCBI register/Museum Catalog No (life stage*)]
Panama, Colón	Portobelo (**Pbelo**)[a]	10	17 (5)	**cs1**: [KM234373/NC_27056 (L), †KM234396 (L), †KM234409 (L), KM234433/NC_27057 (L)]
				cs2: [KM234435/NC_27059(A)]
			18 (3)	**cs1**: [†KM234385(L), †KM234400(L), †KM234429(L)]
			19 (2)	**cs1**: [KM234402/NC_27058 (L), †KM234415(L)]
Colombia, Chocó	Acandí (**Aca**)	2	≠1	**cs3**: [†KM234371(F)]
			2	**cs3**: [†KM229741(F)]
	Jurubidá (**Juru**)	10	≠1 (6)	**cs4**: [KM234376/NC_27055 (F)]
				cs5: [†KM234401(A), †KM234404(A), †KM234405(A), †KM234406(A), †KM234419(A)]
			≠8 (2)	**cs5**: [†KM234398(A), †KM234425(A)]
			12	**cs5**: [†KM234411(A)]
			20	**cs5:** [†KM234387(A)]
	Nuquí (**Nq**)	3	≠8 (3)	**cs6**: [†KM234403/ NC 26137 (M), †KM234432(F)]
				cs7: [†KM234413/ NC 26136 (M)]
	Bahía Solano (**BS**)	21	3 (6)	**cs8**: [KM234375(A), †KM234395(F), KM234397(A), KM234408(A), †KM234434(F)]
				cs9: [†KM234412(F)]
			4	**cs8**: [KM234389(A)]
			5	**cs10**: [†KM234420(M)]
			6 (2)	**cs11**: [†KM234379(M)]
				cs8: [KM234416(A)]
			7	**cs8**: [†KM234381(A)]
			≠8 (2)	**cs8**: [KM234374(A), KM234378(A)]
			10	**cs8**: [†KM234388(F)]
			13 (2)	**cs8**: [†KM234372(M), KM234424(A)]
			14	**cs8**: [KM234417(A)]
			15	**cs8**: [KM234384(A)]
			16 (3)	**cs8**: [KM234380(A), †KM234382(F), KM234391(A)]
	Litoral de San Juan (**LSJ**)	19	≠1 (9)	**cs12**: [†KM234383(F), KM234386(A), KM234394(A), KM234399(A), KM234410(A), KM234426(A), KM234428(A), KM234430(A), †KM234431/NC 26433(F)]
			≠8 (7)	**cs12**: [KM234390(A), KM234393/NC_26391 (A), KM234414(A), †KM234418(F), †KM234421(F), KM234423(A), †KM234427(F)]

Country, Dept.	Municipality	n	Haplotype (n)	Capture site: [NCBI register/Museum Catalog No (life stage*)]
			9	**cs12**: [KM234422(A)]
			11	**cs12**: [KM234407(A)]
			21	**cs12**: [KM234392(A)]

*[L: larvae, A: adult, M: adult Male, F: adult Female].

[a]Type locality for *An. neivai.*

cs: capture site

[†]species previously identified with taxonomy based in morphology,

[‡]haplotypes found in several municipalities.

TABLE 2

Variable sites in *An. neivai* haplotypes (based in a 535 pb *COI* alignment).

Haplotype	n	Municipality	Variable sites (nucleotide position)																													Nd[b]	K2P %[c]
				23*	50	77	101	100	101+	155	167	179	191	200	206	219	245	257	266	267	269	273	293	294	314*	356*	368	380	433*	466	483	512		
17	5	Pbelo[a]	A	G	G	T	C	C	A	T	A	C	T	A	T	T	A	T	C	T	A	T	A	G	C	C	C	T	G	A	G	C		
18	3	Pbelo[a]	.	A	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	0.37
19	2	Pbelo[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	2	0.37
1	16	Aca, Juru,LSJ	T	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	.	7	1.31
2	1	Aca	T	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	A	.	8	1.50
3	6	BS	T	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	T	8	1.50
4	1	BS	T	.	A	.	.	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	T	T	.	A	.	.	T	9	1.68
5	1	BS	T	.	A	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	.	T	T	T	.	A	.	.	T	10	1.87
6	2	BS	T	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	T	T	.	A	.	.	T	9	1.68
7	1	BS	C	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	.	7	1.31
8	14	BS, Nq, Juru, LSJ	T	.	A	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	.	8	1.50
9	1	LSJ	T	.	A	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	T	9	1.68
10	1	BS	T	.	A	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	C	A	.	.	T	10	1.87
11	1	LSJ	T	.	A	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	.	9	1.68
12	1	Juru	T	.	A	.	.	T	G	.	G	.	C	.	C	.	.	.	.	.	.	.	.	.	T	T	T	.	A	.	.	.	11	2.06
13	2	BS	T	.	A	.	.	.T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	A	.	.	.	7	1.31
14	1	BS	T	.	A	.	T	T	G	.	G	.	.	.	.	.	.	.	.	.	.	.	G	.	T	T	.	.	A	.	.	.	10	1.87
15	1	BS	T	.	A	.	.	T	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	T	.	.	A	.	.	.	8	1.50
16	3	BS	C	.	A	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	.	A	.	.	.	6	1.12
20	1	Juru	T	.	A	.	.	T	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	T	T	.	.	A	.	.	.	8	1.50
21	1	LSJ	T	.	A	.	.	T	.	C	.	T	.	G	.	.	.	.	T	.	.	.	.	A	T	T	.	.	A	G	.	.	12	2.24

Municipality acronyms: Pbelo: Portobelo (Panama, Colon); Aca: Acandí, Bahía Solano (BS), Nq: Nuquí, Juru: Jurubidá, LSJ: Litoral de San Juan (Colombia, Chocó).

*Sites that separate Panama from Colombia.

[+] Transversion

[a] Type locality for *An. neivai*.

[b] Nucleotide differences against Haplotype 17.

[c] K2P % distance (%) against Haplotype 17.

Average K2P distance (%) between samples from Colombia and Panama: 1.62; between samples from Colombia: 0.56.