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Research paper

Intraspecific phylogeny of *Anopheles (Kerteszia) neivai* Howard, Dyar & Knab 1913, based on mitochondrial and nuclear ribosomal genes

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ABSTRACT

Three mitochondrial regions and a fragment of a large nuclear ribosomal subunit was used to study the evolutionary patterns of *An. neivai*, a mosquito inhabiting mangroves and tropical forest in the lowland and coastal areas of the Yucatan Peninsula through the Pacific Ecuadorian coast. This species exhibits epidemiological importance regarding Malaria transmission in natural ecosystems, particularly in rural areas of the Pacific Colombian coast. The results based on phylogenetic networks and Bayesian inference showed no robust evidence supporting the existence of previously suggested cryptic species. Diversification patterns in geographically widespread species such as this one, are complex and therefore could impact malaria control strategies. Further studies focused on behavior, morphology, and phylogenomics will improve the understanding of the evolutionary patterns within *An. neivai* and its role as a disease vector.

1. Introduction

Anopheles (*Kerteszia*) *neivai* Howard Dyar & Knab, 1913 (Diptera: Culicidae) is widely distributed from the Yucatan Peninsula in Mexico through the northern pacific coast in Ecuador, inhabiting lowland tropical forests and mangroves in coastal areas (Forattini, 2002; Knight and Stone, 1977; Zavortink, 1973). Epidemiological evidence indicates *An. neivai* serves as a vector for malaria in the Colombian Pacific Coast in several localities such as Charambirá, Santa Bárbara–Iscuandé, and Buenaventura (Escovar et al., 2013; Murillo et al., 1988; Olano et al., 1997).

Despite the reports of primary vectors for this area such as *An. darlingi*, and *An. albimanus* (Montoya-Lerma et al., 2011), the available epidemiological data from *An. neivai* and other secondary culicid vectors suggest these species of mosquitoes should be considered as important vectors for disease within their local range (Sinka et al., 2010). In this context, and for malaria-control purposes, a precise taxonomical identification and knowledge of the vector species is essential (Higa et al., 2010; Müller et al., 2013). There are several sections within the *Anopheles* genus where cryptic species –two or more distinct species classified as a single species– are recognized, thus contributing to the difficulty of proper taxonomic assignment and appropriate control activities (Bickford et al., 2007; Collins and Paskewitz, 1996; Rosa-Freitas et al., 1998). Moreover, the vectorial capacity for transmission of *Plasmodium* spp. may vary across different cryptic species of *Anopheles* and therefore a robust taxonomic assessment of the genus is critical for malaria control efforts (Stevenson and Norris, 2017).

Studying the evolutionary patterns in suspected cryptic species can help to identify recently diverged species within a species complex (Bourke et al., 2013). Nowadays, molecular markers are widely used to examine evolutionary patterns and to reconstruct phylogenies in anopheline mosquitoes at several systematic scales, including intraspecific phylogenies (Moreno et al., 2013; Wilkerson et al., 2005). The purpose of constructing a multilocus phylogeny is to merge all available data sources in order to provide a more robust assessment of species limits. Several strategies for analyzing multilocus datasets exist including matrix concatenation, total evidence, and more recently used, a species tree (Igea et al., 2015; Schlick-Steiner et al., 2010; Yeates et al., 2011).

Regarding tropical anophelines, multilocus phylogenies improved the understanding of the evolutionary patterns in *An. triannulatus*, a vector of malaria in Brazil, with epidemiological importance in Peru and Venezuela. Phylogenetic analyses using mitochondrial *COI*, nuclear

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Table 1
Collection Information for sampled localities.

Country	Location	Species (sample number)	Latitude	Longitude	Altitude (masl[a])
Brazil	Capanema (CAP)	*Anopheles marajoara* (1)	1°11' 45.62” S	47°9' 41.50” W	31
Colombia	Sierra Nevada de Santa Marta (MAG)	*An. pholidotus* (1)	11° 5' 48.0” N	74° 4' 33.8” W	1689
	Acandí (ACA)	*An. neivai* (2)	8° 34' 39.81” N	77° 23' 56.31” W	200
	Bahía Solano (BAH)	*An. neivai* (6)	6° 21' 40.81” N	77° 21' 23.89” W	32
	Nuquí (NUQ)	*An. neivai* (8)	5° 41' 24.1” N	77° 15' 16.7” W	26
	Litoral de San Juan (LIT)	*An. neivai* (5)	4° 16' 14.1” N	77° 29' 34.3” W	8
Panama	Portobelo (POR)	*An. neivai* (6)	9° 29' 59.4” N	79° 41' 30.96” W	16
Guatemala	Puerto Barrios (PUE)	*An. neivai* (4)	15° 40' 23.34” N	88° 41' 27.96” W	912
	Chiquimula (CHI)	*An. neivai* (4)	14° 50' 48.60” N	89° 40' 36” W	1743

[a] Meters above sea level.

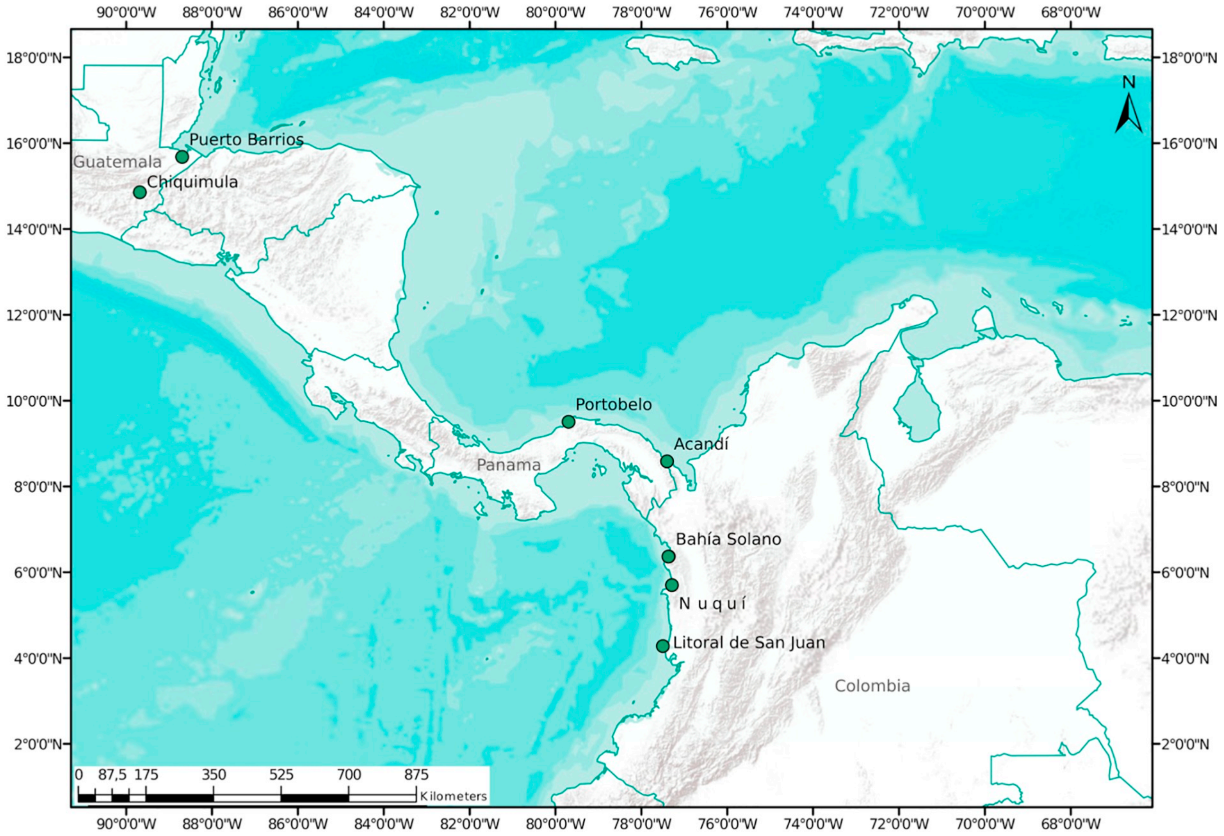

Fig. 1. Collection map for specimens in Colombia, Panama and Guatemala.

ITS2 and *White* DNA sequences revealed two major and well-differentiated groups corresponding to specimens collected from the Amazon and Andean regions (Moreno et al., 2013). In addition, the use of three circadian clock genes and three encoding ribosomal proteins in *An. cruzii*, considered as a secondary malaria vector at the Brazilian Atlantic forest, showed the existence of sibling or incipient species (Rona et al., 2012). In all these studies, the use of multilocus phylogenies were used to identify the smallest diagnosable cluster of individuals, according to the phylogenetic species concept (De Queiroz, 1998).

An. neivai, within the subgenus *Kerteszia*, is proposed as a species complex based on morphological, behavioral, and genetic data including spot scale color and pattern variations in the R_{4+5} vein (Montoya-Lerma et al., 1987), biting activity, and variability in the DNA *COI* barcoding region (Linton, 2009). In particular, the available DNA barcode genetic data suggested at least three different genetic groups for specimens distributed across Guatemala, Colombia, Venezuela and Ecuador (Ahumada et al., 2016; Suaza et al., 2009). However, the evolutionary patterns in *An. neivai*, including topotypic specimens have not been explored.

In the present study, the evolutionary patterns of *An. neivai* in three mitochondrial regions and a ribosomal gene fragment were explored to determine the presence of possible cryptic species, using total-evidence and taxonomic congruence approaches.

2. Materials and methods

Specimens of *An. neivai* were collected from seven locations in South and Central America (Table 1), including the type locality at Portobelo (Panama) (Fig. 1). Adult specimens were collected with Shannon and CDC traps, while immatures (larvae and pupa) were collected in bromeliads and reared under laboratory conditions to collect exuviae as a source of morphological evidence and species confirmation. Morphological identifications were based on the taxonomic keys for adult and immature specimens from (Escovar et al., 2012; González and Carrejo, 2009). In addition, other species from *Kerteszia* (*An. pholidotus*) were included along with specimens from *Nyssorhynchus* (*An. braziliensis* and *An. marajoara*), as this taxon is the recognized sister group of *Kerteszia* (Harbach, 2013, 2007; Sallum et al., 2002). All voucher specimens were

Table 2

Primer sequence and thermal profile used for PCR and cycle sequencing experiments.

Molecular region/ primers	PCR annealing temperature/time
COI (Kumar et al., 2007)/Kum07-F (GGATTTGGAAAT-GATTAGTTCCTT) Kum07-R (AAAAATTTTAATTCCAGTTGGAACAGC)	48–51 °C/ 60s
ND4 (Gorrochotegui-Escalante et al., 2000)/ND4+ (GTDYATTTATGATTRCCTAA) ND4- (CTTCGDCTTCCWADWCGTTC*)*	48 °C/36s
Cytb–TRNASer–IG1–*ND1* (Ready et al., 1997)/CB3FC (CAYATTCAACCWGAATGATA) NINFR GGTAYWTTGCCTCGAWTTCGWTATGA)	47 °C/30s
D1–D2 (Porter and Collins, 1996)/ CP12 (GTGGATCCAGTCGTGTTGCTTGATAGTGCAG) CP15 (GTGAATTCTTGGTCCGTGTTTCAAGACGGG)	62 °C/34.8s

deposited at the Francisco Luís Gallego Entomological Museum (MEFLG), National University of Colombia, Medellín. Field permit for collecting trips were authorized under decree 1376, June 27th 2013, Ministerio de Medio Ambiente, República de Colombia, collecting permits for Guatemala and Panama were obtained through USDA importation and transit of vectors permit issued to the Centers for Disease Control and Prevention and collecting permit issued to Gorgas Institute for Health Research respectively (available upon request).

For DNA extraction purposes, two legs on each adult specimen were removed as well as abdominal segments for those in larval stage. The DNA extraction procedure was performed using the macerate buffer protocol for insects (Collins et al., 1987; Uribe et al., 1998). Subsequent Polymerase Chain Reaction (PCR) for amplifying Cytochrome c oxidase subunit I (*COI*) corresponding to the DNA *COI* barcoding region, NADH dehydrogenase subunit 4 (*ND4*), a fragment between Cytochrome b and NADH dehydrogenase subunit 1 (*Cytb–tRNASer*–IG1–*ND1*) and two variable regions from the *28S* ribosomal RNA (*D1–D2 loops*) were performed separately using 33 µL molecular grade H_2O; 5 uL Buffer 10×; 2.5 µL dntp [5 mM]; 3 µL $MgCl_2$ [50 mM]; 1 µL [10 mM] for each primer set and 0.3 µL of Taq Bioline® (London, United Kingdom). Primer sequences and their respective thermal profiles are provided in Table 2. All PCR products were evaluated using agarose gel electrophoresis (Lonza Gelstar®; Basel, Switzerland). For the successful products, subsequent purification was performed on a Millipore multiscreen HTS vaccuum Manifold® (Billerica, USA).

DNA sequencing reaction was performed for each region, using the primers described in Table 2 with the following conditions: 0.5 µL [10 mM] of primer, 2 µL of purified PCR product, 4.5 µL molecular grade H_20, 2 µL ABI® 5× sequencing buffer, 0.5 µL Applied Biosystems BigDye Terminator® V1.1 (Waltham, USA). The thermal conditions for cycle sequencing were as follows: 96 °C/ 60 s (denature), 50 °C/4 s (anneal), and 60 °C/120 s (extension), for 25 cycles. For the purification of cycle sequencing products, removal of salt excess and unincorporated terminators, we used Applied Biosystems BigDye Xterminator® (Waltham, USA). All purified products, were analyzed in an Applied Biosystems ABI 3500XL® capillary electrophoresis sequencer (Waltham, USA) at the Centers for Disease Control and Prevention (CDC), Atlanta, USA.

Raw chromatograms, were analyzed in terms of quality estimators such as the Continuous Read Length CRL (CRL) and Quality Value over 20 (Qv20+), using Applied Biosystems Sequence Scanner 1.0 (Waltham, USA). The resulting DNA consensus sequences from high quality chromatograms were built using the CAP 3 assembly program (Huang and Madan, 1999) available in Geneious® 9 (Kearse et al., 2012). A Basic Local Alignment Search Tool (BLAST) was performed for each sequence to verify positional homology for the sequenced regions. The absence of nuclear mitochondrial DNA segments (NUMT) (Lopez et al., 1994) were verified following previously published procedures (Hlaing et al., 2009). Searches for publicly available *An. neivai COI* sequences were performed using GenBank (Benson et al., 2013) and the barcoding of Life Database (BOLD) (Ratnasingham and Hebert, 2007). These sequences were used as a complementary tool for the taxonomic

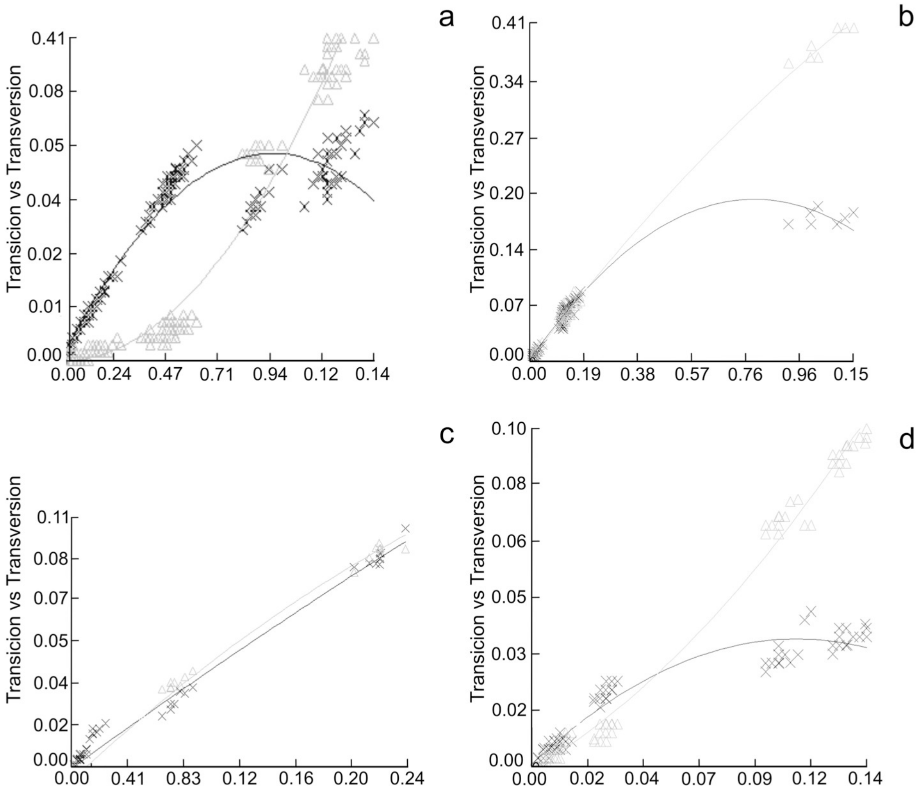

Fig. 2. Transition (Xs) vs. Transversion (▲v) rates over a distance (K80) among mitochondrial and nuclear ribosomal regions. a) *COI*, b) *ND4*, c) *D1–D2*, d) *Cytb–TRNASer*–IG1–*ND1*.

Table 3
Congruence values from distance matrices (CADM) for *COI*, *D1–D2*, *Cytb–tRNASer*–IG1–*ND1* and *ND4* regions.

Region	CADM[a] (W)[b]
COI, *Cytb–tRNASer*–IG1–*ND1*	0.921
COI, *D1–D2*	0.886
D1–D2, *Cytb–tRNASer*–IG1–*ND1*	0.851
COI, *D1–D2*, *Cytb–tRNASer*–IG1–*ND1*	0.848
Cytb–tRNASer–IG1–*ND1*, *ND4*	0.653
COI, *Cytb–tRNASer*–IG1–*ND1*, *ND4*	0.649
COI, *D1–D2*, *ND4*, *Cytb–tRNASer*–IG1–*ND1*	0.648
D1–D2, *ND4*	0.646
COI, *ND4*	0.636
COI, *D1–D2*, *ND4*	0.630
D1-D2, *Cytb–tRNASer*–IG1–*ND1*, *ND4*	0.621

[a] **Statistical hypothesis for CADM**
H$_0$: Distance matrix is not congruent among regions.
H$_1$: Distance matrices are congruent among regions.
[b] Kendall's concordance estimator (Kendall and Smith, 1939; Legendre, 2005).

identification of the specimens collected herein.

The phylogenetic signal for each region was evaluated based on the substitution saturation index (Xia et al., 2003), and the transition/transversion divergence plot based on the Kimura distance model (Kimura, 1980), available in DAMBE 6 (Xia, 2017). Prior to concatenation, a set of congruence tests were implemented to provide statistical support for all possible combinations of markers, including the Incongruence Length Difference (ILD) test (Farris et al., 1994) implemented in the HOMPART command with an heuristic search and 560 homogeneity replications in PAUP* 4.0b10 (Swofford, 2003), the *Likelihood Ratio Test* (LRT) (Huelsenbeck and Bull, 1996) with 100 bootstrap replicates in Concaterpillar 1.7 (Leigh et al., 2008), and the Congruence Among Distance Matrices (CADM) (Legendre and Lapointe, 2004) test implemented in R (R Developement Core Team, 2015) using the APE package (Paradis et al., 2004) and the Kimura distance model (Kimura, 1980). We examined the molecular model of evolution for each of the selected regions from the concatenated matrix using JModeltest 2 (Darriba et al., 2012). We explored three analyses to estimate the intraspecific phylogeny. First, a phylogenetic network was calculated using Splitstree 4 (Huson and Bryant, 2006) and the Neighbor-Net method (Bryant and Moulton, 2004) with Maximum Likelihood based distances (Schmidt et al., 2002). Subsequently, a Bayesian Inference approach was used for a molecular clock under the Yule speciation model hypothesis (Aldous, 2001; Yule, 1925), and a multilocus species tree approach under coalescence (Heled and Drummond, 2010), both estimated using BEAST 2 (Bouckaert et al., 2014). In addition, the estimation for each separate region used the same criteria for the network approach and the Bayesian inference as above.

3. Results

A total of 35 specimens of *An. neivai*, one *An. marajoara* and one *An. pholidotus* were studied using three molecular regions. *COI* exhibited a length of 596 bp fragment with 65 polymorphic sites, supporting the existence of 17 haplotypes. For the other mitochondrial regions such as *Cytb–tRNASer*–IG1–*ND1,* 12 polymorphic sites were found, with 15 haplotypes in a 415 bp fragment. In the *ND4* region, there were 58 polymorphic sites within a 357 bp fragment, with 13 haplotypes. In contrast, the *D1–D2* nuclear ribosomal gene fragment, consisted of a 469 bp with 11 polymorphic sites and seven haplotypes. In terms of transition and transversion rates over genetic distances, *ND4* exhibited higher substitution rates when compared to *COI, Cytb–tRNASer*–IG1–*ND1 and D1–D2,* which showed the lower rates among all regions (Fig. 2). Accession records for each sequence are available in Table S1.

Comparison of the newly sequenced specimens to available *COI* records from GenBank, resulted in the identification of a single cluster

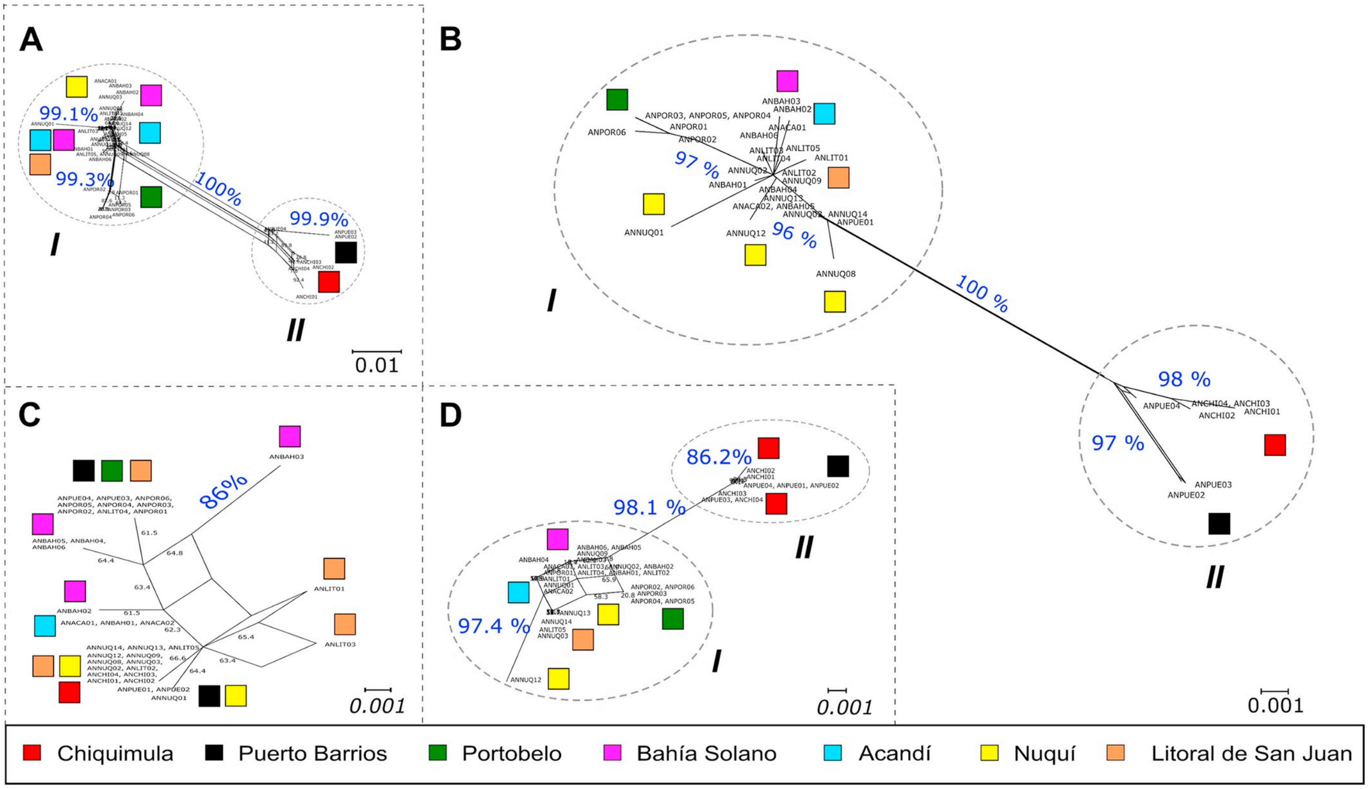

Fig. 3. Phylogenetic network (1000 bootstrap replicates and 95% credibility) for *COI* (A), *Cytb–tRNASer*–IG1–*ND1* (B) *D1–D2* (C) and the concatenation of *COI*, *Cytb–tRNASer*–IG1–*ND1*, *D1–D2* (D).

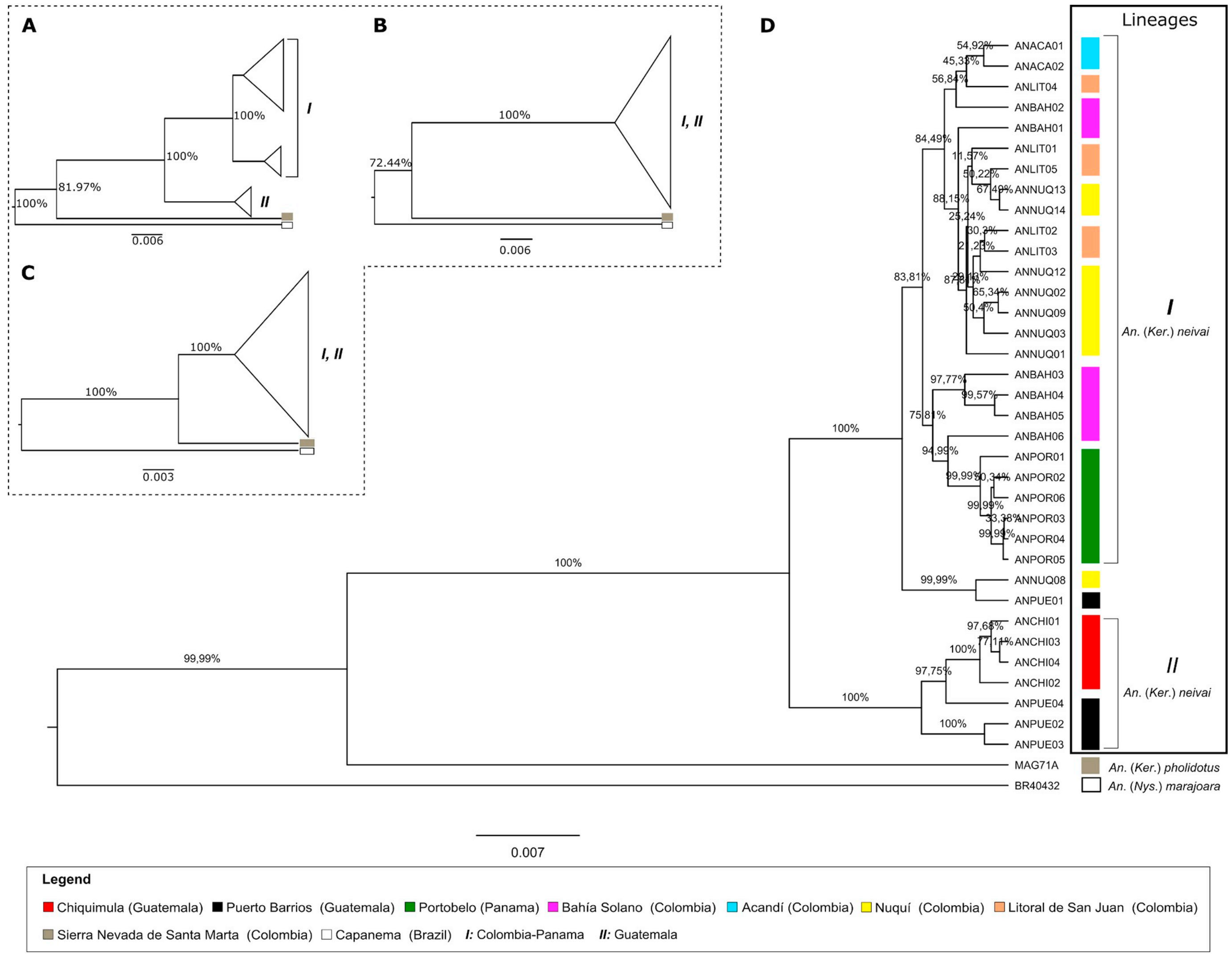

Fig. 4. Bayesian inference tree using Yule speciation model for *COI* (A), *Cytb–tRNASer*–IG1–*ND1* (B), *D1–D2* (C) in separate and concatenated analysis (D).

comprised of sequences generated from topotypic specimens collected in Portobelo (Panama), with small sub clusters in northern (Guatemala) and southern locations (Buenaventura) (S1 Fig).

Based on the polymorphic sites and the statistical parameter of each test, CADM was chosen over the remaining tests based on its reported accuracy on type I errors and robustness (Campbell et al., 2011) (S2 table). The final concatenated dataset consisted of at least two mitochondrial regions and the ribosomal *D1–D2* fragment, with a resulting concatenation for the *COI*, *Cytb–tRNASer*–IG1–*ND1* and *D1–D2* alignment. (Table 3).

The initial phylogenetic network revealed two statistically supported groups: Colombia-Panama (group *I*) and Guatemala (group *II*), with several local variants for Colombia (Fig. 3B). This topology was also identified in network analyses based on *COI* and *D1–D2* (Fig. 3A, C, D). Similarly, the resulting trees for *COI*, *Cytb–tRNASer*–IG1–*ND1* and *D1–D2* using Bayesian inference (Fig. 4C), showed agreement with the topologies from the phylogenetic network and a Bayesian tree inferred from *COI* sequence data (S2 Fig); in contrast, there was disagreement from the concatenated set with topologies from *Cytb–tRNASer*–IG1–*ND1* and *D1–D2* (S3 and S4 Fig). Furthermore, the intra-specific clades in the concatenated set tree showed weak support for the specimens in the Colombian localities (below 90%), with no relation from these inner groups to a geographical distribution, except for specimens collected at the locations in Panama and Guatemala. Despite these results and based on the general topology from the concatenated Bayesian tree (Fig. 4), a scenario for the existence of two possible entities *An. neivai* s.s, and *An. neivai A*, tested by estimating a maximum credibility clade tree, which revealed strong support only for the entire *An. neivai* (100% support) clade, and low support for the grouping of three inner entities in *An. neivai* A (only 86.72% support) (Fig. 5).

4. Discussion

Phylogenetic studies are migrating from single gene to multilocus analyses (Yoshida et al., 2017) and this shift is accompanied by specific advantages, including more accurate assessments of species boundaries and increased resolution of phylogenetic relationships using probabilistic methods. Moreover, such methods also have the potential to elucidate speciation processes through the availability of more accurate species trees rather than those inferred from single genes (Kubatko et al., 2011). However, some drawbacks such as inconsistences between gene and species trees become problematic when studying closely related species with large distributions or populations (Heled and Drummond, 2010). There is also evidence of differences in mutation rate patterns between nuclear and mitochondrial DNA (mito-nuclear discordance) which can lead to inaccurate hypotheses (Denton et al., 2014).

The evolution of genes at the intraspecific level cannot be appropriately represented based only on bifurcating trees, because relationships among individuals from the same species are not hierarchical as in

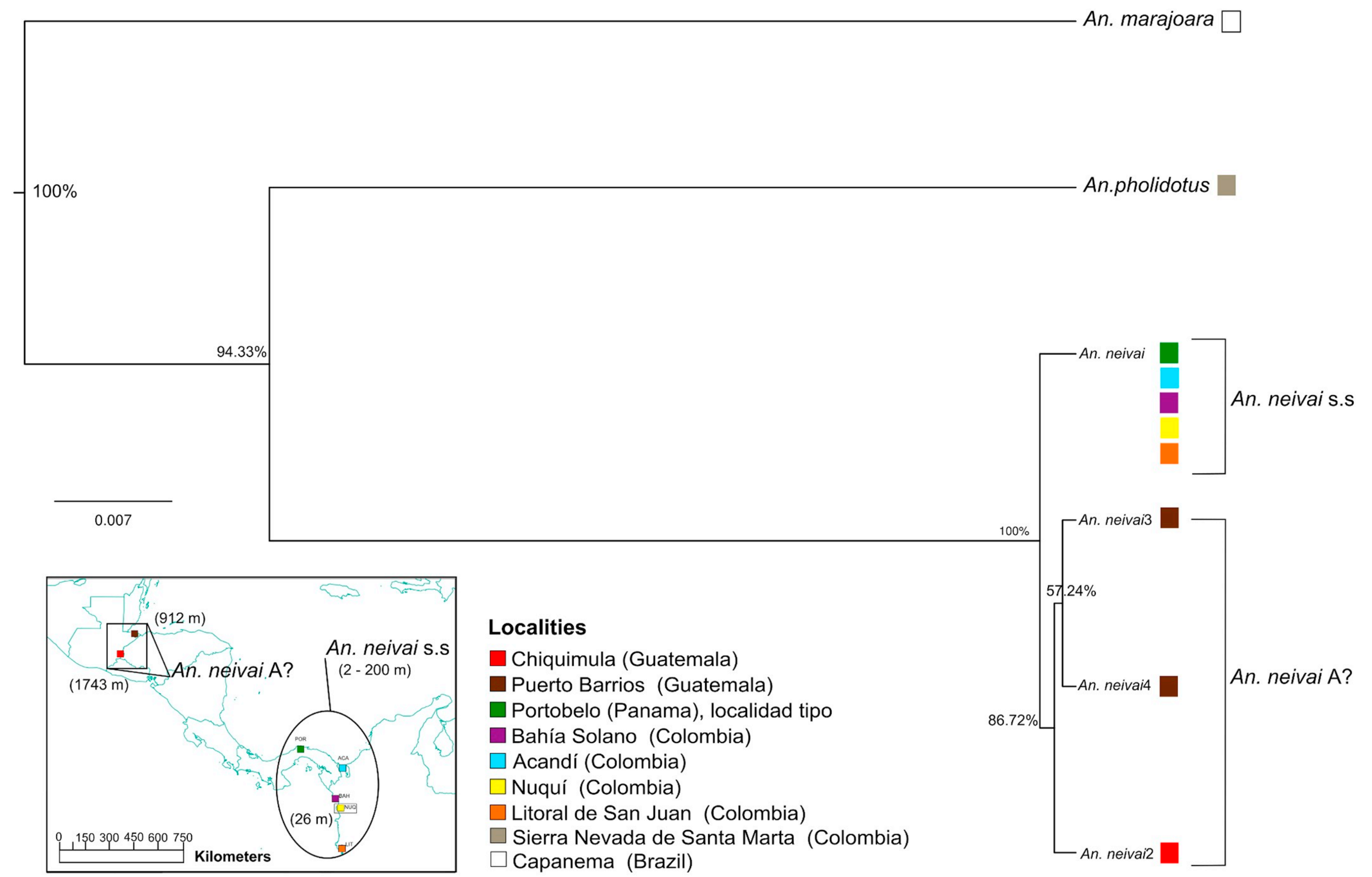

Fig. 5. Maximum credibility clade for possible cryptic species in *An. neivai* based in a coalescence multilocus approach.

traditional phylogeny, in this sense, phylogenetic networks offer more robust information (Posada and Crandall, 2001). For cryptic species, the discontinuity in genetic variability and reduced to non-existent gene flow among populations, is indicative of species level separation, represented in a network topology as separate segments or subnetworks (Ahmed et al., 2012). For example, in anophelines the use of parsimony networks based on a *COI* fragment in *An. minimus* revealed lack of connectivity among haplotypes as consequence of the existence of two lineages, suggesting the presence of cryptic species (Chen et al., 2011).

Our results from a neighbor-net topology, based on concatenation of two mitochondrial regions and a nuclear ribosomal gene fragment, showed overall topological agreement. From this topology, the specimens from Guatemala, Panama and Colombia were part of the same entity: *An. neivai.* The presence of reticulate vertex patterns in networks from separate analyses in *Cytb–tRNASer*–IG1–*ND1* (Fig. 3B) and *D1–D2* (Fig. 3C) suggests high variability at the sampled locations. In addition, the loops –represented as squares inside the networks –found on individual neighbor and concatenated net topologies (Fig. 3C, D), as result of including *D1–D2,* suggest of possible recombination events or reverse mutations (Posada and Crandall, 2001). Previous studies on the phylogeny of Anopheles, showed the *28S* large ribosomal subunit to exhibit lower mutation rates when compared to mitochondrial genes (Krzywinski et al., 2001). Despite these rates, the differences found in *D1–D2* sequences, were quite informative at species level for *An. neivai.*

Recent developments in mathematical modelling offer the alternative of estimating multispecies in coalescent trees. In this model, no horizontal gene transfer, admixture from different species, and orthologous genes is assumed, hence the topology from a species tree define barriers for gene flow, which can represent species at a taxonomical rank (Heled and Drummond, 2010)

Differences in topologies from the Bayesian gene trees under the Yule speciation model, were evident as a consequence of different mutation rates, even among mitochondrial regions (Figs. 1,4A, B). On the other hand, the concatenated gene tree (Fig. 4C) and the multilocus species tree (Fig. 5) showed an overall topological agreement with the concatenated network topology (Fig. 3C), suggesting the same two lineages: Colombia-Panamá (*I*) and Guatemala (*II*) for the majority of specimens examined. In addition, the support values below 95% from maximum credibility clade in the multilocus species tree for lineage *II*, suggests these represent a single entity: *An. neivai.*

5. Conclusion

Despite of local scale variability and different evolutionary rates from examined mitochondrial and nuclear regions, the results from the Bayesian multilocus species tree under coalescence support the existence of a single entity: *An. neivai.* Inferring the intraspecific history of this vector is important for a better understanding of the biology of the species and its future impact on vector control purposes, in special at the Colombian Pacific coast due to its epidemiological importance.

6. Final recommendation

Further integrative taxonomy studies including environmental factor such as precipitation, relative humidity, organic matter, and air precipitation in bromeliad tanks over vector population dynamics; and also studies involving, behavior, immature morphology and phylogenomics in *An. neivai* and relationships with other species from *Kerteszia*, will provide new insights of the evolution patterns in *An. neivai* and perhaps elucidate cryptic species with implications in disease transmission.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.10.013.

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