Distribution of Anopheles species in malaria endemic areas of Honduras in an elimination setting

Denis Escobar
  Microbiology Research Institute, Universidad Nacional Autonoma de Honduras

Krisnaya Ascencio
  Microbiology Research Institute, Universidad Nacional Autonoma de Honduras

Andrés Ortiz
  Microbiology Research Institute, Universidad Nacional Autonoma de Honduras

Adalid Palma
  Microbiology Research Institute, Universidad Nacional Autonoma de Honduras

Gustavo Fontecha
  Universidad Nacional Autonoma de Honduras - Microbiology Research Institute

---

Research

Keywords: Anopheles spp., Phylogeny, COI, ITS2, Honduras

Abstract

Background: *Anopheles* mosquitoes are the vectors of malaria, one of the most important infectious diseases in the tropics. More than 500 *Anopheles* species have been described worldwide, and more than 30 are considered a public health problem. In Honduras, information on the distribution of *Anopheles* spp. and its genetic diversity is scarce. This study aimed to update information on the diversity of *Anopheles* mosquitoes in Honduras with a morphological and molecular approach.

Methods: Mosquitoes were captured in 8 locations in 5 malaria endemic departments during 2019. Two collections methods were used. Adult anophelines were captured outdoors using CDC light traps and by aspiration of mosquitoes at rest. The morphological identification was performed using taxonomic keys. Genetic analyses included the sequencing of a partial region of the cytochrome oxidase I gene (COI) and the ribosomal internal transcribed spacer 2 (ITS2).

Results: A total of 1320 anophelines were collected and identified through morphological keys. Seven *Anopheles* species were identified. *An. albimanus* was the most widespread and abundant species (74.02%). To confirm the morphological identification of the specimens, 175 and 122 sequences were obtained for COI and ITS2 respectively. Both markers confirmed the morphological identification. COI showed a greater nucleotide diversity than ITS2 in all species. High genetic diversity was observed within the populations of *An. albimanus* while *An. darlingi* proved to be a highly homogeneous population. Phylogenetic analyses revealed clustering patterns in *An. darlingi* and *An. neivai* in relation to specimens from South America. New sequences for *An. crucians*, *An. vestitipennis*, and *An. neivai* are reported in this study.

Conclusions: Here we report the distribution of *Anopheles* species in endemic areas of malaria in Honduras. According to our results, both taxonomic and molecular approaches are useful tools in the identification of anopheline mosquitoes. However, both molecular markers differ in their ability to detect intraspecific genetic diversity. These results provide supporting data for a better understanding of the distribution of malaria vectors in Honduras.

Background

According to the World Health Organization (WHO), more than 228 million cases of malaria occurred worldwide in 2018. The WHO Region of the Americas accounted for less than 0.5% of all malaria cases, as declines have been recorded in most of the endemic countries on the continent, except mainly for Venezuela [1]. Nine countries in Central America and Hispaniola are taking part in a sub-regional initiative to eliminate malaria over the next years [2]. As a signatory to this agreement, Honduras has managed to reduce vectorial transmission by more than 96% since 2004, reporting only 651 cases in 2018 [1]. This reduction can be attributed in part to the integrated control of Anopheles species.

The genus Anopheles includes more than 500 formally recognized species and several unclassified members (incertae sedis), some of them grouped into species complexes [3]. Based on molecular markers such as ITS2, both dominant vector species (DVS) and secondary vectors of malaria in the Americas are grouped into three sub-genera: Anopheles (Anopheles), An. (Nyssorhynchus), and An. (Kerteszia) [4, 5]. Approximately 70 species of these three sub-genera are capable of transmit malaria parasites [6], and of those, 30 to 40 have sufficient vector capacity to be considered as public health problems [7, 8]. There are discrepancies on the literature with regards to the number of dominant Anopheles species in Mesoamerica. According to a global map of dominant malaria vectors published in 2012, there are at least seven species reported on the isthmus. Anopheles pseudopunctipennis and An. albimanus are the most prevalent species, whereas An. darlingi shows more focalized distribution patterns. An. aquasalis is predominant in the coastal areas of southern Central America and with lower vector capacity [9]. Other authors point out that the most relevant species of malaria vectors recognized in Mesoamerica are Anopheles albimanus, An. pseudopunctipennis, An. darlingi, An. vestitipennis, and An. punctimacula [2].

Scientific information regarding malaria vector species in Honduras is scarce. The first partial record of anophelines in the country dates from 1930, when Dr. Antonio Vidal described seven Anopheles species from four ecological regions [10]. Vidal´s report was followed by a brief description in 1998 of the local species on the island of Utila (Bay Islands) [11]. Additionally, some specimens of anophelines collected in Honduras and other countries have been used in order to determine their genetic diversity [12]. Other authors have described extensively the composition of Anopheles species in the Neotropics [13], or have made notable efforts to predict the distribution of the DVS of malaria in the Americas through intensive literature searches and an evidence-based approach [9, 14]. Despite these efforts, there are still important information gaps about Anopheles species in Honduras, and the only verifiable data on their distribution in the country are internal reports by the Ministry of Health, which publishes them as part of routine entomological surveillance since 2013. According to those reports, 12 species of anophelines have been identified through morphometric keys: Anopheles albimanus, An. albitarsis, An. apimacula, An. argyritarsis, An. crucians, An. darlingi, An. gabaldoni, An. grabhami, An. neomaculipalpus, An. pseudopunctipennis, and An. punctimacula. Another information gap in Honduras is the lack of molecular markers data that support the classification of mosquitoes based on morphometric keys. Molecular markers are critical to distinguish between evolutionarily close or cryptic species, even using immature specimens [15, 16].

To optimize the limited resources available for vector control strategies in Honduras, it is necessary to know in depth the distribution and relevant bionomic aspects of DVS and other anophelines considered secondary vectors of malaria. This study aims to provide an update on the diversity of the Anopheles mosquitoes in Honduras, supporting its distribution in morphological data, as well as in two molecular markers.

Methods

Study sites

Entomological captures were carried out in 8 sites in 5 departments of the country (Atlántida, Colón, Comayagua, El Paraíso, and Gracias a Dios) from February to October 2019 (Table 1). The departments of Atlántida, Colón and Gracias a Dios are classified as very humid tropical ecosystems, while Comayagua and El Paraíso are considered as subtropical dry. The average temperature varies between 25ºC and 33ºC, and the relative humidity ranges from 40–91% in all sites depending on the season of the year. The population's livelihood in the selected areas is mainly based on agricultural and livestock activities. The study sites are those monitored by the Ministry of Health of Honduras to undertake routine entomological surveillance as they remain endemic to malaria by Plasmodium vivax. Malaria due to P. falciparum malaria is reported almost exclusively in Gracias a Dios. Geographical coordinates and altitude of the collection sites are shown in Table 1.

Table 1
Anopheles specimen collection sites.

Department	Location	Coordinates	Altitude (m.a.s.l.)	Month of collection
Atlántida	La Ceiba	15.748587, -86.900546	7	February
Atlántida	La Ceiba	15.758790, -86.867092	7	February
Colón	Iriona	15.938416, -85.058888	4	March
Colón	Iriona	15.773889, -85.134556	27	March
Colón	Sonaguera	15.629846, -86.287587	82	April
Colón	Tocoa	15.655448, -86.04725	38	April
El Paraíso	Morocelí	14.103168, -86.917882	600	August
Comayagua	Comayagua	14.439279, -87.689953	588	August
Gracias a Dios	Tikirraya	15.018379, -83.641264	13	October
Gracias a Dios	Kaukira	15.309131, -83.565868	8	October

Mosquito Collection

A single collection of anophelines was carried out per site. Atlántida and Colón were visited during the dry season of the year (February to April), and El Paraíso, Comayagua and Gracias a Dios were visited in the rainy season (August to October) (Table 1). Two collection methods were used at each site to capture the greatest amount and diversity of Anopheles species. The first method used outdoor CDC light traps, with 3 to 5 traps per site in a period from 18:00 pm to 6:00 am. The second method was by aspiration of mosquitoes resting outdoors, during the period from 18:00 pm to 21:00 pm [17]. After collection, mosquitoes identified as anopheline were placed on a Petri dish with silica gel and transported at room temperature to the laboratory in Tegucigalpa where they were stored at -20 °C until later morphological identification [18].

Morphological Identification

The morphological identification was performed using keys for anophelines of Central America and Mexico proposed by Wilkerson & Strickman [19]. The integrity of the mosquito´s anatomical structures was verified individually. Subsequent mounting and identification were carried out with the help of a stereoscope. Mosquitoes were counted and classified by species and sex. After morphological identification, wing and leg vouchers of each species were preserved as a reference in the Center for Genetic Research of the National Autonomous University of Honduras. Each mosquito was then stored individually at -20 °C for subsequent molecular tests.

COI Gene

A subset of morphologically identified specimens were chosen for molecular analysis. DNA was extracted from each specimen according to the protocol provided by the AxyPrep MAG Tissue-Blood gDNA Kit, Axygen® (Corning Incorporated, Life Sciences, Tewksbury, MA, USA). Preliminarily, the mosquitoes were macerated with a pestle in a 1.5 ml conical tube together with 50 µl of lysis solution provided by the kit. DNA was stored at − 20 °C until further use. Molecular analyses were performed on Anopheles mosquitoes to confirm species and calculate genetic variation within species. Two molecular markers were used: cytochrome c oxidase I gene (COI), and the internal transcribed spacer 2 (ITS2). The following primers were used to amplify a fragment of COI: LCO1490 GGTCAACAAATCATAAAGATATTGG and HCO2198 TAAACTTCAGGGTGACCAAAAATCA [20]. Reactions were carried out in a volume of 50 µl, with 25 µl of Taq Master Mix 2X (Promega, Madison, Wisconsin), 2.0 µl of each primer (10 µM), 2 µl of acetylated bovine albumin (BSA) (10 mg/ml), 4 µl of DNA, and nuclease-free water. The PCR program was as follows: 1 cycle at 95ºC for 10 minutes, 37 cycles at 94ºC for 1 minute, 48ºC for 1 minute, 72ºC for 1 minute, and 1 cycle at 72ºC for 7 minutes.

Some mosquito specimens that could not be amplified with the pair of primers described above were amplified using LCO1490 and a reverse primer described by Kumar et al [21]: AAAAATTTTAATTCCAGTTGGAACAGC (Fig. 1), with the following reagents and concentrations: 25 µl of Taq Master Mix 2X (Promega, Madison, Wisconsin), 1 µl of each primer (10 µM), 2 µl of DNA, and 21 µl of nuclease-free water. The cycling conditions were: 1 cycle at 95ºC for 5 minutes, 5 cycles at 94ºC for 40 s, 45ºC for 1 minute, 72ºC for 1 minute, 37 cycles at 94ºC for 1 minute, 54ºC for 1 minute, 72ºC for 90 s, and a final extension at 72ºC for 10 minutes. The PCR products were separated by electrophoresis in 1% agarose gels with ethidium bromide.

ITS2 Ribosomal Region

For ITS2 amplification, PCR reactions were performed using the universal primers [22]: 5.8S ATCACTCGGCTCGTGGATCG and 28S ATGCTTAAATTTAGGGGGGTAGTC. Reagents concentrations were as follows: 25 µl of Taq Master Mix 2X (Promega, Madison, Wisconsin), 2 µl of each primer 10 µM, 2 µl of DNA, and water for a total reaction volume of 50 µl. PCR amplifications were performed with the following conditions: 94ºC for 2 min, 34 cycles of 94ºC at 30 s, 57ºC at 30 s, 72ºC at 30 s, and final extension of 72ºC at 10 min.

Sequence Analysis

The amplification products of both COI and ITS2 markers were sequenced on both strands using the same primers of the PCR. A representative subset of mosquitoes of all species and all collection sites was selected for sequencing. Purification and sequencing services were provided by Psomagen (https://www.macrogenusa.com). The sequences were edited with the Geneious® 9.1.7 software (Biomatters Ltd. Auckland, New Zealand) and were deposited in two databases: Barcode of Life Data System (BOLDSYSTEMS, http://www.boldsystems.org), and in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). Barcode Index Numbers (BINs) and accession numbers were obtained for each sequence. All sequences were submitted as queries to NCBI through the BLAST tool [23] under default parameters to identify the most similar sequences in the GenBank nucleotide collection.

Nucleotide Diversity (π) And Number Of Haplotypes

In order to calculate the nucleotide diversity (π), the sequences of both molecular markers were analysed separately and by species. The sequences were aligned using the MUSCLE algorithm. MEGA v10.0 software with 1000 Bootstrap replicas was used to calculate the pairwise distance using the Maximum Composite Likelihood substitution method, and 95% as the site coverage cut-off. The percentage of identical bases for each species and between species (intra- and inter-specific similarities) was calculated in order to demonstrate the reported "barcoding gap".

The haplotype diversity was calculated with R through the function hap.div of pegas (v0.12 package) and using the Nei and Tajima´s method [24]. Haplotype frequencies were calculated using the Haplotype function with default parameters, and the haplotype network was computed with the haploNet function using an infinite site model, pairwise deletion missing data, and probability of parsimonious link [25].

Phylogenetic Analysis

Nucleotide sequences were trimmed and manually corrected using the Geneious® 9.1.2 software. The ClustalW tool was used to align sequences. Phylogenetic trees were constructed using the Tamura-Nei distance model, the Neighbor-Joining method and a bootstrap of 1,000 replicas with no outgroup. Length, identical sites and pairwise % identity were calculated for each molecular marker and each species.

To calculate the phylogenetic relationships between specimens collected in Honduras with those collected in other countries of the Americas, analogous COI and ITS2 sequences for all available *Anopheles* species were downloaded from the GenBank database. Sequences were aligned and phylogenetic trees constructed under the same parameters described above.

Results

Distribution of Anopheles species

Eight locations with active foci of malaria [8] were visited to collect anopheline mosquitoes. A total of 1320 adult individuals of seven Anopheles species were collected and identified by a taxonomic key (Fig. 2): Anopheles (Nyssorhynchus) albimanus Wiedemann, An. (Nys.) darlingi Root, An. (Anopheles) vestitipennis Dyar & Knab, An. (An.) crucians Wiedemann, An. (An.) pseudopunctipennis Theobald, An. (An.) punctimacula s.l. Dyar & Knab, and An. (Kerteszia) neivai Howard, Dyar & Knab (Table 2). More morphological details of the vouchers can be observed in the project "CIGAN Bionomy of Anopheles sp. in Honduras" of the BOLD database.

Distribution of Anopheles species according to capture site and geographic region

Department	Location	An. albimanus	An. darlingi	An. vestitipennis	An. crucians	An. pseudopunctipennis	An. punctimacula	An. neivai	Total (%)
Atlántida	La Ceiba 1	307	61	1	-	-	1	8	378 (28.64%)
Atlántida	La Ceiba 2	21	17	2	-	-	-	-	40 (3.03%)
Colón	Iriona 1	7	-	-	-	2	-	-	9 (0.68%)
Colón	Iriona 2	8	-	-	-	-	-	-	8 (0.60%)
Colón	Sonaguera	-	-	-	-	10	-	-	10 (0.76%)
Colón	Tocoa	96	14	-	-	-	1	-	111 (8.41%)
El Paraíso	Morocelí	23	-	-	-	-	-	-	23 (1.7%)
Comayagua	Comayagua	294	-	-	-	1	-	-	295 (22.34%)
Gracias a Dios	Tikirraya	44	-	-	-	-	-	-	44 (3.3%)
Gracias a Dios	Kaukira	177	-	92	132	-	-	1	403 (30.5%)
		977 (74.02%)	92 (6.97%)	95 (7.2%)	132 (10.0%)	13 (0.98%)	2 (0.015%)	9 (0.07%)	1320 (100%)

Most specimens were identified as An. albimanus (74.02%), An. crucians (10%), An. vestitipennis (7.2%), and An. darlingi (6.97%). The remaining 3 species accounted for less than 1% of the total. An. albimanus was found in all locations except Sonaguera (Colón). The highest diversity of species (n = 5) was found in La Ceiba (Atlántida) followed by Kaukira (Gracias a Dios) (n = 4). Moreover, five other localities reported only one to three species. An. crucians was only found in Gracias a Dios. The highest density of mosquitoes was obtained in Gracias a Dios (33.8%), Atlántida (31.67%), and Comayagua (22.34%) (Fig. 3). An. darlingi was only present in Atlántida and Colón.

Nucleotide Sequences

A total of 160 COI sequences and 122 ITS2 sequences were obtained for six out of seven Anopheles species. No sequences of An. neivai were obtained for either of the two markers. A second set of primers for COI (Fig. 1) was able to produce 5 sequences of An. neivai and 10 sequences of four other species: An. albimanus, An. darlingi, An. punctimacula, and An. vestitipennis.

All COI and ITS2 sequences were deposited in the BOLD system database and the following BINs were assigned: CIGAN001-19 to CIGAN067-19, CIGAN068-20 to CIGAN178-20. These sequences were also deposited in GenBank under the following accession numbers: MT033921 – MT034050, MT040803 – MT040831, MT048394 – MT048399, MT049952 – MT049958, MT053086, MT062520, MT066404, MN998028 – MN998149.

The COI intra- and inter-specific percentage of identity for the six species were non-overlapping, averaging 99.04% (98.35 to 100.0) and 88.52% (86.51 to 91.60), respectively. Inter-specific pairwise genetic distances greater than 3% support the "barcoding gap" between the Anopheles species reported in this study.

COI sequences were analysed with the NCBI BLAST tool in order to confirm the morphological identification of the species. An. albimanus, An. darlingi, An. pseudopunctipennis, and An. punctimacula were correctly identified by BLAST with identity percentages of 95.6–99.7%. Sequences of An. crucians, An. vestitipennis, and An. neivai could not be identified by BLAST due to the absence of sequences of these species in the databases, making them the first COI sequences reported for the three species in the GenBank. All species were correctly identified by ITS2 with identity percentages of 99.63–100% with the exception of An. vestitipennis, whose sequences were not available in the databases. This is also the first report of ITS2 sequences for An. vestitipennis. In summary, the morphological identification coincided with the molecular identification of both markers for the species with sequences previously reported in the databases.

Nucleotide diversity and haplotypes

Intraspecific variation was calculated for both markers. COI showed a higher level of polymorphism than ITS2. According to COI, the species with the highest nucleotide diversity was An. crucians (π = 0.05), followed by An. vestitipennis (π = 0.03) (Table 3). The species with the lower diversity was An. darlingi. An. albimanus revealed a high number of haplotypes (n = 55). An. pseudopunctipennis showed the highest proportion of COI haplotypes with respect to the number of sequences analysed (11/11) and An. darlingi revealed the lowest haplotype index (3/16). ITS2 showed a low number of haplotypes (1–4) in all species (Table 3) (Fig. 4).

Table 3
Intraspecific comparison of nucleotide sequences and number of haplotypes for COI and ITS2 in 5 species of Anopheles of Honduras

COI	An. albimanus	An. crucians	An. darlingi	An. pseudopunctipennis	An. vestitipennis
Length	712	711	684	684	681
N	103	14	16	11	14
Identical sites	659	600	682	654	596
Identical sites (%)	92.6%	85.3%	99.7%	95.6%	87.5%
Pairwise % identity	99.1%	95.8%	99.9%	98.9%	97.7%
π	0.01	0.05	0.00	0.01	0.03
Nº haplotypes	55	13	3	11	10
Haplotypes/N	0.53	0.93	0.19	1.0	0.71
ITS2	An. albimanus	An. crucians	An. darlingi	An. pseudopunctipennis	An. vestitipennis
Length	566	380	596	567	576
N	76	13	10	7	14
Identical sites	552	367	593	549	567
Identical sites (%)	97.7%	99.5%	99.5%	97.0%	98.6%
Pairwise % identity	99.9%	99.9%	99.9%	99.1%	99.7%
π	0.0	0.0	0.0	0.0	0.0
Nº haplotypes	3	1	1	3	4
Haplotypes/N	0.04	0.08	0.1	0.43	0.29

Phylogenetic analysis

Three analyses were performed to infer phylogenetic relationships between sequences. The first analysis included all the sequences of each marker for six Anopheles species. Both dendrograms (COI and ITS2) showed that the species clearly separated into clades (Fig. 5).

The second analysis included sequences of An. albimanus classified according to geographic region. Phylogenetic relationships based on COI sequences showed only one separate cluster that included 11 out of 14 sequences of mosquitoes collected in Gracias a Dios. The other sequences were not clustered (Fig.6). ITS2 sequences did not reveal any clustering according to geographical origin. This analysis was not performed for other Anopheles species due to the low intraspecific variation.

The third phylogenetic analysis included the COI sequences of five species obtained in this study (An. albimanus, An. darlingi, An. pseudopunctipennis, An. punctimacula, and An. neivai) together with analogous sequences available in GenBank in order to understand the relationships between individuals from Honduras with mosquitoes from other countries in the Neotropical region. The same analysis was performed separately with the ITS2 sequences of five species from Honduras (An. albimanus, An. darlingi, An. pseudopunctipennis, An. punctimacula, and An. neivai) and sequences from specimens of other countries. The phylogenetic tree of An. albimanus included 12 COI sequences of mosquitoes from Colombia and 103 sequences of mosquitoes from Honduras, however the sequences of Colombia clustered together with the majority of sequences from Honduras. Eleven sequences of mosquitoes captured in Gracias a Dios formed a well-supported clade (Fig.7a). For An. darlingi 16 sequences from Honduras, 6 sequences from Colombia, 5 sequences from Brazil, and 4 sequences from Peru were analysed. According to this analysis the population was divided into two clusters, one including all the sequences of Honduras, and another with the sequences of South America (Fig.7b). In addition, 12 sequences of An. pseudopunctipennis from Honduras and nine sequences from Colombia were analysed. For the analysis of An. punctimacula, seven sequences from Brazil, 14 sequences from Colombia and one sequence from Honduras were included. No clusters were detected for both species (Fig.7c, 7d). The analysis for An. neivai included three sequences from French Guiana, six sequences from Colombia, and five sequences from Honduras. The specimens of the three countries showed a defined separation according to geographical origin (Fig.7e).

The phylogenetic analysis of the ITS2 sequences included a total of eight countries of the Americas, including Honduras, Colombia, Brazil, French Guiana, Panama, Nicaragua, Ecuador and Belize. None of the trees could demonstrate separation of populations based on geographic origin (Fig.8).

Discussion

This study provides updated information on the distribution of Anopheles species in endemic malaria regions of Honduras. Seven Anopheles species were found. An. albimanus was the most common species and the most widely distributed. This is consistent with the existing literature. An. albimanus has been described as the dominant species in Central America, the Caribbean and some coastal regions of northern South America [9, 12, 13]. This has been demonstrated through studies conducted in Colombia [26], Panama [27], Belize [28], and Guatemala [29]. The predominance of this species - considered as a generalist species - can be attributed to the wide range of habitats, feeding preferences, and heights in which the larvae can develop [30, 31]. In this study, mosquitoes were collected at eight geographical sites. In seven sites, An. albimanus was the most frequently captured species despite the ecological differences between all locations. Five of the eight sites are classified as wet coastal ecoregions (Atlántida, Colón, and Gracias a Dios), with less than 550 m.a.l.s., while two sites (Comayagua and El Paraíso) are classified as Piedmont, with heights above 550 m.a.l.s. and drier ecosystems [13]. La Ceiba (Atlántida) and Kaukira (Gracias a Dios) yielded greater diversity of species (4 to 5), similar to reports from Cordoba, in the coastal region of the Colombian Caribbean [26], and in a study carried out in Papua New Guinea describing the abundance and composition of anophelines [32]. Such biological diversity of anopheline species could be due to the fact that both sites have tropical ecosystems and are regions rich in lagoons, coral reefs, mangroves, beaches, rivers and abundant flora and fauna. In addition, Kaukira is located in the department of Gracias a Dios, also known as La Mosquitia, the region with less anthropogenic intervention in the country.

The second most abundant species collected in Kaukira was An. crucians. This finding is remarkable since this species was not registered anywhere else in this study. Its abundance is probably related to the high density of phanerogam plants that have been previously associated with the development of their larvae [28]. An. crucians has been recognized as one of the five most important malaria vectors in the country [33], and has been reported as one of the most frequent species in Belize, Guatemala, Honduras and Nicaragua [28, 34]. Since La Mosquitia is the main region with permanent transmission and the highest number of malaria cases in the country throughout the year, it would be interesting to further explore the importance of this species in the malaria transmission. On the other hand, An. darlingi was collected only in two coastal departments (Atlántida and Colón), consistent with previous reports [35]. This species is known for its preference to inhabit areas of high rainfall and where the tropical forest is close to the ocean [13, 36].

In addition to the morphological identification of specimens, sequences of the COI gene and the ITS2 ribosomal region were obtained for all species. Four and six species of anophelines were properly identified by BLAST of the COI and ITS2 sequences, respectively. Up to the moment of the analysis, there were no analogous sequences available of COI for An. crucians, An. vestitipennis and An. neivai, nor any sequences of ITS2 for An. vestitipennis in the GenBank database. Consequently, these would be the first sequences reported. This findings support the barcode strategy as a useful tool to confirm the correct assignment of misidentified or unidentified species using morphology [26, 37, 38, 39].

When comparing the individual ability of both markers to identify or confirm Anopheles species in Honduras, it seems that both are informative enough and fulfil their purpose [39, 40, 41]. Some authors report problems to solve and identify species when those markers are used individually [42], and they suggest that a multi-locus approach might have a greater power of discrimination [43, 44]. However, our study shows that both molecular markers are useful separately and are a good complement to the identification of Anopheles based on taxonomic keys [45].

Intraspecific variation was calculated for five Anopheles species. A greater nucleotide diversity (π) and number of haplotypes with COI than with ITS2 were observed. According to this result, COI would be more informative to decipher the intraspecific phylogenetic relationships. Some authors reported different findings when analysing the phylogeny of the Anopheles Hyrcanus Group using ITS2 sequences downloaded from GenBank. They concluded that ITS2 would be more reliable than COI as a phylogenetic analysis tool among very close taxa. This discrepancy could be attributed to the fact that the Hyrcanus Group includes at least 25 species widely distributed in a large geographic area [15, 16]. Discrepancies between markers are expected since there are different evolutionary processes that act differently on mitochondrial and nuclear genes [46]. Nevertheless, COI could be considered a more useful marker for evidencing intraspecific genetic diversity between Anopheles spp. in Honduras.

The species with the lowest genetic diversity was An. darlingi when 16 COI sequences were analysed. Although the number of sequences studied is low, it is possible to say that the population is relatively homogeneous. High homogeneity within the population could be attributed to the fact that the geographical area in which the mosquitoes were collected was small. Similar results were reported in a study conducted in Darien, at the border between Panama and Colombia, with 40 individuals who showed low nucleotide diversity (π = 0.0006) [47].

On the other hand, when the phylogenetic relationship of An. darlingi specimens collected in the Caribbean of Honduras was analysed together with 15 sequences obtained from mosquitoes from Colombia, Peru and Brazil, the resulting Neighbor-Joining tree showed two well differentiated clades between the populations of South America and the population of Honduras. This could support the theory of geographic and reproductive isolation between the populations of northern Central America and South America. There are several studies that analyze the population continuity of An. darlingi throughout Central and South America. Several researchers report that An. darlingi populations in Central and South America reveal significant differences through the use of morphological and behavioural markers [48], RAPDs [49], COI [50], and microsatellite loci [51]. It has been

hypothesized that this geographic isolation could be attributed to the absence or low population densities of An. darlingi in Nicaragua and Costa Rica [14, 47].

An. neivai was the second species that showed well separated clades within the dendrogram. One clade included five sequences from Honduras, a second clade included three sequences from French Guiana, and a third clade consisted of six sequences from Colombia. A recent study analysed four mitochondrial and ribosomal sequences of 35 specimens from Guatemala, Panama, and the southern Pacific coast of Colombia. Phylogenetic networks showed two clusters well differentiated by geography [52]. Although the authors concluded that their results support the existence of a single taxonomic entity, sequences from Guatemala clearly separate from those of the rest of Panama and Colombia. This result is consistent with what was found in our study and supports the hypothesis of the existence of two possible entities: An. neivai sensu stricto in South America, and An. neivai "A" in Central America [52].

Phylogenetic analyses and haplotype networks for An. albimanus detected 55 haplotypes without any clustering pattern based on geographical origin. This suggests high genetic diversity and the existence of gene flow between populations. This finding suggests that there is no evidence of isolation that could lead to the generation of divergent lineages in An. albimanus. The only lineage that showed a low to moderate bootstrap support (64.8) was composed of 11 sequences of mosquitoes captured in La Mosquitia. This result is interesting given that this region is socially isolated from the rest of the country by the Río Plátano biosphere reserve. However, this hypothetical isolation should be confirmed in the future by more robust and informative molecular markers such as microsatellite loci [53]. Future sampling should also include specimens from other geographical regions, particularly from the Honduran islands in the Caribbean.

Conclusions

In this study, the distribution of Anopheles species in malaria endemic areas of Honduras has been described through a morphological approach and two molecular markers. Conventional taxonomy, COI, and ITS2 proved to be useful tools for the correct identification of anopheline species. However, both molecular markers differ in their ability to detect intraspecific genetic diversity. According to phylogenetic analyses, the only two species that seem to show some level of structuring with respect to South American lineages are An. darlingi and An. neivai. An. albimanus was the most abundant and widely distributed species and there is no evidence of disruption in gene flow between populations of different geographical areas. In summary, our results contribute to the development of a sequence-based confirmation tool for anopheline identification in Honduras, which is an important step for the monitoring and integrated control of malaria vectors. Future work should be aimed at a wider sampling of other geographical regions and in the use of microsatellite markers to assess the population structure of these anopheline species.

Abbreviations

WHO
World Health Organization
COI
Cytochrome oxidase subunit I
ITS
Ribosomal Internal Transcribed Spacer
CDC
Centers for Disease Control and Prevention, USA
PCR
Polymerase Chain Reaction
BINs
Barcode Index Numbers
BOLD
Barcode of Life Data System
NCBI
National Center for Biotechnology Information
BLAST
Basic Local Alignment Search Tool
m.a.l.s.
Meters Above the Level of the Sea
RAPDs
Random Amplified Polymorphic DNA

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Funding for this study was provided by the Scientific Research Office of the UNAH (DICU-UNAH/2018), Honduras, and the office of the Pan American Health Organization in Honduras. The funding institutions did not participate in the design of the study and the collection, analysis and interpretation of the data or in the drafting of the manuscript.

Authors' contributions

GF conceptualized the study; DE and GF contributed with the study design; DE, KA and AP performed the collection of mosquitoes and morphological identification; DE and KA performed the laboratory experiments; AO and GF performed bioinformatic analyses; DE, KA, AO and GF organized and cured the data; all authors wrote, reviewed, read and approved the manuscript; supervision, project administration, and funding acquisition were on charge of GF.

Acknowledgements

The authors acknowledge the support given by the staff of the Ministry of Health of Honduras, in particular Benjamín Bonilla, Eduardo Martínez, Fernando Argeñal, Allan García, and Víctor Ciliezar. We appreciate the help provided by Dr. Ana Sánchez in the revision of the final manuscript.

References

1. World Health Organization: World Malaria Report 2019. Geneva: World Health Organization; 2019: 232.

2. Herrera S, Ochoa-Orozco SA, Gonzalez IJ, Peinado L, Quinones ML, Arevalo-Herrera M. Prospects for malaria elimination in Mesoamerica and Hispaniola. PLoS Negl Trop Dis. 2015;9 5:e0003700; doi: 10.1371/journal.pntd.0003700. https://www.ncbi.nlm.nih.gov/pubmed/25973753.

3. Harbach R: Genus ANOPHELES Meigen, 1818. Mosquito Taxonomic Inventory. Book Genus ANOPHELES Meigen, 1818 Mosquito Taxonomic Inventory City 2011. http://mosquito-taxonomic-inventory.info/genus-anopheles-meigen-1818# (2008). Accessed July 5 2018 2018.

4. Freitas LA, Russo CA, Voloch CM, Mutaquiha OC, Marques LP, Schrago CG. Diversification of the Genus Anopheles and a Neotropical Clade from the Late Cretaceous. PLoS One. 2015;10 8:e0134462; doi: 10.1371/journal.pone.0134462. https://www.ncbi.nlm.nih.gov/pubmed/26244561.

5. Marrelli MT, Sallum MA, Marinotti O. The second internal transcribed spacer of nuclear ribosomal DNA as a tool for Latin American anopheline taxonomy - a critical review. Mem Inst Oswaldo Cruz. 2006;101 8:817-32; doi: 10.1590/s0074-02762006000800002. https://www.ncbi.nlm.nih.gov/pubmed/17293975.

6. Service MT, H. . The Anopheles vector. In Essential Malariology., Fourth edition. edn. London: Arnold: ; 2002.

7. Hay SI, Sinka ME, Okara RM, Kabaria CW, Mbithi PM, Tago CC, et al. Developing global maps of the dominant anopheles vectors of human malaria. PLoS Med. 2010;7 2:e1000209; doi: 10.1371/journal.pmed.1000209. https://www.ncbi.nlm.nih.gov/pubmed/20161718.

8. World Health Organization: A framework for malaria elimination. Geneva. 2017.

9. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. Parasit Vectors. 2012;5:69; doi: 10.1186/1756-3305-5-69. https://www.ncbi.nlm.nih.gov/pubmed/22475528.

10. Vidal A. Anopheles mosquitoes in Honduras. Revista Médica Hondureña. 2010;78 3. http://www.bvs.hn/RMH/pdf/2010/pdf/Vol78-3-2010-12.pdf.

11. Taylor DS, Turner RL. Notes on mosquito collections from Utila, Bay Islands, Honduras. J Am Mosq Control Assoc. 1998;14 2:214-5. https://www.ncbi.nlm.nih.gov/pubmed/9673926.

12. De Merida AM, Palmieri M, Yurrita M, Molina A, Molina E, Black WCt. Mitochondrial DNA variation among Anopheles albimanus populations. Am J Trop Med Hyg. 1999;61 2:230-9; doi: 10.4269/ajtmh.1999.61.230. https://www.ncbi.nlm.nih.gov/pubmed/10463672.

13. Rubio-Palis Y, Zimmerman RH. Ecoregional classification of malaria vectors in the neotropics. J Med Entomol. 1997;34 5:499-510; doi: 10.1093/jmedent/34.5.499. https://www.ncbi.nlm.nih.gov/pubmed/9379453.

14. Sinka ME, Rubio-Palis Y, Manguin S, Patil AP, Temperley WH, Gething PW, et al. The dominant Anopheles vectors of human malaria in the Americas: occurrence data, distribution maps and bionomic precis. Parasit Vectors. 2010;3:72; doi: 10.1186/1756-3305-3-72. https://www.ncbi.nlm.nih.gov/pubmed/20712879.

15. Fang Y, Shi WQ, Zhang Y. Molecular phylogeny of Anopheles hyrcanus group (Diptera: Culicidae) based on mtDNA COI. Infect Dis Poverty. 2017;6 1:61; doi: 10.1186/s40249-017-0273-7. https://www.ncbi.nlm.nih.gov/pubmed/28478763.

16. Fang Y, Shi WQ, Zhang Y. Molecular phylogeny of Anopheles hyrcanus group members based on ITS2 rDNA. Parasit Vectors. 2017;10 1:417; doi: 10.1186/s13071-017-2351-x. https://www.ncbi.nlm.nih.gov/pubmed/28882174.

17. PAHO: Manual de referencia para la vigilancia, el seguimiento y la evaluación de la malaria. 2018: 208.

18. Ahumada ML, Orjuela LI, Pareja PX, Conde M, Cabarcas DM, Cubillos EF, et al. Spatial distributions of Anopheles species in relation to malaria incidence at 70 localities in the highly endemic Northwest and South Pacific coast regions of Colombia. Malar J. 2016;15 1:407; doi: 10.1186/s12936-016-1421-4. https://www.ncbi.nlm.nih.gov/pubmed/27515166.

19. Wilkerson RC, Strickman D, Litwak TR. Illustrated key to the female anopheline mosquitoes of Central America and Mexico. J Am Mosq Control Assoc. 1990;6 1:7-34. https://www.ncbi.nlm.nih.gov/pubmed/2324726.

20. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3 5:294-9. https://www.ncbi.nlm.nih.gov/pubmed/7881515.

21. Kumar NP, Rajavel AR, Natarajan R, Jambulingam P. DNA Barcodes Can Distinguish Species of Indian Mosquitoes (Diptera: Culicidae). Journal of Medical Entomology. 2007;44 1:1-7; doi: https://doi.org/10.1093/jmedent/41.5.01.

22. Djadid ND, Gholizadeh S, Aghajari M, Zehi AH, Raeisi A, Zakeri S. Genetic analysis of rDNA-ITS2 and RAPD loci in field populations of the malaria vector, Anopheles stephensi (Diptera: Culicidae): implications for the control program in Iran. Acta Trop. 2006;97 1:65-74; doi: 10.1016/j.actatropica.2005.08.003. https://www.ncbi.nlm.nih.gov/pubmed/16188214.

23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215 3:403-10; doi: 10.1016/S0022-2836(05)80360-2. https://www.ncbi.nlm.nih.gov/pubmed/2231712.

24. Nei M, Tajima F. DNA polymorphism detectable by restriction endonucleases. Genetics. 1981;97 1:145-63. https://www.ncbi.nlm.nih.gov/pubmed/6266912.

25. Templeton AR, Crandall KA, Sing CF. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics. 1992;132 2:619-33. https://www.ncbi.nlm.nih.gov/pubmed/1385266.

26. Gonzalez C, Molina AG, Leon C, Salcedo N, Rondon S, Paz A, et al. Entomological characterization of malaria in northern Colombia through vector and parasite species identification, and analyses of spatial distribution and infection rates. Malar J. 2017;16 1:431; doi: 10.1186/s12936-017-2076-5. https://www.ncbi.nlm.nih.gov/pubmed/29078770.

27. Loaiza JR, Bermingham E, Scott ME, Rovira JR, Conn JE. Species composition and distribution of adult Anopheles (Diptera: Culicidae) in Panama. J Med Entomol. 2008;45 5:841-51; doi: 10.1603/0022-2585(2008)45[841:scadoa]2.0.co;2. https://www.ncbi.nlm.nih.gov/pubmed/18826025.

28. Grieco JP, Johnson S, Achee NL, Masuoka P, Pope K, Rejmankova E, et al. Distribution of Anopheles albimanus, Anopheles vestitipennis, and Anopheles crucians associated with land use in northern Belize. J Med Entomol. 2006;43 3:614-22; doi: 10.1603/0022-2585(2006)43[614:doaaav]2.0.co;2. https://www.ncbi.nlm.nih.gov/pubmed/16739424.

29. Juárez Sandoval JA: **Bionomía del Vector de la Malaria Anopheles vestitipennis Dyar & Knab (Diptera:Culicidae) en la cuenca del rio polochi, Alta Verapaz, Guatemala, C.A.** *Academic.* Monterrey, NL: Universidad Autónoma de Nuevo León; 1994.

30. Fuller DO, Ahumada ML, Quinones ML, Herrera S, Beier JC. Near-present and future distribution of Anopheles albimanus in Mesoamerica and the Caribbean Basin modeled with climate and topographic data. Int J Health Geogr. 2012;11:13; doi: 10.1186/1476-072X-11-13. https://www.ncbi.nlm.nih.gov/pubmed/22545756.

31. Vazquez-Martinez MG, Rodriguez MH, Arredondo-Jimenez JI, Mendez-Sanchez JD, Bond-Compean JG, Cold-Morgan M. Cyanobacteria associated with Anopheles albimanus (Diptera: Culicidae) larval habitats in southern Mexico. J Med Entomol. 2002;39 6:825-32; doi: 10.1603/0022-2585-39.6.825. https://www.ncbi.nlm.nih.gov/pubmed/12495179.

32. Keven JB, Katusele M, Vinit R, Koimbu G, Vincent N, Thomsen EK, et al. Species abundance, composition, and nocturnal activity of female Anopheles (Diptera: Culicidae) in malaria-endemic villages of Papua New Guinea: assessment with barrier screen sampling. Malar J. 2019;18 1:96; doi: 10.1186/s12936-019-2742-x. https://www.ncbi.nlm.nih.gov/pubmed/30909928.

33. Secretaría de Salud de Honduras: Norma de Malaria en Honduras. vol. N.PNM.01.01.09.10. Tegucigalpa, Honduras2010.

34. Brennan JM. The occurrence of Anopheles crucians in Guatemala. Am J Trop Med Hyg. 1951;31 1:138; doi: 10.4269/ajtmh.1951.s1-31.138. https://www.ncbi.nlm.nih.gov/pubmed/14799721.

35. Rivera-Núñez LA: **Algunos aspectos de comportamiento de Anopheles darlingi (Diptera: Culicidae) de la Ceiba, Atlantida, Honduras**. Panama City, Panama: University of Panama; 1990.

36. Brochero HL, Rey G, Buitrago LS, Olano VA. Biting activity and breeding sites of Anopheles species in the municipality Villavicencio, Meta, Colombia. J Am Mosq Control Assoc. 2005;21 2:182-6; doi: 10.2987/8756-971X(2005)21[182:BAABSO]2.0.CO;2.

https://www.ncbi.nlm.nih.gov/pubmed/16033120.

37. Gomez GF, Correa MM. Discrimination of Neotropical Anopheles species based on molecular and wing geometric morphometric traits. Infect Genet Evol. 2017;54:379-86; doi: 10.1016/j.meegid.2017.07.028. https://www.ncbi.nlm.nih.gov/pubmed/28774799.

38. Laboudi M, Faraj C, Sadak A, Harrat Z, Boubidi SC, Harbach RE, et al. DNA barcodes confirm the presence of a single member of the Anopheles maculipennis group in Morocco and Algeria: An. sicaulti is conspecific with An. labranchiae. Acta Trop. 2011;118 1:6-13; doi: 10.1016/j.actatropica.2010.12.006. https://www.ncbi.nlm.nih.gov/pubmed/21172298.

39. Lobo NF, St Laurent B, Sikaala CH, Hamainza B, Chanda J, Chinula D, et al. Unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions using molecular tools. Sci Rep. 2015;5:17952; doi: 10.1038/srep17952. https://www.ncbi.nlm.nih.gov/pubmed/26648001.

40. Beebe NW. DNA barcoding mosquitoes: advice for potential prospectors. Parasitology. 2018;145 5:622-33; doi: 10.1017/S0031182018000343. https://www.ncbi.nlm.nih.gov/pubmed/29564995.

41. Carter TE, Yared S, Gebresilassie A, Bonnell V, Damodaran L, Lopez K, et al. First detection of Anopheles stephensi Liston, 1901 (Diptera: culicidae) in Ethiopia using molecular and morphological approaches. Acta Trop. 2018;188:180-6; doi: 10.1016/j.actatropica.2018.09.001. https://www.ncbi.nlm.nih.gov/pubmed/30189199.

42. Carter TE, Yared S, Hansel S, Lopez K, Janies D. Sequence-based identification of Anopheles species in eastern Ethiopia. Malar J. 2019;18 1:135; doi: 10.1186/s12936-019-2768-0. https://www.ncbi.nlm.nih.gov/pubmed/30992003.

43. Bourke BP, Oliveira TP, Suesdek L, Bergo ES, Sallum MA. A multi-locus approach to barcoding in the Anopheles strodei subgroup (Diptera: Culicidae). Parasit Vectors. 2013;6:111; doi: 10.1186/1756-3305-6-111. https://www.ncbi.nlm.nih.gov/pubmed/23597081.

44. Foster PG, Bergo ES, Bourke BP, Oliveira TM, Nagaki SS, Sant'Ana DC, et al. Phylogenetic analysis and DNA-based species confirmation in Anopheles (Nyssorhynchus). PLoS One. 2013;8 2:e54063; doi: 10.1371/journal.pone.0054063. https://www.ncbi.nlm.nih.gov/pubmed/23390494.

45. Chan A, Chiang LP, Hapuarachchi HC, Tan CH, Pang SC, Lee R, et al. DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasit Vectors. 2014;7:569; doi: 10.1186/s13071-014-0569-4. https://www.ncbi.nlm.nih.gov/pubmed/25498759.

46. Loaiza JR, Scott ME, Bermingham E, Sanjur OI, Rovira JR, Dutari LC, et al. Novel genetic diversity within Anopheles punctimacula s.l.: phylogenetic discrepancy between the Barcode cytochrome c oxidase I (COI) gene and the rDNA second internal transcribed spacer (ITS2). Acta Trop. 2013;128 1:61-9; doi: 10.1016/j.actatropica.2013.06.012. https://www.ncbi.nlm.nih.gov/pubmed/23806568.

47. Loaiza J, Scott M, Bermingham E, Rovira J, Sanjur O, Conn JE. Anopheles darlingi (Diptera: Culicidae) in Panama. Am J Trop Med Hyg. 2009;81 1:23-6. https://www.ncbi.nlm.nih.gov/pubmed/19556561.

48. Charlwood JD. Biological variation in Anopheles darlingi Root. Mem Inst Oswaldo Cruz. 1996;91 4:391-8; doi: 10.1590/s0074-02761996000400001. https://www.ncbi.nlm.nih.gov/pubmed/9070397.

49. Manguin S, Wilkerson RC, Conn JE, Rubio-Palis Y, Danoff-Burg JA, Roberts DR. Population structure of the primary malaria vector in South America, Anopheles darlingi, using isozyme, random amplified polymorphic DNA, internal transcribed spacer 2, and morphologic markers. Am J Trop Med Hyg. 1999;60 3:364-76; doi: 10.4269/ajtmh.1999.60.364. https://www.ncbi.nlm.nih.gov/pubmed/10466962.

50. Mirabello L, Conn JE. Molecular population genetics of the malaria vector Anopheles darlingi in Central and South America. Heredity (Edinb). 2006;96 4:311-21; doi: 10.1038/sj.hdy.6800805. https://www.ncbi.nlm.nih.gov/pubmed/16508661.

51. Mirabello L, Vineis JH, Yanoviak SP, Scarpassa VM, Povoa MM, Padilla N, et al. Microsatellite data suggest significant population structure and differentiation within the malaria vector Anopheles darlingi in Central and South America. BMC Ecol. 2008;8:3; doi: 10.1186/1472-6785-8-3. https://www.ncbi.nlm.nih.gov/pubmed/18366795.

52. Lopez-Rubio A, Suaza-Vasco JD, Solari S, Gutierez-Builes L, Porter C, Uribe SI. Intraspecific phylogeny of Anopheles (Kerteszia) neivai Howard, Dyar & Knab 1913, based on mitochondrial and nuclear ribosomal genes. Infect Genet Evol. 2019;67:183-90; doi: 10.1016/j.meegid.2018.10.013. https://www.ncbi.nlm.nih.gov/pubmed/30395997.

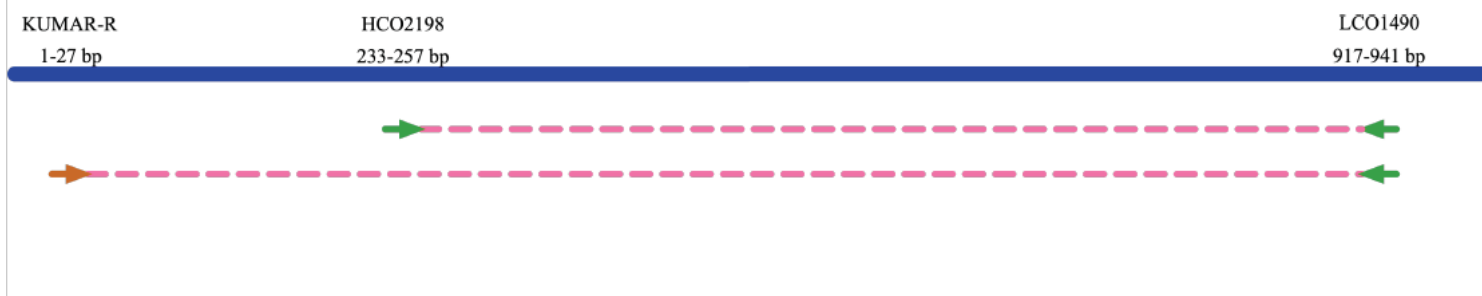
53. Gutierrez LA, Naranjo NJ, Cienfuegos AV, Muskus CE, Luckhart S, Conn JE, et al. Population structure analyses and demographic history of the malaria vector Anopheles albimanus from the Caribbean and the Pacific regions of Colombia. Malar J. 2009;8:259; doi: 10.1186/1475-2875-8-259. https://www.ncbi.nlm.nih.gov/pubmed/19922672.

Figures

Figure 1

Scheme of a region of the COI gene. Target sites of the primers used in the PCR are shown with arrows

a1	a2	b1	b2	c1
c2	d1	d2	e1	e2
f1	f2	g1	g2	

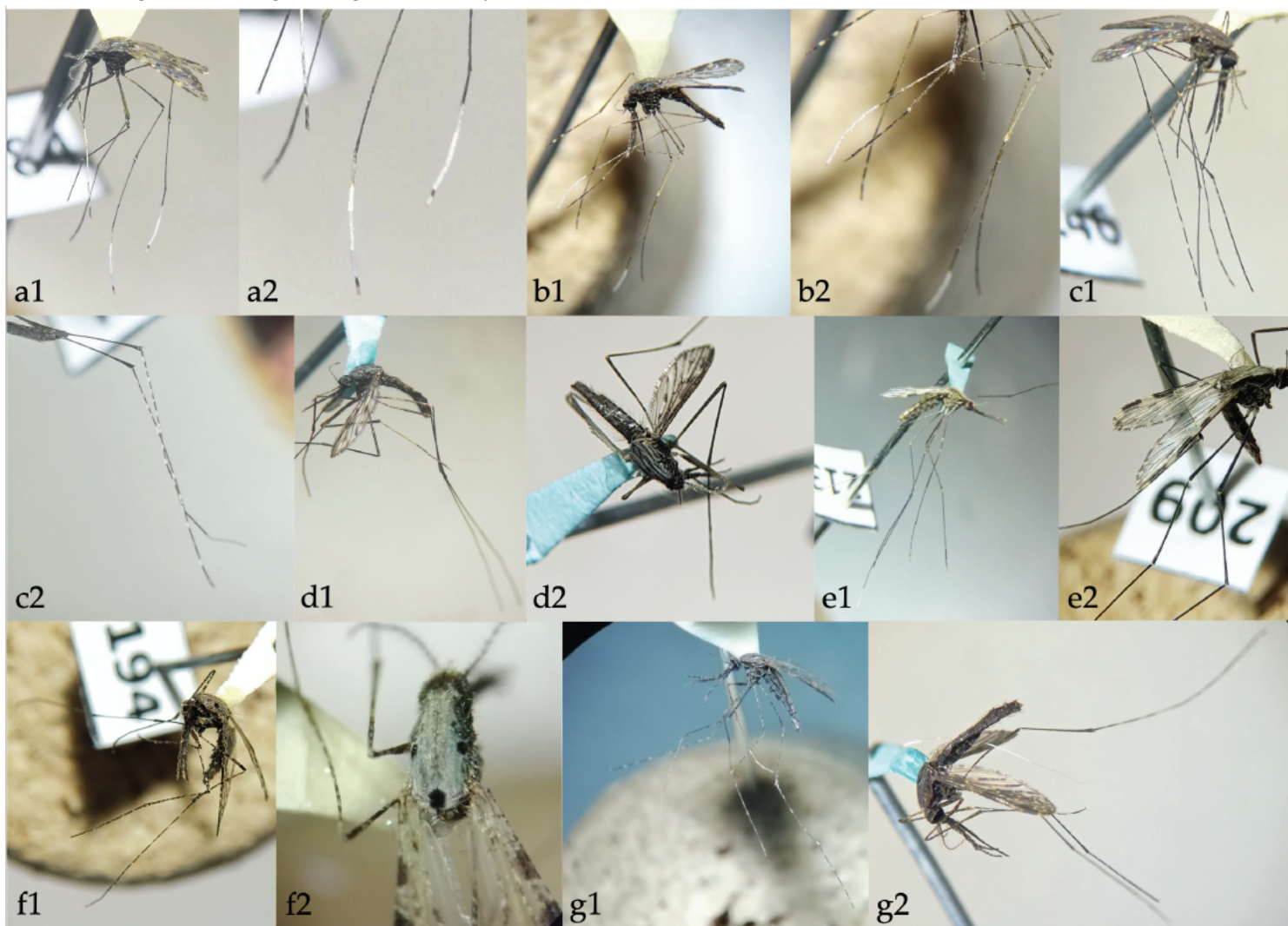



Figure 2

Anopheles specimens from Honduras. (a) An. albimanus (b) An. darlingi (c) An. vestitipennis (d) An. crucians (e) An. pseudopunctipennis (f) An. punctimacula (g) An. neivai

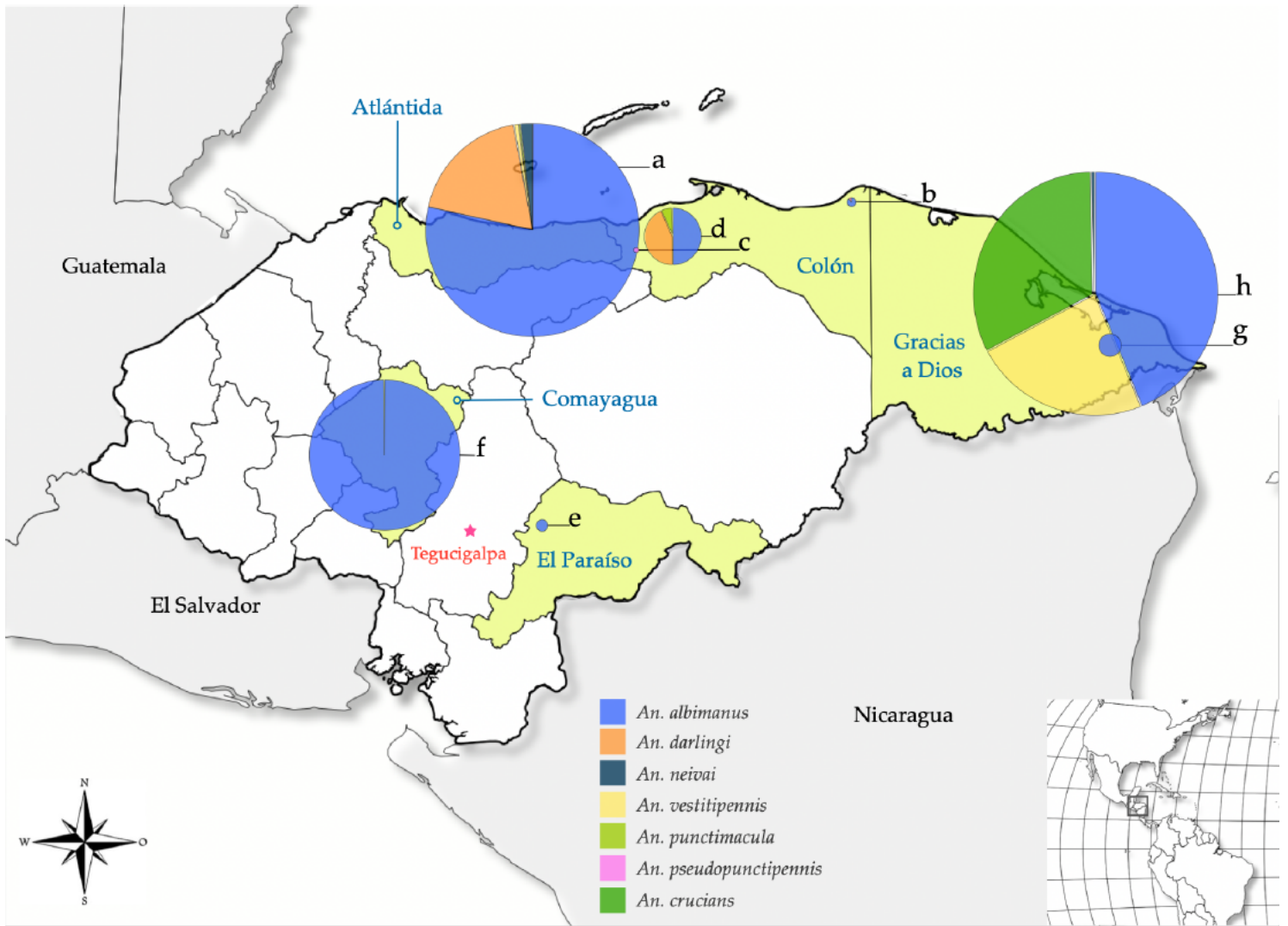



Figure 3

Map of Honduras showing eight collection sites. The pie charts show the proportion of Anopheles species collected at each site. The size of the charts is proportional to the number of specimens collected. (a) La Ceiba (Atlántida), (b) Iriona (Colón), (c) Sonaguera (Colón), (d) Tocoa (Colón), (e) Morocelí (El Paraíso), (f) Comayagua (Comayagua), (g) Tikirraya (Gracias a Dios), (h) Kaukira (Gracias a Dios)

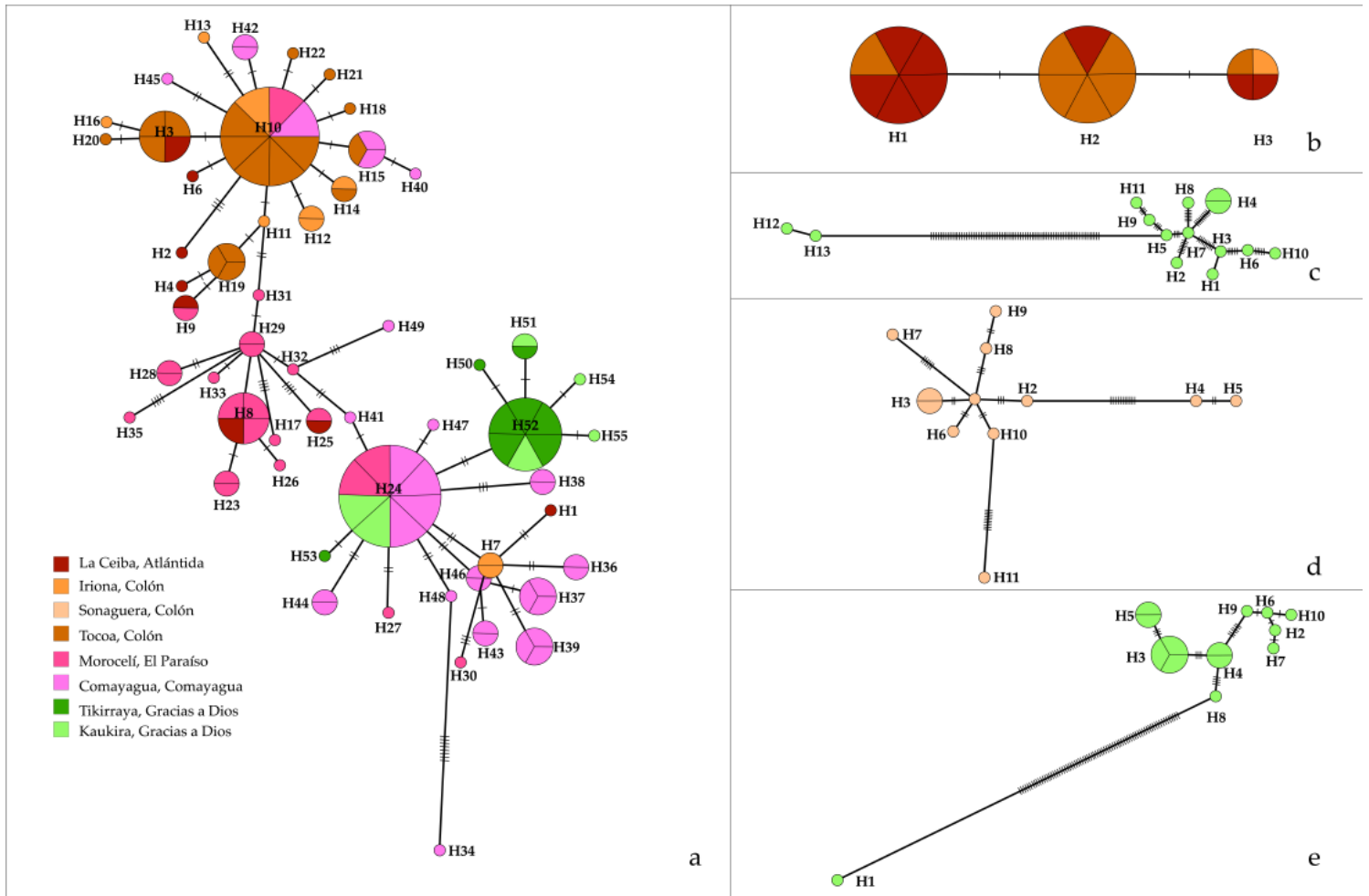



Figure 4

COI haplotypes networks for Anopheles spp. collected in 8 different locations of Honduras. (a) An. albimanus, (b) An. darlingi, (c) An. crucians, (d) An. pseudopunctipennis, (e) An. vestitipennis

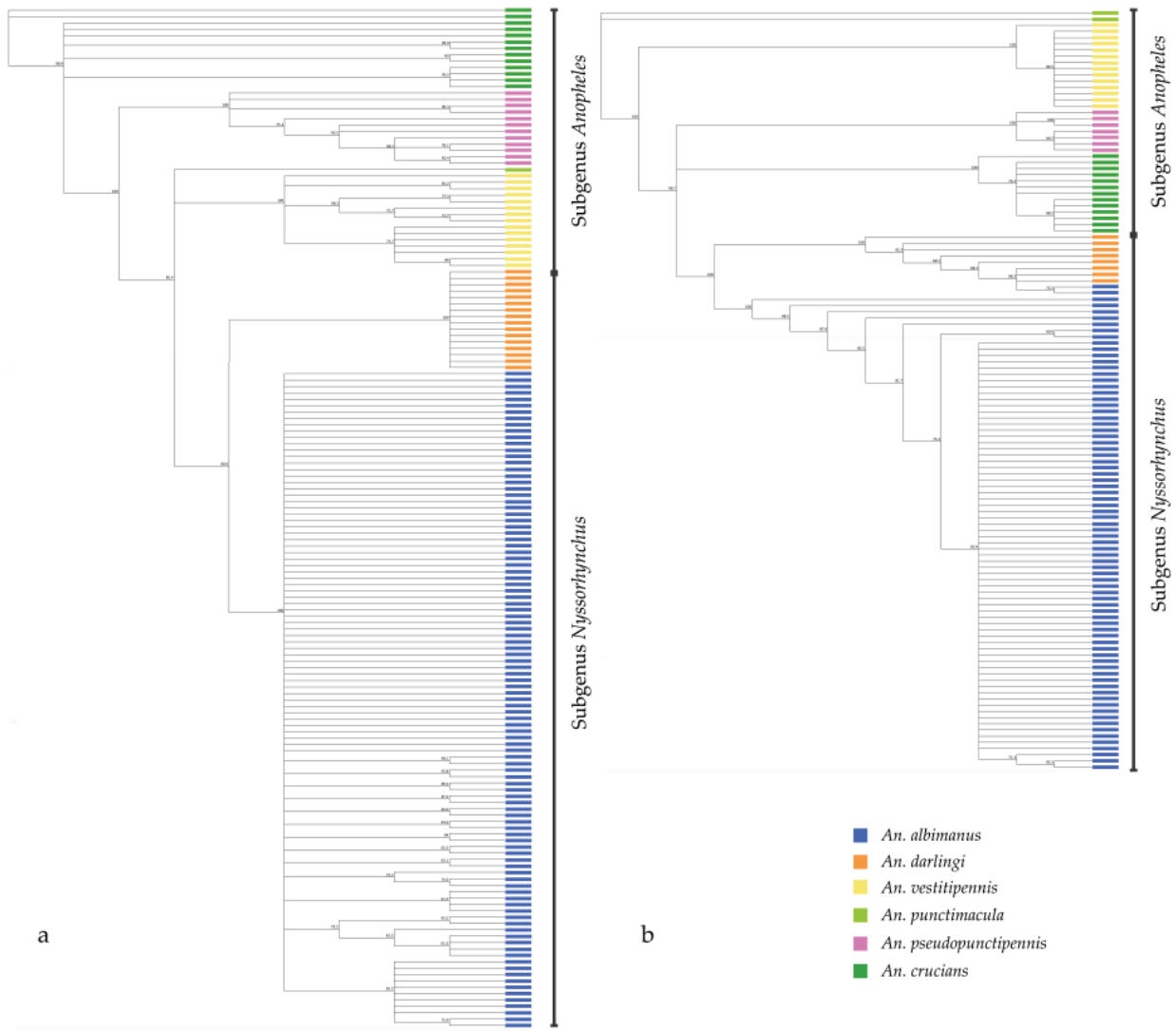



Figure 5

Phylogenetic analysis of (a) COI and (b) ITS sequences of six Anopheles species. Dendrograms were constructed using the Neighbor-Joining method and the Geneious 9.1.7 software with a bootstrap of 1000 replicates

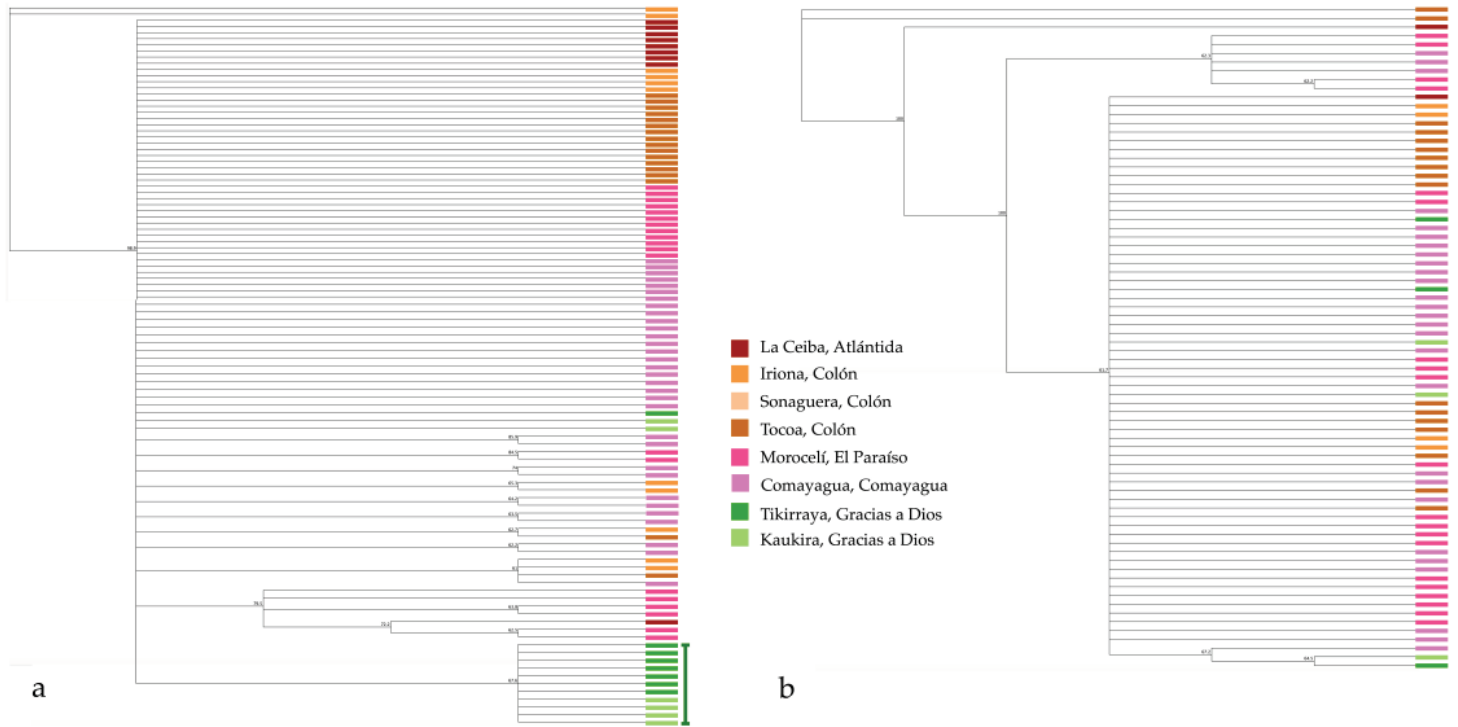



Figure 6

Phylogenetic analysis of (a) COI and (b) ITS sequences of Anopheles albimanus. Coloured¬ boxes indicate the geographic region where the insects were captured. Dendrograms were constructed using the Neighbor-Joining method and the Geneious 9.1.7 software with a bootstrap of 1000 replicates

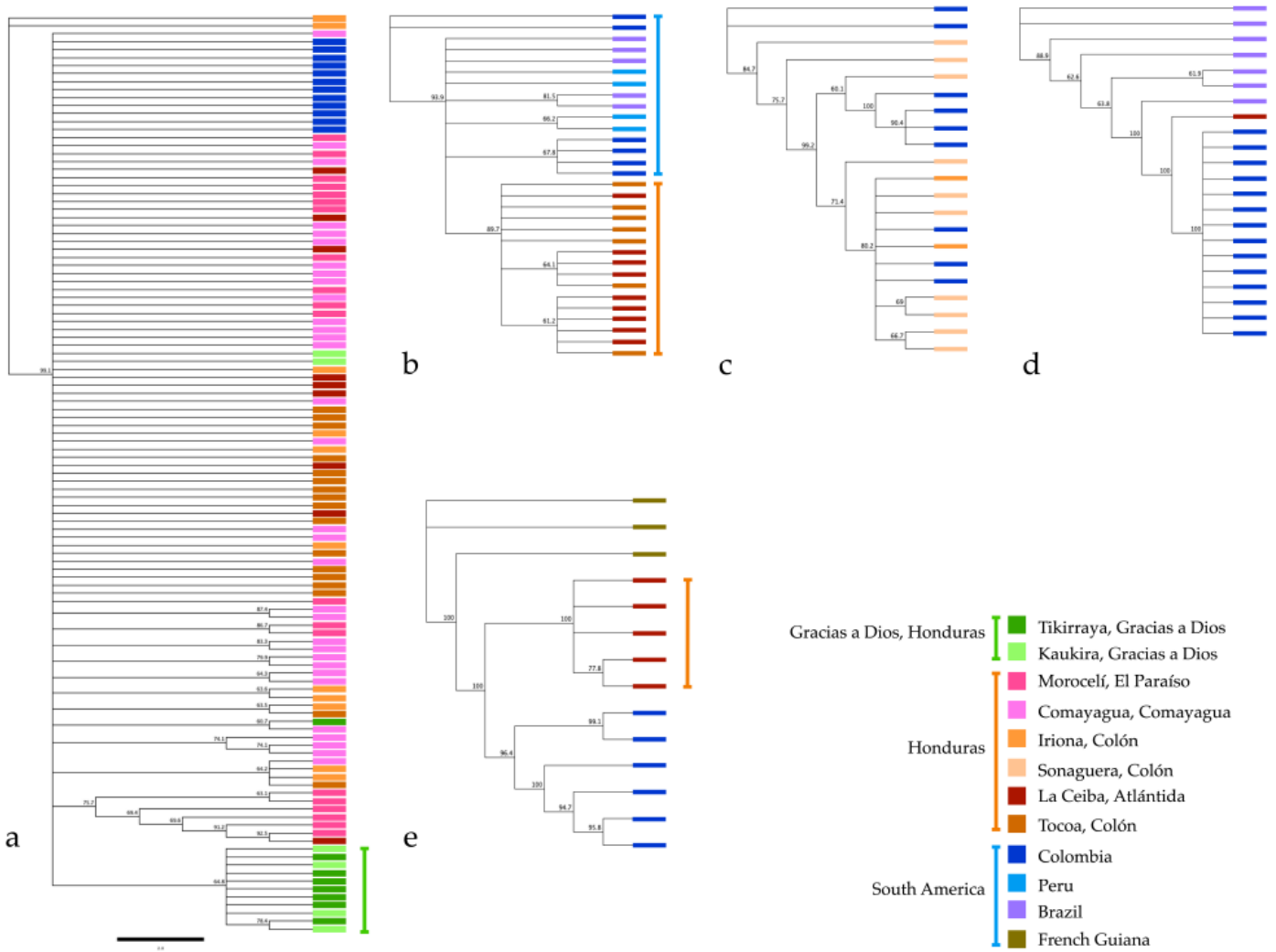



Figure 7

Phylogenetic analysis of the COI gene of Anopheles spp. from Honduras and four South American countries. Coloured¬ boxes indicate the geographic region where the insects were captured. (a) Anopheles albimanus, (b) An. darlingi, (c) An. pseudopunctipennis, (d) An. punctimacula, (e) An. neivai. Trees were constructed using the Neighbor-Joining method and the Geneious 9.1.7 software with a bootstrap of 1000 replicates

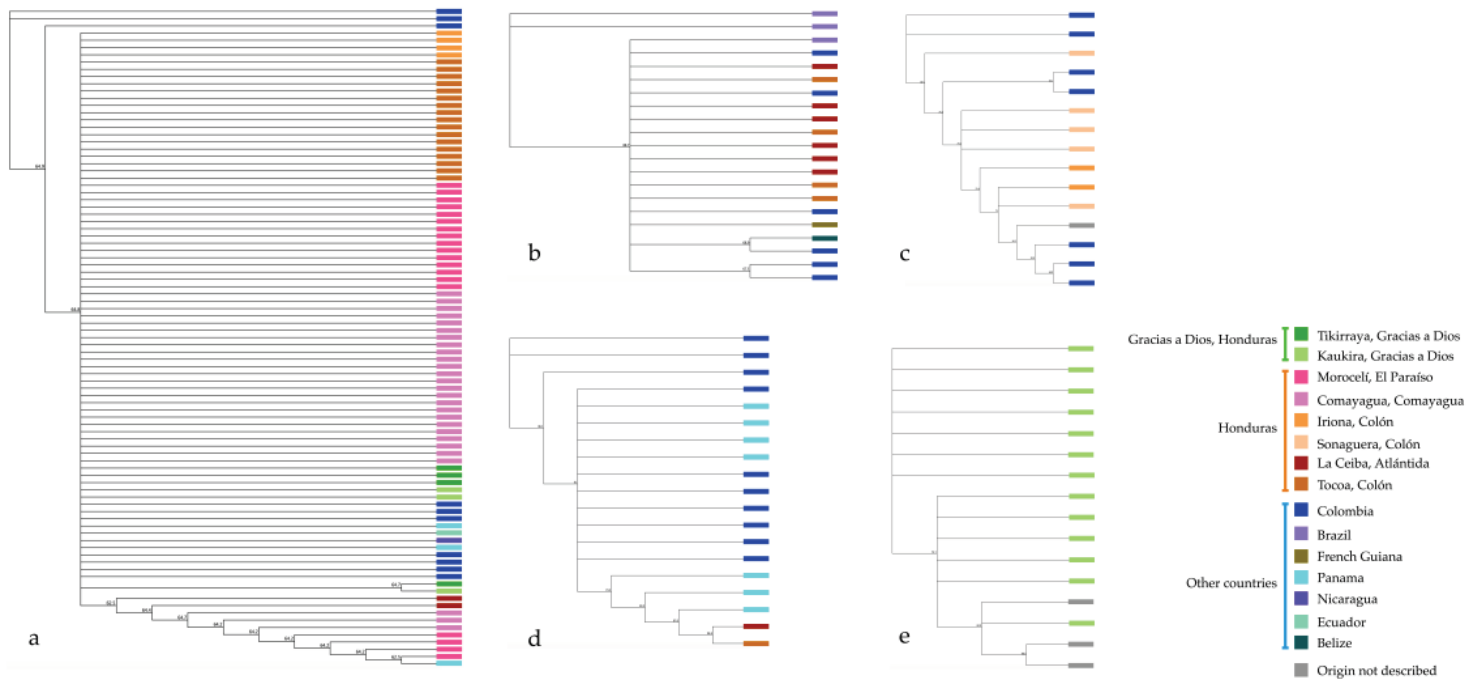



Figure 8

Phylogenetic analysis of the ITS2 region of Anopheles spp. from Honduras and other seven countries. Coloured¬ boxes indicate the geographic region where the insects were captured. (a) Anopheles albimanus, (b) An. darlingi, (c) An. pseudopunctipennis, (d) An. punctimacula, (e) An. crucians. Trees were constructed using the Neighbor-Joining method and the Geneious 9.1.7 software with a bootstrap of 1000 replicates