Natural Plasmodium infection of Anopheles benarrochi B (Diptera: Culicidae) in native communities of the Province of Condorcanqui, Amazonas-Peru

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Research Article

Keywords: Plasmodium Anopheles, COX1, kdr, Malaria, Native communities, Amazonas, Peru

Posted Date: October 9th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3409121/v1

Abstract

Background

Malaria is a severe health problem in the native communities of Condorcanqui in the Amazonas Department of Peru. Recently, the number of malaria cases has increased considerably following a *Plasmodium falciparum* outbreak in 2019. However, there is no information on the *anopheline* species acting as *Plasmodium* vectors in this area or its insecticide resistance status. This study aims to: i) to molecularly characterize the anopheline population from the district of Rio Santiago; ii) to determine their incrimination in malaria transmission; and iii) to evaluate mutations associated with resistance to pyrethroid insecticides and DDT in the mosquito population.

Methods

Mosquitoes were collected between March and September 2022, using Shannon traps, CDC light traps, and mouth aspirators. Only those morphologically identified as *Anopheles* sp. were subjected to molecular confirmation by PCR amplification and sequencing of the COX1 barcode region. Additionally, specimens that were molecularly confirmed as *Anopheles* were analyzed for the kdr region of the VGSC gene related to insecticide resistance. Likewise, the presence of human blood as a food source was detected using the β-globin marker, and the presence of *P. falciparum* and *Plasmodium vivax* was determined through a nested PCR.

Results

A total of 453 mosquitoes were captured, of which ninety-four were morphologically identified as female anophelines. Of the latter, sixty-six (~ 70%) specimens were molecularly confirmed as anophelines and were grouped into four species: *An. benarrochi* B, *An. triannulatus, An. costai* and *An. nimbus*. The sixty-six anophelines were analyzed for human β-globin and *Plasmodium*. It was found that twenty-three samples of *An. benarrochi* B (~ 35%) and one specimen of *An. triannulatus* were positive for human β-globin. Likewise, six (~ 9%) samples of *An. benarrochi* B were positive for *Plasmodium* parasites (four for *P. falciparum* and two for *P. vivax*). It is worth noting that four specimens tested positive for *Plasmodium* parasites and human blood simultaneously, making this a robust outcome to incriminate *An. benarrochi* B as the main malaria vector. No specimens presented mutations associated with insecticide resistance in the kdr region.

Conclusions

An. benarrochi B is the dominant anopheline species in this study and plays an important role in malaria transmission. Further studies are needed to understand its feeding behavior and activity during dry and

rainy seasons to fully incriminate it with malaria transmission and implement targeted vector control programs.

BACKGROUND

In Peru, 26,652 malaria cases were reported in 2022, with ~ 84% *Plasmodium vivax* and ~ 15% *P. falciparum* cases [1], nevertheless, malaria infections remain uncertain in remotes areas because control activities have been postponed owing to COVID-19. Although Loreto is one of the most affected departments in the country (89% of malaria cases) [2], Amazonas, a northeastern department of Peru, has reported a significant increase in the number of malaria cases for the past six years, from 710 cases in 2018 to 1657 in 2022 [3]. In 2019, a 2.5-fold increase was reported due to an outbreak of autochthonous *P. falciparum* cases in native communities of Rio Santiago, in the Condorcanqui Province of Amazonas, this outbreak was triggered by an index case of *P. falciparum* imported from Loreto, which subsequently spread to other communities during that year [3]. However, there is limited information about the circulating vectors involved in malaria transmission in these communities.

In the Peruvian Amazon, the species recognized as the main malaria vector is *Anopheles darlingi* Root [4]; this highly anthropophilic species has been reported in the departments of Madre de Dios [5], Loreto [6, 7], Ucayali and San Martin [8]. Other potential malaria vectors, *An. triannulatus* (Neiva & Pinto) and *An. benarrochi* (Cova García & López) have been reported in eastern and in western Loreto, respectively [9]. Previous studies suggested that *An. benarrochi* is a species complex consisting in four distinct species (*An. benarrochi* B, *An. benarrochi* G1, *An. benarrochi* G2 and *An. benarrochi*) [10], from which *An. benarrochi* B has been identified as a malaria vector in northern Loreto and Madre de Dios [11].

Efforts to eliminate malaria transmission in native communities of Rio Santiago have focused on rapid diagnosis, case management, insecticide-treated nets (ITNs), and indoor and outdoor residual spraying programing by the MoH. However, this progress can be undermined by parasites´ resistance to antimalarial, and also by mosquitoes' resistance to insecticides and vector´s behavior. According to the World Malaria Report 2022, a total of eighty-eight countries reported insecticide resistance in the last decade. Among these countries, twenty-nine documented resistance to four primary classes of insecticides (pyrethroids, organophosphates, carbamates, and organochlorines) at various locations within their territories [12]. One of the primary mechanisms related to pyrethroid resistance, known as knockdown resistance (kdr), involves mutations at the kdr region of the voltage-gated sodium channel gene (VGSC), which is the primary target of synthetic pyrethroids [13]. In South America, *An. darlingi* resistance to dichlorodiphenyltrichloroethane (DDT) and pyrethroids has been reported in western Colombia [14], while on the northwestern coast of Peru, *An. albimanus*, has shown cross-resistance to all classes of insecticides [15]. In this study, a molecular characterization of mosquitoes collected in native communities of Rio Santiago was evaluated in order to identify species of *Anopheles* mosquitoes, incriminate them as malarial vectors, and evaluate insecticide resistance by the kdr region marker.

METHODS

Study area and mosquito collection

Approximately, 99% of malaria cases (1633 malaria cases in 2022) [16] in the Amazonas Department were reported in the Rio Santiago District (Condorcanqui Province). This is a remote impoverished area where native communities live on the banks of the river, with no electricity, drinking water, or road access, with the rivers as the primary means of transportation [3].

Mosquito collections were conducted in four native communities of Rio Santiago: Alianza Progreso (AP), Nueva Esperanza (NE), Chapiza (CH), and Caterpiza (CT) (Fig. 1), located within the ecosystem of the Amazonian Humid forests with temperatures that can reach 35ºC, an average annual rainfall of around 4,800 mm, and a relative humidity of above 90% [3, 17].

Adult mosquitoes were collected for six nights during the rainy season between March and September of 2022.Mosquitoes were captured with three Shannon traps and then, with the help of mouth aspirators, the mosquitoes were transferred to cryovials. Likewise, two light traps from the Centers for Disease Control and Prevention (CDC) were used, last two traps placed ~ 10 m from the houses and ~ 10 m from the forest entrance between 18:00 and 22:00 [18, 19].

Anopheles mosquitoes were identified in the field based on their morphological characteristics using entomological keys [20], then they were stored in 1.5ml cryovials containing 70% ethanol. Samples were classified and stored by date and location; additional information such as temperature and relative humidity was also collected.

DNA isolation, amplification, and sequencing of COX1 and kdr

Genomic DNA was extracted from the whole body of each specimen using a DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions. The 710bp barcode region of the mitochondrial Cytochrome Oxidase I gene (COX1) was amplified for all samples using the LCO1490 and HCO2198 primers [21]. The 25 µl PCR reaction included 1ul of extracted DNA, 0.5 µM of each primer, 1 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2mM dNTP, 1X PCR buffer (Invitrogen), 2.5mM $MgCl_2$ and nuclease-free water. Thermocycling conditions were used according to the literature [22].

PCR amplification of segment 6 of domain II (IIS6) of the VGSC gene (approximately 225 bp), corresponding to the kdr region, was amplified using AAKDRF2 and AAKDRR primers [23]. The 25 µl PCR reaction included 5 µl of extracted DNA, 0.5 µM of each primer, 1 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM dNTP, 1X PCR buffer (Invitrogen), 2.0 mM MgCl2 and nuclease-free water. Thermocycling conditions were used according to the literature [24].

Amplicons were visualized on 2% agarose gels stained with SafeView™ Classic (Applied Biological Materials). PCR products were purified using the Exo-CIP™ kit (New England Biolabs), and sequencing was performed on a 3500 Genetic Analyzer (Applied Biosystems™).

Bioinformatic analysis

The sequence data was analyzed using the Geneious Prime (version 2022.2.1, Biomatters Inc, Newark) (https:/www.geneious.com), and Basic Local Alignment Search Tool (BLAST) searches were carried out for species identification with sequences available in the GenBank database. Species were determined based on query coverage (QC) and percentage identity (PI). All anopheline sequences were aligned using the MUSCLE tool, implemented in the MEGA software (version 7.2.6.1). In addition, 77 previously reported sequences of different anopheline species were also included in the phylogeny (Additional file 1: Table S1) [10, 25, 26, 27, 28, 29]. A Maximum Likelihood (ML) tree was constructed using a Kimura 2-parameter model (K-2P) and the Bootstrap method with 1000 bootstrap replicates to examine phylogenetic relationships in MEGA X software [30]. The *Aedes aegypti* COX1 sequence (NC035159) was used as an outgroup to root the tree. *An. benarrochi* haplotypes from this study and from 59 previously reported sequences (Additional file 1: Table S2) were determined using DnaSP v6 [31], and the haplotype network was constructed using the Median-Joining algorithm in PoPART software [25]. For kdr analysis, *An. darlingi* (MN062262) and *An. albitarsis* (MW315118) were used as references.

Human blood meal identification and Plasmodium detection

Source of mosquitoes blood meal was assessed using a human β-globin protocol previously reported [32]; the thermal profile consisted of an initial denaturation step at 94°C for 7 min; followed by 35 cycles at 94°C for 1 minute for denaturation, 53°C for 1 minute for hybridization, and 72°C for 1 minute for extension; and then a final extension at 72°C for 5 minutes.

The detection of *P. falciparum* and *P. vivax* infections in mosquitoes was performed using the 18S rRNA subunit nested PCR technique according to the protocol previously described [33].

RESULTS

Molecular identification of mosquitoes

The environmental conditions during the collection dates were very similar, with a temperature range from 25ºC to 28ºC and humidity levels between 75% and 87% (Table 1). A total of 453 individuals were captured using Shannon traps and then transferred into cryovials using mouth aspirators. None individuals were captured using CDC-LT traps. Ninety-four females were initially identified as *Anopheles* based on morphological characteristics (Table 1) and the remaining species were identified morphologically as Culicidae (detailed data not shown); After molecular confirmation, it was found that only sixty-six individuals belonged to the *Anopheles* genus (AP = 55, CT = 5, NE = 5, and CH = 1) and the remaining twenty-eight mosquitoes were molecularly confirmed as other non-anopheline Culicidae.

Table 1
Mosquito specimens (n = 94) collected in Rio Santiago, Condorcanqui province, 2022. Alianza Progreso (AP = 81), Nueva Esperanza (NE = 5), Chapiza (CH = 1), and Caterpiza (CT = 7)

Site	Coordinates		Date	T° (°C)	RH (%)	Number of specimens	Molecular Identification
NE	196280.9	9581345.6	02/03/2022	27	75	5	*An. benarrochi* B
CH	199060.7	9587806.4	02/03/2022	27	87	1	*An. benarrochi* B
AP	195051.2	9592391.3	01/03/2022	26	75	13	*An. benarrochi* B
			01/03/2022	26	75	2	*An. triannulatus*
			23/05/2022	28.1	83	13	*An. benarrochi* B
			23/05/2022	28.1	83	15	*Coquillettidia venezuelensis*
			24/05/2022	26	86	15	*An. benarrochi* B
			24/05/2022	26	86	5	*Coquillettidia venezuelensis*
			25/05/2022	25.3	87	9	*An. benarrochi* B
			25/05/2022	25.3	87	2	*An. costai*
			25/05/2022	25.3	87	1	*An. nimbus*
			25/05/2022	25.3	87	6	*Coquillettidia venezuelensis*
CT	198182.6	9566709.0	20/09/2022	25.3	87	5	*An. benarrochi* B
			20/09/2022	25.3	87	1	*Sabethes sp*
			20/09/2022	25.3	87	1	*Culex bastagarius*

An. benarrochi B was found to be the predominant species (include %, 51/66 specimens) with the presence of *An. triannulatus* (2/66), *An. costai* (2/66) and *An. nimbus* (1/66) (Additional file 1: Table S3). Among non-anopheline mosquitoes, *Coquillettidia venezuelensis (26/28)*, one *Sabethes* sp, and one *Culex bastagarius specimens* were also molecularly identified (Additional file 1: Table S4). According to the median-joining haplotype network based on COX1 (Fig. 2), there were thirty-six *An. benarrochi* B haplotypes, from which six were found in Amazonas. Haplotype 1 was found in Amazonas and also in Ecuador, which is expected due to proximity. Haplotypes 3, 5 and 6 were only found in Amazonas, while haplotypes 2 and 4 were found in Amazonas and Loreto Department. A ML K-2P tree (Fig. 3) further supported the correct identification of the specimens in this study. Additionally, the species clade assignment corresponded to the traditional subgenus classification [34]; specifically, species such as *An. benarrochi B, An. benarrochi, An. rangeli, An. konderi, An. albimanu*s and *An. triannulatus* are included in

the *Nyssorhynchus* subgenus, while *An. nimbus* and *An. kompi* belonged to the *Stethomyia* subgenus group and *An. costai* to the *Anopheles* subgenus group.

Blood meal source and Plasmodium identification in mosquitoes Anopheles spp.

During morphological identification, it was observed that certain specimens presented an enlarged abdomen, which was attributed to their blood meal (Additional file 2: Fig. S1). Of the sixty-six anophelines molecularly confirmed, *An. benarrochi* B (twenty-three samples) and *An. triannulatus* (one sample) were positive to human β-globin. Furthermore, six specimens of *An. benarrochi* B were found to be positive for *Plasmodium* (four *P. falciparum*, and two *P. vivax*) (Additional file 1: Table S3). Notably, four samples exhibited the presence of *Plasmodium* parasites and human blood, suggesting the incrimination of *Anopheles benarrochi* B in malaria transmission in the region.

Identification of insecticide-resistance genotype in the kdr region

Out of the total sixty-six anophelines, sixty-four successfully underwent amplification and genotyping for the kdr region (Fig. 4, Additional file 1: Table S3). These sequences were further examined to identify mutations in codons 1010, 1013, and 1014 [24]. No missense mutations were detected in any of the sequences. All sequences of *An. benarrochi* B and *An. triannulatus* showed the GTT codon for valine at position 1010 (V1010), AAC codon for asparagine at position 1013 (N1013), and TTA codon for leucine at position 1014 (L1014), suggesting that the collected anopheline population has wild-type genotypes related to susceptibility to DDT and pyrethroid insecticides. Only the *An. nimbus* sequence showed a synonymous mutation at position V1010 (GTA). On the other hand, two *An. benarrochi* B haplotypes differed in a C/A substitution (at position 45); however, it should be noted that these mutations correspond to synonymous substitutions in codons unrelated to insecticide resistance. Similarly, the two *An. triannulatus* sequences differed in an A/G substitution in the intronic region. Further analysis of the intron downstream kdr revealed size variations among the species. The intron of *An. nimbus* (90 bp) was found to be larger than that of *An. benarrochi* B (72 bp) and *An. triannulatus* (75 bp), which also differed from the reference sequences (*An. darlingi* wildtype and *An. albitarsis* mutated at 1014 codon).

DISCUSSION

This study is the first report on the molecular characterization of anopheline species circulating in native communities in Condorcanqui – Amazonas Department, Peru. The primary objective was to identify the malaria vector species in the area to maximize the impact of malaria control strategies. In the eastern region of Peru, *An. darlingi* has been recognized as the main malaria vector; however, it is plausible that other *Anopheles* species could be transmitting malaria in endemic areas where *An. darlingi* is absent [8]. For instance, *An. triannulatus* was reported as the dominant vector in eastern Loreto, while *An. benarrochi* was mostly found in western Loreto [8, 35].

Two anopheline species, *An. benarrochi* B (56/66) and *An. triannulatus* (2/66), both belonging to the *Nyssorhynchus* subgenus, were successfully identified in this study. Furthermore, the network analysis and ML phylogenetic tree showed that *An. benarrochi* B sequences were strongly linked to previously reported sequences in Ecuador, Colombia [25], Peru [11, 29, 25] and Brazil [10].

The presence of *An. benarrochi* B as a malaria vector in an endemic area is usually associated with the absence of *An. darlingi* [36]. Our findings further support this hypothesis, since *An. benarrochi* B was identified as the predominant species in all four communities in all sampling periods. Likewise, it should be emphasized that *An. benarrochi* B has already been identified in other regions of Peru [25, 11, 35], and with the present study, it extends its distribution to the northwest of the country, including three new haplotypes.

Malaria cases in these communities have been significantly on the rise in the last years. In 2020, the Chapiza Health Establishment reported a high Annual Parasite Index (API) of 122.8, which further increased to 224.8 by 2022. The cases included infections caused by both *P. falciparum* and *P. vivax*. Here, we determined the presence of these parasites in specimens of *An. benarrochi* B from the Alianza Progreso community, which belongs under the jurisdiction of the Chapiza Health Center. A similar situation was observed at the Caterpiza Health Establishment, where the API was 9.7 in 2020 (considered a moderate risk) and it spiked to 812.7 (classified as high risk) with only *P. vivax* cases in 2022. Interestingly, the anophelines captured in Caterpiza did not test positive for *Plasmodium*, but they did test positive for the human B-globin marker, confirming the anthropophilic behavior of *An. benarrochi* B. Moreover, a total of six out of sixty-six anophelines were found to be infected with *Plasmodium*, which represents a highly significant percentage compared to what was reported previously [11, 35, 37]. Considering that the specimen's collection was made after the rainy season, with high mosquitoes' density and in areas with APIs of moderate to high risk, it may explain the high number of infected mosquitoes found in the study. A limitation of this study is the low number of entomological surveys in all communities (one sampling night in each one) preventing the calculation of reliable entomological indices. Nevertheless, the data provides evidence of the importance of *Anopheles benarrochi* B as a malaria vector in this unexplored area, although implication of other anopheline species should be investigated. Furthermore, since 2019, there has been a significant shift in the epidemiological landscape of malaria in Condorcanqui, given the emergence of *P. falciparum* [3]; it is suspected that alterations in vector composition, the high human biting rate and peak biting time (~18:00–20:00) [35] could have also contributed to this event.

On the other hand, the genetic diversity of *An. costai* and *An. nimbus* has received limited attention in previous studies. The lack of research on these species may be attributed to their lack of implication as significant malaria vectors. They have been predominantly observed in forest habitats and have often been misidentified as *An. mediopunctatus*, which has been found to carry *P. vivax* [38].

There is limited information regarding insecticide resistance in the most significant malaria vectors in Latin America [24] and conducting insecticide resistance studies in the Peruvian Amazon region is

challenging due to limited accessibility, inadequate laboratory capacity, and a shortage of trained personnel [39]. In this study, genotypes linked to resistance to DDT and pyrethroids were not detected within the kdr region of any of the sixty-six *Anopheles* specimens captured in the Rio Santiago district. Further investigations involving a larger number of mosquitoes are essential to evidence the susceptibility status of main malaria vectors to develop successful vector surveillance programs in Peru. Additionally, noteworthy findings include the identification of synonymous mutations in *An. benarrochi* B (in the exonic region) and *An. triannulatus* (in the intronic region) and variations in the intronic size within the species. The analysis of intron sequences, including nucleotide positions and sizes, may support the hypothesis of interspecific differences and offers potential as a tool for taxonomic classification [23, 24].

Regarding other *Anopheline* species, several studies have reported the absence of mutations in the kdr region associated with insecticide resistance. Wild-type kdr alleles were found in *An. nuneztovari s.l. An. darlingi*, and *An. albimanus* from Valle del Cauca, Colombia [24] and in *An. albimanus* populations from Guatemala, Ecuador, and Colombia [23]. On the other hand, specific mutations at codons 1010, 1014 and 1014 in the kdr region have been associated with pyrethroid resistance [40]. In Peru, resistance to pyrethroids was reported in *An. darlingi* in Loreto [41] and a high frequency of TCG (L1014S) and TGT (L1014C) associated with resistance in *An. albimanus* was revealed in Tumbes through RNA-seq analysis [42].

CONCLUSION

This study reveals the presence of *Anopheles benarrochi* B and its natural infection with *Plasmodium* in unexplored native communities in the Amazonas region highlighting its role as a major malaria vector in this area, although other anopheline species might be also involved. This research also evidences the wider distribution of *An. benarrochi* B in northern Peru. Furthermore, the fraction of mosquito population analyzed showed wild-type/susceptible kdr genotypes in native communities of the province of Condorcanqui, providing a base line for insecticide surveillance and control interventions in the area. More entomological surveys in these communities of the province of Condorcanqui are required to characterize vector bionomics and behavior across seasons to unveil the species involved in malaria transmission. This will help in the design and implementation of specific programs to combat malaria by the Condorcanqui Health Network.

Abbreviations

BLAST
Basic Local Alignment Search Tool
CDC
Centers for Disease Control and Prevention
COX1
Mitochondrial Cytochrome Oxidase I gene
DNA

Deoxyribonucleic acid
ELISA
Enzyme-linked immunosorbent assay.
IET
Instituto de Enfermedades Tropicales
KDR
Knockdown resistance
PCR
Polymerase chain reaction.
PI
Percent identity
rRNA
Ribosomal ribonucleic acid.
QC
Query coverage
VGSC
Voltage-Gated Sodium Channel gene

Declarations

Acknowledgements

The authors express their sincere thanks to colleagues of the Institute of Tropical Diseases (IET-UNTRM) and Dirección Regional de Salud de Amazonas.

Funding

Proyecto METAVEC N°: 050-2021-FONDECYT

Ethics declaration

National Forestry and Wildlife Service. Authorization code No AUT-IFS-2021-071 and Ethics committee approval from UNTRM CIEI-N° 011.

Contributions

SC and RT conceived and designed the study. LG, MM and CG, advised on interventions, study communities and coordination with local and national authorities. SC, RT, LR and LG implemented the study. MVP and JZ analysed the data. MVP, JZ, SC and RT interpreted the data. MVP & JZ wrote the first draft of the manuscript. SC, MM & CG critically revised the manuscript for important content. All authors read and approved the final version of the manuscript.

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Figures

Figure 1

A. Map of Peru and the geographical location of Amazonas. B. Map of Amazonas indicating the province of Condorcanqui and the district of Rio Santiago. C. Mosquito collection sites in the native communities of Rio Santiago. D. Landscape of Alianza Progreso, 2022.

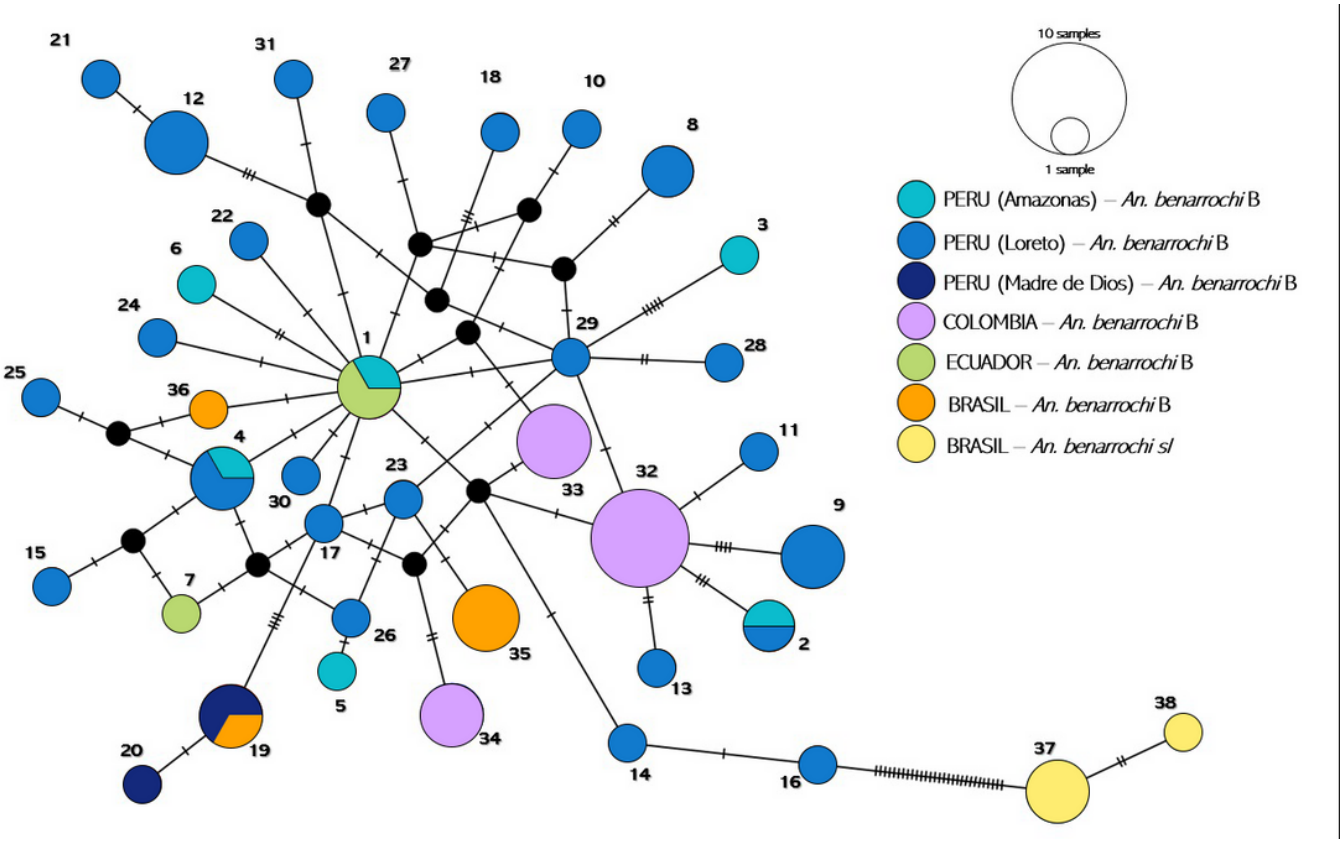

Figure 2

Anopheles benarrochi B MJ network. Circles represent unique haplotypes; the size of the circle is proportional to the number of individuals sharing the haplotype. Black nodes indicate theoretical missing haplotypes, and hash marks represent mutation steps between haplotypes.

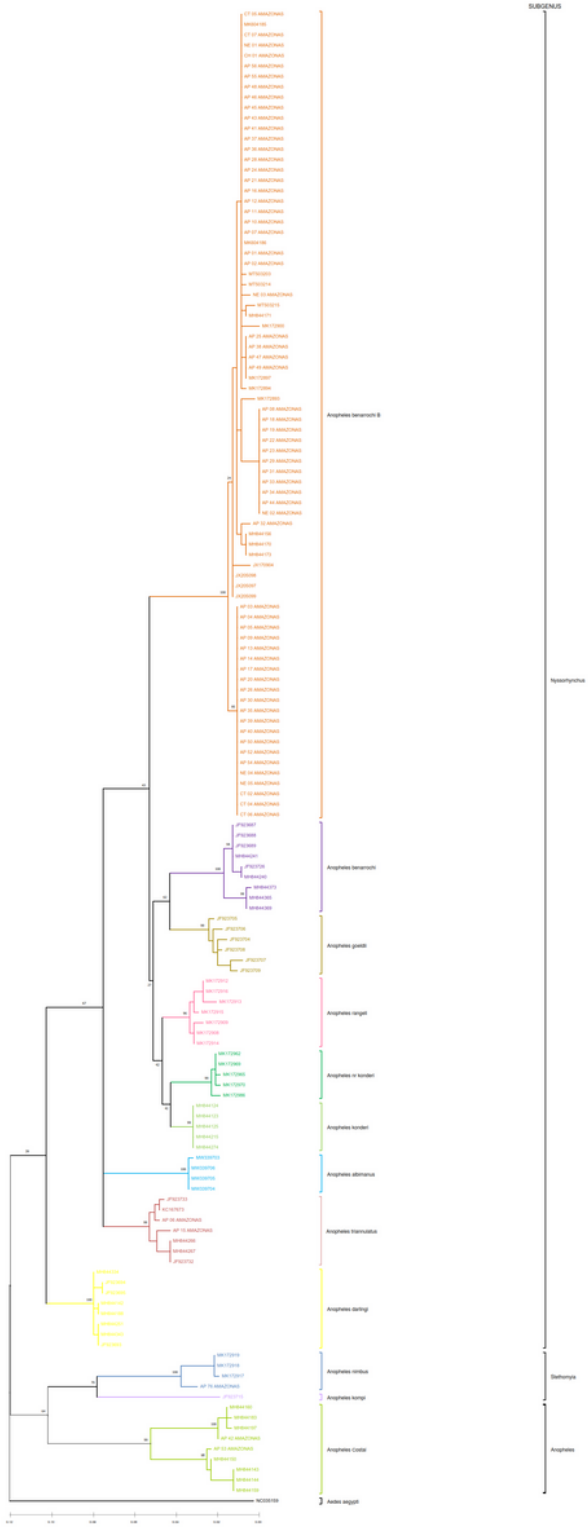

Figure 3

Maximum likelihood (ML) phylogenetic tree using the COX1 gene (467 bp) with a total of 144 sequences.

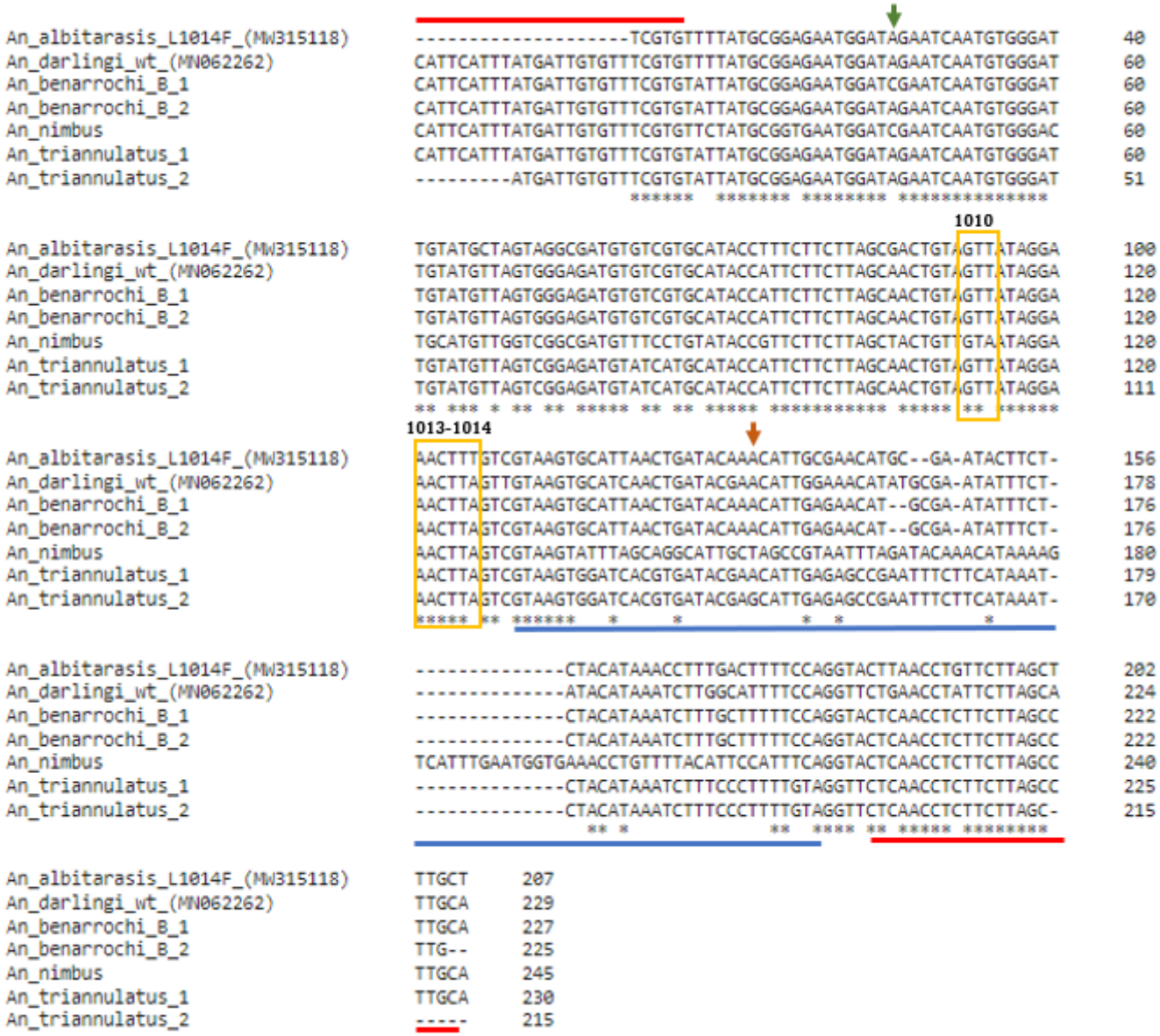

Figure 4

Alignment of the kdr sequences, including the *An. albitarsis* mutant L1014F (MW315118) and the *An. darlingi* wild type (MN062262). Mutation sites reported are enclosed in a yellow box. The blue line below indicates an intron. Primers AAKDRF2 and AAKDRR used to amplify the segment are indicated by red arrows. SNPs detected in the intron for *An. triannulatus* are indicated with an orange arrow, SNPs detected in the exon are indicated with a green arrow.

Supplementary Files

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- AdditionalFile2.FigureS1.png